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Statins, HMGCR and Breast Cancer

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Statins, HMGCR and Breast Cancer

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Abstract

Breast cancer is the most common cancer in women and the second most common cause of cancer-related deaths. Breast cancer treatments are improving but still, breast cancer recurrences, disease-associated morbidity and mortality are challenges that need to be addressed continuously, i.e., through new treatments options.

Statins, or HMGCR inhibitors, are a group of per oral drugs that lowers the cholesterol levels in the blood by inhibiting HMGCR, the rate-limiting enzyme of the cholesterol biosynthesis pathway. Statins are most often well tolerated, have few side effects and are inexpensive. In addition to lowering the cholesterol level in the blood, statins have pleiotropic, or cholesterol-independent, mechanisms that have shown anti-tumoral effects *in vitro, in vivo*, and in phase II clinical trials. In addition, results from observational studies have demonstrated that statin use decreases the risk of breast cancer recurrence and breast cancer-specific mortality.

Paper I-III are based on the MAST trial. The MAST trial is a phase II clinical trial applying the window-of-opportunity study design. A total of 50 women with primary invasive breast cancer were included. After inclusion, a tumor biopsy was taken, thereafter treatment with atorvastatin 80 mg daily for two weeks was initiated, followed by the planned breast cancer surgery. At the surgery, renewed tumor sample was taken.

In paper I, the results showed that, overall, statins did not decrease the tumor proliferation, which was used as a biomarker for treatments effect. In patients that expressed the rate-limiting enzyme of the cholesterol biosynthesis pathway, HMGCR, a significant decrease in tumor proliferation was seen. In addition, the expression of HMGCR in the post-treatment tumor samples was significantly increased following statin treatment.

In paper II, a whole genome expression profiling of the paired tumor samples was done to study statins effect on the transcriptional level. The results showed significant changes on the transcriptional level and suggested pro-apoptotic events and inhibition of the MAPK-pathway. In breast cancer cell lines, anti-proliferative effects were seen as well as an up-regulation of genes involved in the cholesterol biosynthesis pathway.

In paper III, the effect of statins on the cell cycle regulators cyclin D1 and p27 were investigated. After statin treatment the expression of the oncogene cyclin D1 was decreased and the expression of the tumor suppressor p27 was increased, suggesting that these cell cycle regulators have a role in the anti-proliferative mechanisms of statins.

In paper IV, the associations between cholesterol-lowering medication (CLM) use, HMGCR expression and breast cancer-specific mortality (BCM) in the large, prospective, population-based Malmö Diet and Cancer study was investigated. High expression of HMGCR was associated with unfavorable tumor characteristics. Use of CLM was associated with moderate reduced BCM, but with weak evidence. A trend was seen for lowering BCM in CLM users with tumors that expressed HMGCR weakly or not at all.

In conclusion, these studies demonstrate some mechanisms of statins anti-cancer effects on breast cancer. To further study statins role in breast cancer patients, a large clinical trial is needed.

Key words breast cancer, statins, HMGCR, cyclin D1, p27, clinical trial, gene expression profiling, cholesterol-lowering-medication

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Statins, HMGCR and Breast Cancer

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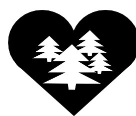

I think I can. I think I can. I think I can

(The little Engine That Could)

To "my boys", Freyr, Stefán Bjarni og Benedikt Dagur

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List of original papers

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. **Bjarnadottir O**, Romero Q, Bendahl PO, Jirström K, Rydén L, Loman N, Uhlén M, Johannesson H, Rose C, Grabau D, Borgquist S. Targeting HMG-CoA reductase with statins in a window-of-opportunity breast cancer trial.
Breast Cancer Research and Treatment 2013; 138(2):499-508

II. **Bjarnadottir O**, Kimbung S, Johansson I, Veerla S, Jönsson M, Bendahl PO, Grabau D, Hedenfalk I, Borgquist S. Global transcriptional changes following statin treatment in breast cancer. *Clinical Cancer Research 2015; 21 (15): 3402-11*

III. Feldt M, **Bjarnadottir O**, Kimbung S, Jirström K, Bendahl PO, Veerla S, Grabau D, Hedenfalk I, Borgquist S. Statin-induced anti-proliferative effects via cyclin D1 and p27 in a window-of-opportunity breast cancer trial.
Journal of Translational Medicine 2015; 13:133

IV. **Bjarnadottir O**, Feldt M, Inasu M, Elebro K, Bendahl PO, Kimbung S, Borgquist S. Cholesterol-lowering medication use, HMGCR expression, and breast cancer survival – The Malmö Diet and Cancer Study.
Manuscript

Abbreviations

AI	Aromatase inhibitor
ALNI	Axillary lymph node involvement
BCM	Breast cancer-specific mortality
BMI	Body mass index
BRCA	Breast cancer gene
CDK	Cyclin dependent kinase
CI	Confidence interval
CLM	Cholesterol-lowering medication
ER	Estrogen receptor
GnRH	Gonadotropin-releasing hormone
HER2	Human epidermal growth factor receptor 2
HMGCR	3-Hydroxy-3-metylglutaryl coenzyme-A reductase
HR	Hazard ratio
IHC	Immunohistochemistry
LDL	Low-density lipoprotein
MAST	The MAmmary cancer and STatin trial
MDCS	Malmö Diet and Cancer Study
MDT	Multidisciplinary team
MVP	Mevalonate pathway
NHG	Nottingham histological grade
OFS	Ovarian function suppression

PgR	Progesterone receptor
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RCT	Randomized clinical trial
RFS	Recurrence free survival
SCAP	SREBP cleavage activating protein
SERM	Selective estrogen receptor modulator
SREBP	Sterol-regulatory element-binding protein
TF	Transcription factor
TFBS	Transcription factor binding site
TNBC	Triple negative breast cancer
TMA	Tissue microarray
WHO	World Health Organization
WOO	Window-of-opportunity
27-HC	27-Hydroxycholesterol

Abstract

Breast cancer is the most common cancer in women and the second most common cause of cancer-related deaths. Breast cancer treatments are improving but still, breast cancer recurrences, disease-associated morbidity and mortality are challenges that need to be addressed continuously, i.e., through new treatments options.

Statins, or HMGCR inhibitors, are a group of per oral drugs that lowers the cholesterol levels in the blood by inhibiting HMGCR, the rate-limiting enzyme of the cholesterol biosynthesis pathway. Statins are most often well tolerated, have few side effects and are inexpensive. In addition to lowering the cholesterol level in the blood, statins have pleiotropic, or cholesterol-independent, mechanisms that have shown anti-tumoral effects *in vitro, in vivo*, and in phase II clinical trials. In addition, results from observational studies have demonstrated that statin use decreases the risk of breast cancer recurrence and breast cancer-specific mortality.

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In conclusion, these studies demonstrate some mechanisms of statins anti-cancer effects on breast cancer. To further study statins role in breast cancer patients, a large clinical trial is needed.

Introduction

Breast cancer

Epidemiology

Breast cancer is the most common cancer among women in Sweden, and 9,730 Swedish women were diagnosed with breast cancer in 2014 [1]. Worldwide, breast cancer is the second most common cancer in the world, and it was estimated that there were 1.7 million new breast cancer cases in 2012 [2, 3]. For Swedish women, the cumulative risk of being diagnosed with breast cancer before the 75th birthday is 11% [1]. In Sweden, the breast cancer incidence is still increasing, while breast cancer mortality has decreased. However, in 2013, almost 1,500 women died in Sweden due to breast cancer [4]. At the same time, more than 100,000 Swedish women are still alive after their breast cancer diagnosis [5]. Since the 1960s, the 5-year survival for breast cancer has increased from 60% to 90% in Sweden [5]. Worldwide, breast cancer ranks as the fifth cause of cancer death, and among women, breast cancer is the second cause of cancer death, after lung cancer [2].

Risk factors

The biggest risk factors for developing breast cancer are being a woman and getting older. Most breast cancer cases are considered to be sporadic, as they are not caused by any known genetic aberration, such as *BRCA1* and *BRCA2*. Only 5-10% of breast cancers are considered to be due to inheritance/genes, but approximately 20-25% of breast cancer patients have a positive family history [6]. Several risk factors that increase the risk of being diagnosed with breast cancer have been identified. Among the hormone exposure and reproductive factors are early menarche, nulliparity, high age at the birth of the first child, later onset of menopause and the use of hormone replacement therapy [6, 7]. In addition, alcohol consumption and radiation exposure increase the breast cancer risk, as does obesity, predominantly in post-menopausal women [6-8]. Increased breast density was also recently identified as a risk factor for developing breast cancer [9]. There

is some evidence that regular exercise can be protective against developing breast cancer, while metabolic syndrome, type II diabetes and hypercholesteremia have demonstrated to increase risk [10].

Hallmarks of cancer

The development of cancer is complicated, and the process involves multiple biological steps. A cancer cell has many characteristics and biological capabilities that a normal healthy cell does not have. Hanahan and Weinberg's ground-breaking work described in "Hallmarks of Cancer" was first published in 2000 and revised in 2011 with the next generation of cancer hallmarks. Those authors described the properties of a cancer cell that are required to build a tumor [11, 12]. In the revised version from 2011, the six hallmark capabilities of the cancer cell are described. The six established hallmarks are the cancers cell's ability to sustain proliferative signaling, evade growth suppressors, resist cell death or apoptosis, allow replicative immortality, stimulate angiogenesis and activate invasion and metastasis [11]. In addition to these six hallmarks of cancer, Hanahan and Weinberg propose that cancer cells should have two enabling characteristics: genome instability and mutation- and tumor-promoting inflammation. Finally, the cancer cell's potential to avoid immune destruction and reprogram energy metabolism are described as two emerging hallmarks [11]. The hallmarks of cancer and their therapeutic targeting possibilities are illustrated in Figure 1.

The cell cycle, cyclin D1 and p27

In a normal cell, several cyclin-dependent kinases (CDKs) and cyclins control and regulate the action of the cell cycle by forming cyclin-CDK complexes. CDKs are serine/threonine protein kinases, and like their name implies, CDKs are dependent on cyclins to perform their function [13]. Through different phases of the cell cycle, these complexes are activated and inactivated via phosphorylation. Between nuclear division (mitosis, M-phase) and DNA synthesis (S-phase) is the first gap phase, the G1 phase [14]. In G1 phase, the cell receives and interprets many signals that influence its fate, such as cell division, further growth or death [14]. Between S-phase and M-phase is another gap, the G2 phase, which allows for DNA repair and damage control [15]. Quiescent cells that are not active are in G0 phase [15]. In cancer cells, normal, healthy cell regulation is lost. This loss can occur at several levels and leads to increased and atypical cell proliferation. When cyclin D1 binds to the CDK4/CDK6 complex, phosphorylation occurs and inactivates the tumor suppressor protein Rb [14, 16].

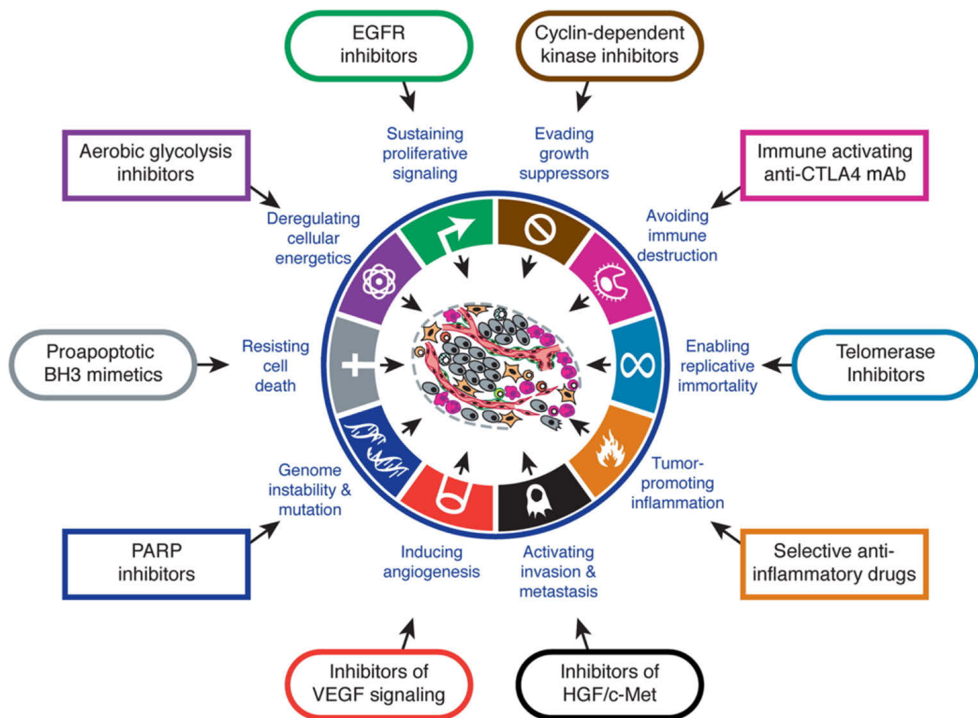

Figure 1. Hallmarks of Cancer.
Reprinted from The Cell, volume 144, issue 5, Hanahan D, Weinberg RA, Hallmarks of Cancer: The Next Generation, 646-674. Copyright (2011), with permisson from Elsevier (Hanahan & Weinberg, 2011).

Until a stage late in the G1 phase, which is known as the restriction point, cell cycle progression is dependent on stimulation by growth factors. After the restriction point, cells are refractory to these signals until they return to G1 [15]. Cyclin D is active during the S phase of the cell cycle, through the restriction point, but after that point, the cell is on its own to continue with proliferation. The oncogene cyclin D1 is encoded by the gene *CCND1,* and at the protein level, cyclin D1 is overexpressed in up to 50% of human breast cancer tumors, with or without accompanying gene amplification [14, 17, 18].

The cyclin-CDK complexes are regulated and inhibited by CDK inhibitors. p27, which is also named kip1, is one of these CDK inhibitors and is encoded by the *CDKN1B* gene [13]. p27 regulates G0-S phase in the cell cycle, in part by inhibiting the cyclin E-CDK2 and cyclin D/CDK4/6 complexes [19, 20]. But the role of p27 is considered rather complex and some studies have suggested that p27 role is not always as an inhibitor [21]. p27 is often deregulated in cancer, via either

reduced protein levels or the mislocalization of the protein. This deregulation is associated with poor prognosis [19].

Prognostic and treatment predictive factors

Age

Breast cancer incidence increases with age. Until menopause, breast cancer cases double every 10 years, but after menopause, the rate slows down [7]. In Sweden, the incidence of breast cancer is currently highest in the age group 60-69 years old, although this figure was previously highest among the oldest women [5].

Even though breast cancer is not common among women younger than 40 years of age, young women with breast cancer have a higher degree of morbidity than older women. Young women with breast cancer also have an increased risk of disease recurrence and higher mortality rates [22]. Their disease is often diagnosed when it is more advanced, and a higher proportion of young women are diagnosed with breast cancer that exhibits unfavorable characteristics, such as triple-negative receptor status or HER2 amplification [22].

TNM classification

One of the most important prognostic tools in breast cancer is the TNM classification. The T stands for tumor size, where T1 is assigned to tumors ≤2 cm in diameter, T3 is assigned to tumors >5 cm, and T4 is assigned to tumors of any size that are growing into the chest wall and/or the skin (ulceration or skin nodules) [23]. N indicates the involvement of axillary lymph nodes at different levels (N0-N3), and M stands for the presence of distant metastases (M0 and M1) [23]. Both tumor size and lymph node status are well established prognostic factors [24]. When a distant metastasis is diagnosed, the disease has become disseminated and is no longer considered curable.

Histological classification

Histological classification of invasive breast carcinoma is performed according to the WHO classification [25]. Invasive ductal carcinoma is the most frequent type (40-75%), followed by invasive lobular carcinoma, which accounts for 5-15% of cases [25]. The other types are more infrequent, including medullary carcinoma (1-7%), pure tubular carcinoma (approximately 2%), neuroendocrine tumors (2-5%), mucinous producing tumors (around 2%) and cribriform (0.8-3.5) [25].

Nottingham histological grade

In 1991, Elston and Ellis introduced the Nottingham histological grading system for breast cancer, which is a modified version of Bloom and Richardson´s earlier

method [26]. The histological tumor grade in breast cancer is assessed by the formation of a tubule, the pleomorphism of the nuclei and the number of mitotic events. When >75% of the tumor forms a tubule, one point is given, when between 10 and 75% of the tumor forms tubule, two points are given, and when <10% of the tumor forms a tubule, three points are given. Small and regular nuclei score one point, nuclei that exhibit a moderate difference in size and shape score two points, and very large and bizarre nuclei score three points. Mitotic counts up to 9 per 10 fields score one point, 10-19 mitotic events scores two points and more than 20 mitotic events scores three points. In that way, grade I tumors are well-differentiated tumors with 3-5 points, grade II tumors are moderately differentiated tumors with 6-7 points and grade III tumors are poorly differentiated tumors with 8-9 points. With their work, Elston and Ellis demonstrated that patients with poorly differentiated tumors had worse recurrence-free intervals and overall survival than patients who were diagnosed with well-differentiated tumors [26].

Estrogen receptor and progesterone receptor

The estrogen receptor (ER) is a nuclear receptor that exists in two main forms, the more studied one ERα and ERβ, which are encoded by the genes *ESR1* and *ESR2,* respectively [27]. The classic, or genomic activity, of ER occurs when estrogen has diffused into the cell and binds the ER, which dimerizes with another estrogen receptor. The dimer then attracts coactivator and corepressor complexes to bind the estrogen response element regulatory sequence in the promoter regions of target genes [27, 28].

ER is a positive prognostic factor. In Sweden in 2015, 85% of all breast cancer patients were ER-positive, and the most common sub-type defined by hormone receptors was the ER-positive/PgR-positive/HER2 normal, which accounted for 77% of cases [29]. ER is also a predictive marker for the response to endocrine treatment. For a breast cancer patient to benefit from ER-targeted treatment, ER expression should be positive. In Sweden, ER is considered to be positive when more than 10% of the tumor cells exhibit staining [30]. ER-positive breast cancer is more common among post-menopausal patients.

Progesterone receptor, PgR, is also a nuclear receptor. The role of PgR is not as clear as ER, but they both are positive prognostic factors and studies suggest combined together, ER and PgR are often better prognostic factors [31, 32]. In a meta-analysis by Early Breast Cancer Trialists´ Collaborative Group (EBCTCG), the relative risk reduction of breast cancer recurrences and breast cancer deaths following tamoxifen treatment among ER-positive patients were independent of PgR status [33]. PgR is one of the factors used to distinct between luminal A and luminal B type breast cancer, where luminal A has PgR expression higher than 20% and better prognosis [34]. In Sweden PgR is positive on immunohistochemistry when more than 10% of the cells are stained [30].

Human epidermal growth factor receptor 2 (HER2)

Human epidermal growth factor receptor 2 (HER2) is a tyrosine kinase and a member of the epidermal growth factor receptor family. In primary breast cancer, HER2 is overexpressed or amplified in 15-20% of cases [35-38]. The gene that encodes the protein (*HER2/neu/c-erbB2*) is an oncogene. Before the arrival of HER2-targeted therapy, the presence of this gene was a negative prognostic factor; that is, patients with HER2-positive tumors generally have a shorter time to relapse and worse overall survival [38]. HER2-positive tumors are normally more aggressive, with a higher tumor grade, increased proliferation and the patients are more likely to present early systemic disease as compared to patients with HER2 normal breast cancer [35, 39].

Even though HER2-positive status is a negative prognostic factor, it is a positive predictive factor for the response to HER2-targeted treatment. Therefore, HER2 testing is recommended at all stages of primary breast cancer, in cases of recurrence and when the disease becomes metastatic. It is recommended that HER2 status be evaluated via either protein expression using immunohistochemistry (IHC), with a scale from 0 to 3+ to estimate circumferential membranous staining, or gene amplification using gene expression (ISH or similar), with a positive or negative score. HER2 expression is considered to be positive when IHC is 3+ or ISH is positive. With an IHC score of 0 or 1 and ISH-negative status, HER2 expression is negative. When the IHC score is equivocal at 2+, normally ISH is performed, or a new biopsy is taken [35]. In Sweden, ISH is performed for tumor cases with IHC scores 2+ and 3+ [30].

Ki67

One of the hallmarks of cancer is uncontrolled proliferation, and in recent years, the estimation of Ki67 expression has become a commonly used method to evaluate proliferation in breast cancer [40]. In the early 1980s, the Ki67 antigen was identified by Gerdes *et al.* in Kiel, Germany. The name arises from the location of the research group, Kiel University, and 67 was the clone number on the 96-well plate [41, 42]. Cells express Ki67 in the G1, S, G2 and M phases of the cell cycle, but not in the resting G0 phase. The expression level varies throughout the cell cycle, with a peak level during mitosis and low expression in G1 and S phase [42].

Immunohistochemistry is normally used to evaluate cell proliferation with Ki67, and the assessment is reported as the Ki67 index or the percentage of stained cells [43]. The antibody MIB-1 (Molecular Immunology Borstel) is the most commonly used antibody, and a Ki67-positive cell has nuclear staining, regardless of the intensity [40, 43].

In the adjuvant setting, Ki67 has been studied as a prognostic marker, but the results have not been undisputed. In the neo-adjuvant setting, Ki67 is used primarily as an intermediate or end-of-study endpoint [40]. In pre-surgical studies, the change in Ki67 is often used as a dynamic marker to evaluate the effect of treatment on proliferation in cancer cells [40]. When evaluating treatment efficacy in comparative studies, the reduction in the Ki67 index as a percentage is often considered to be the most appropriate end-point [40].

The great variation and lack of standardization of Ki67 validation has made the use of Ki67 in breast cancer management challenging [40]. In contrast to modern Swedish pathological breast cancer assessment, the Ki67 expression is not part of the standard international breast cancer evaluation, (i.e., the USA). One of the issues is the cut-off value for low vs. high Ki67 expression. There is no consensus about the optimal cut-off for high vs. low Ki67 expression, but in many studies, the cut-off has been approximately 10-20% [40]. There is still a need to standardize the Ki67 score, and a quality assurance program must to be continued by laboratories [44].

Molecular subclasses

Since gene expression analysis entered the breast cancer scene, breast cancer is divided into different subgroups. Depending on their mRNA expression levels, the four groups show differences in prognosis and treatment response. The work of Sørlie, Perou *et al.* in this field is the basis for this classification [45, 46]. Luminal A- and luminal B-type tumors are ER-positive and predict the response to endocrine treatment. Generally, luminal B tumors have higher proliferation and worse outcomes than luminal A tumors and have more use of chemotherapy [47]. The third subgroup, HER2/ERBB2-enriched, highly express genes on the ERBB2 amplicon. Finally, the fourth subgroup is called basal-like, which has negative expression of ER and PgR and normal HER2 status [45].

In Sweden, the molecular/ “intrinsic” subtypes are not assigned based on gene profiling but are adapted by using surrogate IHC markers, based on the recommendations from the St. Gallen International Expert Consensus Conference [48, 49]. Luminal A breast cancer tumors are ER-positive and have low proliferation, that including all grade I tumors and grade II tumors with low Ki67 expression or grade II tumors with intermediate Ki67 expression and PgR expression > 20%. Luminal B tumors are also ER-positive, but exhibit high proliferation, including all grade III tumors and grade II tumors with high Ki67 expression or grade II tumors with intermediate Ki67 expression and PgR expression less than 20%. HER2-enriched tumors exhibit HER2 amplification or scores of 3+ on IHC/positive ISH analyses. Basal-like, or triple-negative, breast cancer has ER and PgR expression of less than 10% and has a normal HER2 status [50, 51].

In 2010, the South Sweden Cancerome Analysis Network – Breast (SCAN-B) consortium was initiated as a multicenter prospective study with the aim to analyze breast cancers with next-generation genomic technologies. The long-term goal of the project is to develop new diagnostic, prognostic as well as treatment-predictive clinical test to improve breast cancer diagnosis and treatment [52, 53].

Clinical breast cancer

Diagnosis of breast cancer

A new lump in the breast should always be approached with the following triple assessment: physical examination, radiographic imaging and tissue sample of the lump. Physical examination includes palpation of the breasts and the lymph nodes. Mammography is often the first choice of radiographic imaging, but ultrasound is also commonly used, and in cases such as young women and high breast cancer risk patients, magnetic resonance imaging is performed. Fine needle aspiration is often the first step in tissue sampling from a breast tumor, but this approach is often supplemented by core needle biopsy for further analyses. In Sweden, almost half of breast cancer cases are diagnosed with mammography screening [29].

Multidisciplinary cancer conference

Multidisciplinary team (MDT) meetings for patients with primary breast cancer started more than 25 years ago in some parts of Sweden and are now part of standard care in many countries [51, 54, 55]. The purpose of the MDT is to discuss newly diagnosed breast cancer cases and to decide the best possible treatment for the patient in a multidisciplinary manner. Discussing cases in a well-organized MDT meeting improves coordination for the patient and improves communication and decision-making between health-care workers [54-56]. In a meta-analysis performed by Wright *et al.*, MDT meetings had a positive effect on patient outcomes including survival, patient satisfaction and diagnosis and/or treatment planning [55].

In southern Sweden, the MDT meetings are normally held at least once per week, and the participants are a medical breast oncologist, a breast cancer surgeon, a breast radiologist, a pathologist with a specialty in breast cancer, and if possible, breast cancer nurses. In Sweden, every new breast cancer case should be discussed at an MDT conference as a part of the decision-making process regarding diagnosis and treatment. In southern Sweden, 99% of primary breast cancer cases

are discussed at MDT meetings [29]. Yet, MDTs are still not part of standard care for patients with metastatic breast cancer.

Treatment of breast cancer

Surgery is still considered to be the only curative treatment for invasive breast cancer. Depending on tumor size and other prognostic and predictive factors, the members of the MDT conferences decide if, when and which treatment to offer the respective patient. Treatment that is given after breast cancer surgery is called adjuvant treatment. Adjuvant treatment is administered to women who are considered to be free of their breast cancer to decrease their risk of recurrence, by treating plausible free cancer cells/micrometastasis. Neo-adjuvant treatment is given before planned cancer surgery. In addition to the aim of decreasing the risk of recurrence, neo-adjuvant treatment is also given to down-stage or de-escalate the tumor before surgery and to monitor the treatment response and estimate the relevant patient's follow-up and prognosis. Adjuvant and neo-adjuvant treatments for breast cancer will be described below, but treatment in an advanced or metastatic setting will not be addressed.

Surgery

If it is possible to remove the tumor radically with good cosmetic results, breast-conserving surgery (also called partial mastectomy and lumpectomy) is the surgical method of choice. When that outcome is not possible, all breast tissue is removed with radical mastectomy. Mastectomy is predominantly performed when tumors are very large or multifocal, when radiation therapy is not possible or when the patient strongly prefers mastectomy [50, 51]. In recent years, the oncoplastic surgery technique has become more popular. This technique combines resection of the tumor with different types of plastic surgery techniques to achieve better cosmetic results [57].

The sentinel node technique is used to identify lymph node metastasis in the axilla. Before this technique, axillary dissection was the standard procedure for detecting and remove positive axillary lymph nodes. Today, axillary dissection is performed when the sentinel node is positive and the metastasis is larger than 2 mm. Patients in southern Sweden who have a micrometastasis (tumor in the lymph node bigger than 0.2 mm but smaller than 2 mm) and are undergoing partial mastectomy with adjuvant radiation therapy and adjuvant chemotherapy are not recommended for axillary dissection [50, 51].

Radiation therapy

Radiation therapy following surgery is given to decrease the risk of local recurrence. After breast-conserving surgery, the remaining breast tissue is treated with radiation therapy. When at least one lymph node is positive, both the remaining breast tissue and lymph node stations are irradiated. After mastectomy, only patients with positive axillary lymph nodes or tumors bigger than 5 cm receive radiation towards the thorax and lymph node stations. For T3 or multifocal tumors, the thorax is treated with radiation [50, 51].

Chemotherapy

The characteristics of the breast cancer tumor are used to decide if a patient needs adjuvant or neo-adjuvant chemotherapy. All cases should be discussed at an MDT conference. The standard chemotherapy given in Sweden is a polychemotherapy regimen, based on the results obtained from many years of clinical trials [58, 59]. In southern Sweden, the standard chemotherapy in the adjuvant setting is three cycles of the anthracycline ebirubicin together with cyclophosphamide, given intravenously every three weeks, followed by taxane, either three cycles of docetaxel every three weeks or paclitaxel weekly for 9 to 12 weeks [50, 58, 59]. In southern Sweden, women who have HER2-negative tumors larger than 10 mm in diameter and who are either younger than 35 years of age or have a luminal B-type tumor or a luminal A-type tumor with at least four positive lymph nodes are recommended for chemotherapy. Chemotherapy is also recommended for women with triple-negative breast cancer with a tumor diameter larger than 5 mm or a positive lymph node status. For HER2-positive patients, chemotherapy in combination with HER2-targeted therapy (see below) is recommended for invasive tumors larger than 5 mm and/or for a positive lymph node status. All young women (especially women younger than 40 years old) who are still pre-menopausal when receiving chemotherapy should be informed about the benefits of ovarian function suppression (OFS), i.e., with a gonadotropin-releasing hormone (GnRH) agonist, to increase the chance of future fertility, reduce the risk of early menopause and improve survival [22, 51].

Neo-adjuvant chemotherapy is recommended for patients who have inoperable or inflammatory breast cancer at diagnosis, as long as the cancer is not metastatic (cT4cN0-3M0). Neo-adjuvant chemotherapy should be considered for patients with lymph node metastasis when diagnosed, cT3 tumors and cT2cN0 tumors that are either TNBC- or HER2-positive.

Endocrine treatment

For endocrine treatment to be useful, the patient's ER status must be positive. In Sweden, ER positivity is assigned when more than 10% of the cancer cells are stained via immunohistochemistry. In southern Sweden, endocrine treatment is

recommended for all luminal B breast cancer patients and for patients with luminal A tumors that are larger than 10 mm or a positive lymph node [50, 51].

Tamoxifen is a selective estrogen receptor modulator (SERM), which binds the ER and antagonizes the effects of estrogen on specific genes [28]. Tamoxifen has ER agonist effects on other genes and tissues [28]. On a relative scale, tamoxifen reduces recurrences and contralateral disease in more than one-third of cases and reduces mortality by 30% among ER-positive women [33]. Tamoxifen can be used by pre-menopausal and post-menopausal women, as well as by men. In southern Sweden, ovarian function suppression (OFS) with either a GnRH agonist or oophorectomy is recommended for all women younger than 35 years with lymph node-positive disease or luminal B tumors with a negative lymph node status. OFS can also be suggested for women 35 years and older, who are still pre-menopausal and have unfavorable tumor characteristics [50].

Another type of endocrine treatment used in the adjuvant and neo-adjuvant setting is aromatase inhibitors (AI) (i.e., letrozole, anastrazol and exemestan). AIs work by blocking the conversion of weak androgens produced by the adrenal gland to estrogen in breast cancer tissue as well and other peripheral tissues [28]. AIs are therefore only useful for post-menopausal women. Large randomized clinical trials comparing AIs and tamoxifen treatment showed reduced recurrence and improved survival in favor of AIs; therefore, AIs should be the first choice of endocrine treatment for post-menopausal women when possible [49, 60, 61].

The side effects differ between tamoxifen and AIs. With tamoxifen thromboembolic disease and endometrial cancer are among important side effects, while osteoporosis and joint disorders, such as joint pain, arthritis and arthrosis, are described with AI treatment [51]. Among similar side effects with both tamoxifen and AIs are hot flushes, nausea, and depression. The recommended time for endocrine treatment is typically five years. With lymph node positivity or T3-T4 disease, prolonged therapy for an additional five years is recommended [22, 49, 50, 62].

Unfortunately, many ER-positive patients develop resistance against endocrine treatment, leading to treatment ineffectiveness and the recurrence or progression of cancer disease [63, 64].

HER2-targeted treatment

Women who are HER2-positive and have stage pT1bpN0 and higher cancer or positive lymph nodes are recommended HER2-targeted therapy (i.e., trastuzumab) in combination with chemotherapy [49, 50]. Trastuzumab (Herceptin®) is a humanized monoclonal antibody that binds the extracellular domain of HER2 and inhibits HER2 dimerization [36, 65]. In the (neo)-adjuvant setting, trastuzumab is given either intravenously or subcutaneously, every three weeks for one year.

Large randomized clinical trials of trastuzumab use among HER2-positive patients have demonstrated prolonged disease-free survival and overall survival, even with a long follow-up [36, 37, 39]. The treatment is recommended in adjuvant, neo-adjuvant and metastatic settings [36, 49].

The most serious side effects of trastuzumab treatment is increased risk of congestive heart failure and decline in left ventricular ejection fraction [36, 66]. That is why all patients that are candidates for trastuzumab treatment are recommended echocardiogram or MUGA (Multigated acquisition) before start, during and after treatment.

Patients with a higher risk of relapse due to either lymph node involvement or hormone-receptor negativity can be administered dual blockade with trastuzumab and pertuzumab [65]. Pertuzumab is another humanized monoclonal antibody that binds to the extracellular HER2 dimers and inhibits HER2 heterodimerization with other HER2 family receptors [65]. In southern Sweden, dual blockade is offered in neo-adjuvant setting [50].

Bisphosphonate adjuvant treatment

Bisphosphonate is a drug that normally is used to prevent the loss of bone mass, i.e., osteoporosis. Recently, bisphosphonate was added to adjuvant treatment in breast cancer, primarily for postmenopausal women [49, 50]. A recent meta-analysis showed that post-menopausal women, naturally or induced menopause, who were treated with bisphosphonate, exhibited reduced breast cancer recurrence, including both distant and bone recurrence, and experienced decreased breast cancer mortality [67]. A study of premenopausal women, where menopause was induced by goserelin, treated participants with either tamoxifen or an aromatase inhibitor with or without zoledronic acid. The results showed that the group treated with zoledronic acid had improved disease-free survival [68]. In South Sweden, adjuvant bisphosphonate treatment is recommended lymph node positive patients, both post-menopausal women and pre-menopausal women receiving GnRH agonist [50]. The anti-RANK ligand antibody denosumab has also been studied, but this antibody is still not included in the standard clinical treatment [49, 69].

Clinical study designs

In clinical studies, several study designs are used. The choice of study design helps to evaluate the study's level of evidence and its strengths, limitations and biases [70]. Roughly, clinical studies can be divided into two categories: clinical trials and observational studies.

Clinical trials

The best experimental procedure for assessing the effectiveness of an intervention, often medication, is with a well-conducted clinical trial [71]. The aim of a clinical trial is to improve the existing standard methods in medical practice and to find better treatment options than the treatments that already are available. Human clinical trials are most often divided into four temporal phases: phases I-IV [71]. Clinical trials are obligated to follow a comprehensive clinical trial protocol that includes, among other parameters, a detailed study design, background, aims, intervention, inclusion and exclusion criteria for patients and an assessment of adverse effects [71]. The trials must also be approved by a local ethical committee and a medical products agency. In addition, clinical trials should be registered in the European Clinical Trials database (EudraCT, https://eudract.ema.europa.eu) and at ClinicalTrials.gov. Since the Declaration of Helsinki, every participant or guardian must sign an informed written consent form [72].

In phase I trials, humans are given the test drug for the first time. Before entering phase I, the test drug has usually been tested in cell lines *(in vitro*) and animal models (*in vivo*). The phase I participants are often healthy individuals but can also be patients who have not responded to standard therapy and are lacking treatment options, like cancer patients. The aim of a phase I trial is to estimate tolerability and investigate the pharmacokinetics and pharmacodynamics of the drug. These trials often have small sample sizes, and the participants are often given the drug in escalating doses until the optimal tolerated dose is found that can be used in phase II [71, 73].

When drugs enter phase II, the purpose is to evaluate the possible biological activity and effects. Phase II trials are most often used to decide whether a drug should be further developed to reach phase III. A variety of factors affect the decision, including the estimated beneficial and adverse effects, feasibility, and event rates in the target population [71]. The results of phase II trials are often used to design and start phase III trial.

Phase III studies are designed to assess the value of an intervention in clinical practice, to assess the effectiveness of new interventions or existing interventions with new indications and to examine adverse effects [71]. Phase III trials are larger than phase I and II trials and can include from 100 to several thousand patients. Drugs are often approved after phase III trials. In Europe, drugs are approved by the European Medicines Agency (EMA), and in the United States of America, drugs are approved by the Food and Drug Administration (FDA). Phase IV involves long-term surveillance and safety studies conducted after approval from the regulatory agency [71].

The ideal clinical trial design is a double-blind randomized clinical trial (RCT), which is the preferred method for medical intervention and drug development [71]. Double-blind refers to the method, in which both the investigator and the participant are kept unaware of which treatment the participant is receiving in order to decrease bias [74]. Randomization, which is most often conducted using a computer, web-based tool or other randomized manner, is also used to select which treatment the participant will receive and to minimize bias. Placebo treatment, or treatment that is not intended to have any biologic effect outside the offer of treatment itself, is also used to facilitate blinding for the comparison group [74]. In oncology, the new test drug is most often compared to the best standard oncology therapy because the use of placebo is not considered to be ethical [74]. However, RCTs also have limitations. Such studies are expensive and take a long time to plan, implement and analyze. Sometimes new drugs have already been introduced into the market before the results from RCTs have become public. Short study periods or small study populations can lead to missed severe adverse effects. RCT are impractical for rare diseases and urgent situations and may not account for effects beyond the study population [75, 76].

To overcome some of the limitations of traditional phase II-IV trials and RCTs, some new study designs have been introduced in cancer research to study more than one or two treatments in more than one patient type. In basket trials, the aim is to study a single targeted therapy, for which patients are screened, in the context of multiple diseases or disease subtypes. An umbrella trial investigates many targeted therapies, which are often defined by a particular biomarker, in the context of one disease [77]. The window-of-opportunity trial design is another design model.

Window-of-opportunity trials

In window-of-opportunity (WOO) trials, the time from cancer diagnosis to planned standard surgery, which is normally a preparation or waiting time for the patient with no planned intervention, is used to investigate the effect of an intervention (e.g., medication). Figure 2 illustrates the design of WOO trials. At the time of diagnosis, a tumor sample, often core needle biopsy, is taken. After the planned intervention, a tumor tissue sample is obtained from the surgery sample. To estimate treatment effects, change in Ki67 expression is often used as a surrogate marker for proliferation changes in comparative WOO studies and is often considered to be the most appropriate end-point [40]. The tumor tissues obtained before and after the given therapy are then used for further molecular analyses [78]. Other types of assessment for the intervention's effect are possible instead of tumor tissue (i.e., blood samples or radiographic imaging).

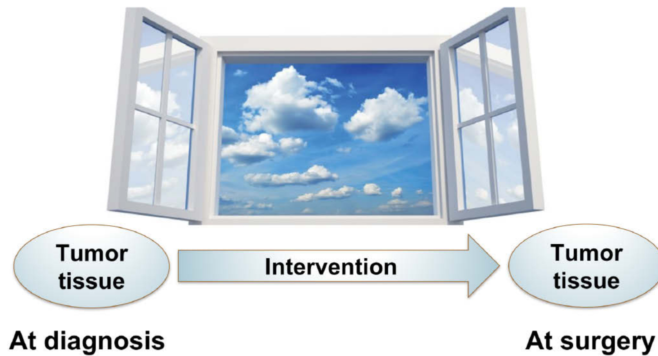

Figure 2. Window-of-opportunity trial design.

WOO trials are not the same as neo-adjuvant trials because the time of the intervention is determined beforehand and is often very short, even though both types of trials are both pre-surgical trials. The aim of the WOO trial is to further investigate the drug's effect on cancer and is rarely to treat potentially anti-tumor effects, in contrast to neo-adjuvant treatment, which has stronger treatment intention.

It is believed that the first WOO trial with endocrine treatment was performed in 1993 in Manchester, England when 103 breast cancer patients were treated with tamoxifen from diagnosis to planned surgery [79]. WOO studies are performed in humans, and the patients have often not been exposed to chemotherapy or relevant drugs before. Thus, the results give patient information in comparison to pre-clinical studies that use *in vitro* or animal models [78, 80]. WOO trials can help in the search for predictive biomarkers, improve understanding at the molecular and/or transcriptional levels, and in some cases, provide insights into clinical efficacy. Further, WOO trials can be useful to study novel therapies when no adequate pre-clinical models exist [78, 80]. As for any other clinical trial, WOO trials are obligated to follow Good Clinical Practices to ensure patient safety. In addition, WOO trials should never delay the time to a planned cancer operation. Among the challenges in WOO studies are the quality of tissue samples, particularly tumor biopsies. WOO trials also require multidisciplinary teamwork and a high level of logistics [80]. Tumor heterogeneity can be a challenge, and it can be difficult to validate the real impact on cancer outcomes when a standardized surrogate marker is lacking [80].

The hope is that WOO trials can help in the drug development process by improving our understanding of the biological efficacy of the test drug, validating potential predictive biomarkers that may predict subsets of patients who could

benefit from treatment and leading to subsequent clinical trials that are powered to find changes in clinical outcome [78, 81].

Observational studies

An observational study is a study that does not have any active intervention and in which no experiment is being performed [71]. There are several types of study designs; here the focus will be on prospective population-based cohort studies, case-control studies and cross-sectional studies.

A population-based prospective cohort study is a study in which the participants are relatively healthy when included and the participants are observed and followed over time, often a long time, until some participants develop outcomes (i.e., disease) [70]. Cohort studies have several advantages. Baseline factors (potential risk factors) are collected before the outcomes have occurred, which makes temporal relationships certain and avoids recall bias. This design can be used to assess multiple outcomes and rare exposures. Because such studies often sample people from the general community, the results are often generalizable to a wider population [70, 75]. It is often convenient with cohort studies to investigate many different disease outcomes in relation to a given exposure [74]. One of the disadvantages of prospective cohort studies is that the results can be affected by loss to follow-up and confounding factors, and prospective cohort studies are often time-consuming and expensive to conduct [70, 75].

In case-control studies, the participants are selected based on outcomes. The case subjects, people who have had the outcome/disease, are compared to people who are similar in many ways except they have not had the outcome/disease (called control subjects). Case-control studies are useful for investigating many different exposures in relation to a single disease [74]. Choosing the control subjects is probably the biggest challenge for case-control studies. Other shortcomings are recall bias and confounding factors. The advantages are that case-control studies are the most efficient design for rare outcomes and are relatively inexpensive, easy and quick [70].

In cross-sectional studies, risk factors and outcomes are measured at a single time. These studies provide valid estimates of risk factor prevalence and outcomes in a particular population and can often be generalized for larger populations. Since risk factors and outcomes are measured at the same time, the results cannot be used to ascertain whether a given risk factor actually preceded the outcome but to uncover risk factors associated with duration/survival. Nonresponse bias, recall bias and confounding factors are among the other disadvantages. Like case-control studies, cross-sectional studies are relatively inexpensive, easy and quick [70, 74].

Cholesterol and HMGCR

Cholesterol is an essential component of cell membranes, and for normal cell function, cholesterol homeostasis is very important. Cholesterol participates in many membrane mechanisms and transmembrane signaling processes between cells [82]. At the same time, excessive levels of circulating cholesterol can be unhealthy, leading to atherosclerotic plaques that in the worst cases, lead to heart attacks and cardiac death [83].

In a normal cell, cholesterol is obtained in two ways: through low-density lipoprotein (LDL) receptor-mediated uptake from the circulation or through the *de novo* biosynthesis pathway [83]. In the cholesterol biosynthesis pathway, which is also called the mevalonate pathway (MVP), 3-hydroxy-3-metylglutaryl coenzyme-A reductase (HMGCR) is the rate-limiting enzyme and transforms HMG-CoA to mevalonate [84]. HMGCR is a transmembrane glycoprotein that is located in the endoplasmic reticulum in all cells. In addition to the production of cholesterol, the mevalonate pathway produces several other products, such as steroid hormones, ubiquinone, bile acid and isoprenoids [84]. Figure 3 illustrates the main steps and products of the mevalonate pathway. Farnsylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) are isoprenoids that play important roles in attaching lipids during the posttranslational modification of a variety of proteins. Among the important proteins are Ras, Rho and Rab. These proteins are small guanosine-triphosphate (GTP)-binding proteins that are members of the GTP superfamily and are dependent on isoprenylation to function appropriately [85-87]. The isoprenylation of proteins enables the covalent binding, subcellular localization and intracellular trafficking of membrane-associated proteins that are essential for the cell [85].

Because cholesterol is hydrophobic, it is transported as an LDL particle in the body. LDL has a hydrophobic cholesterol-ester core coated by polar phospholipids and a large apolipoprotein B protein [83]. With receptor-mediated endocytosis via the LDL receptor, the LDL is delivered to lysosomes and hydrolyzed. The majority of cholesterol is reutilized, but the part that leaves the body does to through the liver, which converts cholesterol to bile acids, which are then excreted from the body [84]. The cellular cholesterol level and HMGCR activity are maintained in a strict manner due to a feedback loop, while extracellular serum cholesterol concentrations vary [83]. When cellular cholesterol levels are high, cellular LDL

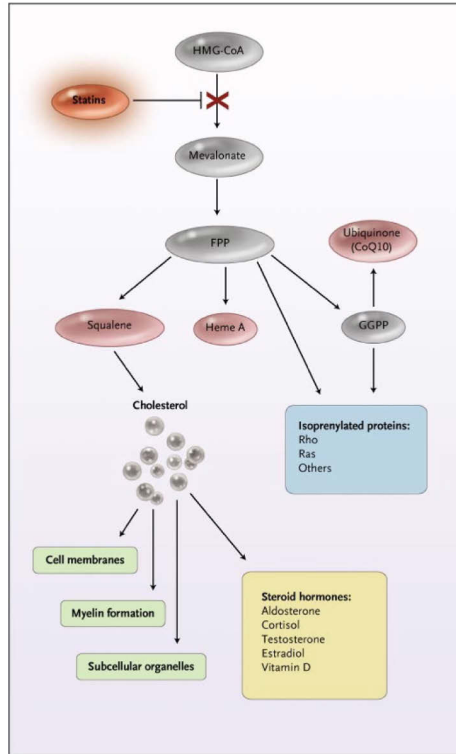

Figure 3. The Mevalonate Pathway.
Inhibition of the mevalonate pathway by a statin (red) showing some of statins pleiotropic effects. FPP: farnesyl pyrophosphate, GGPP: geranylgeranyl pyrophosphate, HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A. Arrows may reflect more than one enzymatic reaction. Reproduced with permisson from New England Journal of Medicine, Copyright Massachusetts Medical Society [88].

uptake and cholesterol biosynthesis are inhibited by a negative feedback loop. When the intracellular level of cholesterol is low, the amount of LDL, HMGCR and other enzymes important for the cholesterol biosynthesis pathway increases. The regulation of cholesterol levels is complex, but among the key players are Scap (SREBP cleavage activating protein), which is a protein of the endoplasmic reticulum membrane that senses the level of cholesterol in the membrane, and the transcription factor sterol-regulatory element-binding protein (SREBP), which regulates cholesterol biosynthesis and binds to the sterol regulatory element promoter-enhancer in the nucleus [83, 89-91]. In 1985, Goldstein and Brown were awarded the Nobel Prize in Physiology and Medicine for their work on the regulation of cholesterol metabolism [91, 92].

Cholesterol, HMGCR and Breast Cancer

Highly proliferative cells, such as cancer cells, must produce cell membranes rapidly, and increased cholesterol synthesis activity is part of the carcinogenic process [93]. The cholesterol biosynthesis pathway is tightly regulated in normal cells, whereas in cancer cells, the pathway can be dysregulated via different mechanisms [94].

Clendening *et al.* suggested HMGCR as a candidate metabolic oncogene and proposed that the dysregulation of the mevalonate pathway promotes transformation [95]. Clendening also associated high mRNA levels of HMGCR and other mevalonate pathway genes with a worse patient prognosis and reduced survival among breast cancer patients [95]. It has also been implied that the mevalonate pathway is a possible therapeutic target for tumors with mutations of the tumor suppressor p53 [96]. The mevalonate pathway is both necessary and sufficient for the phenotypic effects of mutant p53 in the breast tissue architecture. In part via the transcription factor SREBP, mutant p53 associates with sterol gene promoters [96]. *In vivo* studies have also suggested that HMGCR activity is higher in mammary tumors than in normal mammary glands, and the tumors are resistant to feedback regulation by sterols [97].

The rate-limiting enzyme HMGCR is differentially expressed in breast cancer [98], and its expression was previously associated with favorable prognostic clinicopathological parameters, such as a smaller tumor size, a low histological grade, a low Ki67 index, ER positivity, high p27 expression, HER2 negativity and less axillary lymph node involvement [98-100]. In one study, the expression of HMGCR was associated with significantly prolonged recurrence-free survival [99]. One study did not find any significant associations between HMGCR expression and short-term disease-free survival, distant metastasis-free survival or overall survival, using univariable or multivariable models [100]. Similar findings were seen among ER-positive patients only.

27-hydroxycholesterol (27-HC) is a metabolite of cholesterol that is produced by the alternative, or bile-acid, pathway when CYP27A1 hydroxylates cholesterol [101, 102]. The concentration of 27-HC is increased locally in ER-positive breast cancer patients, both in the normal breast tissue and even more so in the tumor [103]. This increase does not appear to be associated with higher serum concentrations [103]. 27-HC has been shown to promote tumor growth in ER-positive models, both *in vitro* and *in vivo* [101, 103, 104]. In breast cancer cell lines, CYP27A1 expression was similar to control levels, but CYP7B1, which metabolizes 27-HC, was decreased in cancer cells [103]. In the TCGA dataset, low *CYP7B1* expression was associated with poorer survival [103]. Nelson *et al.* also demonstrated that 27-HC increases LXR-dependent lung metastasis *in vivo* [104].

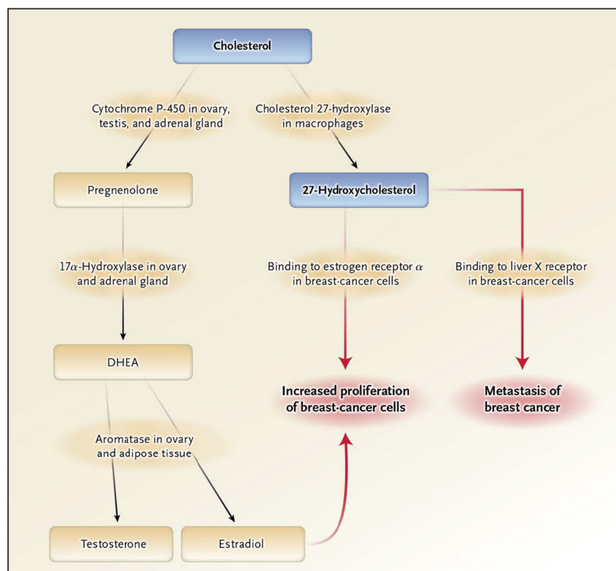

Figure 4. Pathways of Cholesterol Metabolism and 27-Hydroxycholesterol.
Cholesterol is metabolized by enzymes in the gonads and adrenals to produce the hormones testosterone and estradiol, showed on the left side of figure. The right side of the figure illustrates the suggested role of 27-HC in breast cancer. DHEA denotes dehydroepiandrosterone. Reproduced with permission from New England Journal of Medicine, Copyright Massachusetts Medical Society [105].

Figure 4 illustrates the production of 27-HC and its suggested role in breast cancer.

Simigdala *et al.* found that in breast cancer cell lines with estrogen deprivation, the cholesterol biosynthesis pathway was often upregulated. Silencing the cholesterol synthesis genes caused a 30-50% decrease in proliferation [106]. 25-HC, 27-HC and the cholesterol biosynthesis enzymes were presented as a novel mechanism of endocrine resistance in ER-positive breast cancer [106]. Nguyen *et al.* postulated that long-term low estrogen levels in ER-positive breast cancer cells leads to stable epigenetic activation of the mevalonate pathway and cholesterol biosynthesis. The increased levels of 27-HC were sufficient to activate ER signaling in the absence of exogenous estrogen, driving the activation of genes that promote an invasive cell phenotype [107].

To summarize, the HMGCR enzyme has been suggested as a possible oncogene, and members of the lipid metabolism pathway have been associated with the transformation of breast cells. 27-HC´s role as a tumor growth promoter and a novel mechanism of endocrine resistance in ER-positive cancer cells requires further investigation. The understanding of HMGCR expression, both with protein expression evaluated by immunohistochemistry and mRNA expression, has been

disputed, and further studies are needed to elucidate HMGCR´s role in breast cancer.

Statins

Statins, or HMG-CoA reductase inhibitors, are a group of oral drugs that lower cholesterol levels in the blood, mainly by lowering LDL [83, 108]. These drugs are commonly used in the primary and secondary prevention of cardiovascular diseases and to treat hypercholesteremia [83, 109]. Studies have shown that treatment with one of the most studied statins in the cardiovascular setting, simvastatin, reduces the risk of heart attacks and prolongs life [83, 108]. From 2011-12, nearly 30% of Americans over 40 years old were prescribed statins, and in adults 75 years and older, the prescription rate increased to nearly 50% [110]. In Sweden in 2016, almost 1 million Swedes were prescribed statins [111].

Statins are a group of relatively new pharmaceutical drugs that are either fungal-derived or produced from synthetic compounds [112]. Statins' affinity for the active site of HMGCR is approximately 1,000-fold stronger than HMGCR itself, leading to strong competitive inhibition of the HMGCR enzyme [113]. In Tokyo in 1976, Akira Endo discovered the first inhibitor of HMG-CoA reductase, which was named mevastatin (Compactin®) [114]. Lovastatin (Mevacor®) was the first statin approved for human use. Lovastatin entered the market in 1987 and was later followed by simvastatin, pravastatin, fluvastatin, atorvastatin and rosuvastatin [83, 109]. Statins, together with fibrates, resins, ezetemib and other medications that decrease cholesterol in the blood, are often referred to as cholesterol-lowering medications (CLM).

All statins have different clinical pharmacokinetic properties, such as half-life, bioavailability, maximum plasma concentration and lipophilicity [115, 116]. The bioavailability of the statins being used is generally quite low, from less than 5% to around 20% [112, 117]. Lipophilic statins are more likely than hydrophilic statins to use passive diffusion to enter endothelial cells [85]. Lipophilicity is determined from the logD and IC50 [118]. Lipophilicity is a continuous scale, and there is not a set threshold between hydrophilic and lipophilic statins. The most common lipophilic statins include simvastatin, lovastatin, atorvastatin and fluvastatin, whereas pravastatin and rosuvastatin are considered to be hydrophilic [112, 115, 118]. In the USA, simvastatin is the most frequently prescribed cholesterol-lowering medication and is used by more than 40% of patients, followed by atorvastatin (20%) and pravastatin (around 10%). Rosuvastatin and lovastatin are used by less than 10% of patients [110].

Statins are typically safe and well-tolerated drugs [109, 112]. Most of the side effects are dose-dependent and vary between different statin types [119]. Among the side effects are nausea, abdominal discomfort and elevated liver transaminase effects [119, 120]. All statins can cause myopathy and rhabdomyolysis, which are serious side effects; fortunately, these side effects are very uncommon, and the risk is mostly dose- and drug-dependent [112, 119]. In a systematic overview of randomized clinical trials, there was no significant absolute risk of myalgia (muscle pain), creatine kinase elevation, rhabdomyolysis or discontinuation due to any adverse event among the most common statins [120]. The only statin associated with a significantly higher incidence of rhabdomyolysis was cerivastatin, which was withdrawn from the market in 2001 due to reports of rhabdomyolysis [109, 120].

Statins lower the levels of circulating cholesterol by hindering endogenous cholesterol biosynthesis. They achieve that outcome by inhibiting HMGCR, the rate-limiting enzyme of the cholesterol biosynthesis pathway, which happens predominantly in the liver. The inhibition of HMGCR then leads to an increased number of LDL-receptors that take up LDL particles, which lowers LDL levels in the blood [83, 85].

In addition to cholesterol-lowering effects, statins also exert cholesterol-independent or "pleiotropic" effects [85]. Improved endothelial dysfunction, the stabilization of atherosclerotic plaque and reduced inflammatory and thrombogenic responses are among the pleiotropic effects [85, 121]. When statins inhibit HMGCR, they also inhibit the production of the important isoprenoid intermediates farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) that are used in isoprenylation, which is essential for many cell functions [85]. For example, Ras translocation from the cytoplasm in endothelial cells is dependent on farnesylation, whereas Rho translocation is dependent on geranylgeranylation [85]. When the normal breast epithelial cell line MCF10A was treated with a statin (simvastatin or lovastatin), reduced levels of prenylated H-Ras in the membrane fraction were seen in a dose-dependent manner, as well as increased unprenylated H-Ras in the cytosolic fraction, suggesting that by preventing the isoprenylation of H-Ras, statins inhibit membrane localization [122]. Statins also inhibit H-Ras induced invasion, and this effect can be reversed by FPP. Simvastatin and lovastatin inhibited the activation of the signaling molecules Raf, MEK, ERK-1/2, Rac1, PI3K and p38 MAPK in H-Ras MCF10A cells in a dose-dependent manner. This outcome indicates the role of simvastatin and lovastatin in preventing the activation of H-Ras downstream signaling molecules, possibly via the inhibition of the membrane localization of H-Ras [122].

Atorvastatin is a synthetic reversible inhibitor of the HMGCR and belongs to the second generation of statins [123]. When given orally, atorvastatin is given as the calcium salt of the active hydroxyl acid, but *in vivo,* it is in balance with its lactone form. The acid form consists of both a lipophilic part and a hydrophilic part [123]. The elimination half-life for atorvastatin acid is 14 hours, and the bioavailability after intake is 12-14%. Its elimination is accomplished via biliary secretion and direct secretion from the blood to the intestines [112, 117, 123]. Atorvastatin is well tolerated at doses of up to 80 mg/day, which do not increase the risk of myopathy [119].

Breast cancer and statins

The suggested associations between breast cancer and statins have been investigated for years in pre-clinical studies, observational studies, and clinical trials.

As cancer cells need cholesterol for growth and survival, lowering intracellular cholesterol biosynthesis appears to be a promising anti-cancer strategy [93]. Studies have suggested that cancer cell cholesterol utilization is an important feature of carcinogenesis [124, 125]. Cancer cells that are proliferating rapidly have an increased cholesterol demand for the cell membrane and up-regulate cholesterol synthesis as a part of the carcinogenic process [93, 124]. Lowering plasma levels of cholesterol with statins lowers the availability of cholesterol for cancer cells. However, statins probably influence both cholesterol-independent and cholesterol-dependent mechanisms when they affect breast cancer. Many of the suggested mechanisms have been elucidated with pre-clinical studies.

Pre-clinical studies

The effects of statins on breast cancer have been investigated in several pre-clinical studies. The mechanisms of action of statins in cancer cells are still not comprehensively understood. More information is being sampled continuously.

In vitro studies have shown that breast cancer cell sensitivity to statins differs. In a study with fluvastatin and 19 different types of breast cancer cell lines, increased sensitivity was found in cell lines with ER negativity and a basal-like tumor subtype [126]. In fluvastatin-sensitive cancer cell lines, acinar morphology and cell death were seen following treatment [126]. Often, the most statin-sensitive cell lines are the more aggressive ones, such as ER-negative, HER2-positive or triple-negative lines [127]. In work performed by Gopalan *et al.*, MDA-MB-231

(ER-/PgR-/HER2-) cancer cells were more sensitive to simvastatin than MCF-7 (ER+/PgR+/HER2-) breast cancer cells, whereas normal breast epithelial cells seemed more resistant to simvastatin treatment [128].

Several studies have shown anti-proliferative and apoptotic effects on breast cancer cells after treatment with statins, and different mechanisms of action have been suggested. Lovastatin and cerivastatin have been shown to inhibit cell growth with G1 arrest [129-132]. With cerivastatin, increased levels of the CDK-inhibitor p21 and inhibition of RhoA prenylation were proposed as mechanisms [131]. In another work with cerivastatin, the down-regulation of cyclin D1 was seen at both the transcriptional and protein levels, as well as increases in p21 [133]. In the MDA-MB-231 (ER-/PgR-/HER2-) cell line, simvastatin induces apoptosis via NF-κB, as estimated based on reduced mRNA for the anti-apoptotic factor BCL-2, increased levels of the apoptosis marker Caspase-3 and DNA fragments and delocalized/deactivated isoprenoid RhoA in the cytosol [134]. In a study performed by Spampanato *et al.*, the MCF-7 (ER+/PgR+/HER2-) cancer cell line was one of five cancer cell types for which simvastatin induced apoptosis, with increased expression of the pro-apoptotic gene *BAX* and decreased expression of the anti-apoptotic gene *BCL-2* [135]. Another mechanism of simvastatin is to induce apoptosis in breast cancer cell lines by up-regulating death receptor 5 (DR5), CHOP and JNK 2/1; this mechanism is mediated by JNK [128]. Results from Koyuturk *et al.* also showed JNK-mediated cell arrest and apoptosis in both ER-positive (MCF-7) and ER-negative (MDA-MB-231) cell lines [136].

In two ER-positive (MCF-7 and T47D) and two ER-negative (MDA-MB-231 and BT-549) breast cancer cells treated with simvastatin, apoptosis was seen to significantly increase the cleavage of caspase-8, caspase-3 and PARP. Reduced proliferation was also seen, with decreases in c-myc and cyclin D1 expression and increased expression of p21 and p27, as assessed by western blotting [137].

The activation of Akt kinase, which is a downstream product of the PI3K pathway, has been associated with increased survival and anti-apoptosis effects in cancer cells, and the pathway is negatively regulated by the tumor suppressor protein PTEN. In a study performed by Ghosh-Choudhury *et al.*, simvastatin inhibited Akt activation in MDA-MB-231 cell lines and animal models [138]. The expression of the anti-apoptotic factor Bcl_{XL} was repressed through simvastatin-induced NF-κB inhibition, and PTEN expression increased, with derepression by NFκB, which inhibited MDA-MB-231 cell proliferation *in vivo* [138]. Park *et al.* also reported statin-mediated inhibition of TNBC through the PI3K pathway [139].

Campbell *et al.* showed that lipophilic statins induce decreased proliferation and increased apoptosis in a number of cell lines; both ER-negative (MDA-MB-231) and ER-negative/HER2-positive cell lines (SKBr3) were more sensitive than the ER-positive MCF7 cell line [127]. In mouse models, tumor growth was

significantly inhibited by lipophilic statins, with stronger effects observed for fluvastatin than simvastatin. In the examined tumor tissue, the apoptotic caspase-3 expression was increased, and Ki67 was reduced in treated animals in comparison to controls [127]. Campbell *et al.* also showed that SKBr cells treated with statins (fluvastatin, simvastatin and lovastatin) for 48 hours showed a decline in various MAPK proteins, as well as a reduction in cyclin D1 associated with increased p21 levels [127].

Freed-Pastor *et al.* found that the mevalonate pathway (MVP) is a possible therapeutic target in breast cancer patients with mutation of the tumor supressor p53 [96]. In the triple-negative MDA-MB-231 breast cancer cell line, simvastatin increased cell death and decreased cell growth and invasiveness in the same way as the depletion of the p53 mutation [96]. Mutant p53 has been suggested to act as a co-activator with SREBPs in the up-regulation of MVP genes, and geranylgeranylation is a vital part of the mechanism [96]. Higher expression of MVP genes was associated with worse prognosis and correlated with a higher rate of p53 mutations [96].

In a study performed by Denoyelle *et al.* with MDA-MB-231 cell lines, cerivastatin inhibited invasion [131]. The effect was suggested to occur through RhoA inhibition but not Ras inhibition and could be related to an NFκB mechanism [131, 133].

Wolfe *et al.* showed in triple-negative cell lines and *in vivo* model, that simvastatin inhibits metastatic behavior in breast cancer. Mice treated with simvastatin showed fewer lung and brain metastases than controls [140]. In that study, the authors reported a possible connection to the suppressor gene and transcription factor FOXO3, which was regulated by simvastatin. Based on patient data, patients with lower FOXO3 mRNA expression had shorter metastatic-free survival than the group with higher FOXO3 expression [140]. In another study performed by Farina *et al.*, lovastatin inhibited the ability of breast cancer cells to form lung metastases and altered the organization of the cytoskeleton *in vivo* [141].

Breast cancer that is ER-positive is driven by estrogen to continue further growth and development. In ER-positive breast cancer cells, both *in vitro* and *in vivo* studies suggested that the oxysterol metabolite 27-HC promotes tumor growth and induces lung metastasis in estrogen similar manners [102-104]. Results from Kimbung *et al.* based on the window-of-opportunity trial from our institution (the MAST trial) suggested that short-term statin treatment decreased serum levels of 27-HC, in addition to lowering serum total cholesterol and LDL [142]. These alterations were not associated with a decrease in Ki67. CYP27A1, also known as sterol 27-hyroxylase and hydroxylates cholesterol, was up-regulated at the protein level but not the transcriptional level.

In another study, Kimbung *et al.* demonstrated that four types of cell lines (MDA-MB-231, SKBR, MCF-7, BT474) showed up-regulation of the cholesterol biosynthesis pathway, irrespective of statin sensitivity, with higher fold changes in less statin-sensitive cell lines (MCF-7 and BT474). The defined "cholesterol biosynthesis signature" and HMGCR baseline expression were significantly lower in tumors with a reduced proliferation index after statin treatment. High expression of the "cholesterol biosynthesis signature" was associated with significantly shorter recurrence-free survival and overall survival, especially in ER-positive tumors. This pattern was also seen in adjusted models and the Cancer Genome Atlas (TCGA) cohorts [143]. Goard *et al.* reported work on 19 different breast cell lines and suggested that a 10-gene mRNA signature is predictive of fluvastatin sensitivity, but further pre-clinical and clinical studies are needed [126]. In another study with the MDA-MB-231 and MCF-7 breast cancer cell lines, knock-down of the transcription factor sterol regulatory element binding protein 2 (SREBP2) increased fluvastatin sensitivity, estimated as anti-proliferative and pro-apoptotic effects, but SREBP2 knockdown on its own did not affect cell growth [94].

To identify genes and pathways essential for carcinogenesis, transcriptional profiling is often used. In work performed by Hirsch *et al.*, transformed normal breast epithelial cells (MCF-10A) were treated with simvastatin, which led to the inhibition of tumor growth and cellular transformation through NF-κB inhibition [144]. This effect was not seen in non-transformed MCF10A cells. In addition, the transformed MCF10A cells caused tumors that became inhibited when the oxidized LDL receptor 1 (OLR1) gene was silenced. Interestingly, OLR1 and other lipid metabolic genes were identified as important genes for the transformed state [144]. The results suggested that in breast cancer tissues, lipid metabolism genes are often overexpressed, and high expression is associated with more aggressive, metastatic tumors [144].

In TNBC cancer cells, simvastatin suppressed the transcription factor *ETS1*, which plays a role in proliferation and normal cell development, and increased expression of the MAPK-pathway inhibitor *DUSP4* [145]. When *DUSP4* was silenced, decreased simvastatin-induced apoptosis was seen. In a study with 22 breast cancer cell lines, TNBC cell lines showed significantly higher expression of several cell cycle-related genes in comparison to non-TNBC cell lines, including *CCNA1*, *CCND2* and *CDK6,* among others. After treatment with simvastatin, cell-cycle related genes and the MAPK-pathway genes *MAP2K3* and *MAP2K6* were down-regulated [145].

In summary, pre-clinical studies suggest that the sensitivity of breast cancer cell lines to statins differs between drugs and that all statins are not created equal. ER-negative cancer cells are often more sensitive to statins, and more lipophilic statins have shown the most pronounced effects. Interesting mechanisms have been

demonstrated regarding the effects of statins on breast cancer cells, including the ability to induce apoptosis and inhibit proliferation and metastasis. The role of 27-HC in breast cancer and the effect of statins on 27-HC requires further study.

Statins and breast cancer risk

Randomized clinical trials (RCT) that have investigated statin use and cancer risk were performed to estimate the preventive effect of statins against cardiovascular diseases (CVD) as the primary endpoint, not investigate the effect of statins on cancer. Among the secondary endpoints were overall cancer mortality and overall and cancer-specific cancer incidence [146]. Normally, the trials had relatively small sample sizes and short-term follow-up, resulting in few cancer cases. In addition, these studies were designed to investigate CVD, which limits their ability to assess cancer outcomes [146].

In a meta-analysis of seven RCTs (cardiovascular events as the primary endpoint) and 9 observational studies (5 case-control and 4 cohort studies), no associations were seen between statin use and breast cancer incidence [147].

In a case-control study performed by Boudreau et al., statin use did not increase breast cancer risk [148]. Current users of statins for more than 5 years exhibited 30% reduction in risk (95% CI 0.4-1.0) [148]. Boudreau found no associations between statin use and breast cancer risk in another retrospective cohort study [149].

In the Women´s Health Initiative (WHI) study, the use of lipophilic statins reduced the breast cancer incidence by 18% (HR 0.82, 95% CI 0.7-0.97, P=0.02) [150]. In an updated version of the same study with more cases and a longer follow-up, Desai et al. saw no associations between statin use and breast cancer risk [151]. Borgquist *et al.* observed no associations between the use of any statin and the risk of invasive breast cancer in the Nurses' Health Study [152]. The analysis of specific statins and solubility class showed no associations between current use and invasive BC incidence [152]. Another study demonstrated that after statin use more than one year before the breast cancer diagnosis, patients were less likely to develop ER-negative breast cancer [153]. Generally, ER-negative breast cancer is more difficult to treat than ER-positive breast cancer and has fewer treatment options, so that finding could be of interest concerning the role of statins in cancer.

The results obtained from studies that explored statin use and breast cancer risk have been inconsistent regarding the associations between statin use and breast cancer risk. Although the design of some of the studies can be questioned, the most important finding from these studies is that statins do not appear to increase the risk of breast cancer.

Statins and breast cancer prognosis

In contrast to the field of statins and breast cancer risk, breast cancer prognosis has consistently been shown to benefit from statin treatment. In a large-scale Danish study performed by Ahern *et al.,* patients taking lipophilic statins, especially simvastatin, showed reduced recurrence rates of breast cancer [154], whereas patients on a hydrophilic statin had the same risk of recurrence as non-statin users. Over a 10-year follow-up, women taking simvastatin experienced almost 10 fewer recurrences per 100 women [154]. Another smaller study showed a similar trend of decreased recurrence among lipophilic statin users, but the observed difference was not statistically significant [155]. Another Danish study investigated statin use in patients with several cancer types and reported that statin use was associated with the reduction of cancer-related mortality by up to 15%. These associations were also evident for breast cancer survival [156]. Consistent with those results, a nationwide cohort study from Finland showed that both pre-diagnostic and post-diagnostic statin use were associated with a reduced risk of breast cancer death [157].

In the large-scale international BIG 1-98 endocrine trial that investigated endocrine treatment in the adjuvant setting, it was recently demonstrated that the initiation of cholesterol-lowering medication (CLM), including statins, during endocrine therapy was associated with improved disease-free survival, breast cancer-free survival and distant recurrence-free interval [158]. All patients included in the study had hormone receptor-positive early-stage breast cancer, and the observed associations held true for endocrine treatment in general, although the effect was most pronounced for patients who received letrozole alone (5 years), followed by the other regimens: tamoxifen-letrozole, letrozole-tamoxifen and tamoxifen single treatment.

In the Women's Health Initiative study, an association was seen between the use of lipophilic statins and the diagnosis of late-stage breast cancer (HR 0.80, 95% CI 0.64-0.98, P=0.035). This pattern was also observed in sub-analyses with ER-positive women [159]. A trend was seen towards lower breast cancer mortality among statin users, but that difference was not statistically significant [159]. Another more recent WHI study also associated current statin use with a 22% lower risk of cancer death (HR 0.78, 95% CI 0.71-0.86, P<0.001) and with all-cause mortality. The effect was also seen among lipophilic statin users. Breast cancer deaths were among the cancer deaths protected by statin use (HR 0.60, HR 0.42-0.85) [160]. Some studies failed to show a protective effect of statin use. In an Irish study, Smith *et al.* found no association between post-diagnostic statin use and both breast-cancer specific and all-cause mortality [161].

In a recent meta-analysis, statin use was associated with lower (breast) cancer-specific mortality and all-cause mortality; lipophilic statins are associated with decreases in both types of mortality, but hydrophilic statins are only associated with a decrease in all-cause mortality [162]. In another meta-analysis of observational studies, post-diagnostic statin use was associated with lower cancer-specific mortality in breast cancer patients, and pre-diagnostic statin use was protective for both all-cause mortality and cancer-specific mortality [163].

The majority of studies that investigated statin use and breast cancer prognosis reported a protective effect of statins on breast cancer recurrence and mortality. Some strong data support this conclusion, but larger prospective clinical trials are needed.

Window-of-opportunity trials

No large randomized clinical trials have been designed to investigate the effect of statins on cancer. However, at least three window-of-opportunity (WOO) trials have studied the effect of statins on breast cancer.

Garwood *et al.* performed a phase II WOO clinical trial in which 45 patients with newly diagnosed DCIS and low-grade invasive cancer were treated with fluvastatin before planned cancer surgery. In that study, a reduction of Ki67 and an increase in apoptosis (CC3) were seen predominantly in high-grade tumors [164]. No significant changes were seen for tumor size, as measured using volume analysis based on MR imaging. Descriptively, however, high-grade tumors decreased in volume by a median of 24.8%, whereas the volume of lower grade tumors increased by a median of 23% (P=0.02).

In another window-of-opportunity trial performed by Wang *et al.*, simvastatin use significantly increased apoptosis and caused a trend towards reduced Ki67. The PI3K/Akt/mTOR pathway was inhibited, with increased PTEN expression and decreased phosphorylation of Akt and S63P. In patients taking simvastatin for more than 14 days, the MAPK/ERK pathway was deactivated, as indicated by a decrease in the phosphorylation of c-Raf and ERK1/2. These findings were confirmed in two ER-negative and two ER-positive breast cancer cell lines [137].

In this thesis, the results from the MAST (MAammary cancer and STatins) WOO trial that was conducted at Skånes University Hospital will be introduced and discussed. In this WOO trial, we studied changes in tumor proliferation, as determined by immunohistochemical expression of Ki67, as well as changes in the expression of HMGCR, cyclin D1 and p27, after two weeks of statin treatment [165, 166]. We also studied changes on the transcriptional level following statin treatment [167].

Taken together, the results obtained from pre-clinical studies, observational studies and clinical trials have elucidated the effects of statins and their mechanisms in breast cancer cells *in vitro*, *in vivo* and in patients. Several findings are interesting and clarify the role of statins in breast cancer. The obtained results support further studies to explore the potential role of statins in breast cancer, preferably in an adjuvant or metastatic setting. To achieve that goal, large prospective clinical trials are essential.

Aims of the thesis

The primary aim of this doctoral thesis is to improve our understanding of the effects of statins on breast cancer and breast cancer-specific mortality and to study HMGCR as a predictive and prognostic marker.

Paper I

This paper aimed to study the effect of statins on proliferation by using Ki67 as a surrogate marker for changes in proliferation. Another aim was to investigate the potential of HMGCR as a short-term predictive marker for statin treatment.

Paper II

Using gene expression profiling, this paper aimed to study the effect of statins on transcriptional levels and to further explore the effect of statins on breast cancer.

Paper III

This paper aimed to explore tumor expression of the cell cycle regulators cyclin D1 and p21 after statin treatment. We also aimed to investigate the effect of statins on the clinically established biomarkers ER, PgR and HER2.

Paper IV

This paper aimed to study HMGCR as a prognostic factor for breast cancer-specific mortality and to investigate whether the use of cholesterol-lowering medication affects breast cancer-specific mortality.

Materials and methods

The Mammary Cancer and Statin Trial (Papers I, II and III)

The MAmmary Cancer and STatin (MAST) trial is a phase II window-of-opportunity clinical trial that was performed at Skåne University Hospital from February 2009 to March 2012. In the MAST trial, women with newly diagnosed primary breast cancer were prescribed 80 mg of atorvastatin daily for two weeks, and planned standard cancer surgery was then performed. Before atorvastatin treatment was initiated, blood tests and a tumor biopsy were taken from the breast tumor. Two weeks later, during the planned surgery, renewed blood tests and tumor tissue were sampled from the surgically removed tumor. As planned before the trial started, 50 women participated in the MAST trial, and 42 women completed all parts of the study. Figure 5 is a flow-chart illustrating the study enrollment.

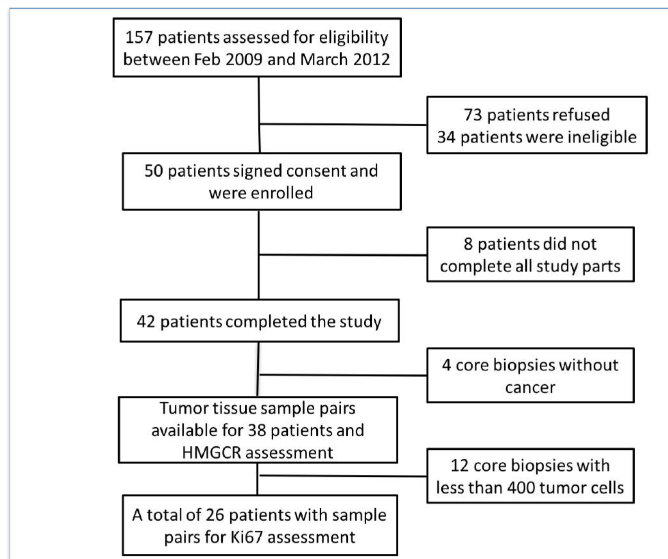

Figure 5. Flow-chart showing enrollment of patients in the MAST trial.

The trial was approved by the Ethical Committee in Lund (Dnr 2008/529) and the Swedish Medical Products Agency. The trial was registered at ClinicalTrials.gov (ID number: NCT00816244, NH). All participants signed a written informed consent form.

The Malmö Diet and Cancer Study (Paper IV)

The Malmö Diet and Cancer Study (MDCS) is a population-based prospective cohort study that was performed in Malmö, Sweden from 1991 to 1996 [168]. Men and women who were living in Malmö at the time were invited to participate in the study. Excluded from enrollment were subjects with an insufficient understanding of the questionnaire due to lower mental abilities or insufficient Swedish language skills [168]. Approximately 40% of the source population participated in the study, and a total of 17,035 women joined the study [169, 170]. A flow-chart showing the enrolment is illustrated in Figure 6. The MDCS was initiated to better understand the relationship between diet and cancer, i.e., to investigate the effect of diet on cancer development [168]. In the part of this study that was included in this thesis, the aim was to study the associations among HMGCR expression, cholesterol-lowering medication use (CLM) and breast cancer-specific mortality. The study

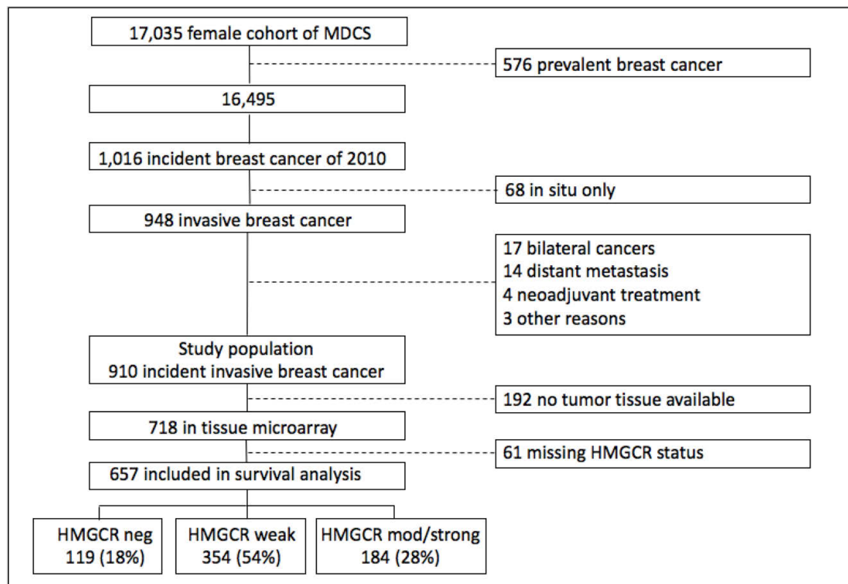

Figure 6. Flow-chart showing the study population in the Malmö Diet and Cancer Study.
Dotted lines represent reason for missing patients.

was approved by the Ethical Committee at Skåne University Hospital (Dnr 652/2005, Dnr 166/207). All participants signed a written informed consent form.

Methods

Tissue microarray

The tissue microarray (TMA) technique is a method that is well established in the research field, since it was introduced in 1998 [171]. To construct a TMA, tumor biopsies are taken from several independent "donor" paraffin-embedded tumor blocks and accurately added to a new "recipient" tumor block in a coordinate-specific manner [171]. The new "recipient" tumor block is then sliced thinly for further analyses (i.e., immunohistochemistry or *in situ* hybridization). In that way, the tumor tissue is used sparsely, and many cases can be studied simultaneously, with different tumor markers used on each microscope slide. Figure 7 illustrates how TMAs are constructed.

Herain, TMAs were used to assess the expression levels of different proteins in papers I and III (post-treatment samples: HMGCR, cyclin D1 and p27) and in paper IV (HMGCR expression in the study population).

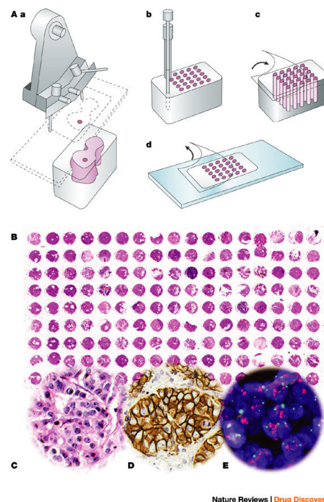

Figure 7. Illustrates construction of Tissue Microarray (TMA).
Reprinted with permission from the Nature Publishing group [172].

Immunohistochemistry

Immunohistochemistry (IHC) is a widely used technique for evaluating protein expression in tissue. In IHC, an antibody specific for the antigen of interest binds to the antigen in the tissue, and the antigen-antibody complex is visualized through an enzymatic assay that produces a brown-colored substrate.

In paper I, antibodies raised against Ki67 (MIB1, Cat. No M7240, Dako Denmark A/S, diluted 1:150) and HMGCR (Cat. No HPA008338, Atlas Antibodies AB, Stockholm, Sweden, diluted 1:150) were used. Ki67 was evaluated on a continuous scale, and HMGCR was evaluated using a four-grade intensity scale (negative, weak, moderate or strong).

In paper III, antibodies against the cell cycle regulator cyclin D1 (Dako M3635, diluted 1:40) and the tumor suppressor p27 (Dako M7203, diluted 1:100) were used. The expression of both cyclin D1 and p27 was evaluated based on the fraction of stained nuclei, using a five-grade scale (i.e., 0-1%, 2-10%, 11-50%, 51-75%, and >75% of stained cells), and based on both nuclear and cytoplasmic intensity, using a four-grade scale (i.e., negative, weak, moderate or strong). In addition, the well-established biomarkers ER, PgR, HER2 and Ki67 were used, according to clinical guidelines.

In paper IV, a novel monoclonal HMGCR antibody (AMAb90619, CL0260, Atlas Antibodies AB, Stockholm, Sweden, diluted 1:100) was used.

In papers I and III, conventional microscopy was used to evaluate expression via IHC. In paper IV, the web-based pathological platform PathXL Xplore (http://www.pathxl.com, PathXL, Ltd., UK) was used.

Cell lines and cell culture

In paper II, four different human breast cancer cell lines with distinctive receptor expression were used: MCF7 (ER+/PgR+/HER2-), BT474 (ER+/PgR+/HER2+), SKBR3 (ER-/PgR-/HER2+) and MDA-MB-231 (ER-/PgR-/HER2-) [173]. For 48 hours, the cells were exposed to either atorvastatin or vehicle (DMSO). Thereafter, total RNA was extracted and subjected to whole-genome transcriptional profiling. Three biological replicates per treatment condition were assayed.

RNA extraction

In papers II and III, total RNA was extracted from fresh-frozen tumor samples and cell lines using an Allprep DNA/RNA mini kit (QIAGEN) in a QIAcube (QIAGEN), according to the manufacturer´s instructions. Before extracting total RNA from fresh-frozen tumor tissue, it is highly recommended to determine tumor

cellularity by evaluating H/E-stained sections. In paper II, in approximately 70% (14/21) of evaluable cases, the tumor cellularity was greater than 50%. RNA quantification was performed using a NanoDrop ND-1000 (NanoDrop Products), and RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent). For further analyses, only samples with RIN values ≥7 were included.

Gene expression profiling

In cancer research, microarray technology is frequently used to 1) classify tumors, 2) search for novel biomarkers and 3) detect transcriptional changes in response to a given therapy.

DNA microarrays are created by robotic machines that arrange minuscule amounts of hundreds or thousands of gene sequences on a single microscope slide. In response to extrinsic or intrinsic stimuli, a cellular mechanism is activated to transcribe specific parts of the DNA that encode the relevant gene(s), producing messenger RNA (mRNA), the template for producing proteins. To investigate which genes are active or inactive in a given cell, the total mRNA present in that cell must be collected for quantification using techniques such as quantitative polymerase chain reaction (Q-PCR) or microarray. For microarray analysis, the mRNA is first reverse-transcribed into complementary DNA (cDNA) by the enzyme reverse transcriptase, and the cDNA is labeled with fluorescent nucleotides during this process. Next, the labeled cDNA is hybridized onto the microarray slide containing complementary synthetic oligonucleotides. To measure the fluorescence intensity for each spot/area on the microarray slide, a laser scanner is used [174, 175]. With higher expression of a gene and presumably higher gene activity, more labeled cDNAs will hybridize onto the complementary oligonucleotide sequence on the microarray slide and generate a brighter fluorescence intensity. Highly expressed or up-regulated genes are often represented as a red spot on a gene matrix/Heatmap. Genes that are less active produce fewer mRNAs and thus fewer labeled cDNAs, which leads to a weaker fluorescence signal. Down-regulated genes are often represented as a green spot on a gene matrix/Heatmap. The absence of a fluorescent signal for a given gene indicates that no mRNAs are available, either because the gene is inactive (shot down) or due to technical failures in the hybridization experiment.

In papers II and III, labeled cDNA from tumor samples and breast cancer cell lines was hybridized to Human HT-12 v4.0 Expression BeadChips (Illumina, Inc.) in the sciblu Genomics Center at Lund University, Sweden (www.lu.se/sciblu). At a later time point, the RNA extracted from the cell lines was processed in one batch.

Microarray data processing and analyses

The Quantile Normalization method was used to initially preprocess and normalize the microarray data [176]. The GenomeStudio Software V2011.1 was used to perform these analyses. The R package illuminaHumanv.db [177] was used to reannotate the Illumina probes. The excluded probes were probe sets with signal intensities below the median of the negative control intensity signals in more than 80% of the samples. The expression of genes represented by more than one probe on the microarray was summarized by taking the median of the signal intensity of the replicate probe sets. By performing a principal component (PC) analysis, associations between technical factors and the main principal components were revealed, whereupon a batch effect was found to be related with the 7th PC. ComBat, which is a supervised empirical Bayes method, was unable to resolve this technical artifact, but since this artifact was not associated with the main PCs, we believe the effects on the final results are marginal. To identify differentially expressed genes between paired pre- and post-treatment samples, a Significant Analysis of Microarrays (SAM) was performed using 500 permutations [178].

Pathway analysis

The transcription factor-binding analysis program Systematic Motif Analysis Retrieval Tool (SMART) was used, as previously described [179], to search for enriched transcription factor-binding site (TFBS) motifs among the differentially expressed genes. Briefly, the promoter regions of the genes that were differentially expressed after statin treatment were scanned for TFBSs. Genomic intervals between -1,500 and +500 base pairs, relative to the putative transcription start site, were defined as promoter regions. Two criteria were used to define significance: significantly present TFBS in terms of the fraction of promoters with binding sites and significantly enriched TFBS in terms of the fraction of promoters. To identify the significant TFBS, a resampling procedure was performed in which the query gene set was compared with 10^5 gene lists of similar size that were randomly drawn from the TFBS/promoter database.

The GeneGo Pathways Software (MetaCore) was used for functional annotation and pathway analysis, and Literature Vector analysis (LitVAn) was used for gene module functional analysis. For paper II, the collection of data is illustrated in Figure 8.

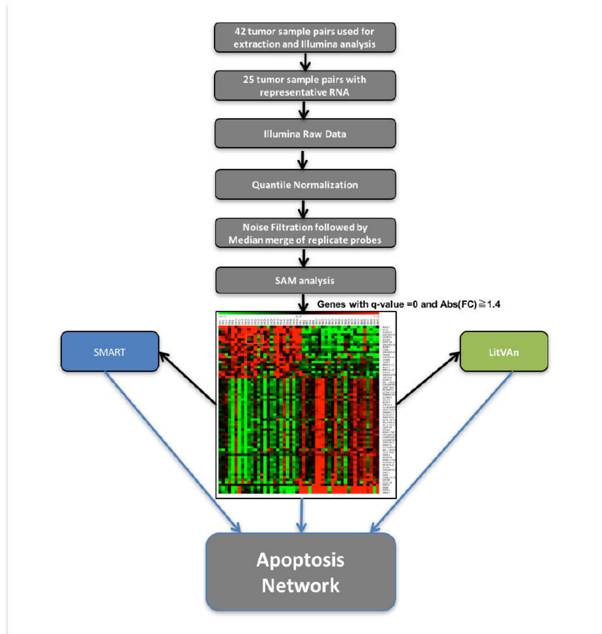

Figure 8. Flow-chart of the data anlyses conducted with patients included in the MAST trial.

qRT-PCR

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is an alternative method for quantifying levels of gene expression. In this thesis qRT-PCR was used to validate the results obtained from microarray analyses regarding the expression of interesting mRNAs/genes that were highlighted from the global approach for gene expression analysis using microarray technology. Further, the validation of antibodies against HMGCR for paper IV also used the qRT-PCR method. Three steps are combined in qRT-PCR. First, the reverse transcriptase (RT)-dependent conversion of RNA into complementary DNA (cDNA) occurs. Second, PCR is used to amplify the cDNA. Finally, the real-time detection and quantification of amplification products is performed [180].

In paper II, qRT-PCR was used to validate the expression levels of *HMGCR* (exon 6-7 and exon 13-14), *DUSP1*, *RHOB*, *JUN* and *FOS* in the clinical samples. A total of 1 µg RNA was reverse-transcribed (Quantitect Reverse Transcription Kit, Qiagen) into cDNA. With a hydrolysis probe (Life Technologies), cDNA corresponding to 5 ng of total RNA (Quantitect Probe PCR kit, Qiagen) was used as a template in the qPCR. *PUM1*, *SLU7*, *PPIG* and *ACTB* were used as endogenous reference genes. As a normalizer for clinical breast cancer samples, cDNA pooled from six normal breast tissue specimens were used. All samples

were run in triplicate. To verify the reaction efficiency, a standard curve was included in each run, and no template controls was used. The $2^{-\Delta\Delta Ct}$ formula was used to calculate the relative expression ratio for each gene in each sample. For statistical analysis of related samples, the Wilcoxon signed-rank test was applied.

Statistical analyses

Paper I

Both a linear scale (i.e., absolute change) and a log scale (i.e., relative scale) were used to assess changes in tumor proliferation after statin treatment. A paired t-test was used to perform direct comparisons of changes on both scales. The average relative change was defined as the geometric mean of the Ki67 ratios. The McNemar-Bowker test was used to evaluate the support for a shift in the ordered categorical variable HMGCR intensity following statin treatment. In analyses comparing the odds of a reduction in proliferation in HMGCR-negative versus HMGCR-positive cases, logistic regression was used. The Mann-Whitney U test (i.e., for two groups) or the Kruskal-Wallis test (i.e., for three groups) was used to evaluate subgroup differences in the distribution of the ordered categorical HMGCR intensity scores. To quantify the correlation between Ki67 and HMGCR, the Spearman correlation (r_s) was used. All tests were two-sided. For the primary and secondary endpoints, differences with P-values below 5% were considered to be significant.

Paper II

The statistical methods used in this paper are described above in the sections Microarray data processing and analyses and qRT-PCR.

Paper III

All evaluated immunohistochemical tumor variables were measured on an ordinal scale. The Wilcoxon matched-pairs signed-rank test was used to evaluate statin-induced changes in pre- and post-treatment protein expression for ER, PgR, HER2, cyclin D1 and p27. To measure the correlation between changes in Ki67 and cyclin D1 or p27, Spearman´s rho was used. The linear-by-linear association was used to test for subgroup differences. All tests were two-sided. Differences with P-values below 5% were considered to be significant.

Paper IV

Associations between patient and tumor characteristics associations and cholesterol-lowering medication (CLM) use were evaluated and presented as numbers and percentages. Continuous variables were summarized using mean, min., and max. values. Between the two groups (any CLM and never CLM use), distributional differences were evaluated with the X^2 test or linear regression (X^2 test for trend), as appropriate. The same methods were used to assess associations between HMGCR expression and patient and tumor characteristics.

Breast cancer-specific mortality (BCM) was used as a clinical endpoint when evaluating the association between CLM use, HMGCR expression and prognosis. BCM was defined as the incidence of breast cancer related deaths. Follow-up time was calculated from the time of breast cancer diagnosis to the date of the first of the following events: breast cancer-related death, death from another cause, emigration or the end of the follow-up time (December 31st, 2014).

Cause-specific Cox regression, yielding hazard ratios (HR) with 95% confidence intervals, was used to analyze the associations between HMGCR expression and the time to breast cancer-related death. The follow-up time was censored at the date of death from a cause not related to breast cancer (so-called competing event). HRs should therefore be interpreted in a hypothetical world in which all other causes of death have been eliminated [181]. Three multivariable models were fitted in addition to crude analyses:

- Model 1 was adjusted for age at diagnosis (continuous).
- Model 2 was adjusted for age at diagnosis and tumor characteristics (tumor size >20 mm (yes/no), metastatic lymph nodes (yes/no), histological grade (grade 1, 2 and 3, 2 degrees of freedom), and ER status (positive/negative)).
- Model 3 included the same covariates as model 2, with the addition of planned adjuvant treatments (endocrine treatment (yes/no), chemotherapy (yes/no) and radiotherapy (yes/no)).

Because the Swedish Prescribed Drug Register was first initiated in July 2005, the prognostic impact of CLM use was evaluated for patients diagnosed 2006 onwards. To evaluate the relationship between CLM use and BCM, the same strategy for crude and adjusted analyses was used as described above. In an exploratory analysis, the predictive value of HMGCR regarding the association between CLM use and BCM the survival analyses were stratified by HMGCR expression.

All data analysis was performed using Stata (StataCorp LP, College Station, TX, 2012; Version 12.1 and 14.1) and SPSS Statistics (IBM, version 19.0 and 24.0).

Results

Paper I

The primary purpose of this part of the trial was to investigate whether statins change proliferation in breast cancer. Ki67 expression was used as a surrogate marker for proliferation. The secondary aim was to evaluate the effect of statins on HMGCR expression.

Out of 50 patients included in this clinical trial, 42 women completed all parts of the study. The mean age was 63 years, nearly 90% of the tumors were ER-positive, and 40% of the patients had positive nodal status.

There were 26 pairs of pre- and post-treatment tumor samples with sufficient quality and at least 200 cancer cells to estimate Ki67 expression. Inferior quality of the pre-treatment core needle biopsies with less than 200 cancer cells was the reason why 16 pairs were not available for comparison. The mean Ki67-index at baseline was significantly lower in ER-positive, PgR-positive and HER2-negative patients. Tumors with a higher grade, tumors with a higher mitotic index and tumors that had triple-negative receptor status had a significantly higher Ki67-index at baseline. No association was seen between tumor characteristics and the change in Ki67 after statin treatment.

The primary endpoint was to investigate the effect of statins on tumor proliferation, that is Ki67 expression. Ki67 was decreased in 15 post-treatment samples in comparison to pre-treatment samples and increased in 11 cases. In the pre-treatment samples, the average expression of Ki67 was 24.0%, and in the post-treatment samples, the average expression of Ki67 was 21.9%. The average absolute reduction was 2.1 percentage points, and the average relative reduction was 7.6%.

For the evaluation of HMGCR expression, the requirement of 200 cancer cells was not applied for this homogenously expressed biomarker and thus 38 pairs were available for comparison of tumor biopsies.

HMGCR was not expressed in 14 of the pre-treatment samples, 11 samples showed weak staining, 10 samples showed moderate staining and three samples had strong HMGCR expression. In the post-treatment samples, one patient had

negative HMGCR staining, seven patients had weak staining, 20 patients had moderate staining and 10 patients had strong HMGCR expression. Comparing the pre-treatment samples with post-treatment samples, HMGCR expression was higher in 26 post-treatment samples, lower in three samples and unchanged in nine samples. These differences were highly significant (P=0.0005).

In tumors with any HMGCR expression in the pre-treatment sample, the treatment predictive value of HMGCR for proliferation was investigated. Among the 24 patients with HMGCR expression prior to statin treatment, the average absolute reduction in Ki67 was 4.6% (P=0.03) and the average relative reduction was 24% (P=0.02). The 14 cases that did not express HMGCR in the pre-treatment sample had a non-significant moderate (0.9%) increase in Ki67 expression. Comparing the two HMGCR-defined subgroups, the difference in the change in Ki67 was significant on the relative scale (P=0.02) but not on the absolute scale (P=0.12).

If the size of the change in Ki67 was ignored, the odds of a Ki67 reduction were 7.3 times higher in the HMGCR-positive tumors than in the HMGCR-negative tumors (OR=7.3, 95%CI 1.3-42, P=0.03). When assuming a linear trend in Ki67 changes over the four HMGCR categories, the average decrease was 4.0% per category, and the relative decrease was 20% per category (P=0.04 and P=0.02, respectively). Post-treatment Ki67 expression was inversely correlated with post-treatment HMGCR expression (rs=-0.42, P=0.03).

Paper II

The aim of this study was to investigate the effect of statins in breast tumors at the transcriptional level using whole-genome expression profiling.

Based on sufficient mRNA quality, there were 25 pre- and post-treatment pairs available for gene expression profiling. After using SAM analysis to identify genes that were differentially expressed in pre-and post-statin treatment tumor samples, 407 genes were differentially expressed: 32 up-regulated and 375 down-regulated. With further filtration of the so-called LOC-genes, which are genes with undefined function located at certain loci, 323 genes remained. After further filtration using a fold change of ≥ 1.49, 67 genes remained, which represent the genes used in the subsequent analysis; 21 genes were up-regulated and 46 genes were down-regulated.

Among the up-regulated genes were Dual-specificity phosphatase 1 (*DUSP1*), Ras homolog family member B (*RHOB*), growth arrest and DNA-damage-inducible beta (*GADD45B*), and the regulator of G-protein signaling 1 (*RGS1*). The

transcription factors *FOS*, *FOSB*, *JUN* and *JUNB*, which are members of the AP-1 transcription factor complex, were also significantly up-regulated.

Among the significantly deregulated biological processes and pathways identified by gene ontology and pathway analyses were GnRH signaling, the immune response, PI3K/AKT, MAPK and apoptosis.

With a vector-based literature search tool, LitVAn, further exploration of the potential functional significance of the altered genes was performed. Interestingly, the LitVAn-derived network converged toward MAPK, ERK, JNK, and p38, which are key genes in the MAPK signaling pathway.

A sub-analysis of the patients with decreased Ki67 after statin treatment, as determined by IHC, provided no additional information beyond what was observed for the whole cohort.

Then, further investigation was performed to determine whether the genes that were altered after statin use were potentially co-regulated by specific groups of transcription factors. For that investigation, the transcription factor binding site (TFBS) analysis program SMART was used. TFBSs were identified with hits >50% and significant enrichment (<0.005) and included cAMP-responsive element binding protein 1 (CREB1), octamer transcription factor (OCT), activating transcription factor (ATF) and serum response factor (SRF).

From the up-regulated genes from the microarray profiling, five genes that were considered to be the most interesting were selected and validated with qRT-PCR. Four of these genes (*DUSP1*, *RHOB*, *JUN* and *FOS),* were found to be upregulated after statin treatment via qRT-PCR, showing trends similar to those observed for the fold changes via microarray profiling. The expression of *HMGCR* was increased in 12 cases and decreased in the remaining 13. No significant changes in *HMGCR* were seen in analyses stratified based on the decrease in Ki67 or the change in HMGCR at the protein level.

In the *in vitro* studies, all four breast cancer cell lines showed significant up-regulation of several key genes involved in the cholesterol biosynthesis pathway when exposed to statins for 48 hours. A significant down-regulation of genes involved in cell proliferation and cell-cycle progression was also seen, especially genes essential for the G2-M phase. In some cell lines, the up-regulation of many pro-apoptosis genes was seen. In a subset of cell lines, consistent with the clinical data, we observed a significant up-regulation of specific *DUSP* genes (*DUSP*4 and *DUSP6*), which regulate MAPK activity, *RHOB* and the AP-1 transcription factor *JUN*.

Paper III

Herein, we aimed to investigate possible changes induced by statin treatment in the cell cycle regulators cyclin D1 and p27, at both the protein and transcript levels. We also investigated whether statin treatment changed the expression of the established clinical biomarkers ER, PgR, and HER2.

Annotations of ER and PgR expression were available for 30 tumor pairs, and annotations of HER2 were available for 29 pairs. No significant change in any of these three markers was seen after statin treatment.

IHC evaluation of cyclin D1 was available in 30 of 42 tumor pairs, and cyclin D1 was expressed in the majority of tumor samples. The nuclear intensity of cyclin D1 was significantly decreased (P=0.008, Wilcoxon matched-pairs signed-rank test) after statin treatment, but the cyclin D1 nuclear fraction and cytoplasmic staining intensity did not change significantly. After statin treatment, no significant association was seen between the change in cyclin D1 and pre-treatment tumor characteristics. Correlation analysis between cyclin D1 and Ki67 showed that decreases in Ki67 corresponded positively with decreases in the cytoplasmic intensity of cyclin D1 (N=25, P=0.03, Spearman´s rho=0.43), but no significant change was seen in the nuclear fraction or the nuclear intensity of cyclin D1.

For p27, IHC annotation was possible in 33 tumor pairs, and the protein was expressed in all pre-treatment samples. After statin treatment, there was a significant increase in both the nuclear fraction of tumor cells expressing p27 and the cytoplasmic intensity of p27 (P=0.03 and P=0.02, respectively). Following statin treatment, no significant change was seen in the nuclear intensity of p27, and no association was found between pre-treatment tumor characteristics and changes in p27. No association was seen between changes in Ki67 and changes in any of the p27 annotations.

At the transcriptional level, no significant change in the expression of *CCND1* and *CDKN1B* after statin treatment was observed. In a sub-analysis, according to change in Ki67 (15 pairs decrease and 10 pairs increase in Ki67), the expression of *CCND1* in the pre-treatment samples was significantly correlated with cell proliferation responses (P=0.02; Mann-Whitney). Similarly, marginally lower *CCND1* expression was observed in the post-treatment samples among the tumors with decreased Ki67 in comparison to tumors with increased Ki67 (P=0.08, Mann-Whitney). For *CDKN1B*, no significant change was seen between tumors that responded with a Ki67 decrease and tumors that did not.

Paper IV

In paper IV, the purpose was to investigate breast cancer-specific mortality both according to HMGCR expression as a prognostic factor and according to the effect of cholesterol-lowering medication (CLM) use.

By the end of follow-up on December 31st, 2010, 1,016 breast cancer cases had been diagnosed. When women diagnosed with cancer *in situ*, bilateral breast cancer and distant metastases were subtracted from the cohort, a total of 910 invasive breast cancers were identified and represented the study population. The construction of TMAs was possible from 718 patients, but another 61 patients were not assessable for HMGCR expression due to a lack of tumor tissue in the TMA core or inferior staining quality. Therefore, 657 samples were available for the evaluation of HMGCR expression; 119 (18%) had negative expression, 354 (54%) had weak expression, 169 (26%) had moderate expression and 15 (2%) had strong expression.

From the study population of 910 patients, 326 patients had been prescribed CLM during the years 2005-2014. A total of 74 patients were prescribed their first CLM before the breast cancer diagnosis (pre-diagnostic CLM), and 252 patients were prescribed the first CLM after (post-diagnostic CLM). For 584 patients, CLM had not been prescribed. In comparison to the post-diagnostic and never CLM groups, a higher proportion of patients in the pre-diagnostic CLM group had grade III tumors, high Ki67 and higher HMGCR expression. When comparing with patients who were never prescribed CLM, any CLM users had a higher BMI at baseline, and their tumors were more often PgR-positive ($P<0.001$ and $P=0.004$, respectively). Tumors with moderate/strong HMGCR expression were associated with tumors with a higher histological grade, high Ki67 and ER negativity (all $P<0.001$).

Four HMGCR antibodies were validated in MCF-7 cells. Following siRNA transfection, *HMGCR* mRNA levels were significantly decreased by approximately 1.7-fold in comparison to controls. Statin treatment, however, significantly up-regulated *HMGCR* mRNA expression by approximately 1.6-fold in comparison to controls. In western blotting studies, all antibodies tested detected a protein band at the expected molecular weight of HMGCR, or approximately 100-kDa. However, the HMGCR antibody ab174830 did not reveal any difference in expression between siRNA silenced cells or statin-treated cells and controls, indicating that HMGCR ab174830 may be recognizing a different target with a molecular weight similar to HMGCR. The antibodies AMAb90619, AMAb90618 and A-9 reliably captured the differential effects of HMGCR down- and up-regulation, and we confirmed their specificity for the target protein by showing a positive reaction in the additional positive control cell lines. These three

antibodies, especially after statin exposure, showed reactivity with a protein of approximately 55-kDa. Based on availability for testing on TMA-containing breast cancer tissue and cell lines, the HMGCR AMAb90619 antibody was chosen for IHC analyses. As expected, positive reactivity was seen in the liver, and in the breast cancer cell lines, the expression of HMGCR was heterogeneous. Further, to test the validity of the IHC assay, AMAb90619 was used to stain a TMA, including primary tumors for which corresponding gene expression data for HMGCR expression were available. Remarkably, four of the five tumors that showed moderate/strong expression of the HMGCR protein also had the highest mRNA expression, confirming the validity of this antibody for IHC. The AMAb90619 HMGCR antibody was used for IHC analyses of HMGCR expression in this study.

Since the Swedish Prescribed Drug Register was not initiated until July 2005, analyses of associations between CLM use and breast cancer-specific mortality (BCM) were restricted to patients diagnosed with breast cancer from January 1st, 2006 onwards. CLM use was associated with a 36% reduction in BCM ($HR_{age\text{-}adj.}$ 0.64), but the evidence for an effect was weak (95% CI 0.25-1.60, P=0.34). In models adjusted for tumor characteristics and adjuvant treatment, similar results were seen. We also performed exploratory analyses stratified for HMGCR expression. For patients with tumors with negative/weak HMGCR expression, CLM use appeared to be more protective regarding BCM ($HR_{age\text{-}adj.}$ 0.16, 95%CI 0.02-1.40, P=0.10) in comparison to patients with moderate/strong HMGCR expression ($HR_{age\text{-}adj.}$ 0.68, 95% CI 0.11-4.01, P=0.67), but the evidence for a differential effect was weak (test for interaction, P=0.27). In analyses stratified based on ER status, a modest trend towards protective effects of CLM use was seen for patients with ER-positive breast cancer, although this difference was not statistically significant ($HR_{age\text{-}adj.}$ 0.75, 95%CI 0.23-2.48, P=0.64).

For the study population with valid HMGCR expression (n=657), the possible prognostic role of HMGCR for breast cancer was evaluated, revealing no evidence of associations. Survival analyses suggested that breast cancer patients with moderate/strong HMGCR expression might have higher BCM than patients with negative/weak HMGCR expression. Although the results were not statistically significant, BCM analyses restricted to patients with ER-positive breast cancer showed that higher precision was obtained in the measurement of BCM in comparison to all patients ($HR_{age\text{-}adj.}$ 1.66, 95%CI 0.81-3.41, P=0.17).

Discussion

Herein, we have used a translational approach to reveal some of the effects exerted by statins in breast tumors and among breast cancer patients. Much remains unknown about the mechanisms by which statins function in breast cancer. For women, breast cancer is the most common form of cancer and the second leading cause of cancer death. Even though treatment options are improving, too many women suffer and die from breast cancer each year. Therefore, more treatment options are needed. Statins are most often safe and inexpensive, have minimal side effects and could be an interesting choice for breast cancer treatment.

The primary endpoint of the MAST trial was the change in Ki67 expression after statin treatment. Reduced Ki67 expression was observed in patients who expressed HMGCR in the pre-treatment sample, but irrespective of HMGCR expression, significant changes in Ki67 were not seen. Currently, Ki67 is the most commonly used marker to assess proliferation in breast cancer, but the lack of standardization and validation has made it difficult to confirm the role of Ki67 in breast cancer assessment and treatment. Ki67 is often used as a surrogate marker for proliferation, particularly in neo-adjuvant and window-of-opportunity trials [164, 182, 183]. In the MAST trial, Ki67 was counted as an overall average score in the whole tumor, according to the recommendations from the International Ki67 in Breast Cancer Working Group [40]. The same guidelines recommend comparing the same type of tissue sample when evaluating treatment effects, but that approach was not plausible in the MAST trial due to that fact that the post-treatment samples were sections from the tumor rather than needle biopsies. Even though biopsy samples are recommended, it can be argued that for the patient, it is less problematic to avoid performing a new biopsy and to instead collect samples during the planned surgery. In addition, the logistics of the extra biopsy, which must be performed by a pathologist or radiologist before the operation, can be challenging and time consuming in comparison to surgeons or pathologists taking a tumor sample from the breast cancer specimen after the operation.

The rate-limiting enzyme of the cholesterol biosynthesis pathway, HMGCR, was up-regulated via immunohistochemistry in the post-treatment samples after two weeks of statin treatment in comparison to the pre-treatment samples. *In vitro* and *in vivo* studies have shown that statins can increase HMGCR protein expression and that HMGCR activity is higher in breast tumors than in normal breast tissue,

and some tumors are resistant to feedback regulation by sterols [90, 91, 97]. HMGCR is overexpressed in several tumors, which may support the idea that to maintain high proliferation in cancer cells, the activity of the mevalonate pathway is increased [124]. Work performed by Clendening *et al.* suggested that HMGCR is a metabolic oncogene and that the dysregulation of the mevalonate pathway (MVP) promotes transformation [95]. At the transcriptional level, investigated in paper II, using both microarray and qRT-PCR analyses, no significant change in *HMGCR* expression was seen following statin treatment in the clinical samples. However, in all four breast cancer cell lines treated with statins, a significant up-regulation of genes involved in the cholesterol biosynthesis pathway was observed, including *HMGCR*. This finding is in line with a previously reported homeostatic feedback response of the mevalonate pathway, in which the inhibition of HMGCR leads to increased transcriptional activity of the transcription factor sterol-regulatory element-binding protein (SREBP) [91].

Results obtained *in vitro* and *in vivo* studies that demonstrated differences in cancer cell sensitivity to statins, and highlighted the need for a predictive marker of statin efficacy. In paper I, the results suggest HMGCR as a probable predictive marker for statin therapy. Patients with tumors that expressed HMGCR in the pre-treatment samples showed a significant decrease in Ki67 expression, but when looking at the whole group, irrespective of HMGCR expression, the changes were not significant. With a heterogeneous disease like breast cancer, the identification of a predictive biomarker is essential for determining the likely treatment efficacy, even for novel therapies such as statins. *In vitro* studies have suggested the use of gene signatures as biomarkers for beneficial statin responses; both a 10-gene mRNA signature and a cholesterol biosynthesis signature have been reported [126, 143]. Others studies have suggested assessments of p53 mutation, PTEN loss and Akt activation as plausible predictive markers for the response to statin treatment [113, 139].

In paper IV, after investigating HMGCR expression as a prognostic marker in the Malmö Diet and Cancer Study, the patients with high HMGCR expression had more aggressive tumor characteristics (i.e., tumor grade III, ER negativity and high Ki67), and trended towards higher breast cancer-specific mortality. That finding is in line with results from Clendening *et al.*, which suggested that high levels of HMGCR mRNA are associated with poor patient prognosis and reduced survival [95]. In some previous publications, HMGCR protein expression has been associated with favorable tumor characteristics, such as low histological grade, the expression of estrogen and progesterone receptors and less axillary lymph node involvement [98-100]. The use of a new novel monoclonal antibody, such as the antibody used in paper IV, could explain part of this difference, in addition to breast cancer heterogeneity.

One of the challenges in this work has been to choose an appropriate highly specific and selective HMGCR antibody that can be used reliably in IHC to detect and evaluate HMGCR expression. Previously, our group has used polyclonal antibodies from different producers, among others, from Atlas Antibodies [165], which were not sufficiently characterized for target specificity and selectivity. More importantly, the previously used antibodies were out of stock and suppliers have discontinued sales. There was therefore a need for a new HMGCR antibody for the analysis performed in paper IV. To this end, several commercially available anti-HMGCR monoclonal antibodies were subjected to an in-depth validation procedure. The chosen antibody, the AMAb90619 antibody from Atlas Antibodies, demonstrated very high target specificity and selectivity and remarkable validity for the IHC assay.

The protein expression of Ki67, HMGCR, cyclin D1 and p27 was assessed via immunohistochemistry before and after statin treatment. Differences were seen in the number of pre- and post-treatment pairs between the different endpoints, from 25 pairs in Ki67 to 38 pairs in HMGCR. When evaluating Ki67, at least 200 cancer cells had to be counted. This strict requirement was required for the primary endpoint being change in tumor cell proliferation and given the clinical guidelines used for Ki67 assessment. That was not the case for the secondary endpoints when assessing the biomarkers HMGCR, cyclin D1 and p27, for which clinical assessment requirements are not available. Additionally, the quality of the pre-treatment needle biopsies was a limiting factor, and great variation was observed. The biopsies with inferior quality were more sensitive to tissue handling and staining.

In papers I-III, the suggested anti-tumoral effects of statins on breast cancer have been partially elucidated, but much remains unclear regarding the mechanism of action. To date, few clinical trials have studied the effect of statins on breast cancer. In addition to the findings reported in papers I-III, one window-of-opportunity trial suggested that simvastatin deactivated the PI3K/Akt/mTOR pathway via increased PTEN expression and affected the MAPK/ERK pathway via the dephosphorylation of c-Raf and ERK1/2 [137]. In breast cancer cell lines, different mechanisms of action have been suggested regarding the ability of statins to inhibit proliferation, induce apoptosis, and inhibit invasiveness and metastasis. Among the mechanisms involved in apoptosis are the activation of the JNK-pathway, the transcriptional down-regulation of the anti-apoptotic protein BCL2 via RhoA-dependent retention of the NF-κB transcription factor, reduced production of AKT1 and the induction of apoptosis through the activation of the pro-apoptotic JNK/CHOP/DR5 pathway [128, 134, 136]. At both the mRNA and protein levels, one study demonstrated that statin induced apoptosis, with decreased expression of the anti-apoptotic gene *BCL2* and increased expression of the pro-apoptotic gene *BAX* [135]. In our gene expression data for breast cancer

patients, we found no up-regulation of these genes or pathways; however, in the triple-negative MDA-MB-231 breast cancer cell line, a decrease in *BCL2* expressions was seen and the up-regulation of *BNIP3,* which is a strong apoptosis inducer, was seen in all four cell lines used. Gene expression analysis of the cell cycle regulators cyclin D1 (*CCDN1)* and p27 (*CDKN1B)*, after statin treatment revealed no significant changes. At the protein level, however, increased expression of p27 and a simultaneous decrease in cyclin D1 were observed, suggesting that the statin-induced effects could be driven by the cell cycle regulatory effects of cyclin D1 and p27. Statins have been shown to lead to G1 arrest and thus inhibit cell growth [129, 131]. The regulation of the transition from G1 to S phase is partially controlled by cyclin D1, through its complex with CDK4 and CDK6, and by p27 through interactions with the complexes CDK2/cyclin E, CDK2/cyclin A, and CDK4/6-cyclin D. When the level of cyclin D1 decreases, the role of p27 changes, as the protein is released from the CDK4/6-cyclin D complex to inhibit CDK2, thereby inhibiting proliferation and promoting cell cycle arrest [21]. The results reported by Wang *et al.* also indicated a decrease in cyclin D1 expression and an increase in p27 expression after simvastatin treatment [137]. These results support our findings in paper III.

To our knowledge, potential whole-genome expression changes after statin treatment in breast cancer patients and breast cancer cell lines have been reported here for the first time. Earlier studies on the effects of statins on transcriptional level in breast cancer cells have focused on one or a few specific genes. The results obtained from paper II suggests that statins inhibit the MAPK pathway via the up-regulation of the pathway inhibitor DUSP1. The MAPK pathway is a pathway that is often uncontrolled in cancer development, with increased proliferation and survival of cancer cells [184]. DUSP1 is a member of the dual-specificity phosphatases, which inactivate MAPK via dephosphorylation [185]. In a study performed by Jung *et al.* in TNBC cell lines, simvastatin was suggested to affect some transcriptional factors, including DUSP4, and increased expression of DUSP4 partially explained simvastatin's antitumor activity [145]. Further, simvastatin down-regulated genes of the cell cycle and genes of the MAPK-pathway, specifically *MAP2K3* and *MAP2K6*. Our results also indicated increased apoptosis, with up-regulation of the pro-apoptotic gene *GADD45B*. GADD45s play roles in various cellular process, including growth control and apoptosis [186].

The gene *RHOB*, which is a member of the Ras superfamily of proteins with GTPase activity and is often expressed in different tissues, was also up-regulated. Statins inhibit the production of isoprenoids, which leads to reduced isoprenylation, resulting in inactivation of small GTPs that are important for cell signaling. Compared to family members RhoA and RhoC, which are often increased in many types of cancer, the expression of RHOB is often down-

regulated in human cancer. In lung and gastric cancer, the expression of RHOB inhibits proliferation, migration and invasion [187]. Statin-mediated inhibition of isoprenoid production has been suggested as an anti-cancer mechanism of statins, and particularly RhoA has been studied in this context [131, 133, 134]. Treating TNBC cell lines with cerivastatin decreased RhoA translocation from the inactive form in the cytosol to the active form on the plasma membrane, this mediated through inhibition of geranylgeranylation [133]. Another member of the isoprenoid family, Ras, has been shown to regulate the expression of the cell cycle regulators investigated in paper III, p27 and cyclin D1, via a Ras-dependent pathway [15].

In the Malmö Diet and Cancer study, analyses of ER-positive patients showed a trend towards the idea that patients with moderate/strong HMGCR expression have higher breast cancer mortality than patients with negative/weak HMGCR expression. However, irrespective of ER status, treatment with cholesterol-lowering medication (CLM) was associated with lower breast cancer mortality among patients with tumors that had negative or weak HMGCR expression. These results may suggest that HMGCR expression is more prognostically important for ER-positive patients and that for ER-positive patients, CLM use can be more preventive of recurrence. In a recent publication based on breast cancer patients with ER-positive disease that evaluated the prognostic impact of CLM use together with endocrine treatment, the investigated treatment improved disease-free survival and distant recurrence-free intervals among breast cancer patients who started CLM use during endocrine treatment. These results suggest a preventive effect of combining endocrine and CLM treatment to prevent breast cancer recurrence and improve disease-free survival [158]. In ER-positive breast cancer cell lines cultured under estrogen deprivation, Simigdala *et al.* proposed that cholesterol biosynthesis pathway genes were often up-regulated together with the cholesterol metabolites 27-hydroxycholesterol (27-HC), and 25-HC. These changes were suggested as a possible mechanism of endocrine resistance [106]. Estrogen resistance is an immense problem in breast cancer treatment, and new therapies are needed. It would therefore be of interest to further investigate the potential role of statins in combination with ER-targeted treatment.

The patient's ER status is also interesting in light of the recent finding related to the oxysterol 27-HC. The concentration of 27-HC is increased in the breast cancer tumor [103]. Studies performed both *in vitro* and *in vivo* have shown 27-HC to induce ER tumor growth in ER-positive breast cancer and to increase lung metastasis through the LXR-receptor [103, 104]. Recently, our lab reported results from the MAST trial, where short-term statin use not only decreased serum levels of total cholesterol and LDL but also decreased serum 27-HC levels [142]. The ability of statins to decrease 27-HC, rather than just decrease cholesterol *per se*, could be a plausible mechanism for the beneficial effect of statins on ER-positive

breast cancer patients [142, 188]. Results reported by Kumar *et al.* demonstrated that women who were taking statins for more than one year before their breast cancer diagnosis were less likely to develop ER-negative breast cancer, which is a disease with fewer treatment options and generally more difficult to treat than ER-positive breast cancer [153]. Interestingly, simvastatin was also shown to inhibit lung and brain metastasis *in vivo* [140], suggesting another possibility for the use of statins in the breast cancer settings, but further research is warranted.

The majority of patients in the MAST trial were ER-positive. However, the majority of studies using cell lines have shown that breast cancer cell lines that do not express ER are often more sensitive to statins; therefore, it would have been of great interest to have had more ER-negative patients included in the MAST trial, but that was not achievable. ER-negative patients often have more aggressive disease at the same time that their breast cancer treatment options are more limited than ER-positive patients. Therefore, better understanding of the effect of statins on ER-negative breast cancer patients would be of great interest.

In most *in vitro*, *in vivo* and observational breast cancer studies, lipophilic statins have showed greater anti-cancer effects than hydrophilic statins. Lipophilic statins, like atorvastatin, are thought to enter endothelial cells more easily than hydrophilic statins via passive diffusion [85]. In the MAST trial, patients tolerated 80 mg of atorvastatin daily for two weeks, and no serious adverse events were reported. Only one participant discontinued the trial due to side effects. In large studies comparing the safety of different doses of a variety of statins, atorvastatin 80 mg was as well tolerated as lower doses of simvastatin and pravastatin [119].

In the MAST trial, patients were taking atorvastatin for exactly two weeks, and tumor tissue was obtained before start of atorvastatin and two weeks later. Therefore, all the statin-induced changes in paper I-III were limited to one time point. On both the protein and the transcriptional level, it is unclear how long treatment time is optimal when assessing tumor biological changes after intervention. With the WOO trial design, it was impossible to obtain tumor samples at a later time point. Both due to the strict phase II clinical trial protocol and also it would have been unethical to delay planned standard cancer surgery.

Statins are not the only inhibitor of the mevalonate pathway that has been investigated in cancer research. Bisphosphonates, that inhibits one of the down-stream enzymes of the mevalonate pathway, farnesylpyrophosphate synthase, recently became a part of adjuvant treatment for post-menopausal breast cancer patients with lymph node positive disease [49, 189]. The prenyltransferase inhibitors, farnesyltransferase inhibitors (FTIs) and geranylgeranyl transferase inhibitors, have also been studied, where FTIs showed some benefit in acute myelogenous leukemia [87, 187]. Knock-down of the SREBP2 gene in two breast cancer cell lines (MBA-MD-231 and MCF7) suggested increased fluvastatin

sensitivity, evaluated as increased anti-proliferative and pro-apoptotic effects, suggesting SREBP2 as an anti-cancer target together with statins [94]. To further explore if statins effects on cancer can be increased by inhibiting other parts of the mevalonate pathway simultaneously, additional studies are needed.

In paper IV, we found no association between CLM use and breast cancer-specific mortality. The associations between CLM use, including statins, and breast cancer-related mortality has been studied for some time, and the majority of the results show that statin use can decrease breast cancer recurrence and mortality. Ahern *et al.* investigated the effect of statin use on breast cancer recurrence in a Danish cohort, and found that breast cancer patients taking simvastatin had 10 fewer breast cancer recurrences per 100 women after 10 years of follow-up and significantly better breast cancer-free survival [154]. Nielsen *et al.* reported reduced overall cancer-related mortality as well as reduced breast cancer-related mortality among statin users [156] in agreement with other studies showing lower risk of breast cancer-related mortality [157, 190]. Recent study from Ireland, however, was not able to confirm these associations [161]. In paper IV, we were unable to confirm that CLM use, including statin use, reduces breast cancer mortality, and we did not have access to breast cancer recurrence rates for patients in the Malmö Diet and Cancer study. In addition, the information about CLM use was limited to prescriptions from July 2005 forward, which is when the Swedish Prescribed Drug Register started. That fact may have contributed to the lack of power to detect differences between users and non-users.

Although breast cancer treatments are improving, breast cancer recurrence and mortality are still major health issues and new treatment options are needed. The results from several pre-clinical studies, observational studies and clinical trials are promising regarding the effect of statins on breast cancer, but larger, preferably targeted, clinical trials are needed. Given statins pleiotropic effects, the few side effects and low costs, statin treatment could be an interesting option in breast cancer. Newer breast cancer treatments are often expensive and in countries less developed the possibilities for chemotherapy or HER2-targeted treatment e.g., are limited. To design a clinical trial where statin treatment is combined with other cancer treatments could be an attractive solution, gaining both patients and the scientific development in breast cancer. Due to the heterogeneity of breast cancer, the identification of a predictive marker for statin treatment, by designing a targeted trial, would be of great value. The future aim is therefore to design a biologically relevant clinical trial, while making it possible to detect groups of patients that could benefit the most from statin treatment.

Strengths and limitations

Paper	Strengths	Limitations
I-III	Phase II clinical trial. Majority of participants completed the trial. Atorvastatin 80 mg well tolerated. Detailed patient and tumor information.	Small trial sample size. No control group. Core biopsies vs tumor sample. TMAs used to evaluate expression on tumor samples. Inferior quality of some needle biopsies. Just change at one time point. Mostly ER-positive post-menopausal patients. The HMGCR antibody.
IV	Large, prospective, population-based study with good follow-up. Detailed patient and tumor information. Validation of HMGCR antibody. Multivariable analyses adjusted for other prognostic factors.	Possible selection bias due to higher education and better health among participants. Histopathological analyses most often based on TMAs. Some missing TMAs tissues. Information about CLM use from 1st of July 2005. CLM use adherence. Patients missing from survival analysis decreasing power. Risk of misclassification of data (register). Information about recurrences or distant metastasis missing.

Conclusions

The results from this thesis indicate that statin could have a role in future breast cancer treatments which is in line with previous pre-clinical and observational studies, but large clinical trials are needed. The main conclusions from this thesis are:

- Overall, statin treatment did not significantly decrease Ki67 expression. When HMGCR expression was seen in the pre-treatment samples, a significant decrease in Ki67 was seen.
- HMGCR expression was up-regulated after statin treatment.
- At the transcriptional level, two weeks of statin treatment caused changes in global tumor gene expression profiles, indicating pro-apoptotic events and inhibition of the MAPK pathway.
- In cell lines treated with statins, the up-regulation of pro-apoptosis genes and the down-regulation of proliferation genes were seen. Several genes from the cholesterol biosynthesis pathway were up-regulated.
- Statin changed expression of cell cycle regulators by increasing expression of the tumor suppressor p27 and decreasing expression of cyclin D1. This suggests that cyclin D1 and p27 play roles in the anti-proliferative effects of statins.
- In the Malmö Diet and Cancer study, HMGCR expression was associated with unfavorable tumor characteristics, but an association with breast cancer-specific mortality was not seen.
- The use of cholesterol-lowering medication was associated with reduced breast cancer-specific mortality, but the evidence was weak.
- An interesting trend was seen toward a lower breast cancer-specific mortality in patients who were using cholesterol-lowering medication and had no or weak HMGCR expression.

Future perspectives

In this thesis, further understanding on statins biological effects in breast cancer has been obtained in a translational manner by combination of clinical studies and functional laboratory work. The results from previous statin and cancer studies and trials are encouraging, but the need for a large prospective clinical trial is essential, to further investigate statins putative role in breast cancer.

The design of the large clinical trial is very important. In the light of the suggested role of 27-HC in ER-positive breast cancer, a clinical trial with statins in combination with endocrine treatment seems to be a promising setting to study the potential role of statins in breast cancer.

Results from cell line studies show, however, that the greatest statin-sensitivity is often in the more aggressive cell lines with negative ER status. That also suggest statins as a treatment option for ER-negative patients, which are often patients with more aggressive breast cancer, which is more difficult to treat with fewer treatment options available. A trial with statins in combination with standard chemotherapy may be a future possibility. A clinical trial in less developed country were the breast cancer treatment options are limited could be an opportunity.

Some of statins advantages are that they are most often well-tolerated, inexpensive and have been on the market for long time. At the same time, all statins are not created equal, and it is important to choose the most effective statin for future trials. In our work we used atorvastatin, well-tolerated and potent statin, but several others have used simvastatin, which is the statin most commonly used.

It is therefore essential to identify a possible predictive marker that can help to identify the patients that would benefit the most from statin treatment. The identification of a predictive marker is one of the important challenges the potential use of statins in breast cancer trials is facing. Another challenge is to decide which statin to use and which dose is best suited to further elucidate statins proposed anti-cancer potential.

Populärvetenskaplig sammanfattning (Summary in Swedish)

Bröstcancer är den vanligaste cancersjukdomen bland kvinnor och var nionde kvinna riskerar att få bröstcancer någon gång i livet. År 2014 drabbades nästan 10 000 kvinnor av bröstcancer i Sverige och antalet fall ökar. Men överlevnaden har förbättrats och i Sverige lever fler än 90 % av bröstcancerkvinnorna efter 5 år. Det är de bästa överlevnadssiffrorna i världen. Första steget i behandlingen av bröstcancer är operation. Beroende på tumörens egenskaper behövs i vissa fall tilläggsbehandling i form av strålbehandling, endokrinbehandling (behandling riktat mot östrogen receptorn), cellgiftsbehandling och/eller antikroppsbehandling. Ändå drabbas många av återfall av sjukdomen och fler behandlingar behövs i kampen mot bröstcancer.

Statiner är läkemedel i tablettform som i första hand används av patienter med höga blodfetter och hjärt- och kärlsjukdomar. Statiner har använts i många år, de är billiga och har få biverkningar. Tidigare studier har visat att användande av statiner leder till färre dödsfall bland hjärtsjuka. Statiner minskar produktionen av kolesterol, en fett-sort som bildas i kroppen. Därför är statiner bra när man har för höga kolesterolvärden (för höga blodfetter). Bildningen av kolesterol minskas genom att statinerna bromsar ett ämne (ett så kallat enzym), som kallas 3-hydroxy-3-metylglutaryl coenzym-A reduktas (HMGCR). I cancerstudier har det visats att cancerpatienter som tar statiner har lägre risk för återfall och lägre risk att dö i bröstcancer. Även i laboratorieförsök med tumörceller och försöksdjur har det visats att tumörerna växer långsammare och dör vid behandling med statiner.

MAST (MAmmary cancer and STatins) är en klinisk studie med cancerpatienter, en s.k. fas II klinisk prövning, som genomfördes vid Skånes universitetssjukhus i Lund från 2009 till 2012 och omfattade 50 bröstcancerpatienter. För att undersöka den eventuella tumörbromsande effekten av statiner fick kvinnor med nydiagnostiserad bröstcancer statiner i två veckor innan de blev opererade. Upplägget av MAST studien var en ”window-of-opportunity” studie-design, som innebär att patienten i det ”behandlingsfria fönstret” mellan diagnos och operation erhåller studieläkemedlet, i detta fall atorvastatin 80 mg tablett dagligen. Blodprover och tumörprov togs både innan statinbehandlingen påbörjades och sedan efter avslutad statinbehandling vid själva operationen. Dessa prov ligger till

grund för arbete I-III i denna avhandling, där vi undersöker statiners effekt på ett flertal olika tumörfaktorer genom att studera tumörmaterial före och efter statinbehandling.

I arbete I undersöktes om statiner påverkar hur fort tumörcellerna delar sig (som mättes i form av proteinet Ki67) samt proteinuttrycket av målenzymet för statiner, HMGCR. I arbete I kunde vi inte visa att två veckors statinbehandling sänkte alla bröstcancercellernas delningshastighet. Däremot såg vi att uttrycket för HMGCR steg. I de tumörprover där HMGCR uttrycktes redan innan start av statinbehandlingen såg vi att Ki67 sjönk och HMGCR kunde alltså förutsäga effekten av statinbehandlingen.

I arbete II studerades om olika geners aktivitet ändrades vid statinbehandling. Resultaten visade ökad aktivitet för gener som har med bland annat celldöd att göra. I försök med bröstcancercellinjer sågs också ökad aktivitet hos gener som påverkar celldöd, men även ökad aktivitet hos gener viktiga för kolesterolproduktionen.

I arbete III undersöktes om statiner påverkar regleringen av cellcykeln genom att påverka cellcykelns regulatorer cyclin D1 och p27. Dessa påverkar cellcykeln på olika sätt, ökad mängd av cyclin D1 kan öka cancercellens förmåga att växa medan p27 inhiberar tillväxten. Efter statin behandling observerades minskat proteinuttryck av cyclin D1 och ökat hos p27, som möjligtvis kan förklara del av statinernas förmåga att hindra tumörtillväxt.

Arbete IV baserades på Malmö Kost Cancer studien som är en stor studie med friska individer inkluderade i Malmö från 1991 till 1996. I Malmö Kost Cancer studien utforskades om uttrycket av HMGCR och användning av kolesterolsänkande läkemedel (statiner samt andra läkemedel som sänker kolesterolnivån i blodet) påverkade antal dödsfall i bröstcancer. Resultaten visade att högt uttryck av HMGCR var förknippat med dåliga tumöregenskaper, som högt Ki67 och ett mer elakartat växtsätt. Patienter som använde kolesterolsänkande läkemedel hade färre antal bröstcancerdödsfall, men svaga bevis låg bakom de resultaten. Hos patienter som helt saknade eller hade mycket lågt HMGCR uttryck observerades en trend att kolesterolsänkande läkemedel minskade risken att dö i bröstcancer.

Dessa studier har gett oss ny insikt i statiners påverkan på bröstcancerbiologin, både på protein- och på gennivå, som förhoppningsvis kan bana väg för framtida statinstudier inom bröstcancer. Resultaten är lovande men begränsande på grund av få patienter. Trots nuvarande cancerbehandlingar påverkas dagens patienter av återfall och cancerdöd. Det är därför angeläget att hitta ännu fler behandlingar i kampen mot cancer. Statiner är billiga, välprövade läkemedel med få biverkningar. För att hitta de patienter som har mest nytta av statinbehandling är identifiering av

en prediktiv markör viktig, och med hjälp av prediktiva markörer skulle statinbehandling kunna ges till de kvinnor som har mest nytta av behandlingen. En större klinisk studie med statiner, t.ex. i kombination med endokrinbehandling, skulle kunna vara nästa steg för att utforska statiners roll inom bröstcancer.

Samantekt á íslensku (Summary in Icelandic)

Brjóstakrabbamein er algengasta tegund krabbameins hjá konum og reikna má með að um það bil níunda hver kona sé í áhættuhópi á að fá brjóstakrabbamein einhvern tímann á lífsleiðinni. Árið 2014 greindust nær 10 þúsund konur með brjóstakrabbamein í Svíþjóð og fer tilfellum fjölgandi. Lifunin hefur batnað, en í Svíþjóð eru meira en 90% kvenna á lífi fimm árum eftir greiningu brjóstakrabbameins, sem er með því besta sem gerist í heiminum. Skurðaðgerð er aðalmeðferðin við brjóstakrabbameini. Háð eiginleikum krabbameinsins er síðan ráðlögð geislameðferð, krabbameinslyfjameðferð, marksækin meðferð og andhormónameðferð. Því miður fá margar konur seinna endurkomu meinsins og því er þörf á fleiri meðferðarmöguleikum í baráttunni við brjóstakrabbamein.

Blóðfitulækkandi lyf, oft nefnd statin, eru lyf sem eru aðallega notuð til að lækka blóðfiturnar, þ.e. kólesterólið í blóði. Statin eru einnig notuð í forvarnarskyni hjá sjúklingum með hjarta- og æðasjúkdóma. Statin hafa verið lengi á markaðnum, þolast oftast vel og hafa fáar aukaverkanir. Klíniskar rannsóknir hafa sýnt fram á færri dauðsföll hjá hjartasjúklingum sem tóku statin. Statin virka á þann hátt að þau hemja ensímið HMGCR, sem er hraðatakmarkandi ensímið í ferlinu sem nýmyndar kólesteról. Í krabbameinsrannsóknum hafa margar afturskyggnar rannsóknir sýnt fram á að notkun statins geti minnkað líkurnar á endurkomu brjóstakrabbameins og leitt til færri dauðsfalla tengdum brjóstakrabbameini. Í frumu- og dýrarannsóknum hefur verið sýnt fram á að statin geta hamið æxlisvöxt og valdið frumudauða.

MAST (e. MAmmary cancer and STatins) er fasa II klínisk rannsókn sem framkvæmd var á háskólasjúkrahúsinu í Lundi í Svíþjóð á árunum 2009 til 2012 og 50 konur tóku þátt í. Til að rannsaka æxlishemjandi áhrif statins fengu konur með nýgreint brjóstakrabbamein statin í tvær vikur, áður en fyrirhuguð skurðaðgerð fór fram. Hönnun rannsóknarinnar kallast „tækifærisglugga“ rannsókn (“window-of-opportunity”). Í slíkum rannsóknum er tíminn frá greiningu að sjálfri skurðaðgerðinni, venjulega biðtími fyrir sjúklinginn, nýttur til að rannsaka áhrif lyfs, í þessu tilviki atorvastatin 80 mg, sem tekið var daglega. Áður en statin meðferðin hófst var grófnálarsýni tekið úr brjóstakrabbameininu ásamt blóðprufum. Að lokinni statin meðferðinni gengust konurnar undir skurðaðgerð á

hefðbundinn hátt þar sem sýni frá æxlinu var tekið úr skurðsýninu ásamt nýjum blóðprufum. Þessi vefjasýni liggja til grundvallar í greinum I-III í þessu doktorsverkefni, þar sem skoðuð eru áhrif statins á ólíka eiginleika krabbameinsfrumna með því að bera saman krabbameinsvefjasýnin fyrir og eftir statin meðferðina.

Í grein I var rannsakað hvort notkun statins leiddi til breytinga á frumufjölgun (mælt með prótíninu Ki67) auk breytinga á prótíntjáningu (e. protein expression) HMGCR ensímsins, sem statin hemja. Niðurstöðurnar sýna að ekki sást minnkun á frumufjölgun eftir statin meðferðina fyrir alla þátttakendurna. Fyrir þá sjúklinga sem tjáðu HMGCR í vefjasýninu, sem tekið var fyrir upphaf statin meðferðar, sást marktæk lækkun á Ki67 sem gefur hugmynd um að tjáning á HMGCR gæti spáð fyrir um svörun við statin meðferðinni. Almennt jókst prótíntjáning HMGCR marktækt við notkun statina.

Í grein II var skoðað hvort statin meðferð hefði áhrif á tjáningu virkra gena. Niðurstöðurnar sýna að statin jók virkni nokkurra gena meðal annars gena sem tengjast frumudauða. Í frumurannsóknum sást það einnig, auk þess sem aukin virkni gena sást sem tengjast nýmyndun kólesteróls.

Í grein III voru rannsökuð áhrif statins á tvo stjórnendur frumuhringsins, cyclin D1 og p27, sem hafa áhrif á ólíka þætti frumuhringsins. Cyclin D1 getur aukið tilhneigingu til krabbameinsmyndunar á meðan p27 hemja tilhneiginguna. Eftir meðferð með statinum sást minnkuð prótíntjáning á cyclin D1 og aukin tjáning á p27, sem gæti verið ein af mögulegum aðferðum statins við að hemja krabbameinsfrumur.

Grein IV byggist á stórri sænskri framskyggnri rannsókn Malmö Kost Cancer studien (Mataræðis- og krabbameinarannsóknin í Málmey) þar sem þátttakendur voru íbúar í Málmey árin 1991 til 1996. Hér var skoðað hvort prótíntjáningin á HMGCR og notkun blóðfitulækkandi lyfja, meðal annars statina, hefði áhrif á dauða af völdum brjóstakrabbameins. Niðurstöðurnar sýndu að aukin próteintjáning á HMGCR var tengd óhagstæðari eiginleikum brjóstakrabbameinsins. Sjúklingar sem notuðuð blóðfitulækkandi lyf höfðu færri dauðsföll af völdum brjóstakrabbameins, en þetta var ekki marktækt. Hjá sjúklingum sem tjáðu ekki HMGCR eða tjáðu það veikt sást vísbending um að blóðfitulækkandi lyf fækkaði þeim dauðsföllum sem rekja mátti til brjóstakrabbameins.

Þetta doktorsverkefni hefur leitt í ljós nýja vitneskju um áhrif statina á hegðun brjóstakrabbameina, bæði á tjáningu prótína og gena, og eru niðurstöðurnar lofandi en þó takmarkandi vegna smæðar rannsóknarhópsins. Þrátt fyrir núverandi meðferðir eru alltof margir brjóstakrabbameinssjúklingar sem fá endurkomu sjúkdóms og deyja. Það er því mikilvægt að finna fleiri meðferðir í baráttunni

gegn brjóstakrabbameini. Statin er ódýrt lyf, hefur verið á lyfjamarkaðinum lengi og hefur tiltölulega fáar aukaverkanir. Til þess að finna þá sjúklinga sem statin meðferð myndi gagnast best væri ákjósanlegt að finna próf sem gæti spáð fyrir um svörun við statin meðferð, til að meðhöndla einungis þær konur sem hefðu gagn af statinum. Það er því ástæða til að framkvæma stærri klíniska rannsókn, mögulega á statinum með andhormónameðferð, til að rannsaka frekar hlutverk statina í baráttunni gegn brjóstakrabbameini.

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Paper I

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CLINICAL TRIAL

Targeting HMG-CoA reductase with statins in a window-of-opportunity breast cancer trial

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Abstract Lipophilic statins purportedly exert anti-tumoral effects on breast cancer by decreasing proliferation and increasing apoptosis. HMG-CoA reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway, is the target of statins. However, data on statin-induced effects on HMGCR activity in cancer are limited. Thus, this pre-operative study investigated statin-induced effects on tumor proliferation and HMGCR expression while analyzing HMGCR as a predictive marker for statin response in breast cancer treatment. The study was designed as a window-of-opportunity trial and included 50 patients with primary invasive breast cancer. High-dose atorvastatin (i.e., 80 mg/day) was prescribed to patients for 2 weeks before surgery. Pre- and post-statin paired tumor samples were analyzed for Ki67 and HMGCR immunohistochemical expression. Changes in the Ki67 expression and HMGCR activity following statin treatment were the primary and secondary endpoints, respectively. Up-regulation of HMGCR following atorvastatin treatment was observed in 68 % of the paired samples with evaluable HMGCR expression ($P = 0.0005$). The average relative decrease in Ki67 expression following atorvastatin treatment was 7.6 % ($P = 0.39$) in all paired samples, whereas the corresponding decrease in Ki67 expression in tumors expressing HMGCR in the pre-treatment sample was 24 % ($P = 0.02$). Furthermore, post-treatment Ki67 expression was inversely correlated to post-treatment HMGCR expression (rs $= -0.42$; $P = 0.03$). Findings from this study suggest that HMGCR is targeted by statins in breast cancer cells in vivo, and that statins may have an anti-proliferative effect in HMGCR-positive tumors. Future studies are needed to evaluate HMGCR as a predictive marker for the selection of breast cancer patients who may benefit from statin treatment.

Keywords HMGCR · Ki67 · Statins · Breast cancer · Mevalonate pathway

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Introduction

Statins are peroral drugs that historically have typically been prescribed as cholesterol-lowering agents. However, a growing body of literature has addressed their cholesterol-independent pleiotropic effects and suggested favorable preventive effects independent of cholesterol levels on both cardiovascular diseases [32, 41, 42] and cancer [1, 9, 14, 31].

Epidemiological support for the anti-neoplastic properties of statins has been mixed. Several studies have suggested a lower cancer incidence among statin users [9, 13, 24], whereas others have failed to confirm a decreased cancer risk [3, 6, 19, 45]. Recently, a reduced cancer mortality of 15 % was demonstrated among statin users [38]. However, prospective trials are warranted to clarify the impact of statins as an anti-cancer drug [10, 27, 44].

Lipophilic statins purportedly exert anti-tumoral effects on breast cancer by decreasing proliferation and increasing apoptosis [8, 11, 12, 22]. Although the biologic mechanisms for these actions are not fully elucidated, hydroxy-methyl-glutaryl coenzyme A reductase (HMG-CoA reductase or HMGCR) is the well-recognized target of statins [20, 21, 26, 29]. HMGCR acts as the rate-limiting enzyme of the mevalonate pathway, which produces cholesterol, steroid-based hormones, and non-sterol isoprenoids [23, 35]. The isoprenoids demonstrate tumor-suppressive properties as regulators of important hallmarks of cancer, such as proliferation, migration, and angiogenesis [35, 37, 46]. In normal cells with a well-regulated mevalonate pathway, statin-induced HMGCR inhibition triggers a homeostatic feedback response that restores the mevalonate pathway [23]. In tumor cells, the mevalonate pathway may be deregulated by the deficient feedback regulation of HMGCR or increased HMGCR activity [11, 12]. Previous studies have demonstrated intertumoral variation of HMGCR protein expression in human breast cancer [4, 5, 7], thereby suggesting that HMGCR may be a positive prognostic marker and a potential predictive marker for tamoxifen response [5, 7]. Moreover, in response to statin treatment, the HMGCR activity revealed an adaptive induction of HMGCR expression in MCF7 breast cancer cells [18], lung cancer cells [2], and leukemia cells [47]. Currently, no in vivo statin-induced effects on HMGCR activity have been reported.

In total, the literature on statins and cancer indicates the likelihood of an association mediated by the mevalonate pathway with HMGCR as a key player. The aim of this window-of-opportunity study was to investigate the anti-proliferative impact of a 2-week, high-dose statin therapy in patients with invasive breast cancer while assessing the potential of HMGCR as a predictive marker for statin-induced alterations in tumor proliferation.

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 breast cancer diagnosis and surgical tumor resection is used to study the biologic effects of a certain drug. In this study, atorvastatin, a lipophilic statin, was prescribed to the participants for 2 weeks pre-operatively. As a non-randomized trial, all patients received an equal daily dose of 80 mg of atorvastatin for 2 weeks. The trial was conducted as a single center study at Skåne University Hospital in Lund, Sweden. A power calculation showed that a sample size of 43 patients is sufficient to achieve 90 % power to detect a 0.5 standard deviation geometric mean Ki67-difference with a two-sided test at the alpha-level of 0.05. To safeguard against a power drop due to non-evaluable patients, a sample size of 50 was chosen. The Ethical Committee at Lund University and the Swedish Medical Products Agency approved this trial. The study has been registered at ClinicalTrials.gov (i.e., ID number: NCT00816244, NIH). The study adheres to the REMARK criteria [36].

Patients

Women diagnosed with primary invasive breast cancer who had a tumor measuring at least 15 mm and were candidates for radical surgery were eligible for participation in this study. Moreover, a performance status below two according to the European Cooperative Oncology Group (ECOG) and normal liver function as evidenced by normal levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were required at the beginning of the study for eligibility. All patients signed an informed consent form. The exclusion criteria included pregnancy, on-going hormonal replacement therapy, cholesterol-lowering therapy (i.e., including statins, fibrates, and ezetimibe), a medical history of allergic reactions attributed to compounds with a similar biologic composition to that of atorvastatin, and a history of hemorrhagic stroke. The study was opened for recruitment in February of 2009, and the pre-planned number of 50 patients was achieved in March of 2012.

Of the 50 patients enrolled in the study, a total of 42 patients completed all portions of the study. Two of the 50 patients discontinued their participation for personal reasons. One patient was excluded due to elevated levels of serum ALT before treatment initiation, and another patient was excluded because her serum ALT increased beyond the maximum reference levels following 1 week of statin treatment. Another two patients could not complete the pre-planned 2 weeks of statin treatment because their date of surgery was rescheduled to earlier dates. One patient was excluded because the diagnosis of invasive breast cancer was questioned; thus, further investigations were warranted. Finally, one patient left the study due to side effects from the treatment, i.e., nausea and dizziness.

Endpoints and tumor evaluation

The primary endpoint was a statin-induced tumor response measured by the change in tumor proliferation (i.e., Ki67 expression). The secondary endpoints were to study the potential predictive role of HMGCR expression before statin treatment evaluated by change in proliferation as well as the change in HMGCR expression after the administration of pre-surgical atorvastatin during a 2 week "window-of-opportunity" [16, 17]. Following inclusion, the participants underwent a study specific core biopsy before statin treatment initiation. Core biopsies were formalin-fixed immediately. Subsequent to the 2-week statin treatment, breast surgery was performed according to standard surgical procedures, and tumor tissue was retrieved from the primary tumor at the Department of Pathology at Skåne University Hospital, Lund, Sweden.

Formalin-fixed and paraffin-embedded tumor tissue from core biopsies and surgical samples were cut into 3–4 µm sections and transferred to glass slides (Menzel Super Frost Plus), dried at room temperature, and baked in a heated chamber for 2 h at 60°C. Deparaffinization and antigen retrieval were performed using PT Link (Dako Denmark A/S) and a high pH buffer. Staining was performed in an Autostainer *Plus* Dako Denmark A/S) using a di-amino-benzidine (DAB)-based visualization kit (K801021-2, Dako Denmark A/S). Counterstaining was performed using Mayer's hematoxylin with antibodies against Ki67 (MIB1, Cat. No M7240, Dako Denmark A/S, diluted 1:500) and HMGCR (Cat. No HPA008338, Atlas Antibodies AB, Stockholm, Sweden, diluted 1:150). All slides were stained in one batch. Western blot experiments using HPA008338 and UT-1 cell line extracts demonstrated that this antibody recognized a band migrating to ~90 kDa, which is the expected molecular weight of HMGCR (data not shown).

Tumor tissue evaluation for Ki67 was performed via manual counting by one senior breast pathologist (DG), who was blinded to other tumor data on the same specimen and to the corresponding Ki67 staining in the sample pair. A fixed number of 400 tumor cells in both core biopsies and surgical samples were counted from representative areas of the tumor. In a similarly blinded manner, HMGCR expression was evaluated via cytoplasmic intensity using a four-grade scale (i.e., negative, weak, moderate, or strong) as previously described [4, 5, 7]. Two observers simultaneously performed the HMGCR evaluation (OB and SB).

From the 42 patients who completed all portions of the study, paired tumor samples were available from 38 patients because tumor tissue was not found in the core biopsies of four cases. For the analyses of Ki67, a minimum of 400 invasive tumor cells in both the core needle biopsies and surgical specimens were required, which was the case for the samples from 26 patients (Fig. 1).

Statistical analysis

Changes in tumor proliferation following statin treatment were evaluated on both the linear scale (i.e., absolute change) and the log scale (i.e., relative change). Analysis on the linear scale was performed by direct comparison of changes in proportions using a paired t test. After log transformation of the proportions, the same test was used also in the latter case. The average relative change was defined as the geometric mean of the Ki67 ratios. To test for differences in the ordered categorical variable, i.e., the HMGCR intensity before and after statin treatment, the McNemar-Bowker test was used. Logistic regression was used in an analysis comparing the odds of proliferation reduction in HMGCR-negative versus HMGCR-positive cases. Subgroup differences in the distribution of the ordered categorical HMGCR intensity scores were evaluated with the Mann–Whitney U test (i.e., for two groups) or with the Kruskal–Wallis test (i.e., for three groups). Spearman correlation (r_s) was used for quantification of the correlation between Ki67 and HMGCR. All tests were two-sided. For the primary and secondary aim, differences with p-values below 5 % were considered significant, whereas a more stringent cut-off is appropriate for the exploratory subgroup analyses presented in the tables. No adjustment for multiple testing was, however, performed. Two software packages, i.e., Stata version 12.1 (StataCorp LP, College Station, TX, 2012) and IBM SPSS Statistics Version 19, were used for the data analysis.

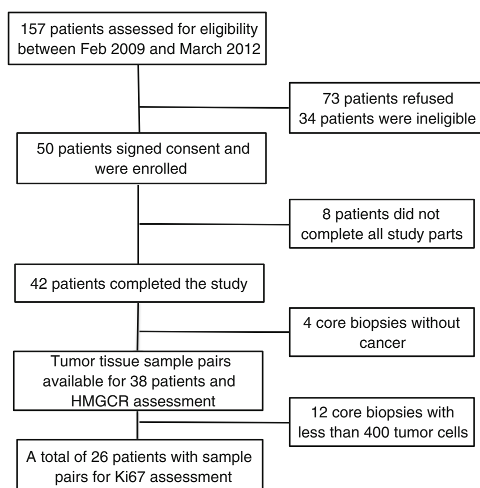

Fig. 1 Flow-chart showing study enrollment

Results

The average age of all 50 patients at the time of inclusion was 63 years with a range from 35 to 89 years, and a similar age distribution was seen among the 42 patients who fulfilled all portions of the study. All the 42 tumors that were examined were indeed invasive breast cancers with an average pathological tumor size (pT) of 21 mm and ranged from 6 to 33 mm. A vast majority of the tumors were estrogen receptor (ER) positive, human epidermal growth factor receptor 2 (HER2) normal, and histologic grade II or III; moreover, most had a low mitotic index. The tumor characteristics were similar for the cohort of 42 patients who completed all portions of the study and the cohort of the 26 patients for whom Ki67 was evaluable (Table 1). For the 26 complete Ki67 pairs, the mean Ki67-index at baseline was positively and significantly associated with both tumor grade and mitotic index (i.e., $P = 0.003$ and $P < 0.001$, respectively) (Table 2). Furthermore, baseline Ki67 was significantly higher in ER negative, progesterone receptor (PgR) negative, HER2 positive, and triple-negative samples. The change in Ki67 following treatment was not associated with the baseline tumor characteristics. The associations between tumor characteristics and HMGCR expression at baseline, HMGCR expression at surgery, and the change in HMGCR expression are shown in Table 3. Baseline HMGCR expression and the change in HMGCR expression were not associated with the tumor characteristics, whereas HMGCR expression in post-atorvastatin samples was positively associated with hormone receptor status.

The primary endpoint in the study, i.e., a change in the Ki67 index following 2 weeks of atorvastatin treatment, was adequately evaluated in 26 paired tumor samples. The Ki67 index had declined in the post-treatment surgical samples in 15 cases and increased in 11 cases as compared to the pre-treatment biopsy samples (Fig. 2a). In the core biopsies, the Ki67 index showed an average of 24.0 % (i.e., with a range of 4.5–87.3 %); in comparison, the average Ki67 index in the surgical samples was 21.9 % (i.e., with a range of 3.0–80.3 %). Therefore, the average absolute reduction was 2.1 percentage points ($P = 0.24$), and the average relative reduction was 7.6 % ($P = 0.39$).

The expression of the target enzyme of statins, i.e., HMGCR, and the potential statin-induced change in expression was the secondary end-point in this study. A total of 38 sample pairs were sufficiently stained and evaluable for scoring of HMGCR intensity. Among the core biopsies collected before statin treatment, HMGCR was not expressed in 37 % of the 38 evaluated samples, weakly expressed in 29 %, moderately in 26 %, and strongly in 8 % of the samples. In contrast, HMGCR expression in surgical samples from the corresponding post-statin treatment tumors was absent in 3 %, weakly expressed in 18 %, moderately expressed in 53 %, and strongly expressed in 26 %. Out of the 38 evaluated cases, the HMGCR scores remained unchanged for nine patients; in contrast, 29 cases were discordant between the core biopsies and surgical samples, and 26 cases demonstrated an increased intensity following statin treatment (Fig. 2b). This change in HMGCR intensity score was highly statistically significant ($P = 0.0005$).

The treatment predictive value of HMGCR was tested in the analyses of tumors with any HMGCR expression in the pre-treatment biopsy samples (Fig. 3a). In this subset of patients (i.e., $n = 24$), the average absolute reduction in the Ki67 index following statin treatment was 4.6 % ($P = 0.03$), and the average relative reduction was 24 % ($P = 0.02$). Cases with absent HMGCR in the pre-treatment biopsy samples (i.e., $n = 14$) had a non-significant, slight average increase in the Ki67 index corresponding to 0.9 % ($P = 0.77$) and a non-significant 15 % increase on the relative scale ($P = 0.33$; Fig. 3b). The change in the Ki67 index in the two HMGCR subgroups was significantly different on the relative scale ($P = 0.02$) but not on the absolute scale ($P = 0.12$). Ignoring the size of the change in the Ki67 index, the odds of a reduction in the Ki67 index was 7.3 times higher in the HMGCR-positive tumors as compared to the HMGCR-negative tumors (OR = 7.3, 95 % CI: 1.3–42, $P = 0.03$). Assuming a linear trend in the Ki67 index changes over the four HMGCR categories (i.e., negative, weak, moderate, or strong), the average decrease was found to be 4.0 % ($P = 0.04$) per category, and the corresponding average relative decrease was 20 % per category ($P = 0.02$). Furthermore, post-treatment Ki67 expression was inversely correlated to post-treatment HMGCR expression (rs = –0.42; $P = 0.03$).

Analyses stratified for histologic grade (i.e., grade I/II vs grade III) and irrespective of HMGCR status showed no statin-induced change in the Ki67 index for grade I/II tumors ($P = 0.95$) and a non-significant absolute reduction of 5.7 % ($P = 0.10$) and a non-significant average relative reduction of 19 % ($P = 0.17$) for grade III tumors (Fig. 3c, d).

Discussion

Herein, we evaluated changes in tumor proliferation following a pre-operative, short-term administration of high-dose atorvastatin and observed a significant, however modest, decrease in proliferation in HMGCR-positive breast cancer. Statin effects were limited to patients with the pre-treatment expression of HMGCR, i.e., the target enzyme for statins. This study indicates that HMGCR may be a predictive marker for statin therapy as the anti-proliferative effect was insignificant in the non-stratified analyses of all tumors.

The potential to use statins as anti-cancer agents in breast cancer has been addressed in previous publications both from an epidemiological point of view [1, 3],

Table 1 Patient and tumor characteristics

	Completed all study portions $n = 42$	HMGCR/Ki67 complete pairs $n = 26$
Mean age (range)	63 (35–89)	63 (35–82)
Tumor size, mm (range)	21 (6–33)	22 (13–32)
Positive nodal status	17 (41 %)	14 (54 %)
Tumor grade (NHG)		
I	9 (21 %)	5 (19 %)
II	17 (41 %)	10 (39 %)
III	16 (38 %)	11 (42 %)
Mitotic index		
1	23 (55 %)	14 (54 %)
2	5 (12 %)	3 (12 %)
3	14 (33 %)	9 (35 %)
ER positive	37 (88 %)	23 (89 %)
PgR positive	33 (79 %)	20 (77 %)
HER2 amplified	7 (17 %)	5 (19 %)
Triple-negative	4 (10 %)	2 (8 %)

Mitotic index according to Nottingham criteria

Triple-negative if ER negative, PgR negative, and HER2 negative

NHG Nottingham histologic grade I-III, *ER* estrogen receptor, positive if >10 %, *PgR* progesterone receptor, positive if >10 %, *HER2* human epidermal growth factor receptor 2

in vitro/in vivo models [8], and in one previous human study [22]. Considering these results in conjunction with recent reviews, the need for prospective trials that consider the anti-cancer potential of HMGCR inhibitors is emerging [10, 12, 44]. As previously demonstrated, HMGCR is differentially expressed showing an intertumoral heterogeneity in human breast cancer [4, 5, 7]. These findings led to the hypothesis that statins may serve as a potential-targeted therapy in breast cancer. This study was designed as a window-of-opportunity study that allowed for the evaluation of the tumor-biologic response following an interventional therapy [16, 17]. In accordance with previous window trials, tumor response as indicated by the change in tumor proliferation measured by the Ki67 index was the primary endpoint [17, 22, 39]. Ki67 is the most widely used marker of tumor proliferation; however, several controversies regarding the counting strategies used with this marker have been raised and were recently addressed in a consensus report for Ki67 assessment [15]. In line with the recommendations from the International Ki67 in Breast Cancer Working Group, this study applied a counting strategy that is applicable for both pre-operative core biopsies and surgical samples. More specifically, we applied a strategy designed to count the average proliferation from across the entire tumor sample, not just the periphery, which is likely to be a highly proliferative zone [15]. In all surgical samples and in 26 out of 42 core biopsies, the objective of counting 400 tumor cells was achieved. However, the number of counted tumor cells might be questioned. Previously reported data have indicated that counting a total of 400 tumor cells is sufficient for the establishment of a valid proliferation index [40]. In our previous report using tumor samples from an untreated cohort, the Ki67 indices in core biopsies and surgical samples were analyzed. The results revealed an absolute higher mean proliferation value of 3.9 % in core biopsies as compared to surgical samples. However, no consistent pattern emerged; i.e., in some cases, the Ki67 index in surgical samples would exceed the index in core biopsies. Consequently, a "correction factor" could not be developed [40]. In our previous study, Ki67 was first evaluated in hotspots. However, the Ki67 consensus report, which was published shortly after our previous study, recommended that Ki67 should be scored as an overall average score for the purpose of consistency while awaiting more robust data from the International Ki67 in Breast Cancer Working Group. In this study, that recommendation was followed, thus making any comparison to our previous hotspot-based counting method difficult. Comparing different sample types for treatment evaluation may not be optimal, and the preferable approach is to compare core biopsies taken at the time of surgery to pre-surgical core biopsies [15]. This study does not have access to core biopsies from surgery; therefore, we applied the recommendation from the consensus report, i.e., with the intention of scoring the surgical sample from fields across the entire tumor [15].

In this study, all patients received an equal dose of the lipophilic statin atorvastatin at the maximum recommended dose to optimize the chances of drug delivery into the breast cancer cells. High-dose atorvastatin was well-tolerated during the two-week administration as evidenced by the fact that only one patient withdrew from the study due to side effects. No serious adverse events were observed. In a previous window-of-opportunity trial on lipophilic statins in breast cancer, a randomized trial design in which patients received either 20 or 80 mg of fluvastatin during a period ranging from 21 to 50 days was applied [22]. All patients in the present study were treated for a period of 2 weeks. The results from the fluvastatin trial and this present study cannot be used to determine whether the duration of statin treatment influences the tumor proliferation results or not. Nevertheless, the results of the two studies were similar despite differences in statin dose and duration. Garwood et al. [22] reported a significant reduction in the Ki67 index in grade III tumors, whereas no significant reduction was demonstrated in the remaining analyses, including all of the 29 sample-pairs. The latter finding corresponds with our results. Regarding the results for the grade III tumors in the present study, we

Table 2 Association of tumor characteristics and baseline Ki67 and change in Ki67

	n	Ki67, % mean (*SD*) pre-atorvastatin	*P*	Change in Ki67 % (*SD*) post–pre atorvastatin	*P*
Age[a]	26	–	(−0.37) 0.06	–	(0.54) 0.005
Tumor size					
≤20 mm	12	25.8 (27.1)	0.64	−3.1 (7.5)	0.54
>20 mm	14	22.4 (13.3)	–	−1.2 (10.1)	–
Nodal status					
Positive	14	19.1 (13.3)	0.29	−1.6 (9.3)	0.92
Negative	12	29.7 (26.0)	–	−2.6 (8.6)	–
Tumor grade (NHG)					
I	5	13.8 (7.9)	0.003	−2.1 (3.8)	0.25
II	10	12.0 (5.7)	–	1.9 (7.5)	
III	11	39.5 (23.0)	–	−5.7 (10.5)	
Mitotic index					
1	14	12.7 (6.5)	<0.001	0.3 (6.8)	0.20
2	3	21.9 (11.8)	–	−6.3 (12.2)	–
3	9	42.3 (24.5)	–	−4.4 (10.6)	–
ER					
Positive	23	18.8 (12.1)	0.02	−1.3 (8.6)	0.40
Negative	3	63.8 (30.9)	–	−7.4 (11.1)	–
PgR					
Positive	20	15.9 (8.6)	<0.001	−0.8 (8.2)	0.30
Negative	6	51.0 (24.3)	–	−6.2 (10.6)	–
HER2 amplified					
Yes	5	33.8 (10.6)	0.03	−6.8 (10.6)	0.20
No	21	21.7 (21.7)	–	−0.9 (8.3)	–
Triple-negative					
Yes	2	81.2 (8.5)	0.02	−5.8 (15.2)	0.70
No	24	19.2 (12.0)	–	−1.8 (8.6)	–

Test of linear trend for variables with three ordered categories and Mann–Whitney *U* test for variables with two categories

Triple-negative if ER negative, PgR negative, and HER2 negative

Mitotic index according to Nottingham criteria

NHG Nottingham histologic grade I-III, *ER* estrogen receptor positive if >10 %, *PgR* progesterone receptor positive if >10 %, *HER2* human epidermal growth factor receptor 2

[a] Spearmans Rho

demonstrated a non-significant 19 % relative reduction in proliferation. However, grade III tumors were significantly associated with high Ki67 expression, which is in agreement with other previous studies [15, 43].

HMGCR is the rate-limiting enzyme in the mevalonate pathway, which is a pathway required for generating a number of fundamental end-products, including cholesterol, isoprenoids, isopentenyladenine, dolichol, and ubiquinone [23]. Deficient feedback control of HMGCR and increased HMGCR expression and activity in tumor cells has been reported in other studies [11], and in this study 2 weeks of statin treatment, resulted in a significant increase in tumor-specific HMGCR expression. This is interpreted as the activation of the negative feedback loop controlling cholesterol synthesis within the mevalonate pathway [11, 12] and corresponds with findings from previous in vitro studies [18]. Furthermore, the demonstrated increase in HMGCR expression subsequent to statin treatment indicates sufficient drug delivery to the breast cancer cells despite atorvastatin's high first-pass metabolism in the gut wall and the liver with an oral bioavailability of 14 % [34].

Interestingly, a recent review by Thurnher et al. [44] addressed the role of statins as an anti-tumor agent through altered protein prenylation from the isoprenoids produced by the mevalonate pathway. Statin-induced inhibition of HMGCR blocks down-stream products in the mevalonate pathway, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Both products are central for protein prenylation [44]. Inhibition of protein prenylation may induce a cellular stress response, thereby generating danger signals and subsequently an

Table 3 Tumor characteristics in relation to HMGCR pre- and post-atorvastatin and change in HMGCR expression

$n = 38$	HMGCR pre-atorvastatin					HMGCR post-atorvastatin					Change in HMGCR			
	Negative	Weak	Moderate	Strong	*P*	Negative	Weak	Moderate	Strong	*P*	Decreased	Unchanged	Increased	*P*
Age[a]	–	–	–	–	(−0.38) 0.02	–	–	–	–	(0.09) 0.58	–	–	–	(−0.08) 0.62
Tumor size														
≤20 mm	5	5	6	2	0.21	1	2	10	5	0.72	2	5	11	0.90
>20 mm	9	6	4	1	–	0	5	10	5	–	1	4	15	–
Nodal status														
Positive	8	4	4	0	0.15	0	3	8	5	0.59	0	3	13	0.90
Negative	6	7	6	3	–	1	4	12	5	–	3	6	13	–
Tumor grade (NHG)														
I	1	4	3	0	0.38	0	0	7	1	0.40	0	2	6	0.16
II	7	3	4	1	–	0	3	7	5	–	2	2	11	–
III	6	4	3	2	–	1	4	6	4	–	1	5	9	–
Mitotic index														
1	7	7	5	1	0.72	0	2	13	5	0.17	1	4	15	0.53
2	2	0	3	0	–	0	1	2	2	–	1	1	3	–
3	5	4	2	2	–	1	4	5	3	–	1	4	8	–
ER														
Positive	12	9	10	2	0.83	0	5	18	10	0.03	2	7	24	0.83
Negative	2	2	0	1	–	1	2	2	0	–	1	2	2	–
PgR														
Positive	9	9	9	2	0.31	0	3	16	10	0.01	2	6	21	0.71
Negative	5	2	1	1	–	1	4	4	0	–	1	3	5	–
HER2 amplified														
Yes	3	1	2	0	0.60	0	3	2	1	0.21	0	1	5	0.98
No	11	10	8	3	–	1	4	18	9	–	3	8	21	–
Triple-negative														
Yes	2	1	0	1	0.80	1	2	1	0	0.02	1	2	1	0.70
No	12	10	10	2	–	0	5	19	10	–	2	7	25	–

Mitotic index according to Nottingham criteria

Triple-negative if ER negative, PgR negative and HER2 negative. *P*-values from exact Mann–Whitney test (two categories) or Kruskal–Wallis test (three categories)

NHG Nottingham histologic grade I-III, *ER* estrogen receptor, positive if >10 %, *PgR* progesterone receptor, positive if >10 %, *HER2* human epidermal growth factor receptor 2, amplified if index with FISH ≥2

[a] Spearmans Rho

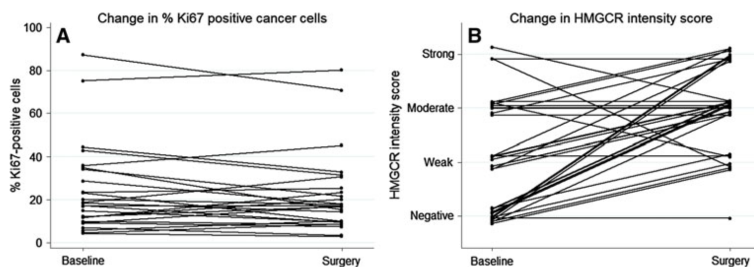

Fig. 2 Change in tumor expression of Ki67 and HMGCR from baseline (i.e., before atorvastatin treatment) to time of surgery (i.e., after atorvastatin treatment). **a** Ki67 ($n = 26$); **b** HMGCR ($n = 38$). Random noise, uniformly distributed over the interval -0.15 to $0.15x$, where x is the arbitrary distance between adjacent categories of the HMGCR intensity scale, has been added to each pair of intensities to visually separate identical otherwise completely overlapping trend lines. This operation does not affect the slopes of the lines

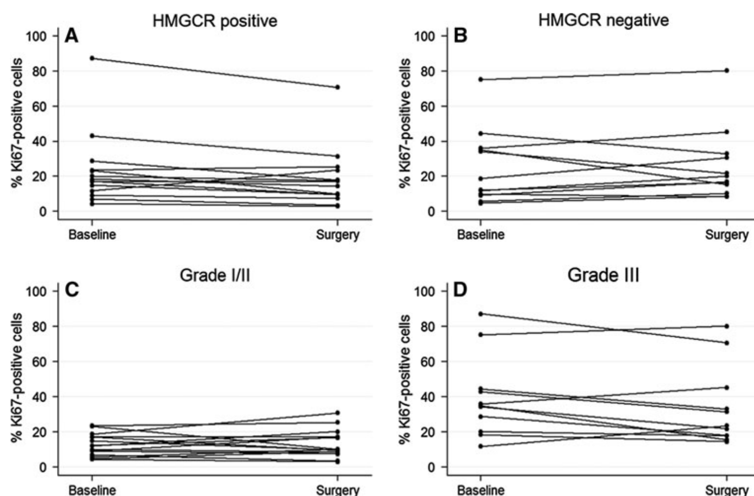

Fig. 3 Change in the Ki67 index measured at baseline (i.e., before atorvastatin treatment) and at surgery (i.e., after atorvastatin treatment) stratified for HMGCR and grade. **a** HMGCR positive; **b** HMGCR negative; **c** grade I/II; **d** grade III

immunological response against the tumor cell [25]. As for the statin-induced anti-proliferative effects indicated in this study, geranylgeranylated proteins may play a central role because they are believed to be essential for cancer cell progression into S-phase [10]. Thus, the mechanisms behind the anti-proliferative effects of statins may depend upon a blockage of the transition of G1-S in the cell cycle [30], which could potentially be mediated by an upregulation of two cyclin-dependent kinase inhibitors, i.e., p21 and p27 [28, 33].

In conclusion, results from this window-of-opportunity trial suggest an upregulation of HMGCR in breast cancer samples following 2 weeks of atorvastatin treatment. The results indicate that HMGCR is targeted in the tumor, and consequently the HMGCR protein is over-expressed depending on feedback loop controlling cholesterol synthesis within the mevalonate pathway. In tumors expressing HMGCR before treatment with atorvastatin, a modest decrease in tumor proliferation was observed. Future studies selecting HMGCR-positive breast cancers may shed further light on the potential anti-proliferative effects exerted by statins.

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Conflict of interest K. Jirström and M. Uhlén hold pending intellectual property in relation to HMGCR as a predictive biomarker in

the treatment of breast cancer. The other authors disclosed no potential conflicts of interest.

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Paper II

Clinical Cancer Research

Global Transcriptional Changes Following Statin Treatment in Breast Cancer

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Abstract

Background: Statins purportedly exert antitumoral 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.

Experimental Design: This window-of-opportunity phase II trial enrolled 50 newly diagnosed breast cancer patients prescribed atorvastatin (80 mg/day) for 2 weeks presurgically. Pre- and posttreatment 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 MDAMB231) 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 upregulated and 375 downregulated genes. Restricted filtration (fold change $\geq$1.49) resulted in 21 upregulated and 46 downregulated genes. Significantly upregulated genes included *DUSP1*, *RHOB1*, *GADD45B*, and *RGS1*. Pooled results from gene ontology, LitVAn and SMART analyses identified statin-induced effects on the apoptotic and MAPK pathways among others. Comparative analyses of gene expression profiles in breast cancer cell lines showed significant upregulation of the mevalonate and proapoptotic pathways following atorvastatin treatment.

Conclusions: We report potential statin-induced changes in global tumor gene expression profiles, indicating MAPK pathway inhibition and proapoptotic events. *Clin Cancer Res; 21(15); 3402–11. ©2015 AACR.*

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-metylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway (1). Furthermore, 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 anticancer 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 and colleagues showed significant changes in high-grade breast cancer tumors, including reduced proliferation and increased apoptosis (5). The complexity of the anticancer 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 biologic 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.

We have previously reported changes in the protein expression of Ki-67 and HMGCR in tumors from breast cancer patients treated with high-dose statins for 2 weeks before 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 pretreatment and posttreatment samples in order to deepen the insight into the molecular mechanisms of statins in breast cancer.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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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 antitumoral 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 2 weeks presurgically. Global gene expression profiling was performed on the pre- and posttreatment tumor sample pairs. This study demonstrates highly significant changes in the expression of genes related to the MAPK 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 anticancer drugs in addition to current treatment guidelines.

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 biologic effects of a certain drug. In this trial, atorvastatin, a lipophilic statin, was prescribed to patients with primary breast cancer for 2 weeks preoperatively. As a nonrandomized trial, all patients received an equal daily dose of 80 mg of atorvastatin for 2 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 preplanned number of 50 patients was achieved in March 2012. Of the 50 patients enrolled in the study, a 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., Ki-67 protein expression). The secondary endpoints included change in tumoral HMGCR expression as well as changes in gene expression following presurgical atorvastatin during a 2-week "window-of-opportunity" period. At the time of enrollment, all participants underwent study specific core biopsies before 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 2-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 ATCC and maintained in culture as recommended by the vendors. Atorvastatin was purchased from Sigma and diluted in 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 DNA/RNA mini kit (QIAGEN) in a QIAcube (QIAGEN) according to the manufacturer's instructions. Before 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), and RNA quantification was performed using a NanoDrop ND-1000 (NanoDrop Products). 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.) 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 reannotated 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 preprocessed 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 $\geq$80% of the samples were excluded. Replicate probe sets were merged by the median of signal intensity values. A principal component analysis 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 because it was not associated with main PCs, we believe the effects on the final results are minimal.

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 posttreatment 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 $-1,500$ and $+500$ 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; ref. 17). A flow diagram outlining the data processing and analyses is shown in Supplementary Fig. S1.

qRT-PCR

Expression levels of *HMGCR* (exon 6–7 and exon 13–14), *DUSP1*, *RHOB*, *JUN*, and *FOS* in the clinical samples were validated using qRT-PCR. Briefly, 1 μg of total RNA was reversely transcribed (Quantitect Reverse Transcription Kit, Qiagen), and cDNA corresponding to 5 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 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, the 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 posttreatment 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 pathologic tumor size was 22 mm ranging from 13 to 32 mm. The majority was ER positive (96%) and 16% were HER2 positive at baseline. HMGCR protein expression was present in 60% of the samples before treatment and 15 out of the 25 tumor pairs demonstrated a decrease in tumor proliferation following statin treatment; assessed by IHC staining for Ki-67 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 upregulated, whereas 375 were downregulated (Supplementary Table S2). Following filtration of 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 $\geq$1.49, a total of 67 genes remained; 21 upregulated and 46 downregulated (Fig. 1), which represent the genes used in all subsequent analyses. Dual-specificity phosphatase 1 (*DUSP1*), also identified as MAPK phosphatase-1, a well-known inhibitor of the MAPK pathway (18), was significantly upregulated. Other highly significant upregulated 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 upregulated 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 biologic 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 toward key genes in the MAPK signaling pathway, including *MAPK*, *ERK*, *JNK*, and *p38*, as visualized in the network.

A subanalysis 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 Ki-67) 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).

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

The TFBS analysis program SMART was applied to further investigate whether genes altered upon statin treatment were potentially coregulated 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).

qRT-PCR validation of candidate genes in pre- and post-statin–treated clinical samples

The results from the qRT-PCR analyses showing the expression ratios of five candidate genes: *HMGCR*, *DUSP1*, *RHOB*, *JUN*, and *FOS* between the pretreatment samples and the posttreatment samples are shown in Supplementary Fig. S2. *DUSP1*, *RHOB*, *JUN*, and *FOS* were found to be upregulated in

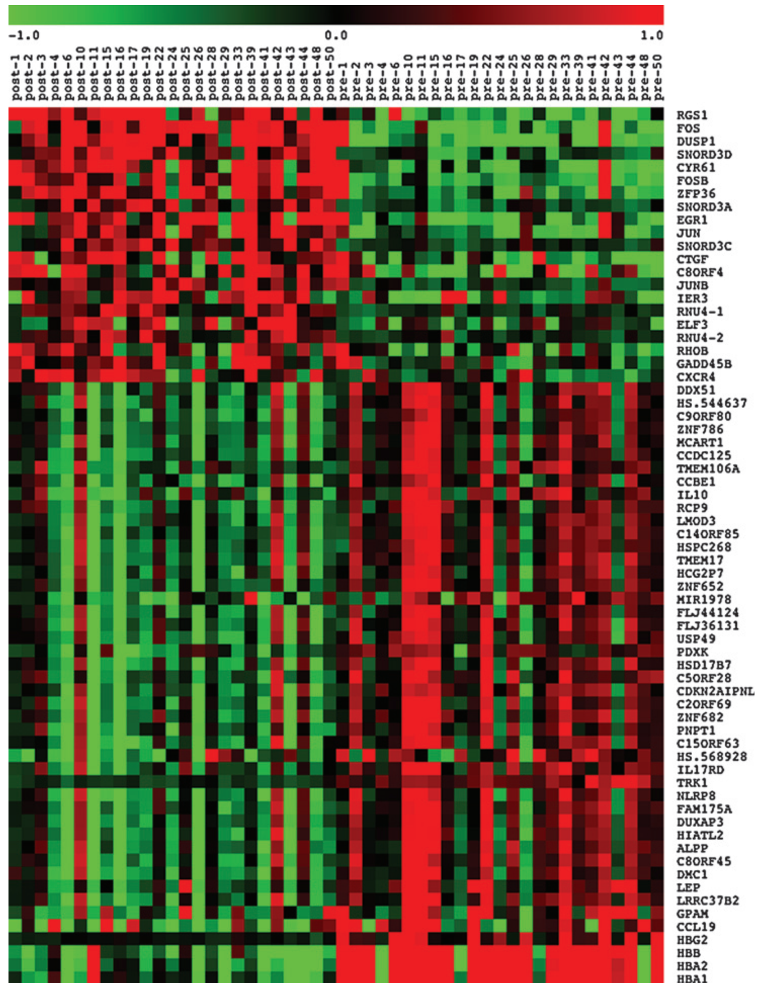

Figure 1.
Heatmap demonstrating the transcriptional changes in pre- and posttreatment samples after 2 weeks of statin therapy. Data generated from SAM analyses followed by filtering out of genes with uncertain functions (LOC genes) and fold change <1.49. Red in the heatmap represents upregulation, and green represents downregulation.

the posttreatment samples by qRT-PCR, displaying relatively similar trends in fold changes as was observed by microarray profiling (Supplementary Fig. S2A). Although *DUSP1*, *RHOB*, *JUN*, and *FOS* were consistently upregulated 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 Ki-67, 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 whether the observed gene expression changes in the clinical samples were directly associated with statin treatment, total RNA extracted from four human breast cancer cell lines was subjected to whole-genome transcriptional profiling following 48-hour atorvastatin treatment. Compared with vehicle (DMSO)-treated controls, 48 hours of statin exposure was found to consistently and significantly upregulate several key genes involved in the cholesterol biosynthesis pathway in all four cell lines (Fig. 4A). Furthermore, a significant downregulation of genes involved in cell proliferation and cell-cycle progression; particularly genes necessary for the G_2–M phase was noted. In addition, a significant upregulation of many proapoptosis genes was noted in some cell lines. Of note, and consistent with the clinical data, a significant

Table 1. Gene ontology and pathway analyses identified deregulated biologic processes using significantly differentially expressed genes following statin treatment as input

	Pathway maps	Total	*P*	FDR	In data	Network objects from active data
1	Transcription_Role of AP-1 in regulation of cellular metabolism	38	1.03926E−13	1.99538E−11	8	c-Jun/c-Fos, AP-1, FosB/JunB, HBB, JunB, c-Jun, c-Fos, Alpha1-globin
2	Reproduction_GnRH signaling	72	3.60723E−13	3.46294E−11	9	c-Jun/c-Fos, FosB, EGR1, AP-1, FosB/JunB, JunB, c-Jun, c-Fos, MKP-1
3	Immune response_MIF-induced cell adhesion, migration and angiogenesis	46	3.71739E−09	2.37913E−07	6	c-Jun/c-Fos, AP-1, c-Jun, c-Fos, MKP-1, CXCR4
4	Immune response_IL3 activation and signaling pathway	31	2.01536E−06	9.67374E−05	4	c-Jun/c-Fos, EGR1, AP-1, c-Fos
5	Immune response_Oncostatin M signaling via MAPK in mouse cells	35	3.3261E−06	0.000127722	4	EGR1, AP-1, c-Jun, c-Fos
6	Immune response_Oncostatin M signaling via MAPK in human cells	37	4.17812E−06	0.000127818	4	EGR1, AP-1, c-Jun, c-Fos
7	Immune response_Human NKG2D signaling	38	4.66002E−06	0.000127818	4	c-Jun/c-Fos, AP-1, c-Jun, c-Fos
8	Development_Growth hormone signaling via PI3K/AKT and MAPK cascades	42	7.0081E−06	0.000149506	4	EGR1, JunB, c-Jun, c-Fos
9	Immune response_Murine NKG2D signaling	42	7.0081E−06	0.000149506	4	c-Jun/c-Fos, AP-1, c-Jun, c-Fos
10	Development_Activation of Erk by ACM1, ACM3 and ACM5	44	8.46451E−06	0.000156068	4	c-Jun/c-Fos, Cyr61, EGR1, c-Fos
	Biological processes					
1	Apoptotic process	1,806	3.571E−07	0.000029	10	CYR61, RHOB, EGR1, IER3, GADD45B, CXCR4, JUN, CTGF, DUSP1, C8orf4
2	Positive regulation of cell death	482	7.331E−08	0.00001042	7	CYR61, RHOB, EGR1, GADD45B, JUN, CTGF, DUSP1
3	Regulation of cell cycle	860	0.000003646	0.000112	7	RHOB, IER3, GADD45B, JUN, JUNB, CTGF, DUSP1
4	MAPK cascade	653	5.765E−07	0.0000345	7	CYR61, FOS, GADD45B, CXCR4, JUN, CTGF, DUSP1

upregulation 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 biologic processes and pathways in the cell lines is presented in Fig. 4B.

Discussion

Despite a plethora of novel anticancer 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 anticancer effects are

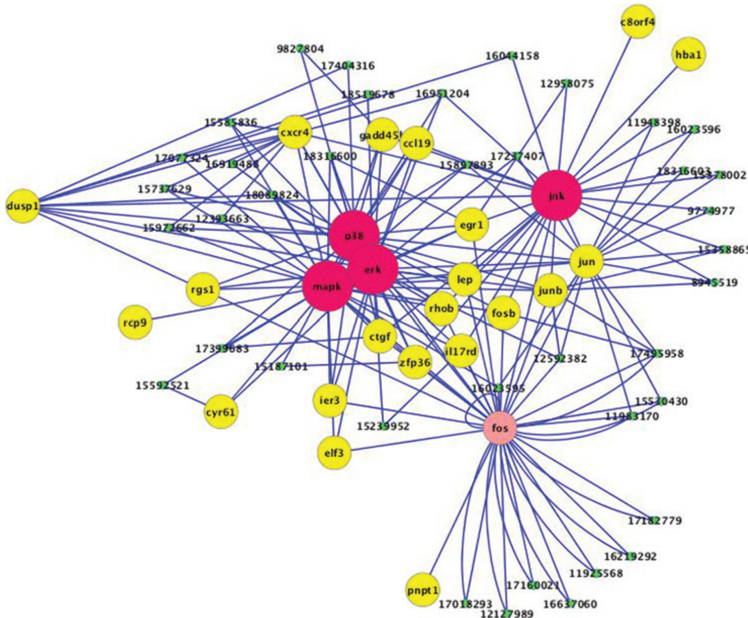

Figure 2.
The suggested gene function modality affected by 2 weeks of statin therapy as visualized by LitVAn.

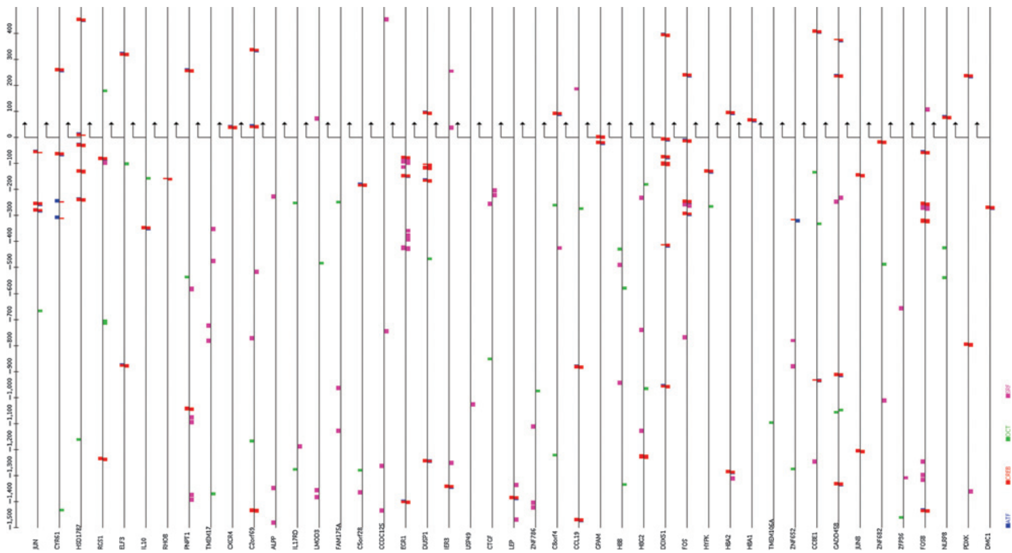

Figure 3.
Illustration of the binding sites for transcription factors identified by SMART analyses. Enriched TFBS were identified using the −1,500,+500 portions of the promoters. Colored boxes indicate TFBS for the respective genes from the gene expression analysis.

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). Furthermore, the mechanisms behind the anticancer effects induced by statins are not fully understood, and translational studies within clinical trials addressing the biologic 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 2 weeks of presurgical statin treatment, suggesting inhibition of the MAPK pathway and increased apoptosis. A number of significant upregulated 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 upregulation 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 toward *DUSP1* itself. In this context, several studies addressing the role of *DUSP1* in cancer have reported conflicting results. In a study performed by Pervin and colleagues, 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 downstream products is necessary. In this study, the expression of *DUSP1* was highly upregulated in the clinical samples after statin treatment, whereas *in vitro*, upregulation of other DUSPs (*DUSP4* and *DUSP6*) was observed. Of note, *DUSP*s specifically dephosphorylate threonine and tyrosine residues on MAPKs and render them inactive. Further studies are warranted to elucidate the importance of *DUSPs* in breast cancer and their role in mediating the anticancer effects exerted by statins.

Apoptosis was found to be significantly upregulated following statin treatment both *in vivo* in clinical samples and *in vitro*. *GADD45B*, an important proapoptotic gene, was upregulated in the clinical samples. *GADD45s* play important roles in a plethora of cellular processes, including growth control and apoptosis (30). The MAPKs p38 and JNK have complex roles in the regulation of *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 antiproliferative target genes upon GnRH receptor activation in cell lines, *DUSP1*, *JUNB*, *FOS*, and

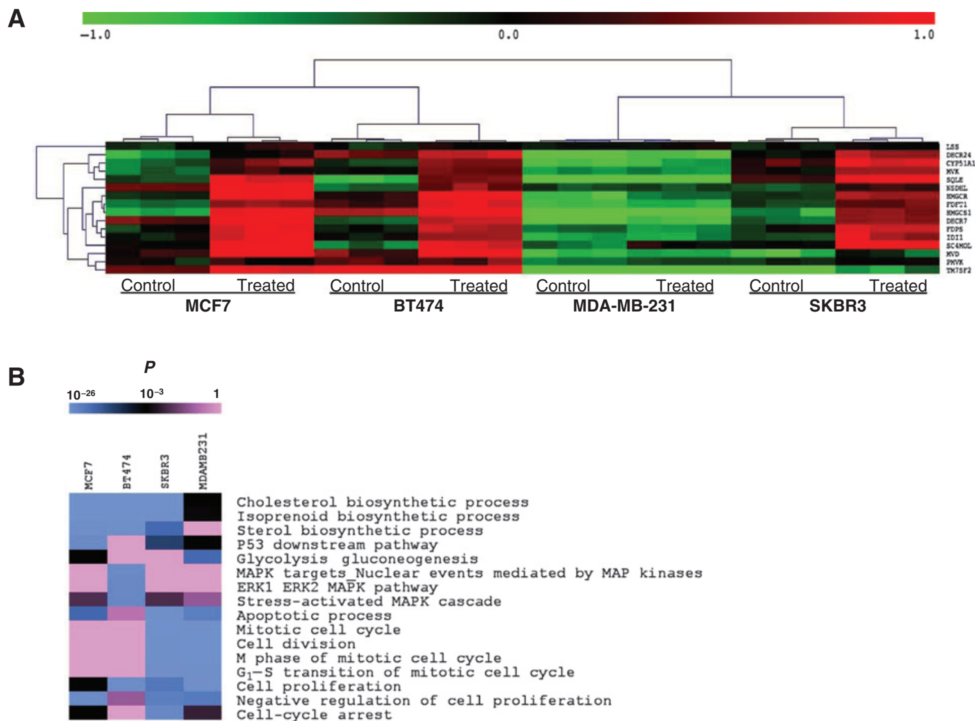

Figure 4.
In vitro statin induced transcriptional changes. A, upregulation of genes involved in cholesterol biosynthesis (mevalonate pathway) was observed in four breast cancer cell lines following 48-hour atorvastatin treatment. B, summary of altered biologic processes and pathways following 48-hour atorvastatin treatment in breast cancer cell lines.

FOSB were among the upregulated genes (32). Wu and colleagues showed that GnRH type II induces apoptosis in human endometrial cancer cells by activating *GADD45A* (33). Furthermore, 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 upregulated 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 are 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 downstream products such as isopreonids. The inhibition of isoprenylation results in the inactivation of small GTPase proteins. Unlike its family members RhoA and RhoC, which are often overexpressed in different types of cancers, RhoB has been reported to be downregulated 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 and colleagues showed downregulation of AP-1 and NF-κB 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 upregulated 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.

The mechanism of statin-induced antiproliferative and proapoptotic effects in cancer cells is currently not clear. Functional *in vitro* and *in vivo* studies have demonstrated mutant *p53*-dependent upregulation of genes in the mevalonate pathway in *p53*-mutant breast cancer cells (36). Furthermore, induction of RhoA-dependent retention of the transcription factor NF-κB, leading to transcriptional downregulation of the antiapoptotic protein BCL2, and reduced production of AKT1 upon statin treatment has been reported (37). Induction of apoptosis via activation of the *JNK/CHOP/DR5* proapoptotic pathway has also been shown

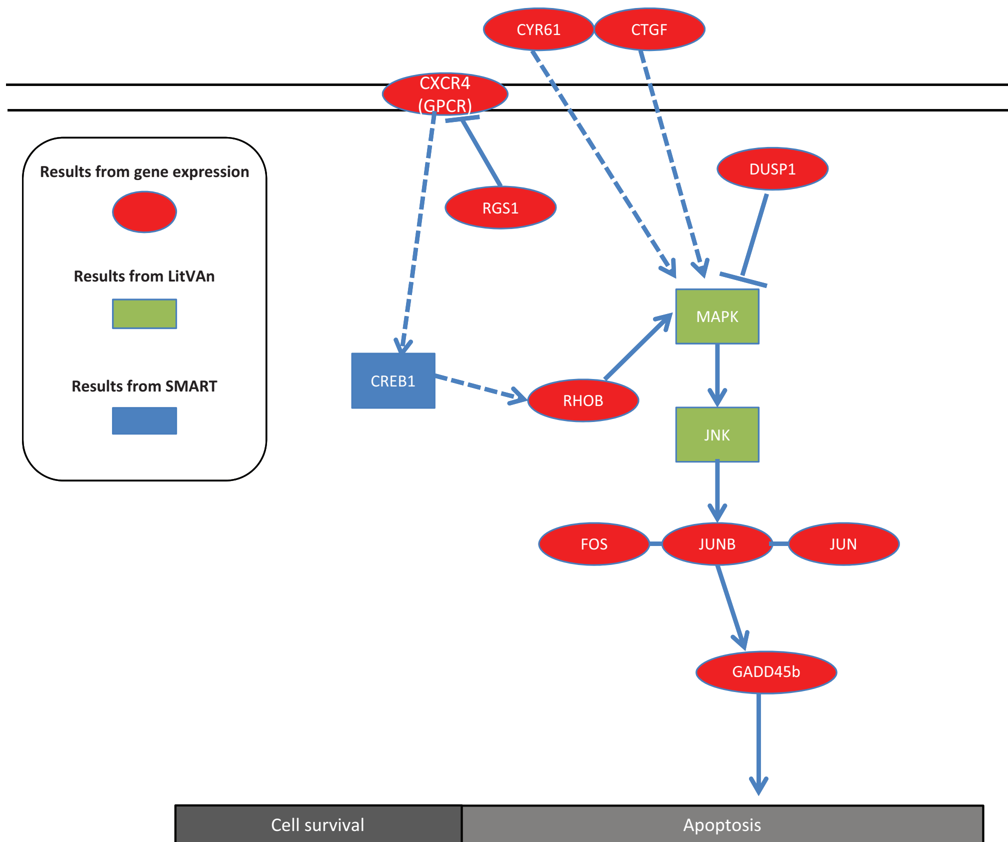

Figure 5.
The hypothetical apoptosis network of combined data from the gene expression, LitVAn and SMART analyses. Red ovals are upregulated genes from the gene expression analysis. Green squares show results from the LitVAn analysis, and blue squares show TFBSs from the SMART analysis. → indicates expression/activation, and ⊥ indicates inhibition. Solid lines represent connections that have been shown in the literature, and dotted lines represent hypothetical connections.

(38). Koyuturk and colleagues 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 anticancer 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). Spampanato and colleagues demonstrated that statins induced apoptosis in breast cancer cell lines, with increased expression of the proapoptotic gene *BAX* and decreased expression of the antiapoptotic 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 upregulation 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 MAPK lead 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 (TIL) 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 pretreatment samples showed a significant decrease in Ki-67 after statin treatment (11). Both microarray and qRT-PCR analyses showed no significant differences in *HMGCR* mRNA expression between the pre- and posttreatment samples. However, some sample pairs showed a clear upregulation, whereas others displayed downregulation 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 upregulation of genes involved in the mevalonate pathway, including *HMGCR*, was observed. This upregulation 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 transcription factor (1). The diverse results on mevalonate pathway inhibition seen in the clinical samples compared with 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 apoptotic effects. We investigated transcriptional changes after 2 weeks of statin treatment, which limits the study to one time point. The treatment time required to translate transcriptional activities to a measurable biologic 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P.-O. Bendahl, S. Borgquist
Development of methodology: S. Kimbung
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Kimbung, D. Grabau, S. Borgquist
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Bjarnadottir, S. Kimbung, I. Johansson, S. Veerla, P.-O. Bendahl, D. Grabau, I. Hedenfalk, S. Borgquist
Writing, review, and/or revision of the manuscript: O. Bjarnadottir, S. Kimbung, I. Johansson, S. Veerla, P.-O. Bendahl, D. Grabau, I.A. Hedenfalk, S. Borgquist
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O. Bjarnadottir, S. Kimbung, M. Jönsson, S. Borgquist
Study supervision: S. Borgquist

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Paper III

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Statin-induced anti-proliferative effects via cyclin D1 and p27 in a window-of-opportunity breast cancer trial

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Abstract

Purpose: Cholesterol lowering statins have been demonstrated to exert anti-tumoral effects on breast cancer by decreasing proliferation as measured by Ki67. The biological mechanisms behind the anti-proliferative effects remain elusive. The aim of this study was to investigate potential statin-induced effects on the central cell cycle regulators cyclin D1 and p27.

Experimental design: This phase II window-of-opportunity trial (Trial registration: ClinicalTrials.gov NCT00816244, NIH) included 50 patients with primary invasive breast cancer. High-dose atorvastatin (80 mg/day) was prescribed to patients for two weeks prior to surgery. Paired paraffin embedded pre- and post-statin treatment tumor samples were analyzed using immunohistochemistry for the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and the cell cycle regulators cyclin D1 and p27. Corresponding frozen tumor sample pairs were analyzed for expression of the genes coding for cyclin D1 and p27, CCND1 and CDKN1B, respectively.

Results: Forty-two patients completed all study parts, and immunohistochemical evaluation of ER and PR was achievable in 30 tumor pairs, HER2 in 29 tumor pairs, cyclin D1 in 30 tumor pairs and p27 in 33 tumor pairs. The expression of ER, PR and HER2 did not change significantly following atorvastatin treatment. Cyclin D1 expression in terms of nuclear intensity was significantly decreased ($P = 0.008$) after statin treatment in paired tumor samples. The protein expression of the tumor suppressor p27, evaluated either as the fraction of stained tumor cells or as cytoplasmic intensity, increased significantly ($P = 0.03$ and $P = 0.02$, respectively). At the transcriptional level, no significant differences in mRNA expression were detected for cyclin D1 (CCND1) and p27 (CDKN1B). However, CCND1 expression was lower in tumors responding to atorvastatin treatment with a decrease in proliferation although not significantly ($P = 0.08$).

Conclusions: We have previously reported statin-induced anti-proliferative effects in breast cancer. This study suggests that cell cycle regulatory effects may contribute to these anti-proliferative effects via cyclin D1 and p27.

Keywords: Cyclin D1, P27, Ki67, Statins, Breast cancer

Background

Statins, a major class of drugs for treatment of hypercholesterolemia, are widely used due to a notable prevention of cardiovascular disease, and accumulating evidence proposes a promising role of statins in breast cancer [1]. Statins act by inhibiting 3-hydroxy-3methylglutaryl coenzyme-A reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway, thereby reducing intracellular cholesterol production [2]. In addition to their lipid-lowering capacity, statins exert several other effects mediated by different products of the mevalonate pathway. These lipid-independent effects include inhibition of inflammatory responses, immunomodulatory actions, apoptotic and anti-proliferative effects, which might contribute to the suggested anti-tumoral effects of these agents [3,4]. The epidemiological evidence projecting statins as anticancer agents is variable, depending on the particular type of cancer in question as well as the class of statin used [5-9].

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Recent data suggest that lipophilic statins may be preferable over hydrophilic statins as anticancer agents [10,11]. In breast cancer, previous studies have shown lipophilic statin use following a breast cancer diagnosis to be associated with a decreased risk of disease recurrence and with reduced breast-cancer mortality [8,12,13]. Results from a phase II study with statins prescribed in the pre-surgical setting have demonstrated reduced tumor cell proliferation and increased apoptosis in patients with high grade *in situ* breast cancer [14]. The anti-proliferative effects of statins were confirmed in invasive breast cancer, as reported in a previous publication from the same trial on which this study is based [15]. In both studies, the anti-proliferative effects were described in terms of decreased intra-tumoral levels of Ki67 [14,15]. However, the comprehensive biological mechanisms behind this anti-proliferative effect are currently not clear. Ki67 is the most widely used clinical biomarker for assessing the proliferative status of a breast cancer. Ki67 is expressed during all active phases of the cell cycle (G1, S, G2, M), but is absent in resting cells (G0) [16,17]. The cell cycle is a complex and strictly controlled series of events, driving cell division and replication of DNA. In normal cells, progression through the cell cycle is controlled by the cyclin dependent kinases (CDKs), a family of serine/threonine kinases [18]. The CDKs form complexes with their regulatory units, cyclins, thereby activating the CDKs, leading to phosphorylation of the cell cycle regulatory proteins that initiate and regulate progression through the different phases of the cell cycle [19]. In breast cancer cells, the cell cycle control system is deregulated at multiple levels, leading to abnormal cell proliferation [20]. Cyclin D1 is a vital regulator of the G1/S transition, as illustrated in Figure 1. The interaction of cyclin D1 with CDK4 and CDK6, leads to phosphorylation and thereby inactivation of the Rb-protein and its G1-maintaining function, which culminates in the expression of proliferation-associated target genes [21,22]. Cyclin D1 is overexpressed at the protein level in up to 50% of all primary breast cancers, in part due to amplification of the cyclin D1 gene, CCND1 [23]. The CDK inhibitor p27, also known as Kip1, is involved in the regulation of the G0-to-S-phase transition. p27 interacts with CDK2-cyclin E, CDK4/6-cyclin D, and CDK2-cyclin A complexes, thereby regulating these complexes strictly [24,25]. The tumor suppressor p27 is frequently deregulated in breast cancer, and reduced p27 expression has been associated with increased proliferation, high tumor grade, HER2 amplification as well as estrogen receptor (ER) and progesterone receptor (PR) negativity [25,26].

The aim of this study was to investigate potential statin-induced effects on the central cell cycle regulators cyclin D1 and p27, to improve the understanding of the statin induced anti-proliferative effects previously reported. A secondary aim was to evaluate the expression of clinically established biomarkers, such as the estrogen receptor, progesterone receptor and HER2 before and after atorvastatin treatment, hypothesizing no statin-induced changes of their expression. These aims were addressed in a phase II window-of-opportunity trial with two-week, pre-operative high-dose atorvastatin therapy in 50 patients with primary invasive breast cancer.

Materials and methods

Trial design

The trial was designed as a window-of-opportunity study, in which the participants were prescribed the lipophilic statin atorvastatin for two weeks, during the treatment-free window between breast cancer diagnosis and surgery. The study was opened for recruitment in February 2009, and the pre-planned number of 50 patients was achieved in March 2012. In this non-randomized phase II trial, all patients received an equal dose of 80 mg atorvastatin daily. The trial was conducted as a single center study at Skåne University Hospital in Lund, Sweden.

The Ethical Committee at Lund University and the Swedish Medical Products Agency approved this trial. The study has been registered at ClinicalTrials.gov (i.e., ID number: NCT00816244, NIH). The study adheres to the REMARK criteria [27].

Patients and tumors

Patients diagnosed with primary invasive breast cancer with a minimum tumor size of 15 mm measured by ultrasound, who were candidates for radical surgery, were eligible for participation in this study. A performance status below 2 according to the European Cooperative Oncology Group (ECOG) and normal liver function were also required for inclusion. Pregnancy, on-going hormonal replacement therapy, cholesterol lowering therapy (i.e., including statins, fibrates, and ezetemibe), a medical history of hemorrhagic stroke or allergic reactions attributed to compounds with a similar biological composition to that of atorvastatin encompassed the exclusion criteria. Complete information regarding the study inclusion and exclusion criteria, as well as clinical and pathological characteristics of the patients and tumors have been described in detail previously [15]. Following inclusion, the participants underwent study specific tumor core biopsies prior to statin treatment initiation with one core biopsy being formalin-fixed immediately and one being fresh frozen at −80°C. Subsequent to the two-weeks statin treatment, breast surgery was performed according to standard surgical procedures, and tumor tissue was retrieved from the primary tumor at the Department of Pathology at Skåne University Hospital, Lund, Sweden. Of the 50 patients enrolled in the study, a total of 42 patients completed all study parts. Two patients were excluded from the trial since

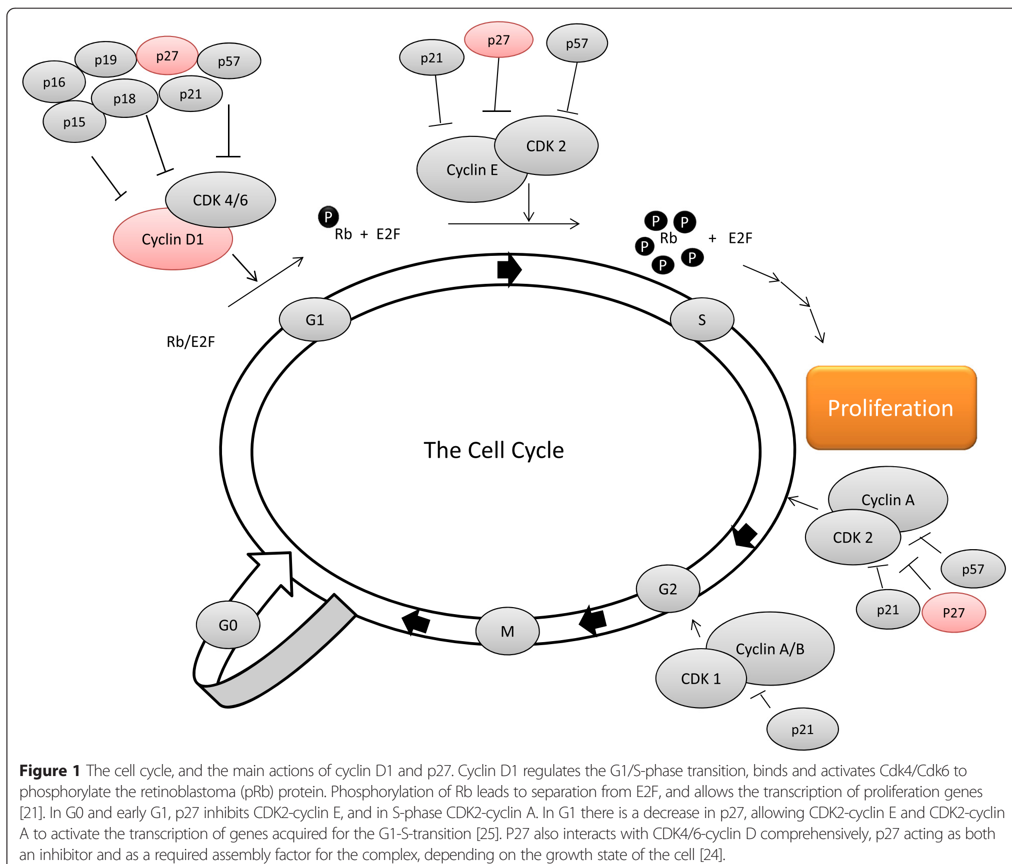

Figure 1 The cell cycle, and the main actions of cyclin D1 and p27. Cyclin D1 regulates the G1/S-phase transition, binds and activates Cdk4/Cdk6 to phosphorylate the retinoblastoma (pRb) protein. Phosphorylation of Rb leads to separation from E2F, and allows the transcription of proliferation genes [21]. In G0 and early G1, p27 inhibits CDK2-cyclin E, and in S-phase CDK2-cyclin A. In G1 there is a decrease in p27, allowing CDK2-cyclin E and CDK2-cyclin A to activate the transcription of genes acquired for the G1-S-transition [25]. P27 also interacts with CDK4/6-cyclin D comprehensively, p27 acting as both an inhibitor and as a required assembly factor for the complex, depending on the growth state of the cell [24].

date of surgery was pre-scheduled after enrollment. Two patients were excluded due to elevated serum levels of alanine aminotransferase. One patient was excluded since the diagnose of invasive breast cancer was questioned, one patient left the study due to nausea and dizziness and two patients left due to personal reasons.

Endpoints and tumor evaluation

The primary endpoint of the clinical trial was statin-induced anti-proliferative tumor response measured by a decrease in Ki67 expression, as previously reported [15]. The purpose of this sub-study was to investigate potential effects of statin treatment on the expression of ER, PR, and HER2 as well as the expression of the cell cycle regulators cyclin D1 and p27.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor tissue from core biopsies and surgical samples were cut into 3 to 4 μm sections and transferred to glass slides (Menzel Super Frost Plus), dried at room temperature, and baked in a heated chamber for 2 hours at 60°C. De-paraffinization and antigen retrieval was performed using PT Link (Dako Denmark A/S) using a high pH buffer. Staining was performed in an Autostainer *Plus* (Dako) using a di-amino-benzidine (DAB) based visualization kit (K801021-2, Dako). Counterstaining was performed using Mayer's hematoxylin with antibodies against ER (SP1, Thermo Scientific, diluted 1:200), PR (Dako M3569, diluted 1:200), HER2 (4B5, Ventan BenchMark Ultra, Ventana Medical Systems, Inc. Tucson, Arizona, R.U.), cyclin D1 (Dako M3635, diluted 1:40), and p27 (Dako M7203, diluted 1:100).

Tumor biomarker assessment

ER and PR expression was evaluated as the fraction of stained nuclei, using a five-grade scale (i.e. 0-1%, 2-10%, 11-50%, 51-75% and >75% of stained cells). HER2 was evaluated using the HercepTest guidelines (DAKO, Carpinteria, CA) for scoring of HER2. No staining observed in <10% of the tumor cells was scored 0, faint staining observed in >10% of the tumor cells was scored 1+, weak to moderate staining in >10% of the tumor cells was scored 2+, and strong staining in >10% of the tumor cells was scored 3+, according to the guidelines. Assessment of cyclin D1 and p27 protein expression was evaluated by considering the fraction of stained nuclei, using a five-grade scale (i.e. 0-1%, 2-10%, 11-50%, 51-75% and >75% of stained cells), and nuclear intensity and cytoplasmic intensity, using a four-grade scale (i.e. negative, weak, moderate or strong) (Additional file 1: Figure S1 and Additional file 2: Figure S2). For Ki67 assessment, 400 tumor cells were evaluated and Ki67 expression recorded as the fraction of positive nuclei using a continuous scale from 0 to 100 [15].

RNA extraction

Total RNA was extracted from fresh frozen tumor samples using the Allprep DNA/RNA mini kit (QIAGEN, Valencia, CA) in a QIAcube (Qiagen) according to the manufacturer's instructions. 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). The 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.lth.se/sciblu). The Illumina probes were re-annotated using the R package illuminaHumanv4.db [28]. The microarray study was conducted within another sub-study of the trial and comprehensive analyses of the data are subject of currently unpublished work. Thus, in this study only analyses concerning the expression of the probes representing cyclin D1 and p27 are reported herein.

Statistical analysis

All assessed immunohistochemical tumor variables were measured on ordinal scales. Changes in ER, PR, HER2, cyclin D1 and p27 protein expression between pre- and post-atorvastatin treatment samples were evaluated using the Wilcoxon matched-pairs signed-rank test. Spearman's rho was used as a measure of the correlation between change in cyclin D1 and Ki67, and p27 and Ki67, respectively. To test for subgroup differences, the Linear-by-linear association was used. All tests were two-sided and differences with P-values below 5% were considered significant. The software packages Stata version 12.1 (StataCorp LP, College Station, TX, 2012) and IBM SPSS Statistics Version 19, were used for the data analysis.

For microarray data analysis, all data were initially pre-processed and normalized using the Quantile Normalization method [29]. The GenomeStudio Software V2011.1 was used for the analyses. Probe sets with signal intensity below the median intensity of negative control signals in 80% of the samples were excluded. Replicate probe sets were merged by the median of signal intensity values. A Significance Analysis of Microarrays (SAM) analysis was performed using the TMeV v4.9 software to identify differences in expression of CCND1 and CDKN1B between paired pre- and post-statin treatment samples. Furthermore, changes in the expression of CCND1 and CDKN1B between tumor pairs stratified into two groups according to statin-induced changes in Ki67 expression were evaluated using the Mann–Whitney U-test. Changes in tumor proliferative rate, quantified by IHC analysis of the expression of Ki67, have been previously reported [15].

Results

Patient characteristics and tumor data

Fifty patients entered the trial; a total of 41 patients were reported as postmenopausal and nine patients as pre-menopausal. Forty-two patients completed all study parts. No serious adverse events were reported. At the time of diagnosis, the average age among all 42 patients was 63 years (range 35–89 years). The average pathological tumor size was 21 mm, ranging from 6 to 33 mm and all 42 tumors were invasive breast cancers. Most tumors were ductal cancers, and the majority of tumors were histological grade 2 or 3 (Table 1).

Changes in the expression of ER, PR, HER2

The evaluation of ER and PR was achievable in 30 tumor pairs and HER2 in 29 pairs, respectively, whereas the remaining pre-treatment biopsies showed insufficient amount of tumor tissue for immunohistochemical evaluation of these markers. The baseline expression of ER, PR and HER2 is shown in Table 1. When contrasting the pre- and post-treatment samples, neither ER, PR nor HER2 changed significantly (Wilcoxon matched-pairs signed-rank test $P = 0.68$, $P = 0.19$, and $P = 0.08$ for ER, PR and HER respectively; Table 2) and the null hypothesis of equal expression before and after statin treatment was retained.

Changes in the expression of cyclin D1

Immunohistochemical evaluation of cyclin D1 was achievable in 30 of the 42 paired samples restricted by insufficient amount of tumor tissue in the remaining core biopsies. Table 3 shows cyclin D1 expression in the pre-treatment samples. A comparison of the expression of cyclin D1 between pre-and post-treatment samples is

Table 1 Patient- and tumor characteristics

Patients completed all study parts		n = 42
Age years (mean, range)		63 (35–89)
Tumor size mm (mean, range)		21 (6–33)
Positive nodal status		17 (41%)
Tumor grade (NHG)		
	I	9 (21%)
	II	17 (41%)
	III	16 (38%)
Mitotic index		
	1	23 (55%)
	2	5 (12%)
	3	14 (33%)
ER (n = 30)		
	Positive	27 (90%)
	Negative	3 (10%)
PR (n = 30)		
	Positive	24(80%)
	Negative	6 (20%)
HER2 (n = 29)		
	0	7 (24%)
	1+	10 (34%)
	2+	7 (24%)
	3+	5 (17%)
Ki67 index (n = 26)		
	Low	15 (58%)
	High	11 (42%)
HMGCR (n = 38)		
	Positive	24 (63%)
	Negative	14 (37%)

NHG Nottingham histologic grade I-III (post-treatment pathological report), Mitotic index according to Nottingham criteria (post-treatment pathological report).
Baseline tumor data (pre-treatment): ER (estrogen receptor), PR (progesterone receptor), HER2 (human epidermal growth factor receptor 2), Ki67 high if >20%, HMGCR positive if any cytoplasmic staining.

shown in Figure 2. In general, the majority of samples expressed cyclin D1. However, the nuclear intensity of the protein expression was significantly decreased (P = 0.008, Wilcoxon matched-pairs signed-rank test) following statin treatment (Table 2). Furthermore, cyclin D1 expression was assessed regarding the fraction of stained nuclei as well as the intensity of cytoplasmic staining, but neither the nuclear fraction nor the cytoplasmic intensity changed significantly following treatment. No significant association was found between the pre-treatment tumor characteristics in relation to the change in cyclin D1 following atorvastatin treatment (Additional file 3: Table S1).

Changes in the expression of p27

Immunohistochemical evaluation of p27 could be performed for 33 of the 42 paired tumor samples. Prior to atorvastatin treatment, all samples demonstrated tumor cells expressing p27 to a different extent as shown in Table 3. Following atorvastatin treatment there was a significant increase in the fraction of tumor cells expressing p27 (P = 0.03, Wilcoxon matched-pairs signed-rank test, Table 2 and Figure 3). The nuclear intensity of p27 did not change significantly (P = 0.35). Further, the cytoplasmic intensity of p27 was significantly increased after atorvastatin treatment (P = 0.02, Wilcoxon matched-pairs signed-rank test). Baseline tumor characteristics in relation to the change in p27 expression following atorvastatin treatment are summarized in Additional file 4: Table S2, for which no significant associations were found.

Correlation between change in Ki67 and change in cyclin D1 or p27

Spearman´s correlation was used to evaluate whether a change in the expression of cyclin D1 or p27 was accompanied by a change in proliferation as determined by Ki67. We observed that a decrease in Ki67 corresponded positively with a decrease in cytoplasmic intensity of cyclin D1 (N = 25, P = 0.03, Spearman's rho = 0.43), as illustrated in Figure 4. No significant associations were detected between the decrease in Ki67 and the change in nuclear fraction or nuclear intensity of cyclin D1, or the change in Ki67 and the change in p27 irrespective of cellular localization or staining intensity.

mRNA expression of proliferation associated genes

Initially, we compared the expression of CCND1 and CDKN1B between paired pre-and post-treatment samples. Good quality gene expression data were available for twenty-five tumor pairs; no statistically significant difference in the expression of these genes was noted. Next, a sub-analysis comparing the mRNA levels of CCND1 and CDKN1B was performed after dividing samples into two groups based on changes in Ki67 expression as assessed by IHC. Ki67 expression was decreased in 15 samples while 10 samples showed an increased expression as previously reported [15]. Separate analyses were performed for the pre- and post-treatment samples. As illustrated in Figure 5A, the expression of CCND1 in the pre-treatment samples was significantly correlated to response in tumor cell proliferation (P = 0.02; Mann–Whitney). Correspondingly, in the post-treatment samples, a marginally lower CCND1 expression was observed among the tumors responding with a decrease in Ki67 compared to tumors with an increase in Ki67 (Figure 5B; P = 0.08; Mann–Whitney). CDKN1B mRNA expression did not differ significantly between tumors responding with a Ki67 response or not (Figure 5C-D; P = 0.3, 0.06: Mann–Whitney).

Table 2 Change in tumor expression from baseline (i.e. before atorvastatin treatment) to time of surgery (i.e. after atorvastatin treatment)

	Complete pairs	Decreasing	Unaltered	Increasing	P-value
ER	*n* = 30	2	25	3	0.68
PR	*n* = 30	3	21	6	0.19
HER2	*n* = 29	7	20	2	0.08
Cyclin D1 nuclear fraction	*n* = 30	4	19	7	0.12
Cyclin D1 nuclear intensity	*n* = 30	14	13	3	0.008*
Cyclin D1 cytoplasmic intensity	*n* = 30	10	14	6	0.48
p27 nuclear fraction	*n* = 33	2	22	9	0.03
p27 nuclear intensity	*n* = 33	9	18	6	0.35
p27 cytoplasmic intensity	*n* = 33	3	18	12	0.02

P-values from Wilcoxon matched-pairs signed-ranks test.
ER estrogen receptor, PR progesterone receptor, HER2 human epidermal growth factor receptor 2.
*Significant even after Bonferroni adjustment for multiple testing within the marker, P = 0.02.

Table 3 Cyclin D1 and p27 tumor expression in the pre-treatment setting

Patients completed all study parts	n = 42
Cyclin D1 nuclear fraction (*n* = 30)	
Negative	2 (7%)
Low (1-50%)	12 (40%)
High (51-100%)	16 (53%)
Cyclin D1 nuclear intensity (*n* = 30)	
Negative	2 (7%)
Weak	5 (12%)
Moderate	14 (33%)
Strong	9 (30%)
Cyclin D1 cytoplasmic intensity (*n* = 30)	
Negative	11(37%)
Weak	14 (47%)
Moderate	2 (7%)
Strong	3 (10%)
p27 nuclear fraction (*n* = 33)	
Negative	0
Low (1-50%)	8 (24%)
High (51-100%)	25 (76%)
p27 nuclear intensity (*n* = 33)	
Negative	0
Weak	3 (9%)
Moderate	18 (55%)
Strong	12 (36%)
p27 cytoplasmic intensity (*n* = 33)	
Negative	17 (52%)
Weak	14 (42%)
Moderate	2 (6%)
Strong	0

Discussion

In the present study, we investigated the effects of short-term administration of a high-dose of atorvastatin on the conventional breast cancer pathological markers ER, PR, HER2, as well as the cell cycle regulators cyclin D1 and p27. Our results indicate that ER, PR and HER2 expression remain stable following treatment with atorvastatin. However, a significant decrease in cyclin D1 expression and a significant increase in p27 expression were observed, indicating that the anti-proliferative effects of statins may be driven by the cell cycle regulatory effects of cyclin D1 and p27.

There is a rising interest in statins, due to their effects extending beyond their well-known lipid-lowering capacity [3]. As previously reported from this trial, a significant decrease in tumor proliferation, in terms of decrease in Ki67 expression, was noted especially in the sub-set of tumors expressing HMGCR at baseline [15]. This difference in proliferation may be driven by changes in the cell cycle regulators cyclin D1 and p27, as has been addressed in this study. It has been proposed that the anti-proliferative and pro-apoptotic effects of statins are due to the inhibition of downstream isoprenoid intermediates, such as farnesyl-pyrophosphate (FPP) and geranyl-geranyl-pyrophosphate (GGPP) [30-32]. FPP and GGPP are molecules which post-translationally modify a number of proteins by creating a hydrophobic domain, thereby allowing the proteins to anchor to cell membranes and perform their normal functions, a process known as protein prenylation [33]. Protein prenylation is necessary for the activation of many proteins participating in signaling pathways on which tumors depend, such as the RAS/Rho superfamily. RAS-dependent pathways regulate the expression of both p27 and cyclin D1, the assembly of cyclin D1 with CDK4/6, and the growth factor-induced regulation, transcription, and stabilization of cyclin D1 [34].

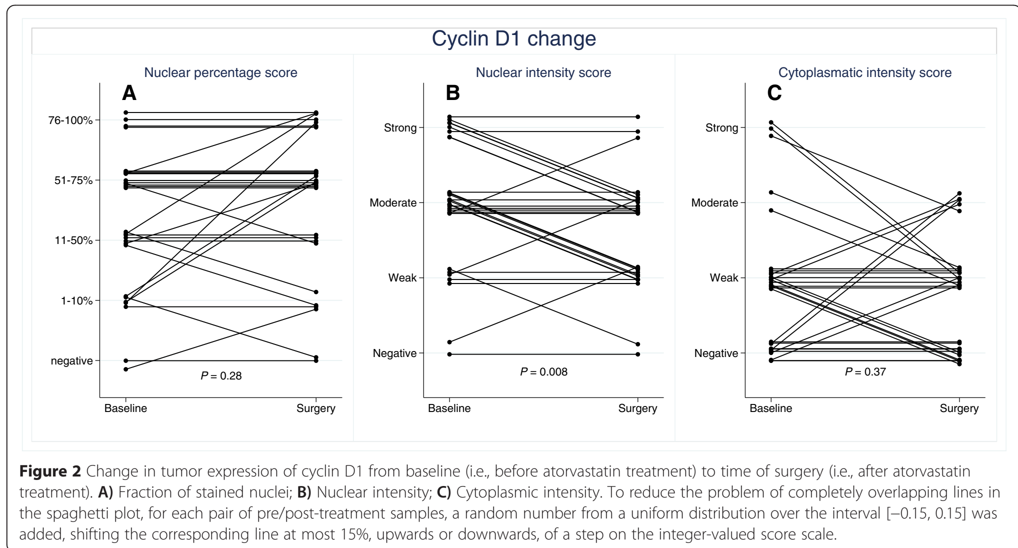

Figure 2 Change in tumor expression of cyclin D1 from baseline (i.e., before atorvastatin treatment) to time of surgery (i.e., after atorvastatin treatment). **A)** Fraction of stained nuclei; **B)** Nuclear intensity; **C)** Cytoplasmic intensity. To reduce the problem of completely overlapping lines in the spaghetti plot, for each pair of pre/post-treatment samples, a random number from a uniform distribution over the interval [−0.15, 0.15] was added, shifting the corresponding line at most 15%, upwards or downwards, of a step on the integer-valued score scale.

In concordance with our results, statins have been shown to inhibit cell growth, with G1 arrest, leading to reduced transition to the S and G2/M phases of the cell cycle [35]. Both cyclin D1 and p27 are involved in the regulation of these transitions, cyclin D1 through the association with CDK4 and CDK6, and p27 by interacting with the CDK2/cyclin E, CDK2/cyclin A, and CDK4/6-cyclin D complexes. A decrease in cyclin D1 entails that p27 is released from the CDK4/6-cyclin D complex, and instead able to assemble with, and inhibit CDK2, thereby promoting cell cycle arrest and inhibit proliferation [24]. This suggests that a statin induced cell cycle arrest at G1 could be

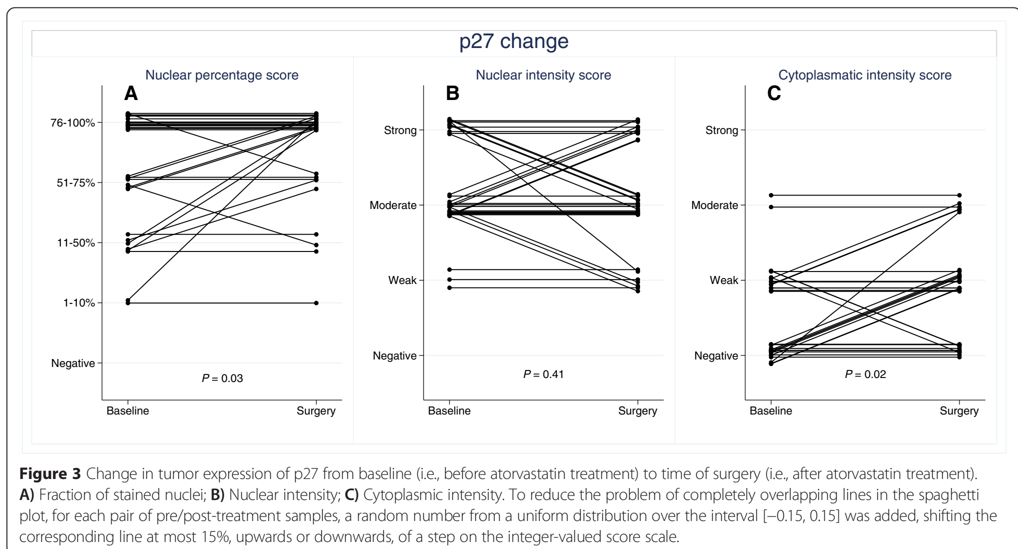

Figure 3 Change in tumor expression of p27 from baseline (i.e., before atorvastatin treatment) to time of surgery (i.e., after atorvastatin treatment). **A)** Fraction of stained nuclei; **B)** Nuclear intensity; **C)** Cytoplasmic intensity. To reduce the problem of completely overlapping lines in the spaghetti plot, for each pair of pre/post-treatment samples, a random number from a uniform distribution over the interval [−0.15, 0.15] was added, shifting the corresponding line at most 15%, upwards or downwards, of a step on the integer-valued score scale.

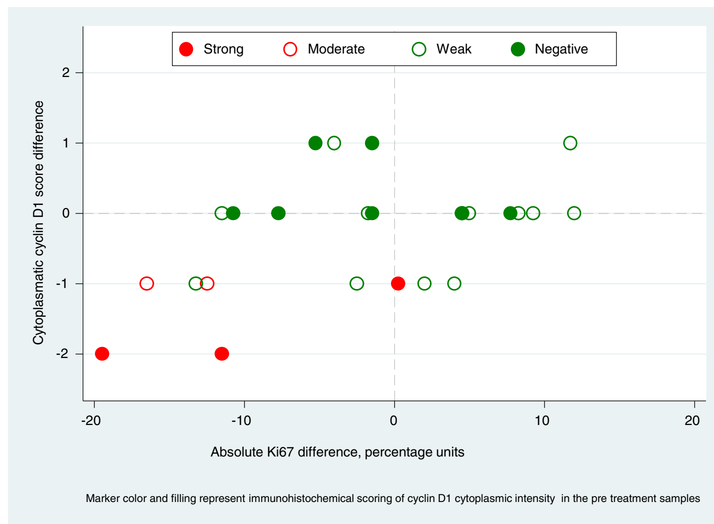

Figure 4 Correlation between change in Ki67 and change in cyclin D1 (cytoplasmic intensity) after treatment with atorvastatin. Marker color and filling represents immunohistochemical scoring of cyclin D1 cytoplasmic intensity in the pre-treatment samples; filled red circles (strong cyclin D1 intensity), red empty circles (moderate cyclin D1 internsity), green empty circles (week cyclin D1 intensity), green filled circles (no cyclin D1 expression).

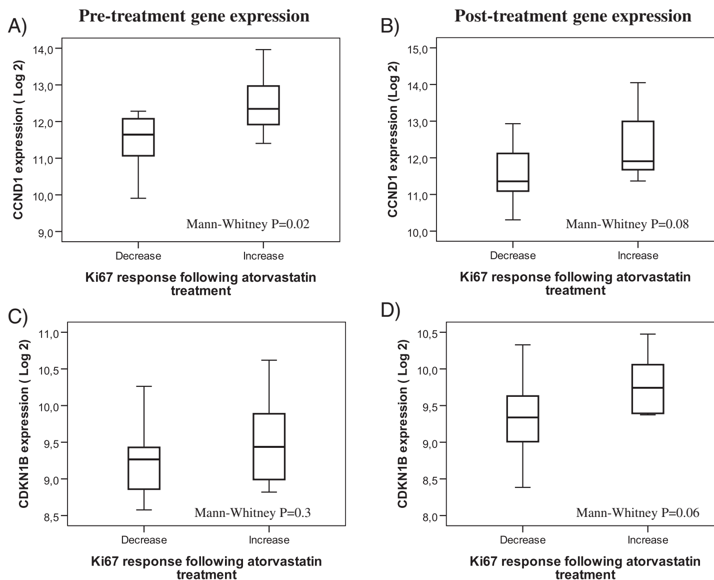

Figure 5 Expression of CCND1 and CDKN1B pre- and post-atorvastatin treatment, divided into tumors responding with a decrease or increase in proliferation (Ki67) following statin treatment. **A)** Pre-treatment CCND1 expression, **B)** Post-treatment CCND1 expression, **C)** Pre-treatment CDKN1B expression, **D)** Post-treatment CDKN1B expression.

the result from a decrease in cyclin D1 expression, and a corresponding increase in p27 as suggested by our data. Previous *in vitro* studies have shown similar results with a statin induced up-regulation of p27 [36-38] and reduced levels of cyclin D1 [39] in various tumor cell lines. Cyclin D1 and p27 are both regulated by a plethora of different signal transduction pathways [25,40], and the underlying mechanisms of the observed decrease of cyclin D1 and increase of p27 in this study is not evident. Given the suggested effects of statins on cell cycle regulators and the recent approval of a CDK4/6-inhibitor for first-line treatment of advanced ER positive breast cancer [41], further studies examining the potential synergistic effects of statins and CDK4/6 inhibitors would be of clinical importance.

The expression of clinically established biomarkers such as ER, PR and HER2 was evaluated in both pre-and post-treatment samples to establish whether these markers were affected by statin treatment. The vast majority of samples pairs remained unchanged. Recently, the cholesterol metabolite 27-hydroxycholesterol (27HC), has been revealed to increase ER-dependent growth in mouse models of breast cancer [42]. In the endocrinological field of research, 27HC has been linked to a decrease in bone mineral density, in part due to its ability to bind to and modulate the transcriptional activity of ER [43]. An *in vitro* study showed that simvastatin exerted osteoinductive effects, partly achieved through an increase in ER expression [44]. Regarding HER2, signaling through this receptor is dependent on the cholesterol content of the lipid rafts [45]. Thus, statins may theoretically enable changes in the expression of both steroid receptors and HER2. Such changes were not detected in this study. However, the treatment duration of only two weeks might be insufficient to índuce changes in ER or PR expression, due to their relatively stable nature [46,47]. The absence of a significant change in the expression of ER, PR and HER2 might be of clinical interest, indicating that statin treatment can be administrated safely to breast cancer patients without altering clinically used prognostic and treatment predictive markers. In the immunohistochemical evaluations of cyclin D1 and p27, expression was scored for both percentage of positive nuclei, nuclear intensity and cytoplasmic intensity. Currently, established scoring systems for immunohistochemical evaluation of cyclin D1 and p27 are not available. In a review by Chu *et al.*, [25] most prognostic studies scored p27 based on the percentage of positive tumor nuclei, with various cut-offs. Others scored both the percentage of positive nuclei and the intensity of the staining. Most studies however omitted scoring of cytoplasmic expression of p27. In this study, immunohistochemical evaluations demonstrated significant changes regarding the cyclin D1 nuclear intensity, fraction of p27 stained cells, and the cytoplasmic intensity of p27. Both cyclin D1 and p27 exert their effects on the G1/S transition control when localized to the nucleus [25,48] A decrease of cyclin D1, results in p27 no longer being sequestrated by the CDK4/6-cyclin D complex to the same extent. Data suggest that the cell favours maintainance of low levels of p27 in the nuclear space, and subsequently mislocalize p27 to the cytoplasmic compartment when levels of nuclear p27 are increased [24], which may explain the concurrent increase in expression of p27 in both the nuclear and cytoplasmic compartments in this study. Importantly, data from functional studies suggest that cytoplasmic translocation of p27 can change its function in tumor cells [49], thus promoting other functions opposite to its tumor suppressor role, e.g. cell migration [50]. A review by Guan *et al.* concluded that further studies were needed to understand the role of cytoplasmic p27 in breast cancer [26]. However, the significance of the cellular localization of p27 cannot be explained based on the results from this study. The cytoplasmic intensity of cyclin D1 was associated with Ki67 (Figure 4), although expression did not change significantly during treatment. During G1, cyclin D1 accumulates in the nucleus, but is exported to the cytoplasmic space when the cell enters S-phase [48], possibly implying a more intense cytoplasmic cyclin D1 staining in high proliferating aggressive tumors, a correlation found in pancreatic adenocarcinoma [51], and suggestively explaining the positive correlation with Ki67. Further, gene expression analyses of paired tumor samples were performed. Only marginal changes in CCDN1 and CDKN1B expression were observed following two weeks of statin treatment. However, the cell cycle-dependent changes in cyclin D1 and p27 can both ensue through other mechanisms, including post transcriptional deregulation. [23,25]. The gene expression of CCND1 was found to be significantly correlated to response in tumor cell proliferation, indicating a difference in the response to statins between cancers with or without CCND1 overexpression.

Whether and how the dose or duration of statin treatment influences the here presented results is unclear and cannot be further elucidated from this trial, as all patients in the study were given atorvastatin for two weeks at the maximum recommended dose to optimize the drug delivery into the breast cancer cells. No serious adverse events were observed, and only one patient withdrew from the study due to side effects, indicating that the treatment with high-dose atorvastatin was well-tolerated during the two-week administration. To gain more insight regarding the statin-induced effects on expression of cell cycle regulators, prolonged treatment duration may be neccessary to demonstrate the maximal effect on cell cycle regulators. However, due to ethical considerations, this window-study was not able to extend the time from diagnosis to surgery, which restricted the duration of statin treatment to two weeks. Thus, as

implied in the trial design and purpose of window-trials, these trials can generate adequate hypotheses which should preferably be evaluated in larger phase III trials [52]. As recently proposed by Ahern et al., the existing evidence supporting a protective effect of statins on breast cancer prognosis, is considered sufficient to launch a clinical phase III trial with statins in the adjuvant setting [1].

Conclusions

In conclusion, the results from this window-of-opportunity study indicate a statin induced effect on central cell cycle regulators, in terms of an up-regulated expression of the tumor suppressor p27 and down-regulated expression of the oncogene cyclin D1 in breast cancer. The results are concordant with previous trial results, and suggest that cell cycle regulatory effects may be contributing to the anti-proliferative effects via cyclin D1 and p27.

Additional files

Additional file 1: Figure S1. Examples of immunohistochemical cyclin D1 staining with negative nuclear and cytoplasmic expression **(a)**, weak nuclear and cytoplasmic expression **(b)**, moderate nuclear and cytoplasmic expression **(c)**, and strong nuclear and weak cytoplasmic expresssion **(d)**, respectively.

Additional file 2: Figure S2. Examples of immunohistochemical p27 staining with weak nuclear and negative cytoplasmic **(a)**, moderate nuclear and weak cytoplasmic **(b)**, moderate nuclear and cytoplasmic **(c)**, and strong nuclear and moderate cytoplasmic **(d)** expression, respectively.

Additional file 3: Table S1. Core biopsy tumor characteristics in relation to the change in cyclin D1 nuclear fraction, nuclear intensity and cytoplasmic intensity, pre- to post-atorvastatin.

Additional file 4: Table S2. Core biopsy tumor characteristics in relation to the change in p27 nuclear fraction, nuclear intensity and cytoplasmic intensity, pre- to post-atorvastatin.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MF contributed to the immunohistochemical analyses and interpretation of data, and in drafting the manuscript. OB participated in the gene expression analyses and in drafting the manuscript. SK contributed to the analysis and interpretation of data, and in drafting the manuscript. KJ has contributed to the conception and design of the trial, and in revising the manuscript. PB carried out the statistical analyses, and revised the manuscript. SV contributed to the analysis and interpretation of the gene expression data. DG contributed to the collection of immunohistochemical data and revision of the manuscript. IH contributed to the acquisition of gene expression data and analyses, and revision of the manuscript. SB was conducting the trial and contributed to the acquisition, interpretation and analyses of the data and in drafting the manuscript. All authors have read and given their approval of the final manuscript to be published.

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Paper IV

Cholesterol-lowering medication use, HMGCR expression, and breast cancer survival – The Malmö Diet and Cancer Study

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Running title: Cholesterol-lowering medication use in breast cancer

Keywords: cholesterol-lowering medication, HMG-CoA reductase, breast cancer, prognosis, mortality

Conflicts of Interest: S. Borgquist reports having received consultant fees from Novartis and Roche. No conflicts of interest were disclosed by the other authors.

Abstract

Background

Cholesterol-lowering medication (CLM), including statins, are commonly used to treat hypercholesterolemia and prevent cardiovascular events. Recent studies have suggested statins as possible anti-cancer agents, but the identification of a predictive marker is essential. The 3-hydroxy-3-metylglutaryl-coenzyme-A reductase (HMGCR) inhibited by statins may serve as such.

Methods

Based on a population-based prospective cohort study, the Malmö Diet and Cancer Study, tumor expression of HMGCR was assessed by immunohistochemistry on tissue microarrays from 910 women diagnosed with primary breast cancer between 1991-2010. HMGCR antibody validation through siRNA knockdown tested four different HMGCR antibodies for their specificity. Information on CLM use was retrieved from the Swedish Prescribed Drug Register, and cause of death from the Swedish Death Registry. Breast cancer specific mortality (BCM) according to CLM use and HMGCR expression was analyzed using Cox regression models.

Results

Of 910 breast cancer patients, 326 had been prescribed CLM during the years 2005 through 2014, 74 patients before and 252 after their breast cancer diagnosis. HMGCR expression was assessable for 657 women; 119 (18%) showed negative, 354 (54%) weak and 184 (28%) moderate/strong expression. The evidence for association between moderate/strong HMGCR and higher histological grade, high Ki67 and ER negativity, respectively, was strong (all $P<0.001$). HMGCR expression was, however, not independently associated with BCM. CLM use was associated with a 36% reduction in BCM ($HR_{age\text{-}adj.}$0.64), but the evidence for an effect was weak (95%CI 0.25-1.60, P=0.34). No evidence of associations between HMGCR expression and BCM was found, although a plausible protective effect of CLM use was seen in the no/weak HMGCR group ($HR_{age\text{-}adj.}$0.16, 95%CI 0.02-1.40, P=0.10).

Conclusions

HMGCR expression was associated with prognostically adverse tumor characteristics. Among breast cancer patients on CLM, no or weak HMGCR expression appeared favorable. These suggested associations need to be tested in larger cohorts.

Introduction

Statins are a group of cholesterol-lowering medications (CLM) most commonly used by patients with cardiovascular diseases and hypercholesterolemia. Cholesterol is produced by the mevalonate pathway, a metabolic pathway that also produces precursors for steroid hormones and isoprenoids (1). Statins exert competitive inhibition of the rate-limiting enzyme of the mevalonate pathway, 3-Hydroxy-3-methylglutharyl-coenzyme A reductase (HMGCR), an enzyme that has been found to be differentially expressed in breast cancer tumors (2). In recent years, attention in cancer research has been drawn to the mevalonate pathway since statins have been found to exert pleiotropic intratumoral effects such as induction of apoptosis and inhibition of proliferation, suggesting a possible effectiveness in cancer (3). In breast cancer, statins have also demonstrated a number of anti-neoplastic properties in preclinical studies of breast cancer cells (4-7). These findings are supported by epidemiological data showing protective associations between statin use and breast cancer recurrence and thus prognosis (8-10). However, biomarkers for selection of patients that may benefit from statins are needed.

Previous studies have explored the correlation between tumor cell expression of HMGCR and breast cancer prognosis as well as the association with statin treatment. One study found that high levels of HMGCR tumor expression in breast cancer was associated with favorable clinicopathological characteristics such as smaller tumor size, low histological grade, estrogen receptor (ER) positivity, and low proliferation (2). Data were confirmed in another study cohort demonstrating HMGCR as an independent prognostic marker, associated with an improved recurrence-free survival, particularly in ER-positive tumors (11). In a study based on a prospective cohort, The Breast Cancer (BC) Blood study, HMGCR expression was associated with less aggressive tumor characteristics (12). Yet another study correlated HMGCR expression as a predictor of response to tamoxifen (13). These findings evaluating HMGCR protein expression have, however, been challenged by HMGCR mRNA expression data, which have showed HMGCR to be inversely associated with breast cancer recurrence rates (14, 15).

The specificity of the HMGCR antibodies previously used may impact on these controversies, and herein we have applied a novel and extensively validated anti-HMGCR monoclonal HMGCR antibody recently developed. Thus, the aim of this study was to explore and clarify the association between CLM use, HMGCR expression based on a novel antibody, and breast cancer prognosis.

Materials and Methods

The Malmö Diet and Cancer Study (MDCS)

To examine associations between life-style factors and cancer a population-based prospective cohort study, the Malmö Diet and Cancer Study, was initiated in Malmö, Sweden and enrolled healthy volunteers between 1991-1996 (16). All inhabitants living in Malmö by 1st of January 1991 and born 1926-1945 (in year 1995 extended to men born 1923-1945 and women born 1923-1950) were invited to participate. Subjects with insufficient understanding of the questionnaire due to lower mental abilities or insufficient Swedish language skills were excluded from enrollment. Approximately 40% of the source population participated in the study (17), and from the female population a total of 17,305 women joined the study. At baseline, data were collected from interviews, questionnaires, and health examinations. In addition, measures of body constitution and blood samples were taken. Written informed consent was obtained from all participants. Ethical permission was obtained from the Ethical Committee at Lund University (Dnr 472/2007).

Study population, patient characteristics, and follow-up

Through linkage of the MDCS female cohort with the Swedish Cancer Registry and the South Swedish Tumor Registry, a total of 1,016 women were identified with an incident breast cancer diagnosed during follow-up until December 31st, 2010. This study only includes primary breast cancer cases and thus the study population has excluded women with primary incident breast cancer diagnosed prior to enrollment in the MDCS (n=576). Information regarding vital status and cause of death was retrieved from linkage of the MDCS breast cancer database to the Swedish Cause of Death Registry with end of follow-up as by December 31st, 2014.

Cholesterol-lowering medication

Information on CLM use, including expedition date, was obtained from the Swedish Prescribed Drug Register from July 2005, when the registry was initiated, through 2014. With the Anatomical Therapeutic Chemical (ATC) Classification, the use of statins and other cholesterol-lowering agents (e.g. fibrate and ezetimibe) were identified. In the register, statins comprised almost 94% of all cholesterol-lowering prescriptions.

Tumor and histopathological analyses

Information on tumor histological type, size, Nottingham grade and axillary lymph node involvement (ALNI) was retrieved from pathology reports for tumors diagnosed from 2005 and onwards, whereas immunohistochemical (IHC) assessed markers were obtained from tissue microarrays (TMAs) from 1991-2007 (2), and from pathological reports from 2008 and onwards for estrogen receptor (ER), progesterone receptor (PR), proliferation index (Ki67) and human-epidermal growth factor-2 (HER2) status. Breast cancers diagnosed before 2006 were reevaluated regarding invasiveness, histological type, and grade (18). For ER and PR, a fraction of stained nuclei >10% was used as cut-off for positivity, in accordance with South Swedish clinical guidelines. For Ki67, a 10% threshold was used given the non-defined level for high or low expression at the current time of Ki67 evaluation. When available, *in situ* hybridization (ISH) was used for HER2 evaluation. When ISH-information was not available, HER2 IHC was considered positive (HER2+) when annotated as 3+ and negative (HER2-) for 0 or 1+. HER2 2+ scoring was considered missing if not confirmed to be either amplified or normal in the ISH analysis.

HMGCR antibody validation

The specificity of several anti-HMGCR antibodies to the HMGCR antigen was validated by genetic and pharmacological strategies that alter the expression of HMGCR. Briefly, to decrease the expression of HMGCR, the MCF-7 breast cancer cell line was transfected with 25nm of ON-TARGET plus HMGCR siRNA pool (L-009811-00-0005, Human HMGCR 3156 siRNA, Dharmacon) constituted in DharmaFECT (Dharmacon)- transfection reagent. Following 24hr of transfection, cells were maintained in full growth medium for another 48h after which total protein and total RNA were extracted for western blotting and RT-qPCR respectively as previously described (15). Alternatively, to upregulate HMGCR expression, MCF-7 cells were exposed to 5µM atorvastatin (Sigma-Aldrich) for 24 hrs after which total protein and total RNA extracted for western blotting and RT-qPCR respectively. Furthermore, HMGCR protein expression was assessed in the HEPG2 Liver cancer cell line and the Chinese hamster ovary cell lines CHO-K1 and UT-1 (derived from CHO-K1 following prolonged exposure to mevastatin) as additional positive controls. The following anti-HMGCR antibodies were screened; HMGCR AMAb90619 and HMGCR AMAb90618 (mouse monoclonals, 1:1000, Atlas Antibodies, Sweden), HMGCR ab174830 (rabbit monoclonal, 1:300, Abcam, Sweden) and HMGCR A-9 (mouse monoclonal, 1:500, Kindly donated by Dr Linda Penn, Princess Margaret Cancer Centre, Toronto, Ontario, Canada). GAPDH

expression served as a loading control for western blotting and RT-qPCR experiments. The antibody AMAb90619 was selected for IHC staining.

HMGCR expression

The HMGCR annotation with immunohistochemistry (IHC) was performed on TMAs that were constructed with duplicate 1-mm cores from each tumor (Beecher, WI, USA). Sections of 4 µm were cut from the TMAs. Prior to immunostaining, the sections were baked at 50°C overnight and de-paraffinized in xylene and graded ethanol. Antigen retrieval was then performed using citrate buffer pH6 (ThermoFisher Scientific, Waltham, MA, USA) in decloaking chamber (Biocare Medical, Walnut Creek, CA, USA). Sections were stained with the mouse monoclonal antibody against HMGCR (AMAb90619, CL0260, Atlas Antibodies, Stockholm, Sweden) diluted 1:100 in Autostainer 480S (ThermoFisher Scientific, Waltham, MA, USA) using a commercial kit (UltraVision LP HRP polymer®, Primary Antibody Enhancer, Ultra V Block and DAB plus substrate system®, ThermoFisher Scientific, Waltham, MA, USA). Slides were counterstained with hematoxylin and mounted using Pertex. Images of the stained slides were taken using an automated system (VSlide, Metasystems).

The web-based digital pathological platform PathXL Xplore (http://www.pathxl.com, PathXL Ltd., UK) was used for microscopy evaluations. The expression of HMGCR was evaluated based on cytoplasmic intensity using a four-grade scale; negative, weak, moderate and strong. After the annotation, the strongly stained group of tumors was merged with the moderately stained group due to few numbers of patients with strongly stained HMGCR (n=15), resulting in three groups (negative, weak and moderate/strong). The HMGCR annotation was performed independently by two observers (OB and MF), both blinded to other clinical or pathological data. When the score was divergent the respective core was estimated again together to reach consensus. In cases where the assessment of the two cores from a particular tumor was disagreeing, the highest score was always used. The study obeys to the REMARK guidelines (19).

Statistical analysis

Associations between patient and tumor characteristics with CLM use was evaluated and presented both as numbers and percentages. Continuous variables were summarized by mean, min., and max. values. Distributional differences between the two groups any CLM use and never CLM use were assessed with X^2 test or linear regression (X^2 test for trend) as appropriate.

The same methods were used to evaluate associations between patient/tumor characteristics and HMGCR expression.

The association between CLM use, HMGCR expression and prognosis was evaluated using breast-cancer specific mortality (BCM) as clinical endpoint. BCM was defined as the incidence of breast cancer-related deaths, both when breast cancer was considered the direct cause or the contributing cause of death. Follow-up time was calculated from the time of breast cancer diagnosis to the date of one of the following events; date of breast cancer-related death, date of death from another cause, date of emigration or the end of follow-up as of December 31st, 2014.

The associations between HMGCR expression and time to breast cancer related death was analyzed by cause-specific Cox regression, yielding hazard ratios (HR) with 95% confidence intervals. The follow-up time was censored at the date of death from a cause not related to breast cancer – a so-called competing event. HR:s should therefore be interpreted in a hypothetical world where all other causes of death have been eliminated (20). In addition to crude analyses, three multivariate models were fitted. Model 1 adjusted for age at diagnosis (continuous). Model 2 adjusted for age at diagnosis and tumor characteristics (tumor size >20 mm (yes/no), metastatic lymph nodes (yes/no), histological grade (grade 1, 2, and 3) and ER status (positive/negative)). Model 3 included the covariates of model 2 with the addition of planned adjuvant treatments (endocrine treatment (yes/no), chemotherapy (yes/no) and radiotherapy (yes/no)).

The prognostic impact of CLM use was evaluated for patients diagnosed with breast cancer from 2006 and onwards since the CLM data were retrieved from the Swedish Prescribed Drug Register, which was initiated in July 2005. For evaluation of the relationship between CLM and BCM, the same strategy for crude and adjusted analyses were done as described above. In an exploratory analysis, the predictive value of HMGCR regarding the association between CLM use, BCM was evaluated through analyses stratified by HMGCR expression (HMGCR negative/weak and HMGCR moderate/strong, respectably). Statistical analyses were performed in SPSS 24.0 (IBM) and Stata version 14.1 (StataCorp LP, College Station, TX, USA).

Results

CLM use, HMGCR expression, patient and tumor characteristics

At the end of follow-up time for identification of incident breast cancers by the 31st of December 2010, a total of 1,016 breast cancers were diagnosed. After subtraction of patients diagnosed with cancer *in situ*, bilateral or distant metastatic breast cancer, 910 patients with invasive breast cancer were identified (Fig. 1). In 192 cases, tumor tissue was not available. The TMAs were thus constructed of biopsies from 718 patients, whereof 61 were not possible to evaluate for HMGCR expression due to either inferior staining quality or lack of tumor tissue in the TMA core. In the end, 657 samples were available for annotation of HMGCR expression; 119 (18%) showed negative expression, 354 (54%) weak expression, 169 (26%) moderate and 15 (2%) strong (Fig. 1).

A total of 326 patients from the study population of 910 had been prescribed CLM during the years 2005 through 2014. Hereof, 74 patients were prescribed their first CLM before (pre-diagnostic CLM) and 252 women after (post-diagnostic CLM) the breast cancer diagnosis, whereas 584 women had not been prescribed CLM. Table 1 shows patient and tumor characteristics according to CLM use. The distributions in the four groups (pre-diagnostic CLM, post-diagnostic CLM, any CLM and never CLM) were similar regarding body mass index (BMI) at baseline, tumor size, lymph node and ER status. Proportionally, more patients in the pre-diagnostic CLM group were diagnosed with grade III tumors, high Ki67 and higher HMGCR expression compared to the post-diagnostic and never CLM groups. In comparison to patients never receiving CLM, the patients in any CLM group had higher BMI at baseline and their tumors were more often PR positive ($P<0.001$ and $P=0.004$ respectively).

Table 2 shows the patient and tumor characteristics according to the HMGCR expression. The mean age at diagnosis was higher in patients with HMGCR moderate/strong tumors compared to the patients with HMGCR low or negative tumors. HMGCR moderate/strong tumors were associated with tumors with higher histological grade, high Ki67 and ER negative tumors ($P<0.001$, $P<0.001$ and $P<0.001$ respectively, Table 2).

HMGCR Antibody Validation

siRNA interference and statin treatment were used to downregulate and upregulate HMGCR expression respectively in MCF-7 cells. As illustrated, *HMGCR* mRNA levels were significantly reduced by about 1.7-folds relative to the controls following siRNA transfection (Fig. 2A). Likewise, statin treatment significantly upregulated *HMGCR* mRNA expression by about 1.6-folds relative to the controls (Fig. 2A). In western blotting, all antibodies tested detected a protein band at about 100-kDa, which is the expected molecular weight of HMGCR (Fig. 2B-2E). However, the differential modulation of HMGCR expression by siRNA or statin treatment was accurately tracked by all antibodies except HMGCR ab174830,

which did not show any differential expression between siRNA silenced cells or statin treated cells and controls (Fig. 2D). This data suggests that HMGCR ab174830 antibody may be recognising a different protein of a similar molecular weight as HMGCR. AMAb90619 and HMGCR AMAb90618 and HMGCR A-9 reliably captured the differential effects of HMGCR knock-down and upregulation and showed a positive reaction in the additional positive control cell lines, confirming their specificity to the target protein. These antibodies also showed reactivity with a protein of approximately 55KDa, especially after statin exposure, the identity of which is being investigated. For IHC analyses, AMAb90619 was selected based on availability for testing on a TMA containing a small collection of breast cancer tissue and cell lines, including liver tissue to serve as positive control for the staining. The expression of HMGCR was heterogeneous in the breast cancer cell lines and tissues and a positive reactivity was seen in the liver as expected. This antibody was therefore used for IHC analyses of HMGCR expression on the TMA including all incident breast cancers from the MDCS as reported herein.

Breast cancer mortality by CLM use

Analyses of associations between CLM use and breast-cancer related deaths were restricted to patients diagnosed with breast cancer from January1st, 2006 and onwards, given the period when information from The Swedish Prescribed Drug Register was available from. CLM use was associated to a 36% reduction in BCM in the age-adjusted analysis ($HR_{age\text{-}adj.}$ 0.64), but the evidence for an effect was weak (95% CI 0.25-1.60, P=0.34, Table 3). Similar results were seen in the models adjusted for tumor characteristics and adjuvant treatment (Table 3). In the exploratory analyses stratified for HMGCR expression, CLM use seemed more protective regarding BCM in patients with tumors with negative/weak HMGCR expression ($HR_{age\text{-}adj.}$ 0.16, 95% CI 0.02-1.40, P=0.10, Table 3) compared to patients with moderate/strong HMGCR expression ($HR_{age\text{-}adj.}$ 0.68, 95% CI 0.11-4.01, P=0.67, Table 3), but the evidence for differential effect (interaction) was week (P=0.27). Stratified analyses for ER status showed a modest trend towards protective effects of CLM among patients with ER positive breast cancer although not statistically significant ($HR_{age\text{-}adj.}$ 0.75, 95% CI 0.23-2.48, P=0.64).

Breast cancer mortality by HMGCR expression

The possible prognostic role of HMGCR expression in breast cancer was evaluated for the entire study population with valid HMGCR expression (n=657) and showed no evidence of associations (Table 4). Compared to patients with HMGCR negative tumors, the survival analyses suggested that breast cancer patients expressing

HMGCR moderate/strongly might have a higher BCM ($HR_{age\text{-}adj.}$ 1.32, 95% CI 0.74-2.37, P=0.34, Table 4). When restricting the analyses to ER positive breast cancer only, this association was measured with higher precision compared to all patients ($HR_{age\text{-}adj.}$ 1.66, 95% CI 0.81-3.41, P=0.17), but not statistically significant in any of the models.

Discussion

In this population-based prospective cohort study, we have investigated BCM according to CLM use and studied HMGCR as a prognostic factor. Breast cancer patients prescribed a CLM showed no evidence of reduced BCM compared to never users. There was, however, a trend towards lower BCM among patients with no or weak HMGCR expression compared to patients with moderate or strong expression of HMGCR. Irrespective of CLM use, HMGCR expression was significantly associated with more aggressive tumor characteristics although no significant associations were observed for breast cancer related mortality.

The associations between statin use and cancer-related mortality have been studied for some years and the results have not been undisputed. Nielsen *et al* have shown reduced cancer-related mortality among statin users, who were prescribed a statin before their cancer diagnosis (9). In a nationwide cohort study from Finland both pre-diagnostic and post-diagnostic statin use were associated with lowered risk of breast cancer death, findings were dose-dependent especially for pre-diagnostic statin use (10). In breast cancer patients, Cardwell *et al* found some evidence for reduced breast-cancer and all-cause mortality among post-diagnostic statin users (21), while Smith *et al* found no association (22). Smith *et al* also investigated hydrophilic or lipophilic statin use and effect modification by ER status and found no association between statin use and breast-cancer specific mortality (22). In a Danish study by Ahern et al looking at using breast cancer recurrence as clinical endpoint, breast cancer patients taking simvastatin had a significantly improved breast-cancer free survival with 10 fewer breast cancer recurrences per 100 women after 10 years of follow-up (23). In this study, we did not have access to recurrence rates and were unable to confirm that CLM use reduced BCM. Insufficient power in this cohort may be responsible for this lack of confirmation since reliable data on CLM use was only valid from July 1st, 2005 when the Swedish Prescribed Drug Register was initiated.

Even though not statistically significant, analyses of ER positive patients, demonstrated a trend for ER positive patients with moderate/strong HMGCR expression to having higher BCM than the patients with negative or weak HMGCR expression. Irrespective of ER status, however, negative/weak HMGCR expression

in tumors of patients treated with CLM was associated with lower BCM. These findings indicate that HMGCR expression is more prognostically important for ER positive patients and that CLM use could be more preventive for ER positive patients. A recent publication investigated the prognostic impact of CLM use in combination with endocrine treatment in ER positive patients and showed improved disease-free survival and distant recurrence-free interval for breast cancer patients that initiated CLM use during endocrine treatment, suggesting that combined endocrine and CLM treatment could have a preventive role by improving disease-free survival and preventing breast-cancer recurrence (24).

Different mechanisms of actions have been proposed as the anticancer effects of statins. Some studies have suggested that cholesterol utilization by cancer cells is an important feature of carcinogenesis (25, 26), suggesting that the statin lowering of plasma levels of cholesterol consequently lowers the availability of cholesterol for the cancer cells. Another study found a link between the cholesterol metabolite 27HC and cancer growth (27). Inhibition of the mevalonate pathway blocks the synthesis of isoprenoid molecules farnesyl pyrophosphate and geranylgeranyl pyrophosphate, molecules involved in protein prenylation, which is necessary for the activation of many proteins participating in signaling pathways on which tumors depend (28).

The rate limiting enzyme of the mevalonate pathway, HMGCR, has been studied as a possible predictive marker for patients that would benefit from statin treatment in a cancer setting. We have earlier published the results from a window-of-opportunity breast cancer trial, where a decrease in tumor proliferation was seen only in tumors expressing HMGCR (29). In previous studies evaluating the role of HMGCR in breast cancer, HMGCR expression has consistently been associated with prognostically beneficial tumor characteristics; i.e. low histological grade, expression of estrogen- and progesterone receptors, less axillary lymph node involvement (2, 11, 12). In this study however, patients with moderate/strong expression of HMGCR more often had tumors with grade III, ER negative and high Ki67 compared to patients with no or weak HMGCR expressing tumors. The use of different antibody and breast cancer heterogeneity can partly be at plausible reason to these differences. In this study, a novel monoclonal antibody recently developed by Atlas Antibodies (https://atlasantibodies.com) was used to evaluate HMGCR expression. The ideal antibody has to be reproducible, perform well in the correct setting and be specific for the target protein (30). Previously we have used polyclonal antibodies from different producers (2, 11, 29), but in this study, we aimed to identify the most appropriate HMGCR antibody by performing in-depth validation of four different antibodies. The antibody chosen for this study, was one of three antibodies that showed specificity to the desired HMGCR protein by capturing the differential effects of HMGCR knock-down and up-regulation, and in addition showed a positive reaction in the positive control cell lines.

The prognostic impact of HMGCR in breast cancer has been evaluated previously by IHC in two independent cohorts, a consecutive breast cancer cohort where patients with tumors expressing HMGCR had a significantly prolonged recurrence-free survival, also when adjusted for established prognostic factors (11). In a population-based cohort of primary breast cancer patients in Sweden, HMGCR expression was not associated with disease-free survival (12). In this study, we did not find a statistical association between HMGCR expression and BCM.

Our study has some limitations. As previously mentioned, the Swedish Prescribed Drug Register started 1st of July 2005, almost fifteen years later than the first breast cancer case in 1991. In our analysis, we also gave the CLM use extra sixth months marginal time for plausible treatment effect by only selecting breast cancer cases from 1st of January 2006. Due to this, when evaluating CLM effect on BCM, the breast cancer cases diagnosed up until the 31st of December 2005 were not used in the analyses, resulting in few cases for the survival analyses. Additionally, we do not know how and if the patients actually took the CLM they were prescribed and purchased. One study on statins use in elderly population showed that after six months almost half of the patient stopped taking their tablets (31). Although our study population was younger we can probably assume that not all patients took their medication as prescribed. Lastly, clinical outcome was restricted to breast cancer related mortality as data on disease recurrence was unavailable, which has been the preferred outcome in previous studies.

In this study, we have investigated the association of CLM use and HMGCR expression on BCM. We observed associations between high HMGCR expression and unfavorable tumor characteristics such as high tumor grade and high Ki67 although no independent association with BCM. Even though our findings were not statistically significant, an interesting trend was seen for lower BCM for breast cancer patients with tumors expressing HMGCR to no or a lesser degree and using CLM. This should be further investigated in a larger observational study and ultimately in a clinical trial.

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Figure 1

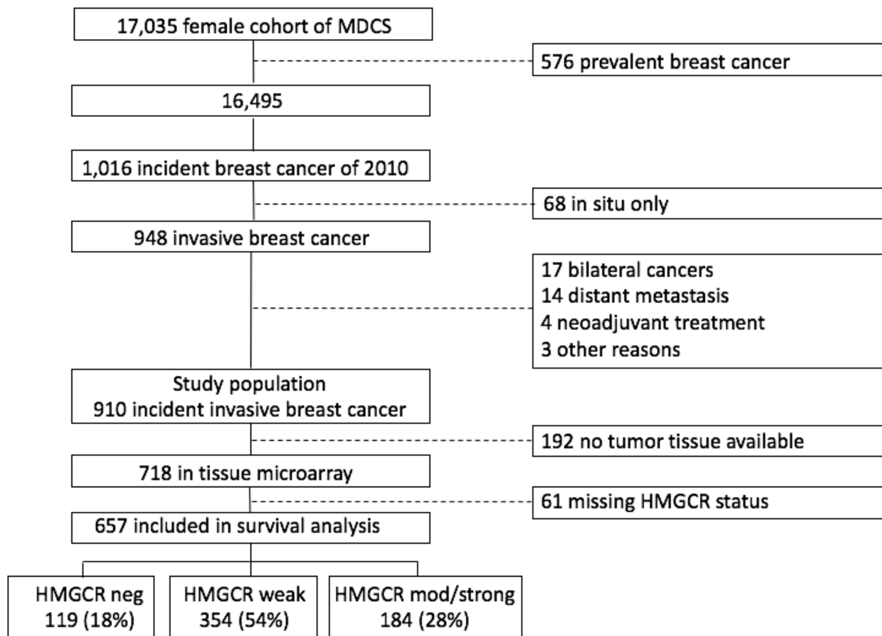

Figure 1. Flow chart showing the study population in the Malmö Diet and Cancer study. Dotted lines display reason for missing patients.

Figure 2

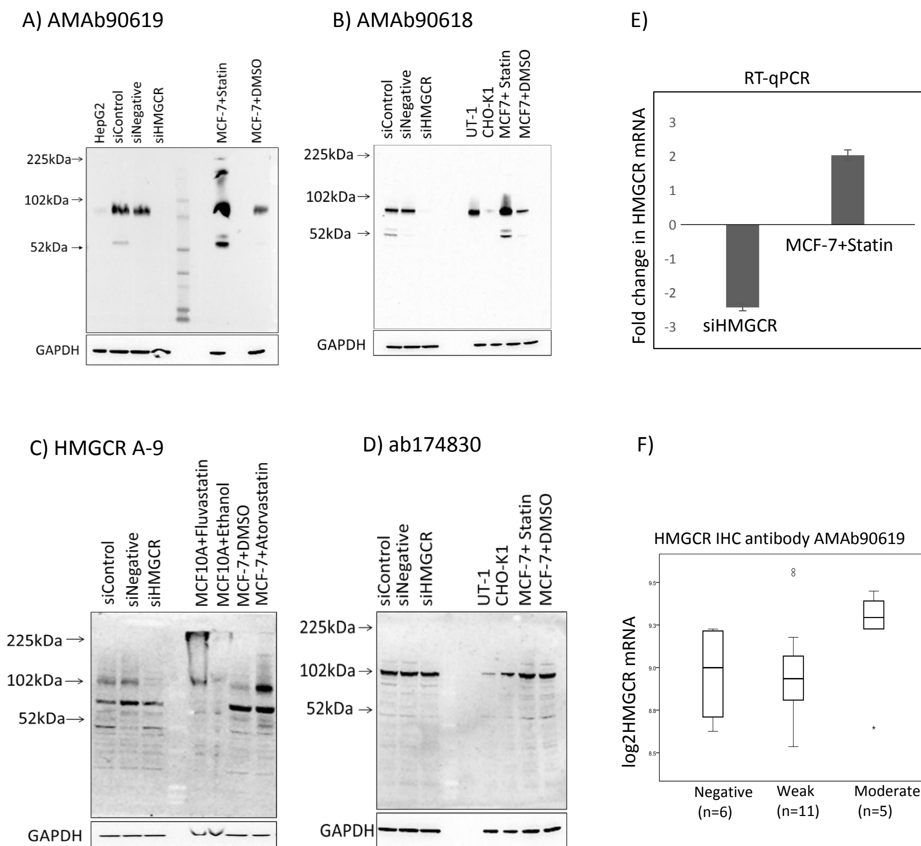

Figure 2. Validation of anti-HMGCR antibodies. A-D) Western blots showing the expression of HMGCR after respective treatments tracked by different antibodies A) HMGCR AMAb90619, B) HMGCR AMAb90618, C) HMGCR ab174830 and D) HMGCR A-9. Human breast cancer MCF-7 cell line was the main test cell line, while the HEPG2 Liver cancer cell line and the Chinese hamster ovary cell lines CHO-K1 and UT-1 (derived from CHO-K1 following prolonged exposure to mevastatin) served as additional positive controls. E) RT-qPCR was performed to evaluate the efficiency of downregulating HMGCR with siRNA or upregulating HMGCR with statin treatment. F) Correlation of gene and protein expression measured by antibody AMAb90619 in tumors.

Table 1: Patient and tumor characteristics according to cholesterol-lowering medication (CLM) use

All n=910 Factor		Pre-diagnostic CLM n=74 n (%) or mean (min-max)	Post-diagnostic CLM n=252 n (%) or mean (min-max)	Any CLM n=326 n (%) or mean (min-max)	Never CLM n=584 n (%) or mean (min-max)	P-value any vs never CLM use
Age at baseline						
years (n= 910)	56.4 (44.7-73.0)	56.3 (46.8-71.5)	57.1 (44.9-72.8)	56.9 (44.9-72.8)	56.2 (44.7-73.0)	0.149[a)]
Age at diagnosis						
years (n= 910)	65.5 (45.7-87.3)	71.1 (59.6-85.6)	64.8 (48.4-84.7)	66.2 (48.4-85.6)	65.0 (45.7-87.3)	0.030[a)*]
BMI at baseline (n=910)						
<25	466 (51)	29 (39)	105 (42)	134 (41)	332 (57)	
≥25 and <30	310 (34)	29 (39)	97 (38)	126 (39)	184 (31)	
>30	134 (15)	16 (22)	50 (20)	66 (20)	68 (12)	<0.001[b)*]
Tumor size (n= 887)						
≤20 mm	637 (72)	51 (72)	186 (75)	237 (74)	400 (70)	
>20 mm	250 (28)	20 (28)	62 (25)	82 (26)	168 (30)	0.219
ALNI (n=819)						
Positive (≥1 metastatic node)	262 (32)	23 (33)	70 (31)	93 (31)	169 (32)	
Negative	557 (68)	47 (67)	157 (69)	204 (69)	353 (68)	0.754
NHG (n=835)						
I	227 (27)	12 (17)	67 (28)	79 (26)	148 (28)	
II	392 (47)	32 (46)	116 (50)	148 (49)	244 (46)	
III	216 (26)	26 (37)	51 (22)	77 (25)	139 (26)	0.843[b)]
ER status (n=760)						
Positive (>10%)	671 (88)	58 (87)	191 (88)	249 (88)	422 (88)	
Negative (<10%)	89 (12)	9 (13)	25 (12)	34 (12)	55 (12)	0.841
PR status (n= 689)						
Positive (>10%)	378 (55)	49 (77)	113 (57)	162 (62)	216 (51)	
Negative (<10%)	311 (45)	15 (23)	85 (43)	100 (38)	211 (49)	0.004[*]
HER2 status (n=593)						
Positive	52 (9)	6 (15)	15 (8)	21 (10)	31 (8)	
Negative	541 (91)	33 (85)	168 (92)	201 (90)	340 (92)	0.646
Ki67 (n=633)						
Low (≤10%)	419 (66)	17 (40)	145 (72)	162 (67)	257 (66)	
High (>10%)	214 (34)	25 (60)	56 (28)	81 (33)	133 (34)	0.842
HMGCR expression (n=657)						
negative	119 (18)	5 (8)	36 (19)	41 (16)	80 (19)	
weak	354 (54)	33 (51)	108 (57)	141 (56)	220 (53)	
moderate/strong	184 (28)	26 (41)	46 (24)	72 (28)	117 (28)	0.534[b)]
Planned adjuvant treatments						
Endocrine therapy						
In all patients (n=878)						
None	409 (47)	18 (26)	124 (50)	142 (45)	267 (48)	
Any (TAM/AI)	469 (53)	52 (74)	123 (50)	175 (55)	294 (52)	0.425
In ER+ only (n=671)						
None	254 (38)	9 (16)	84 (44)	93 (37)	161 (39)	
Any (TAM/AI)	408 (62)	48 (84)	107 (56)	155 (63)	253 (61)	0.722
Chemotherapy (n=823)						
Yes	126 (15)	11 (17)	29 (12)	40 (13)	86 (16)	
No	697 (85)	54 (83)	204 (88)	258 (87)	439 (84)	0.257
Radiotherapy (n=825)						
Yes	499 (60)	43 (67)	148 (63)	191 (64)	308 (58)	
No	326 (40)	21 (33)	85 (37)	106 (36)	220 (42)	0.092
Death during follow-up (n=256)		16	61	77	179	
Breast cancer cause	132 (52)	4 (25)	25 (41)	29 (38)	103 (58)	
Other causes	124 (48)	12 (75)	36 (59)	48 (62)	76 (42)	0.004[*]

CLM: cholesterol-lowering medication; BMI: body mass index; ALNI: axillary lymph node involvement, NHG: Nottingham histological grade; ER: estrogen receptor, PR: progesterone receptor;
HER2: Human epidermal growth factor 2; TNBC: tripple negative breast cancer; HMGCR: HMG-CoA reductase; TAM: tamoxifen; AI: aromatase inhibitors
Pearson X2 test if not specified otherwise a) linear regression b) Linear-by-Linear association. P-value between any CLM treatment and never CLM treatment. *P<0.05 statistically significant
Pre-diagnostic CLM patients treatad with CLM before breast cancer diagnos. Post-diagnostic CLM patients prescribed CLM after breast cancer diagnos.
Any CLM patients treated with CLM at any point; either pre-diagnostic CLM and/or post-diagnostic CLM use. Never CLM patients never treated with CLM according to drug register .

Table 2: Distribution of patient and tumor characteristics of the study population, according to HMGCR expression

All n=910 Tumor in tissue microarray, n (%)		Yes, 718 (79)					No, 192 (21)
HMG-CoA reductase (HMGCR) expression assessable, n (%)		Yes, 657 (92)				No, 61 (8)	
HMGCR expression; negative, weak, moderate/strong		HMGCR negative 119 (18)	HMGCR weak 354 (54)	HMGCR moderate/strong 184 (28)			
Factor	n (%) or mean (min-max)	n (%) or mean (min-max)	n (%) or mean (min-max)	n (%) or mean (min-max)	P	n (%) or mean (min-max)	n (%) or mean (min-max)
Age at baseline, years (n= 910)	56.4 (44.7-73.0)	56.1 (44.7-72.4)	55.8 (44.9-73.0)	57.1 (46.0-73.0)	0.176 a)	55.1 (45.7-72.7)	57.7 (44.8-72.8)
Age at diagnosis,years (n= 910)	65.5 (45.7-87.3)	64.1 (48.5-81.3)	65.1 (45.7-84.7)	67.3 (48.6-87.3)	<0.001 a)*	63.1 (49.4-84.4)	66.0 (47.8-85.1)
BMI at baseline (n=910)							
<25	466 (51)	72 (60)	163 (46)	98 (53)		36 (59)	97 (50)
≥25 and <30	310 (34)	33 (28)	124 (35)	64 (35)		18 (29)	71 (37)
>30	134 (15)	14 (12)	67 (19)	22 (12)	0.708	7 (12)	24 (13)
Tumor size (n= 887)							
≤20 mm	637 (72)	77 (65)	243 (69)	129 (70)		49 (82)	139 (80)
>20 mm	250 (28)	41 (35)	109 (31)	55 (30)	0.402	11 (18)	34 (20)
ALNI (n=819)							
Positive (≥1 metastatic node)	262 (32)	42 (36)	124 (37)	57 (32)		7 (14)	32 (23)
Negative	557 (68)	74 (64)	207 (63)	124 (68)	0.322	45 (86)	107 (77)
NHG (n=835)							
I	227 (27)	29 (25)	101 (29)	27 (15)		23 (42)	47 (35)
II	392 (47)	72 (61)	170 (49)	69 (38)		20 (36)	61 (46)
III	216 (26)	16 (14)	76 (22)	86 (47)	<0.001*	12 (22)	26 (19)
ER status (n=760)							
Positive (>10%)	671 (88)	101 (92)	297 (91)	137 (79)		35 (85)	101 (93)
Negative (<10%)	89 (12)	9 (8)	30 (9)	36 (21)	<0.001	6 (15)	8 (7)
PR status (n= 689)							
Positive (>10%)	378 (55)	60 (60)	165 (56)	84 (53)		17 (47)	52 (53)
Negative (<10%)	311 (45)	40 (40)	131 (44)	75 (47)	0.263	19 (53)	46 (47)
HER2 status (n=593)							
Positive	52 (9)	2 (2)	22 (9)	21 (15)		2 (5)	5 (6)
Negative	541 (91)	85 (98)	224 (91)	119 (85)	0.001*	36 (95)	77 (94)
Ki67 (n=633)							
Low (≤10%)	419 (66)	80 (83)	190 (71)	68 (47)		20 (56)	61 (69)
High (>10%)	214 (34)	16 (17)	79 (29)	76 (53)	<0.001*	16 (44)	27 (31)
Cholesterol-lowering medication							
Pre-diagnostic CLM use	74 (8)	5 (4)	33 (9)	26 (14)		1 (2)	9 (5)
Post-diagnostic CLM use	252 (28)	36 (30)	106 (30)	43 (23)		18 (29)	49 (25)
Any CLM use	326 (36)	41 (34)	139 (39)	69 (37)		19 (31)	58 (30)
Never CLM use	584 (64)	78 (66)	215 (61)	115 (63)	0.687★	42 (69)	134 (70)
Planned adjuvant treatments							
Endocrine therapy							
In all patients (n=878)							
None	409 (47)	44 (38)	143 (41)	68 (38)		41 (67)	113 (66)
Any (TAM/AI)	469 (53)	73 (62)	206 (59)	111 (62)	0.951	20 (33)	59 (34)
In ER+ only (n=671)							
None	254 (38)	35 (35)	107 (36)	34 (25)		20 (57)	58 (58)
Any (TAM/AI)	408 (62)	64 (65)	187 (64)	100 (75)	0.077	15 (43)	42 (42)
Chemotherapy (n=823)							
Yes	126 (15)	12 (11)	58 (18)	36 (21)		5 (9)	15 (9)
No	697 (85)	97 (89)	266 (82)	133 (79)	0.033*	49 (91)	152 (91)
Radiotherapy (n=825)							
Yes	499 (60)	70 (64)	214 (65)	103 (62)		24 (44)	88 (53)
No	326 (40)	39 (36)	113 (35)	64 (38)	0.592	31 (56)	79 (47)
Death during follow-up (n=256)		32	93	53		16	62
Breast cancer cause	132 (52)	18 (56)	43 (46)	32 (60)		6 (37)	33 (53)
Other causes	124 (48)	14 (44)	50 (54)	21 (40)	0.506	10 (63)	29 (47)

CLM: cholesterol-lowering medication; BMI: body mass index; ALNI: axillary lymph node involvement, NHG: Nottingham histological grade; ER: estrogen receptor, PR: progesterone receptor; HER2: Human epidermal growth factor 2; TNBC: tripple negative breast cancer; HMGCR: HMG-CoA reductase; TAM: tamoxifen; AI: aromatase inhibitors
Pre-diagnostic CLM patients treatad with CLM before breast cancer diagnos. Post-diagnostic CLM patients prescribed CLM after breast cancer diagnos.
Any CLM patients treated with CLM at any point; either pre-diagnostic CLM and/or post-diagnostic CLM use. Never CLM patients never treated with CLM according to drug register .
Linear-by-linear association if not specified otherwise a) Linear regression. *P-value <0.0 5statistically significant
▪p-value with uncertainty due to 2 cells with low expected count ★p-value between any vs never CLM use

Table 3: Cholesterol-lowering medication (CLM) effect on breast-cancer specific mortality for breast-cancer patients diagnosed after 1st of January 2006. All cases and in subgroups for HMGCR expression.

	Crude					Model 1					Model 2					Model 3				
All cases	HR	95% CI	P	Number patients	Breast cancer death	HR	95% CI	P	Number patients	Breast cancer death	HR	95% CI	P	Number patients	Breast cancer death	HR	95% CI	P	Number patients	Breast cancer death
Never CLM use		ref					ref					ref					ref			
CLM use	0.70	0.28-1.75	0.44	293	25	0.64	0.25-1.60	0.34	293	25	0.60	0.21-1.68	0.33	255	20	0.79	0.27-2.36	0.68	222	18
	Crude					Model 1					Model 2					Model 3				
HMGCR negative/weak	HR	95% CI	P	Number patients	Breast cancer death	HR	95% CI	P	Number patients	Breast cancer death	HR	95% CI	P	Number patients	Breast cancer death	HR	95% CI	P	Number patients	Breast cancer death
Never CLM use		ref					ref					ref					ref			
CLM use	0.28	0.03-2.30	0.24	148	8	0.16	0.02-1.40	0.10	148	8	0.42	0.03-5.71	0.52	137	7	0.02	0.0002-2.96	0.13	117	7
	Crude					Model 1					Model 2					Model 3				
HMGCR moderate/strong	HR	95% CI	P	Number patients	Breast cancer death	HR	95% CI	P	Number patients	Breast cancer death	HR	95% CI	P	Number patients	Breast cancer death	HR	95% CI	P	Number patients	Breast cancer death
Never CLM use		ref					ref					ref					ref			
CLM use	0.64	0.11-3.82	0.63	83	6	0.68	0.11-4.01	0.67	83	6	0.67	0.09-4.93	0.70	80	5	Data missing due to few patients				

Crude: unadjusted analysis; Model 1: adjusted for age; Model 2: adjusted for age, tumor size, tumor grade, lymph node involvement, ER status; Model 3: Model 2 and planned adjuvant treatment (chemo-, endocrine- and radiation treatment). HR: Hazard ratio; CI: Confidence interval; HMGCR: HMG-CoA reducta

Table 4: Breast cancer mortality according to HMGCR expression in crude and adjusted models.

	Total (n)	Events (n)	Crude			Model 1			Model 2			Model 3		
All patients	657	93	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
HMGCR negative	119	18		ref	0.141		ref	0.287		ref	0.810		ref	0.792
HMGCR weak	354	43	0.93	0.54-1.62	0.798	0.91	0.53-1.58	0.746	0.93	0.52-1.68	0.811	0.96	0.53-1.74	0.893
HMGCR moderate/strong	184	32	1.46	0.82-2.61	0.201	1.32	0.74-2.37	0.353	1.10	0.57-2.12	0.775	1.15	0.59-2.25	0.676

Crude: unadjusted analysis; Model 1: adjusted for age; Model 2: adjusted for age, tumor size, tumor grade, lymph node involvement and ER status
Model 3: Model 2 and planned adjuvant treatment (chemo-, endocrine- and radiation treatment). HMGCR: HMG-CoA reductase; HR: Hazard ratio; CI: Confidence interval