# Cholesterol metabolism in breast cancer: Prognostic factors and optimizing treatment

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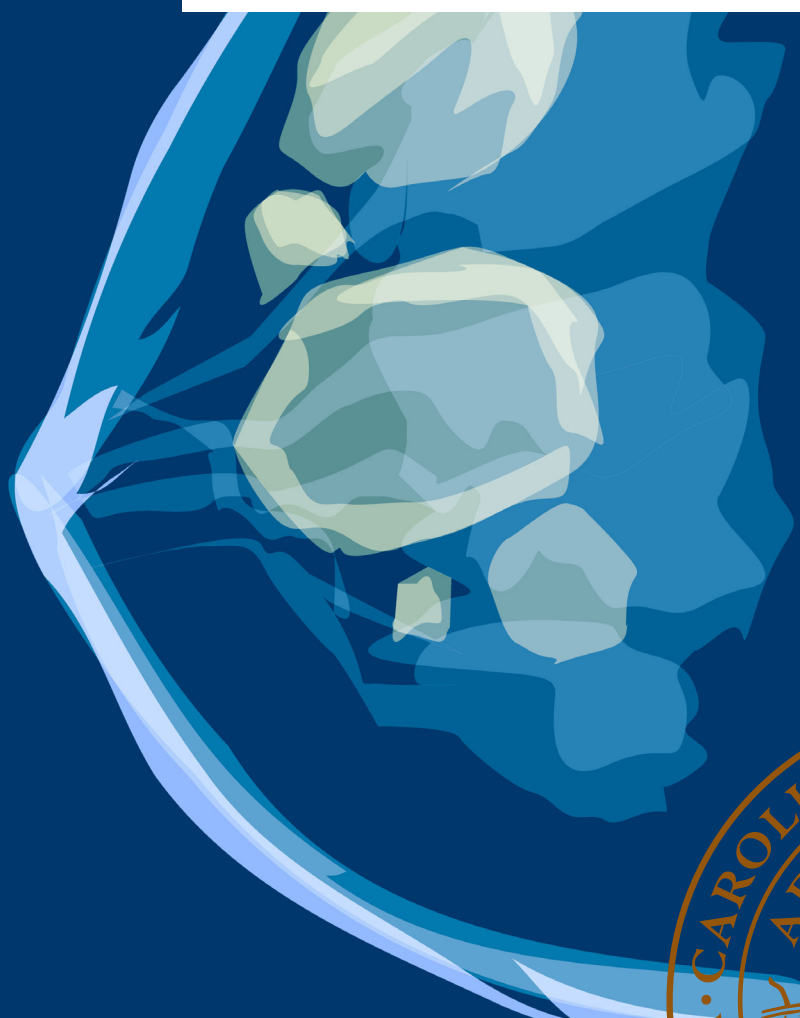

# Cholesterol metabolism in breast cancer: Prognostic factors and optimizing treatment

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LUND UNIVERSITY

DOCTORAL DISSERTATION

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# Cholesterol metabolism in breast cancer: Prognostic factors and optimizing treatment

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*Dream big, be grateful, give love and laugh lots!*

# Table of Contents

# List of original papers

This thesis is based on the following papers.

I. **Insensitivity to atorvastatin is associated with increased accumulation of intracellular lipid droplets and fatty acid metabolism in breast cancer cells**
Barbara Lettiero, **Maria Inasu**, Siker Kimbung & Signe Borgquist
Scientific Reports 2018 8:5462 doi: 10.1038/s41598-018-23726-3

II. **Statin use and patterns of breast cancer recurrence in the Malmö Diet and Cancer Study**.
**Maria Inasu**, Maria Feldt, Helena Jernström, Signe Borgquist, Sixten Harborg
Breast 2022 Feb; 61:123-128. doi: 10.1016/j.breast.2022.01.003

III. **CYP27A1 expression is associated with risk of late lethal estrogen receptor-positive breast cancer in postmenopausal patients**
Siker Kimbung, **Maria Inasu**, Tor Stålhammar, Björn Nodin, Karin Elebro, Helga Tryggvadottir, Maria Ygland Rödström, Karin Jirström, Karolin Isaksson, Helena Jernström & Signe Borgquist
Breast cancer research 2020 Nov 11; 22(1):123 doi: 10.1186/s13058-020-01347-x

IV. **High CYP27A1 expression is a novel biomarker of favorable prognosis in premenopausal patients with estrogen receptor positive primary breast cancer**
**Maria Inasu**, Pär Ola Bendahl, Mårten Fernö, Per Malmström, Signe Borgquist & Siker Kimbung
NPJ Breast Cancer 2021 Sep 23; 7(1):127. doi: 10.1038/s41523-021-00333-6

V. **Targeting cholesterol metabolism in endocrine therapy-resistant breast cancer**
**Maria Inasu**, Somayeh Khazaei, Ann Rosendahl, Helena Jernström, Siker Kimbung & Signe Borgquist
(Manuscript)

# Abbreviations

| | |
|---|---|
| 27HC | 27-hydroxycholesterol |
| AI | aromatase inhibitors |
| BC-blood | breast cancer blood study |
| BMI | body mass index |
| BRCA1 | breast cancer 1 |
| BRCA2 | breast cancer 2 |
| CYP27A1 | cytochrome P450 Family 27 Subfamily A Member 1 |
| EGFR | epidermal growth factor receptor |
| ER | estrogen receptor |
| FFPE | formalin-fixed paraffin-embedded |
| FISH | fluorescence in situ hybridization |
| HER2 | human epidermal growth factor receptor 2 |
| HMGCR | 3-hydroxy-3-methylglutaryl-CoA reductase |
| HR | hazard ratio |
| IGF-1R | insulin-like growth factor-1 receptor |
| IHC | immunohistochemistry |
| IR | incidence rate |
| LDL | low density lipoprotein |
| LDLR | low density lipoprotein receptor |
| MDCS | Malmö Diet and Cancer Study |
| PALB2 | partner and localizer of BRCA2 |
| PI3K | phosphatidyl-inositol-3 OH kinase |
| PgR | progesterone receptor |
| SCD | stearoyl-CoA desaturase |
| STR | short tandem repeat |
| TMA | tissue microarray |
| TNBC | triple negative breast cancer |
| qRT-PCR | quantitative real time polymerase chain reaction |

# Popular science summary

Breast cancer is the most commonly diagnosed cancer in women worldwide. The first step in the treatment of breast cancer is usually surgery. Depending on the characteristics of the tumor, most patients will need additional treatments like chemotherapy and/or anti-hormonal treatment. In some cases, the disease progress and new treatment options are thus warranted. In Sweden, close to 20% women regularly take common cholesterol-lowering medications (statins). Statin use has been linked to a good outcome in some breast cancer patients, but not all breast cancer patients benefit from statin treatment; thus, our goal here was to identify biological markers, i.e., biomarkers, which can be used to select patients that would benefit from the addition of statins to their regular cancer treatment.

The mechanisms by which statins prevent breast cancer cell growth is an area that needs further investigation in the laboratory. In the first study presented here, we treated various breast cancer cell lines with statins. The cell lines had varying response to the anti-cancer effect of statins: Some cell lines were sensitive and others resistant. We then compared how different proteins changed between the sensitive and resistant cell lines. Fatty acids are a lipid component of human cells. We identified a protein involved in the production of fatty acid that was increased in statin-insensitive cell lines. Future studies will test the expression of this protein in clinical studies to understand if it can be used to select patients that can be treated with statins to prevent the return of breast cancer.

The second study is based on the Malmö Diet and Cancer Study cohort. We selected 360 breast cancer patients diagnosed with breast cancer from 2005-2014 and compared the breast cancer recurrence between the patients who used statins with those who were non-users. Breast cancer can either recur loco-regionally, i.e., the disease reappears in the breast, chest wall, armpit, skin near the original tumor, lymph nodes around the chest, neck, and under the breast bone. It can also recur distantly, i.e., in other parts of the body. Our study showed that statin users experienced fewer recurrences to distant sites compared to the non-statin users.

Most breast cancer cells require estrogen for growth, and the supply of estrogen can be blocked by anti-hormone treatment. CYP27A1 is a protein that helps maintain stable cholesterol levels in the cell. When cholesterol is broken down in the cells by CYP27A1, it creates a molecule called 27-hydroxycholesterol that can act as estrogen. When cancer cells are deprived of estrogen they depend on this new molecule for growth. Statins reduce cholesterol levels in the cells, thereby reducing the supply of this estrogen substituted (27-hydroxycholesterol). In the third and fourth studies, we investigated the role of CYP27A1 in breast cancer prognosis, in postmenopausal and premenopausal breast cancer patients, respectively. The third study was based on two patient cohorts of postmenopausal women from southern Sweden: The results showed that high expression of CYP27A1 is not a good

prognostic marker. The fourth study is a combination of patient and laboratory studies. We showed that high CYP27A1 expression is a good prognostic marker in young breast cancer patients and that high 27-hydroxycholesterol reduced breast cancer proliferation.

One of the mechanisms by which cancer cells develop resistance to regular anti-hormonal treatments is by increasing cholesterol production in the cells. Statins block cholesterol synthesis in the cells. Thus, we wondered if we could use statin treatment to block cholesterol synthesis and thus stop resistance mechanism. To answer this question, the fifth study exposed breast cancer cells to anti-hormonal treatments together with statins. The drugs were administered alone or in combination for a prolonged time period. Statin treatment inhibited the upregulation of the proteins involved in the cholesterol-production mechanism. This study suggests that statins can be included as an additional treatment option—especially for postmenopausal women.

The results conclusively show that statins reduce breast cancer recurrence in a subgroup of women. Future work will identify biomarkers to determine which patients will benefit the most from using statins in addition to standard adjuvant therapy. This thesis offers improved insight into the impact of the statin combination treatment in breast cancer leading us one step closer to understanding if statins can be an addition to existing treatment options in breast cancer therapy.

# Background

## Cancer

The recorded history of the disease cancer dates back to the Hippocrates period (460-370 BC). Hippocrates used the term carcinos and carcinoma (both terms meaning crab/crayfish in Greek) to describe non-ulcer-forming and ulcer-forming tumors.

After almost two thousand years of clinical observations, studies, and research, humankind have delineated a very complex picture of this disease. In 2000, Hanahan and Weinberg published an article 'The hallmarks of cancer' describing six key steps necessary for a normal cell to transform to a cancer cell[1]. This list was later updated with two 'next-generation hallmarks' and two enabling characteristics [2]. The hallmarks currently comprise the acquired capabilities for sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing/accessing vasculature, activating invasion and metastasis, reprogramming cellular metabolism, avoiding immune destruction, genome instability and tumor-promoting inflammation. The latest update in 2022 proposed two more emerging hallmarks and enabling characteristics[3] as depicted in Figure 1.

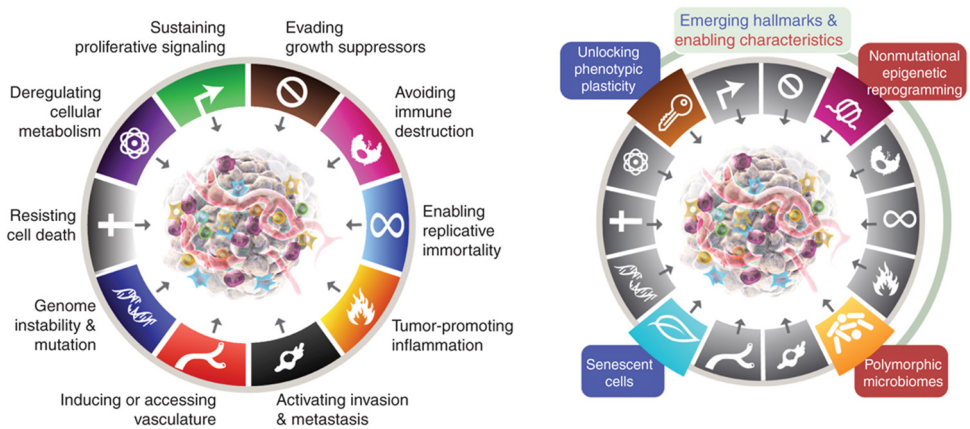


**Figure 1: Hallmarks of cancer. Left: the hallmarks of cancer proposed in 2011. Right: latest proposed hallmarks in 2022.**
Reprinted from Cancer discovery, 2022, 12(1), 31-46, Hanahan D, Hallmarks of Cancer: New dimensions; with permission from AACR.

# Breast cancer

## Epidemiology

Breast cancer is the most commonly diagnosed cancer in women worldwide[4]. In 2020, about 2.3 million cases were diagnosed globally[4] with 10,043 incident cases reported in Sweden[5]. Figure 2 shows the age-standardized breast cancer incidence in Sweden from 1970 to 2020 as well as the age-specific incidence in 2020. Disease incidence varies between countries, with the highest incidence rates (>80 per 100,000) in Australia/New Zealand, Western Europe, Northern America, and Northern Europe. The lowest rates (<40 per 100,000) are in Central America, Eastern and Middle Africa, and South Central Asia[6]. This difference is attributed to various reproductive and lifestyle risk factors, which are described further in the 'Risk factors' section below.

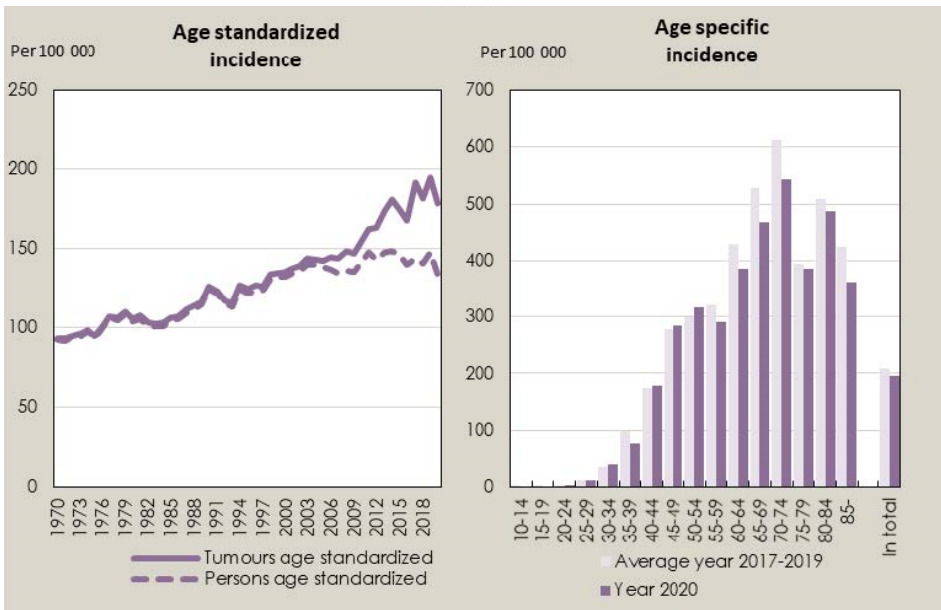


**Figure 2. Breast cancer incidence across time and age groups in Sweden.** Source: the Swedish Cancer Register and The National Board of Health and Welfare.

## Biology of disease onset

The evolution of a pre-neoplastic cell into a detectable tumor is a multistep process. Tumor formation is facilitated by changes at the molecular and cellular levels allowing the cells to acquire the 'core hallmarks' and 'enabling characteristics' of

cancer[3]. At the molecular level, two major pathways have been suggested to be involved in the transformation process: low-grade-like pathways and high-grade-like pathways. Each are characterized by specific chromosomal aberrations. The gene expression signature of the low-grade-like pathway is associated with the estrogen receptor positive phenotype, diploid, or near diploid karyotypes as well as a low tumor grade. The luminal A subtype and to some extent the luminal B subtype fall into this pathway[7]. The high-grade-like pathway has an expression signature of genes involved in the cell cycle and cellular proliferation. Tumors composed of intermediate to high grade, including human epidermal growth factor receptor 2 (HER2) positive tumors and triple negative breast cancers, fall into this pathway[7].

## Risk factors

Increased risk of breast cancer has been attributed to various genetic and lifestyle factors. Women with germline mutations in the BReast CAncer 1 or 2 genes (*BRCA1* or *BRCA2*) have a 70% increased risk of being diagnosed with breast cancer[8]. Monoallelic germline mutations in *PALB2* (partner and localizer of *BRCA2*) also lead to increased risk of being diagnosed with breast cancer[9].

Several other risk factors are associated with an increased life-time breast cancer—mostly centered around increased lifetime exposure to endogenous estrogen, i.e., early menarche, late menopause, nulliparity, low parity, and older age at first full-term pregnancy[10].

Apart from genetic- and menstrual/pregnancy-related factors, there are also lifestyle factors like obesity, smoking, alcohol consumption, and low physical activity that contribute to an increased lifetime risk of breast cancer[11]. These factors are also sometimes referred to as modifiable factors.

*Obesity*

Obesity is defined as a body mass index equal to or greater than 30 kg/m$^2$ and is associated with multiple comorbidities like type II diabetes, hypertension, non-alcoholic fatty liver disease, and dyslipidaemia[12]. In postmenopausal women, obesity is associated with increased risk of breast cancer incidence as well as worse prognosis [13-15]. However, the link between premenopausal breast cancer incidence and obesity is less clear. Various biological mechanisms have been proposed to explain the association between obesity and breast cancer risk. In post-menopausal women, the ovaries cease to produce estrogen and the main estrogen source is adipose tissue. In obese post-menopausal women, heightened aromatase activity leads to increased estrogen synthesis[16] consequently increasing breast cancer risk[17]. Other mechanisms like altered insulin resistance [18] and signaling from adipokines like leptin[19] also mediate the link between obesity and breast cancer.

## Prognostic and treatment predictive factors

Predictive and prognostic factors are of high relevance in the management of breast cancer. These markers guide treatment decisions and care planning. Apart from the classical clinico-pathological features (age, tumor size, lymph node status, metastatic involvement and histological grade), cardinal biomarkers routinely assessed in the clinical setting are estrogen receptor alpha (ER), progesterone receptor (PgR), and HER2.

*Age*

Breast cancer biology is dependent on age, the risk of getting diagnosed with breast cancer increases with age. However, breast cancers diagnosed in younger women <40 years, are often associated with worse prognostic features like hormone receptor negativity and higher histological grade[20].

*TNM classification*

TNM classification is a staging system used to determine the clinical stage of the disease. T defines the size of the primary tumour, N describes the degree of regional lymph node involvement, and M describes the presence of distant metastasis[21]. All of these factors - larger size of the primary tumor, higher number of lymph nodes involved and extend of metastatic spread, are indicators of poorer prognosis in breast cancer.

*Histological grade*

The Nottingham histological grading system, widely used to assess the grade of solid tumors was developed by Elston and Ellis[22]. In this system factors like tubule formation, nuclear pleomorphism and mitotic count are considered to classify tumors in to three categories I–III. Grade I tumors are well-differentiated, grade II moderately differentiated and grade III tumors are poorly differentiated. Grade III tumors are associated with poor prognosis.

*Ki67*

Ki67 is a protein expressed in the cell nuclei of proliferating cells and is used to assess the proliferative status of a tumor. Ki67 status is assessed by quantifying the percentage of tumor cell nuclei that stain positive in a specified area of a tumor. In Sweden, Ki67 is routinely assessed in the clinic and patients are stratified to low, intermediate or high Ki67, where high Ki67 indicate aggressive tumors[23].

*Estrogen receptor*

The majority (70-80%) of the diagnosed breast cancers express ER and depend on proliferative signaling via estrogen. The first human ER was cloned using RNA from the breast cancer cell line MCF-7[24]. In the classical mechanism of estrogen

signaling, estrogen binds at the ligand-binding domain of ER. The ligand-bound ER is then translocated to the nucleus where it binds to the chromatin and regulates the expression of various downstream genes[25]. ER signaling is the main growth and proliferative stimuli for ER+ breast cancers, and this receptor is the main target of endocrine therapies.

*Progesterone receptor*

PgR is also a hormone-dependent nuclear transcription factor. Progesterone is the main ligand of PgR and signaling is involved in mammary gland development. Most tumors have a concordant expression of PgR and ER; tumors expressing PgR but not ER are rare[26].

*HER2*

HER2 expression (amplification) was initially a poor prognostic marker, but later became a treatment-predictive marker. HER2 is a transmembrane tyrosine kinase receptor. A specific ligand for this receptor has yet to be identified, but overexpression of this receptor leads to constitutive signaling induced by interaction with other tyrosine kinase receptor partners leading to cellular proliferation[27].

## Subtypes

Breast cancer is a heterogeneous disease. Histological, immune-pathological and molecular criteria have been used to subtype breast cancer.

*Histological subtypes*

The most common histological subtype is invasive ductal carcinoma (also known as no special type) followed by invasive lobular carcinoma. The pre-invasive counterparts of these lesions are called ductal carcinoma *in situ* and lobular carcinoma *in situ,* respectively. Invasive ductal carcinomas are characterized by a fibrous response to produce a mass. These cancers tend to metastasize via lymphatic and blood systems. However, invasive lobular carcinomas have minimal fibrous response and are more likely to metastasize to viscera[28].

*Immuno-pathological subtypes*

Breast cancers are clinically classified based on the expression of hormone receptors ER and PgR, as well as HER2. Based on these markers, breast cancer is broadly divided into three main subtypes: hormone receptor positive (ER+ or PgR+ and HER2-), HER2 positive (HER2+ regardless of ER or PgR status), and triple negative breast cancer (TNBC) (ER- and PgR- and HER2-).

*Intrinsic molecular subtypes*

In 2000, Sørlie *et al.* identified gene expression patterns that distinguish breast cancer subtypes with clinical implications. These gene signatures largely recapitulated the existing immuno-pathological classification. The main intrinsic subtypes are Luminal A, Luminal B, HER2 enriched, and basal like. There are other rare, clinically relevant subtypes such as Claudin-low and a normal-breast like group[29-31] .

The Luminal A subtype is characterized by a high expression of ER-related genes with a concomitant low expression of genes linked to cell proliferation[32]. The increased dependence on ER-regulated genes makes this subtype more sensitive to endocrine manipulation. The Luminal A subtype is associated with a favorable prognosis.

Relative to Luminal A tumors, Luminal B subtype tumors are characterized by an increased expression of cell proliferation markers. Even if the expression levels of the estrogen receptor is similar in both luminal subtypes, the expression of several luminal related genes/proteins (PgR, FOXA1) is lower in the luminal B group[33].

As the name suggests, the HER2-enriched subtype has an increased expression of the HER2 gene. The HER2 status is assessed at the protein level by immunofluorescence or at the gene level by fluorescence in situ hybridization (FISH)[34]. This subtype features an intermediate expression of luminal-related genes and a high number of mutations across the genome; 72% and 39% of HER2-enriched tumors are TP53 and PIK3CA mutated, respectively[28, 33].

The basal subtype is characterized by the absence of hormone receptors or HER2 amplification and a high expression of proliferation related genes. These tumors are usually presented with a high mutational load. The basal-like subtype is associated with a worse prognosis with a higher short-term recurrence rate and higher mortality rates[35].

*Surrogate subtypes*

The application of gene expression analyses in daily clinical practice is limited by the high technical expertise and costs. Thus, a surrogate clinic-pathological system using four biomarkers (ER/PgR/HER2 and Ki67) was derived to subtype tumors in the clinic.

**Table 1.** Clinicopathological surrogate subtypes of breast cancer

| Instrinsic subtype | Surrogate subtype | Characteristics |
|---|---|---|
| Luminal A | Luminal A- like | ER/PgR positive<br>HER2 negative<br>Ki67 low |
| Luminal B | Luminal B- like / HER2 negative | ER positive<br>HER2 negative<br>At least one of the following<br>Ki67 high or low or negative PgR |
| | Luminal B- like/ HER2 positive | ER positive<br>HER2 positive<br>Ki67/ PgR any |
| HER2 enriched | HER2 positive | ER/PgR negative<br>HER2 positive |
| Basal like | Triple negative | ER/PgR/HER2 negative |

## Treatment of early-stage breast cancer

*Neoadjuvant therapy*

According to the 2021 St. Gallen guidelines, pre-operative neoadjuvant systemic therapy is recommended for women presenting with grade II or III, HER2 positive or triple negative breast cancers. The neoadjuvant therapy aids in down-staging the tumor and helps with surgical intervention as well as starting systemic therapy as early on as possible following diagnosis, and to provide a biological treatment response. In certain cases, the response to neoadjuvant treatment is used as a treatment-predictive biomarker for adjuvant treatment[36].

*Local-regional therapy*

Surgery is the primary therapeutic intervention for most women diagnosed with early-stage breast cancer. The tumor mass is surgically removed either by mastectomy or breast-conserving surgery depending on the patient and tumor characteristics. Axillary lymph nodes are the primary site of a metastatic spread, and sentinel node or axillary resection is also performed to stage the lymph nodes. Lymph node positivity status is used to guide further treatment decisions[36]. Breast-conserving surgery is usually followed by local radiation therapy to prevent local metastatic spread of the disease and thereby reduce the risk of loco-regional recurrences.

*Systemic treatment*

After surgery, systemic treatment is given to reduce the risk of distant metastatic spread of the disease. The adjuvant treatment regimen is decided based on various prognostic biomarkers/risk factors like the anatomic stage and subtype of the tumor and menopausal status of the patient.

Adjuvant endocrine therapy sometimes preceded by chemotherapy is recommended for ER-positive/HER2-negative tumors. If chemotherapy is deemed necessary after the risk assessments, then adjuvant chemotherapy is initiated a few weeks after the surgery. Most common agents are anthracyclines and taxanes[28]. Endocrine therapy (tamoxifen/aromatase inhibitors) is used to inhibit ER signaling. Tamoxifen is a selective estrogen receptor modulator (SERM): It binds to the ER, thus modifying the receptor structure and blocking the ligand binding and subsequent downstream signalling[37]. Aromatase inhibitors prevent estrogen synthesis by blocking the aromatase enzyme[38]. HER2-targeted therapy in combination with chemotherapy is recommended to treat patients with HER2-positive tumors. Monoclonal antibodies (trastuzumab/pertuzumab) bind to HER2, which is a receptor tyrosine kinase that disrupts downstream cell signalling[39].

## Metastatic breast cancer

Despite receiving standard local and adjuvant treatments, around 30% of breast cancer patients will eventually experience disease relapse. Metastatic breast cancer is still incurable with only palliative treatment options. To metastasize, tumor cells from the primary tumor must escape the physical barriers of the primary site, intravasate into the lymphatic and/or vascular system, infiltrate, and then proliferate in the distant organ[40]. The entire metastatic cascade is a complex process of many different genetic and epigenetic alterations of the tumor cells and surrounding stroma of the primary site as well as the distant organ. There are two main proposed models of tumor progression—the linear and the parallel progression model. The linear progression model considers metastatic dissemination to be a late event where various sub-clones in the primary tumor undergo a sequential selection process to form metastases[41]. The parallel progression model postulates that cancer cells acquire metastatic potential and is seeded at the distant sites already during the primary tumor formation[42]. The most common sites of breast cancer metastases are bone, lung, brain, and liver[43].

## Endocrine therapy resistance

Even if most patients do well on adjuvant endocrine treatments, 20 - 30% of patients experience disease relapse usually presenting with an endocrine therapy-resistant disease. Metastatic disease is incurable and one of the major clinical challenges. The main mechanisms underlying therapy resistance are discussed below.

*Loss of ER expression/ function*

Loss of receptor expression in advanced breast cancer leads to endocrine therapy resistance for obvious reasons. This phenotypic change has been observed in various clinical and in vitro studies[44, 45]. A more common way of tumors becoming

refractory to endocrine treatments is by acquiring mutations in the *ESR1* gene. Genomic analyses of advanced/metastatic breast cancers revealed that approximately 20% of these tumors harbor mutations in the ligand binding domain of the *ESR1* gene. The most common *ESR1* mutations are located at two amino acids Y537N/C/S and D538G in the ligand-binding domain. These mutations have been detected in circulating tumor DNAs[46] and also has been studied widely in *in vitro* cell line models[28, 47, 48]. These mutations confer ligand-independent constitutive activation of the receptor-inducing endocrine-therapy resistance.

*Cross-talk with other signaling pathways*

Growth factor signaling pathways are usually a cascade of tyrosine kinase receptors whose activation leads to cell proliferation. Cancer cells hijack these signaling pathways to induce aberrant cell proliferation and transformation. Even though ER+ breast cancer cells rely largely on ER signaling for proliferation and survival, other growth factor signaling pathways are also active. Figure 3 shows that various cellular kinases can phosphorylate the ER and its co-regulatory proteins. Ligand-bound ER has been shown to bind and activate growth factor receptors like insulin-like growth factor-1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR). These receptor activations subsequently can lead to the activation of other key signaling molecules and pathways including members of the Src family, matrix metalloproteinase, G-proteins, and the regulatory subunit of phosphatidyl-inositol-3 OH kinase (PI3K). The upregulation of other signaling pathways is an important resistance mechanism in ER+ breast cancer due to this wide cross talk between cellular signaling pathways[49, 50].

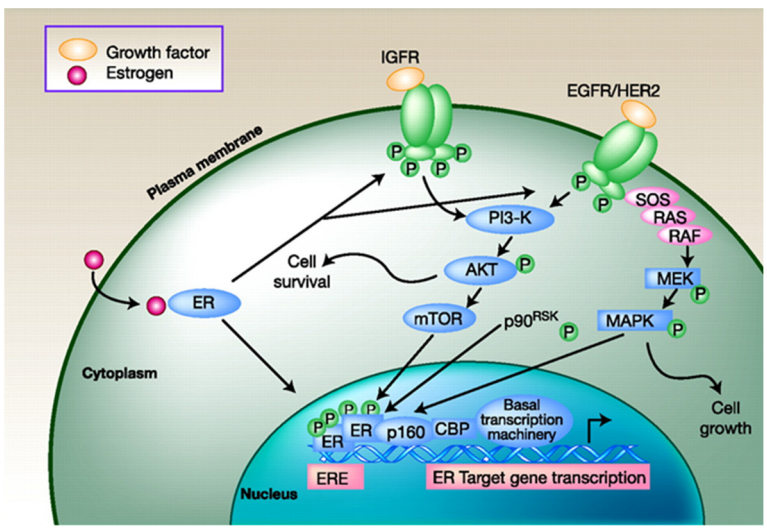


**Figure 3. Cross-talk between ER signaling and signal transduction pathways.**
Reprinted from Clinical Cancer Research, 2010, 16(7), 1979-87, Stephen R.D. Johnston, New strategies in estrogen receptor positive breast cancer with permission from AACR.

*Upregulation of cholesterol biosynthesis*

Laboratory and clinical studies have shown that breast cancer cells upregulate the genes in the lipid metabolism/cholesterol biosynthesis to acquire endocrine therapy resistance[51-53,54, 55]. Integrative analyses of aromatase inhibitor resistant cells by Nguygen *et al.* identified a stable upregulation of genes involved in cholesterol biosynthesis as a mechanism of endocrine therapy resistance[52]. Their data suggest that long-term estrogen-deprived cells convert increased cholesterol production to 27-hydroxycholesterol (27HC). Twenty seven hydroxycholesterol is an endogenous SERM, which can act as an ER ligand in long term estrogen deprived cells[56, 57]. Du *et al.* later studied the impact of long-term estrogen deprivation (to mimic aromatase inhibitors) in invasive lobular carcinoma cell line models. This study also reported an up-regulation of cholesterol biosynthesis genes in these aromatase inhibitor-resistant cell lines[54]. In 2018, Hultsch *et al.* generated tamoxifen-resistant cell lines and used RNA sequencing to identify differentially expressed genes/pathways. Long-term tamoxifen treatment led to differential expression of cholesterol genes in T47D breast cancer cells: These changes were phenotypically observed by a marked increase in the intracellular cholesterol levels[51]. The changes in cholesterol metabolism were also observed in three metastatic patient samples[51].

# Cholesterol

Cholesterol constitutes the single major sterol species of the vertebrate cell membranes. In addition to its functional role in cell membranes, cholesterol acts as the precursor for steroid hormones and bile acids[58]. Cholesterol found in a particular tissue can either be synthesized endogenously within the cells or absorbed exogenously from circulating lipoprotein cholesterol via the low-density lipoprotein receptor (LDLR). The *de novo* cholesterol synthesis pathway, also known as the mevalonate pathway, begins with the synthesis of HMGCoA from acetyl CoA molecules. A series of reactions (Figure 4) lead to the bulk synthesis of cholesterol. The mevalonate pathway is tightly regulated to maintain the intracellular cholesterol levels (Figure 4)[59, 60].

Cholesterol degradation to bile acids in the liver can be initiated by either cholesterol 7α-hydroxylase (CYP7A1) of the classic (neutral) pathway or by mitochondrial sterol 27-hydroxylase (CYP27A1) of the alternative (or acidic) pathway. Oxysterols are oxygenated derivatives of cholesterol synthesized as by-products during bile acid pathway[61]. Oxysterols were once considered to be simple metabolites. But recent research implicate that oxysterols are involved in other functions like autocrine and paracrine cellular signalling[62]. One of the main means by which the oxysterols regulate other cellular functions is by binding to nuclear receptors. Independent studies have identified 27HC—an oxysterol synthesized during the

alternative bile acid pathway by the CYP27A1 enzyme—as the link between cholesterol and breast cancer[63, 64]. This link is described in detail in the section below.

The nuclear receptor superfamily consists of 48 ligand-inducible transcription factors. Lipophilic ligands capable of crossing the plasma membrane can bind to these intracellular receptors. Ligand binding triggers the nuclear receptors to bind to their respective DNA response elements, thus releasing corepressors and recruiting coactivators to promote transcription of downstream target genes[65]. In terms of cholesterol homeostasis, liver X receptors (LXRs) are thought to be a major target of oxysterols. Specifically, LXR activation leads to the induction of target genes involved in cholesterol efflux (ATP binding cassette (ABC) transporters A1/G1/G5/G8)[66].

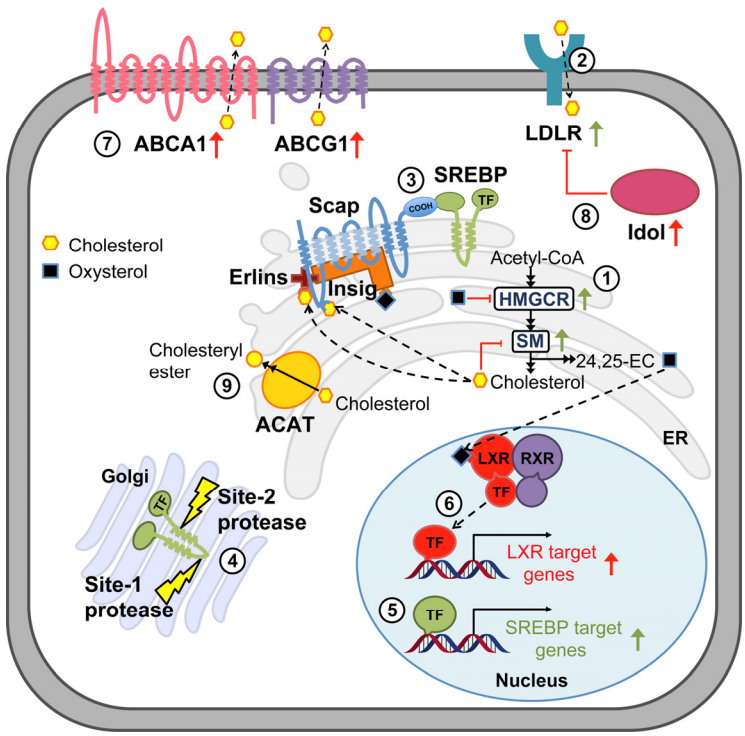


**Figure 4. Cholesterol homeostasis mechanism.**
Cholesterol is synthesized from acetyl-CoA in the endoplasmic reticulum or taken up from circulation via the LDLR. When intracellular sterol levels are low, Insig dissociates from Scap and Scap enable the translocation of SREBP to the Golgi for processing by the proteases. This results in the release of a SREBP transcription factor that translocate to the nucleus leading to the upregulation of the SREBP target genes HMGCR, LDLR, etc. (indicated by the green arrows). High intracellular cholesterol levels induce a negative feedback loop via HMGCR. Cholesterol binds to Scap and oxysterols bind to Insig causing the retention of Scap/SREBP in the endoplasmic reticulum. Oxysterols also act as ligands for the LXR upregulating transcription of LXR target genes (indicated by red arrows). These genes include ABCA1 and ABCG1, which synergize to export cholesterol from the cell. Excess cholesterol can also be esterified by ACAT for storage in an inactive form. Reprinted from Chemistry and Physics of Lipids, 199, Howe V *et al*, Cholesterol homeostasis: How do cells sense sterol excess? 170-178., 2016, with permission from Elsevier.

## Cholesterol metabolism and breast cancer

Cholesterol is an essential component of cell structures and a precursor molecule of estrogen; thus, one would expect to observe a direct correlation between serum cholesterol levels and breast cancer incidence/recurrence. However, epidemiological studies have been inconclusive with studies reporting null findings[67-71], positive associations[72-76], or negative associations[76, 77]. Laboratory studies investigating the link between cholesterol and breast cancer have yielded more conclusive findings. Llaverias and Alikhani *et al.* independently showed that the time required for tumor formation in mice that were on high cholesterol diet were shorter than the control group[78, 79]. ER-negative (ER-) breast cancer cell lines were more dependent on lipids for proliferation and migration[80-82]. Further mechanistic studies identified the oxysterol 27HC as the connecting link between ER-positive breast cancer and cholesterol.

## 27-hydroxycholetserol, CYP27A1, and breast cancer

27HC is an oxysterol synthesized during the alternative bile acid pathway. In 2007 and 2008, two independent studies showed that 27HC is a selective ER modulator (SERM)[56, 57]. Umetani *et al.* conducted a mechanistic study to assess the ER-stimulation properties of various oxysterols. This study reported that the oxysterols 4S-hydroxycholesterol, 25-hydroxycholesterol, and 27HC were capable of inhibiting the actions of estradiol [57]. In a follow-up study, DuSell *et al.* reported that upon binding of 27HC, the ER assumed a structural conformation unique from tamoxifen or estradiol binding[56]. The ER modulatory effects of 27HC are tissue/cell type specific: In models of cardiovascular diseases, it behaves as an ER antagonist while in models of ER+ breast cancer it is a partial agonist[56, 57, 83]. Further *in vitro* and *in vivo* studies investigated the link between cholesterol and breast cancer with a focus on 27HC. Two independent studies showed that 27HC treatment induced tumor growth in MCF7 xenograft mouse models. These studies also showed that this tumor-promoting effect of 27HC was mediated by the expression of the CYP27A1 enzyme. Ablation of CYP27A1 expression in these transgenic mouse models attenuated the tumor-promoting effects of 27HC[63]. However, a later study that unraveled the role of cholesterol biosynthesis in endocrine therapy resistance showed that breast cancer cells were insensitive to the proposed growth-promoting effects of 27HC[55]. Recently it was also shown that 27HC promote breast cancer cell migration and invasion in ER positive as well as negative cell lines mediated by ERβ signaling[82].

As mentioned before, the LXRs are a major target of oxysterol binding. 27HC is an LXR agonist—their activation leads to reduced cellular proliferation. This scenario presents a competition between 27HC signaling via these two (ER and LXR) known receptors. Mechanistic studies show when either of the receptors are

downregulated/antagonized, 27HC can signal via the other available receptor[63, 83]. This also means that the balance between proliferative or anti-proliferative effects of 27HC signaling can be influenced by other biochemical factors like the abundance of estradiol.

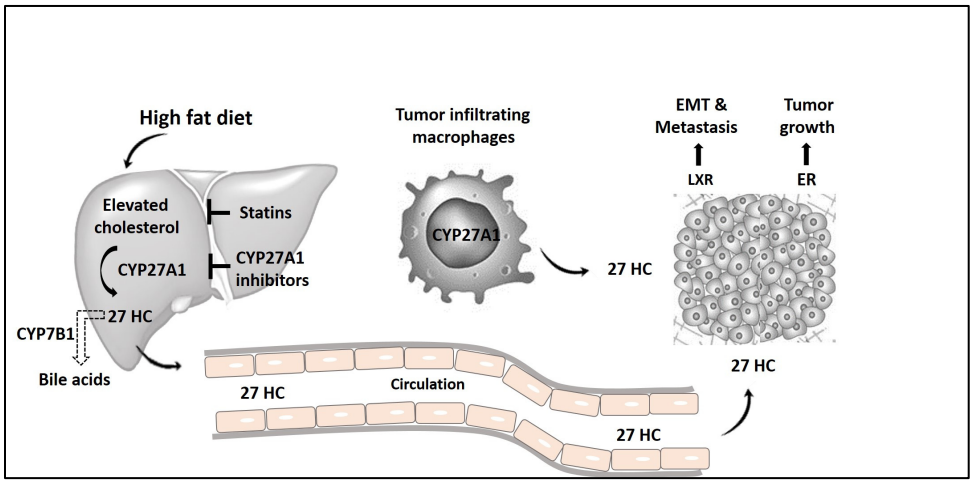


**Figure 5. Role of 27HC in breast cancer.** 27HC is produced by CYP27A1 during the bile acid synthesis pathway. This pathway primarily active in the liver cells is also functional in other cell types like macrophages and tumor cells. 27HC binds to and signal via nuclear receptors ER (α, β) and LXR.

CYP27A1 synthesizes 27HC and hence has been used in various studies as a surrogate biomarker for pathological actions of 27HC. The first study to assess tumoral expression of CYP27A1 in breast cancer did not find any prognostic role[63]. However further studies identified a significant prognostic role for CYP27A1 expression in breast cancer. In 2017, Kimbung *et al.* showed that high CYP27A1 expression was associated with increased recurrence-free and overall survival times in ER+ breast cancer patients who were younger than 50 years of age[84]. These findings were further validated by Nelson *et al.* using an online Kaplan-Meier Plotter tool, which also indicated that high CYP27A1 expression was a good prognostic marker[85]. These findings were counterintuitive to the theory that 27HC promotes BC cell proliferation because one would expect that high CYP27A1 expression would mean poor prognosis. The speculation was that high 27HC binding abrogated the effects of estrogen signaling in pre-menopausal women because the amount of circulating estrogen is higher.

## Cholesterol lowering medication – Statins

Identification of statins as the first class of cholesterol-lowering medications is considered to be a therapeutic milestone in the field of cardiovascular diseases[86]. Statins inhibit HMG-CoA reductase activity, thus preventing *de novo* cholesterol synthesis. To maintain intracellular cholesterol homeostasis, cells upregulate LDLR-mediated endocytosis of plasma LDL, thus reducing plasma cholesterol[87]. Cholesterol is linked to tumor proliferation and treatment resistance, and thus statins are generally well-tolerated drugs; nevertheless, there is great interest in repurposing statins as anti-cancer agents.

## Statins and breast cancer

*In vitro studies*

The impact of statin treatment on breast cancer cells has been investigated since the early 90s. The anti-proliferative impact of statin on breast cancer cells could be attributed to the reduction of the mevalonate derivative proteins farnesyl pyrophosphate and geranylgeranyl pyrophosphate—these are isoprenoids involved in prenylation of RAS and Rho proteins necessary for cell signaling[88]. In a pioneering study, Keyomarsi *et al.* treated MCF7 breast cancer cell line with lovastatin and reported that lovastatin treatment arrested the cell cycle in the G1 phase[89]. Further studies from the same group showed that lovastatin treatment induced expression of p21 and p27 in different BC cell lines[90]. Later in 2001, Denoyelle *et al.* showed that statin treatment reduced the tumorigenic and migratory properties of triple negative breast cancer cells by inhibiting its Rho-dependent pathway[88]. Later studies identified that not all breast cancer cell lines were equally sensitive to the anti-proliferative effects of statins. Cell lines with activated Ras or ErbB2 (HER2) pathways as well as cells that lacked ER expression were more sensitive to the anti-proliferative effects of statins[91, 92]. Due to this heterogeneous response to statin treatment, studies were designed to identify predictive biomarkers for clinical utility. Goard *et al.* and Kimbung *et al.* identified gene expression signatures that could predict statin sensitivity[92, 93]. High expression of cholesterol biosynthesis genes was found to be a marker of statin insensitivity[93]. *In vivo* mouse models were used to show that atorvastatin treatment selectively suppressed the epithelial mesenchymal process and prevented liver and lung metastases[94, 95].

*Epidemiology and Clinical studies*

Even if there is limited evidence for an association between statin use and breast cancer risk, epidemiological studies have been more consistent in showing an inverse association between statin use and reduced disease recurrence. In 2011, a prospective study conducted in Denmark with 18,769 participants reported a significant reduction in breast cancer recurrence after 10 years of follow-up

(adjusted HR=0.70 (0.57–0.86))[96]. Both pre- and post-diagnosis statin use was associated with a lower breast cancer death HR=0.54 (0.44–0.67) and HR=0.46 (0.38–0.55), respectively [97]. When the association between cholesterol-lowering medication and disease recurrence during endocrine therapy was investigated in the BIG 1-98 trial[98], the use of cholesterol lowering medication was found to improve disease-free survival (HR=0.79 (0.66–0.95)), breast cancer-free interval (HR=0.76 (0.60–0.97)), and distant recurrence-free interval (HR=0.74 (0.56–0.97))[99]. A Swedish nationwide study also showed a lower risk of breast cancer-related deaths among statin users including both pre-diagnostic and post-diagnostic statin use (HR=0.77 (0.63-0.95)) and (HR=0.83(0.75–0.93)), respectively[100]. Other studies found no association between statin use and disease recurrence[101, 102].

To the best of my knowledge, only three window-of-opportunity trials have been conducted in clinical settings to assess the impact of statin use in breast cancer. All of these studies consistently showed a decrease in tumor proliferation (assessed by Ki67 status) in post statin treatment samples versus pre-treatment samples[103-105].

# Background to this thesis

The link between cholesterol and breast cancer prognosis is not completely understood. Evidence from pre-clinical, clinical, and epidemiological studies consistently show that the cholesterol-lowering medication (statins) might have a protective role against breast cancer recurrence. A biomarker to select for patients that would benefit from adding statins to their adjuvant treatment regime is lacking. Laboratory studies have identified biomarkers that are differentially expressed in breast cancer cells with varying sensitivity to growth inhibition by statins. However, the treatment-predictive clinical utility of these biomarkers is still unclear.

Upregulation of cholesterol biosynthesis is a mechanism of endocrine therapy resistance in breast cancer cells. 27HC is an oxysterol abundant in the circulation that has been identified as one of the links that connects cholesterol to breast cancer pathology. 27HC is an endogenous SERM with differential ER regulatory properties depending on the available estrogen levels. CYP27A1 is an enzyme that synthesizes 27HC and has been used as a proxy biomarker to study the impact of 27HC in breast cancer. Statins inhibit cholesterol biosynthesis and thus reducing 27HC levels and in turn could abrogate ER activation in breast cancer cells.

# Hypotheses

- *In vitro* statin treatment might induce differential response in cholesterol/fatty acid metabolism in statin insensitive versus sensitive cell lines.
- Post-diagnosis statin use is protective against disease recurrence in postmenopausal breast cancer patients.
- Tumor-specific expression of CYP27A1 has differential prognostic impact based on the menopausal status of the patients.
- Long-term statin combination with estrogen deprivation downregulates the CYP27A1 expression and epithelial to mesenchymal markers in breast cancer cell lines.

# Aims

## Overall aim

- The overall objective was to investigate breast cancer progression while emphasizing the cholesterol-breast cancer linkage in estrogen-receptor-positive breast cancer patients.

## Specific aims

- Investigate and compare the response in cholesterol and fatty acid metabolic pathways in breast cancer cell lines displaying varying sensitivity to statin treatment.
- Study the prognostic impact of statin use regarding breast cancer prognosis.
- Characterize CYP27A1 expression in primary invasive breast cancers in relation to tumor pathological features and study its prognostic impact in pre- and post-menopausal breast cancer patients.
- Study the molecular impact of long-term concomitant statin and endocrine therapy on breast cancer cell lines.

# Patient cohorts

Papers II, III, and IV in this thesis are based on the following patient cohorts.

## The Malmö Diet and Cancer Study (Papers II and III)

The Malmö Diet and Cancer Study (MDCS) is a population-based prospective cohort study initiated to study the associations between diet and cancer[106]. The inclusion criteria were Swedish language proficiency and mental ability to understand the extensive questionnaires. A total of 68,905 eligible individuals comprising both men and women were invited and 28,098 enrolled individuals completed all study parts. Among the individuals who completed all the study parts, 17,035 were women born between 1923 and 1950. During 1991-1996, all of these women visited the study center for baseline examinations where body measurements and blood samples were collected. On a yearly basis, linkage to the South Swedish Regional Tumor Registry, the Swedish Cancer Registry, and the Swedish Cause of Death Registry was performed to identify incident breast cancer cases, vital status, and cause of death[106].

In the MDCS cohort there were 1,240 incident breast cancer cases diagnosed during the years 1991-2014. The Swedish prescription registry was initiated in 2005, and hence the medication history of the patients diagnosed before 2005 is not available; thus, these patients were excluded from subsequent analyses. Patients who had in situ carcinoma, bilateral cancers, were premenopausal at the time of diagnosis, or those who had pre-diagnostic statin use were also excluded (Figure 6).

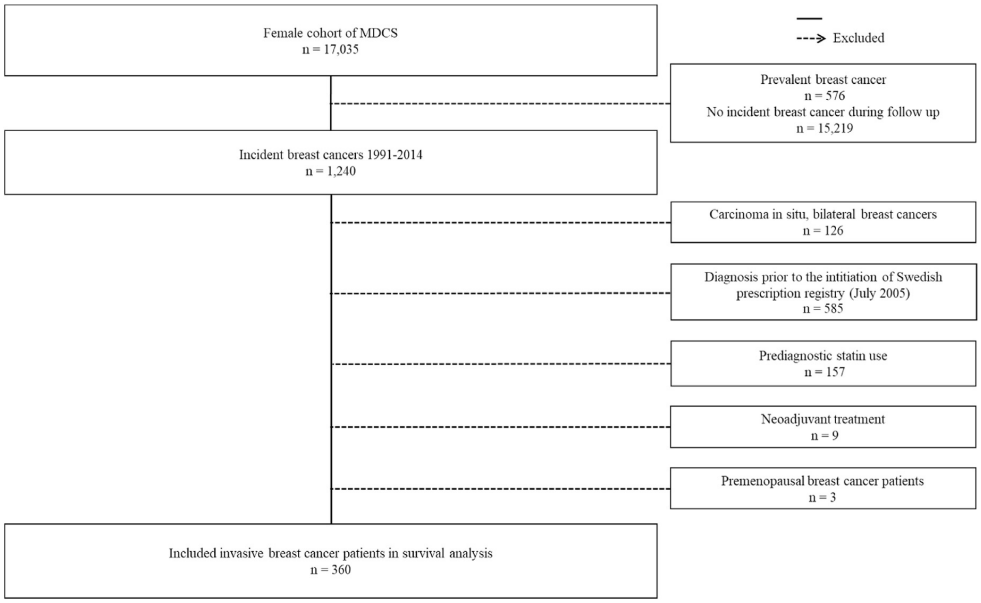


**Figure 6.** CONSORT diagram of study II. Flowchart showing numbers of patients included in and excluded from the analyses

Paper III included invasive breast cancer cases diagnosed during 1991-2010. The exclusion criteria detailed in the paper led to 645 cases evaluable for CYP27A1 expression (Figure 7).

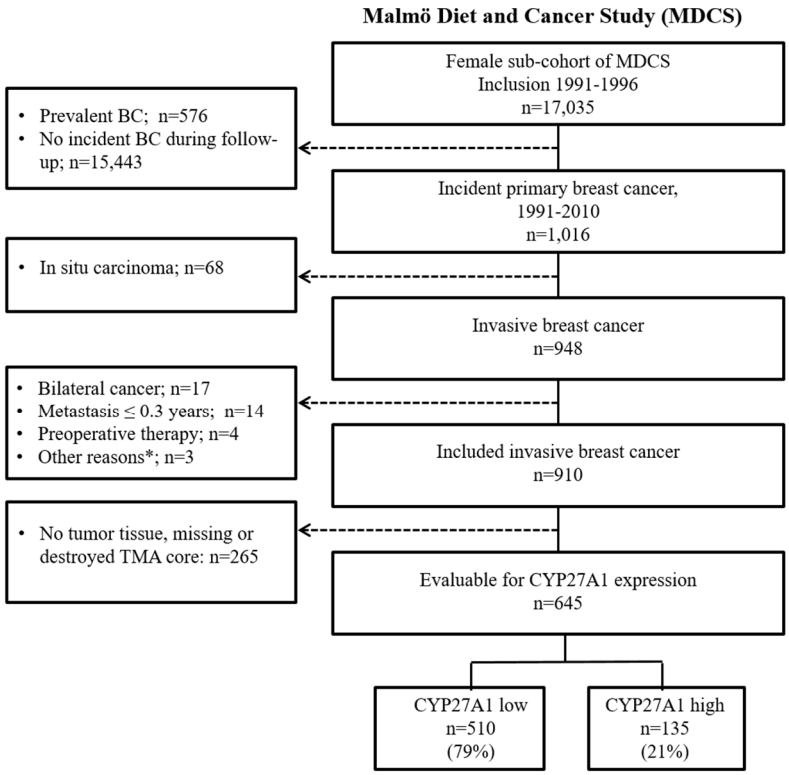


**Figure 7. Consort diagram of MDCS included in study III.** Flowchart showing numbers of patients included in and excluded from the analyses

# The Breast Cancer and Blood cohort

The Breast Cancer and Blood (BC-blood) cohort is a prospective cohort study initiated in October 2002 in Lund, Sweden[107, 108]. The main aim was to understand the impact of genetic and lifestyle factors on breast cancer prognosis. Women with primary breast cancer were invited to participate. Patients who had a previous cancer (within the past ten years) were excluded. Patients filled in a questionnaire pertaining to lifestyle factors, reproductive history, as well as medication and exogenous hormone use at study inclusion during the perioperative hospital visit. Anthropometric body measurements were collected by a certified research nurse at study inclusion. Follow-up questionnaires were filled at subsequent review visits at three to six months, seven to nine months, and annually for the subsequent three years and biannually (by mail) thereafter. Clinicopathological information,

treatment history, and breast cancer events were collected from hospital records. Information regarding new cancers and deaths were retrieved from the South Swedish Regional Tumor registry and the population registry respectively. To analyze the CYP27A1 expression in BC-blood cohort, we included breast cancer patients with invasive disease included during the October 2002- June 2012. After excluding patients who received preoperative treatment, patients included in the MAST trial and those who relapsed within four months of primary diagnosis 987 patients were eligible to be included for the current study. Of these, CYP27A1 expression was evaluable in 813 patients.

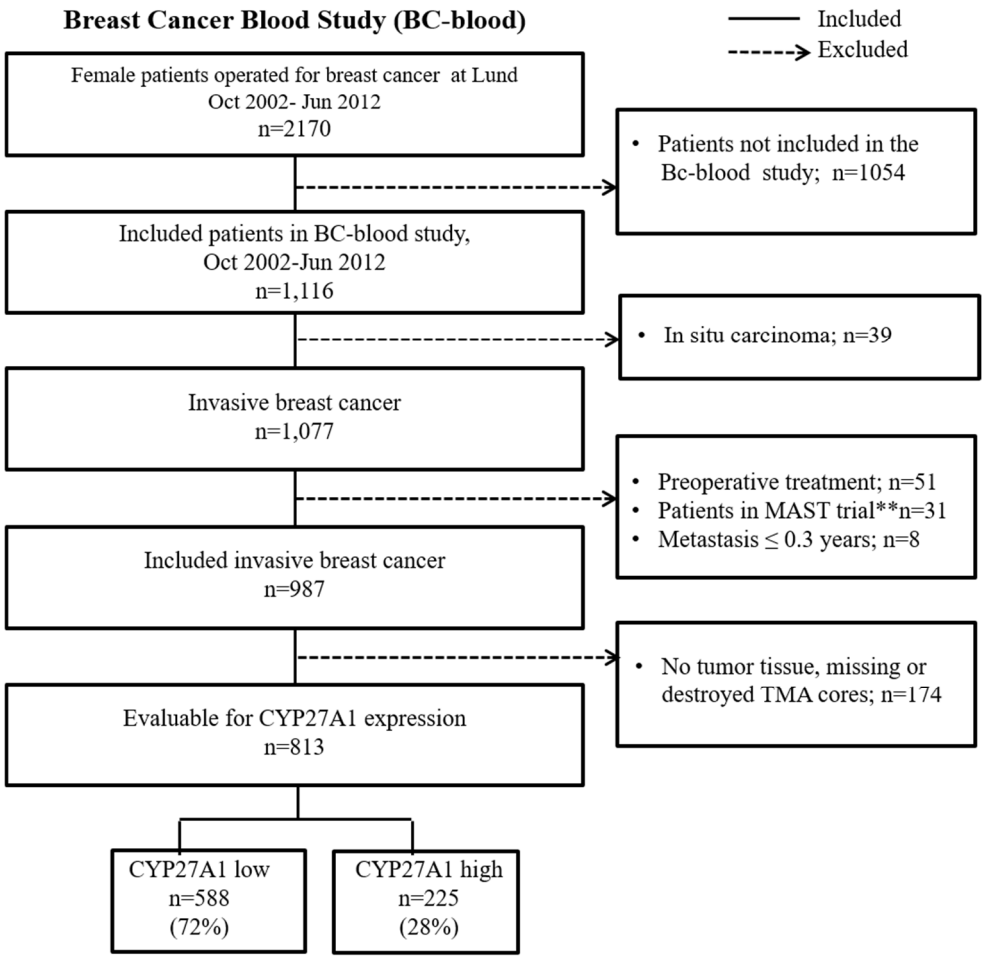


**Figure 8. CONSORT diagram of BC-blood cohort included in the study III.** Flowchart showing numbers of patients included in and excluded from the analyses.

# SB91B cohort

The SB91B study is a prospective study that investigates the prognostic value of an index based on tumor proliferation (S-phase fraction) PgR status and tumor size in breast cancer. The original study included 237 premenopausal breast cancer patients with node-negative disease diagnosed between 1991 and 1994. After excluding cases that were not evaluable for CYP27A1 expression (mRNA and protein) due to the non-availability or poor quality of tumor cores, 193 patients were included in the current study (Figure 9).

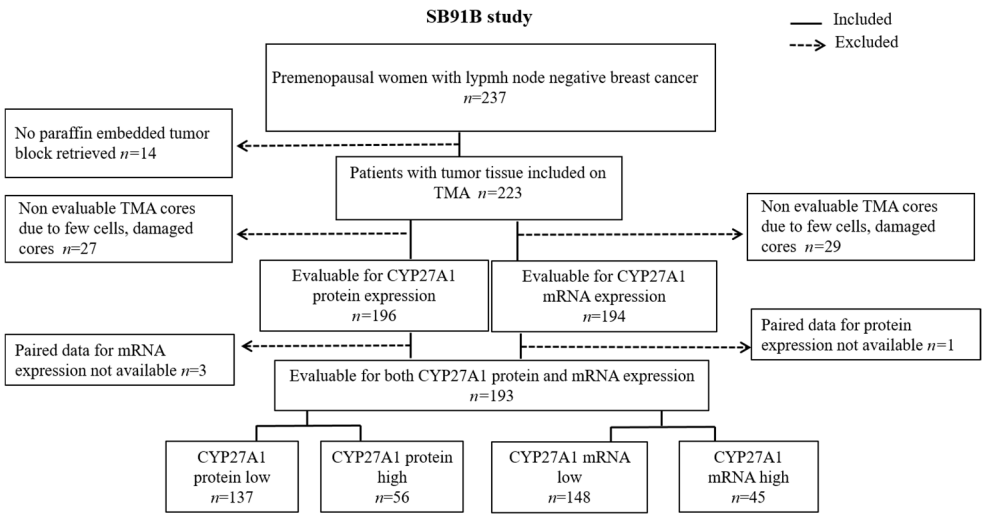


**Figure 9. CONSORT diagram of study IV.** Flowchart showing numbers of patients included in and excluded from the analyses.

# Methodological considerations

## In vitro studies.

### Cell lines

Six human breast cancer cell lines were used: T47D, MCF7, BT474, SKBR3, MDA-MB-231 and CAMA1. These cell lines have different hormone receptor status and molecular features that encompass the heterogeneity of breast cancer[109]. Table 2 summarizes the main molecular features of each of these cell lines. All cell lines were originally isolated from pleural effusions. One of the major advantages of using cultured cell lines in cancer research is the unlimited supply of nearly homogenous experimental systems.

Selection of the right cell lines to address a specific research question is a critical aspect in the design of an experimental study. In paper I, the primary aim was to study the heterogeneous response to statin treatment. Here, we selected a panel of cell lines with varying statin-sensitivity. The lipid-droplet staining experiments were limited to two representative cell lines due to time constraints.

For the *in vitro* parts of paper IV, the main criterion for cell line selection was the expression of the ER because that study investigated the impact of 27HC, which is a SERM. The T47D cells were more sensitive to alterations in the cholesterol/lipid metabolism than MCF7 cells, which were more sensitive to the presence or absence of estrogen. This heightened sensitivity to the presence or absence of estrogen may mask the impact on other pathways like the cholesterol biosynthesis pathway. We also included triple negative MDA-MB-231 cells as a negative control for the proliferation studies (data not shown in manuscript). Interestingly, MDA-MB-231 cells did not proliferate when cultured in lipid-depleted medium, thus indicating that this cell line depends on the availability of exogenous lipids for proliferation.

In study V, one of the secondary aims was to study the impact of 27HC in endocrine therapy resistant cells. 27HC acts as a SERM, and the ability of resistant cell lines to retain the expression of ER was considered to be the main selection criteria.

**Table 2. Breast cancer cell line characteristics.**

| Cell line | Surrogate subtype | Hormone receptor status | Phenotypes | Genetic mutations | Statin sensitivity | Tamoxifen sensitivity | AI sensitivity |
|---|---|---|---|---|---|---|---|
| T47D | Luminal A | ER+,PgR+,HER2- | Epithelial | PIK2CA, TP53 | Resistant | Sensitive | Sensitive |
| MCF7 | Luminal A | ER+,PgR+,HER2- | Epithelial | PIK3CA, CDKN2A | Resistant | Sensitive | Sensitive |
| BT474 | Luminal B | ER+,PgR+,HER2+ | Epithelial | PIK3CA, K111N | Resistant | Resistant | Sensitive |
| SKBR3 | HER2+ | ER-,PgR-,HER2+ | Epithelial | TP53 | Moderate S | Resistant | Resistant |
| MDA-MB-231 | Triple negative | ER-,PgR-,HER2- | Mesenchymal | BRAF, TP53,CDKN2A,KRAS,NF2 | High S | Resistant | Resistant |
| CAMA1 | Luminal A | ER+,PgR+,HER2- | Epithelial | PTEN, TP53 | Resistant | Resistant | Sensitive |

R: resistant, S sensitive

## Lipid droplet staining

Oil Red O staining was used to stain and quantify lipid droplets after statin treatment. Oil Red O selectively stains neutral lipids and cholesteryl esters but not the polar lipids found in the cellular membranes[110]. The statin-treated cells were fixed with 3% paraformaldehyde and followed by incubation in 60% isopropanol. Finally, the cells were stained with Oil Red O-working solution. Excess dye was removed by multiple washing steps in 60% and 10% isopropanol. Next, 100% isopropanol was used to extract the bound dye for a semi-quantitative measurement of the lipid droplet abundance via absorbance at 518 nm. The limitations of using Oil Red O staining are that it cannot be used to stain formalin-fixed paraffin-embedded (FFPE) tissue because the deparaffinization process extracts most lipids from the tissue sections. It is also a quantitative method, more advanced chromatography techniques are required to characterize or perform qualitative analysis of lipid droplets[110].

## Sulforhodamine B (SRB) cell proliferation assay

Cell proliferation assays are generally used to assess the impact of drugs or cytotoxic compounds on the viability of cells. There are a variety of assays developed and based on various cell function principles like enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. These assays also vary based on the time point at which the cell viability is measured: continuous real-time monitoring or end-point monitoring[111].

The SRB assay is based on the ability of the protein dye Sulforhodamine B to bind to basic amino acid residues in an electrostatic and pH-dependent manner via trichloroacetic acid in fixed cells[112]. The dye can bind to proteins in the cell under mildly acidic conditions; bound dye can be extracted from the cell particles by increasing the pH to mildly basic conditions. The extracted dye is then further quantified using a spectrophotometer. There are several advantages to using the SRB assay: i) relatively easy to perform, ii) can be scaled up or down to various plate formats, iii) does not require advanced equipment and iv) robust reproducibility. The main limitation of using SRB assay is that it is an endpoint assay thus only give information about the cell proliferation at a single time point.

## Gene expression microarrays

Gene expression microarrays are a high throughput sequencing technique used to assess the transcriptional activity in cells at a given time point. One of the main applications of gene expression microarrays is to identify the underlying mechanism

of drug response[113]. Briefly, a microarray glass slide contains thousands of spots with copies of identical DNA molecules corresponding to a single gene. A key step in the process of gene expression arrays is the isolation of quality RNA from the source. The RNA molecule is reversely transcribed to synthesize complementary DNA (cDNA), and the nucleotides are labelled with fluorescent dyes. The labelled cDNA is then hybridized onto the microarray glass slide with immobilized oligonucleotides. The fluorescent intensity corresponds to the abundance of a particular gene facilitating quantification. This gene quantification technique generates large amounts of data requiring bioinformatics expertise for data analysis and interpretation. Unlike the newer whole genome sequencing technique, the quality of the data obtained from gene expression microarrays largely rely on oligonucleotide probes.

## Immunoblotting

Immunoblotting or western blotting is a technique widely used in experimental studies to assess protein expression in cells[114]. The total cell lysate is obtained by lysing the cells using lysis buffer. The total amount of protein in the samples is then quantified, and equal amounts of protein (per sample) is resolved by electrophoresis. The protein separated by the molecular mass is then transferred to nitrocellulose membranes. These membranes are then blotted with specific primary antibodies. They are subsequently stained with secondary antibodies to visualize the protein bands. The bands can be quantified using densitometry via imaging software. The results of immunoblot experiments largely depend on the availability of good quality antibodies.

## qRT-PCR

The real time polymerase chain reaction (qRT-PCR) is a technique commonly used to assess gene expression in cells. This technique can be used either to test the presence/absence or to quantify the target gene expression[115]. The first step in this method is to isolate total RNA and then quantify and translate it to its complimentary DNA (cDNA). TaqMan probes were used to amplify the cDNA molecules in studies I, IV, and V. TaqMan probes consist of a fluorophore and a quencher. The quencher molecule quenches the fluorescence emitted by the fluorophore. Taq DNA polymerase was used to synthesize new strands using unlabeled primers and the template. When the polymerase reached a TaqMan probe, its endogenous 5' nuclease activity cleaves the probe, thus separating the fluorophore from the quencher resulting in the emission of fluorescent signal[116]. The use of TaqMan probes increases the specificity and yield of PCR reactions[117].

# Biomarker studies

## Tissue microarray

Tissue microarrays (TMA) are a high throughput technique to evaluate the expression of biomarkers (RNA, protein) conveniently in large materials. TMAs are constructed by multiple extracted cylindrical tissue core biopsies obtained from representative parts of FFPE tumor blocks. These cores are further embedded into a common recipient block—the TMA. The recipient block is then sliced into thin sections and mounted on glass slides[118]. The sections on the glass slides are then stained with antibodies or oligonucleotide probes for evaluation of protein or RNA expression, respectively. The TMA blocks can be stored for many years and stained with many different antibodies to assess the expression of various targets. One of the main advantages of using TMAs versus whole tissue sections is the reduced handling time and resources (tissue, reagents and antibodies). A major disadvantage of using the small cores is the difficulty in capturing tumor heterogeneity. This disadvantage is often circumvented by obtaining multiple cores from the same patient.

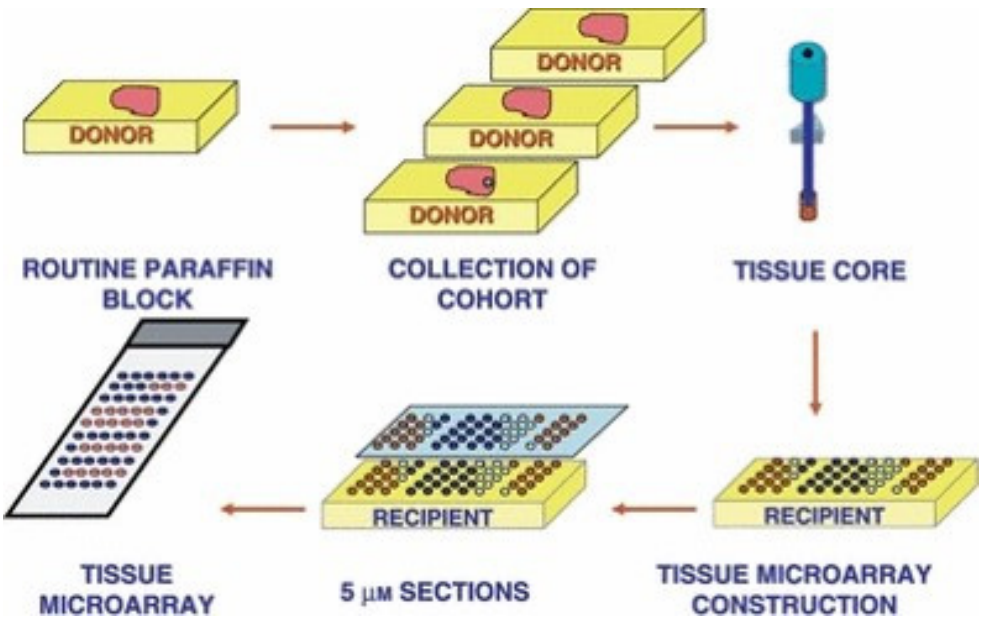


**Figure 10. Workflow of tissue microarray construction.**
Reprinted with permission from J Oral Pathology Medicine, Volume: 37, Issue: 3, Pages: 166-176, first published: 03 February 2008, DOI: (10.1111/j.1600-0714.2007.00606.x).

## Immunohistochemistry

Immunohistochemistry (IHC) visualizes protein in the cells/ tissue. IHC is routinely used for diagnosis in pathology division as well as in biomarker research. The basic principle of IHC relies on the specific binding of antibodies and antigens (protein of interest). In the general IHC protocol, the FFPE tissue is stained with the primary antibody. A secondary antibody raised against the primary antibody with high specificity is added next. The secondary antibody is labelled with an enzyme, and finally a chemical substrate is added to react with the enzyme to create a colored precipitate.

The major methodological consideration in IHC is the specificity, selectivity, and sensitivity of the antibody. This work used a monoclonal CYP27A1 antibody (Abcam, [EPR7529], ab126785) that had been thoroughly validated in a previous study using HEPG2 (human liver cancer cell line) cells and normal human liver tissue as external positive controls. The blocking peptide technique was used to ablate the signal and served as a negative control[63]. The staining protocol has been described in detail in previous work and the publications included in this thesis[84].

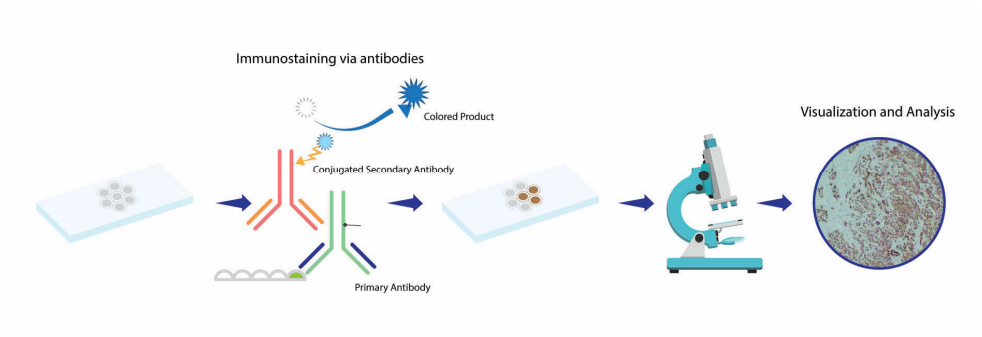


**Figure 11. Immunohistochemistry workflow.**

## RNA *in situ* hybridization

The fourth study used a commercially available RNA *in situ* hybridization technique called RNAscope. This technique visualizes single RNA molecules in cells or tissues[119]. The workflow is similar to IHC. The cells are initially fixed and permeabilized to allow the target probe access after which oligonucleotide probes are hybridized. The signal from the probe is then amplified by addition of multiple amplification molecules. Finally, detection reagents are used to visualize the amplified signal from target RNA (Figure 12). The slides can be visualized under a standard bright field microscope. This RNA quantification technique has a major advantage over other gene expression techniques because the workflow and conditions are similar to IHC; it also allows for a direct comparison between target

expression assessed at different levels (protein and RNA). Compared to the standard RNA in situ hybridization technique, RNAscope uses target specific probes, thus minimizing non-specific signal amplification.

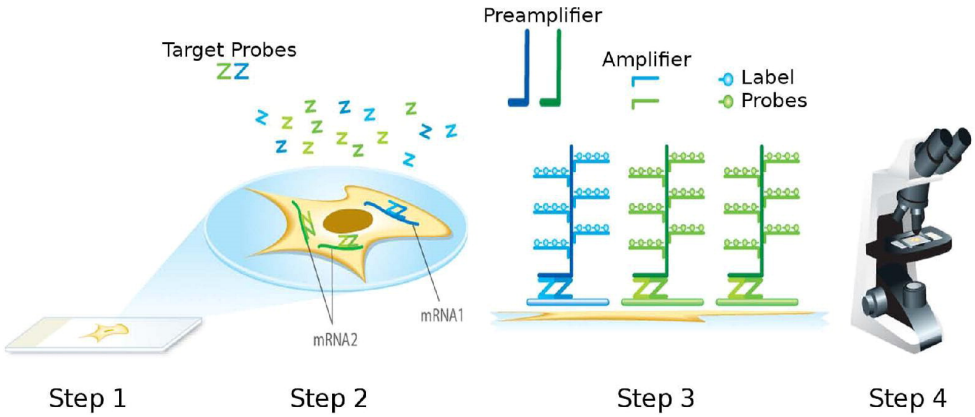


**Figure 12. Workflow of RNAscope and RNA in situ hybridization.**

# Data analysis and statistics

This part is focused on the data analysis and statistics for the epidemiology studies (II-IV).

### Survival analyses

In study II, the incidence rates for an event (respective endpoints) depending on statin use was computed using Cox regression models. In these models, statin use was used as a dichotomous time-varying variable to avoid the immortal time bias. In studies IV and V, Kaplan-Meier plots were used to visualize the difference in event free survival probabilities between patient sub groups with different levels of intra-tumoral CYP27A1 expression. Log-rank tests were used to estimate and compare the events (for respective endpoints analyzed) between different categories. Log-rank tests can only be used to test if there is a significant difference between different categories—it does not provide information regarding the effect size. A major disadvantage of the log-rank estimates is that it is a univariable analysis method; hence, it is not possible to adjust for possible confounders.

In studies III and IV, Cox regression models were used to calculate the adjusted hazard ratios. Cox proportional hazards models are used to estimate the hazard ratio, which is the magnitude of risk of an event happening. Cox proportional models are multivariable models and can be adjusted for multiple confounders. This gives the

test a particular advantage of testing the independent prognostic value of different factors, e.g., the independent prognostic value of the CYP27A1 biomarker assessed in our studies. The main assumption of the Cox proportional hazards models is the proportionality of hazards meaning that the ratio of event rates should be constant during follow-up time. The assumption of proportional hazards can be formally tested by Schoenfeld's test. Schoenfeld's test for proportional hazards was done for the MDCS cohort in paper III, and the assumption was not violated. It was somewhat surprising to see that the assumption was not violated because the Kaplan Meier plots for the MDCS cohort began to separate at five-year mark. One would expect to see a non-proportional hazard ratio over the complete follow up time. This helps explain why KM plots should not be relied on for assessment of proportional hazards.

An important factor to consider when fitting a multivariable model is the number of adjusted covariates. A general rule suggests that there be at least five to ten events per covariate that is adjusted in the model.

## Endpoints

Different endpoints were used in the different studies included in the thesis. The primary endpoint in paper II was breast cancer recurrence. Secondary analyses also analyzed loco-regional recurrences, distant recurrence, and overall survival. When studying the prognostic impact of statin use in breast cancer, disease recurrence is considered to be a more robust endpoint than endpoints that include deaths (due to any cause). One major disadvantage of observational studies is that clinico-pathological factors may not be equally distributed between exposed and unexposed groups and may lead to confounding by indication. This is especially important when we assess the prognostic impact of drugs like statins in the breast cancer setting. Statin is prescribed as a secondary preventive to lower serum cholesterol levels in patients with higher cardiovascular risk. Thus, using a more cancer-specific endpoint minimizes the impact of other confounding issues with comorbidities like cardiovascular diseases and obesity directly related to statin use and mortality.

In the third study, the primary endpoint was overall survival. Other endpoints were breast cancer-specific survival (MDCS) and recurrence-free survival (BC-blood study). Recurrence free survival included deaths due to any cause as events. However, the results might have been more comparable between the cohorts if we had included deaths only with preceding breast cancer events. The fourth study evaluated three distinct clinical endpoints: recurrence-free survival, distant recurrence-free survival, and overall survival. The women in this cohort were pre-menopausal at inclusion, and it is expected that almost all deaths are breast cancer related.

## Systemic errors or biases

In observational studies, systemic errors/biases can be classified into three main categories: selection bias, information bias, and confounding[120]. A confounding variable is defined as a factor that influences exposure and outcomes. Confounding is an important issue in the epidemiological studies (II, III and IV) included in this thesis, as in observational studies potential confounders might not be equally distributed among different patient subgroups. To minimize the impact by confounding variables, we included established prognostic factors in multivariable analyses. The multivariable models were adjusted for known prognostic factors like age, body mass index, tumor histological grade, lymph node status, etc. to assess the independent prognostic value of CYP27A1. Of course, there could be other residual confounders such as high cholesterol levels for which there was no data available. The impact of unknown confounders should also be considered when interpreting the results.

## Validity

Validity generally refers to the accuracy of the measurements/findings obtained from a study. Validity can be broadly classified in to two parts: internal and external validity.

Internal validity refers to the accuracy of findings within the study population. To ensure internal validity in the CYP27A1 biomarker studies, we used a well-validated antibody as described in detail above. In papers II and III, anthropometry measures were obtained via a trained research nurse. However, the anthropometric measure (BMI) used in the MDCS cohort was obtained during the study inclusion, and this data during breast cancer diagnosis might have been more accurate.

External validity is also known as generalizability and is the degree to which the findings from a study can be extrapolated to a broader context. The two cohorts (MDCS and BC-blood) included in this thesis are population-based cohorts, and our findings are generalizable to breast cancer patients in the underlying population from which these cohorts were selected. Most premenopausal patients included in the SB91B cohort did not receive any adjuvant endocrine treatment, but most of these patients will be subjected to endocrine treatments in the current clinical scenario. These differences between cohorts could impair the generalizability of cohort studies.

# Ethical considerations

Patient data from three different cohorts is used in this thesis. In these projects, we used pseudonymous (coded) patient data. All studies have been approved by the ethics committee. For the original study inclusions, all patients signed a written informed consent before entering the study.

The advantages must outweigh the disadvantages to get ethical approval. All patients were included in observational studies, and hence the risks posed by an intervention were minimal. Performing biomarker studies is an important research step to identify prognostic- and treatment-predictive biomarkers to tailor the best treatment plan for each patient. When the TMAs are constructed care is taken not to exhaust the tumor material, in case it is needed for future clinical prognostication purposes. Our studies aim to assess the prognostic potential of these biomarkers with the hope that the findings will help to optimize the best treatment, and thus the benefits outweigh risks for the subjects included in the study[121].

For the long-term endocrine therapy treated cells, we intend to perform the short tandem repeat (STR) profiling to ensure the identity of the cells. Performing STR sequencing on cell lines recently developed from primary cells could be an ethical concern because the profile could be used to re-identify the donor or related family members. However, we are using cell lines that have been long-established and have been used in research for decades; thus, identification of the donor source should not be a problem here.

# Results and Discussion

## Paper I

*Results*

In this study, a panel of breast cancer cells were treated with increasing concentrations of atorvastatin and were classified as sensitive or insensitive to statin treatment in terms of cell proliferation. Cell lines T47D, MCF7, and BT474 were considered insensitive; SKBR3 was moderately sensitive; and triple negative MDA-MB-231 was extremely sensitive to growth inhibition by atorvastatin treatment. To understand the statin-resistant mechanisms in the insensitive cell lines, we investigated the lipid-accumulating phenotype in two representative cell lines with varying statin sensitivity: statin-insensitive T47D and statin-sensitive MDA-MB-231. Atorvastatin treatment induced a dose- and time-dependent accumulation of lipid droplets in T47D cells and not in MDA-MBA-231 cells. To further explore the phenotypic difference of lipid accumulation, we investigated differences in the expression of genes involved in cholesterol and fatty acid metabolism. Stearoyl-CoA desaturase (SCD) genes involved in the unsaturated fatty acid metabolism and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) gene in the cholesterol synthesis pathway were significantly upregulated upon statin treatment in the insensitive cell lines.

*Discussion*

The strong preclinical and clinical evidence [88, 91, 96, 122-124] regarding the anticancer potential of statins has shown a great interest in repurposing cholesterol-lowering statin drugs as anti-cancer agents. Cancer cells are known to show varying sensitivity to growth inhibition by statin treatment, and certain molecular features can be used for patient stratification. It has been shown that breast cancer cells show differential cholesterol/lipid metabolic features at baseline[81, 125]. Thus, we hypothesized that the differences observed at baseline might also reflect a differential response to statin treatment. We thus focused our study on the lipid-accumulating phenotype in breast cancer cells.

Expression of various targets in the cholesterol biosynthesis pathway has been widely studied in the aspect of statin sensitivity in breast cancer cells [93, 105, 126]. In line with previous findings, our study also showed that ER+ breast cancer cells MCF7 and T47D had a high baseline expression of HMGCR and also responded

with the strongest negative feedback loop induction of HMGCR. These cell lines were also insensitive to statin treatment in terms of proliferation. The prognostic impact of HMGCR expression in tumors has yielded mixed results and has been attributed to the varying specificity of antibodies used in different studies. Earlier studies reported that moderate/strong HMGCR expression was associated with better tumor characteristics and prognosis[127-129], but a recent study reported that HMGCR was associated with aggressive tumor characteristics[130]. This later study also looked at the prognostic impact of statin use on breast cancer mortality, thus stratifying patients based on differential HMGCR expression and reporting a null finding.

SCD is the key enzyme in the synthesis of mono-unsaturated fatty acids. The more pronounced upregulation of SCD in response to statin treatment in the insensitive cell lines was a novel observation. We also tried to perform immunoblotting to further explore the impact of this protein expression. However, a lack of a reliable antibody prevented us from validating the mRNA data at the protein level.

Of note, atorvastatin is a lipophilic statin, and the lipid droplet accumulation observed in the T47D cells lines might act to sequester the compound and thus prevent it from disrupting proliferative processes. Further lipidomic analyses is warranted to address this speculation.

SCD is strongly induced in relatively insensitive cells by statin treatment. SCD may be a potential biomarker of statin resistance. Future biomarker studies are warranted to assess the treatment predictive potential of SCD.

# Paper II

*Results*

The purpose of this study was to evaluate the prognostic impact of post-diagnosis statin use on breast cancer recurrence in a prospective population-based MDCS cohort. There were 363 women diagnosed with invasive breast cancer from 2005-2014 of which 91 patients initiated statin use post-diagnosis. Statin users were older than non-users and had higher waist-to-hip ratios and fewer lymph nodes. They were more likely to undergo lumpectomy and receive adjuvant radiation therapy. Regarding prognosis, statin users had low incidence rates for disease recurrence both in crude and adjusted Cox regression models ($HR_{crude}$ = 0.53; 95% CI (0.29-0.95); $HR_{adj}$ = 0.36; 95%CI (0.16-0.84)). Interestingly, when the analyses were stratified based on the pattern of recurrence (loco-regional vs distant), we found that statin users were particularly protected against distant recurrences.

*Discussion*

The impact of statin use on breast cancer prognosis has been studied in various epidemiological settings and outcomes assessed for different endpoints. The most common endpoints were overall survival, breast cancer-specific survival[97, 100, 130], and breast cancer recurrence[96, 101, 124, 131]. Interestingly, studies that assessed breast cancer recurrence as an endpoint consistently showed a protective impact of statin use versus the mixed findings observed for the other end-points studied. The impact of statins on reduced recurrences can be attributed to different biological mechanisms. The most abundant oxysterol in serum, 27HC, has been shown to be associated with tumor growth and metastasis in ovariectomized mice,[63, 64, 132] and we have shown that statin treatment potently reduces the serum 27HC in breast cancer patients[84]. Maybe this is one of the mechanisms by which statins reduce recurrence in these patients. Another mechanism could be the impact of stains on epithelial to mesenchymal transition of the cancer cells. As depicted by the experimental studies[92, 94, 103, 133] statins can potently inhibit the epithelial to mesenchymal transition of the cancer cells. By inhibiting the epithelial to mesenchymal transition of tumor cells, statins play role in preventing the outgrowth of breast cancer metastasis[134]. Statins could be an addition in the adjuvant cancer treatment in the clinic if validated in larger clinical trials.

# Papers III and IV

*Results*

Paper III assessed the prognostic impact of CYP27A1 in two population-based cohorts—the MDCS and the BC-blood study while focusing on the postmenopausal patient group. The same biomarker was evaluated in paper IV in the SB91B cohort at both the protein and mRNA levels—this cohort is a premenopausal primary breast cancer cohort. Paper IV also features *in vitro* experimental data to extend the findings of the cohort epidemiology data.

Twenty one percent (n=135) of tumors in the MDCS had high CYP27A1 protein expression. In this cohort, tumors with high CYP27A1 expression were more likely to be hormone receptor (ER, PgR) negative and of high histological grade (NHG III). The prognostic impact was evaluated in all patients as well as pre-specified subgroups of patients who were older than 55 years and presented with ER-positive tumors. Multivariable Cox regression analyses showed that high CYP27A1 expression indicated poor prognosis for overall as well as breast cancer-specific survival specifically after five years of follow up.

In the BC-blood study, 28% (n=225) of the evaluable tumors had high CYP27A1 protein expression. Elevated expression of CYP27A1 was positively associated with hormone receptor negativity, NHG III, and larger tumor size. These patients were

also more likely to receive chemotherapy and less likely to receive endocrine therapy. After adjusting for potential confounding factors, CYP27A1 expression was not an indicator of prognosis for patients older the 55 years with ER positive tumors in this cohort. Surprisingly, however, high CYP27A1 expression was associated with increased risk of recurrence during the first five years of follow up in patients younger than 55 years of age.

In the SB91B cohort, 29% (n=56) and 23% (n=45) of the evaluable tumors had a high CYP27A1 protein and mRNA expression, respectively. CYP27A1 expression, evaluated at both levels, was associated with Ki67 expression and NHG III. In multivariable Cox regression analyses, high CYP27A1 expression was an independent prognostic factor for better distant recurrence-free survival. Interestingly, even in high histological grade (NHG III) and Ki67 high tumors, the high-CYP27A1-expression subgroup showed better prognosis than the low-CYP27A1 subgroup. *In vitro* studies showed that 27HC treatment reduced breast cancer cell proliferation by downregulating ER signaling in the absence of other serum lipids and presence of estradiol.

*Discussion*

We used three different cohorts to evaluate the associations between CYP27A1 expression and tumor pathological features and prognosis. Generally, high CYP27A1 was associated with aggressive tumor pathological features in all cohorts—positive association to NHG III was consistent across all cohorts included in this thesis as well as in previously published studies[84, 135]. As mentioned previously, in vitro mechanistic studies showed that 27HC acts as a SERM promoting breast cancer cell proliferation in estrogen-low experimental conditions[63, 64, 135]. From these findings, we hypothesized that high CYP27A1 expression in postmenopausal women (low circulating estrogen) would be a poor prognostic marker. As expected, high CYP27A1 expression predicted poor prognosis for breast cancer specific and overall survival in the MDCS cohort particularly after five years of follow up. However, these findings could not be replicated in the BC-blood study. This is most likely due to the shorter follow up in this cohort. The median follow-up of the MDCS cohort is 10.8 years vs seven years in the BC blood study. CYP27A1 is a marker of late lethality in postmenopausal women, and thus the shorter follow up in the BC blood study might explain the null findings.

Another striking finding from paper III was the poor prognosis observed in premenopausal patients during the early follow-up period. This was indeed intriguing because it was previously shown that high CYP27A1expression—analyzed by gene expression in women younger than 50 years of age—predicted better overall and recurrence free survival[84]. One of the reasons for this contradictory finding could be the methodological differences in assessing the

biomarker (IHC vs gene expression), which motivated the research questions in paper IV.

In paper IV, we assessed the CYP27A1 gene and protein expression using comparable in situ techniques. The results predicted good prognosis consistent with an earlier published study[84]. Then again, the findings from the BC-blood study regarding premenopausal patients contradicted those in the SB91B cohort. This contradiction could be due to significant differences in treatment regimens. The patients included in the SB91B cohort mostly did not receive any form of adjuvant endocrine treatment whereas the patients in the BC-blood study were more often given endocrine treatment (tamoxifen). Tamoxifen is a SERM-like 27HC, and thus the presence of tamoxifen complicates the entire ER signaling scenario—it is yet not known how the ligands compete for the receptor binding sites or how the downstream signaling is modified in the presence of these three ligands.

Tamoxifen treatment is also known to reduce blood cholesterol levels. In a large study of 8010 postmenopausal breast cancer patients, it was shown that total serum cholesterol levels were reduced during tamoxifen treatment[99]. This study did not measure the 27HC levels specifically. However, the total cholesterol and 27HC levels in circulation are positively correlated. Later a smaller pilot study (n=15) in patients with a median age of 58 years reported that tamoxifen treatment had no effect on 27HC levels but rather significantly reduced other oxysterols like 24HC, 7α HC, and 25HC. A larger study in premenopausal patients treated with tamoxifen is needed to adequately address the impact of tamoxifen treatment on 27HC levels and thus the impact on prognosis.

The *in vitro* experiments in this paper were performed specifically to look at the mechanistic impact of 27HC alone (in the absence of other lipids) on the proliferation of breast cancer cells. While this is a hypothetical scenario that any tumor is unlikely to experience, these experiments are key to understanding the mechanistic impact of 27HC on breast cancer biology. Previous publications that evaluated the impact of 27HC in breast cancer cell lines *in vitro* [56, 64] used cell culture media supplemented with charcoal-stripped serum and not regular cell culture media. Our study used two different kinds of cell culture media as controls: 1) regular cell culture media and 2) phenol red free media supplemented with charcoal stripped serum. Under conditions controlled for estradiol, 27HC treatment did not significantly impact the cell proliferation as reported in previous publications.

# Paper V

*Results*

To understand the molecular changes behind concomitant, long-term statin and endocrine treatment, we generated endocrine therapy-resistant breast cancer cells treated with or without atorvastatin. Atorvastatin treatment was initiated at day 0, day 30, or at first passage of the endocrine treatment. Cells were harvested after the respective treatment periods, and molecular characterization was performed by qRT-PCR and immunoblotting. The molecular characterization had three main focus areas: 1) estrogen receptor/signaling, 2) cholesterol homeostasis, and 3) epithelial mesenchymal transition (EMT). The MCF7 and CAMA1 cells had heterogeneous response to the respective treatments.

Upon long-term estrogen deprivation to mimic the aromatase inhibition setting, the gene expression of ER (*ESR1*) and cyclin D1 (*CCND1*) was significantly reduced in the MCF7 cells, while CAMA1 cells had a significant upregulation of *ESR1* and *CCND1*. Concomitant statin treatment did not impact the expression of *ESR1* in either cell line while *CCND1* was downregulated in both cell lines. The expression of genes involved in cholesterol homeostasis machinery *ABCA1* and *CYP27A1* were significantly upregulated in both long-term estrogen-deprived cell lines (MCF7_AI and CAMA1_AI). The addition of a statin reversed this upregulation in MCF7_AI_Ato cells, while expression of these genes were either increased (*ABCA1*) or unchanged (*CYP27A1*) in CAMA_AI_Ato cells. Long-term estrogen deprivation alone did not alter the expression of the EMT markers E-cadherin (*CDH1*) or Snail (*SNAI1*) in either cell lines. However, the expression of *CDH1* and *SNAI1* was significantly downregulated in MCF7_AI cells concomitantly treated with statins; no changes were observed in the CAMA1_AI cells.

Tamoxifen-resistant MCF7 cells had a significant downregulation of *ESR1* and *CCND1,* while no significant changes were observed in the CAMA1 cells. The addition of concomitant statin treatment in MCF7_TAM cells reversed the downregulation of *ESR1*. The cholesterol transporter *ABCA1* was significantly upregulated in TAM-resistant MCF7 and CAMA1 cells and was downregulated upon statin addition. CYP27A1 levels remained unaltered in MCF7 cells, but it was upregulated in CAMAI_TAM cells and was further upregulated upon statin addition. Expression of *ESR1* in MCF7_TAM_ATO cells were comparable to the untreated parent MCF7 cells. Expression of EMT markers *CDH1* and *SNAI1* were significantly upregulated in the tamoxifen-resistant MCF7 cells that was significantly downregulated during combination (TAM+ATO)-treated MCF7 cells. In contrast, *CDH1* was upregulated in tamoxifen-treated CAMA1 cells—these cells were, not impacted by the addition of atorvastatin. *SNAI1* was downregulated in TAM-resistant CAMA1 cells, which was reversed upon the addition of atorvastatin.

*Discussion*

Long-term atorvastatin treatment induces differential and heterogeneous molecular responses in endocrine therapy-resistant breast cancer cells. In all our cell line models with the exception of MCF7_Tam cells, the expression of *ESR1*, *CCND1*, *CDH1* and *SNAI1* followed a similar trend, suggesting that the impact of endocrine therapy with or without statin treatment is impacting the ER signalling axis. The expression of cholesterol homeostasis machinery genes were upregulated in the long term estrogen deprived cells as expected. However the cell lines MCF7 and CAMA1 responded differentially to the addition of atorvastatin. This differential response could be attributed to the differential p53 status of these two cell lines[136]. In study III we show that high intratumoral CYP27A1 expression is a marker of poor prognosis in post-menopausal breast cancer patients and it was speculated that statin addition might be beneficial for these patients. In this study, long term atorvastatin treatment downregulated the *CYP27A1* expression. This indicate that in the long-term estrogen deprived cell line MCF7 cell models, statins attenuate the upregulation of genes involved in the cholesterol homeostasis mechanism. By doing this statins block the alternative pathway, the resistant cells might have hijacked for continued proliferation under estrogen deprived conditions. The impact of atorvastatin treatment on EMT markers *CDH1* and *SNAI1* followed the expression of *ESR1* and *CCND1* in the majority of our developed cell line models. The exception was tamoxifen resistant MCF7 cells. This together with the proliferation data suggest that, the ER signalling is disrupted in this model.

In conclusion, our results show that the combination of endocrine therapy and statins is a complex scenario that involves the interplay between various signalling (ER, LXR and SREBP) pathways. The heterogeneity of patient and tumor characteristics add another level of complexity to this. These complexities could partially explain the divergent results observed in different epidemiology studies investigating the cholesterol, statin and breast cancer link. Further pre-clinical and clinical studies are warranted to delineate the molecular complexity of statin and endocrine therapy combination in breast cancer.

# Conclusions

## Paper I

- Statin treatment induced dose- and time-dependent lipid droplet accumulation in statin-insensitive cell lines.
- Statin treatment led to a significantly higher upregulation of the key regulator of lipid biosynthesis: Stearoyl-CoA desaturase.
- Members of fatty acid metabolism could be potential statin-treatment predictive markers.

## Paper II

- In the MDCS cohort, post-diagnosis statin use was significantly associated with reduced incidence of distant breast cancer recurrence.
- If confirmed in clinical trials, statins could be used as an adjuvant anti-cancer therapeutic in the clinic in post-menopausal patients.

## Paper III

- High CYP27A1 protein expression was associated with aggressive tumor characteristics in both study cohorts.
- In the MDCS cohort, high CYP27A1 expression was a prognostic marker of late lethal disease in postmenopausal breast cancer patients.
- In the BC-blood cohort, high CYP27A1 expression indicated poor prognosis during the early follow-up period in premenopausal breast cancer patients treated with adjuvant hormonal treatments.
- CYP27A1 might be a potential target for intervention by CYP27A1 inhibitors or cholesterol-lowering medications.

# Paper IV

- High CYP27A1 expression was associated with aggressive tumor characteristics.
- The incidence of disease recurrence and death was significantly lower in premenopausal, lymph node negative, hormonal treatment naïve breast cancer patients.
- 27HC treatment potently inhibited ER+ BC cell proliferation under lipid-depleted conditions regardless of estradiol levels.
- The impact of 27HC on cell proliferation was transcriptionally mediated through the downregulation of ER signaling with a concomitant upregulation of cholesterol export.
- If validated in a larger cohort, high CYP27A1 expression could be a marker for treatment de-escalation in premenopausal patients.

# Paper V

- Concomitant and long-term combination treatment of statin and endocrine treatment induced a heterogeneous response in breast cancer cell lines MCF7 and CAMA1.
- Tamoxifen atorvastatin combination treated MCF7 cells maintained *ESR1* expression comparable to untreated parent cells.
- In the long term estrogen deprived MCF7 cells, upregulation of *ABCA1* and *CYP27A1* was prevented by concomitant statin treatment.
- Statin combination with endocrine treatment did not significantly impact the expression the two EMT markers (*CDH1* and *SNAI1*) in our cell line models.

# Overall Discussion and Future Perspectives

Cancer cells deregulate the cholesterol homeostasis mechanism to meet the increased energy and structural requirements. Cholesterol is the precursor of isoprenoids, steroid hormones and oxysterols, all biologically relevant molecules in breast cancer proliferation. It has also been shown that triple negative breast cancer cells depend on exogenous cholesterol supply for proliferation. Limiting the intracellular cholesterol biosynthesis and consequently the downstream signaling is a promising therapeutic strategy. Originally developed as preventive medication for cardiovascular diseases, statins have been shown to have pleiotropic effects. Statins act by inhibiting the intracellular cholesterol biosynthesis. Considering the tolerability of statins by most patients, it is an appealing strategy to repurpose statin as an anticancer agent.

Experimental studies included in this thesis identified two markers of statin insensitivity, HMGCR and Stearoyl-CoA desaturase. These targets should be further studied in clinical studies to assess their treatment predictive value in the clinical setting. Another key prognostic marker identified is the enzyme CYP27A1, with differential prognostic impact in pre- and postmenopausal breast cancer. The long-term estrogen deprived and atorvastatin treated MCF7 cell line give credence to a mechanistic link between CYP27A1 expression and statin use. However, the clinical utility of this link could not be satisfactorily tested in our biomarker studies. Further clinical studies should assess the treatment predictive impact of CYP27A1 expression in aromatase inhibitor treated, post-menopausal breast cancer patients, specifically for recurrent disease, ideally separately tested the impact on local and distant recurrences, respectively.

Intracellular cholesterol levels are mainly maintained by the action of two transcriptional factors with opposing effects, LXRα and SREBP. Interestingly estrogen signalling is also involved in the regulation of SREBP genes and subsequently on LXRα genes. The common mechanism of statins is to upregulate the SREBP genes and replenish LDL, but in breast cancer cells, particularly in MCF7 cells, the baseline expression of LDLR is low and from preliminary data (unpublished), we speculate that in MCF7 cells the feedback mechanism to upregulate LDLR protein expression is disrupted. This speculation is supported by the findings that supplementing LDL in lipid-depleted medium did not induce levels

of cholesterol esters in MCF7 cells, suggesting that the exogenous lipid uptake is not an efficient mechanism in MCF7 cells[81]. Thus we hypothesize that in ER+ breast cancer cells, statins do not induce an influx of exogenous cholesterol via LDLR. Thus, in the event of estrogen deprivation in combination with statins, the cells face a double blockade of different proliferative signaling pathways, depriving the cells of estrogen, isoprenoids as well as oxysterols. This speculation must be extensively tested in pre-clinical studies. A far fetching extension of this speculation would also be that, a patient sub-group that would benefit the most from statin use will be post-menopausal breast cancer patients with low baseline LDLR receptor expression.

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