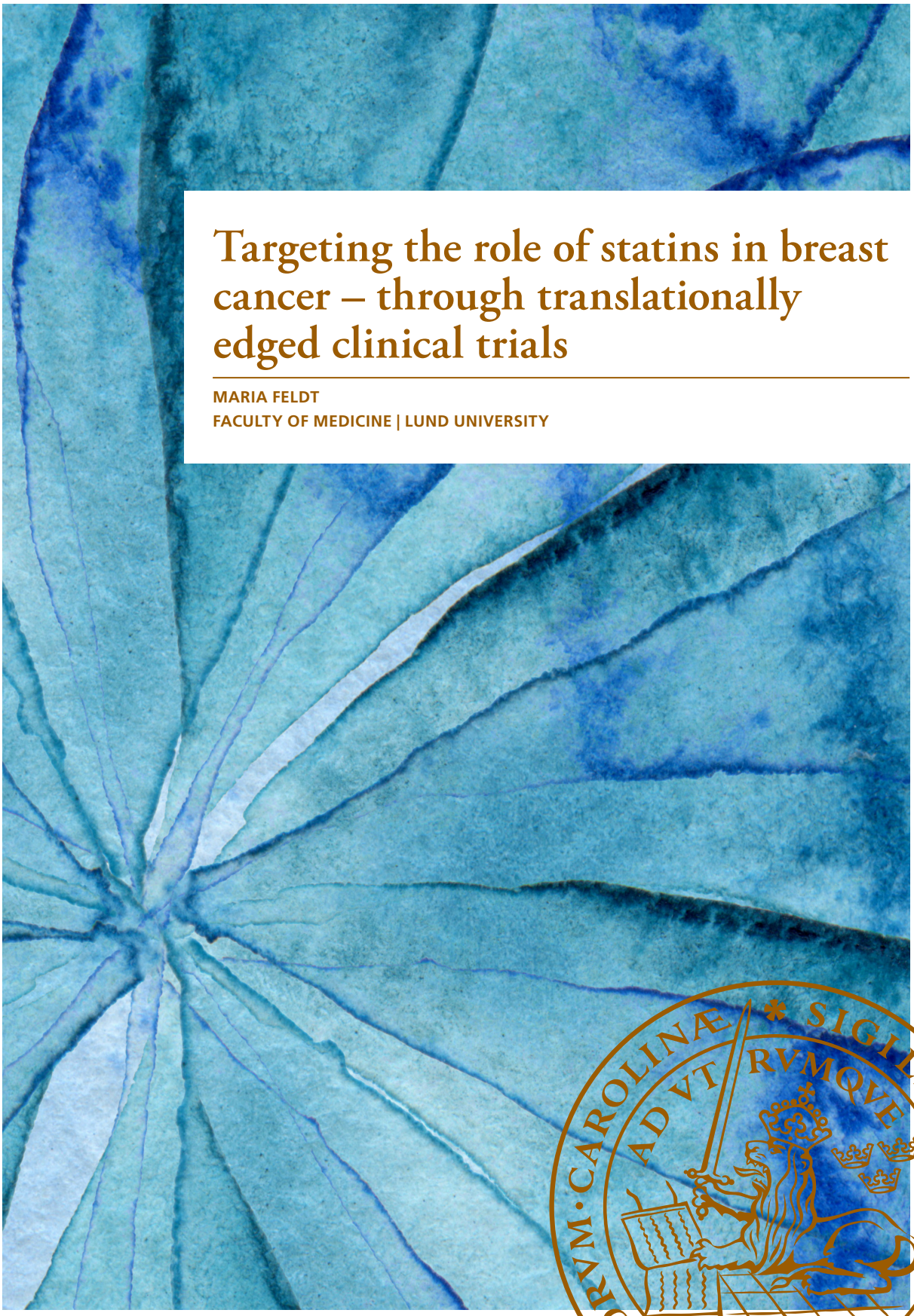

Targeting the role of statins in breast cancer – through translationally edged clinical trials

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Targeting the role of statins in breast cancer – through translationally edged clinical trials

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Abstract

Breast cancer incidence is increasing, and despite major progress in the treatment, breast cancer is still the leading cause of death from cancer among women. Thus, there is a constant need for new treatment options. Statins are peroral drugs that have been widely used since the early 1990s, due to their well-documented effect of lowering plasma cholesterol levels and preventing cardiovascular disease. Statins have also been recognized for their pleiotropic effects extending beyond their plasma cholesterol-lowering properties, and preclinical experiments have shown that statins exert anti-tumoral effects in breast cancer cell lines. Further, epidemiological studies have shown reduced breast cancer recurrence and mortality among statin users.

These findings have led to the conduction of the phase II, window-of-opportunity, MAmmary cancer and STatins trial (MAST), aiming to further explore the statin effects of breast cancer. Papers I and II are based the MAST trial, which included 50 patients who received a high dose of atorvastatin (80mg/day) for two weeks during the treatment-free window between diagnosis and breast surgery. Before the start of atorvastatin treatment, core needle tumor biopsies were taken from the tumors and blood samples were collected. After two weeks of atorvastatin treatment, tumor tissue was retrieved during the standard surgical procedure and, at the same time, new blood samples were collected.

In **paper I,** the protein expression of the cell-cycle regulators cyclin D1 and p27 was evaluated by immunohistochemistry on paired samples of formalin-fixed paraffin-embedded tumor tissue, before and after atorvastatin treatment. Project I revealed a significant down-regulated expression of the oncogene cyclin D1 and a significant up-regulated expression of the tumor suppressor p27 following two weeks of statin treatment.

In **paper II**, fresh frozen paired tumor samples pre- and post-atorvastatin treatment were analyzed by extracting lipids from the tumor samples. Cholesterol levels were then measured using a cholesterol quantification assay in order to evaluate changes in the cholesterol levels. The expression of the LDL-receptor (LDLR) was analyzed by immunohistochemistry on formalin-fixed paraffin-embedded tumor tissue, pre- and post-atorvastatin treatment. Project II revealed a statin-induced up-regulation of the LDLR and preserved intra-tumoral cholesterol levels. *In vitro* experiments on MCF-7 cells treated with atorvastatin were performed for comparison on the cellular level and showed no significant changes in the intracellular cholesterol levels after atorvastatin treatment. There was a higher expression of the LDLR, in agreement with the clinical findings, but it was non-significant.

Paper III is based on the large, prospective population-based Malmo Diet and Cancer Study. Tumor expression of HMGCR, the rate-limiting enzyme of the cholesterol biosynthesis pathway, which is inhibited by statins, was assessed by immunohistochemistry on tissue microarrays from 657 women diagnosed with primary invasive breast cancer between the years of 1991–2010. Tumoral expression of HMGCR was found to be associated with unfavorable tumor characteristics. The associations between statin use, HMGCR expression, and breast cancer mortality were investigated but no statistically significant associations were found.

Paper IV is a descriptive publication of a clinical phase II trial – ABC-SE – in which the effect and tolerability of atorvastatin in combination with endocrine based treatment among patients with advanced breast cancer will be compared to standard endocrine based treatment. The goal of this study is to improve the understanding of the mechanisms behind resistance to endocrine treatment of breast cancer, and also to test the hypothesis that the addition of statins will enhance the effect of the endocrine based treatment.

In conclusion, these results demonstrate new insights into the mechanisms of statins in breast cancer, which together with earlier published studies, and hopefully the results from the ABC-SE trial, will form the basis for future conduction of large, phase III randomized clinical trials, which are needed to clarify the role of statins in breast cancer.

Key words: Statins, HMGCR, cyclin D1, p27, cholesterol, LDL-receptor

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To all my patients

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Manuscript

Thesis at a glance

Paper	Aims	Trial/cohort	Methods	Results & Conclusion
I	To investigate potential statin-induced effects on the cell cycle regulators cyclin D1 and p27 and the clinical biomarkers; ER, PR, and HER2 before and after atorvastatin treatment	MAST trial; a phase II window-of-opportunity breast cancer trial including 50 patients receiving 80 mg atorvastatin daily for two weeks between diagnose and surgery. Tumor tissue and blood samples were collected both before start of atorvastatin treatment and after.	Cyclin D1, p27, ER, PR and HER2 assessed by IHC in FFPE tumor tissue before and after atorvastatin treatment	Following atorvastatin treatment, a significant decrease in cyclin D1 expression and a significant increase in p27 expression were observed, indicating that the anti-proliferative effects of statins may be driven by the cell cycle regulatory effects of cyclin D1 and p27. ER, PR and HER2 expression remained stable.
II	To assess statin-induced changes of the intratumoral levels of cholesterol and the expression of the LDLR, to enhance our understanding of the role of the mevalonate pathway in cancer cholesterol metabolism.		LDLR assessed by IHC in FFPE tumor tissue. Lipids were extracted and cholesterol levels quantified in fresh frozen tumor tissue. *In vitro* experiments on MCF-7 breast cancer-cells treated with atorvastatin were performed for comparison on the cellular level	Following atorvastatin treatment, the expression of LDLR was significantly increased, while the intratumoral levels of total cholesterol remained stable, indicating that LDLR might play a role as a negative regulator in the statin-induced inhibition of breast cancer aggressiveness. The clinical results were supported by functional studies and contribute to the elucidation of the anti-tumoral effects of statins
III	To explore and clarify the association between statin use, HMGCR expression based on a novel monoclonal antibody, and breast cancer prognosis.	MDCS; Out of 17,035 participants, 910 women underwent surgery for primary invasive breast cancer and were followed until the end of 2010.	Tumors were evaluated for HMGCR expression by IHC. Statin use and cause of death data were retrieved from the Swedish Prescribed Drug Register and Swedish Death Registry, respectively.	HMGCR moderate/strong expression was associated with prognostically adverse tumor characteristics. Neither HMGCR expression nor statin use were associated with breast cancer mortality.
IV	To test the hypothesis that the addition of atorvastatin to endocrine based treatment will enhance the efficacy in patients with advanced breast cancer and to improve the understanding of the actions of atorvastatin in breast cancer.	ABC-SE – A randomized phase II trial in the first line metastatic breast cancer treatment setting, comparing standard endocrine based treatment (letrozole in combination with a CDK4/6 inhibitor) with endocrine based treatment plus atorvastatin.	The investigational site is the clinic of Hematology, Oncology and Radiophysics at Skane University Hospital, Lund. The plan for the study is to recruit 126 patients, 63 patients in each treatment arm, over a period of 42 months.	The hope is that ABC-SE trial will provide evidence for the conduction of a future, phase III, multicenter, randomized controlled breast cancer trial with statins, aiming to improve treatment options for patients with advanced hormone receptor positive breast cancer.

Abbreviations: ER: estrogen receptor, PR: progesterone receptor, HER2: Human epidermal growth factor receptor type 2, MAST: Mammary cancer and STatins, IHC: immunohistochemistry, FFPE: formalin-fixed paraffin-embedded, LDLR: Low-density lipoprotein receptor, HMGCR: 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase MDCS: Malmö Diet and Cancer Study, ABC-SE: Advanced Breast Cancer – Statins and Endocrine based treatment Trial

Abbreviations

ABC-SE	Advanced Breast Cancer – Statins and Endocrine based treatment Trial
ACAT	Acyl-CoA: cholesterol acyltransferase
AI	Aromatase inhibitor
BCM	Breast-cancer-specific mortality
BMI	Body mass index
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
CBR	Clinical Benefit Rate
CDKs	Cyclin-dependent kinases
cDNA	Complementary DNA
CI	Confidence Interval
CR	Complete response
DCB	Duration of Clinical Benefit
FFPE	Formalin-fixed, paraffin-embedded
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
GnRH	Gonadotropin releasing hormone
HDL	High-density lipoprotein
HER2	Human epidermal growth factor receptor type 2
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HMGCR	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HR	Hazard ratio
IDL	Intermediate-density lipoproteins
IHC	Immunohistochemistry
ISH	In situ hybridization
LD	Lipid droplet
LDL	Low-density lipoproteins
LDLR	Low-density lipoprotein receptor

LXR	Liver X receptor
MAST	MAmmary cancer and STatins
MDCS	The Malmö Diet and Cancer study
MRM	Modified radical mastectomy
ORO	Oil Red-O
ORR	Overall response rate
OS	Overall survival
pCR	Pathological complete response
PD	Progressive disease
PD-L1	Programmed cell-death 1 ligand 1
PFS	Progression-Free Survival
PR	Partial response
PR	Progesterone receptor
Rb	Retinoblastoma protein
RECIST	Response Evaluation Criteria in Solid Tumors
SCAP	SREBP cleavage activating protein
SD	Stable disease
SERM	Selective estrogen receptor modulator
siRNA	Small interfering ribonucleic acids
SRB	Sulforhodamine B
SREBP-2	Sterol Regulatory Element Binding Protein-2
TMA	Tissue microarray
TNM system	Tumor node metastasis system
TTP	Time to Progression
VEGF	Vascular endothelial growth factor
VLDL	Very-low-density lipoprotein
WOO	Window-of-opportunity
27HC	27-hydroxycholesterol

Introduction

Breast cancer epidemiology

Breast cancer is the most commonly diagnosed cancer form among women, both in Sweden and globally, as well as the leading cancer cause of death among women worldwide. In 2018, approximately 2.1 million women were diagnosed with breast cancer, and 627,000 women with breast cancer died[1], demonstrating the clinical burden of this disease.

As illustrated in Figure 1, there is a great variety in incidence and mortality worldwide, with a higher incidence in high-income countries, but poorer survival in low-income countries. These patterns reflect both the risk factors and feasibility of detection and treatment of breast cancer[2].

Between 1990 and 2016, the incidence rates rose in most countries, with the most rapid increase in low-income countries, where the incidence of breast cancer was relatively low before, but which during this time period underwent economic development, entailing a higher prevalence of known breast cancer risk factors[1].

Globally, the rate of breast cancer mortality has decreased from 17.2 per 100,000 females in 1990 to 14.6 in 2016. The pattern is however very heterogeneous with great variety in different countries. Due to factors such as limitations in the feasibility of screening, early-stage detection, and access to treatment, mortality is often higher in many low- and middle income countries[2,3], despite their lower incidence. Furthermore, data is limited in several world regions, such as Africa.

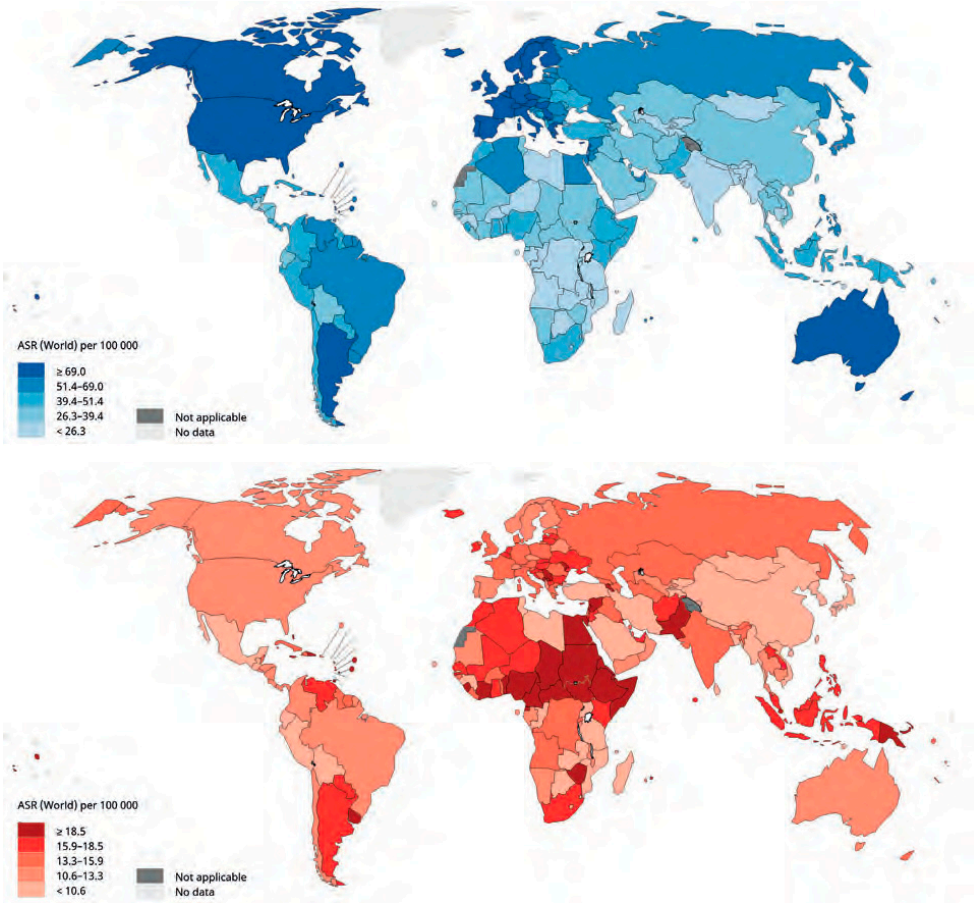

Figure 1. Global distribution of estimated age-standardized (World) incidence- and mortality rates per 100 000 person-years for breast cancer in women, 2018. Reprinted with permission from World Cancer Report 2020, Cancer research for cancer prevention, Chapter 5.9 Breast cancer Multiple, often complex, risk factors, page 384.

The breast

The human female breast, primarily designed to produce milk for offspring, is composed of mammary gland tissue, which is surrounded by adipose tissue and stromal connective tissue. The mammary gland consists of 15-20 lobes in each breast, which are subdivided into lobules, which in turn consist of multiple glandular alveoli. Each lobule is drained via a complex of branching ducts into the opening of the nipple[4]. The functional, milk producing unit of the mammary gland is called the

terminal duct lobular unit, which is composed of a lobule associated with terminal ducts[5]. The mammary gland epithelium consists of the luminal epithelial cells, responsible for the production and secretion of milk; and the basal myoepithelial cells, which have contractile properties and transport the milk toward the branching ducts[4], as illustrated in Figure 2.

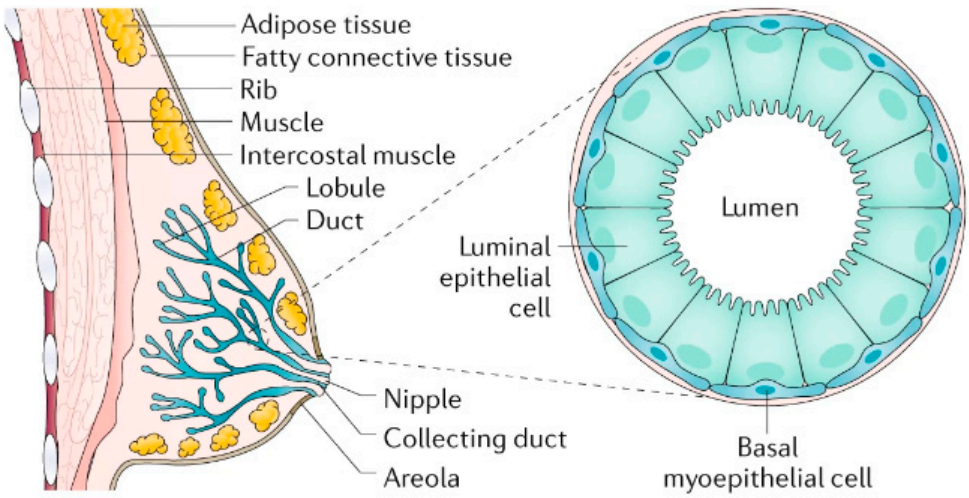

Figure 2. Schematic picture of breast anatomy with details of the mammary gland epithelium. Modified image from "Breast Cancer", Nature Reviews Disease Primers volume 5, Article number: 66 (2019) with permission from Nature Reviews © 2019.

Tumor biology

The initiation of breast cancer is due to genetic changes, most often originating from the epithelial cells in the terminal duct lobular units.

Tumorigenesis is thought to be a multistep process, driven by the accumulation of additional genetic changes, enabling these cells to evade the mechanisms that normally control their proliferation, survival, and migration[6,7]. For most tumors, this transition morphologically reflects an onset from normal breast tissue, via hyperplasia, atypical hyperplasia, carcinoma in situ, and subsequently to invasive cancer[8], even if not all tumors fulfil this continuum[9].

In 2000, Hanahan and Wienberg published an extensive review of the knowledge about tumorigenesis at that time, proposing six hallmarks of cancer, that most human tumors share. These are sustaining proliferative signaling, evading growth suppressors,

resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis[7]. In 2011, in a follow-up review, two new, emerging hallmarks were added; reprogramming of energy metabolism and evading immune destruction, as well as two enabling characteristics; genomic instability and tumor-promoting inflammation[10].

Proliferation and the cell cycle

The most essential feature of cancer cells concerns the ability to withstand a high proliferation rate, which is also an independent prognostic factor in breast cancer, with implications for the choice of treatment[11]. Proliferation is also a central topic of this thesis.

In normal tissues, a well-functioning proliferation is fundamental to maintain a homeostasis of cell number, maintenance, and development of tissue function. The proliferation of each cell ensues through the cell cycle, an organized series of steps that drive DNA replication and cell division. The cell cycle consists of different phases, each precisely programmed and carefully regulated:

- G1 is the stage when the cell is preparing to divide, accumulating its supply of DNA building blocks and associated proteins, as well as increasing the number of organelles and growing in size.
- S phase is the stage of DNA synthesis.
- G2 is a period of rapid cell growth and the synthesizing of proteins necessary for mitosis.
- M is the stage of mitosis, which, in turn, is divided into a series of phases – prophase, prometaphase, metaphase, anaphase, and telophase – that result in nuclear division.
- G0 phase is a quiescent, inactive phase for cells not actively preparing to divide. Some cells leave the cell cycle temporarily, waiting for a signal to re-enter, while others remain in G0 permanently.

To prevent cells from continuing to divide until conditions are favorable, the cell cycle is strictly regulated by cyclin-dependent kinases (CDKs), a family of serine/threonine kinases. The CDKs form complexes with cyclins, their regulatory units, and together they function as checkpoints of the cell cycle regulatory proteins that regulate progression through the different phases of the cell cycle. These checkpoints occur near the end of G1, at the G2-M transition, and during metaphase

of mitosis. In cancer, however, the cell cycle control system is deregulated at multiple levels, allowing for uncontrolled cellular proliferation.

Cyclin D1 regulates the G1/S transition by binding to CDK4/6, which phosphorylates the retinoblastoma protein (Rb). Upon phosphorylation, Rb is inactivated and releases E2F, leading to activation and transcription of proliferation-related genes. Cyclin D1 is overexpressed and/or amplified in many different forms of cancer, including breast cancer[12], and is considered a breast cancer oncogene[13-15]. In breast cancer, cyclin D1 protein overexpression is found in up to 50% of the tumors[16,17], and amplification of the gene, CCND1, in 13-20%[18,19]. The discrepancy between protein and gene expression indicates that protein overexpression of cyclin D1 can occur via additional mechanisms except for gene expression, such as transcriptional and posttranscriptional dysregulation[13,20].

The tumor suppressor p27 acts by binding to, and thereby inhibiting, the CDKs. In G0 and early G1, p27 binds and inhibits the cyclin E-CDK2 complex, and in S-phase CDK2-cyclin A, but as p27 levels decrease during G1, the cyclin E-CDK2 and cyclin A-CDK2 complex can activate the gene transcription required for the G1-S transcription[21-23]. Also, p27 has a dual role in the interaction with CDK4/6-cyclin D, p27 acting as both an inhibitor and a required assembly factor for the complex, depending on the growth state of the cell[24]. Nuclear p27 levels are reduced in many cancer forms, among them breast cancer[25,26]; reduced p27 levels are associated with a poor breast cancer prognosis[23].

Altered metabolism

The high rates and deregulated control of cell proliferation that represent cancer cells require corresponding adjustments of energy metabolism. Altered metabolism is emerging as a hallmark of cancer[10], which is also of importance for this thesis.

The phenomenon of altered glucose metabolism, by increased glucose uptake and excessive lactate formation even in presence of oxygen, was first described by Otto Warburg in 1931[27]. However, there is increasing evidence that cancer cells also show alterations in different aspects of lipid metabolism. Lipids are a group of hydrophobic molecules with varying structures and functions, including triacylglycerids, phosphoglycerids, sterols, and sphingolipids. Lipids have essential roles at the cellular level, as an energy source, as plasma membrane components, and as signaling molecules, for instance. Alteration of lipid metabolism seen in cancer cells includes an enhanced synthesis and uptake of lipids, affecting numerous cellular processes, including cell growth, proliferation, differentiation, and motility[28].

Risk factors

Several risk factors have been established as contributors to breast cancer; the most prominent are female sex and increasing age – 99% of all breast cancers are diagnosed in women and the risk increases steeply with age from 40 until the age of 70 years, then rises more slowly[29].

Reproductive factors connected with a higher number of lifetime hormone cycles and high endogenous levels of estrogen, such as early age at menarche, late age at menopause, nulliparity, late age at first birth, and lack of breastfeeding, all increase the risk of breast cancer[30]. Normal breast development during puberty, menstrual cycles, and pregnancy is stimulated by estrogen and progesterone. During menstrual cycles, the imbalance between estrogen and progesterone may cause DNA damage accumulation, which, after the repetitions of many cycles, can lead to mutations and, subsequently, the development of pre-malignant and malignant lesions[31,32]. The growth of these pre-malignant and malignant cells is stimulated by estrogen through activation of the estrogen receptor, thereby promoting proliferation and survival through transcription of pro-survival genes and the activation of cellular signaling[33]. That high levels of estrogen increase the risk of breast cancer is also reflected by the increase in breast cancer seen followed by the use of hormone replacement therapy during menopause[34,35] as well as an increased breast cancer risk among women who currently or recently used hormonal contraceptives[36-38].

However, these effects differ across breast cancer subgroups, and the association between reproductive factors and breast cancer risk is thought to be more complex and not restricted to estrogen levels. It has also been proposed that post-pubertal breast tissue is relatively undifferentiated before pregnancy and, thus, more susceptible to carcinogenic stimuli. Pregnancy permanently alters gene expression in the mammary gland, with subsequent induction of terminal differentiation of breast cells, altered sensitivity of the mammary gland to later hormonal exposures, and elimination of targets for malignant transformation due to a reduction in the number of stem cells[39]. But the protective effect of pregnancy is not complete, as a transient increase in breast cancer risk occurs around pregnancy, which is thought to be connected to the process of involution post-partum, when the breast regresses to its pre-pregnant state[40]. Lactation, however, decelerates the process of involution, which may reduce breast cancer risk[41], and also increases the proportion of differentiated cells in the breast[42].

Other lifestyle and environmental factors associated with an increased risk for breast cancer are high socioeconomic status, race, ionizing radiation – especially when

exposed at an early age, extensive mammographic density, previous breast disease – both benign and malign disorders, excessive alcohol consumption, physical inactivity, and obesity[29,43-46]. Obesity is associated both with a higher breast cancer risk, particularly in postmenopausal women, and with higher mortality for women of all ages[46].

Also, the risk of breast cancer increases when there is a family history of breast cancer; approximately 10% of breast cancers are inherited[47]. Two high-penetrance tumor suppressor genes, BRCA1 and BRCA2, have been identified, which are responsible for 2–5% of all breast cancer cases[48].

Diagnosis

The most common symptom of breast cancer is a palpable lump in the breast. Other symptoms include skin changes in the breast such as puckering, dimpling, a rash or redness of the skin, mamillar retraction, breast asymmetry, pruritus, mammillary discharge/secretion, or a lump or thickening in an armpit.

A suspected breast tumor is diagnosed with "triple diagnostics", comprising a physical examination, radiographic imaging of the breast and axillar lymph nodes and tissue sampling in the form of a core needle biopsy, where the finding of invasive cancer is central to clinical decision-making. The clinical, radiological and pathological findings will be discussed at a multidisciplinary conference where decisions regarding further management are made.

However, many of the above-mentioned symptoms can be signs of locally advanced disease. With the aim of detecting the disease at an earlier stage for which there is a curative treatment, population screening with mammography has been implemented in most developed countries; in Sweden it is recommended for all women between 40 and 74 years old, every 18 to 24 months[49]. Mammography screening has been estimated to significantly reduce mortality from breast cancer by 20% for the whole population[50] but this effect has been questioned, as improvements in breast cancer treatment over the same period may be part of this mortality reduction[51]. Mammography screening has also been subject to a debate over whether the harms related to mammography screening, particularly overdiagnosis, outweigh the potential benefits[52].

Categorizing a heterogeneous disease

Breast cancer is considered a highly heterogeneous disease, with different biological features, different clinical outcomes, and different responses to treatment across subtypes. To better predict the prognosis and determine treatment modalities for each patient, breast cancer is classified using histology, pathological, and molecular markers.

Histological classification

Invasive breast carcinomas are divided into 19 major subtypes, according to the WHO classification of 2012[53].

About 70% of all breast cancers are categorized as "invasive carcinoma of no special type", formerly called ductal carcinoma. This type has a very varying morphology and does not fit into a specific histotype. The most common special type is lobular carcinoma, which comprises about 20% of all breast cancers. In addition, there are several less common types such as tubular, cribriform, mucinous, metaplastic and micropapillary carcinoma.

The prognosis differs among histological subtypes – especially between some of the rare types – but the utility of this classification system for clinical decision-making is limited due to the rarity of many of the histological subtypes.

Histological grade

A morphological assessment of the tumor is made based on three tumor features, according to the Elston-Ellis Nottingham histological grade[54]. The tumor's tubule formation, nuclear pleomorphism, and mitotic count are each scored, and the final grade is determined by adding the individual scores, with grade I being well differentiated, grade II moderately differentiated, and grade III poorly differentiated.

Tumor grade is a strong prognostic factor, reflecting the aggressiveness of the tumor.

Tumor stage

The tumor node metastasis system (TNM system) is a staging system used for all solid tumors and is a measurement of the extent of the tumor and its spread. For breast cancer, there are three prognostic markers; primary tumor size (and skin or chest- wall invasion) (T), axillary lymph node involvement (N) and distant metastasis (M),

which are combined and categorized into five stages (0–IV) with prognostic significance[55].

Immunohistochemical biomarkers

In the clinic, four immunohistochemical biomarkers – the estrogen receptor (ER), the progesterone receptor (PR), human epidermal growth factor receptor type 2 (HER2) and Ki67 – are recognized by international guidelines as essential for therapy decision-making and are used routinely at diagnosis, on the tissue samples obtained from the pre-surgical core biopsies.

ER is a nuclear receptor that acts primarily as a DNA-binding transcription factor and has two forms, ERα and ERβ, both activated by the steroid hormone estrogen. ERα is a transcription factor for genes associated with cell survival, proliferation, and tumor growth[33]. While the role of ERα in breast cancer is crucial for hormone-dependent growth, the role of ERβ is still controversial, and both proliferative and anti-proliferative roles have been described[56,57]. ER will hereafter be referred to as ERα. About 80% of all breast cancers in Sweden express the ER, which is a favorable prognostic factor as well as a predictive factor for endocrine therapy. In Sweden, a tumor is considered to be ER-positive if 10% or more of the nuclei express ER[58], while internationally, a cut-off value of more than 1% positive nuclei is more common[59].

Similar to ER, PR is a hormone-dependent nuclear transcription factor. PR mediates the effect of progesterone in the development of the mammary gland[60] and PR is expressed in about two-thirds of all ER-positive breast cancers. The impact of PR status in breast cancer is less clear than the ER status; PR is associated with less aggressive tumors but does not drive clinical decision-making to the same extent. It has been hypothesized that PR is a predictor of ER functionality[61], as well as the response to adjuvant tamoxifen treatment[62], but more recent research has shown that there is important crosstalk between ER and PR signaling pathways[63], whereby the activation of one has a significant impact on the other. To be considered PR-positive, 10% or more of the nuclei should express PR in Sweden, and over 1% internationally[59].

About 20% of all breast cancers have an overexpression of HER2, or amplification or activating mutations of the HER2 gene; *erbb2*, which is associated with a more clinically aggressive disease[64-66]. HER2 is a member of the epidermal growth factor receptor family, consisting of an extracellular ligand-binding region, a single membrane-spanning region, and a cytoplasmic tyrosine-kinase-containing domain.

Ligand binding to the extracellular region results in activation of the cytoplasmic kinase domain and subsequent phosphorylation of a specific tyrosine kinase, leading to activation of several intracellular signalling pathways involved in cell proliferation and survival[67]. Because HER2 status also predicts response to HER2 targeted therapy, assessment of HER2 is routinely performed in the clinic. Two different methods are routinely used; immunohistochemistry (IHC) and in situ hybridization (ISH). IHC is a semi-quantitative method identifying the protein on the cell surface and dividing patients into four groups, 0, 1+, 2+, and 3+, and ISH is a quantitative method measuring the number of copies of the HER2 gene present in each tumor cell, which is reported as either positive or negative. ISH is highly reproducible but is comparatively more time-consuming and expensive than IHC, which is the recommended choice for HER2 status evaluation worldwide. Assessed by IHC, a patient is classified as positive when scoring 3+, which is characterized by a strong, complete IHC membrane staining. Tumors scoring 2+, with weak to moderate complete membrane staining observed in > 10% of tumor cells, as assessed by IHC are considered equivocal and are tested with ISH according to Swedish national guidelines[68].

The proliferation marker Ki67 is a nuclear protein, expressed in all phases of the cell cycle except the inactive phase G0[69] Ki67 expression correlates with the proliferative rate of tumor cells, and Ki67 is essential for cell proliferation, though its exact role is still unclear[70]. In breast cancer, Ki67 serves as a prognostic biomarker and as a predictive factor for chemotherapy among hormone receptor-positive, HER2-negative tumors[71]. However, due to inter- and intra-laboratory variabilities of Ki67-scoring and problems with reproducibility, there is no international consensus regarding the most suitable method for Ki67 scoring or cut-offs[72]. According to the Swedish national guidelines, 200 tumor cells in a hotspot region should be counted, with laboratory-specific cut-off values[58].

Molecular subtypes

Novel high-throughput technologies have enabled a new understanding of breast cancer biology and heterogeneity. By studying the combination of multiple genetic alterations, a new independent classification of breast cancer has been revealed, based on different gene expression profiles. In the original study, a rough division between the two distinct mammary gland epithelial cell types – luminal and basal epithelial cells – was made. The luminal subtype is principally ER-positive and the basal-like is ER-negative. Based on differences within these groups, four major molecular subtypes were distinguished; luminal A, luminal B, HER2 enriched, and basal-like[73]. The two

luminal subtypes exhibit deregulation of ER-related genes. In the HER2 enriched subgroup there is a high expression of genes related to the HER2 gene; *erbb2*, and in the basal-like subgroup there is a high expression of proliferation-related genes and mutations of p53[74].

Importantly, these intrinsic molecular subtypes not only differ based on different gene expression profiles but do also present with different prognoses and different responses to therapy. The luminal subtypes are often sensitive to endocrine therapy. Luminal A tumors display a relatively decreased proliferation and generally have a better prognosis than luminal B tumors[75]. The HER2-positive and basal-like subtypes are more aggressive, more likely being grade III, and have a poorer prognosis than the luminal subtypes[75,76]. However, they both tend to respond better to chemotherapy than the luminal subtypes, and the HER2 subtype to HER2-targeted therapy[74].

In clinical practice, an approximate surrogate classification for the determination of the intrinsic molecular subtypes, based on histology and immunohistochemically biomarkers, is used due to the cost and complexity of gene expression analyses. The molecular subtypes and surrogate immunohistochemically biomarkers are presented in Figure 3.

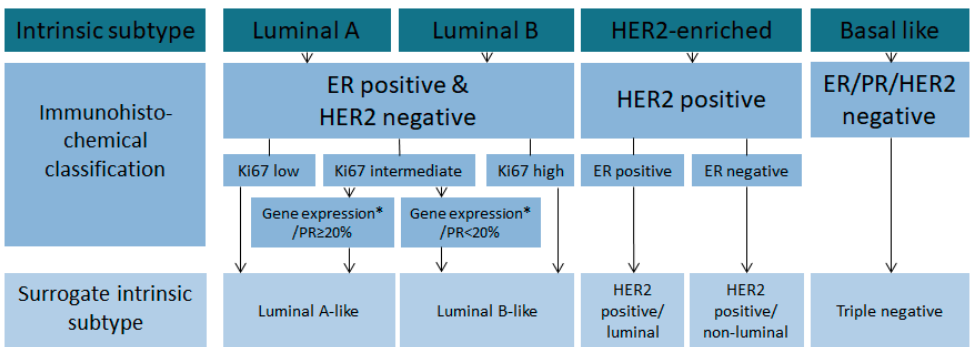

Figure 3. Illustrating the intrinsic molecular subtypes and approximate immunohistochemical surrogate definitions. *In Sweden, gene expression analysis is carried out in women >50 years old, if the result will affect the choice of treatment according to current national guidelines.

Treatment

Primary breast cancer

Since the end of the 19th century, surgery has been the cornerstone of the treatment of primary breast cancer. Subsequently, postoperative radiotherapy was introduced as an additional treatment.

Thereafter, the extended knowledge and categorization of breast cancer that has evolved throughout the past decades have led to the addition of systemic treatments given before (neoadjuvant) or after (adjuvant) surgery, such as chemotherapy, endocrine treatment, and targeted therapies. Both neoadjuvant and adjuvant treatments are given based on the biological properties of the cancer, and on the risk of relapses. For patients with primary, locally advanced tumors, and/or tumors with aggressive prognostic factors, neoadjuvant treatment is offered to shrink the tumor and enable surgery, as well as to provide systemic treatment earliest possible. Adjuvant treatments are given to control or eradicate any remaining cancer cells after surgery, and thereby prevent recurrences.

Surgery

Surgery plays a prominent role in breast cancer treatment. For about 50% of all breast cancers, loco-regional treatment with surgery alone, or in combination with postoperative radiotherapy, leads to a lifelong cure[58].

From the late 19th century until the 1940s, surgery consisted of a radical mastectomy with removal of the entire breast, pectoral muscle, and axillary lymph nodes[77], often with considerable morbidity. The technique of breast cancer surgery gradually developed during the 20th century, first to modified radical mastectomy (MRM) in which the pectoral muscle and lymph nodes are spared[78], and then to breast-conserving surgery, with sector resections. Studies with more than 20 years of follow-up have shown that breast-conserving surgery followed by postoperative radiotherapy has the same survival rate as mastectomy, and is today the primary surgical goal[79,80]. If the tumor size in relation to breast size excludes breast-conserving surgery as a treatment alternative, preoperative systemic treatment to shrink the tumor size is an alternative, or MRM.

Positive surgical margins imply a doubled risk for local recurrence[81], and even if the risk declines following postoperative oncological treatment, it will not completely disappear. Negative margins are defined as no tumor growth in the resection margin, called "no ink on tumor". To reduce the risk of re-surgery due to positive margins, a macroscopic margin of 10mm is recommended during surgery[82].

Sentinel node

The first place to which breast cancer metastasizes is often the axillary lymph nodes, and in early breast cancer, axillary lymph node status is a prognostic factor. The goal of surgery is, besides the removal of a localised breast tumor, to either stage the axillary lymph nodes with sentinel node biopsy or to remove preoperatively determined axillary metastasizing.

With sentinel node biopsy, at least the first lymph node that drains the breast tumor – the sentinel node – is identified with a radioactive isotope, then removed and pathologically analyzed. Sentinel node biopsy has replaced axillary dissection as a standard procedure for the staging of the axilla, as breast cancer outcomes have been shown to be as good[83-85], but with far less of the side effects associated with axillary dissection, such as lymph oedema, mobility restrictions, pain and numbness[86].

In clinically node-negative invasive breast cancer, sentinel node biopsy is performed during surgery, or if the patient is receiving preoperative treatment, after the preoperative treatment is finished.

Axillary dissection is performed when there is a preoperatively determined axillary lymph node engagement, when macro-metastases have been found in sentinel node biopsies, or when there is a viable rest of a metastasis after preoperative treatment[58].

Postoperative radiotherapy

Postoperative radiotherapy is a local treatment, aimed at eliminating any undetected, remaining cancer cells in the radiated area following surgery. Postoperative radiotherapy reduces both the risk of recurrences and the breast cancer mortality[87,88].

Following breast-conserving surgery, postoperative radiotherapy of the residual breast tissue is recommended to all patients as a standard treatment according to Swedish guidelines[58]. However, ongoing research is investigating whether postoperative radiotherapy can be omitted in low-risk tumors. Following mastectomy, postoperative

radiotherapy towards the thoracic wall is mainly recommended when the tumor is larger than 50 mm. Regardless of the surgery method, when there is lymph node involvement, radiotherapy of regional lymph nodes is added to the treatment, including the axillary, supraclavicular fossa, and sometimes the parasternal lymph nodes[58].

Early side effects of radiotherapy are an inflammation of the skin and pneumonitis[89], the latter most often presenting within 1–3 months after the termination of radiotherapy. Late side-effects include lung fibrosis, lymph-oedema of the arm and decreased motility in the shoulder region, lung cancer, and cardiovascular events[90]. To alleviate the side effects and radiation dose to nearby organs, the radiotherapy technique has evolved, and today the respiratory-gated technique is used in locoregional radiotherapy of left sided breast cancers, with a decrease of the radiation dose to the heart and lungs[91,92]. In recent years, conventional fractioning has been changed to hypo-fractioned, using a higher fraction per dose, which shortens the typical duration of the radiotherapy from five to three weeks, but with the same clinical results and similar or even less side effects[93-95].

Chemotherapy

Since chemotherapy was introduced as an adjuvant treatment for breast cancer in the 1970s[96], it has been found to decrease breast cancer mortality by eliminating micro metastasizing by about one-third[97].

Today, chemotherapy can be given both in the neoadjuvant and the adjuvant settings, with similar results on outcome[98]. Neoadjuvant chemotherapy is the standard treatment for patients with a primary inoperable tumor. Also, HER2-positive and triple-negative breast cancers are often treated with neoadjuvant chemotherapy to assess the response of the tumor, which can provide guidance in the adjuvant therapy choice. According to Swedish guidelines, chemotherapy in the adjuvant setting is today recommended for patients with a tumor larger than 10 mm which is considered luminal B or if the patient is younger than 35 years, and to most patients with a lymph node-positive disease. For patients with triple-negative disease, adjuvant chemotherapy is recommended for tumors larger than 5 mm[58].

Comparisons between different combinations of agents have shown anthracycline-based therapy with the addition of a taxane for 4–6 months to be the most efficient regimen and this combination is given in both the neoadjuvant and adjuvant settings[97]. However, the exact administration of chemotherapy, in terms of dose intensity and number of treatments varies depending on different risk factors[58]. For

patients with triple-negative breast cancer receiving neoadjuvant treatment but not achieving pathological complete response (pCR), the addition of adjuvant capecitabine has shown further improved outcome[99].

Chemotherapy is associated with significant side effects, such as bone marrow toxicity, nausea, alopecia, neurotoxicity, and fatigue, and there is a great need for better prediction tools to identify patients less likely to benefit from treatment.

Endocrine treatment

The first observation that estrogen was critical for the growth of some breast cancers was published without knowledge of the existence of the hormone, when the beneficial effects of oophorectomy in patients with inoperable breast cancer were reported by George Thomas Beatson in 1896[100]. In 1923, estrogen was discovered as an ovarian hormone regulating breast tissue[101], and extensive research during the following decades led to the discovery of the estrogen receptor in 1958[102] and tamoxifen in the early 1960s[103].

Tamoxifen is a selective estrogen receptor modulator (SERM), with both estrogen antagonistic and estrogen agonistic effects. Tamoxifen was first approved for the treatment of metastatic breast cancer in the 1970s, which was later expanded to the adjuvant setting[104].

In postmenopausal women – with diminished ovarian function – estrogen is predominantly synthesized in the liver, muscle and fat tissue, via the enzyme aromatase, converting androgens to estrogen[105]. Aromatase inhibitors (AIs) inhibit this conversion and are effective as an adjuvant treatment for postmenopausal women[106].

Adjuvant endocrine treatment is recommended for all patients with luminal early breast cancer and has been found to decrease breast cancer mortality by approximately one-third[107]. AIs have been found to have an improved outcome compared to tamoxifen in postmenopausal women[106].

Currently, according to Swedish guidelines, postmenopausal women with ER-positive breast cancer are recommended AIs for five years or, alternatively AIs for two years, followed by tamoxifen for three years. If the risk of recurrence is high, prolonged treatment with either 2–3 years of AIs or five years of tamoxifen after the first five years of AIs is recommended[58].

For pre- and peri-menopausal women, adjuvant tamoxifen is recommended for five years, or prolonged up to 10 years for women with high risk of recurrence. For

patients younger than 40 years with a recurrence risk high enough to receive adjuvant chemotherapy, ovarian suppression with a gonadotropin-releasing hormone (GnRH) analogue, inhibiting the production of estradiol, is added to the tamoxifen treatment and improves the outcome[108].

Common side effects of tamoxifen are symptoms of menopausal transition such as hot flushes, sweating, vaginal discharge and dryness, but also an increased risk of venous thrombosis/pulmonary embolism and endometrial cancer[109]. For AIs, side effects such as vaginal dryness and joint and muscle pain are common, and there is an increased risk of osteoporosis and bone fractures as well as cardiovascular events[110,111].

HER2-targeted treatment

HER2 was first reported as a proto-oncogene in 1989, and in the 1990s the first HER2-targeted treatment, the monoclonal antibody trastuzumab, was developed. Trastuzumab was initially approved for the treatment of HER2-positive metastatic breast cancer in 2001[112], and since 2005 in the adjuvant setting. Data from randomised controlled trials have shown that the relative risk of recurrence is reduced by 40% among HER2-positive patients after receiving adjuvant treatment with trastuzumab[113].

After the remarkable progress with trastuzumab, other HER2-targeted therapies have been developed. Among these are pertuzumab, another monoclonal antibody binding to a different domain of the HER2 molecule[114], and the antibody-drug conjugate T-DM1, which links the tubulin inhibitor emtansine to trastuzumab. According to Swedish guidelines, neoadjuvant treatment with dual HER2 blockade, preferentially trastuzumab and pertuzumab in combination with chemotherapy is the standard of care for HER2-positive tumors larger than 5 mm in diameter or with lymph node metastasizing[58]. Normally, trastuzumab is given in the adjuvant setting, but for patients not achieving pCR, adjuvant treatment with T-DM1 has been found to significantly and substantially improve outcomes[115].

The most important side effect of trastuzumab is the increased risk of heart failure, and during trastuzumab treatment, systolic heart function should be monitored regularly[116], and is normally not recommended in combination with antracyclins. Pertuzumab side effects include diarrhea and rash[117], and T-DM1 can cause diarrhea and trombocythopenia[115].

Bisphosphonates

Bisphosphonates, osteoclast inhibitors decreasing bone resorption, have been shown to reduce the rate of breast cancer recurrence in the bone and to improve breast cancer survival when given in the adjuvant setting, restricted to postmenopausal patients[118]. Treatment with bisphosphonates also improves bone mineral density and decreases breast cancer treatment-related bone loss, and it is recommended for all postmenopausal, lymph node-positive patients. Because bisphosphonates increase the risk of osteonecrosis of the jaw, dental status should be controlled before the start of treatment.

Loco-regional relapses

A loco-regional relapse can occur in the skin, subcutaneously, in regional lymph nodes, or in the residual breast, if breast-conserving surgery was performed in the primary setting. According to Swedish guidelines, the diagnosis should be verified with a core biopsy, and screening for distant metastases should be performed[58]. All new breast cancers appearing in the former treated breast are classified as local relapses, while relapses in the contralateral breast are classified as new cancers.

The treatment of loco-regional relapses has a curative intention but loco-regional relapses have a worse outcome compared to primary breast cancer. If breast-conserving surgery was performed in the primary setting, mastectomy is normally recommended, but breast-conserving surgery can be repeated and followed by postoperative radiotherapy unless no radiotherapy was given in the primary setting. When mastectomy was performed in the primary setting, a radical excision is recommended, followed by postoperative radiotherapy if not given in the primary setting[58]. Sentinel node should be performed[119].

Adjuvant treatment with chemotherapy, with the addition of HER2-targeted therapy and endocrine treatment when applicable, is strongly recommended[120,121]. Neoadjuvant therapy can be given when the relapse is inoperable. Depending on the results of the neoadjuvant treatment, it is followed by surgery alone or radiotherapy when applicable, and according to the radiotherapy given in the primary breast cancer setting[58].

Advanced breast cancer

Advanced breast cancer includes inoperable locally advanced breast cancer without a spread to distant organs, as well as metastatic breast cancer. Today, there is no cure for advanced breast cancer, and the median overall survival is two to three years. The most common sites of metastases are the bone, lungs, and liver[122].

The treatment of advanced breast cancer is intended to prolong survival, without significantly impairing the patient's quality of life, and to ease the symptoms.

Radiology should be performed for a correct staging of the disease, usually with computer tomography of the thorax and abdomen. The tumor marker CA15-3 can be analysed as a means of predicting therapy response. When possible, a biopsy of metastases should be analysed to verify the diagnosis of advanced breast cancer and for the immunohistochemical markers ER, PR, and HER2, as the expression can diverge from the primary tumor[123].

Surgery

In primary metastasized breast cancer, surgery of the primary tumor has not shown any prognostic benefit in prospective trials[124,125], and resection of metastases is controversial but could be an option for selected patients[126].

Due to metastases in the bone, reduced surgery can be performed prophylactically, or due to a pathological fracture. Surgery of the spine could be performed due to medullar compression[127].

Radiotherapy

Radiotherapy of metastases causes a tumor volume reduction, enabling a restoration of the normal tissues that have been compressed or invaded. It plays an important role in relieving symptoms, especially resulting from bone, brain and soft tissue metastases[128].

Endocrine based treatment

Verified luminal-like metastatic breast cancer should be treated with endocrine therapy as first- and second-line therapy[122]. In addition to tamoxifen and aromatase inhibitors, the anti-estrogen fulvestrant is offered as a treatment choice in the metastatic setting, and at least two endocrine lines of therapy should be used until

there is no longer a response to treatment. For pre- and peri-menopausal women, the endocrine treatment should be combined with ovarian suppression[58,122].

In the first- or second-line treatment, the addition of a CDK4/6 inhibitor to the endocrine treatment is recommended. The CDK4/6 inhibitors act by inhibiting the CDKs 4 and 6, thereby preventing progression through the G1-to-S cell cycle checkpoint, leading to cell cycle arrest[129]. The addition of a CDK4/6 inhibitor to endocrine treatment increases the progression-free survival by about 10 months in the first line setting, and about 5 months in the second line[129-132].

Endocrine treatment can also be combined with an mTOR inhibitor, a peroral drug inhibiting the PI3K/AKT signaling pathway[133], which improves progression-free survival but is not standard-of-care due to a high degree of side effects, primarily in terms of mucositis and pneumonitis[134].

Chemotherapy

For patients with a non-luminal, a luminal but endocrine-resistant, or a biologically or clinically aggressive metastatic breast cancer, chemotherapy is recommended as first-line therapy[58,123].

Normally, sequential monotherapy is recommended to decrease the risk of side effects, but combination therapy can be necessary when there is a rapid disease progression or risk of organ dysfunction[123].

As in the primary setting, anthracycline and taxane therapies are recommended in early lines of treatment, but depending on previous exposure, toxicity profiles, costs, and patient preferences, capecitabine, vinorelbine, eribulin, and gemcitabine are also well-established for the treatment of metastatic breast cancer and can be considered[58,122]. For BRCA mutated triple-negative metastatic breast cancer, a platinum-based treatment regimen is recommended in an early line[58]. Each regimen should continue until progression, or if there are unacceptable side effects.

HER2-targeted therapy

Patients with HER2-positive metastatic breast cancer should start a HER2-targeted treatment in an early line, and the treatment should continue upon progression.

In the first line treatment with trastuzumab and pertuzumab, in combination with chemotherapy, is recommended. In later lines, T-DM1 or trastuzumab in combination with a different chemotherapy agent, and lapatinib, a tyrosine kinase

inhibitor binding to the intracellular domain of the HER2 molecule, could be used. Lapatinib can also be combined with different chemotherapy agents[123,135].

Bone-modifying agents

In addition to standard treatment, patients with bone metastases should receive a bone-modifying agent, either a bisphosphonate or the RANK-L antibody denosumab, to reduce the risk of skeletal-related events[136].

New and emerging therapies

The monoclonal antibody bevacizumab is targeted against vascular endothelial growth factor (VEGF), a key factor in angiogenesis, and has been found to improve progression-free survival when added to chemotherapy in the first line. It is, however, also associated with a higher degree of side effects[137].

For patients with triple-negative breast cancer, addition of the checkpoint inhibitor atezolizumab to the first line of chemotherapy is associated with an increased survival of seven months, restricted to patients with a tumor expression of programmed cell-death 1 ligand 1 (PD-L1) immune cell staining ≥1%[138].

Treatment with PARP inhibitors among patients with a HER2-negative BRCA-mutated metastatic breast cancer, after progression of at least one line of systemic therapy, has shown a superior progression-free survival compared to standard chemotherapy[139].

For patients with ER-positive and HER2-negative breast cancer and a PIK3CA-mutation, with previous progression on aromatase inhibitors, the addition of a PI3K inhibitor to fulvestrant has been found to improve progression-free survival[140] but is not yet approved for standard clinical use. PI3K inhibitors act by inhibiting the PI3K/akt/mTOR signaling pathway, which is critical in many cellular processes controlling cell growth, proliferation, survival, and metabolism.

Therapy resistance

Therapy resistance represents a major obstacle for successful treatment of breast cancer. Some tumor cells are *de novo* resistant and never respond to a given therapy while others develop acquired resistance, which is revealed by a transitory clinical

response, later followed by a relapse. Numerous mechanisms can lead to the development of acquired drug resistance.

Regarding chemotherapy, resistance commonly evolves through increased drug efflux by up-regulation of drug transporter proteins in the cell membrane, or reduced absorption of drugs through mutations of drug transporters involved in drug absorption. Both mechanisms lead to decreased intracellular drug accumulation, and thereby inferior tumor response[141,142]. Other mechanisms include changes in drug metabolism, modifications in the agent targets, enhancement of DNA repair, and epigenetic altering[143].

For targeted therapies, where the therapy inhibits one key pathway in a tumor, the therapy can place selective pressure on the tumor cells, influencing the tumor evolution and leading to resistance. In HER2-positive breast cancer, the main underlying mechanisms of drug resistance to HER2-targeted therapy are thought to be through an altered drug-binding property, alternation of some downstream pathways, and changes in the immune response[144,145].

The development of endocrine therapy resistance is believed to be due to both genetic and epigenetic factors. A minority of patients experience loss of ER expression upon recurrence[146]. Mutations in the ER gene; *ESR1*, or its effectors, some of which are found to cause a conformation change in the ligand-binding domain of the ER, thus lowering the affinity for tamoxifen but also causing cell proliferation and tumor progression without hormone stimulation, are thought to be part of the acquired resistance for endocrine therapy[147]. Due to substantial cross-talk between ER signaling, growth factors, and cellular kinases, endocrine therapy can induce adaptive changes resulting in the activation of alternative signaling pathways, so the cells stop being dependent on ER signaling for proliferation and survival. Other mechanisms such as overexpression of growth factors or their receptors or activation of their downstream signaling can also activate other pathway signaling[148]. Aberrant expression of molecules controlling the cell cycle has been associated with endocrine resistance, such as overexpression of cyclin E1 and D1, or decreased expression of p21 and p27[148]. Also, several components of the tumor microenvironment, as well as inflammatory cytokines, have been implicated in endocrine resistance.

By combining endocrine therapy with different targeted therapies, such as CDK4/6 inhibitors or PI3K inhibitors, endocrine resistance has successfully been delayed and progression-free survival significantly improved[129,133]. However, management of resistance to endocrine therapy is still a challenging aspect in the treatment of breast cancer, with many unidentified factors that contribute to endocrine resistance remaining.

Drug repurposing

There is a constant demand for new, effective cancer drugs. The discovery and development of new cancer drugs is challenged by high attrition rates[149], the long time necessary to bring the new drug to the market, and high costs[150]. In addition, prices of new cancer therapies have escalated in recent years, and the burden of health economics has been significantly increased worldwide[151]. This has led to the strategy of finding new applications for already approved drugs, called drug repurposing. Because much preclinical testing and safety assessment has already been completed, drug repurposing includes advantages such as a lower risk of failure – at least from a safety standpoint – and the fact that the time frame for drug development can be reduced. Depending on the stage and process of the developing drug, less investment might be needed[152]. In the field of oncology , drug repurposing offers the opportunity to test already approved drugs in combinatorial treatments, aiming to improve the efficacy of already existing anticancer treatments and overcoming drug resistance[153].

In this thesis, different aspects of the cholesterol-lowering drugs statins on breast cancer are investigated. Next, the rationale behind statins as a breast cancer drug will be presented.

Cholesterol

Cholesterol is an essential component of human cell membranes, as well as a precursor to several hormones, vitamins, and bile salts. Since the molecular formula of cholesterol was first established in 1888, the interest in cholesterol in the research field was extensive during the 20th century, due to increasing knowledge of the association between cholesterol, atherosclerosis, and cardiovascular disease[154].

Cholesterol can be either *de novo* synthesized, mainly in the liver, or derived from diet intake. Due to its hydrophobic nature, it is transported through the blood inside lipoprotein particles[155]. There are different types of lipoproteins with different purposes; chylomicrons, intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), very-low-density lipoproteins (VLDL), and high-density lipoproteins (HDL). Chylomicrons transport dietary lipids from the intestines to other locations in the body, while IDL is assembled in the liver and is converted to LDL and VLDL in the bloodstream. LDL and VLDL transport cholesterol to the peripheral tissues and HDL conversely transports excess peripheral cholesterol back to the liver for excretion[155]. Clinically, LDL and HDL are important because high LDL

and low HDL increase a patient's risk of atherosclerotic vascular diseases. Different lipoproteins contain different classes of apolipoproteins as important components of their surface, and with roles in lipid transport and metabolism.

The cholesterol biosynthetic pathway – the mevalonate pathway – was determined in the 1950s[156] and is shown in Figure 4. Cholesterol biosynthesis begins with an acetyl-CoA unit, and results, after a sequence of complex reactions, in the production of cholesterol, as well as steroid hormones, vitamin D, ubiquinone (co-enzyme Q10), heme A, isoprenoid intermediates, and many other compounds, as illustrated in Figure 3. The rate-limiting step, when 3-hydroxy-3-methyl-glutaryl-Coenzyme A (HMG-CoA) is irreversibly converted to mevalonic acid, is the third step and is catalysed by 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR).

Extensive research during the following decades gradually revealed the regulation of cholesterol metabolism. Studies in the 1960s showed that if the required cholesterol levels are not met by dietary cholesterol, they are supplemented by *de novo* synthesis, but if dietary cholesterol reaches its required level, the *de novo* synthesis is suppressed, mediated through changes in HMGCR activity[157].

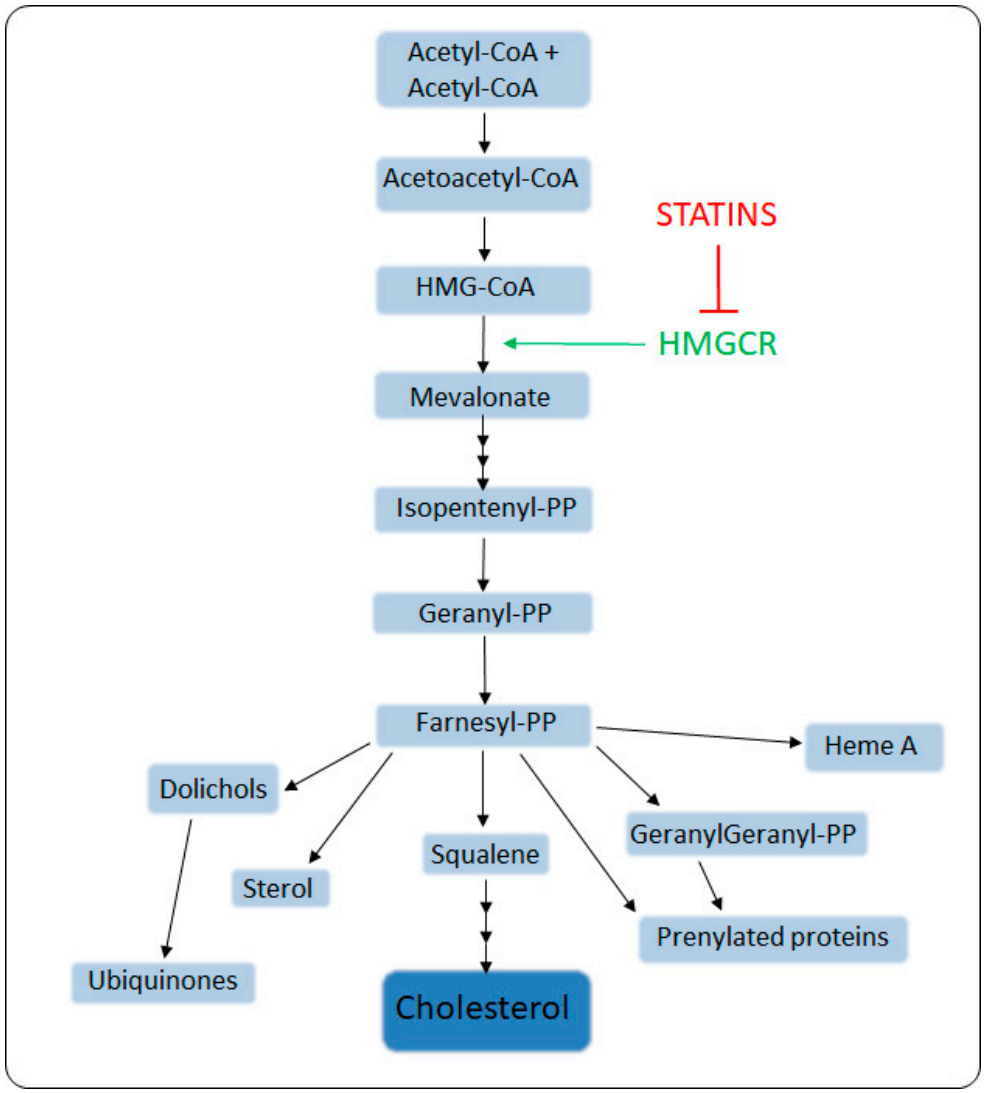

Figure 4. Simple schematic of the mevalonate pathway. The first step is the generation of HMG-CoA from acetyl-CoA units. Then, HMG-CoA is converted to mevalonate by the action of HMGCR, which is the only rate-limiting reaction in the mevalonate pathway and is the target of statins. The end product of the mevalonate pathway is cholesterol.

Despite the invaluable importance of cholesterol, abnormal levels of intracellular free cholesterol are toxic to cells. Thus, intracellular cholesterol homeostasis is strictly regulated by feedback mechanisms at both transcriptional and post-transcriptional levels[158,159], as seen in Figure 5.

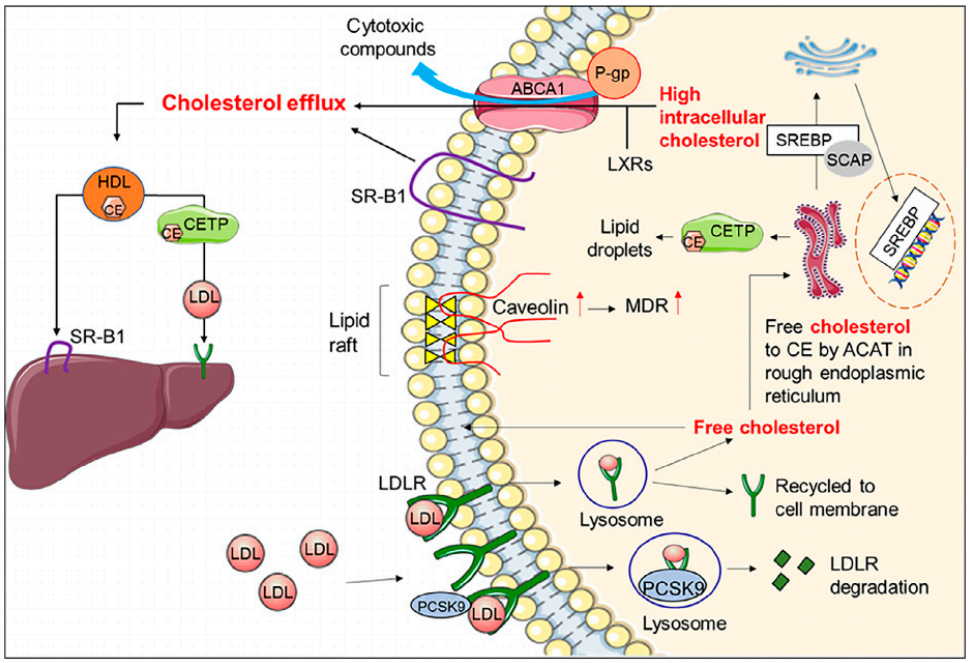

Figure 5. Intracellular cholesterol metabolism. Extracellular free cholesterol is transported extracellularly in LDL, which interacts with the LDLR and enters the cells through endocytosis. The free cholesterol is then dissociated from the receptor in the cell lysosome. The cholesterol is distributed within the cells for different functions, as parts of the cell membrane, or converted to CEs by ACAT in the endoplasmic reticulum and stored as LDs. Furthermore, SREBP-2 regulates intracellular cholesterol synthesis and uptake. Intracellular cholesterol homeostasis is also maintained through efflux, via LXRs. Reprinted from "Targeting cellular cholesterol for anticancer therapy", FEBS Journal, 2019, with permission ©2019 FEBS PRESS.

One of the key regulators of intracellular cholesterol levels is Sterol Regulatory Element Binding Protein-2 (SREBP-2), a transcription factor that functions as a sensor for cholesterol. SREBP-2 is negatively regulated by levels of free cholesterol, and then kept in an inactive state as part of a protein complex in the endoplasmic reticulum. When intracellular levels of cholesterol are low, this complex is disrupted with the help of SREBP cleavage activating protein (SCAP), and SREBP-2 is released to the Golgi, where it is activated. SREBP-2 then coordinates the transcription of HMGCR to increase *de novo* synthesis of cholesterol and activate the transcription

and up-regulation of LDL receptors (LDLR), leading to an increase in cellular cholesterol uptake. LDLRs are cell surface receptors, mediating the delivery of cholesterol in the form of LDL and VLDL from the circulation by receptor-mediated endocytosis[158,160]. When intracellular levels of free cholesterol are high, the cholesterol metabolites known as oxysterols are formed. Oxysterols bind to the nuclear hormone receptors known as liver X receptors (LXRs), which are important transcriptional regulators of genes involved in reverse cholesterol transport, thereby increasing cholesterol efflux[161]. Free cholesterol in the cytoplasm and intracellular membranes is also converted to cholesteryl esters, primarily by the enzyme Acyl-CoA: cholesterol acyltransferase (ACAT). Cholesteryl esters are stored in the intracellular organelles known as lipid droplets (LDs). LDs are composed of a core of triacylglycerides and cholesteryl esters, surrounded by a single phospholipid membrane, with integrated structural and functional proteins. LDs were formerly thought to be simply lipid storage but are now recognised as more complex organelles involved in several pathological conditions including obesity, inflammation, and cancer[162].

After these important findings of the regulation of cholesterol metabolism, the search for HMGCR inhibitors began, with the hope of finding an effective approach to lowering plasma cholesterol, and thereby prevent adverse cardiovascular events.

Statins

In 1973, the HMGCR inhibitor mevastatin (Compactin) was isolated from the fungi Penicillium citrinum by Akira Endo, and is called the first statin[154]. Mevastatin demonstrated good plasma cholesterol-lowering effects in both animal studies and clinical trials, but was however never marketed. The first commercial statin, lovastatin, was given FDA approval in 1987.

After lovastatin, six additional statins have been introduced to the market, including two semi-synthetic statins (simvastatin and pravastatin) and four synthetic statins (fluvastatin, atorvastatin, rosuvastatin, and pitavastatin)[154,163]. All statins are competitive inhibitors of HMGCR, binding to the active site of the enzyme with approximately 10,000 times higher binding affinity than the substrate HMG-CoA, thus inducing a conformational change in its structure and reducing its activity[164]. In general, statins share similar chemical characteristics, but with slightly different structures, kinetic profiles, and metabolic rates[163]. Lovastatin, simvastatin, fluvastatin, and atorvastatin are considered lipophilic, which enable them to enter extrahepatic cell membranes, while pravastatin and rosuvastatin are considered hydrophilic and thereby hepatoselective[163].

Multiple meta-analyses of clinical trials have been consistent: Treatment with statins lowers plasma LDL levels by 25–35% and reduces the frequency of heart attacks by 25–30%[165,166]. Statins are generally well-tolerated and the most common side effects are mild, such as diarrhea, nasopharyngitis, headache, and myopathy. However, more severe adverse events are associated with statin therapy, including hepatotoxicity characterized by elevated serum transaminases, hepatocellular injury, and fulminant liver failure, as well as rhabdomyolysis, which is a condition characterized by massive muscle necrosis that can progress from myotoxicity[167]. Statins are now the most widely prescribed class of drugs in the world, and among them, the most commonly used is atorvastatin.

Atorvastatin is administered perorally with a recommended dose range of 10–80 mg daily. It lowers plasma LDL levels effectively, by one-third to one-half in a dose-related manner. After intake, peak plasma concentration is reached within 1–2 hours. Due to extensive first-pass metabolism, the bioavailability is low, at 14%. Atorvastatin is metabolised by cytochromes P-450 3A4 and P-450 3A5 to the two active metabolites ortho-hydroxy atorvastatin and para-hydroxy atorvastatin, which extend the effect of atorvastatin, resulting in a half-time of HMGCR inhibition of 20–30 hours[167].

Beyond the cholesterol-lowering effects, statins exhibit pleiotropic effects, with anti-inflammatory, antioxidant, anti-proliferative, and immunomodulatory properties. In the cardiovascular field, statin pleiotropy causes improved endothelial function, stabilization of atherosclerotic plaque, and reduced inflammatory and thrombogenic responses, though statin pleiotropy may also significantly affect other medical conditions, such as neurological disorders, autoimmune disorders, thromboembolism, and – of importance for this thesis – cancer[168].

The concept of statin pleiotropy is not fully elucidated, but is thought to occur through other products of the mevalonate pathway, such as the isoprenoid intermediates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). These isoprenoids are important for protein prenylation, a type of posttranslational modification and activation that increase protein hydrophobicity, enabling the proteins to associate with plasma membranes, which is necessary for their function. Examples of proteins dependent on protein prenylation are the Ras and Rho-proteins, which play crucial roles in multiple cellular processes on which tumors depend, such as cell signaling, cell differentiation, proliferation, and cytoskeleton dynamics[169].

Cholesterol and breast cancer

Elevated cholesterol levels can potentially affect carcinogenesis in different ways, primarily by enabling the increased need of cholesterol for membrane synthesis of highly proliferating cells[170]. Further, cholesterol is required for the formation of lipid rafts, parts of the cell membrane containing several signaling molecules. Among these are the signaling molecules of many oncogenic signaling cascades, such as EGFR and HER2 signaling pathways, and differences in membrane cholesterol levels can affect these signaling pathways[171,172]. Another link between cholesterol and breast cancer is that cholesterol is needed for the production of steroid hormones, including estradiol with importance for the growth of hormone receptor positive breast cancer[170].

The relationship between plasma cholesterol levels and the risk of breast cancer has been extensively studied, with inconsistent results from epidemiological studies. One study found an association between high cholesterol levels and breast cancer incidence[173], whereas other studies have not found any associations between cholesterol or LDL levels[174-179], or have found an inverse relationship[179]. However, high cholesterol intake has been found to be positively associated with the risk of breast cancer[180,181], and when investigated in mouse models, a high-fat/high-cholesterol diet has been found to promote breast cancer growth[182,183].

Further, experimental studies have shown that hypercholesterolemia in mice enhanced breast cancer growth, suggesting an association between hypercholesterolemia and breast cancer. Also, the primary oxysterol cholesterol metabolite 27-hydroxycholesterol (27HC) has been identified as acting as an endogenous SERM and was found to promote breast cancer growth and metastasis both *in vitro* and *in vivo*[184,185]. Further, 27HC levels have been found to be elevated within breast tumors compared to normal breast tissue[185], and increased protein expression of the enzyme responsible for its synthesis (CYP27A1) is associated with unfavorable tumor characteristics and impaired survival[186,187], while tumor expression of the enzyme responsible for metabolizing 27HC (CYP7B1) expression is negatively associated with prognosis[185].

HMGCR, the rate-limiting enzyme of the mevalonate pathway, has been found to be constitutively overexpressed in cancer cells[188]. Several studies have examined the relationship between tumor expression of HMGCR in breast cancer, and other tumor characteristics, and have found an association with favorable prognostic clinicopathological parameters[189-191]. In contrast, mRNA levels of HMGCR and other mevalonate pathway genes have been associated with poor prognosis and reduced

survival among breast cancer patients[192]. HMGCR tumor expression in breast cancer will be further investigated in this thesis.

Statins and breast cancer

During the last decades, the possible effect of statins on breast cancer has been investigated in epidemiological studies, preclinical experiments and clinical trials. Effects mediated by the systemic cholesterol-lowering effects, as well as more direct cellular changes induced by statins, have been explored.

Epidemiological studies

Initial epidemiological studies, emerging in the 2000s, showed a decreased breast cancer incidence among statin users[193,194]. Conversely, other studies have not found any association[195-200], and one study even found an increased risk of breast cancer among long-term statin users[201].

In contrast, treatment with statins has more consistently been reported to have an effect in terms of secondary prevention, protecting against breast cancer recurrence and death. In a retrospective study with 6314 participants, Smith et al. found that pre-diagnostic statin use was associated with a reduction in breast cancer-specific mortality (HR=0.81 (0.68–0.96)), with the greatest reduction found among ER-positive patients[202]. In 2011, a Danish prospective study with 18,769 participants was published by Ahern et al. and showed a significant reduction in breast cancer recurrence among patients using simvastatin after 10 years of follow-up (adjusted HR=0.70 (0.57–0.86))[203]. In a study investigating statin use in different cancer types, including breast cancer, statin use was associated with reduced cancer-related mortality[204]. Murtola et al. published a prospective study with 31,236 participants, showing that both post-diagnostic and pre-diagnostic statin use were associated with a lowered risk of breast cancer death (HR=0.46 (0.38–0.55) and HR=0.54 (0.44–0.67)), respectively[205]. Further, initiation of cholesterol-lowering medication in postmenopausal women with early-stage, hormone receptor-positive invasive breast cancer during endocrine therapy was related to improved 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)), as published by Borgquist et al. from the international BIG 1-98 trial[206]. A recent Swedish nationwide study also showed lower risk of breast cancer-related deaths among statin users, both pre-diagnostic and post-diagnostic statin use, (HR=0.77 (0.63-0.95)) and (HR=0.83

(0.75–0.93)), respectively[207]. Consistent with these large-scale studies, several meta-analyses have shown associations between statin use and improved outcomes in terms of breast cancer recurrence and mortality[208-211].

However, some studies have not found an association between statin use and a protective effect regarding breast cancer. The retrospective study by McMenamin et al. found no evidence of an association between statin use and breast cancer-specific death[212] and the already-mentioned retrospective study by Smith et al, found no association between post-diagnostic statin use and either breast cancer-specific or all-cause mortality[202].

In vitro experiments

In addition to the indirect effects of lowering plasma cholesterol, *in vitro* experiments have elucidated mechanisms by which statins exert direct anti-tumor effects. Due to their ability to affect various tissue functions and control specific signaling pathways, the antitumor effects of statins result in the inhibition of proliferation, invasion, migration, angiogenesis, and the induction of apoptosis[213].

Statins have been shown to inhibit breast cancer cell proliferation by affecting the expression and activity of cyclins and CDKs, thus inducing cell cycle arrest at two points, G1/S and G2/M[214,215]. Cell cycle progression is further halted by the inhibition of FPP and GGPP modification and the activation of Ras, Rac, and Rho proteins, which is essential for cells to enter the S-phase[215,216]. Statins have also been shown to influence the transcription of genes that regulate cell proliferation[163,217], thereby reducing breast cancer cell proliferation.

The exact mechanisms and magnitude of how statins induce apoptosis is not established, but statins have been shown to up-regulate the activation of pro-apoptotic molecules such as Bax, Bad, and Caspases 3, 8, and 9[218,219] in different cancer cell lines. Additionally, statins have been shown to promote apoptosis by decreasing phosphorylation and degradation of the regulator of apoptosis Bim[220].

At higher concentrations, statins negatively impact angiogenesis through multiple mechanisms including directly affecting the endothelial cells to reduce tumor angiogenesis, and indirectly, by reducing circulating VEGF concentrations[213].

Statins have been found to reduce the invasiveness and metastatic potential of cancer cells through multiple mechanisms, by destabilizing the cytoskeletal structure of tumor cells in a RhoA/RhoC-dependent manner[215,221], by reducing the expression and activity of the pro-migratory proteases MMP-2, MMP-9, and urokinase, by

inhibiting Ras and Rho activity, and by reducing the expression of the transferrin receptor in breast cancer cells, which causes iron starvation and a reduction in tumor invasiveness [222] Further, the cancer stem cell marker CD44 is down-regulated by statins in breast cancer cells, which reduces cell migration and invasion[223].

Statins have also been shown to inhibit epithelial-mesenchymal transition (EMT), a process in which epithelial cells undergo multiple biochemical changes and lose their ability to retain cellular adhesion, gain migratory properties and assume a mesenchymal cell phenotype. Mesenchymal cells can differentiate into a variety of cell types, giving rise to cancer recurrences. EMT is intensified by FPP and GGPP, intermediate metabolites of the mevalonate pathway, through several signaling pathways[224]. Conversely, lipophilic statins have been shown to inhibit this process, both in breast cancer[224] and other cancer cell types[225].

However, cancer cells differ in their individual statin sensitivity. In breast cancer cell lines, hormone receptor-positive cell lines have shown a relative insensitivity, compared to hormone receptor-negative cell lines, which is thought to be mediated by a regulatory feedback loop via the HMGCR that counteracts the inhibition of the mevalonate pathway[226].

In vivo experiments

Studies on mice xenografts have confirmed statin-induced apoptosis in breast cancer models[227] Also, simvastatin has been shown to prevent tumor growth by reducing Akt phosphorylation and BclXL transcription, while simultaneously increasing the transcription of pro-apoptotic/anti-proliferative PTEN[228].

Clinical trials

Based on the results from epidemiological studies and preclinical experiments, statins have so far been tested in window-of-opportunity (WOO) clinical breast cancer trials. The topic of WOO trials is described in detail in the next section.

Garwood et al. conducted a phase II WOO clinical trial, where 45 patients were included and received either a high dose or low dose of fluvastatin for 2–6 weeks. The results showed a reduced tumor proliferation, as measured by Ki67, and increasing apoptotic activity, restricted to high-grade tumors[229].

Wang et al. published a WOO study in 2016, where 15 female patients with newly diagnosed primary breast cancer were included and received 5–38 days of simvastatin at a dose of 20 mg daily before breast cancer surgery. The results showed a significant

induction of apoptosis and a decreased trend in Ki67 in the post-treatment samples[230].

In this thesis, two papers based on results from the MAmmary cancer and STatins (MAST) WOO trial, are included. The trial was conducted at Skåne University Hospital and will be introduced and discussed thoroughly in the material and methods section. A total of 50 patients with primary breast cancer were included and treated with atorvastatin for two weeks. Primary results showed a significant decrease in proliferation, as measured by Ki67, within the tumors expressing HMGCR before treatment[231]. Another sub-study based on the MAST trial, showed decreased serum 27HC and deregulated CYP27A1 expression in tumors following atorvastatin treatment[186].

These trials are considered early-stage investigations in patients. Larger-scale randomized clinical trials will be required to better elucidate the true clinical efficacy of statins in cancer.

Phase II trials

This thesis is, to a large extent, based on phase II trials. Traditionally, human clinical trials for drug development progress from phase I, small toxicity trials with healthy volunteers, to phase II, trials in a bit larger groups of patients with the target conditions, aiming to assess whether a certain treatment has sufficient biological activity to warrant further investigations in the final phase III trials, which are randomized trials with a large group of patients, aiming to further establish clinical efficacy, outcomes and adverse events.

The gold-standard endpoint in oncological phase III trials is overall survival (OS) which is defined as time from randomization or study enrolment until death from any cause. However, OS requires studies with large patient populations as well as prolonged follow-up of all patients, and consideration of this endpoint alone can delay the evaluation of novel therapies and phase II trials are often insufficient to test such a long-term outcome. In addition, assessment of OS can, in the metastatic setting, be confounded by factors such as crossover to active treatment upon disease progression[232], making it less suitable as an endpoint in phase II trials.

Instead, in phase II oncological trials, an intermediate clinical outcome measure is often used as an endpoint, i.e. time-to-progression. In the adjuvant setting, recurrence is a commonly used endpoint, and in the metastatic setting, either response rate or proportions of progression-free patients at a specific time point, are often used. These

endpoints are generally based on the Response Evaluation Criteria in Solid Tumors (RECIST). RECIST is a standardised means of tumor response assessment, based on changes in metastatic lesions by imaging modalities. At each time the tumor is measured, the patient is categorised as having a complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD)[233]. CR is the disappearance of all target lesions, and PR is defined as a 30% decrease in the sum of target lesions. When there is at least a 20% increase in the sum of diameters of up to five target lesions, the response is called PD, and SD is defined as being compatible with neither PD nor PR.

End-points based on RECIST include:

- Progression-Free Survival (PFS), time from randomization or study enrolment until disease progression or death.
- Time to Progression (TTP), time from randomization or study enrolment until objective disease progression; does not include deaths.
- Overall Response Rate (ORR), the proportion of patients with a reduction in disease burden of a predefined amount, i.e., exhibits a PR or CR.
- Duration of Response (DoR), time from documentation of disease response to disease progression.
- Clinical Benefit Rate (CBR), the proportion of patients whose tumor exhibits SD, PR, or CR.
- Duration of Clinical Benefit (DCB), time from confirmation of SD, PR, or CR, until the disease has been shown to progress following treatment.
- Objective Response Rate (ORR), percentage of patients with PR and/or CR after treatment.

The use of RECIST is limited when tumor measurements on cross-sectional imaging are difficult or uninformative, when there is non-measurable disease, and by the reliance of humans for the measurement. Likewise, these limitations must be taken into consideration when one interprets the endpoints based on the RECIST[234].

Another type of endpoint is biomarkers, which can be used, for example, in trials investigating targeted therapies. The FDA has established means by which to qualify biomarkers for use, but the process of validating a biomarker as an appropriate surrogate study endpoint is both time-consuming and extremely expensive and there is a lack of validated biomarkers[235]. It is also unclear how well biomarkers correctly predict patient and trial outcomes[236].

A discrepancy in response rates between phase II and subsequent phase III trials has been reported, with many promising results from phase II trials not being reproduced in subsequent phase III trials. The reason for this is thought to be multifactorial, but one potential explanation is selection bias from non-randomized phase II trials[237]. Phase II trials can have been conducted as single-arm trials with tumor/metastases shrinkage as an endpoint, but in order to maximize the positive predictive value of phase II trials, an increased use of randomized phase II trials is now recommended, especially for trials of experimental agents combined with standard regimens. Yet, trials of agents anticipated to yield tumor regression, as well as early phase II monotherapy trials aiming to establish a tumor response signal of biological efficacy are still appropriately conducted as single-arm trials[238].

Window-of-opportunity trials

Window-of-opportunity (WOO) trials are a specific form of phase II trials in which the treatment-free window between the time of cancer diagnosis and the initiation of standard therapy, often surgery, is used to test a drug (or other intervention) for a short period of time, usually 2–4 weeks (Figure 6). Typically, biopsies of the tumor and blood sampling are taken before and after trial treatment for analyses and comparison.

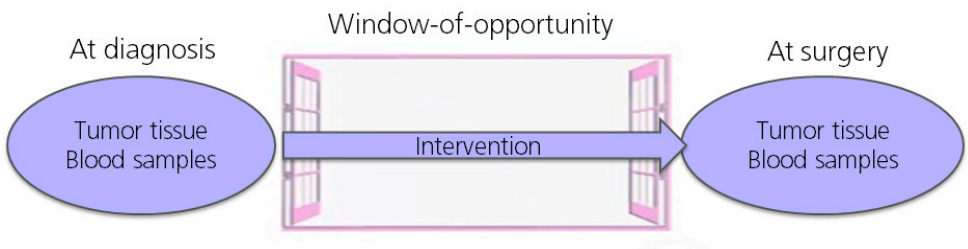

Figure 6. A schematic view of the window-of-opportunity trial design.

WOO trials aim to improve the understanding of the biological effect of a certain intervention, to investigate whether it modulates the target for which it was designed, to validate potential predictive biomarkers, or predict a subset of patients potentially gaining from a therapy[239]. Traditionally, early phase cancer drug trials are performed in patients with heavily pre-treated tumors, when drug activity will be influenced by prior therapy and possible selection of resistant clones. An advantage of WOO trials is the opportunity to perform molecular analyses in treatment-naïve patients[240].

A limitation of the WOO trial design is the shortness of the therapeutic window, which might confine the hypothesized effect and the possibility of detecting it. Tumor shrinkage and downstaging will unlikely be modified in the short time span of a WOO trial, and WOO trials are not intended to prove therapeutic advantage, in contrast to neoadjuvant studies, whose primary endpoint is usually downstaging of the tumor, or pCR[239].

Considerations in WOO trials are the peri-operative setting and consistency of sample handling, to ensure that differences in sample collection at surgery and pre-treatment biopsy do not influence the sample or molecular targets[240]. In the choice of molecular target, using a short-term outcome that may correlate with long-term outcomes is preferable[239].

Aims of the thesis

The overall aim of this doctoral thesis is to further elucidate the mechanisms by which statins potentially affect breast cancer.

Paper I

This study aimed to investigate the mediation of the statin-induced anti-proliferative effects, by analysing the protein expressions of the cell-cycle regulators cyclin D1 and p27. Another aim was to investigate the potential statin-induced effect on the clinically established biomarkers ER, PR, and HER2.

Paper II

This study was performed to study the statin-induced effect on intratumoral cholesterol metabolism. Cholesterol levels and the protein expression of the LDLR have been measured to evaluate any differences following statin treatment.

Paper III

This study aimed to investigate HMGCR as a prognostic factor in breast cancer and to investigate whether statin treatment affects breast cancer mortality.

Paper IV

This is a descriptive publication of a phase II trial aimed at investigating the effect and tolerability of atorvastatin in combination with endocrine-based treatment among patients with advanced breast cancer.

Materials and methods

Trials and cohorts

The MAmmary cancer and STatin trial – MAST trial

Papers I and II of this thesis are based on the MAST trial, a clinical non-randomized phase II breast cancer trial of WOO type. The trial was conducted as a single-center study at Skåne University Hospital in Lund, Sweden. Patients diagnosed with primary invasive breast cancer, with tumors measuring at least 15 mm by ultrasound, and who were candidates for radical surgery were eligible for participation.

Between February 2009 and March 2012, 50 patients were recruited at the time of their breast cancer diagnosis. The patients were prescribed 80 mg of atorvastatin daily for two weeks, between the time of diagnosis and the pre-planned surgery. 42 patients completed all study parts.

Before atorvastatin treatment initiation, core needle biopsies of the tumor were performed, with one core biopsy being formalin-fixed immediately and one being fresh frozen at –80 °C and blood samples were collected. During the planned surgery, tumor tissue was sampled from the surgically removed tumor, and blood samples were collected once more. The trial has been approved by the Ethical Committee at Lund University and the Swedish Medical Products Agency. The study has been registered at ClinicalTrials.gov (i.e., ID number: NCT00816244, NIH). All patients signed an informed consent.

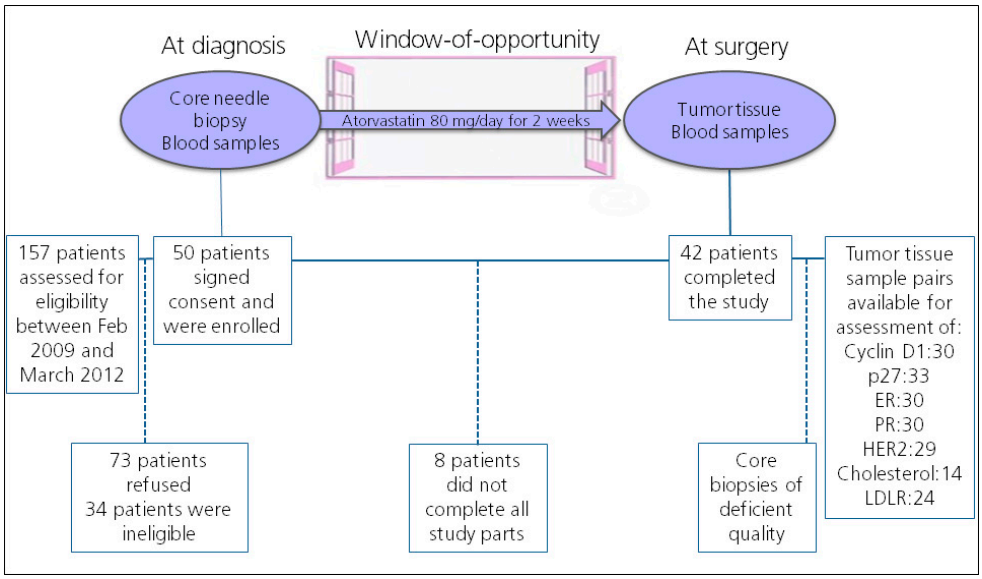

Figure 7. Flow-chart showing the enrollment and interventions of the MAST trial.

A statin-induced tumor response measured by the change in Ki67 expression, as a marker for tumor proliferation, served as the primary endpoint of the MAST trial. The secondary endpoints were to study HMGCR expression as a potential predictive marker before statin treatment, evaluated by the change in proliferation, as well as the change in HMGCR expression after the administration of atorvastatin. The results revealed a decrease in proliferation in HMGCR-positive breast cancer, as previously reported[231].

The Malmö Diet and Cancer study – MDCS

MDCS is a prospective, population-based cohort study with the main objective of investigating the relationship between diet and cancer, but also taking other lifestyle factors into account[241]. Between 1991 and 1996, all men and women from specific birth year cohorts living in Malmö were invited to participate via both a personal letter of invitation and community direct invitation[242]. With a participation rate of 41%, 18,326 women born between 1923 and 1950 were enrolled. Due to incomplete data, 17,035 women were able to join the study[243]. All patients signed a written informed consent form, which included the acceptance of future studies. Every year, linkage to the South Swedish Regional Tumor Registry, the Swedish Cancer Registry, and the Swedish Cause of Death Registry is performed to identify incident breast cancer cases.

In this thesis, paper III is based on MDCS, with the aim to study the associations between HMGCR tumor expression, statin use, and breast cancer mortality. Ethical permission was obtained from the Ethical Committee at Lund University (Dnr 472/2007).

During follow-up until December 31, 2010, a total of 1016 women were diagnosed with an incident breast cancer. Patients diagnosed with cancer in situ, or bilateral or distant metastatic breast cancer were subtracted, and 910 patients identified with invasive breast cancer remained. In 192 cases, tumor tissue was not available. The tissue microarrays (TMAs) were thus constructed of biopsies from 718 patients, though HMGCR expression could not be evaluated in 61 patients due to either inferior staining quality or lack of tumor tissue in the TMA core. In the end, 657 samples were available for assessment of HMGCR expression.

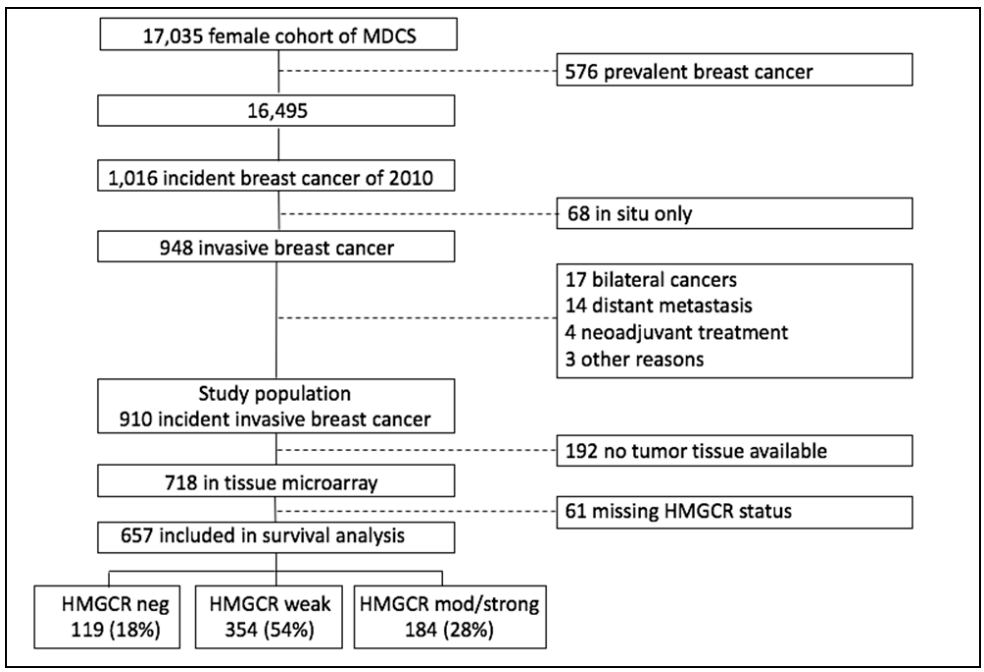

Figure 8. Flow-chart showing the study population in the Malmö Diet and Cancer Study. Reprinted figure from "Statin use, HMGCR expression, and breast cancer survival – The Malmö Diet and Cancer Study", Scientific Reports, 2020. Permission to reprint under the terms of CC BY 4.0 http://creativecommons.org/licenses/by/4.0/.

The Advanced Breast Cancer – Statins and Endocrine based treatment Trial – ABC-SE

ABC-SE is an academic single-center, randomized, open-label, phase II trial in the first-line metastatic setting that will be re-initiated at Skane University Hospital, Lund. Patients with metastatic breast cancer planned for systemic treatment with endocrine based therapy – aromatase inhibitor and a cdk4/6 inhibitor – will be included in this trial. Patients will be randomized to two treatment arms; a standard metastatic treatment with letrozole in combination with a CDK4/6 inhibitor will be compared to treatment with letrozole in combination with a CDK4/6 inhibitor with the addition of atorvastatin. In all, 126 evaluable patients will initially be included over a period of 42 months and randomized to either of the treatment arms.

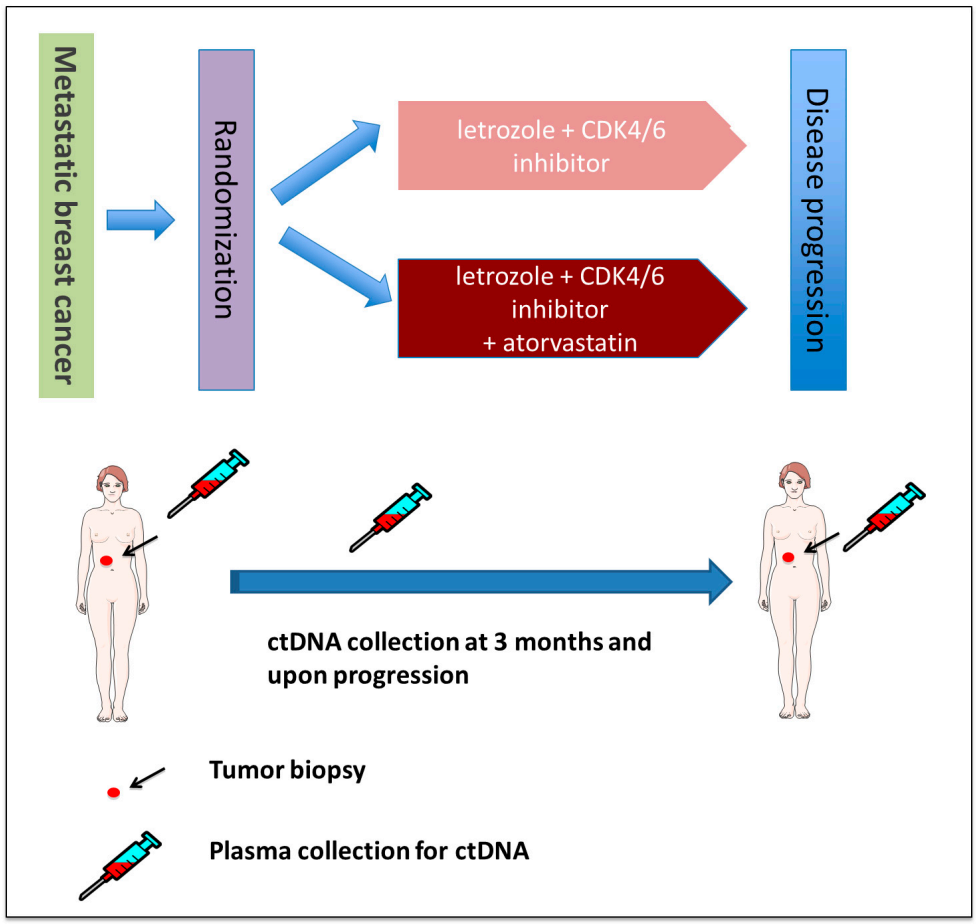

Figure 9. Flow-chart of the ABC-SE trial.

The primary endpoint of the ABC-SE study is CBR, defined as the proportion of all randomly assigned patients who have the best overall response, a complete response, a partial response, or stable disease for at least 9 months. Secondary endpoints are PFS, ORR, TTP, DCB, and OS. Further will the safety and tolerability of atorvastatin in addition to endocrine based treatment, as well as to improve the understanding of the specificity of actions of atorvastatin, and to elucidate mechanisms of resistance to endocrine based treatment be investigated, and serve as additional secondary endpoints. For these issues, translational studies based on the biological samples (tumor tissue, blood samples, and circulating tumor DNA) from the trial will be performed.

Experimental and methodological considerations

This section describes the main techniques and methods used in this thesis. A further detailed description of experimental procedures can be found in the "Materials and Methods" sections in the corresponding papers.

Immunohistochemistry

Immunohistochemistry (IHC) is a microscopy-based technique for visualizing proteins or other macromolecules in tissue samples by taking advantage of the principle of the strong avidity between antibodies and their antigens. IHC is routinely used in the clinic for purposes of diagnosing oncological diseases, as well as in the research field, for example, in the research of biomarkers[244].

First, a tissue section is often formalin-fixed and paraffin-embedded (FFPE). Formalin fixation produces cross-linking of proteins within the tissue, thereby terminating all cellular processes and preventing degradation of cellular organelles and proteins. Thereafter, the tissue section is embedded in paraffin blocks for long-term storage, which can be sectioned into thin slices when required.

Cross-linking might lead to changes in the three dimensional conformation of the proteins and cause reduced immunoreactivity. Therefore, before proceeding to the antibody staining, some of the cross-linking must be reverted, so the antibody binding sites (epitopes) are retrieved. This procedure is called antigen retrieval, in which the tissue slide is treated with digestive enzymes, heat, or detergents.

In the classical IHC assay, the FFPE tissue is stained using a primary antibody that can bind to the specific epitope. In the next step, a secondary antibody capable of

binding the primary antibody with high specificity is added. The secondary antibody is labelled with an enzyme, and next, a chemical substrate is added which reacts with the enzyme to create a colored precipitate[244].

For optimal use in IHC assays, the primary antibody should be both sensitive and specific to the target antigen, preferably with high affinity and low cross-reactivity, and antibodies must be validated for these qualities before use. Antibody validation can be performed by different strategies[245]. One of the strategies involves inducing differential expression (up-regulation or down-regulation) of protein by genetic or pharmacological approaches. Further, the antibody of interest is used to quantify the differential expression of protein by western blotting and/or in the specific intended use. To downregulate the protein expression, knockdown with small interfering ribonucleic acids (siRNAs) can be applied. In this method, siRNA is transfected into a cell, where it binds to a complementary mRNA strand, thereby cleaving it, which hinders translation of the protein for which it encodes. The cells transfected with siRNA will serve as negative controls in the antibody validation; if the antibody still binds to proteins in these cells, that is considered non-specific binding[246]. To verify reproducibility, it can also be of interest to test the antibody in different tissues or cells to ensure that the staining of the antibody is constant and does not change[247].

Antibodies can be either polyclonal or monoclonal. Polyclonal antibodies are a mix of antibodies binding different epitopes on the target, often resulting in a high affinity and robust detection, but also an increased likelihood of off-target binding events. Monoclonal antibodies are identical and bind to the same epitope of an antigen. They are more specific and have a higher reproducibility, but can be hard to interpret if the target epitope is present in low abundance.

IHC have been used in papers I–III. The antibodies used are described in each paper.

Tissue microarrays

Tissue microarray (TMA) is a high-throughput method for the evaluation of protein expression. TMAs are constructed by multiple extracted cylindrical tissue core biopsies, typically 0.6–2 mm, originating from representative parts of different FFPE donor blocks, which are re-embedded into a common recipient block: the tissue microarray. For analysis, the recipient block is sliced into thin sections (4–5 µm) and mounted on microscope glass slides, that can later be used for IHC or ISH[248]. The TMA construction is illustrated in Figure 10.

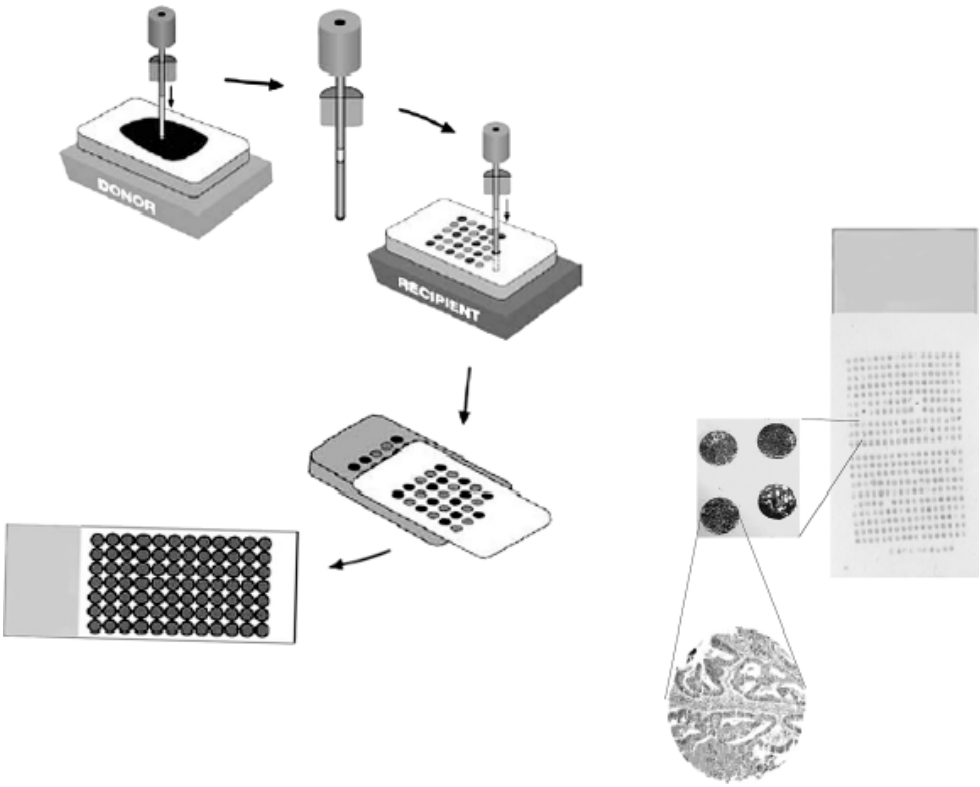

Figure 10. Illustration of the construction of a tissue microarray. Reprinted from "Assessing Expression of Apoptotic Markers Using Large Cohort Tissue Microarrays" Apoptosis and Cancer pp 83–93 2008 with permission ©2008 Springer Protocols.

With the use of TMA, valuable tumor tissue as well as antibodies and reagents are used sparely, and the method is time-efficient[248]. An important consideration regarding the TMA technique is tumor heterogeneity, and that the microarray cores may not be representative of the whole tumor. However, previous research comparing analyses of whole sections and TMAs has found that the results are consistent to a high degree[249], especially with the use of duplicate cores[250].

In papers I–III, TMAs have been used for IHC evaluations.

Gene expression microarrays

Another sub-study of the MAST trial analysed global transcriptional changes following statin treatment [251]. In this thesis, papers I and II include analyses

concerning the expression of the probes representing cyclin D1 and p27 and selected genes of cholesterol homeostasis.

Microarray technology is a method of gene expression analysis that has revolutionized molecular biological research due to the possibility of simultaneous analysis of thousands of gene probes. A microarray is a glass slide containing thousands of spots, each with a few million copies of identical DNA molecules exclusively corresponding to a single gene. The primary step of many molecular biological techniques, such as microarrays is the isolation of RNA. However, isolation of intact RNA is aggravated by the labile nature of RNA and by the occurrence of RNases, which is a group of enzymes that degrade RNA molecules, frequently abundant in both cells but also in the surrounding environment. For quantitative gene expression assays, RNA quantity and quality are essential. After RNA extraction, the RNA molecules are reverse-transcribed into complementary DNA (cDNA) by the enzyme reverse transcriptase, and the nucleotides are then labelled with fluorescent dyes. The labelled cDNA is then hybridized onto the microarray slide, where complementary synthetic oligonucleotides are fixed. The higher the expression of a gene, the more labelled cDNA will hybridize onto the microarray spot, which is measured by fluorescence intensity[252,253].

In papers I and II, RNA was extracted from fresh frozen tumor tissue using the Allprep DNA/RNA mini kit (QIAGEN, Valencia, CA) in a QIAcube (Qiagen) according to the manufacturer's instructions. Determination of tumor cellularity was performed on an H/E-section from the core biopsies, with tumor cellularity greater than 50% found in about 70% (14/21) of evaluable cases[251]. RNA quantification was performed using a NanoDrop ND-1000 (NanoDrop Products). The RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent) for quality control, and a RNA integrity number (RIN) value ≥ 7 was mandatory for inclusion in further analyses. Labelled cDNA from the tumor samples pre- and post-atorvastatin treatment was hybridized to Human HT-12 v4.0 Expression BeadChips (Illumina Inc.) at the Sciblu Genomics Center at Lund University, Sweden (www.lu.se/sciblu). All data were initially pre-processed and normalized using the Quantile Normalization method to adjust for technical variations[254] and analysed using the GenomeStudio Software V2011.1. Complete information about the comprehensive analyses of the data has been described previously[251].

Cell lines

Cancer cell lines are widely used in cancer research as an *in vitro* model system, due largely to their cost-effectiveness and the fact that they provide an almost unlimited

source of biological material[255,256]. Further, ethical considerations are not necessary to the same extent as when using human or animal tissues. However, contamination of cell lines can occur, and when cultured for extensive periods, there is a risk of subpopulations arising and causing phenotypic changes to the cell lines[257]. Other limitations of 2D cell cultures are the lack of tumor microenvironment and interaction with other cell types, and that the effects of *in vivo* drug distribution and metabolism are not always possible to mimic *in vitro*[258].

In paper II, several experiments with the human breast cancer cell line MCF-7 were performed. MCF-7 cells were chosen for these experiments based on their ER-positive and HER2-negative properties, which are in line with the majority of the patients in the MAST trial. The MCF-7 cell line is originally derived from the pleural effusion of a 69-year old woman with metastatic breast cancer in 1970, and has been widely used in research due to its preserved characteristics of mammary epithelium, and for the study of the ER[259].

Cholesterol quantification

Several analytical methods have been developed for analysis of cholesterol from biological samples, classical chemical methods, enzymatic assays, and analytical instrumental approaches such as gas and liquid chromatography, or mass spectrometry. The chromatographic and mass spectrometric methods are more sensitive and accurate than the chemical and enzymatic approaches, but costly and require extensive sample pre-treatment[260]. All methods include lipid extraction as part of sample preparation. To prevent lipid degradation, lipid extraction from human tissue should be performed immediately after the tissue removal, or from fresh frozen tissue[261]. The extraction procedure includes both a polar solvent to separate lipids from proteins, and a non-polar solvent to dissolve lipids, as described by Folch et al.[262].

In paper II, total cholesterol levels from tumor tissue before and after atorvastatin treatment, and from MCF-7 cells exposed to atorvastatin, were quantified using the Abcam Cholesterol Fluorometric/Colorimetric Assay (ab65359), according to the manufacturer's instructions. In the cholesterol assay, cholesteryl esters are first hydrolysed to cholesterol by cholesterol esterase and then oxidized by cholesterol oxidase, yielding hydrogen peroxidase, which reacts with a sensitive cholesterol probe to produce color or fluorescence to quantify total cholesterol. Fluorescent or colorimetric signals are read by a spectrophotometer and plotted against a standard concentration curve.

Lipid droplet staining

Fluorescent lipophilic dyes, which due to their hydrophobic nature, rapidly partition into the nonpolar environment of LDs, are useful LD markers with the capability of detecting both the magnitude and localization of lipids within a tissue. One of these dyes is Oil Red-O (ORO), which stains neutral lipids and cholesteryl esters but not the polar lipids found in biological membranes. ORO staining is feasible in several different tissues, however, FFPE tissues cannot be used for this staining procedure, as the deparaffinization process extracts most lipids from the tissue sections[263]. Another limitation is that no detailed information about the diversity and chemical composition of the lipids can be provided. For that types of analyses, more costly methods can be used, such as label-free microscopy and chromatography methods[264]. Also, the immunofluorescence staining technique requires the fixation of cells, but methods of fixation can impact the LDs' morphology, which must be taken into consideration[265].

In paper II, MCF-7 cells exposed to different concentrations of atorvastatin were stained with ORO to evaluate the amount of lipid droplets.

Proliferation assays

Cellular proliferation is commonly measured to monitor the response and health of cells in culture after treatment with various stimuli. Different assays may measure cell viability, metabolic activity, number of cells over time, number of cellular divisions, or DNA synthesis.

In paper II, a proliferation assay was performed to evaluate the effect of atorvastatin treatment on MCF-7 cell proliferation using the xCELLigence Real-Time Cell Analyzer (ACEA Bioscience, Inc), which involves continuous monitoring of cellular phenotypic changes using impedance as a readout, thereby providing quantitative information about the biological status of the cell, including viability, morphology, and cell number[266].

Western blot

Western blotting is a widely used method for protein detection and analysis. Samples are first prepared to solubilize and denature proteins. The denatured total protein is quantified and separated based on molecular weight through gel electrophoresis. The proteins are then transferred onto a membrane and incubated with a primary protein-specific antibody followed by a labelled secondary antibody. There are several kinds of

labelling, but the enzyme horse radish-peroxidase is often used. A common detection method of the protein bands is by chemiluminescence, and the results are quantified by densitometry and normalized to an internal reference. For accurate identification and quantification of target proteins, western blot relies on antibody specificity as well as careful achievement of all procedures prior to antibody application.

In paper II, western blot was used for the analysis of LDLR expression in MCF-7 cells with and without atorvastatin treatment.

Statistical analysis

Statistics plays a central role in medical research, as an essential tool for interpretation of the data and to obtain answers to scientific questions. Hypothesis testing is fundamental in statistics, where the null hypothesis, indicating no association between the investigated factors or characteristics, is compared to the alternative hypothesis, which does indicate an association between the investigated variables. To reach conclusions about the statistical significance of a research consideration, a widely used statistical term is the p-value.

The p-value is defined as "the probability of obtaining a result at least as extreme as the one that was actually observed in the biological or clinical experiment or epidemiological study, given that the null hypothesis is true". A p-value of less than <0.05 is generally considered statistically significant. However, due to mis- or over-interpretation leading to methodological errors, a more critical approach towards the concept of statistical significance has evolved, warranting a more thoughtful interpretation[267]. In paper II, we have tried to approach this as suggested by Benjamin et al.[268], i.e., suggestive evidence for p-values in the range from 0.005 to 0.05 and significant evidence below 0.005. For the other papers, values of less than 0.05 are considered to indicate statistical significance. All p-values correspond to two-sided tests.

Papers I and II

Distributional differences between groups were calculated as appropriate, depending on data and variable type. In paper I, changes in ER, PR, HER2, cyclin D1, and p27 protein expression, and in paper II, changes in intratumoral cholesterol levels, LDLR protein expression, and gene expression of the cholesterol homeostasis genes between pre- and post-atorvastatin treatment samples were evaluated using the Wilcoxon

matched-pairs signed-rank test, a nonparametric test that is used for matched, ordinal or quantitative data, that do not show normal distribution.

To test for subgroup differences between core biopsy tumor characteristics in relation to the change in cyclin D1 or p27 expression, the linear by-linear association, a test for trends in a larger-than-2x2 table, was used.

For comparison between the normal and cholesterol-rich samples, categorical variables were compared between the grouped samples using Pearson's Chi-square test and ordinal variables were compared between groups with the Mann-Whitney U test.

For the *in vitro* experiments in paper II, changes in cholesterol and lipid droplet content following atorvastatin treatment were evaluated using a two-way ANOVA, comparing the mean differences between groups. The results of the cholesterol levels are expressed as the mean ± standard deviation of three separate experiments and of the lipid droplet content as the geometric mean ± 95% confidence interval of the geometric mean of three separate experiments. Regarding the western blot analysis of the LDLR, results are expressed as the geometric mean ± 95% confidence interval of the geometric mean of three separate experiments. Pairwise comparisons of geometric means were carried out with Student's t-test

Spearman's rho was used as a measure of the correlation between change in cyclin D1 and Ki67, and p27 and Ki67, respectively, in paper I, and between intratumoral cholesterol levels and Ki-67 as well as between the up-regulation of LDLR and Ki-67 in paper II. Spearman's rho is a nonparametric test measuring the strength and the direction of association between two ranked variables.

In papers I and II the analyses have been performed on limited material and are of an exploratory nature, thereby increasing the risk of false positive findings. Different correction methods such as "Bonferroni adjustment for multiple testing" can be used to avoid this, but they increase the risk of false negative findings. We have, to a large extent, performed the analyses we thought to be of interest, though the results should be interpreted with caution and must be validated in further studies.

The software packages Stata version 12.1 (StataCorp LP, College Station, TX, 2012) and IBM SPSS Statistics Version 19, were used for the data analysis in paper I, while IBM SPSS Statistics Version 22, GraphPad Prism 8.3.0, and Stata version 16.0, StataCorp LLC, were used in paper II.

Paper III

Associations between patient- and tumor characteristics with both statin use and HMGCR expression were evaluated and presented both as numbers and as percentages. Distributional differences between the two groups "any statin use" and "never statin use" were assessed with the X2 test or linear regression (X2 test for trend) as appropriate.

The association between statin use, HMGCR expression, and prognosis was evaluated using breast-cancer-specific mortality (BCM) as a clinical endpoint. The associations between both HMGCR expression and time to BCM, as well as statin use and time to BCM, were analyzed by cause-specific Cox regression, a method for survival analysis. The term "regression" refers to a statistical model revealing the association between a dependent variable (outcome) and independent variables (explanatory). Cox regression, also known as proportional hazard regression, is a commonly used approach to regression analysis of survival data, enabling a comparison of survival between two or more groups, and the study of how different predictors affect survival over time.

Yielded effect measures are hazard ratios (HR), with 95% confidence intervals (CIs). The term "hazard" refers to the probability that an individual at any particular time has an event – in this study, BCM. The HR is the ratio of the hazard in the treatment arm/the hazard in the control arm, occurring at a given interval of time. CI is used to measure the precision of the HR and is the range of values that is likely to include the true population.

Follow-up time is defined as the time from baseline until the occurrence of a defined event of interest. When the event of interest is not observed and the follow-up time is incomplete, either due to loss of follow-up or because of death from other causes, these patients are censored. Censored patients contribute to the survival analysis at time points before their censoring time point and are excluded from the analysis thereafter, enabling the use of all available information. Censoring can also occur when the event of interest is not observed during the entire follow-up time. In this study, a patient was censored at the date of death from a cause not related to breast cancer, which is also called a competing event. Thus, the HRs should be interpreted as in a world where only the event of interest exists.

In addition to crude analyses, three multivariate models adjusting for other possibly relevant factors that might affect BCM were fitted stepwise. In an exploratory analysis, the predictive value of HMGCR regarding the association between statin use and BCM was evaluated through analyses stratified by HMGCR expression (HMGCR negative/weak and HMGCR moderate/strong, respectively).

Statistical analyses were performed using SPSS 24.0 (IBM) and Stata version 14.1 (StataCorp LP, College Station, TX, USA).

Paper IV

Power and sample size estimations are used to determine how many patients are needed in a study to answer the research question – whether or not the null hypothesis can be rejected. The study sample size for ABC-SE was calculated by testing the null hypothesis of no difference in CBR versus the alternative hypothesis that the difference in CBR between the control and the study arm is 0.2.

In answering this question, two kinds of errors can occur: type I and type II errors. A type I error is when the null hypothesis is incorrectly rejected, and a type II error is when the null hypothesis is incorrectly accepted. Factors affecting a power calculation are the magnitude of a clinically significant difference, the precision and variance of measurements within any sample, the extent to which type I errors must be avoided, and the type of statistical test used. A major disadvantage of power calculations is the connection to the dichotomous outcome statistical significance, classifying the results as either statistically significant or nonsignificant instead of interpreting the study results in a quantitative way (229).

In the ABC-SE trial, the significance level, α, defined as the probability of rejecting the null hypothesis when it is true, is $\alpha=0.2$ (two-sided). The probability of rejecting the null hypothesis when it is wrong is called the test's power. To reach a power of 0.80 when the true reference proportion of response is at least 0.5, a total of 126 evaluable patients, randomized in a 1:1 ratio, are required to detect a difference of 0.2 between treatment arms, as revealed by the prospective sample size calculation.

The primary endpoint of CBR will be compared in the two groups using a logistic regression model where the absolute differences, odds ratios, and associated 95% CIs and p-values will be reported.

The secondary endpoint of progression-free survival among treatment groups will be analysed in crude analysis using the Kaplan-Meier and Log-Rank test as well as the Cox regression hazards analysis with the latter allowing for confounder-controlled multivariate analysis.

Study design considerations

The MAST trial

Of the 50 included patients, 42 completed all study parts. However, among these, additional tumor tissue was lost due to the bad quality of the pre-treatment core biopsies, which diminished the possibility of performing some analyses. However, this was predominantly due to differences in the performance of core needle biopsies rather than tumoral differences.

In the WOO trial design, a control arm is often not included, though during the work with the MAST trial, a randomized design with a control-arm is believed to have facilitated the interpretation of several analyses, as the natural variance of the endpoints is unknown. Also, outcomes of non-randomised trials depend on the specific characteristics of the patient population included, and there is a risk of selection bias.

The WOO trial design implies a comparison between samples taken at ultrasound-guided breast core biopsy and tissue sampled at surgery. The anaesthetic and surgery can result in significant physiological stress for the patient, and potentially, if the patient is given infusions, might affect host metabolism. Ki67 has been found to vary between samples taken in these different conditions[269], which might also apply to other sensitive markers.

The MDCS

About 40% of the invited women were enrolled in the population-based prospective MDCS[241]. Among the included women, education level was higher and the percentage of foreign-born women was low compared to the women not enrolled, which might limit the generalizability of the trial[243].

Participants in MDCS had a higher breast cancer incidence compared to the general population in the years after inclusion (1991–1996); however, at the same time they had lower breast cancer mortality, which may indicate a higher degree of screening-detected tumors and better health among the participants[243].

The ABC-SE trial

ABC-SE is a randomised trial, which is generally considered a good method to study the effects of treatment. However, bias may arise if the randomization process does not create equal groups, or if there is a large unequal loss of patients during the study. Preferably, trials should be blinded for patients, physicians and researchers but that is not always possible in clinical practice. As discussed in the statistical analysis section, the power of the study is also of importance to detecting a difference between the study arms.

Although gold-standard endpoints of clinical phase III trials are OS or PFS, the goal of phase II trials in the oncological field is often to find agents with sufficient tumoral activity to continue development, and endpoints measuring tumor shrinkage are preferable. Some molecularly targeted agents have, however, shown survival benefit despite very modest tumor shrinkage in phase II trials, which is important to take into consideration. In ABC-SE, OS serves as a secondary endpoint, enabling a possibility of evaluating survival.

Results and discussion

This section presents and discusses the principal results. A more thorough presentation of all results can be found in the original papers. Paper IV constitutes a protocol of a future clinical trial with no results as of now, but will be discussed in terms of future perspectives.

Paper I

The purpose of this study was to investigate potential statin-induced effects on the cell cycle regulators cyclin D1 and p27, and, further, to evaluate the expression of the clinically established biomarkers ER, PR, and HER2, before and after atorvastatin treatment.

Cyclin D1 expression could be annotated for 30 of the 42 paired tumor samples and was assessed for the intensity and fraction of stained nuclei, as well as the intensity of cytoplasmic staining. The nuclear intensity of the protein expression was significantly decreased (P=0.008) following statin treatment but neither the nuclear fraction nor the cytoplasmic intensity changed significantly following treatment.

The expression of p27 was assessable in 33 of the 42 paired tumor samples and was assessed for the intensity and fraction of stained nuclei, as well as the intensity of cytoplasmic staining. Following atorvastatin treatment, there was a significant increase in the fraction of nuclei expressing p27, (P=0.03) but no significant change regarding the nuclear intensity of p27 was found. Further, the cytoplasmic intensity of p27 was significantly increased after atorvastatin treatment (P=0.02). No significant associations were found between the pre-treatment tumor characteristics in relation to the change in either cyclin D1 or p27 following atorvastatin treatment.

These results, indicating a down-regulation of the breast cancer oncogene cyclin D1 and an up-regulation of the tumor suppressor p27, are in line with the reported anti-proliferative effect of statins, from both the MAST trial and other WOO trials [229,231]. Further, in the preclinical field, statins have been shown to inhibit proliferation by inducing cell cycle arrest in different tumor cell lines, including breast cancer cells. In

concordance with the results from our study using atorvastatin, simvastatin has been demonstrated to induce G1 cell cycle arrest through the reduction of CDK 4/6 and cyclin D1[270], while in breast cancer cells BRCA1 overexpression has been shown to sensitize cells to lovastatin treatment through regulation of CDK4/Cyclin D1[214]. Further, statins have been shown to inhibit the proliferation of breast cancer cells by suppressing FPP and GGPP with a subsequent modification and activation of Ras, Rac, and Rho small GTPases[168,271]. Because Rho GTPases are important for p27 degradation, their inactivation can result in the accumulation of p27 and cell cycle arrest[272].

However, the results of this study must be interpreted with consideration given to the small sample size and the fact that the changes of expression were not consistent among nuclear fraction, intensity, and cytoplasmic staining, and, thus, require further validation. At the mRNA level, the expression of *CCND1* and *CDKN1B* between paired pre-and post-treatment samples was compared, but no statistically significant differences between the pre- and post-atorvastatin samples were found, indicating that processes other than gene amplification are responsible for the altered protein expression[13,23].

ER and PR expression could be assessed in 30 tumor pairs, and HER2 in 29 pairs, respectively. None of these three markers were significantly altered following atorvastatin treatment, which was also the hypothesis regarding these three stable clinical markers. However, mechanistic links between the hormone receptors, HER2 and the mevalonate pathway have been reported which motivated these analyses. As mentioned in the introduction section, the cholesterol metabolite 27HC has been found to act as an endogenous SERM and to promote the growth of ER-positive tumors[184,185]. The cholesterol-rich lipid rafts within the plasma membrane are required for HER2 activation and signal transduction[273], and theoretically, changes in cholesterol content can affect both hormone receptors and HER2 signaling, though no statistically significant differences in the expression of either ER, PR, or HER2 were found in this study after two weeks atorvastatin treatment.

In conclusion, the results from this study indicate a potentially statin-induced upregulated expression of the tumor suppressor p27 and down-regulated expression of the oncogene cyclin D1 in breast cancer, suggesting that cell cycle regulatory effects may be contributing to the anti-proliferative effects of statins, via cyclin D1 and p27.

Paper II

This study aimed to assess potential statin-induced changes in cholesterol levels and the expression of LDLR in patient tumors combined with *in vitro* experiments on breast cancer cells, to gain an enhanced understanding of the role of the mevalonate pathway in cancer cholesterol metabolism.

Total cholesterol content in tumor tissue from the clinical samples from the MAST trial was restricted to 14 paired tumor samples. Before atorvastatin treatment, the total cholesterol levels ranged between 3.31 and 35.15 µg total cholesterol/10 mg tissue, and after atorvastatin treatment, the total cholesterol levels ranged between 4.87 and 46.35 µg total cholesterol/10 mg tissue. The median cholesterol level was 10.49 µg total cholesterol/10 mg tissue prior to atorvastatin treatment and 14.1 µg total cholesterol/10 mg tissue after. The tumor tissue total cholesterol content was increased in 11 out of the 14 paired samples following atorvastatin treatment and decreased in the remaining three cases. No statistically significant differences in the levels of total cholesterol pre- and post-treatment were observed.

LDLR expression could be assessed in 24 paired tumor samples. Following atorvastatin treatment, there was a significant increase in the expression of the LDLR compared to paired pre-treatment tumors (P=0.004). No statistically significant differences between patient and tumor characteristics according to baseline LDLR expression were found. The correlation between the change in the LDLR expression and Ki-67 was analyzed to see which patients up-regulated LDLR, and a suggestive, positive correlation between increased LDLR and post-treatment Ki-67 was found (P=0.005, correlation coefficient 0.57), as was a non-significant positive correlation between the change of the LDLR and the change of Ki-67 (P=0.094, correlation coefficient 0.37).

In humans, intracellular cholesterol homeostasis is strictly regulated, but cancer cells are thought to have evolved mechanisms to bypass the strict homeostatic regulation, which might demonstrate a possibility for intervention. In this study, atorvastatin treatment did not cause a statistically significant alteration of intratumoral cholesterol levels, but an up-regulation of LDLR following atorvastatin treatment was found, which can be interpreted as a sign of a preserved intracellular cholesterol homeostasis in breast cancer cells. In another publication by our group, the ability to induce the expression of mevalonate pathway genes via the normal negative feedback loop as a response to statin treatment was seen to a much larger extent in statin-insensitive breast cancer cells as compared to sensitive[274]. In that study, the ER-positive cell lines were considered insensitive and the ER-negative cell lines sensitive[274], which has also

been reported by others[226]. Since the vast majority of patients included in the MAST trial had ER positive tumors, the upregulation of LDLR could be seen as a sign of insensitivity to statins, especially since the upregulation was higher in the tumors not responding to atorvastatin treatment in terms of proliferation. It would have been of great interest – not only regarding these analyses – if the trial had included more patients with ER-negative breast cancer, or to investigate this in a future trial.

Further, in a prostate cancer xenograft model, it was shown that the combination of simvastatin and ezetimibe treatment yielded no changes in tumor growth, despite promising *in vitro* results. Induction of LDLR mRNA was observed in tumor cells, which was interpreted as a possible mechanism of resistance that prostate tumors use to counteract the therapeutic effects of lowering serum cholesterol[275]. The question of whether the up-regulation of LDLR found in our study should also be interpreted as a mechanism of resistance to statin treatment cannot be answered based solely on our results, but it would be of interest to investigate further, as well as to study the combination of statin treatment with the targeting of the LDLR.

Associations between intratumoral cholesterol levels and patient and tumor characteristics were explored by dividing the 42 post-treatment samples into tertiles of intratumoral cholesterol content. Tumor samples in tertiles 1 and 2 served as the joint cholesterol-low group, whereas tertile 3 was considered the cholesterol-rich group of tumors. According to baseline tumor grade, mitotic index, the expression of ER, PR, HER2, HMGCR, or LDLR, or serum lipid levels, there were no statistically significant differences between cholesterol-rich tumors and cholesterol-low tumors.

In the cholesterol-rich tertile, baseline Ki67 levels were higher compared to the cholesterol-low tumors, which was why the correlation between intratumoral cholesterol and Ki67 was analysed. Between pre-treatment intratumoral cholesterol levels and pre-treatment expression of Ki-67, a non-significant correlation was found (P=0.11, correlation coefficient 0.49). Between post-treatment intratumoral cholesterol levels and post-treatment Ki-67, a positive correlation was observed (P=0.003, correlation coefficient 0.46). Cholesterol metabolism is linked to many mechanisms of cancer progression, including cell proliferation, migration and invasion[276], which supports the finding of an association between intratumoral cholesterol levels and proliferation, and illustrates the interest of inhibiting the mevalonate pathway, even if no difference in intratumoral cholesterol levels was found in this study.

In the *in vitro* experiments, a decreased MCF-7 cell proliferation was found upon atorvastatin treatment in a concentration-dependent manner. In line with patient tumor data, LDLR expression, examined by western blot, appeared higher in the

atorvastatin-treated MCF-7 cells compared to controls, though without reaching statistical significance. No significant changes in the total cholesterol levels were found in MCF-7 cells treated with atorvastatin. However, a concentration- and time-dependent increase in the abundance of intracellular LDs was observed in MCF-7 cells exposed to 5 or 10 μM atorvastatin for 24, 48 or 72 h, and compared to control. Attempts were made to assess LD density on cryosections of patient tumors, but were not achievable. LDs are formed in order to avoid lipotoxicity due to an excess of lipids in the cytoplasm and cancer cells accumulate a larger number of LDs in their cytoplasm as compared to normal cells[277]. The increasing number of LDs in the MCF-7 cells following atorvastatin treatment in this study could be due to the simultaneous decrease in proliferation, as well as the uptake of LDL via the up-regulation of LDLR, causing the cells to store lipids in LDs rather than use them for membrane synthesis. It can, however, also be a general stress response of the cells[278]. The role of LDs in cancer is not fully understood, but higher levels of LDs have been associated with higher tumor aggressiveness[279]. How the formation of LDs will later affect the cells cannot be determined from this study.

In conclusion, the results of this window-of-opportunity trial showed no change in intratumoral cholesterol levels following atorvastatin treatment, though an up-regulation of LDLR, in breast cancers with relatively high proliferation, was found, indicating that LDLR might play a role as a negative regulator in the statin-induced anti-tumoral effects.

One of the main objections to the use of lipophilic statins as anticancer drugs is their low bioavailability, and the difficulty involved in increasing the dose due to overall toxicity, which has led to the question of whether observed *in vitro* effects will not be possible to reproduce *in vivo*. The results from papers I and II, as well as from other publications from statin WOO trials[229,231,251,270], indicate that statin treatment is potent enough to induce tumoral effects in humans, even when given within the limited time-frame of two weeks. However, improving the tumoral effect would be of great importance. Interestingly, with the aim of providing a more efficient method of statin delivery in a dose high enough to effectively inhibit cancer progression without causing harmful side effects, studies of nanocarriers of statins are ongoing and have shown promising results *in vitro*. For example, simvastatin encapsulated in nanostructured lipid carriers has been shown to be effective against MCF-7 cells[280], and rosuvastatin and atorvastatin encapsulated in biodegradable polymeric micelles were cytotoxic for MCF-7 cells[281], though more research is necessary to find the most suitable method of drug loading and to determine the dose of statins delivered via a nanocarrier that is effective at inhibiting tumor growth[282].

Paper III

This study aimed to investigate the association between statin use, HMGCR expression, and breast cancer prognosis evaluated in the MDCS. Among the 657 samples available for assessment of HMGCR expression, 119 (18%) showed no expression, 354 (54%) showed weak expression, 169 (26%) showed moderate expression, and 15 (2%) showed strong expression.

The 657 patients evaluated for HMGCR were divided into three groups (HMGCR negative, HMGCR weak, and HMGCR moderate/strong expression) and were associated with patient and tumor characteristics. The mean age at diagnosis was higher in patients with HMGCR moderate/strong tumors than in patients with HMGCR low or negative tumors. HMGCR moderate/strong tumors were associated with tumors with higher histological grade, high Ki67, and ER-negative tumors ($P<0.01$, $P<0.01$, and $P<0.01$, respectively).

The association of high HMGCR expression with more aggressive tumor characteristics – such as ER negativity, tumor grade III and high expression of Ki67 – found in this study is in contrast to previous studies, in which HMGCR expression in breast cancer cells rather has been associated with favorable tumor characteristics[190,191,283]. In the previous studies, polyclonal antibodies were used, in contrast to the novel monoclonal antibody validated and used herein. The antibody AMAb90619 was chosen based on validation with detection of a band at the expected molecular weight of about 100kDa by western blot, significantly reduced HMGCR mRNA levels in MCF-7 cells following down-regulation of HMGCR with siRNA transfection, and significantly up-regulated HMGCR mRNA levels in MCF-7 cells following statin treatment. AMAb90619 was further tested on a TMA containing a small collection of breast cancer tissue and cell lines showing heterogenous HMGCR expression. The use of this new antibody, in addition to tumor heterogeneity, may explain the divergent results. In line with our results, high HMGCR mRNA expression has been found to be correlated with poor breast cancer prognosis[192,274], and HMGCR has also been found to exhibit tumor-promoting effects in other cancer forms[284,285].

Whether differences in HMGCR expression affect tumors' response to statin treatment cannot be determined from this study, but finding a predictive marker of statin therapy is a key issue in the potential introduction of statins as a breast cancer drug. HMGCR is the primary considered candidate and has been investigated as a potential predictive marker for statin treatment in other studies. In the first publication from the MAST trial, a decrease in tumor proliferation was found in

HMGCR-positive tumors, indicating a predictive role[231]. Other biomarkers of the mevalonate pathway, as well as other molecular targets, have been associated with response to statin treatment *in vitro* and in animal models, but further research is required.

The possible prognostic role of HMGCR expression in breast cancer was evaluated for the entire study population with valid HMGCR expression (n=657) and showed no evidence of associations. No statistical significant difference was seen when comparing patients with HMGCR-negative tumors and patients expressing HMGCR moderately/strongly, or when the analyses were restricted to ER-positive breast cancer.

From the study population of 910 patients with invasive breast cancer, 312 patients had been prescribed a statin during the years 2005 through 2014. A total of 74 of these patients were prescribed statins before (pre-diagnostic statin) and 238 women after (post-diagnostic statin) the breast cancer diagnosis. 598 women had never been prescribed a statin. The 910 patients were divided into four groups (pre-diagnostic statin, post-diagnostic statin, any statin, and never statin user) displaying similar distribution regarding body mass index (BMI) at baseline, tumor size, lymph node, and ER status. In the pre-diagnostic statin group, however, proportionally more patients were diagnosed with grade III tumors, high Ki67, and higher HMGCR expression compared to the post-diagnostic and never statin groups. The patients in any statin group had higher BMI at baseline, and their tumors were more often PR-positive ($P<0.01$ and $P=0.01$, respectively) in comparison to patients who never received statins.

Analyses of associations between statin use and BCM were restricted to patients diagnosed with breast cancer from January 1, 2006 onwards, due to the start of the Swedish Prescribed Drug Register in July 2005. No associations between statin use and BCM in any of the multivariate models, adjusted for age, tumor characteristics and adjuvant treatment were found. Statin use was neither found to be associated with BCM in the exploratory analyses, stratified for HMGCR expression, either in patients with negative/weak HMGCR expression, or in patients with moderate/strong HMGCR expression. Neither did the stratified analyses for ER status demonstrate any difference in the protective effects of statins. In the group of patients with negative/weak HMGCR expression, compared to patients with moderate/strong HMGCR expression, patients treated with statins had a lower BCM, but this was not statistically significant.

In the epidemiological field, statin use has been associated with decreased breast cancer recurrence as well as mortality in several large prospective studies and meta-

analyses[203-206]. In this paper, we were unable to confirm an association between statin use and BCM. This may be explained by insufficient power of this study, due to limited number of patients, short follow-up time, and lack of recurrence data. However, epidemiological data has not been entirely consistent, a large nation-wide study from Scotland found no evidence of any associations between post-diagnostic statin use and cancer-specific mortality[212]. Further, the results from observational studies have, to some extent, also been questioned of being subject to biases, such as healthy-user bias, selection bias due to the inclusion of prevalent statin users at the start of follow-up, and immortal-time bias. Together, the current knowledge highlights the need for prospective, randomized clinical trials to further investigate statins in the breast cancer setting[286].

In conclusion, tumor expression of HMGCR was evaluated in the MDCS breast cancer cohort. Neither HMGCR expression nor statin use was found to be associated with BCM. HMGCR expression was found to be associated with unfavorable tumor characteristics, such as high tumor grade, ER negativity, and high Ki67. These suggested associations require further testing in larger cohorts.

Paper IV and future perspectives

Despite major improvements in the treatment of breast cancer, the incidence is increasing and breast cancer is one of the major causes of death among women worldwide[1]. Further improvement of both prevention and treatment of breast cancer is needed. Rather than concentrating only on the development of new therapies, the importance of drug-repurposing has been highlighted, and given the pleiotropic effects of statins, their known safety profile, and low cost, statins have been investigated as a breast cancer drug.

Results from both epidemiological studies, preclinical experiments, and translational results obtained from WOO trials, imply a role of statins in breast cancer, though the exact mechanisms and the magnitude of effect are yet not fully elucidated.

Due to the heterogeneous nature of breast cancer, some of the challenges are to find a reliable predictive statin treatment marker and to determine which patients could benefit from statin treatment. Previous publications have shown divergence in the subtypes of breast cancer and in the treatment settings in which statin treatment has the best effect. Some *in vitro* studies have shown that ER-negative cell lines are more sensitive to statin treatment, suggesting a better effect for non-luminal breast cancer. The fact that statin use has been associated with improved recurrence-free survival

and mortality rather than a decreased incidence in epidemiological studies, together with their shown inhibition of EMT, render statins as potential agents in the adjuvant setting, preventing metastases. Statins have also shown synergistic effects with other cancer therapies[282], why they appear as promising candidates for combined therapies. Regarding breast cancer, the combination of statins with adjuvant endocrine treatment has shown a favorable impact on breast cancer recurrence and mortality[206,207]. These findings, together with the knowledge of cholesterol metabolite 27HC, which may serve as a mechanistic link between ER positive breast cancer and statins, led to the design of the ABC-SE trial, a randomised phase II trial in which the addition of atorvastatin to endocrine-based treatment, as the first-line treatment of advanced breast cancer, is investigated.

ABC-SE is performed to test the hypothesis that the addition of atorvastatin to treatment will enhance the efficacy of the endocrine based treatment in patients with advanced breast cancer. Concordantly, the trial will include translational studies based on the biological samples from the trial to improve the understanding of the specificity of actions of atorvastatin, to explore the potential predictive role of HMGCR tumor expression for atorvastatin efficacy, and to elucidate mechanisms of resistance to endocrine based treatment.

We hope that the ABC-SE trial will result in further understanding of the potential role of statins in breast cancer treatment. More randomised trials, conducted in different breast cancer subtypes and treatment settings, are also warranted to elucidate the potential beneficial effects of statins in breast cancer.

Conclusions

Following two weeks of treatment with atorvastatin, the results from the breast cancer window-of-opportunity trial show:

- An up-regulated expression of the tumor suppressor p27 and down-regulated expression of the oncogene cyclin D1, indicating that the suggested statin-induced antiproliferative effects might occur via these cell cycle regulators.
- No change in the expression of the clinical markers ER, PR, and HER2 was found.
- An up-regulation of LDLR, particularly in tumors with relatively high proliferation, as well as preserved intratumoral cholesterol levels, indicating that LDLR might play a role as a negative regulator in the statin-induced inhibition of breast cancer. The clinical results were supported by *in vitro* experiments and contribute to the elucidation of the anti-tumoral effects of statins.

Results from the population based MDCS show:

- High HMGCR tumor expression, as assessed with a novel monoclonal antibody, was associated with unfavorable tumor characteristics.
- No associations between HMGCR expression and BCM, or between statin use and BCM, were found.

A randomized phase II trial will be initiated to:

- Test the hypothesis that the addition of atorvastatin to endocrine-based treatment will enhance efficacy in patients with advanced ER-positive breast cancer.
- Improve the understanding of the actions of atorvastatin in advanced ER-positive breast cancer.
- Elucidate mechanisms of endocrine resistance to endocrine-based treatment alone or in combination with atorvastatin.

Populärvetenskaplig sammanfattning

Bröstcancer är den vanligaste cancerformen bland kvinnor och man beräknar att ca var nionde kvinna i Sverige kommer att drabbas av sjukdomen under sin livstid. Andelen kvinnor som insjuknar i bröstcancer ökar över hela världen, men å andra sidan har nya behandlingsmetoder och en tidigare upptäckt av sjukdomen med hjälp av mammografiscreening gjort att fler kvinnor kan botas i dag.

Behandling av bröstcancer varierar beroende på tumörens egenskaper men utgörs ofta av en kombination av behandlingsalternativ; operation, strålbehandling, cytostatika, målinriktade och/eller antihormonella läkemedel, för att försöka angripa cancercellerna på olika sätt. Dock är det inte ovanligt att cancercellerna hittar vägar förbi dessa behandlingar, och antingen redan har, eller utvecklar behandlingsresistens efter en tids behandling. Behandlingsresistens är ett betydande problem inom bröstcancerbehandling, och behovet av nya mediciner mot bröstcancer är stort. Att ta fram nya läkemedel mot cancer är ofta en lång och mycket dyr process, varför man på senare år har börjat undersöka om läkemedel som redan finns på marknaden och används för andra tillstånd kan vara till nytta även mot cancer.

Statiner är en grupp läkemedel som sänker kolesterolvärdet i blodet, och som normalt används för att förebygga insjuknande i hjärt- och kärlsjukdomar, såsom exempelvis hjärtinfarkt eller stroke. Statiner intas i tablettform, används världen över, är billiga, och har biverkningar som för de allra flesta är tolerabla. Statiner verkar genom att hämma enzymet HMGCR, som katalyserar kroppens egen bildning av kolesterol. På senare år har man dock upptäckt att statiner har fler, så kallade ”pleiotropa” effekter, och har visat sig kunna påverka även andra sjukdomstillstånd, däribland bröstcancer. Epidemiologiska studier har visat att människor som tar statiner regelbundet har minskad risk att få återfall, eller dö av sin bröstcancer, och laboratorieexperiment gjorda på cancerceller har visat att statiner även kan hämma cancercellernas förmåga att dela sig och låta tumören tillväxa. De exakta mekanismerna bakom de här effekterna är dock ej helt klarlagda.

Det övergripande målet med den här avhandlingen är att undersöka hur statiner kan påverka bröstcancer. Arbete I och II baseras på en klinisk studie som har genomförts vid Skånes Universitetssjukhus i Lund, med syfte att undersöka statiners påverkan på

bröstcancer vidare. Studien heter MAmmary cancer and STatins (MAST-studien) och är en klinisk fas II-studie, av ”window-of-opportunity” typ, där 50 kvinnor inkluderades i samband med att de fick sin bröstcancerdiagnos. Den första delen av en bröstcancerbehandling är ofta operation, och veckorna mellan diagnos och operation brukar normalt sett ingen behandling ges, men i MAST-studien har kvinnorna fått statinbehandling i form av 80 mg atorvastatin dagligen i 2 veckor under den här perioden. Tumörvävnad samt blodprover tagna före och efter statinbehandlingen har jämförts för att få en bättre bild av statinernas effekt på bröstcancer.

I arbete I studeras om behandling med atorvastatin påverkar regleringen av celldelningen, genom att uttrycket av två stycken cellcykelmarkörer, cyclin D1 och p27, analyseras i tumörvävnaden före och efter atorvastatinbehandlingen. Analyserna är gjorda med hjälp av immunhistokemi, som är en metod där man kan påvisa olika cell- och vävnadskomponenter i mikroskopiska preparat med hjälp av antikroppar. Cellcykeln är en mycket kontrollerad serie av förändringar som alla celler genomgår för att kunna dela sig, vilket är nödvändigt för upprätthållandet av friska organs funktion. Hos cancerceller, som kännetecknas av sin snabba tillväxt och delningsförmåga, är kontrollsystemet för cellcykeln ofta satt ur spel, genom att några av de proteiner som reglererar cellcykeln kan vara för högt eller lågt uttryckta. Cyclin D1 är ett protein som ökar celldelningen och som ofta är överuttryckt i bröstcancerceller. p27 är istället ett protein som normalt hämmar cellcykeln och som ofta är underuttryckt i bröstcancerceller. I arbete I sågs en minskning av cyclin D1 och ökning av p27 efter statinbehandlingen, vilket stämmer överens med tidigare studiers resultat, som visat att statiner medför minskad celldelning. Resultaten talar för att den minskade celldelning som statiner kan orsaka sker via cyclin D1 och p27, men detta behöver kontrolleras om i fler studier.

I arbete II undersöktes om statinbehandlingen påverkar tumörernas kolesterolnivåer, genom att analysera kolesterolnivåerna i tumörvävnaden före och efter statinbehandlingen. Statiner verkar genom att hämma ett enzym i den process som tillverkar kolesterol i kroppen, men i vår studie var kolesterolnivåerna i tumörvävnaden inte statistiskt signifikant förändrade efter statinbehandlingen. I studien analyserades även uttrycket av en receptor som kan öka cellers upptag av kolesterol från blodbanan, LDL-receptorn, med immunhistokemi. Efter atorvastatinbehandlingen sågs en uppreglering av LDL-receptorn i tumörvävnaden. Resultaten från det här arbetet talar för att tumörcellerna klarar att hålla jämna nivåer av kolesterol trots att dess egen produktion är hämmad, genom att uttrycka högre nivåer av LDL-receptorn. För att förklara vilken betydelse dessa resultat har i statinernas effekt på bröstcancer behövs fler studier.

Arbete III baseras på Malmö Kost Cancer-studien, som är en stor, populationsbaserad studie där friska individer i Malmö inkluderades mellan åren 1991 och 1996. Totalt 17,035 kvinnor inkluderades, och av dessa insjuknade 910 kvinnor med bröstcancer till och med år 2010. I arbete III har uttrycket av HMGCR (det enzym som statiner hämmar) analyserats med immunhistokemi i tumörvävnaden hos de kvinnor som diagnostiserats med bröstcancer. Analyser har gjorts för att undersöka om det finns ett samband mellan uttrycket av HMGCR, statinanvändning och antalet dödsfall i bröstcancer. Resultaten visar att det finns ett samband mellan ett högt uttryck av HMGCR och dåliga tumöregenskaper, som till exempel tumörer med högre celldelning. Inget samband mellan HMGCR-uttryck och dödlighet i bröstcancer, eller mellan statinanvändning och dödlighet i bröstcancer kunde påvisas.

Arbete IV är en deskriptiv publikation av en studie som planerar att genomföras på Skånes Universitetssjukhus i Lund. Studien heter "Advanced Breast Cancer Statins and Endocrine treatment" (ABC-SE) och är en fas II-studie där kvinnor som diagnostiserats med avancerad bröstcancer, och som är aktuella för behandling med antihormon-baserad behandling kan inkluderas. Avancerad bröstcancer är bröstcancer som inte anses botbar och innefattar bröstcancer som inte kan opereras på grund av sin omfattning, eller som har spridit sig till andra organ. I studien kan patienterna randomiseras till den konventionella antihormon-baserade behandlingen eller till antihormon-baserad behandling som kombineras med statinbehandling.

Dessa fyra arbeten har gett några nya inblickar i statinernas påverkan på bröstcancer. Förhoppningen är att ABC-SE-studien ytterligare kommer att bidra till en fördjupad kunskap om statinernas verkningsmekanismer, och ge svar på om statiner som tillägg till antihormon-baserad behandling ger ett förbättrat svar på behandlingen. Dessa resultat kan, tillsammans med andra publikationer, ligga till grund för att kunna genomföra stora, randomiserade fas III-studier som behövs för att definitivt klargöra huruvida statiner har en roll i bröstcancerbehandling.

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