The Akt/mTOR Pathway and Estrogen Receptor Phosphorylations

– a crosstalk with potential to predict tamoxifen
resistance in breast cancer

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Linköping 2013

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ISBN 978-91-7519-515-5

ISSN 0345-0082

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Printed by LiU tryck, Linköping, Sweden, 2013

Till farmor Nancy

Det droppade en droppe i livets älv
En ensam droppe kan inte flyta själv
Det ställs ett krav på varenda droppe
Att hjälpa till och hålla de andra oppe

-Tage Danielsson-

A dripping drop on a rocking boat
A single drop cannot make it float
A claim; how all drops should behave
Stay close, to keep the boat on the wave

-Freely translated-

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Abstract

Estrogen receptor α content is the primary breast cancer biomarker distinguishing the patients responsive from the non-responsive to endocrine treatments. Tamoxifen is an estrogen competitor with large potential to treat breast cancer patients and prolongs time to recurrence. Despite the estrogen receptor positivity and tamoxifen treatment, many women face recurrence of the disease. An important mechanism of resistance to endocrine treatments is upregulated growth factor signaling, and the subsequent effect on the estrogen receptor, rendering an active receptor that stimulates cell proliferation or reduced estrogen-receptor dependence.

This thesis concerns the investigation of biomarkers, as a complement to the existing markers, for determining optimal treatment for patients with primary invasive breast cancer. Randomized patient tumor materials were used in order to measure variations in gene copies, proteins, and protein phosphorylations and to further relate these variations to time-to-recurrence. Endocrine untreated groups within the patient tumor sets gave us the opportunity to study the prognostic potential of selected markers and to compare tamoxifen-treated patients with endocrine untreated, thus obtaining a treatment-predictive value of each marker or marker combination.

In endocrine-dependent cancer the 11q13 chromosomal region is frequently amplified, harboring the genes encoding the cell cycle stimulator cyclin D1 and the estrogen receptor phosphorylating kinase Pak1, respectively. Amplification of the genes was associated with reduced time-to-recurrence, indicating a prognostic value, whereas *PAK1* gene amplification predicted reduced response to tamoxifen treatment. Moreover, the protein expression of Pak1 tended to predict treatment response, which led to the investigation of this protein in a larger cohort. Together with one of its targets, the estrogen receptor phosphorylation at serine 305, Pak1 predicted reduced response to tamoxifen treatment when detected in the nucleus of tumor cells, suggesting activation of this pathway as a mechanism for tamoxifen-treatment resistance. The estrogen receptor is phosphorylated by several growth factor stimulated kinases. The role of serine-167 phosphorylation has been debated, with inconsistent results. To study the biomarker value of this site the upstream activity of Akt, mTOR, and the S6 kinases were analyzed individually and in combinations. As a prognostic factor, serine 167 indicated an improved breast cancer survival, and as a treatment predictive factor we could not detect a significant value of serine 167 as a single marker. However, in combination with serine 305, and Akt/mTOR-pathway activation, the response to tamoxifen treatment was reduced. The mTOR effector protein S6K1 was found to be associated with HER2 positivity and a worse prognosis. In the group of patients with S6K1 accumulation in the tumor cell nuclei, treatment did not prolong time-to-recurrence, similarly as observed with expression of active S6 kinases. *In vitro*, a simultaneous

knockdown of the S6 kinases in estrogen receptor-positive breast cancer cells resulted in G1 arrest, and tamoxifen-induced G1 arrest was in part S6 kinase dependent.

The results presented herein suggest biomarkers that would improve treatment decisions in the clinic, specifically for estrogen receptor-positive breast cancer and tamoxifen treatment but in a broader perspective, also for other endocrine treatments and targeted treatments.

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. **Bostner J***, Ahnström Waltersson M*, Fornander T, Skoog L, Nordenskjöld B, Stål O. Amplification of *CCND1* and *PAK1* as predictors of recurrence and tamoxifen resistance in postmenopausal breast cancer. *Oncogene*. 2007 Oct 25;26(49):6997-7005
- II. **Bostner J**, Skoog L, Fornander T, Nordenskjöld B, Stål O. Estrogen receptor- α phosphorylation at serine 305, nuclear p21-activated kinase 1 expression, and response to tamoxifen in postmenopausal breast cancer. *Clinical Cancer Research*. 2010 Mar 1;16(5):1624-33
- III. **Bostner J**, Karlsson E, Pandiyan MJ, Westman H, Skoog L, Fornander T, Nordenskjöld B, Stål O. Activation of Akt, mTOR, and the estrogen receptor as a signature to predict tamoxifen treatment benefit. *Breast Cancer Research and Treatment*. 2013 Jan; 137(2):397-406
- IV. **Bostner J**, Karlsson E, Bivik C, Perez-Tenorio G, Franzén H, Konstantinell A, Fornander T, Stål O. S6 kinase signaling and tamoxifen response in breast cancer cells and in two randomized breast cancer cohorts. Manuscript

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Populärvetenskaplig sammanfattning

Kombinationer av biomarkörer med potential att vägleda bröstcancerbehandling

Bröstcancer är den vanligaste cancerformen som drabbar kvinnor. Över 8000 svenskar insjuknar varje år. Idag finns det några få tumörmarkörer för att avgöra vilken behandling en patient ska få och hur prognosen ser ut. Det finns också ett urval av läkemedel som hjälper många patienter att bli friska från sin sjukdom. Likväl återinsjuknar en stor andel av patienterna trots kirurgi och mediciner. Den här avhandlingen syftar till att hitta och validera markörer i tumörerna som kan vägleda i val av läkemedel. De flesta brösttumörer växer med hjälp av kroppens egenproducerade hormon östrogen. När östrogen binder till sin mottagare (receptorn) i cancercellen stimuleras cellen till att växa och överleva. En vanlig behandling mot östrogenberoende bröstcancer är tamoxifen, som motverkar östrogenets effekter på cellen genom att konkurrera ut östrogen vid inbindning till receptorn. När tumören växer trots behandling har resistens uppstått. Orsaken kan vara förändringar i proteiner, som är viktiga delar av cellens kommunikationssystem. Vissa patienter har redan vid diagnos en förändrad signalering i tumörcellerna som gör att de inte kommer att svara bra på behandling, medan andra utvecklar förändringar som reaktion på behandling. I den här avhandlingen har fokus främst legat på att upptäcka förändrade proteiner i primära brösttumörer. Vi har jämfört nivåer och aktivering av proteiner, samt ökat genantal för vissa gener, med tid till ett eventuellt återfall i patientgrupper från kontrollerade studier. Vi har kunnat påvisa att förändringar i specifika delar av arvsmassan och av vissa proteiner påverkar behandlingssvaret. Högt uttryck av några markörer har även visat sig påverka patienternas prognos till det sämre, vilket tyder på att de är drivande för tumörutvecklingen.

Vi har studerat ökning av *CCND1*- och *PAK1*-generna, som ligger i en del av arvsmassan som är förändrad i ungefär 15 % av alla brösttumörer. Tillsammans visade ökning av dessa gener en sämre prognos, och *PAK1* predikterade sämre svar på tamoxifenbehandling. Även förhöjt proteinuttryck av Pak1 pekade åt sämre behandlingsnytta, vilket ledde till att vi undersökte Pak1s proteinuttryck i en större patientgrupp. Pak1 är ett protein som kan förändra formen på östrogenreceptorn i samma del som östrogen och tamoxifen binder in. Receptorn bli då aktiv utan att behöva stimulans från östrogen, vilket gör behandling med konkurrenten tamoxifen verkningslös. Ett förhöjt proteinuttryck av Pak1 i tumörcellens kärna, tillsammans med förändrad östrogenreceptor, förutspådde sämre behandlingssvar än om tumören bara uttryckte en eller ingen av markörerna. I den här studien indikerade resultaten även att förhöjt Pak1-uttryck utanför cellkärnan gav en sämre prognos, vilket skulle kunna förklaras med Pak1s koppling till cellens rörelse och tumörspridning.

Tillväxtfaktorer skapar signalkaskader inne i celler och kan vara en orsak till att tumörceller inte svarar på tamoxifenbehandling. En effekt av dessa signaler är strukturförändring i östrogenreceptorns olika delar. Resultatet kan bli en försämring av behandlingssvaret eftersom en förändring kan göra receptorn mer aktiv. Vi undersökte aktiviteten av proteinerna Akt och mTOR tillsammans med förändringar av östrogenreceptorn, och fann att när minst två av de tre markörerna var förhöjda fick patienterna oftare tillbaka cancer trots behandling. Intressant nog visade en förändring av östrogenreceptorn, på den delen som östrogen inte binder in till, en bättre prognos. Detta skulle kunna förklaras med att dessa tumörer har skapat ett beroende av östrogen genom att stabilt uttrycka östrogenreceptorn och tumörerna antar då en mindre aggressiv karaktär.

För att undersöka Akt/mTOR-signalkaskaden ytterligare tittade vi vidare på två proteiner som aktiveras av mTOR; S6K1 och S6K2 samt aktiviteten av dessa två. Ett sätt att undersöka om proteiner är inblandade i tumörtillväxt och behandlingssvar är att titta på de olika faserna som celler genomgår när de delar sig för att bli fler. Om cellerna stannar längre tid i startfasen tyder det på att de delar sig långsammare. När vi tog bort både S6K1 och S6K2 från cellerna i ett experiment med odlade tumörceller stannade fler celler i startfasen, vilket visar att de två proteinerna är involverade i celledelningsprocessen. Ett ackumulerat uttryck av S6K1 i cellkärnorna, eller en ökad enzymatisk aktivitet av S6K1 och S6K2 påvisade att tamoxifenbehandlingen inte gav någon effekt på återfallsfrekvensen. Istället gick det bättre för både obehandlade och behandlade patienter i den här gruppen. Detta tyder på att patienter skulle kunna besparas biverkningar av en behandling som inte fungerar optimalt och att en annan behandling kan vara intressant för den här gruppen av patienter. Däremot visade resultaten att höga nivåer av S6K1 var förenat med en sämre prognos för de som inte fått tamoxifen.

Sammanfattningsvis visar avhandlingen att det finns markörer i de undersökta signalkaskaderna som till viss del kan förutsäga svar på behandling och prognos. En ensam markör är inte viktig för tillräckligt många patienter, i de undersökta grupperna, att den själv kan avgöra om tamoxifen är en optimal behandling eller inte. Tillsammans, när flera komponenter i en signalkaskad undersöks, kan markörerna däremot bli värdefulla. Pak1-relaterad aktivering av östrogenreceptorn samt Akt/mTOR/S6K-kaskaden är två signalvägar som samtalar med varandra och har potential att vägleda behandling. De består också av proteiner som skulle kunna hämmas med målriktade läkemedel.

Abbreviations

AF	activating function
AI	aromatase inhibitor
Akt	v-akt murine thymoma viral oncogene (PKB)
BSA	bovine serum albumin
CCD	charge-coupled device
<i>CCND1</i>	the cyclin D1 gene
CDK	cyclin dependent kinase
CYP2D6	cytochrome P450 2D6
DAB	3, 3'-diaminobenzidine
DBD	DNA binding domain
DEPTOR	DEP-domain-containing mTOR interacting protein
DNA	deoxyribonucleic acid
estrogen	17 β -estradiol (E2)
eIF3B	eukaryotic initiation factor 3B
eIF4E	eukaryotic initiation factor 4E
EGF	epidermal growth factor
ER	estrogen receptor (ER α , unless otherwise specified)
ERK 1/2	extracellular signal-regulated kinase 1/2 (p42/44 MAPK)
FKBP12	FK506-binding protein 12
FRB	FKBP12-rapamycin-binding site
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HER2	human epidermal growth factor receptor 2 (ErbB2/neu)
HGF	hepatocyte growth factor
HR	hazard ratio
HRG	heregulin β 1
HRP	horse-radish peroxidase
IGF1	insulin-like growth factor-1
IGF-1R	insulin-like growth factor-1 receptor
IHC	immunohistochemistry
IRS-1	insulin receptor substrate-1
LBD	ligand binding domain
LC8	dynein light chain 1
LST8	sec13 protein 8
MAPK	mitogen-activated protein kinase
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NHG	Nottingham histological grade
NPI	Nottingham prognostic index
<i>PAK1</i>	the Pak1 gene
Pak1	p21-activated kinase 1
PDK1	phosphoinositide-dependent protein kinase-1
PgR	progesterone receptor (PR)

PH	plextrin-homology
PHLPP	pleckstrin homology domain leucine-rich repeat protein phosphatase
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PIK3CA	PI3K catalytic subunit p110 α
PIP ₂	phosphatidyl inositol -4, 5- bisphosphate
PIP ₃	phosphatidyl inositol -3, 4, 5- trisphosphate
PKA	protein kinase A
PKB	protein kinase B (Akt)
PKC	protein kinase C
PRAS40	proline-rich Akt substrate 40
Protor	protein observed with rictor 1
PTEN	phosphatase and tensin homolog
PTM	posttranslational modification
PVDF	polyvinylidene difluoride
qPCR	quantitative polymerase chain reaction
RANKL	receptor activator of nuclear factor kappa-B ligand
Raptor	regulatory associated protein of mTOR
Rb	retinoblastoma protein
RFS	recurrence-free survival
Rheb	ras homolog enriched in brain
Rictor	rapamycin-insensitive companion of mTOR
Risc	RNA-induced silencing complex
RPS6K	ribosomal protein S6 kinase
RSK	p90 ribosomal S6 kinase
S6K	S6 kinase
Sin1	stress-activated protein kinase-interacting protein 1
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
TMA	tissue micro array
TSC1	tuberous sclerosis complex 1 (hamartin)
TSC2	tuberous sclerosis complex 2 (tuberin)
VEGF	vascular endothelial growth factor

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Introduction

Clinically established biological markers for estimation of prognosis and for decision of treatment are scarce in the field of breast cancer. The few markers used today are valuable for large groups of patients, but blunt and not always exact for each individual tumor as many patients do not initially respond or eventually develop resistance towards the treatments. This thesis focuses on intracellular signals within the phosphoinositide 3-kinase /v-akt murine thymoma viral oncogene/mammalian target of rapamycin (PI3K/Akt/mTOR) cascade, estrogen receptor α (ER) activation, and the convergence of these two pathways. Key proteins and genes within these pathways have been examined with regard to adjuvant tamoxifen treatment and their prognostic value. Development of reliable clinical markers requires thorough control in large cohorts of randomized patients, validation, and the use of methods applicable in routine clinical practice. We have used three different cohorts of tamoxifen randomized postmenopausal breast cancer patients. Antibodies for quantitative and location-specific detection of proteins and phosphorylated proteins have been validated. Real-time quantitative PCR and immunohistochemistry (IHC), two methods already implemented in the clinical setting, were used in the present studies. In addition, antibody validation and tamoxifen response analysis were conducted in experimental breast cancer cells.

Cancer

The complexity of cancer development, sustention, and spread in the body is far from unraveled. Growth and death turnover of normal cells are strictly regulated, and cells developing into tumors become independent of the normal restrictions in the body, and must go through several changes to overcome these barriers. There are six suggested main barriers that tumors challenge, and two more that are not as well studied, but possibly essential barriers [1]. These are: growth signal restriction, growth suppressor mechanisms, programmed cell death, chromosome shortening, restricted nutrient and oxygen supply in a fast growing cell mass, and contact inhibition. In addition, many tumors change their energy metabolism from oxygen-driven, aerobic, into anaerobe-like metabolism, and finally, cancer cells must find a way to slip through the net of immune defense that normally would destroy aberrant cells.

To determine the prognosis, expected course of disease, of a cancer patient, variables as tumor size, invasion, histological subtype and grade, and protein expression of known prognostic biomarkers are helpful. To validate a biomarker as truly prognostic the only option is to use an untreated cohort, in order to follow up the natural course of the disease without any interfering treatments. This is not an ethical approach, so retrospective cohorts of partly untreated patients are used to evaluate biomarkers previously shown promising in experimental systems.

Breast cancer

Cancer of the breast is the second most common cause of death for women worldwide, following heart disease. It is the most frequent cancer affecting women, with the estimated incidence of 1.3 million cases in 2008. In Sweden, the incidence reached record numbers in 2011 with 8 382 women and 45 men [2]. Most breast tumors originate from the epithelia of the ducts, called ductal carcinoma (75%), or from the epithelia of the lobules, called lobular carcinoma (5-15%) [3]. Other invasive histological subtypes are mucinous, apocrine, medullary, inflammatory, and tubular carcinomas.

Risk factors and prevention

Heredity is a strong risk factor for developing breast cancer. Patients with familial breast cancer account for about 20% of all breast cancer cases. These patients display a younger age, a lower rate of ER positivity, and have a worse prognosis compared with sporadic breast malignancy [4]. Another major risk factor for developing breast cancer is the cumulative dose of hormone over time, with estrogen being the most important hormone [5]. An early menarche, late menopause, and no pregnancy increase the risk, while the opposite seems protective. Although pregnancy, with the massive hormone-burden, may trigger tumor development, a protective effect by the differentiation of breast epithelia during pregnancy and breastfeeding, especially if carried out at a low age, is an established preventive factor. Detection of dense breast tissue is also a strong risk factor for breast cancer [6]. Although the reason is not fully known, preventive tamoxifen treatment reduced the density and the incidence of tumor development in high-risk groups, indicating a hormonal control of breast density [7]. The mammography screening program, available for women at age 40-74 in Sweden, is a major step in prevention of breast cancer related deaths. By early detection of cellular changes in the breast, invasive disease and metastatic spread can be avoided to a larger extent. Physical activity has been shown to be preventive by lowering the risk of obesity and thereby reducing the total number of ovulatory cycles. After menopause, obesity is a risk factor as the production of estrogen partly localizes to fat tissue after the ovulation has seized [8]. Studies on diet and breast cancer risk summarize that low-fat and high-fiber diet gave some protection, whereas high-energy intake and alcohol increased the risk [9].

Treatment

Surgery is the major treatment for primary breast cancer, either mastectomy or breast conserving surgery. Surgery may be followed by radiotherapy, which is implicated to reduce risk of local recurrence. Chemo, endocrine, and targeted therapies are systemic treatments that decrease the risk of local and distant recurrence [10]. Although a tumor is detected at an early stage, undetected micrometastases are common, which are cancer cells remaining after surgical removal of the tumor. To prevent these cells from growing and forming a

recurring tumor, adjuvant systemic therapy is applied after surgery. Pre-surgical therapies are given for tumor shrinkage, and in so called neoadjuvant studies, which provide opportunities to study the tumor response to a treatment. This approach may be a tool for individual targeted therapy evaluation not only in trials, but also implemented to a larger extent in clinical routine in the future.

Biomarkers for prediction of breast cancer outcome and treatment

The breast cancer subtypes, according to histologic appearance, show some difference in prognostic outcome [3]. Tubular carcinoma seems to be the most favorable, whereas inflammatory carcinoma was found to constitute the worst prognosis. The TNM-classification determines the clinical stage of a breast tumor, based on tumor size (T), nodal involvement (N), and distant metastasis (M). The Nottingham histological grade (NHG) shows the aggressiveness of the tumor and is a prognostic factor included in the Nottingham prognostic index (NPI) scoring system [11]. The NHG includes scoring of tubule formation, nuclear irregularity, and number of mitoses, which are combined to a resulting grade 1-3 scale. Histopathologic biomarkers are used for estimating breast cancer prognosis and as therapeutic guidance. The currently available standard biomarkers are IHC staining of the ER, the progesterone receptor (PgR), Ki67, and also HER2, which is followed by fluorescence in situ hybridization or chromogenic in situ hybridization for confirmation of amplification in tumor cells with circumferential membrane staining [12]. The inaccuracy of the scoring is a problem and is in part a result of intratumor heterogeneity, few observed areas, the subjective observer variation, and method variation sensitive antibodies [13]. According to the IHC-staining pattern, tumors are divided into subtypes (**Figure 1**) [14]. Luminal A classified tumors are ER and/or PgR positive with low cell proliferation, whereas luminal B tumors show high proliferation detected with the Ki67 marker, which is exclusively expressed in proliferating cells. Sometimes, analysis of the S-phase fraction has served as proliferation marker. A small subgroup of luminal B tumors is ER positive and HER2 amplified. The HER2 amplified and the triple negative tumors are often highly proliferative. A confirmation of basal carcinoma can be obtained by IHC staining for a basal marker, such as cytokeratin 5/6. Cutoffs for biomarkers are discussed and changed when new and more convincing studies are presented. The cutoff for ER-positive IHC staining is set at 10% positively stained nuclei. The recent call is that patients having tumors with more than 1% ER-positive nuclei may experience a benefit from tamoxifen treatment [15]. These mentioned subtypes are closely related to the classification of breast tumors by micro-array based gene expression profiles, namely; luminal A, luminal B, HER2-enriched, normal-like, and basal-like tumors [16]. Claudin-low tumors have later become a separate subtype, expressing a stem cell- and mesenchymal-like gene profile [17]. Other gene-based tests, such as Oncotype DX® [18], and MammaPrint® [19], are available for prognostic and treatment prediction, in particular for chemotherapeutic decisions.

HE	Intrinsic subtype	ER	PR	HER2	Ki67
Luminal A

Luminal B

Luminal B HER2 amplified

HER2 amplified

Triple negative

Figure 1 Biomarkers for treatment predictive evaluation of breast tumors. Haematoxylin/eosin (HE) staining visualizes the cells of the specimen. ER, PgR (PR), HER2, and Ki67 are the basic immunohistochemical markers. Together with information on tumor size, grade, nodal status, and menopausal status they form the base for post-operative adjuvant treatment decisions. The picture was kindly provided by Dr. Dorte Grabau.

The breast

The female breast undergoes major changes during childhood, puberty, pregnancy, lactation, and menopause [20]. A mammary gland contains 15-20 lobes and within each lobe there are a series of lobules connected with ducts starting in the alveoli and draining into the nipple (**Figure 2**). Each duct is lined with an inner layer of cuboidal epithelial cells surrounded by an outer layer of myoepithelial cells, which can contract the duct. Stroma, containing ligaments, fibroblasts, lymphocytes, and adipocytes, surround the gland. Blood and lymph vessels infiltrate the stroma. The lobular structures are divided into four stages, 1-4 [21]. Type 1 lobules are the most proliferative, measured by Ki67 staining, and the alveoli are large but few. This structure is the predominant structure in women who have not gone through pregnancy. The differentiation of the gland increases slightly upon each menstrual cycle. During pregnancy the glands drastically change and type 2 and 3 lobules become the dominating structures, with type 4 lobules being lactating glands that regress back to type 3 after the lactating period. After lactation, the type 2 and 3 lobules are still the most common, and this differentiation is thought to be one explanation as to why early pregnancy reduces the risk of breast cancer later in life. After menopause the lobules undergo involution to simpler lobules. Individual variation in lobule type was suggested to predict risk of breast cancer in women previously diagnosed with benign breast disease, with a higher degree of involution being protective, even after adjustment for parity and histologic category [22].

Figure 2 The normal breast is composed of grape-like structures of alveoli, clustering together and forming the lobules, which are attached to the ducts. The ductal epithelial cells are the most common origin of breast cancers. Myoepithelial cells surround the epithelial cells to confer contraction upon lactation. When a mass of epithelial tumor cells breaks through the basal membrane it is defined as an invasive breast carcinoma. The picture was kindly drawn by Dr. Veronika Brodin-Patcha.

Steroid hormone receptors in breast

The estrogen and progesterone are the dominating hormones regulating breast development. The nuclear hormone receptor family member estrogen receptor (ER) α is the target for the ovarian steroid hormone 17 β -estradiol (estrogen). It is essential for normal female physiology, regulating important functions during development, and during the menstrual cycle. In normal breast epithelial tissue, only about 10% of the cells express the receptor, while a complete deletion of the receptor seriously disturbs mammary gland development, showing that the ER signaling controls surrounding cells [23]. Upon estrogen binding, the receptor conformation is altered. It undergoes dimerization and is phosphorylated at several residues. A switch in the balance between corepressors and coactivator protein complexes is observed and the receptor is shuttled to the nucleus where it recognizes and binds specific DNA sequences. When activated, the ER exerts its function as a nuclear transcription factor [24]. The classical mode of transcription involves binding of the ER directly to repeated elements in the DNA sequence, namely estrogen responsive elements (ERE), whereas a non-classical transcription involves other transcription factors, such as AP-1, SP-1, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B). In addition, a non-genomic activation of the ER has been described, where a membrane bound ER induced signaling through growth factor cascades, which activated other transcription factors [25]. More than 400 genes are thought to be regulated by estrogen activation of the ER [26]. Positive proliferation regulators are transcribed, such as genes encoding proteins involved in the cell cycle progression and growth factors. *CCND1*, the gene encoding cyclin D1, and the PgR are two of the most studied ER-regulated genes. Other genes are downregulated by the ER, such as antiproliferative and proapoptotic genes. Together, these genes control growth and differentiation of cells in the reproductive machinery, bone, cardiovascular system, brain, and liver.

Knockdown of ER β , a homolog of the ER α , did not disturb normal mammary gland function [27]. Its role in breast cancer is under debate, but it has generally been suggested to be a good factor [28]. Diverse functions of its isoforms, a possible ER α -inhibiting function, and a metastasis inhibiting role, make it an interesting marker in breast cancer [29].

In addition to the ER, the PgR also stimulates normal breast development [30]. It seems more important for side branching than elongation of the mammary gland, which takes place mostly during pregnancy and lactation. PgR stimulates initiation of breast cancer, possibly through paracrine receptor activator of nuclear factor kappa-B ligand (RANKL) signaling to the NF κ B-induced transcription. However, low PgR expression in invasive breast cancer correlates with a more aggressive disease. Being an ER-regulated gene, its expression indicates ER activity, and low PgR levels reflect high growth factor signaling activity [31]. The tamoxifen treatment predictive role of PgR levels has been debated [32]. Data showing a reduced response when PgR levels are low, has not always been confirmed. In the clinical

setting, a detection of PgR in ER-negative tumors may reflect a false-negative ER test. Hence, these patients could respond to adjuvant endocrine treatment.

Endocrine therapy

The majority of breast tumors is dependent on the ER for proliferation and survival of the tumor cells. Depending on the cutoff, normally set at 1-10% of cells stained positive for ER, 75-90% are classified as ER positive (86% in Sweden 2011) and subjected to adjuvant endocrine therapy that targets the ER pathway. The available endocrine therapies are the selective ER modulator tamoxifen, the estradiol reducing aromatase inhibitors (AI) anastrozole, exemestane, and letrozole, the ER downregulator fulvestrant, the estradiol reducing luteinizing hormone releasing hormone agonists, such as goserelin, and ovariectomy, which reduces the endogenous supply of estrogen [33]. All mentioned therapies directly or indirectly inhibit the activity of ER signaling. The most frequent primary choice of endocrine treatment in the premenopausal setting is tamoxifen, and tamoxifen and/or AIs for postmenopausal breast cancer patients [34]. Before menopause, estrogen is mainly produced in the ovaries. AIs do not affect this estrogen production, only the peripheral, which is mainly localized to adipose tissue, liver, muscle, and adrenal glands. Therefore, it is important to know the menopausal stage before treating with AIs. AIs have been found more effective than tamoxifen on reducing postmenopausal breast cancer recurrence, although not significantly effective on breast cancer mortality [35]. Follow-up data on long-term recurrence and mortality will give further information on the benefits of either treatment.

Tamoxifen

Tamoxifen has been used as an adjuvant breast cancer treatment for over 35 years. The use of tamoxifen reduced the number of surgical hormone reducing oophorectomies. The first studies showing an inhibitory effect on breast tumor growth by tamoxifen were published in the beginning of the 1970's [36,37]. It was first approved by the U.S. Food and Drug Administration for advanced breast cancer in 1977, and during the 1980's its effects were further evaluated and its signaling mechanisms were investigated. By 1990, it was established that only ER-positive tumors responded to treatment with the antiestrogen [38]. Thereafter, studies with an endocrine untreated control group among patients with ER-positive tumors were no longer considered ethical. During the early 1990's, tamoxifen came into standard adjuvant treatment of ER-positive breast cancer [39].

The prodrug tamoxifen, Nolvadex®, is metabolized mainly in the liver, where the enzyme cytochrome P450 2D6 (CYP2D6) is highly involved. It is converted to its more potent form, the 4-hydroxy (4-OH) tamoxifen, which is finally converted to the most active form of the drug metabolites, endoxifen. Tamoxifen can also be metabolized into endoxifen by other CYP enzymes, mainly via N-desmethyl tamoxifen. This route seems to be the most extensive

conversion of tamoxifen into endoxifen [40]. The tamoxifen metabolites are competitive ligands of the ER, targeting the ligand binding site where estrogen also binds. They are so called selective ER modulators, which mean that different tissues respond differently to treatment. Skeletal tissue, the cardiovascular system, and several other tissues respond with an agonistic effect, as with estrogen. In breast and ovarian tissue, tamoxifen has an antagonistic effect on the ER, reducing estrogen-stimulated growth in breast cancer cells by rendering a non-functional nuclear receptor complex that fails to stimulate transcription of proliferative genes. Upon stimulation with either estrogen or tamoxifen, the ER is shuttled into the nucleus. Depending on the balance between corepressors and coactivators, the action of tamoxifen is either antagonistic or agonistic [41]. Hence, an oncogenic upregulation of coactivators could disturb the balance in breast cancer cells with the result of a more agonistic tamoxifen effect.

Tamoxifen has clearly reduced breast cancer recurrence and mortality, and is still one major treatment alternative for ER-positive breast cancer patients. The most frequently reported side-effects are menopausal-like symptoms, such as hot flashes, vaginal dryness, and mood swings [42]. More severe, but less frequent, adverse events include thrombosis, pulmonary emboli, and endometrial cancer. Positive effects are reduced risk of osteoporosis, ischemic heart disease, contralateral breast cancer, and lung cancer. As not all treated patients respond, but still face the side-effects of tamoxifen, an important issue is to reduce the use of tamoxifen in non-responders. A common daily dose for adjuvant treatment with tamoxifen is 20 mg given orally. Due to individual changes in uptake and metabolism, the serum concentrations and breast tissue concentration of tamoxifen and its metabolites show large variations. A study showed that 20 mg of daily intake resulted in an average breast tissue tamoxifen concentration of 0.75 µg/mL, with variations spanning from 0.2-2.5 µg/mL [43]. A common concentration of tamoxifen or its metabolite 4-OH tamoxifen *in vitro* is 1 µg/mL, which is a comparable dose with tissue concentrations after oral administration.

Tamoxifen improves the clinical outcome of ER-positive breast cancer in both the adjuvant and the metastatic setting [44]. Five years of treatment with this drug reduced recurrence by 40% at 15 years after diagnosis, when compared with no endocrine treatment, and the benefits remained regardless of menopausal stage or age [45]. The time of treatment is generally five years, which have been shown more efficient in reducing recurrence than two years administration [46]. Recently, studies reported a better outcome of ten years treatment when compared with five years [47]. Although the adverse events were also extended, the benefits are thought to outweigh the risks. An ongoing trial is comparing extended AI treatment after 5 years of AI [48], and switching from tamoxifen to AI after five years is also discussed [49]. The level of ER positivity in the tumor cells has effects on the treatment efficacy, with higher receptor concentrations rendering improved tamoxifen treatment benefit as well as AI treatment benefit [50]. Whether polymorphisms in the CYP2D6 gene, resulting in a poor tamoxifen metabolizing enzyme, are indicative of tamoxifen treatment response is still under debate [51].

Cell cycle progression

Proliferation of breast epithelia is stimulated by mitogens, such as hormones, growth factors, and cytokines, resulting in activation of the cell cycle mediators cyclin D1, cyclin E1, and c-myc, and regulation of the CDK inhibitors p21, and p27 (**Figure 3**). Mitogens will initially promote cells to leave the gap (G) 0 phase, the resting phase, and enter the G1 phase. Cyclin D1 is critical for cell cycle progression, and regulates the transition of the restriction point in G1 to synthetic (S) phase entry [52]. When stimulated it binds and activates the catalytic proteins CDK4 and CDK6. Cyclin D1/CDK4/6, together with cyclin E/CDK2, induces phosphorylation of the retinoblastoma protein (Rb), whereupon Rb releases the inhibition on the transcription factor E2F1 [53]. At this stage, the mitogenic stimuli is no longer necessary and the E2F1 stimulates transcription of additional genes required for the G1 to S-phase transition, where the DNA content of the cell will duplicate, and eventually, the cell will go into mitosis which ends in the cell duplication.

Figure 3 The G1 to S-phase transition is highly regulated. Cyclin D1 protein releases the cyclin E/CDK2 complex from its inhibitor, p21, and also phosphorylates Rb initially. C-myc inhibits p21, and induces transcription of other cell cycle regulatory genes. E2F1 is released from Rb upon Rb hyperphosphorylation by the cyclins and promotes transcription of cell cycle promoting genes.

Interestingly, the cyclin D1, c-myc, and the E2F1 genes are transcriptional targets of the ER (**Figure 4**). Components of the cell cycle machinery have been studied in the context of hormonal treatment resistance. E2F activation was associated with resistance to AIs [54]. Loss of Rb was associated with tamoxifen resistance [55], and progression-free survival was prolonged in recurrent breast cancer when comparing letrozole in combination with CDK4/6 inhibitors with letrozole alone [56].

Figure 4 Estrogen (E2) stimulation of the estrogen receptor (ER) enhances transcriptional gene expression of the cell cycle promoting genes c-myc, cyclin D1, and E2F1.

Gene amplification

An amplicon is a restricted region of a chromosome, which has been repeated, and thereby contains several copies of each gene [57]. Double-strand break at fragile sites in the DNA, allowed to pass through the cell cycle without arrest at the checkpoints, is a possible origin of amplicon generation. The extra DNA can be arranged in so called double minutes not included in the chromosomes, be a repeated part of a chromosome, or scattered throughout the genome. Oncogenes are commonly amplified in breast cancer, and if overexpressed on mRNA and protein level the genes are potential drivers and contribute to the selective advantage of cells harboring the amplification [58]. In an amplicon, often holding many genes, one or more genes could be the driving gene. An amplicon could also lack driving genes, and instead be a passenger of another amplicon.

11q13

The cyclin D1 gene (*CCND1*) is located to the 11q13 amplicon, which contains several potential oncogenes, among others p21-activated kinase 1 (*PAK1*), *EMSY1*, *GAB2*, and *RPS6KB2*. Although the *CCND1* gene has been suggested as a driver of the amplicon, more data is still needed to reveal its role in the amplicon [58]. Amplification of 11q13 and the individual gene *CCND1* in breast cancer has been described as markers of poor prognosis [59,60]. The 11q13 amplicon is not restricted to breast cancer, but also found in other cancer diseases such as ovarian, and head and neck carcinoma, and reported to show decreased disease-free survival [61,62]. In addition, 11q13 and *CCND1* amplification predicted reduced response to endocrine treatment [63,64].

The *PAK1* gene is mapped to a core, separated, but frequently coamplified with the *CCND1* core of the 11q13 amplicon [65]. Amplification of the region harboring *PAK1* in breast tumors has been presented [66]. *In vitro*, cells displaying *PAK1* amplification were dependent on Pak1 for survival [67].

Cyclin D1

Cyclin D1 is a regulator of the cell cycle, promoting the transition from G1 to S phase [68]. Overexpression shortened the time for G1 to S phase transition, and inhibition of cyclin D1 hindered cells from entering the S phase. The protein and mRNA are overexpressed in nearly half of all breast tumors [69], and the gene is amplified in about 12% [70]. Upregulated cyclin D1 and *CCND1* amplification were shown to correlate with ER positivity [71], and with the luminal B subtype [72]. Functionally, the two proteins are connected so that active ER induces *CCND1* transcription, and cyclin D1 is a coactivator of several transcription factors, including the ER [73-75]. Amplification of the *CCND1*, high expression of cyclin D1 mRNA, and overexpression of the protein have been reported in various cancers [76,77]. The prognostic value of cyclin D1 in breast cancer has been debated. Some studies show that high cyclin D1 indicated improved prognosis in non-selected breast cancer and in ER-positive endocrine treated breast cancer patients [59,60,78]. Other studies have found opposing data, showing poor prognosis with high cyclin D1 mRNA expression in ER-positive, but not in ER-negative breast cancer [69], and high cyclin D1 protein expression predicted worse outcome in early stage, ER-positive, endocrine treated patients [79]. A recent meta-analysis concluded that high expression of cyclin D1 is an independent marker for worse prognosis in ER-positive breast cancer [80].

Pak1

The Pak1 serine/threonine kinase was first identified in 1994 [81]. Pak1 is activated through growth factors, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), and heregulin $\beta 1$ (HRG), by binding to the Rho GTPases Rac and cdc42 [82], and GTPase independently through PI3K [83]. Upon growth factor stimulation, Pak1 is recruited to the plasma membrane, to the adapter protein Nck, where it regulates signaling in several pathways (**Figure 5**) [84]. Generation of the Pak1 crystal structure revealed that the inactive Pak1 forms a homodimer, which is separated upon phosphorylation, and followed by release of the catalytic domain inhibition [85]. PDK1 phosphorylates Pak1, thus promoting its kinase activity GTPase independently [86]. Akt has been shown to phosphorylate Pak1 at serine 21, and the phosphorylation of other sites seems important for maintaining the Pak1 activation [87]. Most studies on Pak1 have focused on its role in migration, cell polarity, cytoskeletal interaction, and anchorage independent growth stimulating invasion and metastasis [83,88-90]. Additionally, Pak1 takes part in many functions, such as stimulating angiogenesis through upregulation of vascular endothelial growth factors (VEGF), cell survival through NF κ B and Bad, and tumor progression through activation of the ER [91-94].

To enter the nucleus, Pak1 binds the dynein light chain 1 (LC8) and importin through its nuclear localizing signal sequence [95], and therein participates in mitosis by interacting with histone H3 and Aurora [96,97]. Pak1 has been closely connected to the MAPK pathway, with a regulatory effect rather than stimulatory, and inhibition of Pak1 led to Akt and ERK suppression *in vitro* [98]. Phosphorylation of the threonine 423 on Pak1 seems to be an indicator for Pak1 kinase activity, an effect of autophosphorylation as a consequence of other conformation altering phosphorylations [99].

Figure 5 Pak1 activation by growth factors is involved in a large number of processes within the normal and transformed cell. Different location-specific roles have been demonstrated for Pak1. The multiple roles of Pak1, together with a detected upregulation in many tumors implicate an oncogenic potential.

Rayala *et al.* suggest that tamoxifen triggers Pak1 binding to ER α , but only in tamoxifen resistant cells [100]. Mammary tumor formation in a transgenic mouse model was observed upon hyperactivation of Pak1, and this seemed to modulate cell growth by activating the MAPK and p38MAPK pathways [89]. The tumor suppressor Merlin, suppressing Met phosphorylation, was inhibited by Pak1. In addition to the MAPK pathway, the Merlin-Met pathway was also important for Pak1 induced anchorage-independent growth in breast cancer cells [65].

In vitro, Pak1 phosphorylated ErbB3 binding protein 1, enhancing HER2 levels and thus rendered ER-positive cells resistant to tamoxifen, suggesting a role for cytoplasmic Pak1 in tamoxifen response [101]. The ER was phosphorylated by Pak1 at serine 305 in the ligand binding domain [94], and this site was also shown to be phosphorylated by protein kinase A (PKA) [102]. Regardless of the kinase, the phosphorylated serine 305 stimulated transcriptional activity of the receptor, with and without estrogen, demonstrating a tamoxifen resistance mechanism by growth factor stimulation of breast cancer cells. Taken together, Pak1 plays a central role in diverse functions in tumor progression. Specific inhibitors are developed; however, no pure Pak1 inhibitor is currently available for *in vivo* studies [103].

Estrogen receptor phosphorylations

The three dimensional structure of the ER is altered upon posttranslational modifications (PTM), such as phosphorylation, acetylation, ubiquitination, sumoylation, methylation, and palmitoylation. These changes affect the binding of corepressors and coactivators, altering dimerization, DNA binding, activity, stability, cellular localization, and the genetic target pattern upon receptor stimulation [104,105]. Estrogen binds the ligand-binding domain (LBD) and displaces the ER helix 12 domain from a hydrophobic pocket. Thereby coactivators, such as SRC-1 or AIB1, and p300, are recruited and the transcription activating function (AF) 2 is activated. The following conformational changes in the AF-1 allow for serine-118 phosphorylation by CDK7 [106]. AF-1 and AF-2 are both required, together with the hinge domain, for full transcriptional activation of the ER [107]. The transcription initiation complex is made up by coactivators that modify the chromatin, and recruit RNA polymerase. When antiestrogens, such as tamoxifen and raloxifen, bind the LBD, the hydrophobic pocket is occupied by the helix 12, preventing coactivator binding and instead enhancing corepressor binding. Phosphorylation of the LBD/AF-2 serine 305 by Pak1 or PKA seemed to affect the phosphorylation status of the serine 118 [100], and to recruit coactivators to the LBD/AF-2 in the presence of antiestrogens, thereby conferring resistance [108]. Overexpression of cyclin D1, acting as a receptor coactivator, induced an agonistic effect on the ER upon antiestrogen treatment [109]. The role of the coactivator AIB1 expression has been discussed. High expression is thought to indicate worse prognosis [110], and an improved response to tamoxifen treatment [111,112]. These findings suggest that, not only the estrogen level, but also a balance of coregulators and kinases determines the ER activation status and the effect of antiestrogens.

ER serine 118

Estrogen-induced phosphorylation of serine 118 has been suggested to be dependent on a variety of kinases, such as IKK α , GSK-3, MAPK, and CDK7 [113-115]. This site is also phosphorylated by an active MAPK pathway (**Figure 6**). A mutant ER, which could not be phosphorylated on serine 118, showed decreased transcriptional activity [116]. This was later described as an altered gene expression pattern upon estrogen stimulation [117]. A shift to less non-classical transcription, where ER operates through other bridging transcription factors, but with sustained classical transcription, with direct binding to EREs in the target gene promoters, was observed with the mutant 118 site. The expression levels of serine 118 have shown conflicting data regarding tamoxifen response. Tamoxifen resistant cell lines showed increased serine 118 expression when compared to non-resistant cells [118], whereas no serine 118 change was observed in other tamoxifen-resistant cells [119], and in our hands the serine 118 was reduced in the resistant cells. Culturing conditions and antibody selection may be reasons for the observed variations, but also the cell line specific variations in tamoxifen-resistance mechanisms. Phosphorylated serine 118 has been shown to be a marker of a tamoxifen sensitive ER with reduced DNA binding to target gene

promoters upon tamoxifen treatment in premenopausal breast cancer patients [120], suggesting an estrogen dependent ER. On the contrary, an increase in serine 118 levels was observed in tumors recurring despite tamoxifen treatment [120]. Serine 118 phosphorylation has been shown to correlate with PgR expression, low grade, and high differentiation, thus indicating a functional receptor and a cellular estrogen dependence that successfully can be targeted with antihormonal treatment [121]. In the postmenopausal setting, the role of this biomarker is yet inconclusive, and randomized trials including untreated controls may shed light on its treatment predictive value [122].

ER serine 167

The ER serine 167 residue is located in the N-terminal AF-1 domain, as serine 118, and is phosphorylated by growth factor stimulation through the intracellular kinases p90RSK, Akt, and S6K1 (**Figure 6**), and induced indirectly by estrogen [123-125]. *In vitro*, phosphorylation of serine 167 enhanced DNA binding, coactivator recruitment, transcriptional activity of the ER, and reduced tamoxifen response [126]. Data from clinical trials on the relevance of serine 167 expression levels for endocrine treatment response are somewhat conflicting. In a neoadjuvant AI study, a decreased serine 167 expression along with a decreased p-Akt after treatment indicated treatment response, although a high starting expression of p-Akt indicated improved response [127]. In the adjuvant setting of tamoxifen treated patients, an increased recurrence rate was observed with high serine 118 levels, but not with high serine 167 [128]. In other similar trials, high expression of serine 167 and low serine 118 expression in the primary tumor indicated a better outcome [129,130]. A high expression in the primary tumor was indicative of a longer time to progression in the metastatic setting [131]. These data suggest a divergent role for the serine 167 as a biomarker in breast cancer, possibly dependent on what kinase and pathway dominating its phosphorylation.

ER serine 305

At the border of the hinge region and the LBD, the serine 305 residue is located and has been demonstrated to be a target for phosphorylation by Pak1 and PKA (**Figure 6**) [94,132]. Results from *in vitro* studies and clinical expression studies point out a reduced tamoxifen response upon increased phosphorylation, as well as with high expression of its effector kinases [102,132,133]. A phosphorylation mimicking mutation at this site promoted receptor dimerization, induced ER binding to promoters, and upregulated expression of known ER target genes, like cyclin D1 and PgR [108].

Figure 6 The 19 known phosphorylation sites on the ER, reviewed in reference [104]. The serine 118, 167, and 305 are the most extensively studied and are phosphorylated by kinases such as MAPK, P90RSK, S6K1, Akt, PKA, and Pak1.

Combined ER phosphorylations and other modifications

An ER-phosphorylation code by IHC detection was suggested as a marker of tamoxifen response in ER-positive, tamoxifen-treated patients [134]. High expression of the phosphorylation sites in the AF-1 domain, the DBD, and the hinge domain were shown to indicate a good prognosis, whereas the included markers of the AF-2/LBD indicated a worse prognosis. Unfortunately, the serine 305 residue was not investigated in the study.

Modification of other sites of the ER may also be important for estrogen- and tamoxifen-regulated activation. The serine 294 site in the hinge domain is induced both by estrogen and tamoxifen, but not by growth factors [123]. This phosphorylation was shown to be CDK7 dependent. The tyrosine kinase c-Abl phosphorylated the ER on the two tyrosine residues 52 and 219, in the AF-1 and DBD, respectively, enhancing ER stability and transcriptional activity [135]. A mutation changing the lysine 303 into an arginine has been demonstrated to confer ER hypersensitivity to estrogen and growth factors. This effect was dependent on serine 305 phosphorylation [136]. Acetylation of the lysine 303 by the histone acetyl transferase p300 was estrogen independent and inhibited transcriptional activity of the receptor, whereas acetylation of other sites showed opposite effects [137]. The lysine 303 may also be ubiquitinated, thus preventing degradation when the ER is not stimulated by estrogen and facilitate degradation upon stimulation [138]. Mutations, leading to amino acid substitution in the helix 12 domain, were shown to activate the ER ligand independently, and higher treatment concentrations were required for antagonistic function [139,140]. These mutations were most frequently found in recurring breast tumors, suggesting a role in acquired resistance.

Taken together, the ER is highly regulated at the posttranslational level, both estrogen dependently and ligand independently. Coregulator balance and location specific expressions of active kinases alter the pattern of gene transcription and the cellular response to endocrine treatment. Hence, adding information on ER PTMs to the ER analysis of breast tumors would render valuable information for treatment prediction. Further studies are needed to elucidate the complexity of ER regulation and to find the most important markers for ER PTMs.

Growth factor signaling

A wide variety of growth factors are produced in the body, such as insulin-like growth factor-1 (IGF1), EGF, and HRG. A common feature is that they bind to cell surface receptors, which leads to conformational changes of the receptors and intracellular signaling cascades. These signals enhance growth, survival, and differentiation through complex networks. Aberrations in the receptors and the downstream intracellular signaling proteins are frequent in tumors, resulting in an active signaling pathway without the stimulation of growth factors, i.e. a self-sufficiency of growth stimulation [141]. The EGF receptor family consists of four tyrosine kinase receptors; EGFR, HER2, HER3, and HER4. About 15% of breast tumors are *HER2*-amplified at 17q12, which is a bad prognostic indicator. However, during the past 15 years a monoclonal antibody, trastuzumab (Herceptin®), targeting the extracellular domain of HER2 and thereby inhibiting its signaling, has improved the outcome for this group of patients drastically [142]. The HER2 has no known ligand, but amplifies signals by heterodimerization of the other EGF receptors, and by homodimerization when amplified.

PI3K/Akt

The PI3K is activated downstream of the membrane growth factor receptors (**Figure 7**). It consists of two domains; the p85 inhibitory/regulatory, and the p110 catalytic subunits (PIK3CA) [143]. Mutations in the *PIK3CA* gene, predominantly in exon 9 and 20, are some of the most common aberrations found in breast cancer, especially in luminal A tumors (ER+/PgR+/HER2-) with a frequency of 49% [144,145]. In this subgroup, the mutations have been suggested to be good prognostic factors for endocrine-treated patients [146,147]. A recent study showed no prognostic value of the *PIK3CA* mutations in untreated low-risk patients, or in tamoxifen-treated patients [148]. However, a beneficial outcome was found for AI treated patients. In addition, the mutational status of the gene indicated resistance to trastuzumab treatment in patients with HER2-positive tumors [149]. These data were not confirmed in a recent randomized trial of high-risk patients [150]. The role of the *PIK3CA* mutations in breast cancer remains inconclusive. This may result from its divergent roles in selected subtypes and upon treatment pressure. An active pathway downstream PI3K was rather related to basal-like tumors [148].

The p85 binds to active growth factor receptors whereupon its inhibition of the p110 domain is released [151]. Apart from growth factor receptors, various intracellular proteins affect this signal, such as hormone receptors, Src, insulin receptor substrate-1 (IRS-1), Rac, Rho, and PKC. The active p110 phosphorylates the lipid phosphatidyl inositol -4, 5-bisphosphate (PIP₂) into phosphatidyl inositol -3, 4, 5- trisphosphate (PIP₃), which in turn recruits proteins with plextrin-homology (PH) domains, such as Akt (PKB) and phosphoinositide-dependent protein kinase-1 (PDK1), to the cell membrane and activates them. The PIP₂ to PIP₃ conversion is reversed by the phosphatase and tensin homolog

(PTEN), a tumor suppressor commonly lost in tumors, which at low expression was suggested to confer resistance to tamoxifen [152]. The primary downstream mediator of PIP₃ is Akt. Akt is recruited to the plasma membrane, where it in direct contact with PIP₃ is phosphorylated at threonine 308 by PDK1 and subsequently by mTOR complex 2 (mTORC2) at serine 473 for full activation [153]. Tuberous sclerosis (TSC) 2 is phosphorylated and inactivated by Akt. This disrupts its interaction with TSC1, transforming the GDP-bound Ras homolog enriched in brain (Rheb) into the mTOR activating GTP-bound Rheb [154]. Stimulation with insulin was shown to increase the Rheb-GTP and thereby activate mTOR and its subsequent substrates, the S6Ks and 4EBP1 [155].

Figure 7 Growth factor stimulation of the PI3K/Akt/mTOR pathway can induce ER-regulated transcription. S6K1 is a target gene of ER, which is upregulated upon ER activation.

The Akts are a family of serine/threonine kinases with three separate isoforms (Akt1, Akt2, and Akt3). All three are activated by a large range of growth factors through PI3K and PDK1, and they all share the two phosphorylation sites important for activation; threonine 308 and serine 473 [156]. Akt can directly phosphorylate the serine 167 on the ER [124], and several other substrates, of which some results in the prevention of apoptosis. Bad and the forkhead transcription factor are inactivated and the NFκB, transcribing survival genes, is activated. High p-Akt-t308 or p-Akt-s473 tumor levels predicted worse outcome in endocrine-treated patients [157-161]. This effect seemed mostly related to Akt1, whereas Akt2 has been suggested to be an indicator of improved prognosis in ER-positive breast cancer [160-162]. Phosphorylated Akt was correlated with low proliferation and small tumor size, but whether it is related to ER or HER2 positivity could depend on its localization within the cell.

mTOR

The catalytic serine/threonine kinase mTOR forms two separate complexes. In the mTOR complex 1 (mTORC1), mTOR binds the scaffold protein regulatory associated protein of mTOR (raptor), the inhibitory protein proline-rich Akt substrate 40 (PRAS40), the negative regulator DEP-domain-containing mTOR interacting protein (DEPTOR), and the positive regulator Sec13 protein 8 (LST8). In the mTORC2, mTOR binds rapamycin-insensitive companion of mTOR (rictor), LST8, protein observed with rictor 1 (Protor1), DEPTOR, and stress activated protein kinase interacting protein (sin1). The mTORC1 is the most extensively studied complex and through phosphorylation of its downstream effectors, S6K and 4EBP1, it controls translation and ER activation, and thereby growth and proliferation [163]. mTORC1 is regulated by growth factors, insulin, stress, oxygen levels, amino acids, and energy levels in the cell. As a response to these signals, it controls metabolism through anabolic; protein- and lipid synthesis, and nutrient storage, and catabolic processes; autophagy and energy consumption [164]. In addition to the growth factor-PI3K dependent activation, mTORC1 can also be activated by ERK1/2 and p90S6K, through disruption of the TSC1/2 complex upstream of mTOR [165,166]. A reduction in nutrients and energy results in an inactive mTORC1, the metabolism of the cell slows down, and in many organisms this is associated with prolonged lifespan and reduced cancer incidence. The mTORC2 is less studied, but data show that, similarly to mTORC1, it can be stimulated by growth factors. Akt activation by phosphorylation of serine 473 is a known positive feedback loop controlled by mTORC2 [153,167], and the activation of SGK1 by mTORC2 promotes growth and ion transport in the cell membrane [168]. mTORC2 was also shown to regulate cytoskeleton organization through protein kinase C (PKC) phosphorylation [169].

A large number of mTOR inhibitors have been evaluated, of which some have reached clinical trials. Thus far one, the rapamycin analogue everolimus, has been integrated into clinical practice as a targeted drug in addition to endocrine treatment in the metastatic setting of breast cancer [170]. Recent data suggested everolimus also for HER2-positive recurring breast cancer [171]. Rapamycin and its analogues bind the FK506-binding protein 12 (FKBP12) in the cytoplasm, which then binds to the FKBP12-rapamycin-binding site (FRB) of mTOR and disrupts its kinase activity. This effect is mainly mTORC1 specific, however, long term treatment with rapamycin has shown some inhibitory effects on mTORC2 as well. ATP-competitive kinase inhibitors targeting both mTOR complexes have been suggested to be more efficient than mTORC1 specific inhibitors *in vitro* as this would inhibit feedback activations of Akt and the MAPK pathway [172,173]. On the other hand, temsirolimus, an mTORC1 inhibitor showed better response than KU0063794, a dual mTOR inhibitor, on renal cell carcinoma xenografts, proposing an effect on the microenvironment by temsirolimus not seen with KU0063794 [174]. Active mTOR expression predicted a more aggressive phenotype and a shorter disease-free survival in breast carcinoma and worse outcome in triple-negative breast cancer [175-177].

S6 kinases

Stimulation of cells with growth factors, hormones, or cytokines rapidly activated the S6Ks, whereas mTOR inhibition with rapamycin or TSC2 knockout inhibited S6K activation by all mitogenic stimuli [178]. The threonine 389 residue was phosphorylated by an active mTORC1 and has been shown to be the crucial activation indicator of the S6Ks. The S6Ks are two distinct homologs; the S6K1 (*RPS6KB1*) and the S6K2 (*RPS6KB2*), with a set of isoforms each (**Figure 8**). Yet another S6K, the p90 ribosomal S6K (RSK) (*RPS6KA*), exists. This protein was not activated by mTOR, but by the MAPK pathway, although all S6Ks were dependent on PDK1 for full activation [179]. As S6K1, the p90RSK stimulated ER phosphorylation at serine 167 [180].

Figure 8 An overview of the ribosomal S6 kinases with the homologs and isoforms of the S6Ks.

The S6Ks are involved in translational control in concert with the 4EBP1 (**Figure 9**) [181]. Without mitogenic stimuli the S6K1 binds the eukaryotic initiation factor (eIF) 3B, and 4EBP1 binds eIF4E. When mTOR is stimulated by growth factors or by nutrients, it phosphorylates the S6Ks and 4EBP1. The S6Ks are further phosphorylated by PDK1 and when fully activated, the 40S ribosomal subunit S6 is phosphorylated. The translation initiation factors are then free to form a complex and start the translation of specific proteins, such as c-myc [182].

Apart from regulating translation, the S6Ks control invasiveness, motility, and also angiogenesis through upregulation of matrix metalloproteinase 9 and VEGFs [183,184].

Figure 9 Translation initiation is a multistep process. Growth factors can induce translation through mTORs phosphorylation of the 4EBP1 and S6Ks, leading to dissociation from translation initiation regulators, eIF4E and eIF3B. PDK1 further stimulates S6K activation, whereupon the 40S ribosome subunit S6 is phosphorylated and the assembly of the translation initiation complex can start translation of mRNA into proteins.

Similar and distinct effects of S6K1 and S6K2 have been reported. S6K1 is the most studied homologue. S6K1 deficient mice showed significantly reduced body size, a prolonged lifespan, and in many cells a compensatory upregulation of S6K2 was observed [185,186]. Knockout of both homologs did not render live offspring in mice. A similar effect was seen upon complete mTOR knockout. Knockout of the *RPS6KB2* did not influence the phenotype. At basal conditions, the autoinhibitory C-terminal domain of the S6Ks repressed the internal activity. Insulin and EGF released this repression, and inhibition of the MAPK pathway showed that S6K2 was more dependent on ERK1/2 for the repression release than S6K1 [187]. Interestingly, the S6K1 was shown to stimulate mTOR phosphorylation in a positive feedback loop, at the activation site serine 2448 [188].

An increase in S6K1 and S6K2 at gene and protein levels in breast tumors compared with normal tissue implicates oncogenic roles of the two proteins [189]. S6K1 upregulation predicted worse prognosis [190], and amplification of the 11q13 region harboring the *RPS6KB2* reduced time to recurrence [191,192]. Overexpression of the splicing factor SF/ASF increased the expression of the p31 isoform of S6K1, which has been suggested to be the most potent oncogenic isoform of the S6Ks [193,194]. The gene encoding this splicing factor is located at 17q23, close to the S6K1 gene in a region frequently amplified in breast cancer [195]. Ben-Hur *et al.* suggested that the long S6K1 isoforms, p70/p85, possess tumor suppressing properties, compared with the short isoform, which may comprise the tumor promoting role of S6K1.

Rapamycin-induced feedback loop

Phosphorylation of Akt as a result of mTORC1 inhibition with rapamycin has been observed both *in vitro* and in about 60% of tumors [196,197], although not reported in cultured rapamycin-treated breast cancer tissue [198]. Harrington *et al.* knocked down TSC2, upstream of mTOR, in mouse embryo fibroblasts and described the subsequent upregulation of phosphorylated Akt [199]. Knockdown of S6K1 and S6K2, separately led to increased IRS-1 mRNA, but phosphorylation and degradation of IRS-1 was mostly seen upon S6K1 knockdown. The phosphatase and proposed tumor suppressor PHLPP dephosphorylated both Akt-s473 and S6K1-t389, and a reduction of PHLPP induced the feedback loop [200]. Thus, a stabilization of IRS-1 by TSC knockout, rapamycin treatment, or S6K1 knockdown or deactivation enabled signaling from insulin-like growth factor-1 receptor (IGF-1R) to Akt. This feedback mechanism was described as IGF-1R/IRS/PI3K-dependent, where inactivated S6K1 could not disrupt the IRS-1/IGF-1R binding by phosphorylating IRS-1-s302 (serine 307 on human IRS-1) and no longer target IRS-1 for degradation. On the other hand, in human adipocytes S6K1 was shown not be the kinase required for this phosphorylation [201], suggesting cell specific roles of the S6K1.

Bidirectional crosstalk between growth factor pathways and the ER

A study on ER positive breast tumors, measuring PI3K pathway components on mRNA and protein arrays, showed inverse correlation with ER levels, and inhibition of PI3K in cell lines increased the ER levels [202]. Luminal B tumors showed higher PI3K pathway activity and lower levels of ER-induced genes, such as the PgR. Estrogen stimulation controlled downregulation of components of the growth factor pathway, such as the PI3K, the HER3, and Ras-oncogene family members within the MAPK pathway [26]. The growth factor pathways and the ER pathway seem to regulate each other so that upregulation/stimulation or downregulation/inhibition of one pathway leads to compensatory responses of the other. This is probably an effect remaining from normal cells, in order to balance the signals. It implies that a compensatory activation of the other pathway will take place during pressure by treatment on one pathway, a so called bidirectional crosstalk.

An established mechanism of endocrine-treatment resistance is the overexpression of growth factor receptors, generally the HER2 receptor [203]. ER-positive cells stably transfected with HER2 showed *de novo* tamoxifen resistance and a growth advantage in a low estrogen environment, suggesting also AI resistance [204,205]. HER2/ER-positive tumors are targeted with both anti-HER2 and endocrine therapy. However, only 10% of ER-positive tumors show HER2 amplification and HER2 expression is correlated with ER-negative and PgR-negative tumors [206]. Downstream of growth factor receptors, signaling proteins are commonly mutated, or in other ways altered, in breast cancer. These observations suggest that this pathway is important for tumor development. On the other

hand, mutations in the *PIK3CA* gene did not confer tamoxifen resistance in a patient cohort or in the MCF-7 cell line [146].

Tamoxifen-sensitive breast cancer cells can become tamoxifen resistant upon long-term treatment with tamoxifen, showing that sensitive tumors eventually develop acquired resistance [207,208]. Common features of tamoxifen-resistant MCF7 cells are decreased PgR expression and increased Akt phosphorylation [209]. Previously, it was observed that the resistant cells had upregulated EGFR, increased HER3 phosphorylation, and more ERK1/2, Akt, and P90RSK activation, whereas the ER α , PgR, and ER β expression was reduced [119,210]. IGF-1R is an ER-regulated gene and was upregulated upon estrogen stimulation in MCF-7 cells [211], and reduced but activated in tamoxifen-resistant MCF-7 xenografts [212]. In cultured cells, inhibition with an IGF-1R antibody showed an inhibiting effect on the growth of tamoxifen-sensitive cells, but not on the resistant cells [209]. In addition, a stable induced expression of the IGF-1R made cells hormone independent, thus antiestrogen resistant [213].

A membrane-associated ER has been observed and was suggested to be a marker for endocrine resistance [119]. It binds growth factor receptors and confers their stimulation of downstream signaling pathways of growth and survival, non-transcriptionally [25,214].

These findings indicate a crosstalk between the ER signaling and the PI3K pathway as a mechanism for tamoxifen resistance.

Aims of the thesis

General aims

The main goal with this thesis was to evaluate potential biomarkers for the use in endocrine treatment prediction and prognosis in postmenopausal breast cancer.

Specific aims

Paper I	to investigate the frequency and oncogenic property of 11q13 amplification, with focus on the <i>CCND1</i> and <i>PAK1</i> genes. to investigate the separate and joint contribution of the <i>CCND1</i> and <i>PAK1</i> gene amplifications for tamoxifen response. to evaluate the Pak1 protein expression pattern in breast cancer tissue.
Paper II	to further delineate the role of Pak1 protein expression and one of its targets, the serine 305 residue of the estrogen receptor α, in a large randomized breast cancer cohort.
Paper III	to investigate the crosstalk between mTOR signaling and the estrogen receptor α by analyzing expression of activated proteins within the respective pathways and their role in predicting tamoxifen efficacy.
Paper IV	to investigate the role of the mTOR targeted proteins S6K1 and S6K2 by means of their expression and activation on treatment response. to evaluate antibodies potential for future clinical use.

Comments on Material and Methods

Postmenopausal Stockholm patient cohorts

In 1976, a randomized trial of postmenopausal breast cancer patients was initiated by the Stockholm Breast Cancer Study group [215]. The aim was to compare the treatment response of radiotherapy with chemotherapy in a high-risk group, and tamoxifen treatment with no endocrine treatment in a high-risk and a low-risk group. Inclusion criteria for the high-risk group were a positive node status and/or a tumor size exceeding 3 cm, whereas the low-risk group inclusion criteria were a negative node status and a tumor size not exceeding 3 cm. No evidence on ER status predicting tamoxifen response was available at the time. Hence, both patients with ER-positive and ER-negative tumors were included. This design makes the cohort with collected primary tumors and long-time follow-up data unique, as a true treatment response value of biomarkers can be assessed [216]. The study was closed in 1990, when endocrine treatment for patients with ER-positive tumors came into clinical routine practice.

The two groups were originally randomized to 2 years of tamoxifen or no endocrine treatment. In 1983, a new trial protocol was adapted, where patients without recurrence after 2 years of endocrine treatment were further randomized to an additional 3 years or no continued treatment. The ER status was assessed prospectively using isoelectric focusing until 1988, and thereafter with an enzyme-immunoassay. Retrospectively, ER was detected with IHC, and results were adapted in the following studies. Where no IHC data on ER status was available, previous prospective data was included. Cutoff for ER positivity was set at 25% positive cells or 0.05 fmol/ μ g DNA in paper I, and 10% positive cells or 0.05 fmol/ μ g DNA in the subsequent papers of this thesis. This was a result of an adjustment to current clinical cutoff levels, where ER positivity is set to 10% positively stained nuclei determined by IHC. DNA of 224 tumors and frozen tissue of 101 tumors from the high-risk group were used in paper I, and paraffin embedded tissue of 912 tumors from the low-risk group was used in paper II, III, and IV. For all markers detected in these studies, a portion of samples was missing. This was due to: lost or destroyed cores during processing, samples did not containing enough material for numerous studies, or no tumor cells were distinguishable in the sample. In the supplementary table of paper II, missing samples are compared with the samples on tissue micro array (TMA) and with samples of the original cohort. The results show no bias in the missing cores according to tumor size, ER status, or tamoxifen treatment.

Tamoxifen 5 versus 2 years cohort

In 1983, a trial including five centers in Sweden recruited postmenopausal breast cancer patients for randomization to 5 or 2 years of tamoxifen [217]. The benefit of the longer

treatment was evident in the whole group, and in the ER-positive, but not in the ER-negative group. A subset of 130 formalin fixed paraffin embedded tumors from this trial was available for analysis and used in paper IV. The benefit of the longer treatment in this cohort was used for validation of tamoxifen response value of biomarkers. This is not an optimal cohort for this issue, however, an indication of reduced tamoxifen response with expression of biomarkers can be observed. The small size of the cohort, impeding further subgroup evaluation, is another limiting factor.

Ethical considerations

Data was presented according to the REMARK guidelines of prognostic biomarkers. These guidelines state that a proper scientific biomarker study should be transparently presented so that the reader can value the marker [218]. Ethical approval for cohorts in all papers was from the Karolinska Institute Ethics Council (Dnr KI 97-451), with an approved addition 030201. Ethical approval for the smaller cohort included in paper IV was from the Linköping University Ethics Board (Dnr M132-04). According to the declaration of Helsinki, all subjects included in a clinical trial should sign informed consent and the use of a placebo group can only be used if the effect of the drug is not established, hence, withholding a potentially effective drug is not ethical. Recommendation for previously collected biobank samples, according to Helgesson *et al.*, states that integrity data, such as personal number should be coded [219]. At the time, when trials included in this thesis were conducted, approval for biobanking of tumors was not standard procedure. Tumors in paraffin and frozen tissues were collected retrospectively and subject data are coded. When the ER was defined as a biomarker for tamoxifen-treatment response, the study was closed due to ethical considerations.

Real-time quantitative PCR

Gene amplification can be detected by real-time quantitative polymerase chain reaction (qPCR), fluorescence *in situ* hybridization, comparative genome hybridization, or Southern blotting. We used qPCR for analysis of the *CCND1* and *PAK1* genes, a method in which gene amplification is detected based on the number of cycles needed to amplify the template DNA. The starting copy number of the nucleic acid template is proportional to the increase in fluorescence detected. The oligonucleotide probe has a 5' reporter fluorescent dye and a 3' quencher dye. While the probe is intact, the quencher dye is located close to the reporter resulting in reduced fluorescence emitted by the reporter. This phenomenon is called fluorescence resonance energy transfer. The probe anneals downstream of the primer and as the primer is extended by the 5' nuclease activity of the Taq DNA polymerase, the probe is cleaved. The reporter is separated from the quencher, resulting in a detectable reporter signal. The probe is removed completely from the target strand allowing the primer to extend further. Reporter dye molecules are separated from the quencher with each PCR cycle, leading to increased fluorescence intensity, which will be proportional to the amount

of product produced. We used the standard curve method, with known DNA concentrations from the SKBR3 cell line and the *APP* gene as reference gene, to determine normal and amplified levels of the genes. The limit for amplification was determined using a histogram, showing the frequency of tumor samples for different gene copy ratios. The modal peak was regarded as the normal gene copy ratio, with 2 copies, and the cutoff for amplification was set at 4 copies. A limitation with this method, compared with the *in situ* methods, is the possible dilution with non-tumor cells. However, tissues were selected to contain more than 50% tumor cells previous to DNA extraction, and amplification data was highly reproducible on whole-genome arrays [220].

Tissue processing

Tumor tissue was collected upon surgery of the primary tumor, fresh frozen, and cut in a microtome with freezing temperature to keep the tumor frozen. Sections were mounted onto microscope glass slides and stored in -70°C until analysis.

Tumor tissue later used for TMA was collected at surgical removal of the primary tumor and incubated in formalin for fixation. Thereafter, sections were paraffin embedded and cut into micrometer thin slices for haematoxylin/eosin staining. A pathologist selected three cores of abundant tumor cell content, whereupon the cores were removed and assembled in a recipient paraffin block together with cores from other tumors. TMA slides were stored in -70°C until immunohistochemical analysis.

Immunohistochemistry

Immunohistochemistry (IHC) refers to a technique of protein epitope detection by antibodies in a tissue [221]. It is used for clinical consultation to determine protein content in tumors, an important part of treatment decision and prognosis evaluation. IHC is also used by researchers to find new targets for treatment, and to explore signaling pathways. The technique allows for detection of protein expression levels, localization, and distribution in a tissue and within cells. The relative expression levels of a protein can be compared between different samples. For preparation the tissue is fixed, preferentially in formalin, in some cases in alcohol, dehydrated, and paraffin embedded for easy cutting and storage. These steps are essential to stop naturally occurring autolysis of cells when a tissue is removed from the body. Formalin fixatives will form methylene bridges in the cells, which must be removed by antigen retrieval in order for antibodies to detect epitopes. Alcohol fixation will cause less destruction of the epitopes but may only be used for small samples as the penetration rate is poorer. The tissue is cut in ~4 µm slices and placed on microscope glass slides. Paraffin-embedded tissue is heated to around the paraffin melting temperature to attach the tissue to the slide. To deparaffinize and rehydrate the tissue, xylene and thereafter a decreasing concentration of ethanol is used, as in paper II, or an automated system taking care of the deparaffinization, rehydration, and epitope retrieval can be

applied, as in paper III and IV. To improve the reactivity of the antibody to the proteins, antigen retrieval is important to destroy the cross-links of proteins induced by the fixative. Heat is a common technique for antigen retrieval and the pH of the antigen retrieval buffer can be optimized for each antibody, spanning between pH 6 and pH 10. To inactivate endogenous peroxidase, necessary if the chosen detection method is peroxidase based, slides are incubated in hydrogen peroxide. Blocking of the tissue with a protein solution prevents the antibody from unspecific binding.

For IHC in research purposes, both polyclonal and monoclonal antibodies are used. Tissues are incubated with the diluted antibody, commonly at 4°C overnight in a moisturized chamber, preventing drying, which would destroy the tissue. The optimal antibody dilution is determined by titration of the antibody. A wash step rinses the unbound and weakly bound primary antibodies and keeps the blocking active with the included albumin. Secondary antibodies target antibodies obtained from a specific animal. Hence, if the primary antibody was produced in a mouse, the secondary antibody should be an anti-mouse antibody. The secondary antibody amplifies the signal of the primary antibody, as several secondary antibodies bind one primary. The secondary antibody frequently has a biotin-streptavidin conjugation with an enzyme. We used the enzyme-detection system with horseradish peroxidase (HRP), which catalyzes the oxidation of 3, 3'-diaminobenzidine (DAB) to produce a brown product. The nuclei are counterstained with haematoxylin to identify localization of proteins in the cells. Finally, the slides are dehydrated and mounted with cover glass before visual analysis in a light microscope.

Antibodies

Antibodies, polyclonal or monoclonal, are designed to detect a protein, a part of a protein, or one single epitope on a protein. Polyclonal antibodies are produced by immunizing an animal with a protein sequence. The animal produces different antibodies towards the peptide as the immune system recognizes the foreign substance. Upon bleeding, the antibodies can be retrieved from the serum. An advantage with polyclonal antibodies is if the protein of interest has isoforms of different lengths, all the forms may be detected and if the epitope is destroyed or hidden, the chance to detect the protein increases if more than one epitope can be bound. Monoclonal antibodies are also produced by initially immunizing an animal with a peptide [222]. Immune cells from the spleen are collected and fused with cancer cells to form an immortal hybridoma, which will produce monoclonal antibodies. These antibodies are derived from one clone of B cells and they all bind to the same epitope. Monoclonal antibodies are often highly specific and can be used for producing an unlimited amount of identical antibodies, in contrast to polyclonal antibody production, where the blood volume of one animal is a limiting factor.

Immunohistochemical scoring

Scoring range of the staining was determined for each antibody (**Table 1**). By a visual overview of the stained tissues, a range from negative to strong intensity, with the number of separate steps possible, and if applicable, the percentage of positive cells, was determined. Thereafter, staining of the entire cohort was evaluated by two observers individually, with the set frames for scoring. In the next step, a consensus of scoring by the two observers was reached by a second observation of samples. There were three cores from each tumor on the TMAs and when a variation between the cores was observed, the strongest staining was considered. Samples were excluded if tumors were non-representative or missing. Cutoff for low and high protein expression was determined so that an adequate sample number was found in each group, and after study of current literature, if applicable.

Validation of antibodies

The selection of specific antibodies is crucial for obtaining repeatable results of IHC assays. Positive and negative controls of the epitope in each staining are preferred for validation of an antibody. The optimal strategy is to include a known positive and negative tissue. This may be obtained by knockdown and upregulation of the epitope, respectively, in a cell culture as was performed in paper IV. Results are detected by western blot analysis and on formalin-fixed paraffin-embedded cell pellets. On TMAs, a large number of tumors are mounted on the same slide, and optimal antibody concentration was chosen so that both negative and positive staining could be observed on a test slide. With this approach the tumor staining variability served as control. The peptide used for immunizing the animal for antibody production can serve as a control of specificity in the human tissue. The antibody is incubated with excess of the peptide prior to staining and subsequent IHC results should be negative. For antibodies detecting phosphorylated epitopes, a phosphatase applied to the tissue prior to staining removes all phosphorylations and a negative staining should be observed if the antibody is phospho-specific [223]. To test the secondary antibody, a tissue can be stained without the primary antibody. This should result in a non-stained tissue if the secondary antibody is not reacting with any component of the tissue.

Table 1 Protein expression scoring

Epitope	Scoring	Cutoff (high)	Comment	Paper
Pak1 nucleus	Negative, weak, moderate, strong	Moderate, strong		I
Pak1 cytoplasm	Negative, weak, moderate, strong	Moderate, strong		I
Pak1 nucleus	>10% positive cells	>10% positive cells		II
Pak1 cytoplasm	Negative, weak, moderate, strong	Moderate, strong		II
p-ER-s305 nucleus	<1%, 1-25%, 26-75%, >75%	≥1%		II/III/IV
p-ER-s305 cytoplasm	Negative, positive	Positive		III
p-ER-s167 nucleus	Negative, weak, strong <1%, 1-25%, 26-75%, >75%	Strong intensity in >75%		III/IV
p-ER-s167 cytoplasm	Negative, positive	Positive		III
p-Akt-s473 nucleus	Negative, weak, moderate, strong <1%, 1-25%, 26-75%, >75%	H-score >4	H-score (histological score, 0-6) was obtained by adding intensity with percentage; negative (0), weak (1), medium (2), strong (3), and <1% (0), 1-25% (1), 26-75% (2), >75% (3)	III/IV
p-Akt-s473 cytoplasm	Negative, weak, moderate, strong <1%, 1-25%, 26-75%, >75%	H-score >3		III/IV
p-mTOR-s2448 cytoplasm	Negative, weak, moderate, strong <1%, 1-25%, 26-75%, >75%	Strong intensity in >25%	All intensities were graded as percent stained cells No nuclear staining was observed	III/IV
p-S6K-t389 nucleus	Negative, weak, moderate, strong	Strong	No positive nuclear staining was observed in cohort 2	IV
p-S6K-t389 cytoplasm	Negative, weak, moderate, strong	Strong	Negative and positive were graded in cohort 2	IV
S6K1 nucleus	Negative, weak, moderate, strong	Strong	Medium and strong were considered high in cohort 2	IV
S6K1 cytoplasm	Negative, weak, moderate, strong	Strong	Medium and strong were considered high in cohort 2	IV
S6K2 nucleus	Negative, weak, strong	Weak, strong	Negative, weak, medium, strong were graded in cohort 2, with cutoff set at medium, strong	IV
S6K2 cytoplasm	Negative, weak, medium, strong	Strong	Medium and strong were considered high in cohort 2	IV

Cell culture

In paper IV, antibodies were validated *in vitro*, foremost in the breast cancer cell line ZR75-1 [224]. This cell line was derived from tumor cells in ascites fluid of a postmenopausal woman recurring on endocrine therapy. The cells are ER-, PgR-, and androgen receptor (AR) positive, show a PTEN loss, and are responsive to tamoxifen treatment. We chose this cell line because S6K1 and S6K2 are similarly expressed, compared with the commonly used MCF-7 cell line, which harbors a massive S6K1 amplification. Another reason for choosing this cell line was that this was a cell line where we could detect expression of ER serine 167 after growth factor stimulation. For S6K1 short-isoform detection, the BT474 breast cancer cell line was used. Cells were grown in Optimem media without phenol-red, in 4% fetal bovine serum (FBS). The phenol-red may have estrogenic effects and was therefore omitted.

Tamoxifen-sensitive, and tamoxifen-resistant MCF-7 cells were kindly provided by Dr. Anne Lykkesfeldt [207,210]. These cells were grown in DMEM/F12 media, supplemented with 1% FBS. Tamoxifen-resistant cells were constantly grown with 1 μ M tamoxifen, to maintain the treatment resistance.

Transfection and treatments

Transient transfection of cells with small interfering RNA (siRNA) is an experimental tool to study the role of one specific protein. It is a posttranscriptional regulation process, where the siRNAs are incorporated into the RNA-induced silencing complex (RISC) directed to break down and inhibit translation of a specific mRNA in order to silence its expression [225]. In paper IV, the S6K1 and S6K2 were knocked down using electroporation to force uptake of siRNA into the cells. Cells were seeded at a set density after transfection, and 24 hours later, media was changed. The time to minimal protein expression depends on which mRNA is targeted and turnover rate of its corresponding protein. The lowest mRNA level is often reached before the lowest protein level, as stability and rate of protein degradation is a factor to take into account for protein levels. Of experience, many proteins are sufficiently downregulated at 72 to 96 hours after transfection. Cells were treated with HRG and 4-OH tamoxifen, the more potent form of tamoxifen. Controls were included in every experiment. For siRNA transfection, a random siRNA sequence served as control. HRG was diluted in water supplemented with 0.1% bovine serum albumin (BSA), and 4-OH tamoxifen was diluted in 99.5% ethanol. The dilution media for each treatment was used as a control.

Western blot protein detection

To detect relative protein content, and size of specific proteins, the western blot method was applied. Cells were harvested on ice, with radio immune-precipitation assay (RIPA) buffer, containing detergents, such as sodium dodecyl sulfate (SDS). Protease inhibitors and phosphatase inhibitors were added to the buffer. RIPA lyses the cells and proteins are protected from degradation and dephosphorylation.

Negatively charged denatured proteins were separated according to size by polyacrylamide gel electrophoresis. When distributed throughout the gel the proteins were blotted over to a polyvinylidene difluoride (PVDF) membrane, which was incubated with antibodies similarly as previously described for TMAs. Following a chemiluminescent reaction between detection solution and the secondary antibodies, protein could be visualized as bands with a charge-coupled device (CCD) camera. Western blot results are often presented as an inverted picture, with black bands, in which the number of stained pixels can be measured to obtain a quantitative comparison of band intensity. For equal loading of protein onto electrophoresis gels, the total protein content was measured prior to loading, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β -actin protein detection was used.

Flow cytometry detection of cell cycle distribution

An increase of percent cells in G0/G1 phase was considered as a marker for cell cycle arrest. The flow cytometry method is based on the set of chromosomes changing during the cell cycle [226]. The DNA was stained with the fluorescent dye propidium iodide (PI). In the G0/G1 phase, the cells contain the normal set of chromosomes, whereas during S phase the DNA content is increasing, and in G2/M phase the set is doubled before the cell divides and the two daughter cells are formed. With a DNA histogram, the rate of cells in each phase was distinguished. In addition, apoptotic cells can be detected, rendering a peak with less DNA than the normal set of chromosomes. We aimed to detect effects on the cell cycle distribution pattern after a proper time of downregulated protein. Cells were harvested at 96 hours post-transfection. The prolonged time was due to the delayed siRNA effect, which is not instant at transfection.

Preparation of formalin-fixed paraffin-embedded cells

Western blot is a common method for antibody validation, however not always comparable with IHC as the conformation of the epitopes may differ in the two assays. Therefore, cultured cells were formalin fixed, pelleted, and paraffin embedded before IHC staining with antibodies. With this method the antibodies could be validated prior to use of valuable tumor TMAs.

Crystal violet cell proliferation assay

Crystal violet is a dye that can be used for cell viability and cell proliferation assays [227]. It stains DNA and the color intensity measured in a spectrophotometer is proportional to the cell number. The relative cell number can be obtained when comparing samples with controls. Cells were seeded in 24-well plates at a concentration of 10 000 cells per well. At day 1 and day 3, medium was renewed and treatment was added to four replicates per sample. At day 6, cells were washed, fixed in 4% paraformaldehyde, stained with 0.4% crystal violet in 1% ethanol, carefully washed in tap water, and let to dry. Cells were dissolved with 1% citric acid monohydrate in 50% ethanol and analyzed at 560 nm in a

spectrophotometer. Wells without cells were used as negative controls and detected signal from these wells were subtracted from signals in sample wells.

Statistics

Time to local or distant recurrence after diagnosis was defined as recurrence-free survival (RFS) and visualized with Kaplan-Meier curves. Hazard ratios (HR) with 95% confidence intervals were calculated with Cox proportional hazard regression, showing reduced or increased risk of recurrence with variation in the tested variable. This method was also applied for multivariate analysis with adjustments for variables that may interfere with the result. Treatment prediction was evaluated from two separate figures showing low and high expression level of the tested variable, respectively. To detect potential differences in time to recurrence with regard to the treatment effect, an interaction analysis was performed with the Cox proportional hazards model. For correlations between variables the Pearson χ^2 test was applied in 2x2 analyses and for rank correlations, when expression of any variable was considered in more than two levels, the Spearman test was used. Forest plots were drawn in the Excel 2010 software. A p-value <0.05 was considered significant, and in analyses with multiple comparisons a p-value <0.01 was considered significant to adjust for random significance. For determining statistical significance of cell cycle arrest by flow cytometry measures and proliferation of cells in the crystal violet analysis non-paired t-test was applied.

Results and Discussion

Paper I

In paper I, we studied the 11q13 chromosomal region by determining the copy number status of the two genes *CCND1* and *PAK1* in a series of 224 high-risk breast tumors with real-time quantitative PCR. Additionally, the Pak1 protein expression was evaluated in a subset of the series using IHC.

Amplification of the two genes correlated with each other, showing coamplification in a significant number, but not in all tumors. We did not detect associations between the genes and their corresponding protein products. This was, however, seen in other studies for *CCND1* and *PAK1* gene-to-protein correlation [63-65]. *CCND1* correlated strongly with ER positivity, whereas *PAK1* only tended to correlate with a positive ER status. A large trial of single nucleotide polymorphisms (SNPs) in breast cancer showed that an SNP variant located close to the *CCND1* gene was correlated with a positive ER status and was even stronger correlated with ER/PgR positivity, markers for luminal A breast tumors [228]. *CCND1* amplification in patients with ER-positive tumors indicated worse prognosis than for patients with non-amplified tumors, but this prognostic value was not evident for *PAK1* amplification. However, amplification of any of the two genes on 11q13 showed the strongest prognostic value, and this result remained significant in a multivariate analysis (**Figure 18**). The worse prognosis observed with *CCND1* amplification was later confirmed in patients with ER-positive tamoxifen-treated tumors [229,230].

PAK1 amplification alone predicted reduced response to tamoxifen treatment (**Figure 10**). Analyzing Pak1 protein expression in this small series did not render a significant treatment-predictive value. A tendency towards a reduced tamoxifen benefit was observed, hence, data was only used as an indication to go further in a larger material. A disadvantage with the analysis was that the frozen tissue was old and the staining was difficult to score. Later attempts to stain these tumors with other antibodies have failed, suggesting that paraffin-embedded tissue is better in long-term follow-up studies, despite the risk of some epitope destruction with formalin. An advantage with this cohort was that tumors were at a higher stage than in the cohort used in the subsequent study, rendering more events in a shorter time, thus improving the significance of the smaller cohort.

We conclude that *PAK1* gene status is a candidate predictive factor for tamoxifen treatment and that Pak1 protein requires further evaluation in a larger study. The 11q13 amplicon as well as the *CCND1* gene status alone are potential prognostic factors.

Figure 10 Forest plot illustrating tamoxifen-treatment prediction in high-risk breast cancer patients, hazard ratio (HR) (◆) with 95% confidence interval (bars). PAK1 amplification data include only ER-positive patients. *Italic figures on bar represent the upper 95% confidence interval.*

Paper II

With the background of paper I, we continued to evaluate the Pak1 protein expression levels in a different and larger series of breast tumors in paper II. Studies have shown that Pak1 phosphorylates the ER on serine 305, and this modification activates the receptor, leading to transcriptional upregulation of cyclin D1 and cell proliferation [94,231]. In this study, we aimed to evaluate the tumor protein levels of serine 305 in addition to Pak1 in a large series of tamoxifen randomized low-risk breast cancer patients.

Pak1 protein localization showed different treatment predictive and prognostic roles. When overexpressed in the cytoplasm, Pak1 correlated with large tumors, ER negativity, and indicated a worse prognosis in systemically untreated patients. This was not in line with data from a previous study where no prognostic value of Pak1 was detected in endocrine untreated premenopausal patients [232]. In addition, Pak1 cytoplasmic expression correlated significantly with a positive HER2 status ($p=0.001$). Its known potential to stimulate anchorage-independent growth, and other tumor promoting phenotypes, supports that a high expression of cytoplasmic Pak1 would provide an advantage for tumor cells, thus predicting a worse outcome. On the other hand, high Pak1 levels in the nuclear compartment correlated with small tumors, ER positivity, a negative HER2 status ($p=0.02$), and tended toward a prediction of reduced response to tamoxifen. The Pak1 association with response to tamoxifen was in line with data found in premenopausal breast cancer patients, showing that high nuclear, but not cytoplasmic, expression was indicative of a reduced response to tamoxifen [232].

Adding expression of nuclear phosphorylated ER serine 305 to the Pak1 analysis further reinforced the treatment-predictive value in ER-positive patients (**Figure 19**). The difference in tamoxifen benefit between patients with an inactive Pak1-ER pathway and patients with an active signal in the tumor was significant. Phosphorylation of the ER is complex, with several kinases, coactivators, and corepressors regulating the receptor conformation and activity. Phosphorylation at separate sites of the receptor may indicate diverse response to endocrine treatment [134]. Our data suggest that further investigations of the Pak1/p-ER-s305 pathway would be of interest for endocrine treatment prediction, especially in combination with other markers, such as expression levels of phosphorylated PKA, which was also shown to phosphorylate ER at serine 305 [102]. *In vitro*, this phosphorylation activated ER independently of estrogen. This indicates that, as the site can be phosphorylated in an environment of low estrogen levels, these results do not only apply to tamoxifen resistance, but also to AI-treatment resistance. A pure AI vs. no endocrine treatment study in the primary setting would not be considered in an ethical perspective. Instead, the use of the neoadjuvant “window-of-opportunity” for such a trial may be an option. Biomarker detection in tumors from retrospective tamoxifen vs. AI trials could answer the question of the superiority of AIs over tamoxifen remains in the subgroup with an active Pak1-ER pathway.

In paper II, we conclude that activation of the Pak1-ER pathway indicates reduced response to tamoxifen treatment. These patients would rather be given an alternative adjuvant treatment, such as the ER-degrading fulvestrant. In addition, Pak1 could be of interest as a drug target for a subgroup of patients with Pak1-overexpressing tumors.

In paper I and II, the antibody specifically targeted total Pak1, without any selection for phosphorylated Pak1. For further assessment of Pak1s role in tamoxifen resistance the active state of Pak1 would be interesting to consider. Additional unpublished *in vitro* data showed no sensitization of tamoxifen response by inhibiting Pak1 expression with siRNA in tamoxifen-resistant MCF-7 breast cancer cells (**Figure 11**). However, increased Pak1 expression was observed in three out of four separate clones of these cells compared with the tamoxifen-sensitive parental cells (**Figure 12**).

Figure 11 Knockdown of Pak1 did not sensitize tamoxifen-resistant (TR1) MCF-7 cells. Cells were transfected with siRNA and treated with a gradient of 4-OH tamoxifen for 5 days. Comparable cell density was assessed with crystal violet staining. Cells were also seeded for Pak1 protein detection and harvested at 72 hours posttransfection for western blot protein detection.

Figure 12 Increased Pak1 expression was observed in three out of four acquired tamoxifen-resistant clones (TR1, TR4, TR7, and TR8), compared with the parental tamoxifen-sensitive cell line (S).

Paper III

In paper III, we aimed to investigate potential treatment predictive markers within the crosstalk between growth factor stimulated Akt/mTOR pathway and ER activation. Protein expression of phosphorylated Akt at serine 473 (p-Akt) and phosphorylated mTOR at serine 2448 (p-mTOR) represented growth factor stimulation, and ER phosphorylations at serine 167 and serine 305 (p-ER), represented ER activation (**Figure 13**).

High expression of p-Akt in the cytoplasm was correlated with HER2 positivity and p-ER cytoplasmic expression, whereas both p-Akt in the nucleus and p-mTOR were closer connected to hormone receptor positivity and p-ER in the nucleus. In the treatment predictive analyses only the nuclear p-Akt was included, as the cytoplasmic p-Akt did not show correlation with ER-positive characteristics. Here, we aimed at evaluating a crosstalk in typical ER-positive tumors without the involvement of HER2 induced tamoxifen resistance. HER2 was adjusted for in the interaction analyses. The diverse findings of p-Akt localized to cytoplasm and nucleus is a phenomenon that should be taken into consideration in future studies of Akt. Akt activation is also a promising tool to validate the treatment response of mTOR inhibitors in neoadjuvant studies. In the present study, only the mTORC2 activated, Akt serine 473, site was evaluated. To complete the understanding of Akt activation in the cohort, also the PDK1 induced phosphorylation site, threonine 308, should be taken into consideration for analysis. Antibodies directed towards phosphorylated Akt1 would be useful, in comparison to the p-Akt1/2 antibodies available today. An option is to use the proximity-ligation assay (PLA) (OLINK Bioscience, Sweden), to distinguish the two homologues, as presented in an endocrine-treated breast cancer cohort [161]. This method is yet time-consuming and expensive, as the antibody concentration needed is about ten times higher than for conventional IHC. Therefore, in its present form, it is not applicable in clinical routine.

Nuclear p-Akt in combination with p-ER in paper III was a significant marker for tamoxifen treatment prediction ($p=0.024$) (**Figure 19**). However, in combination with one ER phosphorylation site, the serine 167, in paper IV, the interaction analysis was not significant ($p=0.16$). Akt phosphorylates the ER on serine 167, and may also participate in activating Pak1 [87], which in its active state phosphorylates the ER at serine 305 in some tumors. This would in part explain the additional value of ER serine 305 to the analysis of treatment prediction.

Combining high serine 167 with serine 305 levels was borderline significant in the tamoxifen treatment prediction analysis ($p=0.067$). In paper II, we showed that the single marker serine 305 did not significantly predict reduced treatment benefit ($p=0.24$), hence, the addition of serine 167 seemed to add an effect with reduced response to treatment, despite a detected improved prognosis with high ER serine 167 (**Table 2**).

P-mTOR was a borderline significant marker of lost tamoxifen-response for the ER/PgR-positive patients, and with a combination score of p-mTOR/p-Akt/p-ER the treatment prediction was significant in the ER-positive group ($p=0.029$) (**Figure 19**).

Active mTOR expression predicted a more aggressive phenotype and a shorter disease-free survival in breast carcinoma and worse outcome in triple-negative breast cancer [175-177]. In contrast to these results, we showed no impact on prognosis of p-mTOR expression in breast cancer.

We observed only cytoplasmic p-mTOR staining (**Figure 13**). This indicated an mTORC1-specific detection as mTORC1 was previously shown to predominantly localize to the cytoplasm [233].

Figure 13 Visualization of levels of phosphorylated markers in paper III and IV. Here, p-Akt-s473 shows medium cytoplasmic and strong nuclear staining, p-mTOR-s2448 shows strong cytoplasmic staining in >25% of the tumor cells, p-ER-s167 shows weak cytoplasmic staining and strong nuclear staining, and p-S6K-t389 shows strong cytoplasmic staining and negative nuclear staining.

Paper IV

In paper IV, the aim was to investigate whether S6K1 and S6K2 are involved in tamoxifen response, and to detect any prognostic potential of these markers. Activation of the S6 kinases was also analyzed as a marker for the mTOR pathway. In order to carry out the analyses, we validated antibodies directed towards the downstream mTOR targets S6K1, S6K2, and their phosphorylation at threonine 389 (388 in S6K2). The p-S6K antibody was shown to detect both S6K1 and S6K2. For *in vitro* evaluation of antibodies and tamoxifen response, the ZR75-1 cell line was used. ZR75-1 is an ER-positive, tamoxifen-sensitive breast cancer cell line with a similar expression of the two S6 kinase homologs. To test the phospho-antibodies, cells were stimulated with HRG and upregulation of the components, p-Akt, p-S6K, p-ERK, and p-ER-s167, in the PI3K and MAPK pathway were observed. We did not detect any change in p-mTOR or p-4EBP1. A full activity of mTOR in control cells may be one reason for this observation. The ZR75-1 cells have a downregulated PTEN, which could contribute to high basal levels of downstream signals, and cell media nutrient components may also keep mTOR active.

We found the ZR75-1 cells to respond to 4-OH tamoxifen at increasing concentrations spanning from 10 nM to 1 μ M. Five μ M was a toxic concentration used as control. The cells were treated with a 4-OH tamoxifen gradient in a crystal violet analysis with and without 0.1 μ M HRG (**Figure 14**). Results show a decreased tamoxifen response, with the need for higher treatment concentrations to get similar results in stimulated cells compared with unstimulated cells.

Figure 14 The ZR75-1 breast cancer cell line showed less sensitivity to 4-OH tamoxifen upon heregulin β 1 (HRG) stimulation.

To stimulate the PI3K/Akt pathway and induce tamoxifen resistance, HRG was added to the cell media. At 30 minutes the pathway was activated, and the activation remained at 24 and 48 hours. In addition, HRG induced the p-S6Ks in three other tested breast cancer cell lines (Figure 15).

An initiating increase in p-S6K upon 4-OH tamoxifen stimulation, and a decrease after longer treatment exposure, was detected in ZR75-1 cells. The primary response could be a result of membrane-associated ER responding to 4-OH tamoxifen as an agonist, and the secondary response suggests a transcription regulated response. In clinical trials, AIs reduced Akt and mTOR activation, suggesting that estrogen-induced ER activation stimulates these pathways, possibly through transcription of genes upregulating Akt and mTOR [234,235]. A shift of the total S6Ks on western blot after HRG stimulation indicated that these antibodies also detected phosphorylated S6Ks. The stable p-S6K, p-ERK1/2, and p-Akt expression with 4-OH tamoxifen treatment in HRG-stimulated cells suggests that the HRG signal ran over the tamoxifen effect seen in unstimulated cells. Starvation of cells prior to treatment may enable visualization of differences from 4-OH tamoxifen upon HRG-stimulated tamoxifen resistance.

A comparison of a tamoxifen-sensitive (S) MCF-7 breast cancer cell line with tamoxifen-resistant (TR1) MCF-7 cells showed, in our hands, an upregulated mTOR activity, visualized by p-S6K-t389, a decrease in the total ER and p-ER-s118, a minor increase in p-ER-s305 and an upregulated Pak1 (Figure 16). Acquired tamoxifen resistance thus seems to involve crosstalk between ER and growth signaling.

Figure 16 Crosstalk between ER signaling and growth factor pathways in tamoxifen-resistant (TR1) MCF-7 cells when compared with the parental tamoxifen-sensitive (S) cell line.

Cell cycle distribution assay is a tool to evaluate the proliferation rate. Knockdown of the S6Ks separately did not induce significant G1 arrest; however, a double knockdown stopped the proliferation in a significant number of cells. This showed that the S6Ks are involved in cell cycle regulation in these cells. Tamoxifen stopped the proliferation significantly only when the two S6Ks were intact, indicating that they are needed for a proper tamoxifen response. Upon S6K1 and 2 knockdowns, there are multiple other changes taking place, such as upregulation of the genes encoding the mTORC components rictor and raptor (unpublished data). Thus, the proliferation-assay results could be an indirect response of the knockdown. S6K1 was shown to phosphorylate rictor in the mTORC2, leading to mTORC2 inhibition [236], and upon S6K1 knockdown, one could speculate that the turnover of rictor is disturbed.

The suggested negative feedback loop of S6K1 inhibiting IRS-1 was observed as increased p-Akt in ZR75-1 cells upon S6K1 knockdown, but not with S6K2 knockdown, showing divergent roles of the two homologs. These results were also observed in tamoxifen-sensitive and -resistant MCF-7 cells (data not shown). However, the IRS-1 was not analyzed, and the S6K1-regulated Akt inhibition through mTORC2 may also be involved.

After validation of the antibodies, two cohorts were stained for p-S6K-t389 (**Figure 13**), S6K1, and S6K2. The larger cohort was previously stained for S6K2 with a separate antibody [192]. On a test slide, the two S6K2 antibodies were shown to correlate. According to correlation data on protein expression, two subtypes of tumors were distinguishable. Tumors with high S6K1 were more often HER2 positive, had an overexpression of phosphorylated Akt in the cytoplasm, showed a somewhat increased proliferation rate, and an average tumor size. On the contrary, tumors showing high expression of S6K2 were more often ER positive, with an active mTOR, and nuclear expression of p-Akt and p-ER-s167 was higher. These tumors were significantly smaller and showed low proliferation. We did not observe any prognostic disadvantage with high activity of the S6Ks, nor of the p-mTOR expression level (**Table 2**). High p-S6K nuclear levels rather tended towards a better prognosis. This could be explained by the close connection with ER positivity found in this group. The hypothesis of two distinct tumor variants according to S6K1 or S6K2 expression is in line with a gene amplification analysis showing almost mutual exclusiveness of the large 17q12-23 amplicon, harboring the *HER2* and the *S6K1* genes, with the 11q13 amplicon, including the *CCND1* and the *S6K2* gene [237].

Tamoxifen analyses of the data showed significantly reduced responses with S6K1 nuclear accumulation and with high p-S6K, regardless of location (**Figure 19**). Our research group previously showed a benefit with high S6K2 in the nucleus in the ER/PgR-positive group, and *in vitro* data indicate that growth factor stimuli locates S6K2 to the cytoplasm and S6K1 to the nucleus [238,239]. These results led us to test the effect of location of the two homologs on tamoxifen response in the larger patient cohort. The group with high nuclear S6K1 and high cytoplasmic S6K2 is small, and the p-value for interaction between the two analyses

was not significant, but the results do not disprove our hypothesis. Some markers, such as nuclear accumulation of S6K1, have an effect on the untreated group, resulting in no difference in recurrence between treated and untreated patients. Subgroups with these markers do not benefit from tamoxifen, despite a better outcome than without the marker expressed. Finding these markers could indicate a group that does not need tamoxifen, thus could be spared the side effects. However, there are still patients recurring in the subgroup, suggesting that all patients in this group should be subjected to another treatment as they do not benefit from tamoxifen treatment. To distinguish patients in need for additional or alternative treatment from patients not in need of treatment requires further studies before an implementation in the clinic can be completed.

Combining variables that alone do not reach significant value in treatment response could be an approach to study the activity of the pathway. One marker may be important in a subset of patients, and with several markers a larger group of patients could be included. S6K1 and Akt are both known to phosphorylate the ER on serine 167. We wanted to test the difference in tamoxifen response according to which of the kinases was responsible for the phosphorylation of ER, by combining either p-S6K with p-ER, or p-Akt with p-ER. No one of the two analyses showed significant interaction comparing low with high expressions, although both combinations tended towards a reduced difference in RFS between tamoxifen-treated and endocrine non-treated patients. *In vitro*, phosphorylation of the ER serine 167 has indicated a reduced tamoxifen response with an increase in ER activation [126]. On the other hand, data from clinical trials show the opposite in breast cancer patients, with serine 167 being a possible good marker for treatment prediction, similar to the serine 118 marker [134].

The only prognostic marker in paper IV was high expression of S6K1, and this was observed for both nuclear and cytoplasmic S6K1 (**Figure 18**). In a multivariate analysis, adjusting for ER, PgR, HER2, and tumor size, the prognostic value remained significant. Of note, the prognostic value of S6K1 was only true in the endocrine untreated group, not in the tamoxifen treated group of the large cohort, or in the small cohort were all were given tamoxifen.

We report a worse prognosis with high S6K1, but not with the high expression of the p-S6K-t389. The S6K1 antibody detected all isoforms (**Figure 17**), while the phospho-specific antibody only detected the longer isoforms. These data support the proposed hypothesis of a more oncogenic short isoform, the 31 kDa S6K1 [193,194].

Figure 17 The S6K1 antibody detects the p85, the p70, and the p31 isoforms in the BT474 breast cancer cell line. The short p31 isoform is not affected by growth factor stimulation as it contains only the N-terminal half of the p85S6K1, and therefore it cannot be detected by the p-S6K-t389 antibody and not stimulated by mTOR. The western blot membrane was previously incubated with a p-ERK1/2 antibody, rendering a strong signal in heregulin 81 (HRG) treated cells.

Prognosis, paper I-IV

A prognostic value of a marker could interfere with the treatment predictive analyses. Therefore, it is important to be aware of the prognostic value of a marker prior to analyzing its treatment predictive value [216]. Of all the phosphorylated biomarkers investigated in this thesis, only p-ER-s167 showed a prognostic value, although this was not significant in a multivariate analysis adjusted for tumor size, HER2, ER, and PgR expression (**Table 2**). This marker indicated a better outcome of breast cancer survival. The prognostic biomarkers found in the present studies were 11q13 amplification, high Pak1 protein expression, S6K1 cytoplasmic and nuclear protein expression (**Figure 18**).

Table 2 Cox univariate analysis of endocrine-untreated primary breast tumors with expression levels of phosphorylated proteins in the Akt/mTOR/ER pathway. The endpoint was breast cancer survival. The significant (**bold**) value of p-ER-s167 did not stay significant in multivariate analysis (p=0.73).

Biomarker	HR (95% CI)	P-value
p-ER-s167 (nuclear)	0.49 (0.26-0.96)	0.037
p-ER-s305 (nuclear)	0.75 (0.46-1.22)	0.25
p-Akt-s473 (cytoplasmic)	1.01 (0.69-1.75)	0.69
p-Akt-s473 (nuclear)	0.72 (0.42-1.25)	0.25
p-mTOR-s2448 (cytoplasmic)	0.78 (0.37-1.73)	0.57
p-S6K-t389 (cytoplasmic)	1.11 (0.64-1.93)	0.70
p-S6K-t389 (nuclear)	0.72 (0.44-1.17)	0.18

Figure 18 Prognostic markers found in the present papers. Hazard ratio (HR) (◆) with 95% confidence interval (bars). Amplification results are from high-risk ER-positive patients and the subsequent markers are from systemically untreated low-risk breast cancer patients. The p-value represents multivariate Cox proportional hazard regression analysis using recurrence-free survival as endpoint, for all but CCND1 and p-ER-s167, where only the univariate analyses were significant, and for p-ER-s167 the endpoint was breast cancer survival.

Treatment prediction, paper II-IV

Knowledge of which pathways the tumor is depending on and how these pathways are affected upon treatment is a step towards individual treatment regimens, so called tailored treatment. ER positivity is a known factor for beneficial tamoxifen treatment, and there are a large number of additional markers with potential to predict endocrine-treatment response, within the ER-positive group, that has not yet proven stable enough in large well-defined cohorts to enter the clinic. Here, we present markers in the mTOR/Akt growth factor pathway, along with ER activation by phosphorylation of the ER together with effector kinases of its specific phosphorylation sites, to predict response to tamoxifen treatment (**Figure 19**).

Figure 19 Forest plot illustrating tamoxifen-treatment prediction in low-risk breast cancer patients. Hazard ratio (HR) (♦) with 95% confidence interval (bars). New biomarkers are selected for ER-positive tumors. At HR 1.0, the tested marker indicates no benefit from tamoxifen when compared to non-treated controls.* p-value for interaction is from test for trend. ** p-value for interaction is adjusted for tumor size. *Italic figures on bar represent the upper 95% confidence interval.*

Thesis at a glance

Paper	Hypothesis	Results	Advantages	Limitations
I	Amplification of 11q13 and protein expression of Pak1 interfere with patient outcome and treatment response.	11q13 amplification and <i>CCND1</i> amplification alone, predicted worse prognosis. <i>PAK1</i> amplification and Pak1 protein expression indicated reduced tamoxifen response.	Randomized cohort with endocrine untreated controls. DNA status with qPCR is a stable method.	Few patients and frozen tissue for protein detection.
II	Pak1 to p-ER-s305 signal is a sign of tamoxifen resistance.	Double positive subgroup was confirmed resistant to tamoxifen. Pak1 cytoplasmic localization showed worse prognosis.	Large randomized cohort with systemically untreated controls. IHC makes protein localization detection possible.	Few events in low-risk cohort requires large groups for sufficient power, thus subgrouping is a problem. Phosphorylated epitope detection is not as stable as total protein detection.
III	Active mTOR signaling and crosstalk with the ER is a mechanism of tamoxifen resistance.	Crosstalk was confirmed as a marker of reduced tamoxifen response and a four-marker score is suggested. P-ER-s167 predicted a beneficial prognosis.	Large randomized cohort. Several markers in one pathway include more tumors in the positive group.	Phosphorylated epitopes One cohort
IV	The S6Ks are oncogenic and markers of mTOR activity, thus interfere with proliferation, prognosis, and treatment response	S6K1, not S6K2, was a marker for worse prognosis. S6K1 nuclear accumulation and active S6K1/2 predicted no tamoxifen benefit. Loss of the S6Ks was involved in proliferation.	Two randomized cohorts Extensively validated antibodies	No nuclear p-S6K in the smaller cohort No untreated control group in the smaller cohort One cell line

We suggest that:

CCND1 gene amplification alone predicts worse prognosis, whereas *PAK1* gene amplification alone is a marker for reduced tamoxifen response.

High cytoplasmic Pak1 protein expression is a marker of worse prognosis.

High phospho-protein expression of ER serine 167 predicts an improved prognostic outcome in adjuvant untreated postmenopausal patients.

A high S6K1 expression is a marker of worse prognosis.

Downregulation of the S6 kinases *in vitro* results in a significant G1 arrest, suggesting an involvement in cell cycle progression.

The antibodies demonstrate high specificity, with potentials for use in detection of clinical biomarkers.

Future perspectives

Several biomarkers with potential values for treatment prediction have been proposed, yet none has been proven to the point that it could be implemented into clinical routine for breast cancer since the introduction of ER, PgR, HER2, and Ki67. One major problem is the lack of sufficiently large cohorts with control groups. Another problem is that one single marker does not have the answer for all patients. Combining several markers previously proven important, may be an option, covering more patients. The more markers in combination, the larger cohorts for evaluation of the markers are necessary. Evaluation of markers in high-risk groups with more events and shorter time to an event is an option to reduce the number of patients needed in such a trial. The use of neoadjuvant trials, with biopsies taken before an intervention, together with tumors from surgery after intervention, is a new strategy to evaluate biomarkers and the response to treatment. To analyze the phospho-markers; p-Akt, p-mTOR, p-S6K, and p-ER in such a trial would gain insight into the effect on signals upon treatment. It would also be interesting to validate the markers found in this thesis in a prospective synchronic tumor set, with paired primary tumors and metastases. Especially the Pak1 protein, which has shown promising metastatic involvement, would be of interest for such a study.

Pak1 showed importance for tamoxifen resistance in cohorts and was also overexpressed in a tamoxifen-resistant cell line. It would be interesting to dig deeper into the oncogenic role of Pak1. Development of a specific p-Pak1 antibody and of Pak1 inhibitors will contribute to further understanding of Pak1 as a potential treatment target.

Adding data on more potential biomarkers in the present cohorts will give further clues about the signaling pathways, treatment prediction, and prognostic values. As the tumor material available is not endless, the next approach may be to gain as much information on the tumors as possible, such as using array platforms. However, new and better methods are constantly developed, and waiting with analyses may render improved results in the future. On the other hand, waiting means withholding possibly important information from patients in need of reliable and valuable biomarkers today. Breast cancer patient series, including an ER-positive control group not given any endocrine treatment, are few. Meta-analyses and common methods to evaluate biomarkers in these cohorts are strategies to evaluate biomarkers for clinical prospective trials. The option is to evaluate the impact of the markers on time-to-event in endocrine-treated tumors, although no true control group is available.

The studies in paper IV could be extended with further studies *in vitro*. The cellular localization of the S6Ks seems important for the role of these proteins. The location of the specific isoforms has been under debate, and the diverse suggestions for their role in tumorigenesis could depend on cell type specific localization. It would be interesting to

determine the location of the isoforms of S6K1 and S6K2, and further investigate their oncogenic potential. A cell cycle distribution assay on starved cells may strengthen results on S6K involvement in proliferation and tamoxifen response, as cell lines normally are overfed. In addition, more than one cell line should be tested. It would be interesting to analyze the cell cycle distribution in ER-positive, tamoxifen-resistant cell lines after S6K knockdown and upregulation, respectively, as a complement to ZR75-1 cell analyses.

Tack

Jag vill börja med att tacka alla patienter som deltagit i studierna och som ger forskningen möjlighet att gå framåt. Kirurger, onkologer, patologer och teknisk personal runtom i landet har gjort studierna möjliga, med insamling av material och analyser. Utan ekonomiska bidrag från Cancerfonden, Vetenskapsrådet, Knut och Alice Wallenbergs stiftelse, Läkaresällskapet, Lions Vimmerby, Rotary Borgholm skulle vi inte kunna bedriva oberoende forskning. Tack alla som skänker en slant och alla Ni som arbetar ideellt med insamlingar!

Olle Stål, min huvudhandledare, min chef. Tack för frihet, inspirerande samtal och för att Du tror på mig och gläds åt varje nytt resultat, även de som går åt "fel" håll! Tack för att Du lär mig nya saker varje gång vi ses och varje gång du läst mina texter! Cecilia "**Cissi**" Bivik, min bihandledare som hoppade på tåget halvvägs genom tunneln. Mitt bollplank, min "arbetsterapeut", tack för all hjälp, inte minst för korrektur av både språk och vetenskap! Birgit "**Bibbi**" Olsson, Bryssel, båtmöten, barndiskussioner, jag tog över efter dig, sen lurade jag tillbaka dig, önskar att Du aldrig slutar! Jag är evigt tacksam för att Du täckte upp för mig under skrivandeperioden, ovärderligt! **Marie** Ahnström-Waltersson, min tidigare nära samarbetspartner och medförfattare i Arbete 1, tack för den grunden jag fick och startkapital att utgå från! Cyklin D1 plasmiderna ligger fortfarande på is om du är sugen på att labba lite;). **Karin** Leifler Söderlund, utan din statistiksammanfattning vore vi alla beroende av statistiker. Du saknas på labbet! **Elin** Karlsson, manus och samarbeten som gått upp och ner som gatorna i San Francisco, San Antonio, Amsterdam och London. Många resor har det blivit, tack för sällskapet! Jag beundrar din förmåga att hålla ordning på alla faktorer, variabler och varianter. Du kommer att bli en strålande PhD! **Hanna** Westman, **Stinorna**, **Munees**, **Hanna** Fransén; "mina" studenter som gästspelat på labbet i projekt, det har varit så roligt att jobba med Er, it's been a pleasure! Good luck! **Veronika Brodin-Patcha**, rumskamraten, glädje och skratt, jag älskar din öppenhet och ärlighet! Tack för att du delade med dig av din konstnärliga talang till kappan! **Birgitta Holmlund**, Du är snäll och god, jag gillar om dig! Din tekniska expertis är så värdefull! Tack för stödet och sällskapet på ditt kontor, tillsammans med vänliga, peppande **Kerstin**, under ramskrivandet! **Gizeh**, din fantasi har inga gränser. Håll forskningsglöden och forskningsglädjen vid liv, Du kommer att bli en av de excellenta! **Erik**, våga kliva utanför boxen och lita på dina idéer, Du har "crazy-science" i blodet! Kram på dig! **Agneta**, både vetenskapliga texter och sångtexter är din grej. Våra diskussioner går från "lökar" till lökar. Dr **Tove**, saknar dig! **Cynthia**, bella! Tack för alla borttagna streck! In the world you MEET no limits, bjartar vonir rætast, þetta er ágætis byrjun. **Helena**, coollugn statistiker med ögon för Bonferroni. **Sander**, välkommen till Oncostars! **Linda**, Du anar inte vad din energi gjort för onkologlabbet, och för mig. Du kommer alltid vara på plussidan! Om det är Nobelpriset som är ditt mål, blir jag inte förvånad. Go get it! **Chatarina**, fixar allt! Sprider glädje, man blir glad nära dig! **Suss**, tack för spiken! Du kommer att bli den läkaren jag vågar lämna mina barn i händerna på när de behöver vård. Du har en förståelse som ingen annan. Hela onkologlabgänget inklämda i ett hotellrum på Villa Fridhem, that was fun! **Xiao-Feng**, **Sebastian**, **Ivana**, **Surajit**, **Wen-Jian**, **Atil**, **Annika**, **Åsa**, **Daniella**, **Annelie**, **Vivian**, **Amet**, **Tina**, **Karin**, **Håkan**, **Ammi**, **Bibbi** och resten av KEF-gänget, tack för trevligt sällskap över oändliga kaffekoppar och överdådiga frukostar! **Malin** och **Julia**, räta och aviga och så fika på det! **Christina Ekerfelt**, samordnare för Biomedicinska forskarskolan 2005, och min första kontakt med LiU, nuvarande prefekt för IKE. Tack för att du antog mig då och för att du idag gör IKE till en bra institution att verka på! **Charlotta Dabrosin**, ämnesföreträdare i onkologi, tack för att jag fått möjligheten att fördjupa mig i onkologisk forskning vid Linköpings universitet! Nu går vi vidare och lägger till ytterligare pusselbitar till cancers alla gåtor!

Farmor Nancy, tack för att Du såg mig! När jag ville bli konstnär gav Du mig oljefärger, när jag som nioåring ville stava mitt namn med ph, var Du den som kom ihåg det. Jag saknar dig, Du borde varit här nu, på den här resan! Boken är tillägnad dig, för detta, och för att Du är en av alla de som drabbats.

Mina fina syskon med familjer; **Jennifer, Jonathan o Rebecca**, tack för att jag får låna ut tjejerna till Er ibland, **Oscar**, och **Albin**, och så underbara **August** med sin sprudlande energi och gosiga kramar! Älskar Er! Må vi fortsätta ses över middagar och annat skoj! Moster **Ulla**, som bett om att få läsa mina publikationer och med glada tillrop inför disputationen peppade mig en mörk oktoberkväll. Johnsson-familjen; **Eva, Benna, Sofie, Andreas** och **Hanna** som direkt tog emot mig med öppna armar. Tack för alla trevliga samtal, spel, promenader och omtanke! **Pappa o Karin**, jag önskar vi sågs oftare, hos Er känner jag mig alltid välkommen! **Mamma**, Du är en kämpe! Tack för din trygghet och för friheten Du gav mig. Tack för barnvakt och samtal! Jag älskar dig! **Stefan**, tack för att Du hängde med till Uppsala för grundutbildning, och senare till Linköping för doktorandutbildning. För att Du gav mig två mirakel i livet. Jag önskar dig all lycka! **Marie**, min älskade vän från telefonkiosken, önskar dig närmare i framtiden! Än så länge möts vi halvvägs mellan Kalmar och Linköping; på Gränsö spa, och i Vimmerby. Låt oss göra fotspår i sanden! Barnen; **Alice♥** och **Sigrid ♥** mina mirakel, min glädje, min lycka, min mening, jag älskar Er oändligt! **Robert ♥** Du är inget roder som styr mig, Du är ett segel som för mig framåt. Du är fjärlarna i min mage, Du är stenen jag vilar på när vingarna inte bär. Jag älskar dig obeskrivligt mycket!

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