ALTERATIONS IN THE PI3K/AKT SIGNALING PATHWAY AND RESPONSE TO ADJUVANT TREATMENT IN BREAST CANCER

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Linköping 2008
Cover illustration: “Light shining through the intertwined branches of a signaling pathway”. Picture reproduced with permission of Massimiliano Gentile.

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Permission was obtained to reprint papers I-III.
ISSN 0345-0082
Printed in Sweden by LiU-Tryck, Linköping 2008
To the patients who made possible these studies
To my mother
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Crosstalk between ERs, HER-2 and the phosphatidylinositol 3’ kinase (PI3K)/AKT signaling pathway could be a cause of therapeutic resistance in breast cancer. The PI3K/AKT pathway controls cell proliferation, cell growth and survival, and its members include oncogenes and tumor suppressor genes. Alterations in this pathway are frequent in cancer. In this thesis, we aimed to study the biological significance of some of these alterations in a tumor context as well as their clinical value. PIK3CA gene, encoding the PI3K catalytic subunit, was examined for mutations. The tumor suppressor PTEN, that counteracts PI3K-mediated effects, was studied at the protein level whereas amplification of RPS6KB1 (S6K1) and RPS6KB2 (S6K2) genes, encoding two substrates of the mammalian target of rapamycin (mTOR) acting downstream PI3K/AKT, was also inspected. AKT phosphorylation or activation (pAKT) was determined by immunohistochemistry. Other factors related with this pathway, such as HER-2, heregulin (HRG) β1, the cell cycle inhibitor p21\textsuperscript{WAF1/CIP1}, the pro-apoptotic factor Bcl-2, and cyclin D1, were also considered. These studies were performed in two patient materials consisting of premenopausal patients that received endocrine treatment (paper I) and postmenopausal patients randomized to receive radiotherapy (RT) or chemotherapy (CMF) in combination with tamoxifen (Tam) or no endocrine treatment (papers II-IV). In the first material, we found that pAKT indicated higher risk of distant recurrence among endocrine treated patients. In the second material HRGβ1 induced accumulation cytoplasmic p21 \textit{in vitro} and pAKT was associated with cytoplasmic p21 in the tumors. In addition, p21 cellular localization identified subgroups of ER+ patients with different responses to tamoxifen. Other alterations such as PIK3CA mutations and PTEN loss were positively associated in this material. PIK3CA mutations lowered the risk
for local recurrences while PTEN loss conferred radiosensitivity as a single variable or combined with mutated PIK3CA. PIK3CA mutations and/or PTEN loss was associated with lower S-phase (SPF). Nevertheless, among patients with low proliferating tumors, these alterations predicted higher risk of recurrence in contrast to those with high proliferating tumors. Finally, we found amplification of the S6K1 and S6K2 genes. S6K2 amplification was associated with cyclin D1 gene amplification, predicted poor recurrence-free survival and breast cancer death, and indicated benefit from tamoxifen. On the other hand, S6K1 amplification was associated with HER-2 amplification/overexpression, indicated higher risk of recurrence and was a predictor of poor response to radiotherapy. These results indicate the potential of this pathway as therapeutic source.
SAMMANFATTNING


En sådan receptor är HER-2 vilken överuttrycks i 15-20% vid bröstumörer. HER-2 receptorn kan rekrytera proteiner med enzymatisk aktivitet, till exempel PI3K. PI3K aktiverar ett annat enzym, AKT, vilket är inblandat i en kaskad som leder till tumörtillväxt och tumöröverlevnad (genom till exempel aktivering av östrogenreceptorn). Våra resultat hitills visar att patienter med aktiverat AKT (pAKT) har större risk att få metastaser och därmed sämre överlevnad än patienter utan pAKT, detta trots hormonell behandling. I större material där HER-2 proteinuttrycket korrelerar med pAKT har vi också funnit att patienter med AKT-negativa tumörer kunde dra nytta av både tamoxifen och strålbehandling. Vi har även undersökt PIK3CA genen (som kodar för en del av PI3K) och hittat mutationer i 24% av bröstumörerna. Det är dock ännu oklart hur dessa mutationer ska tas hänsyn till för att kunna bestämma en effektiv behandling. PTEN är ett annat enzym som motverkar PI3K-aktivitet. Bortfall av PTEN förekommer ofta i bröstcancer och har associerats med PI3K/AKT aktivering. I vårt material var PTEN-förlust frekvent (37%) och associerades med PIK3CA mutationer. PTEN förlust som ensam faktor eller tillsammans med PIK3CA mutationer ökade strålkänslighet. Andra proteiner som är inblandade i PI3K signalvägen är S6K1.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AKT</td>
<td>v-akt murine thymoma viral oncogene homolog</td>
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<tr>
<td>pAKT</td>
<td>Phospho AKT or activated AKT</td>
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<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
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<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1</td>
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<tr>
<td>BAD</td>
<td>BCL2-antagonist of cell death</td>
</tr>
<tr>
<td>Bax</td>
<td>BCL2-associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>BRCA1,2</td>
<td>Breast cancer 1 and 2, early onset</td>
</tr>
<tr>
<td>CCND1</td>
<td>Gene encoding cyclin D1</td>
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<tr>
<td>CDK</td>
<td>Cyclin dependent kinases</td>
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<tr>
<td>CHK</td>
<td>CHK checkpoint homolog</td>
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<tr>
<td>c-Myc</td>
<td>v-myc myelocytomatosis viral oncogene homolog</td>
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<tr>
<td>CTTN</td>
<td>Cortactin</td>
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<tr>
<td>4EBP-1</td>
<td>Transcription factor 4E binding protein 1</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor-receptor</td>
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<tr>
<td>eIF4</td>
<td>Eukaryotic translation initiation factor 4A</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FKHR</td>
<td>Forkhead member of transcription factors</td>
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<tr>
<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
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<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HER-2</td>
<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog</td>
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<tr>
<td>HRG</td>
<td>Heregulin</td>
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</table>
ABBREVIATIONS

(17β)HSD1  17β-hydroxysteroid dehydrogenase
I-κB       Inhibitor of nuclear factor κB
IGF        Insulin-like growth factor
IKK        I-κB kinase
ILK        Integrin-linked kinase
IRS-1      Insulin-receptor substrate 1
JUN        Jun oncogene
LRRC32/GARP Leucine rich repeat containing 32
MAPK       Mitogen-activated protein kinase
MEK        Mitogen-activated and extracellular signal-regulated-kinase
Mdm2       Murine double minute 2
MKKK4      Mitogen activated protein kinase kinase-4
mTOR       Mammalian target of rapamycin
NF-κB      Nuclear factor κB
p21\(^{WAF1/CIP1}\)  Cell cycle inhibitor
PAK1       p21/Cdc42/Rac1-activated kinase 1
PCNA       Proliferating cell nuclear antigen
PDGFR      Platelet derived growth factor receptor
PDK        Protein dependent kinase
PgR        Progesterone receptor
PHLPP      PH domain and leucine rich repeat protein phosphatase
PI3K       Phosphatidylinositol 3’ kinase
PIK3CA     Gene encoding the PI3K catalytic subunit p110α
PIK3CR     Gene encoding PI3K p85α subunit
PKB        Protein kinase B
PKC        Protein kinase C
PPAR\(_\text{g}\) Peroxisome proliferator activated receptor g
**ABBREVIATIONS**

PPM1D Human wild type p53-induced phosphatase 1  
PTEN Phosphatase and tensin homolog deleted on chromosome 10  
P_{70}S6K 40S ribosomal protein S6 kinase  
RAD51 RAD51 homolog (RecA homolog, E. coli)  
Ras Rat sarcoma viral oncogene homolog  
RB Retinoblastoma protein  
Rheb GTPase Ras homolog enriched in brain  
RPS6KB Gene encoding p70S6K  
SPRY2 Human sprouty homolog 2  
TBX-2 T box transcription factor-2  
TGF Transforming growth factor  
TSC1/2 Tuberous Sclerosis Complex proteins
This thesis includes the following papers:


The female breast develops progressively stimulated by estrogens, progesterone, and other growth and inhibitory factors. A delicate interplay of all of these stimuli guarantees that some cells proliferate; others rest, while others die in a concerted way. Despite this, at some point, breast cancer may arise. The disease is difficult to define because of its heterogeneity, unknown timing, different primary target cells, as well as the multitude of genes, and signaling pathways involved. Endocrine therapy, especially tamoxifen, remains the most used systemic treatment in breast cancer, with estrogen receptor (ER) expression as the guide for the therapeutic decision. Tamoxifen inhibits ER-mediated gene transcription, leading to cell cycle arrest and apoptosis. However, in spite of a high response rate, tumor resistance may develop over time affecting patient’s survival. Antiestrogen resistance has been explained by several mechanisms, including interactions between growth factor receptors and ER cascades. Especially interesting for us has been the crosstalk between ERs, HER-2 and the phosphatidylinositol 3’ kinase (PI3K)/AKT signaling pathway. The PI3K/AKT pathway controls biological functions such as cell proliferation, cell growth and survival, and its members include oncogenes and tumor suppressor genes. Alterations in this pathway are frequent in cancer, providing the tumor cells with survival and proliferative advantages.
THE NORMAL BREAST

Changes in the female breast are more notorious at puberty when the glandular and the connective tissue develop to ensure milk production. The mammary glandular tissue is composed of a network of ducts that end in the functional units of the breast: the lobules (Figure 1). Each lobule consists of around 20 small glandular structures called acini, alveoli, or ductules (Robert B. Clarke, 2002) which open into the terminal duct called terminal duct lobular unit (TDLU). When an average of 11 acini cluster around the terminal duct, they are called lobule type 1 (lob 1) which become lob 2 and 3 by branching and differentiation. Lob 4 structures appear only after pregnancy.

Figure 1. Diagram of the normal breast representing branches of the ductal network ending in lobules. The putative stem cells appear as a black dot in the lobule 1 structures at the terminal ductal lobular units.
INTRODUCTION

Acini, like ducts, are ring-shaped structures with a layer of epithelial cells lining the lumen. In the adult lactating gland, the acini enlarge and the cytoplasm of the epithelial cells fills with milk-containing vacuoles. Each lobule has a lactiferous duct that allows the passage of milk toward the nipple, where it collects in a widening of the ducts called sinuses. The entire ductal network is called a ductal tree and is composed of three cell lineages: luminal or alveolar epithelial cells lining the lumen in the TDLU, that produce milk, ductal epithelial cells, and a more external layer of contractile myoepithelial cells in contact with a basal membrane, which facilitates milk passage (Figure 2).

Besides the glandular structures, the breast also contains connective tissue with blood and lymphatic vessels, adipose, and nervous tissue, which provide nutrition and support. The mammary gland is a hormone responsive organ, and its development requires estrogen and progesterone, two ovarian hormones acting on their receptors: estrogen receptor and progesterone receptor (PgR). In the normal gland both estradiol and progesterone regulate cell growth in a paracrine fashion (Anderson et al., 1998), by stimulating the local production of growth factors such as transforming growth factor α (TGFα), epidermal growth factor (EGF), insulin-like growth factor (IGF), amphiregulins, and heregulins (HRG). Many of these growth factors share common signaling pathways such as mitogen activated protein kinase (MAPK) and PI3K/AKT, whose activation ultimately leads to cell growth by induction of cell cycle regulators such as cyclin D1 and ER-activation. The action of estradiol is manifested in ductal growth and dichotomous branching. On the other hand, progesterone seems to be more important during pregnancy, when it stimulates epithelial cell proliferation, branching, and lobular differentiation.
INTRODUCTION

However, the effects of progesterone remain controversial, since in vitro studies have shown that progesterone can both stimulate and inhibit cell division (Musgrove et al., 1991).

Most of the ER and PgR- expressing cells are located in the luminal layer of the epithelium, where more than 90% of steroid-mediated cell proliferation occurs. Differences in expression of cytokeratin (CK) and other markers allow segregation of the luminal and basal epithelial cell subpopulations, the latter originating from epithelial stem cells. These cells give rise to luminal ER+ and ER- cells, and to the myoepithelial basal cells (Polyak, 2007). In mice stem cells with self renewal capacity that can generate both the ductal and lobular component of the mammary tree have been identified (Kordon & Smith, 1998) whereas in humans the identity of the normal stem cell remains elusive. Both luminal and basal breast cancers are believed to originate from mammary stem cells or progenitor cells located at the end buds of the TDLU (Polyak, 2001), but other evidence indicates that these cells may be found in the ducts (Villadsen et al., 2007).
INTRODUCTION

Figure 2. Diagram of the TDLU. Arrows indicate the three cell lineages present in the ductal network and the basal membrane. Modified from (Polyak, 2007)

1.1 HORMONES AND RECEPTORS

Estrogens exist in form of estrone (E1), estradiol (E2), and estriol (E3). A two-step reaction catalyzed by the enzymes aromatase and 17β-hydroxysteroid dehydrogenase (17βHSD1) converts androgens to estradiol. Estradiol is thought to be the driving force of the ductal growth in the mammary gland, but also influences endometrial growth and cyclic changes, as well as differentiation of the follicles. The ovaries are the main source of estradiol are in premenopausal women, while in postmenopausal women the peripheral tissues (adipose tissue, skin and muscle) are the primary source. Estradiol exerts its actions through the ER, which belongs to the nuclear receptor family. Upon ligand activation, the ER dimerizes and binds to the DNA, thereby acting as a transcription factor.
INTRODUCTION

Figure 3. Different pathways leading to ER activation. ERE (estrogen responsive element), TF (transcription factor), GF (growth factor), GFR (growth factor receptor), pi (phosphorylation). The bent arrows indicate transcriptional activation. Modified from (Heldring et al., 2007).

In the classical pathway, the ER is directly coupled to the sequence of estrogen response elements (ERE)-containing genes. In the non-classical pathways the ER can also interact with the DNA by means of other transcription factors or be involved in non-genomic actions arising from the cell membrane (Pappas et al., 1995). Moreover, the ER can be activated by phosphorylation in a ligand-independent way (Figure 3)
INTRODUCTION

ER was renamed to ERα after the discovery of the ERβ, cloned in 1996 (Kuiper et al., 1996). Both hormone receptors bind to estradiol but are expressed in different tissues, and seem to have opposite biological effects. In mammals, both ERs are expressed in the luminal cells of the normal breast, but the ERβ can be also found in myoepithelial cells and the surrounding stroma (Speirs et al., 2002). Functionally, ERα regulates normal and malignant cell growth in a paracrine or autocrine fashion, respectively; while the ERβ has been considered a tumor suppressor due to its ability to suppress the transcriptional effect of the ERα, and to its anti-proliferative and pro-apoptotic effects during carcinogenesis (Saji et al., 2005). The progesterone receptor is an estrogen-regulated gene used to indicate the functionality of the ER, for example, patients with ER+/PgR+ tumors receive more benefit from tamoxifen when compared to the ER+/PgR- group (Ravdin et al., 1992). PgR has been detected in some ER- cases indicating a false negative result or poor assay sensitivity. The predictive value of PgR in absence of ER is still under discussion (EBCTCG, 1998).

.2 BREAST CANCER

.2.1 Epidemiology

According to data published in 2006 by the National Board of Health and Welfare (http://www.socialstyrelsen.se/en/), breast cancer represents 29.4% of all female cancers and it is the most common malignancy among
women in Sweden. Only in 2006, 7059 new cases were diagnosed, 60% of them were ≥ 60 years old women and only 3.8% were < 40 years at the time of diagnosis, indicating that breast cancer risk increases with age. More than 82 000 women live with the disease (diagnosed between 1958 and 2006) and approximately 1500 die every year, with breast cancer as the cause of death. Breast cancer in men has also been reported, though it is infrequent. Only 36 men received this diagnosis during year 2006. Despite the high incidence in Sweden among women (a 1.3%/year increase over a 20 year period), the mortality rate has decreased in western countries due to improvements in mass screening, increased use of adjuvant systemic treatment, and introduction of new drugs.

2.2 Etiology

Breast cancer is a heterogeneous disease that develops under a long period following several yet uncharacterized steps. Neither the identity of the first malignant cell nor the decisive genetic alterations that lead to breast cancer are known, which makes it difficult to reach a consensus about the etiology of the disease. In some cases of hereditary cancer the family history plays a decisive role for development of the disease, but mutations of the BRCA1, BRCA2 (breast cancer 1 and 2 respectively), and TP53 genes only accounts for the minority of breast cancers which indicates the existence of some other factors. It is speculated that the likelihood of breast cancer occurrence depends on the number of stem cells at risk, which is determined from the time in the uterus or early in life. In adult life, the interplay of growth factors, hormones, and their receptors stimulate survival and proliferation of mutated cells, whereas pregnancy may cause
INTRODUCTION

breast cancer progression by either disruption of the normal cell microenvironment during the phase of breast involution, or by promoting expansion of the already initiated cells (Bissell, 2007). An etiologic model, including both epidemiological and experimental data, brings some understanding of the causative agents and their timing (Trichopoulos et al., 2005). For example, high mammographic density, high mammary gland mass, big size of the breast, adult height, and birth size are likely to reflect the total number of mammary stem cells present and associated with higher risk. Other factors, such as earlier menarche, late menopause, postmenopausal-overweight, hormone replacement treatment, and alcohol intake are related to the influence of hormonal and growth factors. The risk of breast cancer increases otherwise with age and depends on the lifestyle and environmental conditions.

2.3 Heterogeneity

Breast cancer cells diverge, both genotypically and phenotypically, depending on the molecular alteration that originated the tumor, the cell type that originated the tumor, and the fact that mammary cells have different susceptibility to malignant transformation. Breast cancer is thought to originate after a multistep carcinogenesis. The multistep model of breast cancer proposes a linear development of the disease from hyperplasia to carcinoma in situ and then to invasive and metastatic carcinoma (Figure 4). Progression occurs under the control of hormones/hormone receptors, growth factor/growth factor receptors, and oncogenes/tumor suppressor genes (Beckmann et al., 1997) which upon
genetical alterations equip the epithelial cells with proliferative and survival advantages.

Recently, the disease has been classified into five different entities with specific gene expression profiles (Andre et al., 2007; Perou et al., 2000). The groups, luminal A and B, basal cell-like, HER-2+ and normal-like, have been matched to the known clinical variables (Calza et al., 2006). Luminal A tumors express ER and seem to have a better prognosis compared to the other subtypes. Basal cell-like tumors are negative for ER, PgR, and HER-2, frequently present p53 mutations, are positive for the epidermal growth factor-receptor (EGFR) and express CK 5 and 17. BRCA1 mutated cancers typically represent this group. HER-2+ types overexpress HER-2 and are clearly a distinct subgroup that receives advantage from some therapeutic modalities. The characteristics of the luminal B and the normal-like types are not well defined. The five types are already present at the ductal carcinoma in situ stage (DCIS) (Yu et al., 2004) which may suggest different tumor progression pathways for each of them (Polyak, 2007).
INTRODUCTION

Figure 4. Multi-step model of breast cancer initiation, progression and metastasis. The process is influenced by genetic, epigenetic or environmental alterations. Arrow-heads indicate that the identity of the target genes is still unknown. Modified from (Polyak, 2001).

Moreover, molecular subtypes that differ in their degrees of proliferation and differentiation have been reported, which support once more the idea of breast cancer heterogeneity (Bertucci & Birnbaum, 2008). Differences at the intratumoral levels, based on different primary target cells have been explained by two theories, the clonal evolution and the stem cell hypothesis, which agree on the monoclonal origin of breast cancer and disagree on the identity of the primary target cell. According to the stem cell theory, breast cancer originates in a small stem cell population that persists in the tumor during its initiation, progression, and recurrence. These cells have the ability of cell renewal and differentiation giving rise to all the cells in the tumor and to tumor heterogeneity. On the other hand, the clonal evolution model
supports the idea that breast cancer originates from a normal cell that undergoes multiple mutations, which confer the most aggressive and tumor-driving phenotype to malignant cells. However, newer experimental data support a new model, in which tumor cells could originate from a normal mammary cell or progenitor cell, and then self-renew or undergo a combination of differentiation and clonal selection due to the natural pressure of the environment and mutations. In this way the tumor would be composed of a combination of differentiated and less proliferative cells as well as self-renewing cells with proliferative advantages acquired from the mutations (Campbell & Polyak, 2007).

.3 ONCOGENES AND TUMOR SUPPRESSOR GENES

.3.1 Oncogenes

Oncogenes were first identified in a virus as altered forms of cellular genes (proto-oncogenes) able to transform normal cells by altering their phenotype and conferring tumorigenic properties. Oncogenes can encode growth factors, growth factor receptors, Ser/Thr protein kinases, nuclear transcription factors, GTPases, and other factors related with growth and differentiation. Therefore, these genes are tightly regulated, and when this control fails cancer may arise. Proto-oncogenes can be activated by different mechanisms such as mutations, chromosome rearrangements, increased gene expression, and epigenetic mechanisms, which taken together lead to increased protein expression or constitutive activation of the gene product. A common mechanism in breast cancer is gene amplification, which associates with increased copy number of a certain
INTRODUCTION

gene relatively to the rest of the genome. Examples of chromosome areas affected in breast cancer by amplification are the chromosomal regions 8p12, 8q24, 11q13, 17q12, 17q23 and 20q13 (Letessier et al., 2006; Sinclair et al., 2003).

3.1.1 Amplification in the 11q13

The chromosome locus 11q13 is amplified in up to 15% of breast cancers (Ormandy et al., 2003). This region harbors four distinct cores of amplification. Some of the genes found in these cores are LRRC32 or GARP (leucine rich repeat containing 32) and PAK1 (p21/Cdc42/Rac1-activated kinase 1) in core 1 (Bostner et al., 2007), CCND1 (cyclin D1) in core 3, and EMS1 or CTTN (cortactin) in core 4. High frequency of amplification in some of these regions indicates that important oncogenes may be contained within them. One of the most promising candidates is cyclin D1. Cyclin D1 is a cell cycle regulator that binds cyclin dependent kinases (CDK) 4/6 to drive G1-S progression. Cyclin D1 is frequently amplified and overexpressed in breast cancer (Dickson et al., 1995), and often associated with ER expression. In vitro studies have shown that cyclin D1 promotes ER-activation (Zwijsen et al., 1997). Cyclin D1 overexpression in breast cancer is associated with growth factor independency (Muskgrove et al., 1994) and tumorigenesis in transgenic mice (Wang et al., 1994) and its clinical value to predict both disease-free/overall survival (Bieche et al., 2002) and response to therapy (Ahnstrom et al., 2005; Jirstrom et al., 2005; Muskgrove et al., 1994; Rudas et al., 2008; Wang et al., 1994) has been reported.
.3.1.2 Amplification in 17q12 and 17q23

Chromosomal region 17q12-21 is often amplified in breast cancer and the major oncogene candidate in this area is the HER-2 gene (Yokota et al., 1986). Gains in the 17q22-24 area were first reported in primary breast cancers in 1994 (Kallioniemi et al., 1994) and thereafter in other studies (Sinclair et al., 2003). Gains in the 17q23 area have also been reported in other tumors, but the higher level of amplification predominates in breast cancer. In vitro studies with the breast cancer cell line BT-474 detected two peaks of amplification, the first containing the HER-2 gene and the other that was distally located to the 17q22-24 region (Barlund et al., 1997). Further analysis identified RPS6KB1, T box transcription factor-2 (TBX-2) gene, and the human wild type p53-induced phosphatase 1 (PPM1D) as possible oncogene candidates in the 17q22-24 area due to its amplification and overexpression in MCF-7 cells (Couch et al., 1999; Sinclair et al., 2003). The role of each gene in this amplicon is obscured due to co-amplifications with or without protein overexpression and different cellular contexts.

.3.2 Tumor suppressor genes

Tumor suppressor genes (TSGs) are those genes that cause malignancy by loss of its function. TSGs often hinder malignant transformation due to negative regulatory effect on cell growth or by participating in DNA repair and apoptosis. In a minority of breast cancers, these genes are affected by germline mutations and inherited (present in all the cells of the body) but in sporadic cases, which are the commonest manifestation of breast cancer, the same genes can harbor sporadic somatic mutations (in some cells of the
INTRODUCTION

Body). Opposite to oncogenes, TSG can be inactivated by allelic loss or loss of heterozygosity (LOH) where the part of one chromosome containing the TSG is lost while the other chromosome is unaffected. According to the “two hits” hypothesis, proposed by Knudson (Knudson, 1971), TSGs unmask the malignancy usually by alterations of the two alleles, which may occur by inherited mutation of one allele followed by somatic mutation or loss of the other. This hypothesis, proved to be true for retinoblastoma disease resulting the susceptibility gene (RB1) in the first TSG to be reported. In some cases there are other mechanisms involved in the inactivation of the gene product such as promoter methylation (impairs transcription), increased proteasomal degradation, increase in some other proteins that interferes with its function or cell delocalization and microRNAs. Some examples of TSG in breast cancer are: RB, p16, TP53, BRCA1 and BRCA2, CHK2 (CHK checkpoint homolog 2), ATM (Ataxia telangiectasia mutated) and PTEN (Phosphatase and tensin homolog deleted on chromosome 10) that will be discussed below (Osborne et al., 2004).

.4 PROGNOSTIC AND PREDICTIVE FACTORS

Breast cancer prognosis is generally good and many patients live longer without relapses. However, for some patients the relapses appear already within the first 5 years after the diagnosis but a recurrence may occur even after 10 years or more. Therefore, it is important to divide the patients into different risks groups to treat them efficiently. With help of prognostic factors, it is possible to envisage the natural course of the disease while the predictive factors provide information on the likelihood of the treatment
response. At present, the most important prognostic factor in the clinic is the TNM system (Table I), which allows tumor classification according to the size of the tumor (T), the nodal infiltration (N) and the presence of metastasis (M). Another useful prognostic indicator is the Nottingham grade including the degree of nuclear atypia, the degree of tubular formations, and mitotic activity. In Sweden, and other countries, this grading system is used (Elston & Ellis, 1991). Other factors predicting breast cancer survival and response to treatment are those related with cell proliferation (thymidine labeling index, mitotic index, Ki-67, PCNA, and bromodeoxyuridine labeling). Among them, the S phase fraction (SPF) is a valuable prognostic factor (Stal et al., 1993).

Table I. TNM system.

<table>
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<tr>
<th>STAGE</th>
<th>CRITERIA</th>
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<tr>
<td>0</td>
<td>Carcinoma in situ</td>
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<tr>
<td>I</td>
<td>Tumor ≤ 2cm, axillary lymph nodes not involved</td>
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<tr>
<td>II</td>
<td>Tumor 2-5 cm and/or involved but mobile axillary lymph nodes</td>
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<tr>
<td>III</td>
<td>Tumor &gt; 5 cm and/or fixed axillary lymph nodes; includes inflammatory breast cancer</td>
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<tr>
<td>IV</td>
<td>Distant metastases beyond ipsilateral axillary lymph nodes</td>
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More than 70% of breast cancers express ER which is used to predict patient outcome and response to tamoxifen (Becwoda et al., 1991; Clark & McGuire, 1988; Clark et al., 1984; Heel et al., 1978; Osborne et al., 1980). However, 30% of the ER+ tumors are non-responsive to the treatment (de novo resistance) and many others become refractory (acquired resistance).
INTRODUCTION

in presence of the receptor, indicating that the mere presence of ER is not the ideal predictive marker and other factors are needed (Payne et al., 2008). In an effort to satisfy the individual needs of the patients, newer array based-analysis have been developed. Examples of these are the 70 gene-signature (van 't Veer et al., 2002) that predict short interval to metastasis among the node negative patients, the 231 gene-signature associated with survival (van de Vijver et al., 2002), and the 93 gene-signature (Sotiriou et al., 2003). Many of the genes involved in these signatures are related to cell cycle regulation, invasion, metastasis, angiogenesis, DNA-replication or chromosomal stability. However in order to design the most optimal experiment to be able to choose the appropriate prognostic or predictive marker, among thousands of factors, it is vital to know the genes, proteins and pathways that lie behind the resistance.

.5 TREATMENT

The most common treatments in breast cancer are surgery, radiotherapy, chemotherapy, endocrine treatment and antibodies. Surgery can conserve part of the breast (breast-conserving) or remove the whole gland (mastectomy). Radical mastectomy is preferred in case of large tumors, several tumors, and inflammatory or diffuse cancer among other requisites. In order to control tumor spreading a biopsy is taken from the first nodes that receive lymph from the tumor (sentinel nodes). This technique often replaces the axillary dissection in patients without evident lymphonode infiltration. Surgery is often the initial treatment followed by other auxiliary or adjuvant treatment. Radiotherapy, for example, is often recommended after breast-conserving surgery or to patients with lymphonodal infiltration. The main purpose of this treatment is to reduce the risk for local
recurrences. Patients in this thesis received 46 Gy with 2 Gy/fraction 5 days a week for a total of 4.5 weeks. The standard treatment in South-East Sweden is 50 Gy in 25 fractions. Another adjuvant treatment is chemotherapy also called CMF in this thesis due to the three components comprised in this regime (cyclophosphamide or chlorambucil, methotrexate and fluorouracil). CMF are cytostatic substances mostly affecting the proliferative fraction of tumor cells. The risk for breast cancer-death is reduced when CMF followed by 5 years tamoxifen (see below) is applied directly after surgery compared to surgery alone (Bergh J et al., 2007).

Tamoxifen, a selective estrogen receptor modulator (SERM), is the most common therapy used in the ER+ breast cancers. Tamoxifen (ICI 46,474) (Harper & Walpole, 1967), that had been a failure as a contraceptive agent, was first used in 1971 to treat breast cancer (Clarke et al., 2001; Jordan, 2003). This compound binds to the ligand-binding domain (LBD) of the ER antagonizing the actions of estradiol and the receptor association with co-activators. In addition to these effects, tamoxifen has also agonist properties in other tissues such as heart and bone and is associated with increased risk of endometrial cancer (Riggins et al., 2007). Another class of compounds in use are the aromatase inhibitors (AI) which target the enzyme that converts androgens to estrogens. Both substances are used in postmenopausal women where the principal sources of the hormone are the peripheral tissues. The treatment of choice for premenopausal women, besides tamoxifen, are the gonadotropin releasing-hormone (GnRH) analogs like goserelin (Zoladex). Production of estrogen by the ovaries is stimulated by luteinising hormone (LH) and follicle stimulating hormone (FSH), produced by the pituitary gland. Goserelin stops the production of LH from the pituitary gland, which leads to a reduction of oestrogen. Thus, tamoxifen, AIs and GnRH analogs act through different mechanisms to
deprive the cells of estrogens stimulatory actions. Finally, those tumors that overexpress HER-2 are treated with trastuzumab, a monoclonal antibody, often given in combination with cytostatics.

.6 TAMOXIFEN AND TAMOXIFEN RESISTANCE

Antiestrogen resistance may be explained by several mechanisms, including loss or mutation of ER, increased estradiol level, alterations in antiestrogen metabolism or interactions between growth factor receptors and ER cascades (Clarke et al., 2001; Riggins et al., 2007). These mechanisms, mainly involved in cell proliferation (Doisneau-Sixou et al., 2003), may coexist with those affecting cell death. Increasing amounts of evidence indicates that the mechanisms whereby drugs such as the GnRH analogues, AI and tamoxifen exert the cytotoxic action also include apoptosis (Imai & Tamaya, 2000; Perry et al., 1995; Riggins et al., 2005). Therefore, factors involved in the apoptotic failure may also contribute to the antiestrogen resistance. Among the effects of tamoxifen are reduction in expression of c-myc and cyclin D1, accumulation of hypophosphorylated RB protein, nuclear induction of the cell cycle inhibitors p21WAF1/CIP1 and p27Kip1, inhibition of Bcl-2 and induction of Bax expression. The question is how cancer cells circumvent these effects to survive and proliferate.

One answer could be the crosstalk between the signaling pathways emerging from the ER and other growth factor receptors (Figure 5). For example, EGFR, HER-2 and IGF-1R are often elevated in unresponsive tumors (Johnston et al., 2003; Nicholson et al., 1999). Several other studies have suggested that overexpression of HER-2 in ER+ cell lines confers resistance to the endocrine treatment, being the PI3K/AKT pathway often
in the same picture (to be discussed below) (Kurokawa & Arteaga, 2003; Nelson & Fry, 2001; Zhou et al., 2001).

For some years ago, the PI3K/AKT cascade, which is the major survival pathway for many cell types, was shown to activate the ER protecting the cells from tamoxifen-induced apoptosis (Campbell et al., 2001). Since then the amount of experimental evidence has increased. Overexpression of activated AKT in breast cancer cells induces estrogen independence and resistance to the endocrine treatment while its inhibition causes the opposite effect. Cell lines selected against tamoxifen relay on AKT activation to conserve this phenotype (Frogne et al., 2005). Moreover, the mammalian target of rapamycin (mTOR), is activated by AKT in tamoxifen-resistant cells, that upon rapamycin treatment recover response to tamoxifen. AKT can also sequester p21WAF1 and p27KIP1 (Zhou et al., 2001) in the cytoplasm where these proteins are unable to mediate the cytostatic effects of tamoxifen. On the other side, AKT can also induce the transcriptional activity of ERβ (Duong et al., 2006) indicating that the effects of this signaling pathway may hide some surprises.
Figure 5. Crosstalk between ER and growth factor receptor pathways that activate AKT and ultimately lead to cell proliferation and survival. Modified from (Riggins et al., 2007).

.7 THE PI3K/AKT PATHWAY IN CANCER

.7.1 HER-2

HER-2/c-erbB2 belongs to a family of tyrosine-kinase receptors (TKR) together with EGFR (HER-1), HER-3 and HER-4. HER-2 contributes to malignant growth by activating and recruiting signaling cascades involved in cell proliferation and survival, like for example MAPK and PI3K/AKT
INTRODUCTION

pathways (Grant et al., 2002). The HER-2 gene has been found amplified/overexpressed in 10-30% of breast tumors (Lofts & Gullick, 1992; Singleton & Strickler, 1992; Slamon et al., 1987). This is often associated with more aggressive tumors and poor treatment response (Carломagno et al., 1996; Slamon et al., 1989; Stal et al., 1995). In ER+/HER-2+ cancers the response rate to tamoxifen is reduced in comparison with ER+ tumors with normal HER-2 expression (Nicholson et al., 1990). However, HER-2 is more accepted as a predictor of trastuzumab (Herceptin®) treatment while its predictive value for endocrine treatment is still under discussion (Arpino et al., 2004; Elledge et al., 1998). Although HER-2 does not directly belong to the PI3K/AKT pathway, it is frequently involved in PI3K activation and in breast cancer.

7.2 PI3K

Phosphatidylinositol 3 kinase is a dual kinase that phosphorylates phosphoinositides and serine/threonine residues on proteins. The main substrates are phosphatidylinositol 4P and phosphatidylinositol 4,5P$_2$ (PIP2) that become phosphatidylinositol 3,4P$_2$ or phosphatidylinositol 3,4,5P$_3$ (PIP3) after phosphorylation at the 3' position of the inositol ring (Whitman et al., 1988) and the p85 regulatory subunit (Dhand et al., 1994). The kinase is a heterodimer with a regulatory and a catalytic subunit composed of five structural domains. In its basal state the p110 subunit is bound to and inhibited by the p85 regulatory subunit, whose structure consists mainly of bindings sites for adaptor proteins, PI3K catalytic subunits or TKR. PI3K activation occurs at the cell membrane when the p110 catalytic subunit is in close proximity to its lipid substrates and the
p85-inhibitory effect is released. At the cell membrane, the p85 regulatory subunit can interact directly with phosphotyrosine residues present in activated growth factor receptors or indirectly with the insulin receptor substrates 1 and 2 (IRS-1 and IRS-2). The catalytic p110 subunit can also interact with Ras through its Ras-binding domain (RBD).

The PI3K family is organized in several classes and subclasses based on differences in tissue distribution, structure, substrate affinity, activation and function. The class IA comprises the catalytic subunits p110α, β and δ that can heterodimerize with one of the regulatory subunits p85α/p55α/ p50α or p85β/p55β or p55γ. In this thesis we will concentrate on class IA because this class seems to be more important in carcinogenesis (Denley et al., 2008; Vivanco & Sawyers, 2002).

The PIK3CA gene, situated on chromosome 3q26.3, encodes the p110α catalytic subunit. The protein (110 kD) is composed of five structural domains: a p85 binding domain situated at the N terminal end, a Ras binding domain, a domain called C2 (protein kinase C homology domain 2) proposed to bind cellular membranes, a helical domain of unknown function and the catalytical domain to the C terminal end (Huang et al., 2007). The gene consists of 20 exons and was originally found amplified in cancer (Hui et al., 2001; Ma et al., 2000; Shayesteh et al., 1999). But in 2004, Samuel and collaborators revealed high frequency of mutations in this gene (Samuels et al., 2004). The mutations clustered in >85% of the cases to the exons 9 (helical domain) and 20 (catalytical domain) thereby defined as “hot spots”. The most affected codons were 542, 545 (exon 9) and 1047 (exon 20) (Figure 6).
INTRODUCTION

Figure 6. PIK3CA gene and distribution of its mutations. The orange boxes indicate hot spots of mutated residues situated in the helical (E542 and E545) and kinase domains (H1047). Modified from (Bader et al., 2005).

Due to the evolutionary conservation of the affected residues, the mutations may have an activating nature (see results and discussion). Other reports, studying the mutational status of this gene, have confirmed the high rate of mutations in several cancer types such as breast (Bachman et al., 2004; Barbareschi et al., 2007; Board et al., 2008; Buttitta et al., 2006; Campbell et al., 2004; Lai et al., 2008; Lee et al., 2005; Levine et al., 2005; Li et al., 2006; Liedtke et al., 2008; Perez-Tenorio et al., 2007; Saal et al., 2005; Samuels et al., 2004; Wu et al., 2005), liver (Lee et al., 2005), ovarian (Campbell et al., 2005; Levine et al., 2005), colon (Samuels et al., 2004; Velho et al., 2005), glioblastoma (Hartmann et al., 2005; Samuels et al., 2004), head and neck squamous cell carcinoma (Qiu et al., 2006), brain and gastric carcinomas (Samuels et al., 2004). Genetical alterations have also been reported for the PIK3CR gene encoding the p85α regulatory subunit (Jimenez et al., 1998; Jucker et al., 2002; Philp et al., 2001).
.7.3 PTEN

PTEN, situated on chromosome 10q23.3, was first reported as a protein tyrosine phosphatase and as a tumor suppressor gene mutated in several cancers (Li et al., 1997; Steck et al., 1997) and germline mutations of this gene are associated with hereditary cancer syndromes like Bannayan-Zonana and Cowden’s disease. PTEN has indeed double phosphatase activity on lipids and proteins. The main lipid substrates are the products of PI3K (Maehama et al., 2001) while the protein phosphatase activity is associated with inactivation of focal adhesion kinase (FAK), Src homology 2 domain containing-protein (Shc), platelet derived growth factor receptor (PDGFR) and PTEN itself (Suzuki et al., 2008). PTEN regulation can occur at transcriptional and post-translational levels, by interaction with other proteins or by relocation to different cell compartments. At transcriptional level, PTEN is positively regulated by EGFR, p53, resistin, peroxisome proliferator activated receptor γ (PPARγ), human sprouty homolog 2 (SPRY2) and phytoestrogens. It is negatively regulated by mitogen activated protein kinase kinase-4 (MKKK4), transforming growth factor β (TGFβ) and recently reported, by the proto-oncogenic transcription factor JUN (Suzuki et al., 2008). Post-translational mechanisms include phosphorylation, acetylation or oxidation, all of them leading to PTEN inactivation. Moreover, PTEN interactions with other proteins either stabilize PTEN (Wu et al., 2000), target it for degradation (Tang & Eng, 2006) or decide PTEN location in the cell. PTEN can be recruited to the cell membrane to access its substrates or shuttle between the cytoplasm and the nucleus. The role of PTEN in the nucleus was deduced from the presence of PIP3 in this cell compartment (Caramelli et
Nuclear PTEN seems to be engaged in down regulation of cyclin D1 and phosphoMAPK, which is crucial for cell cycle arrest, whereas cytoplasmic PTEN is required to decrease phospho AKT (pAKT) levels, up regulate the cell cycle inhibitor p27Kip1 and induce apoptosis (Chung & Eng, 2005; Chung et al., 2006). Lost nuclear PTEN has been associated with tumor formation (Perren et al., 1999). Other PI3K-independent functions of PTEN have been found: p53 acetylation in response to DNA damage (Li et al., 2006) and restriction of cell migration. PTEN alterations in cancer manifest in form of loss of heterozygosity (LOH), protein loss, mutations and epigenetic alterations (Ali et al., 1999; Aveyard et al., 1999; Dahia, 2000; Dreher et al., 2004; Forgacs et al., 1998; Li et al., 1997). Hence, low frequency of mutations has been reported in breast cancers. With the introduction of a new technique high frequency of gross PTEN mutations among BRCA1 mutated cancers (Saal et al., 2008), can be found.

7.4 AKT

AKT (v-AKT murine thymoma viral oncogene homolog) also known as protein kinase B (PKB) is the human homolog of the v-AKT oncogene (Bellacosa et al., 1991; Burgering & Coffer, 1995; Jones et al., 1991; Staal, 1987). There are three AKT isoforms encoded by three different genes: AKT1, AKT2 and AKT3. The structure of all three isoforms is conserved through evolution and consists of an amino terminal pleckstrin homology (PH) domain, a kinase domain and a carboxy terminal regulatory domain with certain similarity to this found in AGC kinases (cyclic AMP dependent-protein kinase, cyclic GMP-dependent protein kinase and protein kinase C). All AKT isoforms are distributed ubiquitously in human
INTRODUCTION

tissues and their functions have been deduced in part from knockout studies. For example, AKT1 and AKT3 knockout mice exhibit decreased body size and impaired brain development respectively while AKT2 null mice develop type II diabetes.

All the AKT isoforms are found altered in cancer either by amplification like in the case of AKT1 (Staal, 1987) and AKT2 (Nakayama et al., 2006; Ruggeri et al., 1998), protein overexpression (AKT1, AKT2, AKT3) or activation (AKT1, AKT2) (Ermoian et al., 2002; Gupta et al., 2002; Horiguchi et al., 2003; Hsu et al., 2001; Kanamori et al., 2001; Kreisberg et al., 2004; Kurose et al., 2001; Malik et al., 2002; Min et al., 2004; Nakayama et al., 2001; Nam et al., 2003; Roy et al., 2002; Schlieman et al., 2003; Sun et al., 2001; Terakawa et al., 2003; Tokunaga et al., 2006; Yuan et al., 2000). AKT1 has been also found mutated in breast, colorectal and ovarian cancers (Brugge et al., 2007; Carpten et al., 2007). These alterations have prognostic significance in cancer (Dai et al., 2005; Ermoian et al., 2002; Kreisberg et al., 2004; Min et al., 2004; Nakanishi et al., 2005; Nam et al., 2003; Schlieman et al., 2003; Terakawa et al., 2003; Tsurutani et al., 2006) indicating the potential of AKT as a therapeutic target.

7.4.1 AKT activation and signaling downstream

Ligand-mediated activation of a plethora of TKR and other receptors leads to AKT activation (Hanada et al., 2004). AKT was early reported as a PI3K target (Burgering & Coffer, 1995; Franke et al., 1995) since the PH domain of AKT interacts with the PI3K substrates to be recruited to the cell membrane where it can be activated by phosphorylation (Figure 7). The phosphorylation sites crucial for the full activation of AKT are T308, in the
INTRODUCTION

activation loop, and S473 in the hydrophobic motif (Alessi et al., 1996). The kinase responsible for T308 phosphorylation is protein dependent kinase 1 (PDK1) (Alessi et al., 1996) whereas the identity of a PDK2, responsible for S473 phosphorylation, is not so well defined. Among the PDK2 candidates are integrin-linked kinase (ILK) (Persad et al., 2001), mTOR complex 2 (mTORC2) (Sarbassov et al., 2005) and AKT itself (Toker & Newton, 2000). After activation AKT can be transferred to the cytoplasm or the nucleus where it can phosphorylate its targets. AKT can be negatively regulated by PTEN (Stambolic et al., 1998) and the PH domain and the leucine-rich repeat protein phosphatase (PHLPP) (Gao et al., 2005). AKT activation triggers many biological processes that may be relevant to cancer. For instance, cell survival, through phosphorylation and inactivation of many pro-apoptotic factors such as the BCL2-antagonist of cell death (BAD), which sequesters the apoptotic factors BCL-X1 and BCL-2 in a non-functional complex, caspase-9, and a forkhead member of transcription factors (FKHR), involved in transcription of several pro-apoptotic genes. Indirectly, AKT can also exert a positive effect on the pro-survival nuclear factor κB (NF-κB), by activating the I-κB kinase (IKK) that causes degradation of the NF-κB inhibitor (I-κB). Moreover, AKT can phosphorylate the murine double minute 2 (Mdm2), a negative regulator of the pro-apoptotic tumor suppressor p53, leading to Mdm2 nuclear translocation and better access to p53. Another effect of AKT activation is to increase cell proliferation by inhibiting glycogen synthase kinase 3 (GSK3)-induced cyclin D1 degradation. AKT has also been shown to delocalize p21 and p27 to the cytoplasm inhibiting their function as cell cycle inhibitors. In addition to its role in survival and proliferation, AKT activation is also associated with genetic instability through the DNA
INTRODUCTION

damage checkpoint gene 1 (CHK1) inhibition and increased cell growth, a process mainly controlled by mTOR. AKT activates mTOR indirectly by phosphorylating and inducing degradation of the Tuberous Sclerosis Complex proteins 1/2 (TSC1/2). Normally, the tumor suppressor TSC1/2 is able to drive the GTPase Ras homolog enriched in brain (Rheb) into a GDP-bound inactive state that is not able to phosphorylate and activate mTORC1. Because of mTOR activation, two main substrates are phosphorylated: the 40S ribosomal protein S6 kinase (p70S6 kinase 1 or S6K1) (discussed below) and the eukaryotic translation initiation factor 4E binding protein 1 (4EBP-1) initiating transcription of genes involved in cell proliferation, participating in ribosome biogenesis or regulating cellular metabolism.
Figure 7. Signaling downstream of AKT.
.7.4.1.1 p70S6K1 and p70S6K2

Besides S6K1, there is another kinase S6K2, encoded by a different gene. S6K1 and S6K2 are serine/threonine protein kinases that belong to the family of AGC protein kinases. S6K1 and 2 are able to phosphorylate the 40S ribosomal protein S6 thereby enhancing protein biosynthesis (Jastrzebski et al., 2007), cell growth and cell cycle progression. S6K1 is believed to regulate G1-S transition while S6K2 seems to be more important during G2/M phase (Boyer et al., 2008). Besides these actions, S6K1 and perhaps S6K2 participate in a negative feedback loop where overactivation of the proteins leads to AKT inhibition (Figure 7). Alternative splicing of the S6K1 (RPS6KB1) and S6K2 (RPS6KB2) genes give rise to the p70 (α II) and p85 (α I) isoforms of S6K1 or to the p54 (β II) and p60 (β I) isoforms of S6K2. Both kinases can be found in the nucleus and the cytoplasm and recently S6K2 has been located to the centrosome (Rossi et al., 2007). S6K1 and 2 share 70% homology with >83% in the catalytic domain alone (Gout et al., 1998; Koh et al., 1999) which may suggest redundant biological functions. However knock out models indicated that S6K1 and S6K2 control body size and metabolism through different mechanisms (Jastrzebski et al., 2007; Pende et al., 2004). Moreover, a closer structural inspection reveals that S6K2 contains a C-terminal proline-rich region, absent in S6K1, that may be involved in SH3 protein-protein interactions (Lee-Fruman et al., 1999).

S6K1 activity increases due to sequential phosphorylation upon nutrient or growth factor stimulation. S6K1 can be activated in a PI3K/AKT dependent manner by mTORC1 or independently of PI3K and even mTOR (Jastrzebski et al., 2007). S6K2 and S6K1 activation shares many
features but additionally, PKC and MEK signaling pathways seems to play a more important role in S6K2 activation compared to S6K1 (Jastrzebski et al., 2007).

The genes encoding these kinases are situated relatively close to well known amplicons (17q12-q21 and 11q13) containing the HER-2 and the CCND1 oncogenes respectively. The RPS6KB1 gene has been found amplified in about 9% of breast cancers (Barlund et al., 2000).
AIMS OF THE STUDY

Paper I
1- To determine the frequency of AKT-1 expression and activation in breast cancer.
2- To study the associations of AKT-1 expression and activation with other variables and patient survival after endocrine treatment.

Paper II
1- To study the *in vitro* effects of heregulin β1 upon AKT activation, p21 cellular location and response to tamoxifen.
2- To analyze the expression and localization of p21 in breast tumors, its association with other clinico-pathological variables and AKT activation, as well as its clinical relevance.

Paper III
To determine the frequency of PIK3CA mutations and PTEN loss. To explore whether PIK3CA mutations and PTEN loss are mutually exclusive mechanisms, correlate with other known clinico-pathological markers or have clinical implication in breast cancer.

Paper IV
To study the frequency of S6K 1/2 amplification in breast cancer. Looking for coamplifications with the HER-2 and CCND1 genes, associations with other clinical variables and members of the PI3K pathway. To determine the clinical value of S6K 1/2 amplification.
ETHICAL CONSIDERATIONS

There are a plethora of guidelines and regulations that cover the potential conflict between research interests, patient integrity, autonomy, and the preservation of public trust in biomedical research (Helgesson et al., 2007). Some of the ethical issues that we can identify in our particular studies include the collection of patient samples without explicit consent, the patient’s right to receive feedback of the results of the study, the secrecy in the management of personal data as well as the repetitive use of biological material (tumor tissue or cell lines) from deceased. The patient materials included in these studies are sample collections from the Biobanks at the Karolinska and Linköping University hospitals. Since the patient identity has been protected by a code, it is unavailable for public knowledge and approval from the local ethics committees has been obtained before carrying out the corresponding investigations.
.1 Patient material

In these papers we used frozen sections or DNA from tumors still available after hormone receptor assays. The material was labeled and stored at -70 °C until used. The 93 premenopausal women included in paper I participated in two different trials (Baum et al., 2006; Ryden et al., 2005). All the patients included in the original trials had invasive breast cancer stage II (pT2 N0 M0, pT1 N1 M0 and pT2 N1 M0) and underwent radical surgery in the form of a modified radical mastectomy or breast-conserving surgery with axillary lymph node dissection. The patients with lymph node infiltration (N1) or breast-conserving surgery received radiotherapy. All the patients were also treated postoperatively with tamoxifen, goserelin (introduced after 1990) or both endocrine modalities (see Table II for details).

The postmenopausal patients included in papers II-IV (Table II) had unilateral, operable breast cancer and were required to have either histologically verified lymph node metastases or a tumor diameter, measured on the surgical specimen, exceeding 30 mm. They were randomized to four treatment groups: adjuvant chemotherapy, adjuvant chemotherapy plus tamoxifen, radiotherapy, and radiotherapy plus tamoxifen. Chemotherapy treatment consisted of 12 courses of CMF. Surgery consisted of modified radical mastectomy. Only those tumors judged to have more than 50% of malignant cells in the tumor sections were included in paper IV and the subsets of patients included in the
MATERIALS AND METHODS

different studies showed not bias in comparison with all the 679 postmenopausal patients in the whole trial in terms of tumor characteristics and treatment.
### Table II. General description of the patient material.

|                  | Paper I  
n=93 | Paper II  
n=262 | Paper III  
n=270* | Paper IV  
n=207* |
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<td>36</td>
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<td>Distant</td>
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<td>128</td>
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<td>106</td>
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<tr>
<td>Breast cancer death</td>
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<td>120</td>
<td>95</td>
</tr>
<tr>
<td>Mean follow time</td>
<td>5.3 years</td>
<td>11 years</td>
<td>11 years</td>
<td>11 years</td>
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* PTEN was evaluated in 201 patients
* RPS6K1 was evaluated in 206 patients.
* < 50 years of age.
Cell lines

Attempts to culture breast cancer cell lines started in 1937 but it was not until 1958 that Lasfargues and Ozzello succeeded for the first time with the long-term culture of a breast cancer cell line, BT-20 (Lasfargues & Ozzello, 1958). Other well known breast cancer cells were isolated in the 1970s: SK-BR3 (Trempe & Fogh, 1973), MDA-MB-231 (Cailleau et al., 1974), T-47D (Keydar et al., 1979), ZR-75-1 (Engel et al., 1978), MDA-MB-468 (Cailleau et al., 1978) and BT-483 (Engel & Young, 1978). Among the most famous breast cancer cells are MCF-7 (Soule et al., 1973), where “M” stands for Michigan, “C” for Cancer and “F” for Foundation. The number 7 refers to the number of attempts that were required to perpetuate this cell line from a patient. Another cell line mentioned in paper III is MCF-10A (Soule et al., 1990), isolated in 1990 and derived from a patient with fibrocystic breast disease.
.1 Flow cytometry

Flow cytometry is a powerful technique that allows analysis of multiple parameters in complex cell populations in a very short time. A flow cytometer is divided into different parts: the fluidic system that deliver the samples into the machine, the optical system composed by a laser, which is the light source, objects that direct the light and detectors that receive the emitted light and finally, the electronic and peripheral computer systems which are used to translate the impulses into digital data. The cell samples or particles are delivered in a stream of sheath fluid in a way that only one cell at a time reaches the laser beam. This facilitates the gathering of information related to the size, complexity, cell phenotype, DNA content, viability, etc. While the cell size and complexity are deduced from the forward and side scattered light respectively, the other parameters can be studied by labeling the cells with different fluorescent-conjugates as we will describe below. This technique has a broad spectrum of applications from immunophenotyping to cell sorting. In this thesis we have employed flow cytometry to quantify the fraction of cells in S phase (papers I-IV), to determine the expression of HER-2 (paper I-IV) and to measure apoptosis (paper II).

.1.1 S phase fraction

To quantify the DNA content of single cells different methods have been described. In our lab we have chosen a method that employs the NP-40 detergent in combination with the proteolytic enzyme trypsin to isolate the nucleus (Vindelov et al., 1983). The advantages of this method are based on
the simplicity of the technique, the high reproducibility and the versatility of the material that can be used. The DNA in the cells is visualized by staining with different fluorescent dyes like propidium iodide. Due to the direct relation between the number of incorporated dye molecules and the DNA content (stoichiometry) it is possible to display the DNA content in a frequency distribution graph (histogram) of fluorescent intensity v.s number of cells, where the fluorescent intensity is proportional to the amount of incorporated dye. Since the DNA content is related to the phase of the cell cycle, it is possible to deduce the proliferative status of the cells from the DNA histogram. We have employed the ModiFit software to quantify the S-phase fraction.

1.2 HER-2 content

Determination of HER-2 by flow cytometry combines the high sensitivity of immunofluorescence with the high speed of the flow cytometer. After a rather quick protocol, a primary anti-HER-2 antibody or an unspecific immunoglobulin (negative control) followed by a secondary antibody coupled to a fluorescent dye could indirectly detect the antigen. The HER-2 positive tumors could be determined by a ratio between the fluorescence associated with the specific antibody and the negative control. At the same time, the histogram for PI provided information about the cell cycle distribution of the cells. The clear advantages of this technique, including speed, multiparametric analysis, and quantitative measurements are somehow overshadowed by the fact that the visual inspection of the cells is missing.
.1.3 Apoptosis

Apoptosis and necrosis are two forms of cell death that differentiate in many aspects and can be detected by several techniques. Apoptotic cells can shrink, altering the form and composition of the cell membrane, but keep membrane integrity whereas necrotic cells explode with leakage of the intracellular components causing inflammation. Another feature of apoptotic cells is the non-random DNA fragmentation that can be detected as a ladder pattern in agarose gels, the increased permeability of the mitochondria due to formation of pores and the involvement of proteolytic enzymes that are in charge of degradation of many of the intracellular proteins. Different techniques have been developed for flow cytometry. For instance, these assays can measure DNA fragmentation, alterations in membrane symmetry, release of mitochondrial content and activation of cysteiny1-aspartate-specific proteinases (caspases). Caspases, play a central role in apoptosis and the targets of their cleavage constitute a key to differentiate necrotic from apoptotic cells. The M30-cytodeath antibody is directed against the CK18-NE antigen exposed in CK18 only after caspase cleavage. This event is specific for apoptotic cells and has broad detection windows from the early to the late stages of apoptosis. Several caspases seem to cleave CK18 \textit{in vivo} which may compensate for the absence of a specific caspase in certain cells.

.2 Immunohistochemistry

Immunohistochemistry (IHC) is a technique used to detect proteins in tissues by combining immunological detection with morphological analysis.
MATERIALS AND METHODS

The success of this technique relays on: the section type, fixatives used, procedure to retrieve the antigen and the detection method and evaluation. An essential part of the technique is the preservation of the cells and tissues in a life-like manner. To achieve this tissues are incubated with a fixative. Fixatives are substances used to preserve the antigen structure and conformation from the rigors of the technique and the staining procedure. Among the fixatives used are acetone (paper I-II) that denaturates the proteins by coagulation, and formaldehyde (paper III) that forms crosslinkages. To improve the immunological reaction in the fixed tissue, the antigens can be retrieved by enzymatic digestion, microwave irradiation, autoclaving or pressure-cooking in the appropriate buffer. A common buffer solution is 10 mM citrate buffer at pH 6.0 as described in paper III. However to assure a good quality of the material it is also necessary that the sections are representatives of the whole tumor. The antibodies also play an essential role in this procedure since they link the specific antigens with a dye allowing further visualization in a light or electron microscopy. The antibodies are raised in animals previously immunized with the protein of interest or a part of it (antigen). Depending on their clonal origin the antibodies are classified as polyclonals (secreted by several cell clones) or monoclonals (secreted by a single plasma cell clone). While the polyclonal antibodies are immunochemically dissimilar and react with several epitopes on the antigen, the monoclonals are secreted from a single plasma cell clone, are immunochemically identical and recognize a single epitope on the antigen. Though monoclonal antibodies are expected to be the primary choice in IHC due to their specificity some problems can arise. For example crossreactivity can occur when several antigens share the same epitope or false negative responses if the epitope is lost. To avoid some of the problems related to antibody specificity it is necessary to control the
reaction. The primary antibodies can be replaced by either another irrelevant antibody of the same class and subclass, by affinity-absorbed antiserum or by preimmune serum from the same animal that produced the antibody.

Additionally negative, positive or internal tissue controls can be used. The positive control is the tissue that expresses the antigen in question, the negative control does not contain the antigen and the internal control contains the antigen in the tumor cells and adjacent normal structures.

In these papers we have used an avidin/streptavidin method (paper I-II) using a biotin labeled secondary antibody followed by streptavidin conjugated with horseradish peroxidase (HRP) or a two-step method based on an HRP labeled polymer which is conjugated to the secondary antibody (paper III)

.3 Western blotting

The western blot (WB) was described for the first time in 1979 by Towbin et al. (Towbin et al., 1979) and received this name as the alternative for proteins when there was a “Southern” for DNA and a “Northern” for RNA. The WB discriminates the antigenicity and molecular weight of the proteins. The disadvantage compared to IHC is that the antigens are not visualized in the original tissue. By this method, the protein mixture is run through a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a membrane. In further steps the membrane is blocked in buffers with animal serum, bovine serum albumin (BSA) or milk, incubated with primary antibodies, secondary- enzyme labeled antibodies and finally developed by autoradiography or enhanced
MATERIALS AND METHODS

chemiluminescence (ECL). The technique is not truly quantitative though some attempts have been done to quantify the intensity of the bands by using different computer programs.

.4 Polymerase chain reaction (PCR)

The PCR method generated a biotechnological revolution. It was conceived in 1983 by Kary Mullis (Mullis et al., 1986). The applications are many ranging from detection of mutations to presence of unwanted genetic material, in forensic medicine to archeology. The PCR is a chain reaction of a thermostable polymerase (Taq) that occurs in vitro leading to exponentially amplification of a certain DNA sequence. In the test tube, seven essential components are needed: the DNA polymerase, a primer-pair, deoxynucleoside triphosphates (dNTPs), divalent cations such as Mg$^{2+}$, buffer to maintain pH, monovalent cations and template DNA. Thereby the reaction goes through three basic iterative steps that occur at different temperatures. The first step is denaturation of the template to unzip the two DNA strands. It occurs at 94-95°C for 20 seconds. Then, primer annealing at a temperature usually 3-5°C lower than the calculated melting temperature at which the oligonucleotide primers dissociate from their template. Finally, the extension reaction at 72°C, where the dNTPs are incorporated to the growing DNA strand. The number of cycles required to achieve a detectable PCR product varies depending on the number of copies of template DNA and the efficiency of the Taq polymerase but generally fluctuates between 30-35 cycles. As the reaction proceeds the amount of DNA sequence between primers double
and in a matter of few hours the number of DNA copies can reach a billion.

In paper III we performed an alternative PCR called “touchdown PCR” (Don et al., 1991) which is done by using progressively descending annealing temperatures in a consecutive few cycles. The aim of this PCR variant was to avoid contamination of our sequence target with another nearly identical sequence. At the end of the reaction an aliquot is withdrawn and run in an agarose gel to control the PCR products.

.5 Real-time PCR

The standard PCR is not a quantitative method. The introduction of the real-time PCR technique made possible to monitor and quantify the PCR-products as the reaction occurs. Fluorescent dyes, coupled to a probe or to DNA, are used to detect the PCR products as they accumulate. Briefly, the specific primers are added to the PCR reaction together with the sample DNA and the other reagents. The probes are usually coupled to a reporter and a quencher dye where the reporter can be 5′FAM™ and the quencher either a TAMRA™ fluorescent or a non fluorescent minor groove binder (MGB) at the 3′ end. The MGB probes (paper IV) are shorter and easier to detach from the DNA sequence as the polymerase extend the primed sequence. Once the probe detaches from the DNA and is cleaved by the polymerase, the quencher dye is no longer able to quench the reporter and the emitted fluorescence, proportional to the amount of DNA, is detected (Figure 8). Because the target sequences are amplified and detected in the same instrument there is no need of post PCR steps. A program plots the fluorescence intensity over the number of cycles. Since the PCR reaction is
an exponential process, the cycle number at which the fluorescence reaches the exponential phase of the curve defines a threshold and the point where the fluorescence intercepts this threshold is called \( \text{Ct} \) (cycle threshold). The greater the initial DNA concentration the fewer the cycles required to achieve this \( \text{Ct} \). In \textbf{paper IV} we quantified the gene copy number by using the standard curve method. Fourfold serial dilution of DNA from a cell line was used to prepare the standard curve. The relative gene content in tumor samples was quantified by extrapolating the \( \text{Ct} \) of the sample in the standard curve (log\( C \) v.s. \( \text{Ct} \)).

\textbf{Figure 8.} Diagram of the TaqMan\textsuperscript{®} MGB probe. A reporter (R) dye and a non-fluorescent quencher (NFQ) are bound to an MGB probe situated between the forward (FP) and reverse (RP) primers (A). Under these conditions, the R is quenched by the proximity of the NFQ. In B the probe is displaced as soon as the polymerase elongates the DNA strand. Finally, the polymerase degrades the probe liberating the R that fluoresces (C).
.6 Single-strand conformation analysis

A method to detect single-strand conformation polymorphisms (SSCP) was first described by Orita and collaborators in 1989 (Orita et al., 1989). With the novel technique, it was possible to detect DNA polymorphisms and point mutations at different positions in DNA fragments. The SSCP takes advantage of the mobility shifts caused by small nucleotide alterations that lead to conformational changes of the single-stranded DNA (Figure 9). Each single strand will adopt a certain conformation dependent of the primary structure. Small variations such as mutations, deletions or gains will produce conformational alterations leading to different patterns of migration in a gel compared to the normal sequence. To detect the aberrant bands, the sequence is radiolabeled, denatured and run through a gel. The bands are then visualized by exposing the dried gels to X-rays and detected by autoradiography. The shifted bands can be cut from the gel and sequenced.
Figure 9. Diagram of mutational screening by SSCA. Modified from (Gentile, 2001).

.7 Sequence analysis

The first attempts to sequence DNA were made in the 1970’s. From this period the most renowned method was that of Sanger and collaborators (Sanger et al., 1977). The Sanger sequencing is based on the dideoxynucleotides (ddNTP’s) incorporation in addition to nucleotides to the DNA. Dideoxynucleotides contain a hydrogen group on the 3’ carbon instead of a hydroxyl group (OH). These modified nucleotides, when integrated into a sequence, prevent the addition of further nucleotides. This occurs because a phosphodiester bond cannot form between the dideoxynucleotide and the next incoming nucleotide, and thus the DNA
chain is terminated. Four tubes are prepared each containing one labeled ddNTP (either ddATP, ddGTP, ddCTP or ddTTP); four dNTPs, and the DNA polymerase in the corresponding PCR buffer. As the DNA elongation proceeds in each tube the dNTPs are going to be incorporated. The reactions will stop with the random incorporation of one ddNTP instead of a dNTP to the growing chains. Since all the reactions start at the same nucleotide but ends at specific bases at the end of the reactions there will be a mixture of different length DNA strands that can be run through a polyacrylamide gel. The resolved bands can be detected by exposing the dried gel to UV light or X rays depending on the labeling method (Figure 10).

In paper III the mutations were confirmed by automated sequencing based on a modification of the traditional Sanger method in which ddNTPs are labeled with a fluorescent dye for detection. In this case the ddNTPs are labeled with two fluorescent dyes: fluorescein and four different rhodamines. Fluorescein acts as a donor dye, absorbing energy from the incident laser light and transferring it to the acceptor dye (rhodamine) situated on the same ddNTP (Ju et al., 1995). Each acceptor dye then emits light at its characteristic wavelength for detection, allowing the identification of the nucleotide that terminated the reaction. This method produces a more accurate sequence due to its sensitivity.

.8  STATISTICAL METHODS

In the clinical material, the relationships between different variables were assessed by the Chi square test or the Chi square test for trend (Spearman's rank correlation), when the variables included more than two categories
MATERIALS AND METHODS

(papers I-IV). To estimate the role of one (univariate analysis) or a group of variables (multivariate analysis) in survival analysis the Cox’s proportional hazard regression model was used. The Cox’s proportional hazard regression was also used in the interaction analysis between a certain variable and treatment. The multiple sample test which is a generalization of Gehan’s generalized Wilcoxon test was also used to assess the differences between groups in the survival analysis. The Product-limit method was employed to estimate the cumulative probabilities of recurrence free survival and the differences in recurrence free survival between groups was explored by the Log-rank test.

For the *in vitro* study (paper II) the differences between two groups of treatments was calculated by the Student’s t-test for independent samples. The criterion for statistical significance was *P*< 0.05 and all the statistical procedures are comprised in STATISTICA (data analysis software system), version 8.0. www.statsoft.com.
MATERIALS AND METHODS

**Figure 10.** Sequencing according to Sanger
RESULTS AND DISCUSSION

Most of the findings in this thesis are based on observations from tumor specimens preserved after surgical treatment. Different properties of the tumors were revealed with help of immunological methods to detect protein expression and DNA analysis to determine the copy number and mutational status of different genes. Some of the variables studied in the papers were already known prognostic or predictive markers giving information about the course of the disease or the treatment response. Other variables, including those that belong to the PI3K/AKT pathway were intriguing due to their novelty and almost unknown biological function in the tumor context.

We found correlations between these variables that may be of clinical significance. In order to explore cause–effect relationships we also tested our hypotheses using in vitro methodologies. While in vitro models may not mirror in vivo situations, they do inspire new ideas that can be validated in vivo.

In paper I we explored the expression and activation of AKT in a clinical material consisting of 93 tumors from premenopausal breast cancer patients by immunohistochemistry. From the three AKT isoforms, we chose to study the expression of AKT1 because it was thought to play the most prominent role in breast tumorigenesis compared to AKT2 and AKT3. AKT1 was expressed in 46% of the tumors, with predominant cytoplasmic location, while activated AKT (pAKT) was positive in 54% of the cases. Despite the positive correlation between AKT1 expresion and activation (P=0.04), some AKT1 negative/pAKT positive cases were observed, suggesting the presence of another activated isoform being recognized by
the pAKT antibody. The structural homology of these proteins could facilitate a cross-reaction of the antibody with a different isoform as it was suggested later (Stal et al., 2003). In this study there was a positive correlation between AKT1, AKT2 and pAKT though stronger between pAKT and AKT1. In order to increase the statistical power a new variable was conceived (tAKT). tAKT positive patients were those showing strong tumor staining with at least one of the three antibodies and the frequency of tAKT positive patients (55%) was similar to that found with pAKT in paper I.

The high frequency of AKT activation found in breast cancer suggests an important role for this pathway in the tumor. For example, pAKT has been detected in 22-64% of breast cancers (Schmitz et al., 2004; Shi et al., 2003), employing different antibodies and scoring systems. Recent studies confirm the role of AKT1 in breast cancer progression (Ju et al., 2007) but its transforming activity is only evident together with other alterations, such as HER-2 overexpression/activation or PTEN loss (Dummler & Hemmings, 2007). In our material (paper I), AKT activation was not correlated to HER-2 expression, which is partly explained by the low frequency of HER-2+ cases (n=6, 7%), as expected in a sample mostly conformed by ER or PgR positive tumours. However, in a larger material consisting of 280 postmenopausal patients, we did find this association (Stal et al., 2003).

Interestingly, HRGβ1, the most potent ligand for the activation of HER-2, 3, and 4 receptors, was found associated to pAKT when expressed in stromal cells (P=0.017), which suggests a possible paracrine or juxtacrine mechanism of AKT activation. Tumor-associated fibroblasts have been found to confer morphogenetic and mitogenic induction of epithelial cells (Shekhar et al., 2001) and to induce acceleration of epithelial tumor growth
RESULTS AND DISCUSSION

in vivo (Camps et al., 1990). Conversely to what we expected, pAKT was inversely correlated to SPF (P=0.001) (Table I, paper I). Since our article was published, additional elements have come to explain the divergent roles of AKT kinases in cancer. The three isoforms induce transformation in vitro, however AKT1 and AKT2 seem to have opposing functions in cell proliferation. While AKT1 induces cell proliferation, AKT2 promotes cell cycle exit in nontransformed mammalian cells (Heron-Milhavet et al., 2006). The effects of AKT2 have been explained in part by interactions with the cell cycle inhibitor p21 G1P1 in the nucleus. Moreover, AKT has been reported to decrease the growth rate in cells transfected with mutant PTEN in comparison with the empty vector (McCubrey et al., 2008), and its being found by others to cause ERβ induction (Duong et al., 2006). Still, we found that pAKT was associated to higher risk for distant metastasis among the ER+/endocrine-treated patients, results later confirmed by others (Kirkegaard et al., 2005; Tokunaga et al., 2006(b); Tokunaga et al., 2006(a)) suggesting an interaction between AKT and ER signaling pathways.

In univariate analysis, pAKT positivity predicted a higher risk to develop metastasis with borderline significance (RR=2.4, 95% CI, 1.0-6.2) (Figure 2A, paper I), while multivariate analysis including pAKT and traditional prognostic factors, indicated that pAKT was an independent predictor of distant recurrence (P=0.004) (Table II, paper I). The analysis of the interaction between pAKT and SPF revealed that a low SPF was a favorable feature only if the tumor was in addition pAKT negative. This suggests that distant relapses could arise, probably due to malignant cell survival rather than cell proliferation considering the low proliferative state of the tumor (Figure 2B, paper I).
RESULTS AND DISCUSSION

Nodal status and pAKT had independent prognostic value, with all patients in the group node-/pAKT- having a better 6 years-survival than the node+/pAkt+ group (Figure 2C, paper I).

In an attempt to elucidate the effects of HRGβ1 on cell survival, proliferation, and AKT activation upon endocrine treatment, we used the breast cancer cell line MCF-7. The ER+ MCF-7 cells are hormone dependent and tamoxifen-responsive. In these cells, AKT phosphorylation lasted at least 72h after addition of HRGβ1, which indicates a possible role for HRGβ1 (Figure 1A, paper II). HRGβ1 also stimulated p21 protein expression in the same time-window that AKT activation occurred. Since the small decrease in the levels of p21 observed upon treatment with Tam and E2 disappeared in the presence of HRGβ1 (Figure 1B, paper II), we speculated that HRGβ1 could also stabilize p21. Moreover, upon HRGβ1-treatment, p21 levels increased both in the nucleus and in the cytoplasm as demonstrated by Western blot (Figure 2, paper II) and immunofluorescence (Figure 3, paper II). This modest accumulation of cytoplasmic p21 suggests that HRGβ1 can also mediate p21 cellular location, recently attributed to AKT (Zhou et al., 2001). HRGβ1 also induced cell proliferation and counteract the action of tamoxifen upon cell cycle progression (measured as percentage of cells in S-phase) and cell death (apoptosis), (Figures 4 and 5, paper II). Interestingly, a recent report shows that HRGβ1 is able to induce breast cancer cell proliferation by induction of p21 and cyclin D1 (Yang et al., 2008), but the authors did not specify the cellular location of p21. Other authors found that HRG-transfected cells accumulate p21 in the nucleus, as discussed in paper II. The role of the induction/delocalization of p21 by HRGβ1 during development of tamoxifen resistance is not documented in the literature. In
RESULTS AND DISCUSSION

A 3-D model with MCF-7-derived cells, where tamoxifen inhibited ER-mediated transcription and cell proliferation, the proportion of p21-positive cells decreased upon tamoxifen treatment (Truchet et al., 2008). More in accordance to what is expected, another study showed that p21 loss leads to the tamoxifen growth-induced phenotype. These facts suggest that p21 is an essential player to consider during tamoxifen treatment. Our results obtained in clinical samples from 280 postmenopausal women revealed that p21, in addition to its classical nuclear location, can also be found in the cytoplasm of malignant cells at a higher frequency (26.7%) than in the nucleus (14.5%). When we evaluated the effect of tamoxifen among patients with ER+ tumors we found that patients whose tumors classified as p21 N+/C- benefited significantly from tamoxifen (P=0.0082), as did the patients that had either undetectable p21 levels or double positive phenotype (P=0.034). In contrast the p21 N-/C+ group appeared not to respond to tamoxifen (P= 0.7). This group however contained a small number of patients (Figure 8 and Table II, paper II). It is still unclear how cytoplasmic p21 could affect the response to tamoxifen, but it is known that the cytoplasmic protein can mediate cell survival through binding and inactivation of the apoptosis signal-regulating kinase 1 (ASK1)(Asada et al., 1999). On the other hand, nuclear p21 has been already associated with better survival under antiestrogen treatment (discussed in paper II). Why the p21N+/C- patients presented the worst outcome in comparison with the other groups in absence of the drug is speculated in paper II. A recent report suggests that the favorable prognosis of ER+ breast cancer can be explained by the antiproliferative function of the ER by its physical interaction with p21 in the absence of estrogens. Consequently, this interaction could be favored if tamoxifen is present as the ER cannot bind E2, but not in its absence where the ER-E2
complex could provoke cell proliferation (Maynadier et al., 2008). In general, accumulation of p21 was neither beneficial in the cytoplasm nor in the nucleus. The patients included in the p21 N+/C- group also registered the worst distant recurrence-free survival compared to the other p21 phenotypes (P=0.034) (Figure 7, paper II). Since the patients comprised in this study were randomized to different treatments, we cannot conclude which therapeutic regime failed in the presence of nuclear p21. Poor disease free-survival has been observed among p21+ patients that received CMF, as discussed in paper II, but we could not detect any obvious interaction between p21 and the benefit from CMF versus radiotherapy. Moreover, we could not find a significant association between p21, nodal status, or tumor size. Cytoplasmic p21 was often registered among HER-2+ tumors, but this association was not significant. However, there was an association between cytoplasmic (P=0.00001) and nuclear p21 (P=0.022) with AKT phosphorylation and it was in the same material we previously reported a significant association between pAKT and HER-2+ status (Stal et al., 2003).

Overexpression or constitutive activation of growth factors and growth factor receptors, such as HRGβ1 and HER-2, contribute to AKT activation, and these may coexist with other cellular alterations. Mutations of the PIK3CA gene and PTEN loss are two that we discussed in this thesis. The PI3K is conformed by a regulatory p85 and a catalytic p110 subunit. Most of the PI3K mutations, reported so far in cancer, occur in the gene encoding the p110α subunit. Among the 21 exons that compose this gene, more than 85% of the mutations are concentrated in exons 9, encoding part of the helical domain, and exon 20, encoding part of the kinase domain. In our material (see paper III) we found missense
RESULTS AND DISCUSSION

mutations in 24% of the tumors, in agreement with the literature, where the mean percentage of mutations is 25%. The most frequent mutations were E545K, followed by E542K in exon 9; and H1047R in exon 20 (Table I, paper III). The higher frequency of mutations (67%) reported for cell lines could be partly explained by the more challenging conditions of the in vitro culture, increasing the risk of the cells for more genetical alterations. But these mutations must confer some advantages to the tumor cells since MCF-10A, a non tumorigenic epithelial breast cell line, did not present mutations. In fact, the most frequent mutations described so far (E545K, E542K, and H1047R) are associated in vitro with increased lipid kinase activity, cell growth, growth factor independency, transforming activity and invasive potential (Carson et al., 2008). They are also related to increased AKT activation (Zhang et al., 2007). In vivo studies have also shown that alterations in the p110α helical or catalytic domains results in a more active PI3K pathway, and could raise tumors in animal models (Bader et al., 2006; Zhao et al., 2005). However these results might not be comparable with others using natural mutant cells (Morrow et al., 2005). Those authors suggested that PIK3CA mutations in the helical domain may even lead to reduced rate of PI3K activation.

Associations with other clinical markers could also help to decipher the role of these mutations in vivo. In our material PIK3CA was often related to indicators of good prognosis like ER expression (P= 0.052) and negative HER-2 status (protein, P= 0.013 and gene amplification, P=0.083), which can also be observed in our panel of cell lines often sharing an ER+/HER-2- phenotype when mutated. In addition the PIK3CA mutations were related to small tumor size (P= 0.057). However, PIK3CA mutations are also associated to other factors like PTEN loss (P=0.0024), AKT1+ (P= 0.032), and high expression of cyclin D1 (P= 0.031) which in turn could
RESULTS AND DISCUSSION

indicate a bad course for the disease. PIK3CA mutations did not associate with node status, AKT2 expression, or pAKT. However, pAKT+ (P=0.0033), tAKT positive (P=0.0019), and cyclin D1++ (P=0.031) phenotypes were significantly associated with mutated PIK3CA (PIK3CA mut) and/or HER-2+) in a combined variable.

Other authors have reported association with ER+ tumors but also with presence of normal PTEN and high HER-2 expression (Saal et al., 2005). These authors conclude that PIK3CA and PTEN alterations were redundant at the same time that they found some PIK3CA/PTEN mutants and HER-2 negative cases. In line with our results a recent report also found PIK3CA mutations frequently concordant with PTEN loss in breast cancer (Stemke-Hale et al., 2008).

Why would it be advantageous for a malignant cell to have this “redundant” phenotype? As demonstrated in endometrial carcinoma, where PIK3CA and PTEN mutations coexist, PTEN knockdowns/PIK3CA mutants additively enhanced AKT activity compared with a single alteration (Oda et al., 2005). PTEN protein phosphatase activity, that operates independently of the PI3K pathway, could be another reason to support the non-redundant role of PTEN (Freeman et al., 2003; Okumura et al., 2005) and finally the complexity of this signaling pathway may require more than one input for its full activation.

PTEN protein expression was also studied by immunohistochemistry and considered positive, in 126/201 tumors (63%), and weak or negative in 75 tumors (37%). PTEN was mainly located in the cytoplasm of tumor cells and in some cases in the nucleus (Figure 3, paper III). These results are in agreement with those of other authors that reported loss of PTEN in 27%-50% of invasive cancers (Depowski et al., 2001; Tsutsui et al., 2005). In our material, PTEN loss correlated with ER+ status (P=0.0015), small tumor
RESULTS AND DISCUSSION

size (P=0.022), and low HER-2 expression (P=0.011). Likewise a combined variable PTEN/PIK3CA, was associated with ER+ (P=0.0017), small tumor size (P=0.0075), non-amplified HER-2 (P=0.054), low HER-2 protein expression (P=0.00080), and low SPF (P=0.014) (Table II, paper III). In some studies PTEN loss has been correlated to clinical parameters such as node metastasis, shorter disease free survival, tumor grade, or aneuploidy, but in others it has been found irrelevant to the clinical outcome (Panigrahi et al., 2004).

Based on these clinical correlations it was difficult to predict the prognostic role of PIK3CA mutations and PTEN loss in breast cancer. We found that the risk to relapse with a local recurrence was significantly lower among patients with mutated PIK3CA in comparison with those who carried wild type PIK3CA in a univariate analysis (P= 0.023) (Figure 2A, paper III) and a trend was observed in a multivariate analysis (P=0.07).

PI3K has been proposed to cause cell death mediated by hypoxia, glucose deprivation, or serum withdrawal (see discussion of paper III) which in part challenges the general view of PI3K as a survival pathway. PI3K mutations indicated good prognosis in Japanese women (Maruyama et al., 2007) and have been related to decreased rate of node positive disease among ER+ tumors (Liedtke et al., 2008). Moreover, exon 9 and exon 20 mutations seems to have different functions as demonstrated in vitro and in vivo. Exon 20 has been associated to good prognosis in the same material that exon 9 mutation predicted for worse outcome (Barbareschi et al., 2007), whereas others have found exon 20 mutations coupled to poor prognosis for patients with invasive carcinomas (Lai et al., 2008) or both mutations associated to poor patient survival (Li et al., 2006). We also found that among the tumors with low SPF (SPF<5%), the PIK3CA mutated and/or PTEN negative type predicted for worse recurrence-free
survival (P=0.020) while it indicated better survival among the group with higher S-phase (SPF>10%) (P=0.0073) (Figure 4, paper III). PTEN alone did not provide clinical information regarding distant or local recurrences, or breast cancer survival. The patients in the PTEN negative group tended to benefit more from radiotherapy than from chemotherapy (P=0.02) compared to those in the PTEN + group (P=0.29) and so did the patients with mutated PIK3CA and/or PTEN - type.

The most accepted idea behind PI3K/PTEN alterations is that this phenotype associates with AKT activation and survival of radiation-induced apoptosis. Indeed lack of PTEN sequesters CHK1 and initiates genetic instability (Puc et al., 2005), facilitating other genetic alterations that may confer survival and growth advantages to the malignant cells. Are the PIK3CA mutations some of them? Another possibility could be that the unstable cells will not survive the mutagenic effects of radiation. Interestingly, PIK3CA mutated and PTEN deficient tumors present a low percentage of cells in S-phase. The effect of cell cycle arrest in response to radiation is also a controversial issue. Cell cycle arrest upon radiation can give time to the cells to repair the major damages and survive the effect of radiation (Lees-Miller, 2008) but at the same time it contributes to radiosensitization (Albert et al., 2006). Moreover, PTEN can enhance transcription of RAD51 reducing the incidence of spontaneous double strand breaks, but in PTEN deficient cells RAD51 might be suppressed. A recent report, showing that patients with low expression of BRCA1/BRCA2/RAD51 complex respond well to radiation (Soderlund et al., 2007), allowing us to suggest that PTEN deficient cells are more sensitive to radiation.

Another member of the PI3K/AKT pathway that has been involved in therapy resistance is mTOR. Experimental and clinical evidence indicate
RESULTS AND DISCUSSION

that mTOR inhibitors not only improve the therapeutic effect of tamoxifen and letrozole (Chollet et al., 2006), but are also effective in combination with the dual HER-1/HER-2 inhibitor lapatinib and the HER-2 inhibitor trastuzumab (Vazquez-Martin et al., 2008). Among all the compounds that have been developed to target the PI3K pathway, mTOR inhibitors predominate in clinical trials (LoPiccolo et al., 2008). Taken together, these findings indicate that mTOR level and its downstream effectors could be interesting targets to explore for new alterations with therapeutic impact.

For example, RPS6KB1, a gene encoding the mTOR substrate p70S6 kinase 1, has been found amplified in 8-10% of breast tumors (Barlund et al., 2000; Barlund et al., 1997; Sinclair et al., 2003), while very little has been published in cancer about the other gene (RPS6KB2) encoding the other S6 kinase. This prompted us to explore our material looking for RPS6KB1/2 gene amplification. We found RPS6KB1 and RPS6KB2 amplification in 22/206 cases (10.7%) and in 9/207 cases (4.3%), respectively, but also in cell lines (75% and 50%) (supplement, paper IV). Even though the frequency of amplification in tumors was rather low in comparison with other alterations in this pathway, amplified S6K1 and S6K2 were independent prognostic factors indicating higher risk to develop recurrence. The S6K2 gene was also an independent predictor for breast cancer-related death (Table II, paper IV). Statistical analysis revealed that S6K1 amplification was significantly associated with HER-2 gene amplification and protein expression, while S6K2 was associated with ER+ status and CCND1 amplification (Table I, paper IV).

Interestingly S6K1 and/or S6K2 amplification and mutations in the PIK3CA gene were inversely correlated, (P=0.004), while no association was found with PTEN deficiency or AKT activation. This supports the idea of inter-tumor heterogeneity where some of the tumors are driven by
PIK3CA mutations or PTEN deficiency, where others take advantage from HER-2 and/or S6K1/2 amplification. From those alterations, AKT activation was correlated only with HER-2, indicating the existence of AKT-independent mechanisms in the PTEN negative/PIK3CA mutated tumors. However, the simplest explanation would be that we have only detected the phospho-Ser 473 residue leaving the Thr 308 unexplored.

The possibility of HER-2 and S6K1 co-amplification has been discussed before due to the physical proximity of the 17q12-21 and the 17q22-24 regions, and S6K1 was identified as the first candidate oncogene in the 17q23 region (Couch et al., 1999). In our material, we found that, in terms of locoregional control, the patients without S6K1 gene amplification benefited more from radiotherapy than from chemotherapy (P=0.0038) in comparison with the patients carrying this genetic alteration (P=0.39). 17q amplicon, including S6K1 and/or HER-2 gene amplification, also indicated poor response to radiotherapy (Figure 2, paper IV). Both 17q and S6K1 amplification status interacted significantly with the benefit from radiotherapy (Table III, paper IV).

Other authors have found stimulation of the HER-2/PI3K/AKT signaling pathway to be involved in resistance to radiation-induced apoptosis (Soderlund et al., 2005), and trastuzumab to be a radiosensitizer (Liang et al., 2003). In a previous study (Stal et al., 2003) AKT activation was also associated with poor response to radiotherapy in comparison with chemotherapy. The S6K1/2 proteins share common as well as distinct cellular substrates and functions (Martin et al., 2001; Shima et al., 1998), and both may be involved in a feedback loop leading to AKT inactivation (Manning et al., 2005), suggesting that in absence of AKT these kinases might take over the signaling pathway. In fact, mTOR inhibitors have been introduced in the clinical practice and sometimes the disappointing results
RESULTS AND DISCUSSION

are coupled to a possible AKT activation as a side effect (LoPiccolo et al., 2008). However, in vitro studies have shown that by blocking mTOR it is possible to impair ER-mediated cell proliferation (Chang et al., 2007) and to restore response to tamoxifen in breast cancer cells with aberrant AKT activity (deGraffenried et al., 2004). Interestingly, among the ER+ patients included in this study, an increased number of the S6K2 gene copies indicated better response to tamoxifen in comparison with the S6K2 negative group (Table IV, paper IV).
CONCLUSIONS

**Paper I**
AKT-1 protein expression and activation are frequent events in premenopausal breast cancer. AKT-1 activation is related with low cell proliferation however, pAKT positive patients also register shorter distant recurrence-free survival suggesting that this factor might be used to predict the response to endocrine therapy in premenopausal patients.

**Paper II**
HRGβ1 stimulates AKT activation in MCF-7 cells. It can also induce p21 expression and may induce cytoplasmic accumulation. HRGβ1 can impair the cytostatic and cytotoxic effect of tamoxifen but seems to be more important as a survival factor.

p21 is present in the cytoplasm of the malignant cells more frequently than in the nucleus. Cytoplasmic p21 is associated with pAKT, in line with the hypothesis that AKT induces p21 cytoplasmic delocalization.

Cellular location of p21 may help to identify subgroups with different therapeutic response among ER+ patients.

**Paper III**
PIK3CA mutations and PTEN loss are common alterations in breast cancer and coexist in a proportion of tumors. These alterations are more frequent among ER+, small and low proliferative tumors and this phenotype may differ from that of HER-2 positive tumors. PIK3CA mutations and/or PTEN loss predicts better recurrence-free survival
CONCLUSIONS

among patients with high proliferating tumors while among those with low proliferating disease it indicates a poor outcome.

PIK3CA mutations indicate a lower risk to relapse with local recurrences while low PTEN, as a single variable or combined with PIK3CA mutations tended to confer radiosensitivity.

Paper IV

The S6K1 and 2 genes are amplified in breast cancer and coamplified with HER-2 and CCND1 respectively. S6K1 and/or S6K2 amplifications are inversely correlated with PIK3CA mutations.

S6K2 amplification may be used to predict development of recurrences and higher risk for breast cancer death.

Patients with S6K2-increased gene copy number will benefit from tamoxifen treatment.

S6K1 amplification indicates higher risk to develop recurrences and poor response to radiotherapy.
Our results support the notion of the high frequency of PI3K/AKT pathway alterations in breast cancer. Taking into consideration HER-2 overexpression/amplification, PIK3CA mutations, low PTEN expression or RPS6KB1/RPS6KB2 amplification, the frequency of alterations was more than 75% (76.9%). However, this combined variable did not have an obvious clinical value, which could be explained by the complexity of this signaling pathway, where the hierarchy and biological function of its members is not fully understood. Based on the different clinical associations, two different paths emerged from these studies. One, that seems to be governed by HER-2 amplification or overexpression and RPS6KB1 amplification and the other driven by PIK3CA mutations and PTEN loss. While the first group is related to poor response to radiotherapy in comparison with chemotherapy the second phenotype conferred radiosensitivity. Another aspect to consider is how to stratify the patients in order to improve the accuracy of the diagnosis and treatment. PIK3CA mutated/low PTEN phenotype associated to ER+, small and low proliferating tumors. However, the patients with low proliferative tumors carrying this phenotype often had a shorter recurrence-free survival in comparison with the wild type counterpart while the group with highly proliferating tumors exhibited a longer recurrence-free survival in presence of these alterations. Likewise p21 identified subgroups of ER+ patients with different responses to the endocrine treatment. Regarding AKT activation, we can conclude that it seemed to be involved in response to the endocrine treatment among the premenopausal women while it was linked more to radiotherapy resistance in the postmenopausal patient material.
FUTURE PERSPECTIVES

1- It would be interesting to elucidate the individual role of the three AKT isoforms in a larger patient material from a randomised trial not forgetting to explore the expression of the pThr308 AKT.

2- Whether HRGß1 induces p21 expression and delocalization through the PI3K/AKT signaling pathway and how this could impair the effect of tamoxifen deserves a major study.

3- To characterize the impact of the different PIK3CA mutants in a tumor context may help to understand the role of PIK3CA mutations in breast cancer.

4- Immunohistochemical detection of p70S6K1 and p70S6K2 should be performed in order to look for associations between gene amplification and protein expression. Furthermore, in vitro studies may help to verify the role of these proteins in breast cancer.
ACKNOWLEDGMENTS

My interest in cancer research began at a lecture about monoclonal antibodies. The man who spoke knew how to catch the attention of young students with color slides of the immunological struggle. Jorge Gavilondo, the professor at that time, is unaware that his name has a place in my acknowledgments.

But here I am, in our lab, in Sweden. There are many persons that I would like to thank, like professor Bo Nordenskjöld, you welcomed me so kindly to this country and changed my life. Olle Stål, I feel so lucky having you as my supervisor. You know the secrets of every signaling pathway but I admire you most for your kindness, your sense of justice and generosity. You guided me wisely and gave me so much freedom that sometimes I thought I was alone. However, whenever I needed you I could find you there, firm as a rock. Birgit Olsson, you probably suspect how much I like you. You have opened so many doors for me: to your home, to the lab, to the Swedish language. You have taught me the essentials of Christmas decoration, advanced cooking and Real-Time PCR. I wish I had your energy and a little part of your big heart. To the Ph.Ds. that create such a good atmosphere in the lab. We can talk about EVERYTHING and nothing at the same time. We have seen each other celebrate birthdays and publications, engage and marry, build houses and families. We can meet to discuss the latest article or to see SATC. We can make diet and exercises or jump to a “semla” all with the same enthusiasm. We have shared so much during these years…Daniella Pfeiffer, you that always have time to listen and to help. Åsa Wallin, Little Miss Sunshine, I am sure you will be fine. Karin Söderlund or Leifler, I will buy your first book …perhaps that
ACKNOWLEDGEMENTS

about cell-death? Josefine Bostner, I admire your strength. Marie Ahnström Waltersson, you are almost there! Cissi Bivik, you infiltrated this group bringing with you a good portion of humor and common sense, Agneta Jansson, thanks for good advice in the lab and in the kitchen. Cecilia Gunnarsson and Marie Askmalm, the medical “gurus” in our group, thanks for answering all my questions about medical and non-medical issues. Jingfang Gao, did you know that the keyword of my PC was DUMPLING? 祝你好運, Piiha Lotta, you have something to teach about discipline and determination, Andreas Lewander, good luck with your research! Tove you came later but have already found a place in this group. Pia Wegman and Sten Wingren once were you also part of this group.

To Xiao-Feng Sun, such a gentle person, thanks for our conversations and all these times that you care about my doings and health. To Liliane Ferraud and Birgitta Holmlund, the hidden force behind the ER and PgR, my eternal providers of the Vindelov fluids (without complains). Thanks for welcoming me. You are such nice persons.

To Anna Esguerra Merca, Lisa Alkhori and Elin Karlsson, my students and coauthors. It has been a pleasure working with you. Keep curious! To Håkan W, Kerstin, Irene, Pia, life as a Ph.D. student becomes less miserable and a lot more enjoyable having your help. To all the friendly souls at KEF, for fikas, relaxing conversations, and help.

Almighty Chatarina Malm, thank you so much for your help with all the administrative issues, for your kindness and care. Anette Wiklund, for your endless patience with all my “föräldralöshetsblanketter” and other “spiritual” issues.
Florence Sjögren, once upon a time you unveiled for me the secrets of the flow cytometer and now you corrected my English. Thanks! Anette Molbaek, you helped me to decipher the mystery of the sequences. Till pedagogerna på Vitra förskolan som har vartit så extra-förståndiga med en glömsk, springande och sliten mamma. To Massimiliano Gentile, when I came all confused and scared I found a friend in you. Thanks for your generosity. I am still missing your extraordinary sense of humor and our good chats about films, books, technology and life. To Anneli Karlsson, Camilla Gårdman, Malin Bergman, we have experienced together some “ups and downs”. Fortunately, we have each other to laugh. I hope we will keep in touch. To Ainhoa, Marta, Anke and Daniel, my dear friends, I cannot imagine life without our private jokes, gatherings, small surprises and long phone calls. I always feel that we have each other. Christianne, Jesús, Ana y Fermín, muchas gracias por haberme acogido, a mí y a mi familia bajo sus alas. Siempre serán bienvenidos. To Rosaura, Rafa y Virginia, mis amigos cubanos, que sería de mi sin nuestros encuentros, sin sus sabios consejos. Con ustedes me siento como en casa. Alexandra (Alex), without your help I could not have finished this thesis. I’m happy that we became friends. Hanna Olausson med familj, jag vet inte riktigt hur stort ditt hjärta är Hanna, du som har delat din familj med mig och som har varit där och lyssnat och uppmuntrat. Jag känner mig rik med er som vänner. Maria Elena Faxas, you welcomed me to your lab in Cuba, when there was no lab and then guided me through the scientific and cultural etiquette. We shared the passion for French language and more than one conspiracy.
ACKNOWLEDGEMENTS

We even won a price for finding the coupling between immunology and witchcraft. Carlos García Santana, you introduced me to the basics of the immune-swamp and showed me its beauty. It was at your lab in Cuba that I heard for the first time about Sweden. I admire you for your tremendous knowledge “que abarca desde lo sublime hasta la timba”.

Mis amigos de Cuba, Ofelia Sevy, Eladio Iglesias, Zaima Mazorra, Dmitri Prieto, Karina Mendoza, L. Orlando Pardo… que me acompañaron durante la carrera y ahora estan desperdigados por el mundo haciendo las mas diversas cosas. Les deseo éxito en todo lo que emprendan!

Ana Maria Barral, mi hermana y querida amiga, you found a mountain for me and encouraged me all the way up. I trust you and very much know how good teacher you are. Your students, like your child, will be prepared to leave you but will always come back…

Familjen Hammarberg, ni är alltid välkomna och efterlängtade.

Familjen Johansson, till Kumla vill man alltid åka för där finns det gott om plats och kärlek för en stor familj.

Mi papa, por tu confianza, cariño y ejemplo de trabajo.

Håkan, Sebastian, Beatrice och Mattias, ni är min trygghet och min bästa inspirationskälla… och med er är jag hemma.

Special thanks to the Swedish Cancer Foundation and the Lion Foundation that supported this work.


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