HER2 and TP53 in human breast cancer
Studies of methods and prognostic value

Jenny Bergqvist

Stockholm 2006
Till minne av pappa,

som lärde mig att det finns viktigare saker i livet än begåvning och framgång: **Vänskap och mod.**
ABSTRACT

Prognostic and predictive factors are needed for tailored therapy strategies, aimed at reducing breast cancer relapse and improving survival. The present arsenal of such factors is insufficient. The aims of this thesis were to study the TP53 gene, and the human epidermal growth factor receptor 2 (HER2), also known as c-erbB-2 and HER2/neu, in human breast cancer, especially in relation to outcome after tamoxifen therapy. In order to make the best out of the biological markers’ prognostic and predictive values, optimal methods for their determinations are required, and this is also addressed in the thesis.

We found mutated TP53 to be associated with worse outcome after adjuvant chemotherapy (CMF), but to be of no statistically significant importance with regard to the outcome of adjuvant tamoxifen. However, in the total patient population, women with wild type TP53 tumours had better survival compared with women whose tumours were TP53 mutated. We used sequenced-based methodology for analysis of all TP53 exons, based on indications that this method is superior to immunohistochemistry IHC in prognostication of breast cancer relapse and survival.

The monoclonal antibody trastuzumab, directed against HER2, is one of many examples of emerging targeting therapies, of which tamoxifen was the first. We describe the first patients in Sweden who received trastuzumab on a named patient basis, all with advanced breast cancer failing on conventional therapy. The previously demonstrated efficacy and acceptable toxicity in randomised studies were confirmed in our small population-based patient cohort.

Endocrine therapy with tamoxifen, mediating both anti-oestrogen, and oestrogen-like effects, has been used for more than three decades. Subsequently, tamoxifen resistance is a problem for a large cohort of breast cancer patients. Preclinical as well as clinical studies have hypothesised HER2 to be involved in tamoxifen resistance through cross-talk signalling with the oestrogen receptor, via the mitogen activated protein kinase, extra cellular regulated kinases 1 and 2 (ERK1/2) pathway. We confirmed HER2 to be associated with worse outcome after tamoxifen therapy while, in contrast to other studies, we found that activated ERK1/2 immunohistochcmical staining, prognosticate better outcome.

The increasing number of patients eligible for trastuzumab therapy requires reproducible, cost-effective, and high throughput assays for HER2 determinations. Immunohistochemistry and fluorescence in situ hybridisation are the most frequently used methods for evaluation of HER2 status. We demonstrate quantitative real-time PCR and an RNA expression-based methodology to generate high-quality HER2 assessments with equivalent, and in some aspects improved results, compared with immunohistochemistry and fluorescence in situ hybridisation/chromogenic in situ hybridisation.

Key words: Breast cancer, HER2, c-erbB-2, TP53, tamoxifen, endocrine resistance
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LIST OF PUBLICATIONS AND MANUSCRIPTS

This thesis is based on the following papers, which in the text will be referred to by their Roman numerals (I-IV):


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ABBREVIATIONS

AF-1  activation function 1
AF-2  activation function 2
AIB1  amplified in breast cancer 1
AIs  aromatase inhibitors
ATR  ataxia telangiectasia related
ATM  ataxia telangiectasia mutated
Bax  pro-apoptotic Bcl-2 associated X protein
BCCS  breast cancer corrected survival
CDK  cyclin dependent kinases
CHK  check point kinases
CI  confidence Interval
CISH  chromogenic in situ hybridisation
CMF  cyclophosphamide, methotrexate, fluorouracil
CR  complete response
EGFR  epidermal growth factor receptor
ER  oestrogen receptor
ERαS118  ERα phosphorylated on Ser 118
EREs  oestrogen response elements
ERK1/2  extra cellular regulated kinases 1 and 2
FFPE  formalin-fixed paraffin-embedded
FISH  fluorescence in situ hybridisation
GADD45  growth arrest and DNA damage-inducible 45
HER2  human epidermal growth factor receptor 2
HR  hazard ratio
HRT  hormone replacement therapy
IGF-1  insulin like growth factor 1
IHC  immunohistochemistry
MAPK  mitogen activated protein kinases
MDM-2  murine double minute 2
MDR-1  multi drug resistance 1
MMP  matrix metalloproteinase
OAS  overall survival
p21  cyclin dependent kinase inhibitor p21
PAI-1  plasminogen activator inhibitor
pERK1/2  activated ERK1/2
PI3K/Akt  phosphatidylinositol-3-kinase/Akt
PCR  polymerase chain reaction
PgR  progesterone receptor
PR  partial response
QRT-PCR  quantitative real time PCR
RFS  relapse-free survival
RNA-EP  RNA expression profiles
SD  stable disease
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFα</td>
<td>tumour growth factor α</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
INTRODUCTION

1. BREAST CANCER

1.1. General background

Breast cancer is globally the most common cancer among females and statistically one out of ten women in Sweden will develop breast cancer during their lifetime (Schwartsmann et al. 2002; The national board of health and welfare 2004). In 2004, 6925 breast cancers were diagnosed in Swedish women, which constitute about 30% of all female cancers diagnosed in Sweden at the same time period. Breast cancer mortality is decreasing despite increasing incidence, and presently approximately 1500 women die from breast cancer in Sweden every year (The national board of health and welfare 2004).

According to a summary published in the year 2003, including women diagnosed with breast cancer during 1990-1994 in Europe, Swedish women had the highest five-year survival in Europe with more than 80% breast cancer survival (Coleman et al. 2003). There are still obvious differences in breast cancer survival among the European countries. However, the differences are decreasing, probably due to earlier diagnosis achieved by mammography screening programs and better therapeutic strategies (Coleman et al. 2003). Furthermore, in Sweden the 5-year relative survival rate has increased from 65% (women diagnosed 1964-1966) to 84% (women diagnosed 1994-1996), during 30 years (Talback et al. 2003).

Great efforts are being made to define and improve the use of prognostic and predictive breast cancer markers in order to accomplish more individualised therapy, aiming at further reduction of recurrence rate and mortality, respectively. The fact that the leukocyte nadir and other toxicities has been demonstrated to have a positive correlation with therapy outcome, i.e. those receiving adjuvant therapy without toxicity may have a worse outcome, further underlining the potential importance of tailored dosage strategies (Cameron et al. 2003; Colleoni et al. 1998; Poikonen et al. 1999; Saarto et al. 1997).

The primary selection of patients for adjuvant therapy has to be refined in order to reduce both over- and undertreatment. In order to achieve this, we need better prognostic and therapy predictive factors.

1.2. Etiology

The majority of breast cancers are sporadic and 8 of 9 women with breast cancer lack family history of the disease (Collaborative Group on Hormonal Factors in Breast Cancer 2001).

1.2.1. Ovarian hormones and reproductive history

The first of two peaks in breast cancer incidence is demonstrated in the 5th decade of life, primarily influenced by ovarian hormones and reproductive history. Endocrine related conditions like early...
menarche, late menopause, and nulliparity, which all include prolonged influence by oestrogen, have all been associated with increased breast cancer risk (Table 1) (Kelsey et al. 1993; Veronesi et al. 2005a). The genotoxic effect of oestrogen is probably due to several mechanisms including the induction of cell proliferation and aneuploidy, in addition to an increase of the mutation rate indirectly through the cytochrome P450 system (Russo and Russo 2004).

Furthermore, young age at first labour, several pregnancies, breastfeeding, and castration before menopause, on the other hand, are followed by a risk reduction (Kelsey et al. 1993; Veronesi et al. 2005a).

Prenatal conditions like intra-uterine life as well as early childhood growth has also been discussed, in addition to birth-weight (Ekbo 1998; Ekbo et al. 1992; Kaisser et al. 2001; Macmahon 2005; Ros et al. 2001; Tamimi et al. 2003).

Table 1. A selection of risk factors in breast cancer and the corresponding relative risks for women. Adapted from (Veronesi et al. 2005).

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Relative Risk (RR)</th>
<th>High-risk group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&gt;10</td>
<td>Elderly individuals</td>
</tr>
<tr>
<td>Age at menarche</td>
<td>3</td>
<td>&lt; 11 years old</td>
</tr>
<tr>
<td>Age at menopause</td>
<td>2</td>
<td>&gt; 54 years old</td>
</tr>
<tr>
<td>Age at first full pregnancy</td>
<td>3</td>
<td>First child after the age of 40</td>
</tr>
<tr>
<td>Breastfeeding and parity</td>
<td>4.3% RR reduction for every year of breastfeeding in addition to a 7% RR reduction for every birth</td>
<td></td>
</tr>
<tr>
<td>Use of exogenous hormones:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral contraceptives</td>
<td>1.2</td>
<td>Current users</td>
</tr>
<tr>
<td>Hormone replacement therapy</td>
<td>1.66</td>
<td>Current users</td>
</tr>
<tr>
<td>Family history</td>
<td>≥2</td>
<td>Breast cancer in first-degree relative</td>
</tr>
<tr>
<td>Cancer in the other breast</td>
<td>&gt;4</td>
<td>Previous breast cancer</td>
</tr>
<tr>
<td>Previous benign breast disease</td>
<td>4-5</td>
<td>Atypical hyperplasia</td>
</tr>
<tr>
<td>Geographical location</td>
<td>5</td>
<td>Developed countries</td>
</tr>
<tr>
<td>Body-mass index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopause</td>
<td>0.7</td>
<td>High body-mass index</td>
</tr>
<tr>
<td>Postmenopause</td>
<td>2</td>
<td>High body-mass index</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>1.07</td>
<td>7% increase with every daily drink</td>
</tr>
<tr>
<td>Exposure to ionising radiation</td>
<td>3</td>
<td>Abnormal exposure to young girls &gt;10 years old</td>
</tr>
</tbody>
</table>
1.2.2. Exogenous hormones

The use of contraceptives generates a small increase (Hazard Ratio (HR) 1.2) of the risk being diagnosed with breast cancer, which will disappear 10 years after the treatment has stopped (Table 1) (Macmahon 2005; Veronesi et al. 2005a).

In the 8th decade of life, primarily influenced by androgens converted to oestrogens via aromatization and hormone replacement therapy, the other peak of breast cancer incidence is found (Colditz 2005). According to a publication from the year 2000 approximately 30-40% of Swedish women at the age of 45-55 years old use HRT (Thunell L 2000). The current use of HRT has decreased, very likely reflecting the recent discussions of increased breast cancer risk, caused by HRT. In addition, recent studies have been unable to demonstrate the previously claimed protective cardiovascular effect and other positive effects including some quality of life issues, based on previous observational studies (Beral 2003; Chlebowski et al. 2003; Hays et al. 2003; Manson et al. 2003).

A study based on more than 1 million women, confirmed previous findings that current and recent use of HRT increases breast cancer incidence (Beral 2003). The combination of oestrogen-progestagen was associated with a higher breast cancer risk (HR 2.0, 95% Confidence Interval (CI) 1.9-2.1) compared with compounds containing oestrogen alone (HR 1.3, 95% CI 1.2-1.4) (Table 1) (Beral 2003). Furthermore, the breast cancer risk was increasing with increased total duration of HRT use, with the highest HR demonstrated in women with a current use since ≥10 years. However, 5-10 years after stopped HRT treatment the breast cancer risk was no longer increased (Beral 2003).

The reason for combining oestrogen with progestagen in HRT is the strong reduction of endometrial cancer, while oestrogen alone markedly increases the risk for endometrial cancer (Beral 2003). One possible explanation for the increased development of breast cancer when using a combination of oestrogen and progestagen instead of oestrogen alone, might be that progestagen reinforce the proliferative effect mediated by oestrogen (Cline et al. 1998). However, the effect of progestagen on breast tissue is not clear, and while some studies report an increase in the proliferation of human breast epithelial cells, others support a decrease (Fournier et al. 2005).

1.2.3. Life style factors

The main hypothesis is that high fat intake increases, while high intake of fruits and vegetables decreases breast cancer risk. However, pooled analysis of prospective studies has not resulted in strong statistical evidence to support this (Veronesi et al. 2005a). Furthermore, a high body mass index was proposed to increase the breast cancer risk in postmenopausal women, while the opposite was found in premenopausal women (Table 1) (van den Brandt et al. 2000). Similarly, studies of physical activity in relation to breast cancer development have demonstrated conflicting results, even though it seems like physical activity reduces the risk of breast cancer (Colditz et al. 2003; IARC 2002). Based on the previously discussed data, a healthier lifestyle including weight control and physical activity are recommended by the European Code against cancer (Boyle et al. 2003).
Other life style factors studied in relation to breast cancer are alcohol and cigarette smoking. The results indicate that alcohol consumption is associated with an increased incidence of breast cancer, while cigarette smoking is not (Hamajima et al. 2002). A study, including postmenopausal women with regard to alcohol consumption and the risk for developing breast cancer, showed a statistical significant increased risk, especially for the development of oestrogen receptor (ER) positive and progesterone receptor (PgR) negative tumours, (HR 2.4, 95%CI 1.6-3.6) (Suzuki et al. 2005).

Additional life style factors, discussed in relation to the development of breast cancer are environmental exposures, especially ionizing radiation, which is known to cause malignant transformation of mammary tissue (Table 1) (Macmahon 2005).

1.3. Prognostic and predictive factors

Ideally a prognostic factor prognosticates the progression of the untreated disease, while a predictive factor predicts the most likely therapy response to a certain therapeutic agent. In reality, today almost all women with primary breast cancer receive adjuvant therapy influencing relapse-free (RFS), breast cancer corrected (BCCS) and overall (OAS) survival. Subsequently, breast cancer materials from recent years will not be representative, due to the lack of untreated patients, for investigation of prognostic factors.

Both prognostic and predictive factors are needed to achieve more individualised therapy strategies that, hopefully, will generate improved survival and reduced number of needless adverse events. Nevertheless, the choice of treatment strategies is so far often based on risk identification using prognostic factors due to the lack of predictive factors.

1.3.1. Stage
So far, the tumour-node-metastasis (TNM) staging system is the most important prognostic tool in human breast cancer (Singletary and Greene 2003; Thor 2004). The TNM system is based on primary tumour size, regional lymph node status and if there are distant metastases present (Table 2). The 10-year overall survival in a retrospective study based on approximately 240 000 women with breast cancer showed relative survival as follows: Stage 0 patients 95%, Stage I patients 88%, Stage II patients 66%, Stage III patients 36%, and Stage IV patients 7% (Bland et al. 1998).

Tumour size is one of the strongest prognostic factors in human breast cancer. Women with node-negative breast cancers less than 2 cm, receiving no adjuvant therapy, had a disease-free survival (DFS) rate of 79% and a median time to recurrence of 48 months at 20-years of follow-up, while patients with primary tumours greater than 2 cm had a survival rate of 64% and a median time to recurrence of 37 months (Quiet et al. 1995). Furthermore, another study reported tumour size ≤5mm (Stage T1N0M0) to be associated with a 100% 9-year distant DFS (Joensuu et al. 2003).
The most important prognostic factor in breast cancer, however, is the axillary lymph node status. The 10-year recurrence rate for women with node positive breast cancer is close to 70%, while the corresponding rate for women with node negative breast cancer is only 20-30% (Mirza et al. 2002). In addition, the mortality increases with the number of affected lymph nodes at diagnosis. The 5-year survival for women without lymph node metastases at diagnosis was in a study 72%, compared with 59%, 52%, and 41% for those with 3, 4, and 6-10 metastatic lymph nodes, respectively (Nemeto T 1983). Furthermore, the median survival after relapse decreases with the number of metastatic lymph nodes at primary diagnosis (Rack et al. 2003). The median survival time in women with ≥4 lymph node metastases at diagnosis was 13 months compared with 20 months in patients with 1-3 lymph node metastases, and 42 months in patients with no lymph node metastases at all, respectively, after relapse (Rack et al. 2003).

In patients with small tumours, the sentinel lymph node biopsy has been demonstrated to predict the axillary status equivalently to complete axillary dissection, but with reduced long-term arm morbidity (Veronesi et al. 2005b; Veronesi et al. 2003).

Patients with distant metastases have in general a median overall survival time of approximately 20 months, but the organ site of the metastasis is of outermost importance (Giordano et al. 2004). Worse 5-year survival is presented for the patients with visceral metastases (13%), followed by those with bone metastases (23%), while 41% of the women with soft tissue recurrences are still alive (Giordano et al. 2004).

**Table 2. Tumour stage and TNM classification. Adapted from (Regional Oncologic Center in Uppsala/Örebro region 2006).**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tumour size (T)</th>
<th>Lymph node status (N)</th>
<th>Distant metastasis (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis = Carcinoma in situ</td>
<td>N0 = No regional lymph node metastasis</td>
<td>M0 = No distant metastasis</td>
</tr>
<tr>
<td>Stage I</td>
<td>T1 ≤ 20mm</td>
<td>N0 = No regional lymph node metastasis</td>
<td>M0 = No distant metastasis</td>
</tr>
<tr>
<td></td>
<td>T1 &gt; 20mm, T2 ≤ 50mm</td>
<td>N1 = Movable ipsilateral axillary metastasis</td>
<td>M0 = No distant metastasis</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T2 &gt; 50mm</td>
<td>N1 = Movable ipsilateral axillary metastasis</td>
<td>M0 = No distant metastasis</td>
</tr>
<tr>
<td></td>
<td>T3 &gt; 50mm</td>
<td>N1 = Movable ipsilateral axillary metastasis</td>
<td>M0 = No distant metastasis</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>T4 &gt; 50mm</td>
<td>N2 = Fixed ipsilateral axillary metastasis</td>
<td>M0 = No distant metastasis</td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>Any</td>
<td>N3 = Metastasis in ipsilateral supra- or infraclavicular lymph nodes</td>
<td>M0 = No distant metastasis</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any</td>
<td>Any</td>
<td>M1 = Distant metastasis</td>
</tr>
</tbody>
</table>

**Primary tumour size (T)**
Tis = Carcinoma in situ, T1 = Tumour ≤ 20mm, T2 = Tumour 21-50mm in greatest dimension, T3 = Tumour > 50mm, T4 = Tumour of any size with direct extension to chest wall or skin and inflammatory carcinoma

**Regional lymph nodes (N)**
N0 = No regional lymph node metastasis, N1 = Movable ipsilateral axillary metastasis, N2 = Fixed ipsilateral axillary metastasis, N3 = Metastasis in ipsilateral supra- or infraclavicular lymph nodes, or internal mammary lymph nodes

**Distant metastasis (M)**
M0 = No distant metastasis, M1 = Distant metastasis
1.3.2. Histopathologic grade

Bloom and Richardson presented in 1957 the most commonly used grading system of today, which was later modified by Elston and Ellis (Bloom and Richardson 1957; Elston and Ellis 1991). The grading system is based on the breast cancer nuclear polymorphism, mitotic count and tubule formation, which are assessed and summarised (Table 3). Histologic grade according to Elston and Ellis has been demonstrated to be of an independent prognostic value in some studies (Elston and Ellis 1991; Kronqvist et al. 2002; Page et al. 1998), and women with primary tumours classified as Histological grade 1 have been reported to have a 95% 9-year survival (Joensuu et al. 2003). However, other studies could not prove histopathologic grade to be of statistically significant importance (Janssen et al. 2003; Younes and Laucirica 1997). Furthermore, the issue of intraobserver variability with regard to the scoring system, and lack of robustness is a question of debate. When pathologists at seven pathology departments scored 93 invasive breast cancers, in order to study the reproducibility of the histologic grading system, the obtained overall mean kappa value was 0.54 (Boiesen et al. 2000).

Another prognostic index, similar to the Elston grade, but based on tumour size, histologic grade, and lymph node status is called The Nottingham Prognostic Index (NPI) (Haybittle et al. 1982). This index has been recommended in order to select patients to adjuvant therapy, especially in the good prognostic group (D'Eredita et al. 2001).

We have now discussed the major prognostic factors in breast cancer and will continue to discuss the predictive factors ER and PgR, which mainly are used to select patients for endocrine therapies (Hayes 2005).

**Table 3.** Histopathologic grade according to Elston and Ellis, based on the grading system introduced by Bloom and Richardson. Adapted from (Regional Oncologic Center in Uppsala/Örebro region 2006).

<table>
<thead>
<tr>
<th>Elston grading system</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubules (T)</td>
<td></td>
</tr>
<tr>
<td>Percentage of tumour area composed of tubules</td>
<td></td>
</tr>
<tr>
<td>&gt;75% of the tumour composed</td>
<td>1</td>
</tr>
<tr>
<td>&lt;10% T &lt;75%</td>
<td>2</td>
</tr>
<tr>
<td>&lt;10%</td>
<td>3</td>
</tr>
<tr>
<td>Mitoses (M)</td>
<td></td>
</tr>
<tr>
<td>Mitotic counts in 10 high power fields</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>1</td>
</tr>
<tr>
<td>10&lt; M &lt;20</td>
<td>2</td>
</tr>
<tr>
<td>&gt;20</td>
<td>3</td>
</tr>
<tr>
<td>Nuclear pleomorphism</td>
<td></td>
</tr>
<tr>
<td>Small nuclei, regular outlines, uniformity of nuclear chromatin</td>
<td>1</td>
</tr>
<tr>
<td>Moderately variation in shape and size, visible nucleoli</td>
<td>2</td>
</tr>
<tr>
<td>Pronounced variation in shape and size, large and abnormal nuclei</td>
<td>3</td>
</tr>
</tbody>
</table>

| Summary                  |       |
| Elston score             |       |
| 3-5                      |       |
| 6-7                      |       |
| 8-9                      |       |
| Diffentiation            |       |
| Well differentiated      | I     |
| Moderately differentiated | II    |
| Poorly differentiated    | III    |
| Grade                    |       |
1.3.3. The oestrogen receptor

The oestrogen receptor alpha (ERα) protein consists of six different regions including the DNA, ligand, and co-regulator binding domains. In addition it contains the constitutively activated transcription activation function (AF-1), which is hormone-independent. The other transcription activating function (AF-2), located in the ligand-binding domain, is hormone-dependent and oestrogen inducible (Figure 1) (Jordan and Morrow 1999; Kumar et al. 1987; Montano et al. 1995).

Available data of ER action in human breast cancer tissue probably reflects the action of ERα, as this isoform is considered to be the one that predominates in most cancers (Leygue et al. 1998; Speirs et al. 1999a; Speirs et al. 1999b). According to this, we will from now on in the text use the abbreviation ER in relation to oestrogen receptor data not exclusively related to the ERβ. However, many previous studies of ER are based on two common assays unable to distinguish between ERα and the more recently discovered ERβ (Koehler et al. 2005; Nicolini et al. 2006; Palmieri et al. 2002).

Oestrogen bound ER dimerises, and translocates to the nucleus, where the nucleotide sequence of oestrogen response elements (EREs) affect the following ER conformation. Subsequently, the actual conformational change modulates the recruitment and activity of co-regulators (Figure 2). (aKlinge et al. 2004). ER working as a transcription factor mediates the majority of ER actions, which is referred to as the classical way of oestrogen action (Figure 2) (Nicolini et al. 2006). Non-classical action is mediated without direct DNA binding, instead ER uses other transcription factors like AP-1 (Kushner et al. 2000). Cross-talk with other growth factor pathways is a non-genomic way of ER action where the signalling is going in both directions (Kelly and Levin 2001; Schiff et al. 2003).

![Figure 1](image.png)

**Figure 1.** The functional domains of the oestrogen receptor α (ERα).
A/B: Transactivation domain, C: DNA binding domain, D: Dimerisation domain, Hsp 90-binding, E: Ligand binding domain, F: Modulation transcriptional activation domain, AF1: Activating function 1, AF2: Activation function 2.
1.3.4. The progesterone receptor

Like the ER, the progesterone receptor (PgR) exists in two isoforms, PgR-A and PgR-B, and an equal ratio of the two is found in normal breast tissue while the ratio in breast cancer clearly is deviant (Graham et al. 1995; Kastner et al. 1990). PgR is one of the most studied ER regulated genes and it mediates the effect of progesterone in both the mammary gland and breast cancer (Conneely et al. 2003). Originally, PgR was mainly considered with regard to the hypothesis that it reflect a functional ER. But according to several recent reports, the PgR is suggested to be the superior one of the two hormone receptors able to subgroup ER positive tumours with reference to survival times (Costa et al. 2002; Ryden et al. 2005). Furthermore, different combinations of the hormone receptor expressions can be used to divide breast tumours into subgroups with distinct characteristics (Arpino et al. 2005). Breast cancers positive for both ER and PgR were less likely to be HER2 protein overexpressers compared with ER+/PgR- tumours (Arpino et al. 2005). In addition, the association between HER2 status and endocrine response varied with PgR status, why PgR negativity might predict an aberrant growth factor signalling rather than a non-functional ER pathway (Arpino et al. 2005).

Another biological marker used for therapy prediction in breast cancer is the human epidermal growth factor 2 (HER2) that is used for selection of patients for trastuzumab therapy, which will be discussed in chapter 4. Additional prognostic and predictive markers in breast cancer, not recommended for use in clinical routine, are TP53 (discussed in chapter 3), cyclins, urokinase plasminogen family, and proliferation markers. These factors will now be discussed in brief.

Figure 2. Oestrogen receptor (ER) activity. Adapted from (Schiff et al. 2005).

The **Classic action** of the ER:
The ER binds directly to the DNA sequence called oestrogen response elements (ERE), and recruit co-regulators.

A. The oestrogen (E)-ER complex generally recruits co-activators (CoA), which results in induced transcription.

B. Tamoxifen (T)-ER complex generally recruits co-repressors (CoR), which blocks transcription.

The **Non-classic action** of the ER:
C. The ER induces transcription indirectly by interaction with other transcription factors, such as AP-1, bound to the DNA.
1.3.5. Cyclins

Cyclins and their corresponding partners cyclin dependent kinases (CDK) control cell cycle progression regulating the transition from G1 to S phase. A high level of cyclin E protein has been associated with TP53 mutations and worse BCCS in multivariate analysis (Keyomarsi et al. 2002) (Lindahl et al. 2004).

Furthermore, adverse effect of tamoxifen in a very small subgroup of women with cyclin D amplified tumours was recently reported (Jirstrom et al. 2005), while others found the cyclin D to be of no prognostic value at all (Keyomarsi et al. 2002; van Diest et al. 1997).

1.3.6. Urokinase plasminogen activator family

Several members of the urokinase plasminogen system have been associated with both RFS and OAS in breast cancer. For example, the urokinase-type plasminogen activator (uPA) and the plasminogen activator inhibitor (PAI-1) predicted RFS and OAS in both node negative and node positive women (Foekens et al. 2000). The uPA converts zymogen plasminogen to active plasmin that degrades the extra cellular matrix, a prerequisite step in tumour invasion and metastasis. In addition uPA has been shown to prevent apoptosis, but stimulate angiogenesis, mitogenesis and cell migration, and to be involved in cell addition (Duffy 2002).

1.3.7. Proliferation markers

Increased cell proliferation has been demonstrated to be associated with worse RFS and OAS in breast cancer in some (Brown et al. 1996; Molino et al. 1997), but not in other studies (Rudas et al. 1994). Moreover, proliferation rate has been correlated with chemotherapy sensitivity (Colleoni et al. 1999; Vincent-Salomon et al. 2004).

The conflicting results may be due to the variety of methods, including mitosis counting, S-phase fraction, Ki-67/MIB1 labelling, and cyclin A index, used to estimate tumour proliferation (van Diest et al. 2004) (Vincent-Salomon et al. 2004). S-phase fraction measured by flow cytometry has been demonstrated clinically useful, prognosticating distant recurrence and survival in node negative premenopausal women (Wenger and Clark 1998).

MIB-1 is a commonly used antibody towards the cell cycle specific antigen Ki-67 (Urruticoechea et al. 2005). A recent study showed that patients with tumours presenting > 20% Ki-67 positive cells had a worse 9-year distant DFS of 80%, compared with 90% for those whose tumours consisted of <20% positive cells (Joensuu et al. 2003).

1.3.8. Vascular endothelial growth factor

Angiogenesis is a prerequisite for tumour progression and the vascular endothelial growth factor (VEGF) is the most studied vascular growth factor. The gene is located on chromosome 6p21.3 and at least five isomers of the VEGF protein can be translated (Linderholm et al. 1998; Vincenti et al. 1996). VEGF165 is the predominant isoform which acts in a paracrine way. The FMS-like kinase (Flt-1, VEGFR-1) and the kinase domain receptor (KDR, VEGFR-2) mediate the mitotic and anti-
apoptotic functions of VEGF (Nicolini et al. 2006). VEGF has been demonstrated to be a negative prognostic factor with reference to RFS and OAS (Gasparini et al. 1997; Linderholm et al. 2000; Toi et al. 1995).

1.3.9. Biomolecular markers in blood

The monitoring of circulating tumour cells in blood and their exposed markers may be of interest with reference to diagnosis of recurrent disease and evaluation of therapy response. A number of tumour-derived circulating markers have been studied, but only CEA and CA15.3 so far, have been considered useful (ASCO 1996; Nicolini et al. 1990).

In addition to the previously mentioned tumour markers, there are many other factors under investigation, including circulation HER2 and TP53, which might become clinically useful in the future (Nicolini et al. 2006).

2. BREAST CANCER TREATMENT

After primary surgery, adjuvant chemotherapy, radiotherapy, and endocrine therapy will follow whenever appropriate in due order (Albain 2002; Wallgren et al. 1996).

2.1. Surgery

Today breast-conserving techniques (partial mastectomies) are frequently used for operable breast cancer, the most common being sector resection. Breast conserving surgery supplemented with local radiotherapy, compared with mastectomy, results in similar survival expectations at 20-years of follow-up (Fisher et al. 2002; Veronesi et al. 2002).

Axillary nodal status is still the most critical prognostic factor for patients with early stage breast cancer. Patients with clinical T1N0M0 stage are without lymph node metastases in 75% of subsequent axillary lymph node dissection (Dees et al. 1997). Due to this, and recent trends toward systemic adjuvant therapy in early stage breast cancer, regardless of axillary status, sentinel lymph node dissection (SLND) has emerged as an alternative to axillary lymph node dissection in women with a small breast cancer (Veronesi et al. 2003).

2.2. Radiotherapy

Breast conserving surgery in patients with invasive breast cancer should be followed by radiotherapy to the breast in order to reduce loco-regional recurrences (Liljegren et al. 1999; Malmstrom et al. 2003; Veronesi et al. 2001). Furthermore, postoperative radiotherapy, against the chest wall and regional lymph nodes, after mastectomy in high-risk premenopausal women with breast cancer receiving adjuvant chemotherapy (cyclophosphamide, methotrexate, fluorouracil (CMF)), has been demonstrated to significantly improve RFS and OAS (Overgaard et al. 1997). Likewise, postoperative radiotherapy against the chest wall and regional lymph nodes, after mastectomy in high-risk postmenopausal women with breast cancer receiving adjuvant tamoxifen, generated
decreased risk of loco-regional recurrence and improved survival (Overgaard et al. 1999). Moreover, an overview has a two-third reduction of loco-regional recurrences and an improved 15-year breast cancer survival of 5.4% (absolute figures) after postoperative radiotherapy, both after breast conserving surgery (the majority of patients node-negative) and mastectomy (all node-positive patients) (EBCTCG 2005).

2.3. Chemotherapy

2.3.1. Adjuvant chemotherapy

The aim of systemic adjuvant therapy is to eradicate possible micro-metastases in any part of the body. The combination of drugs, with preferably non-overlapping toxicity profiles, can accomplish synergistic effects by different mechanism of action. According to an overview based on randomised trials of adjuvant systemic therapy, polychemotherapy generated an absolute gain of the 15-year breast cancer survival in women with a node positive breast cancer of 15% and 6%, in women ≤50 years and 50-69 years old at diagnosis, respectively (EBCTCG 2005). A similar analysis of the 15-year gain of breast cancer survival in node negative women, generated by polychemotherapy, was 10% and 5%, in women ≤50 and 50-69 years old at diagnosis, respectively (EBCTCG 2005). The reason for the less marked effects for the older age group is not known, one may speculate that it partly may be due to poor compliance of the prescribed chemotherapy regimens, especially in the earlier studies when potent anti-emetic drugs were not available and not developed. In addition the overview data regarding outcome may be an underestimation of the chemotherapy effects, while a more recent study including pre/perimenopausal women with node positive breast cancer, revealed an absolute survival gain in the receptor negative group of around 15-20%, compared with women receiving goserelin, at five years of follow-up (Jonat et al. 2002). The absolute difference between anthracycline-based and CMF therapy at 10 years of follow-up was demonstrated to be 4% with regard to both recurrence and breast cancer mortality (EBCTCG 2005). The risk reductions in favour for the anthracycline-based therapies were similar irrespective of age.

2.3.2. Palliative chemotherapy

Chemotherapy with single agents shows 10-70% objective response rates in metastatic breast cancer, and the most commonly used combinations of chemotherapy demonstrate an estimated survival improvement of 12-24 months in median (Bergh et al. 2001).

2.4. Endocrine therapy

Endocrine therapy was first described in 1896 when oestrogen deprivation was generated by oophorectomy and has been frequently used since the 60-ies (Beatson 1896). Similar to adjuvant chemotherapy, the purpose of systemic endocrine therapy is to eradicate micro-metastases in any part of the body.
2.4.1. Adjuvant endocrine therapy

2.4.1.1. Ovarian ablation/suppression

Surgery, radiotherapy and gonadotropin-releasing-hormone (GNRH) agonists can be used in order to generate ovarian suppression. Medical ovarian suppression in premenopausal women, generated by luteinizing hormone releasing hormone (LHRH) or GNRH agonists, decreases both breast cancer recurrence, and mortality rate with 4% and 3%, respectively (absolute risk reductions) (EBCTCG 2005).

2.4.1.2. Tamoxifen

Tamoxifen, which targets the oestrogen receptor with subsequent inhibition of oestrogen-mediated cell proliferation, reduces the absolute risk of recurrence and breast cancer mortality with 12% and 9%, respectively, in women with ER positive breast cancer at 15 years follow-up (EBCTCG 2005). These results may be an underestimation of the effects, due to that 20% of the tumours included in the group with ER positive tumours, actually had unknown receptor status. Furthermore, tamoxifen treatment will decrease the incidence of contralateral breast cancer (EBCTCG 2005).

Tamoxifen is well known to mediate both anti-oestrogen and oestrogen agonistic effects, depending on the tissue context. The anti-oestrogen effect in breast tissue is accompanied by oestrogen-like effects on bone, endometrium, and the liver (Fisher et al. 1998; Jordan et al. 1987; Smith 2003; Wilking et al. 1997). Previous data indicating a protective effect on the cardiovascular system mediated by oestrogen have not been confirmed in recent studies (Beral 2003; Chlebowski et al. 2003). In addition, a recent study showed decreased coronary heart mortality in women receiving 5 years of adjuvant tamoxifen versus 2 years, why the oestrogen-induced cardiovascular effects, still are unclear (Nordenskjold et al. 2005). The HRT data on cardiovascular mortality contrast the tamoxifen data. The potential mechanistic discrepancy between HRT and tamoxifen require further investigation.

The forthcoming 15-year mortality, for a middle-aged woman with an ER positive breast cancer, would be approximately halved by 6 months of chemotherapy followed by 5 years of tamoxifen. This conclusion was made in an overview of randomised trials where women younger than 50 years at diagnosis, demonstrated 38% and 31% mortality reductions after anthracycline-based chemotherapy and tamoxifen treatment, respectively, which are summarised to a final mortality reduction of 57% (EBCTCG 2005).

2.4.1.3. Aromatase inhibitors

The aromatase inhibitors (AIs) inhibit the enzyme responsible for the conversion of peripheral androgens to oestrogen. The third generation of aromatase inhibitors are divided in non-steroidal inhibitors like anastrozole and letrozole, and the steroidal inhibitors like formestane and exemestane (Mouridsen and Robert 2005; Santen et al. 2003). Recently, a DFS advantage for AIs compared with tamoxifen has been demonstrated with regard to adjuvant therapy (Coombes et al. 2004; Howell et al. 2005a; Mouridsen and Robert 2005; Thurlimann et al. 2005). However, no significant overall survival gain was demonstrated in any of the single adjuvant studies. Furthermore, a switch after two to three years of tamoxifen therapy to AIs was more effective than continuing tamoxifen therapy for the remainder of the five year treatment period (Coombes et al. 2004; Jakesz et al. 2005). Moreover,
AIs given after completed 5 years of adjuvant tamoxifen was recently demonstrated to improve DFS (Goss et al. 2005).

2.4.2. Palliative endocrine therapy

2.4.2.1. Tamoxifen and Aromatase Inhibitors

According to a review of randomised clinical trials, no survival benefit was demonstrated in women with metastatic breast cancer receiving endocrine therapy in combination with chemotherapy, why the recommendation from this report was to use them separately (Fossati et al. 1998).

In metastatic breast cancer the objective response to tamoxifen in ER and/or PgR positive breast cancer has been reported to be around 20% and the clinical benefit (complete response (CR)+ partial response (PR)+stable disease (SD)> 6 months) 45% (Nabholtz et al. 2000). Based on data in favour for the aromatase inhibitors compared with tamoxifen, the aromatase inhibitors now challenge tamoxifen, the previously recommended first line treatment for women with an ER positive breast cancer relapse. The overall response and the clinical benefit were demonstrated to be more than 30% and 50%, respectively, in these randomised clinical trials, but no overall survival gains were demonstrated (Bonneterre et al. 2000; Mouridsen et al. 2003; Nabholtz et al. 2000).

2.4.2.2. Other endocrine therapies studied in metastatic breast cancer

Fulvestrant is a new type of endocrine agent that binds, blocks and degrades the ER. In addition, it lacks cross-resistance with other ER antagonists as well as it lacks agonistic effects (Dowsett et al. 2005b; Wakeling et al. 1991). In clinical studies, fulvestrant has shown similar efficacy to tamoxifen (first line) and anastrozole (second line), respectively, in postmenopausal women with advanced breast cancer (Howell et al. 2005b; Howell et al. 2004).

The progestin megesterol acetate was in clinical use before the third generation of AIs. Data is conflicting whether it is superseded or not with regard to efficacy in metastatic breast cancer by the third generations of AIs (Carlini et al. 2005; Hamilton and Piccart 1999; Messori et al. 2000). However, the toxicity profiles are divergent and differences in overall toxicity need to be further studied, and taken into account by the physicians.

3. TP53

3.1. Wild-type TP53

The TP53 gene is located on chromosome 17p13.1 (Soussi et al. 1990; Vogelstein and Kinzler 1992) and it encodes the p53 protein, which is a 53kDa nuclear phosphoprotein. Cellular stress like DNA damage, ultraviolet-light, ionizing radiation, and hypoxia increase TP53 activity (Graeber et al. 1996; Hall et al. 1993; Harris 1996; MacCallum et al. 1996; Ponten et al. 2001), which will both induce and repress transcription of several down-stream genes (Figure 3). p53 mediates signalling in various cellular pathways, which are in control of cell survival, cell proliferation, and survey of the genomic integrity (Harris 1996).
The p53 protein consists of 3 domains with separate functions. The DNA binding domain is probably the most indispensable with reference to its tumour suppressor activity. Furthermore, this domain contains most of the mutations, although mutations may occur anywhere (Borresen et al. 1995; Hollstein et al. 1994). In addition to the DNA binding region, p53 contains an amino-terminal transactivation domain and a multifunctional carboxy-terminal oligomerisation domain (Figure 4).

3.2. Mutated TP53

The TP53 gene is one of the most well studied genes and proved to have an impaired function in the majority of human cancers, which results in a proliferative advantage (Hollstein et al. 1991; Levine et al. 1991). In 20-30% of the human breast cancers, mutated TP53 has been demonstrated to be a negative prognostic factor associated with worse RFS and OAS (Pharoah et al. 1999; Soussi and Beroud 2001).

![Figure 3. Schematic presentation of some of the genes up-stream and some of the mediators down-stream TP53. Adapted from Liu et al., Drug development research, 2004).](image-url)
3.3. TP53 regulations and mutations

The main task for wild type p53 is to induce cell cycle arrest (el-Deiry et al. 1993; Kastan et al. 1992; Lin et al. 1992), allowing DNA repair, or if the DNA is extensively damaged, trigger apoptosis (Figure 3) (Amundson et al. 1998; Clarke et al. 1993). The cyclin dependent kinase (CDK) inhibitor p21 and the growth arrest and DNA damage-inducible Gadd45, are examples of factors mediating p53-induced growth arrest and DNA repair (el-Deiry et al. 1993; Kastan et al. 1992). Another result of TP53 activation may be increased levels of the pro-apoptotic Bcl-2 associated X protein (bax), which is followed by release of cytochrome C from the mitochondria and activation of the caspase cascade (Miyashita et al. 1994; Nicholson and Thornberry 1997; Selvakumaran et al. 1994). In addition, p53 decreases the levels of the anti-apoptotic Bcl-2 protein (Haldar et al. 1994; Miyashita et al. 1994). Furthermore, p53-induced apoptosis can follow several additional mechanisms, which will not be discussed within this thesis (Nicolini et al. 2006).

Figure 4. Functional domains of the p53 protein

Evolutionary conserved domains
In addition to mutations, there are several other mechanisms that alter p53 functions. These mechanisms include alterations of the ataxia telangiectasia mutated (ATM), Check point kinases 2 (CHK2) and other genes involved in the phosphorylation, acetylation and ribosylation of p53, which is followed by impaired TP53 function (Borresen-Dale 2003). Moreover, the tumour suppressor TP53 activates the expression of its own negative regulator Mdm2, which both blocks the transcriptional activity of TP53 and mediates its degradation (Haupt et al. 1997). A result of the impaired p53 function discussed previously, including activation of the Multi drug resistance 1 (Mdr-1) protein (Saeki et al. 2005), may cause drug resistance. This hypothesis is based on studies demonstrating that cell cycle arrest and apoptosis, induced by chemotherapeutic agents, are dependent on a functional TP53 pathway (Lowe et al. 1994; O’Connor et al. 1997). However, TP53 status has been demonstrated to predict resistance to antitumour therapies in some studies (Aas et al. 1996; Bergh et al. 1995; Berns et al. 2000; Geisler et al. 2003; Kandioler-Eckersberger et al. 2000; Lowe et al. 1994) but not in others (Archer et al. 1995; Bertheau et al. 2002; Rozan et al. 1998).

Conflicting data with regard to the therapy predictive value of TP53 status is probably in part due to different accuracies of the methods used for TP53 evaluation, and that different chemotherapy regimens with different dose-intensities were used (Aas et al. 1996; Berns et al. 2000; Bertheau et al. 2002; Geisler et al. 2001; Kandioler-Eckersberger et al. 2000; Lai et al. 2004; Olivier et al. 2006). In addition many of the studies have lacked sufficient statistical power needed to demonstrate a significant impact of TP53 status (Borresen-Dale 2003). Recently, an expression signature for TP53 status was demonstrated to be of both prognostic and predictive importance in human breast cancer (Miller et al. 2005). The TP53 mutated signature predicted worse disease specific survival after tamoxifen therapy.

### 3.4. Detection methods

A variety of methods have been used in order to investigate TP53 status. In brief, they can be separated into DNA/RNA and protein-based methods. Different methods are used for screening purposes compared with analysing mutations at a particular position.

A variety of DNA and RNA based assays can detect TP53 mutations in predefined positions. The allele specific oligonucleotide hybridisation, oligonucleotide ligation assay, and the ligase chain reaction are some examples, all requiring knowledge of the sequence for the position of the possible mutation site, as well as the sequence adjacent to it (Barany 1991; Landegren et al. 1988; Saiki et al. 1986). For patient tumour samples, however, screening of the entire TP53 gene is often desired. Single strand conformation polymorphism (SSCP) and denaturing high-performance liquid chromatography (DHPLC) are examples of DNA/RNA based methods visualising different migration patterns for samples with mutated compared with wild type TP53 (Liu et al. 1998; Orita et al. 1989). These methods are unable to show the position of the mutation.

The exact sequence of the TP53 gene can only be investigated by sequenced-based analysis (Elledge 1996), which has been demonstrated to be superior to IHC in prognosticating RFS, BCCS and OAS (Bergh et al. 1995; Berns et al. 2000; Bertheau et al. 2002; Clahsen et al. 1998; Overgaard et al. 2000; Sjogren et al. 1996). Nevertheless, the most frequently used method for determination of TP53 status is immunohistochemical analysis.
3.4.2. Immunohistochemistry compared with sequencing

The immunohistochemical staining generated by an antibody towards p53 protein visualise mutated p53, due to its decreased degradation compared with wild type p53 protein, which is rapidly degraded and normally not possible to detect with IHC (Bartek et al. 1990; Crawford et al. 1984). There are, however, both false negative and false positive results with concern to TP53 status, comparing IHC results with sequencing data. Immunohistochemical staining may be generated by other causes than a mutated p53 protein, including increased levels of wild type p53, and interpretation difficulties evaluating the immunohistochemical stainings, which generate false positive results (Sjogren et al. 1996). The opposite, with false negative results, may occur due to mutations generating a truncated protein, a protein unrecognisable by the antibody, or no protein at all (Brandt-Rauf et al. 1992; Finlay et al. 1988) (Sjogren et al. 1996; Williams et al. 1998).

False positive and negative results may, of course, also occur using the sequencing technique. Contamination of analysed DNA may generate false positive results and a low tumour cell/normal cell ratio may generate false negative results, respectively. Nevertheless, these drawbacks could be minimised by including steps for control of the DNA/RNA quality and quantity.

4. HER2 AND TRASTUZUMAB

4.1.1. The HER2 molecule in breast cancer

The human epidermal growth factor receptor 2 (HER2), also known as c-erbB-2, is homologous to the viral erythroblastosis leukaemia 2, and Epidermal growth factor receptor 2 (EGFR2). HER2 is also homologous to Neu, found in rats, and is why the term HER2/neu is sometimes seen. The 185 kDa transmembrane tyrosine kinase receptor is located on chromosome 17 (17q11.2-q12) and HER2 is one of four members in the Epidermal growth factor receptor (EGFR) family. This family of transmembrane receptors includes the EGFR, HER2, HER3 and HER4. A variety of homo- and heterodimers have been demonstrated between the four family members, regulating their cellular effects (Abd El-Rehim et al. 2004). All members except HER2 have several known ligands. HER3 and 4 binds heregulins while EGFR binds EGF, TGF-α, amphiregulin, epiregulin and betacellulin (Zaczek et al. 2005). The orphan HER2 receptor signals through heterodimer formation with the other three members and is the preferred heterodimer partner (Zaczek et al. 2005). In addition, constitutively activated/phosphorylated HER2 has also been demonstrated (Zaczek et al. 2005).

The frequency of breast cancer tumours with HER2 protein overexpression and/or HER2 amplification vary between 10-34% according to previous literature (Descotes et al. 1993a; Descotes et al. 1993b; Revillion et al. 1998; Ring and Dowsett 2003; Ross and Fletcher 1999; Sjogren et al. 1998; Slamon et al. 1987; Slamon et al. 1989). The overall rate of HER2 positivity was around 20% in a summary of 22 616 breast cancer tumours (Revillion et al. 1998).

Protein overexpression and/or gene amplification of the oncogene HER2 are associated with aggressive biological behaviour and reduced RFS and survival times in both node negative and node positive women (Lohrisch and Piccart 2001; Revillion et al. 1998).
In addition to gene amplification, genetic alterations of HER2 such as splice variants resembling neu mutants, and small in frame insertions or missense substitutions in the kinase domain, have been found in human carcinoma (Siegel et al. 1999; Stephens et al. 2004). Furthermore, proteolytic cleavage and shedding of HER2's extra cellular domain will result in a truncated HER2 with increased kinase activity and enhanced transforming ability, which has been associated with lymph node metastasis in human breast cancer (Molina et al. 2002).

The knowledge of the association between worse breast cancer survival and positive HER2 status led to the development of the humanised monoclonal antibody trastuzumab. HER2 protein expression or amplification should be addressed in order to select patients eligible for trastuzumab therapy. In addition, women with HER2 positive tumours are prone to respond to anthracycline-based therapy, which partly may be due to co-amplification of the topoisomerase II α gene for a subgroup of these patients with a HER2 amplification (Di Leo et al. 2002; Jarvinen et al. 2000b; Yarden and Sliwkowski 2001). Whether HER2 can also be a predictive factor with regard to tamoxifen therapy is more controversial, even though the majority of studies support this (Berry et al. 2000; Elledge et al. 1998; Houston et al. 1999; Wright et al. 1992). Moreover, neoadjuvant therapies with AIs have been found to be more effective than tamoxifen in HER2 positive tumours (Dixon et al. 2004) (Ellis et al. 2001). HER2 is an established prognostic factor in the St Gallen guidelines from 2005 (Goldhirsch et al. 2005). This strategy is also adopted by the Swedish Breast Cancer Groups, which also recommend the selection of an AI instead of tamoxifen, for patients with HER2 amplified cancers (Group 2006).

4.1.2. Trastuzumab therapy

The humanised monoclonal antibody trastuzumab was approved in 1998 for treatment of women with HER2 positive metastatic breast cancer in the USA, and in Europe the drug was approved in 2000. With this antibody it is possible to offer patients with HER2 protein overexpressing breast cancers therapeutic targeting at a molecular level.

The exact mechanism of trastuzumab's anticancer effect is not known. A direct growth inhibitory effect has been demonstrated in HER2 overexpressing human tumour cells in vivo (Drebin et al. 1988a; Drebin et al. 1988b) and several modes of action have been presented (Baselga et al. 2001; Treish et al. 2000):

- Internalisation and degradation of HER2 cause disruption of growth signalling (Sarup et al. 1991). But recently, trastuzumab was demonstrated to inhibit growth irrespective of HER2 down-regulation (Longya et al. 2005).
- Cell cycle arrest induced by accumulation of the cyclin-dependent kinase inhibitor p27(Kip1) and inactivation of cyclin-Cdk2 complexes (Lane et al. 2000).
- Induction of antibody dependent cellular toxicity following the interaction with immunoglobulin G1 Fc region (Clynes et al. 2000).
- Inhibition of HER2 ectodomain cleavage in breast cancer cells, which reduce the amount of active truncated HER2 fragments (Molina et al. 2001).
- Trastuzumab has also been suggested to suppress angiogenesis (Petit et al. 1997; Sliwkowski et al. 1999).
- Recruitment of functional PTEN to the plasma membrane, which reduce PI3K and subsequent Akt activity (Longya et al. 2005).
Moreover, the combination of trastuzumab with other drugs gives additional mechanisms at hand, like interaction with the DNA repair mechanism of cisplatin-induced DNA damage (Pietras et al. 1998).

The first clinical studies of trastuzumab included women with HER2 positive metastatic breast cancer. The monoclonal antibody proved its efficacy as a single agent against metastatic breast cancer in both previously treated and untreated women (Cobleigh et al. 1999; Vogel et al. 2002). Overall response rate and clinical benefit rate was 19%-35% and 33%-48%, respectively, when trastuzumab alone was given as first-line treatment to women with HER2 positive metastatic breast cancer (Baselga et al. 2005; Vogel et al. 2002). Prolonged RFS (3-5,6 months) and OAS (5-8,5 months) were observed when trastuzumab was added to chemotherapy in first line therapy of metastatic breast cancer (Marty et al. 2005; Slamon et al. 2001). The overall response was 50-61% compared with 32-34%, for trastuzumab in combination with chemotherapy, compared with chemotherapy alone, respectively (Marty et al. 2005; Slamon et al. 2001).

Previously the monoclonal antibody trastuzumab was given once a week (2mg/kg) based on pivotal trials that showed a mean terminal half-life of 6-9 days. Later, revision of the pharmacokinetic studies revealed a half-life of 23 days (Leyland-Jones 2001). Subsequently, trastuzumab is now administered once every three-week (6mg/kg), with maintained efficacy and tolerability. The toxicity profile and response rates are around 20% for monotherapy and 60% in combination with chemotherapy, respectively, in women with metastatic breast cancer (Baselga et al. 2005; Marty et al. 2005).

Promising data also in the adjuvant setting of breast cancer therapy was recently reported. Romond and colleagues showed an absolute difference in DFS and OAS of 12% and 2,5%, respectively, in favour for patients receiving trastuzumab in addition to adjuvant chemotherapy compared with those receiving chemotherapy alone with a median follow-up time of 2 years (Romond et al. 2005). Another study investigating adjuvant trastuzumab showed a statistically significant reduction of the number of events (recurrence of breast cancer, contralateral breast cancer, second non-breast malignant disease, or death) in the trastuzumab treated patients (HR 0.5 95% CI 0.4-0.7), however, no OAS gain was demonstrated with only a median follow-up of 1 year (Piccart-Gebhart et al. 2005). In addition, 9 weeks of adjuvant trastuzumab after chemotherapy, resulted in improved RFS (HR 0.4 95% CI 0.2-0.8) (Joensuu et al. 2006). The positive effects of trastuzumab in both primary and metastatic disease, together with good tolerability and a favourable toxicity profile will likely increase the number of patients treated with trastuzumab. Trastuzumab is accompanied by relatively modest adverse events, mostly mild to moderate infusion related reactions, compared with adverse events related to chemotherapy. The important exception is cardiotoxicity that limits its use together or after anthracyclines (Cobleigh et al. 1999; Seidman et al. 2001; Vogel et al. 2002). The recent adjuvant studies present 3-4% of serious cardiac events, of which a minority remains after discontinued treatment (Piccart-Gebhart et al. 2005; Romond et al. 2005; Tan-Chiu et al. 2005). Furthermore, neoadjuvant therapy with trastuzumab together with chemotherapy has been studied in phase II clinical trials and demonstrated pathologic complete response rates of 12-42% in addition to clinical response rates of 70-100% (Baselga et al. 2004; Buzdar et al. 2005).
4.1.2.1. Trastuzumab resistance

Not all HER2 positive tumours respond to trastuzumab therapy. Breast cancers with a strong (3+) HER2 protein overexpression show a better response to trastuzumab compared with cancers that has a moderate (2+) expression (Baselga 2001; Slamon et al. 2001). Nevertheless, all tumours with a strong protein overexpression do not respond to trastuzumab therapy (Cobleigh et al. 1999; Hudelist et al. 2004; McKeage and Perry 2002; Slamon et al. 2001).

Heterogeneity of HER2 status between primary tumours and their corresponding metastases might be one explanation for trastuzumab resistance. The discordant HER2 protein expression between primary and secondary tumours of 4.5% presented in a study (Sekido et al. 2003), was by the authors suggested to probably reflect a heterogeneous gene amplification in the primary tumours. In addition, several previously published reports suggest that HER2 status is stable over time (Cardoso et al. 2001; Carlsson et al. 2004; Simon et al. 2001; Tanner et al. 2001). However, some of those studies include only axillary lymph node metastasis, and some are based on autopsy material, why we think that further studies investigating HER2 status over time are needed (Cardoso et al. 2001; Niehans et al. 1993; Simon et al. 2001; Symmans et al. 1995). However, among paired primary/metastatic tumours, 5 out of 68 cases were discordant in one study, when analysed by FISH (Gancberg et al. 2002), while another study of primary tumours and corresponding metastasis (including both distant and loco-regional metastases) showed complete concordance (Tanner et al. 2001).

Recently, a change of HER2 status over time was reported in a study where 9 of 24 women with HER2 negative primary breast cancer acquired HER2 gene amplification during progression of the disease (Meng et al. 2004). In addition, the development of high concentrations of the extra cellular portion of HER2 has been demonstrated in serum along with breast cancer progression in patients with HER2 negative primary tumours (Kandl et al. 1994; Molina et al. 1996).

These data together with variability of oestrogen- and progesterone receptors, statistically significant discordance of approximately 30% between primary tumours and corresponding metastases has been demonstrated (Franco A 2004), should be an indication for biopsy verification of the receptor and HER2 status in the metastatic lesions before initiating therapy.

Another mechanism for trastuzumab resistance could be that HER2 signalling is counteracted by IGF-I-R signalling (Lu et al. 2004; Lu et al. 2001). Moreover, a better response to trastuzumab therapy has been reported for tumours with activated/phosphorylated HER2 compared with tumours with unphosphorylated HER2 (DiGiovanna and Stern 1995; Hudelist et al. 2003b).

4.1.3. Detection methods

The United States Food and Drug Administration has approved the use of either fluorescence in situ hybridisation (FISH, Figure 5) or immunohistochemical (IHC, Figure 1, 3A, Paper III) tests (using Hercept Test, Dako (polyclonal antibody) or the monoclonal antibody (CB11) for the determination of HER2 expression and/or amplification (Birner et al. 2001). IHC on paraffin-embedded tumour material has so far been the most widely applied test for HER2 evaluation. The most commonly used scoring system is the HercepTest criteria, recommended by Dako, (Glostrup, Denmark): 0 for tumours with <10% of the tumour cells stained, 1+ corresponding to faint tumour cell membranous staining present in >10% of the tumour cells, 2+ corresponding to moderate tumour cell
membranous staining present in $>10\%$ of the tumour cells, and 3+ corresponding to strong tumour cell membranous staining present in $>10\%$ of the tumour cells.

Recently, Ginestier and colleagues compared five different antibodies with FISH and real time PCR on both RNA and DNA, respectively (Ginestier et al. 2004). They suggested improvements of the immunohistochemical technique rather than to use real time PCR, in order to achieve more correct HER2 assessments in the future. However, IHC is significantly influenced by several factors like, fixation protocols, staining procedures, scoring systems and the use of different antibodies (Pauletti et al. 2000; Tubbs et al. 2001). In addition, the interpreting difficulties remain, although a new technique, the Automated Cellular Imaging System (ACIS, ChromaVision Medical Systems, San Juan Capistrano, CA), has been reported to improve the immunohistochemical analysis (Wang et al. 2001). This strategy is to be further investigated.

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**Figure 5.** A. Immunohistochemical analysis of a breast cancer with a strong (3+) HER2 protein overexpression. B. Fluorescence in situ hybridisation analysis of a HER2 amplified breast cancer. By courtesy of Jorma Isola, Tampere University, Finland.
The enzyme-linked immunosorbent assay (ELISA) technique offers quantitative HER2 evaluation well suited for automated procedures, and is relatively easy to perform (van de Vijver 2002; van de Vijver 2001). However, the method cannot be used on formalin-fixed paraffin-embedded tissue, which constitutes most commonly available tumour tissue in the current tumour archives.

According to current recommendations for HER2 testing in the UK, nothing but FISH and IHC should be used for clinical diagnostics, while ELISA, Southern blotting, chromogenic in situ hybridisation (CISH) and polymerase chain reaction (PCR) should be considered experimental (Ellis et al. 2004). The combination of the two tissue based methods IHC and FISH is characterised by high practical acceptance and reproducibility (Bankfalvi et al. 2000; Hanna et al. 1999; Schnitt 2001) and previous studies have reported a high concordance (91%) between the two methods (Jacobs et al. 1999; Jimenez et al. 2000). In addition, a strong HER2 (3+) protein overexpression and HER2 gene amplification have been associated with similar clinical outcome after trastuzumab therapy (Baselga 2001; Vogel et al. 2002). A frequently used HER2 testing algorithm is based on screening for HER2 status using IHC, followed by retesting with FISH or CISH in borderline cases with a moderate (2+) protein overexpression (Figure 6). This is based on data demonstrating that only about one out of four HER2 2+ tumours were amplified according to FISH (Mass 2000a).

**HER2 EVALUATION**

Figure 6. The commonly used algorithm for evaluation of HER2 status.
CISH is an alternative technique to FISH for detection of HER2 amplification to establish whether the tumours are actually HER2 amplified or not (Figure 6). In Finland, CISH is used instead of FISH, based on its equivalent results (Isola et al. 2004; Tanner et al. 2000). In one study, HER2 status generated by FISH and CISH were concordant in 180 of 197 (93.8%) formalin-fixed paraffin-embedded (FFPE) tumour samples and there was no difference in success rates between the methods (Isola et al. 2004). CISH does not require fluorescent dye but uses peroxidase-based immunostaining, which can be visualised using conventional light microscopy (Tanner et al. 2000). In addition, the staining will not decay over time (van de Vijver 2002). Accurate assessments of HER2 status is a prerequisite for finding the breast cancer patients who will show clinical benefit from trastuzumab therapy, but the issue of how to best evaluate HER2 is not known and previous data are conflicting (Pauletti et al. 2000; Seidman et al. 2001; Tubbs et al. 2001). In scientific work, additional methods have been used with different degrees of success. We present results in favour for the use of quantitative real time polymerase chain reaction (QRT-PCR) on DNA, in addition to micro array based analyses using RNA, for the assessment of HER2 in Paper IV.

5. ENDOCRINE RESISTANCE

5.1. Background

Elevated levels of oestrogen as well as prolonged influence by oestrogen have for a long time been considered as risk factors for breast cancer development (Kelsey 1993; Macmahon 2005). Consequently, large efforts have been made to develop anti-oestrogen therapies. The ER and PgR status are used to select patients for endocrine therapy. However, not all women with hormone receptor positive breast cancers respond to anti-oestrogen therapy, why endocrine resistance is a problem (Schiff et al. 2003). Objective response rates of about 20% and clinical benefit (CR + PR + SD >6 months) in approximately 50%, have been demonstrated in women with metastatic breast cancer, treated with tamoxifen or aromatase inhibitors, respectively (Bonneterre et al. 2000; Buzdar et al. 2004; Mouridsen et al. 2003). In addition, the majority of all breast cancer patients, who initially respond to given endocrine therapy, will sooner or later develop tumour progression, and in most instances therapy failure is due to secondary resistance.

5.2. Tamoxifen resistance

There are several possible mechanisms of tamoxifen resistance, such as changed ER and or PgR expression or function, changed metabolism of tamoxifen, and changed oestrogen response. Furthermore, the tumour specific supply of co-regulators of the ER, and an increased growth factor receptor signalling will affect the outcome of oestrogen induced signalling as well as tamoxifen therapy.
5.2.1. Modified ER and or PgR expression or function

The response to tamoxifen therapy has clearly been demonstrated to be associated with the ER and PgR expression with only a few percent responders in patients with tumours lacking both of the hormone receptors (Dowsett et al. 2005a; Osborne 1998). The capital mechanism of de novo resistance to tamoxifen therapy is probably lack of the ERα. However, the emergence of ERβ, may explain tamoxifen response and/or resistance in ways different from those associated with ERα expression (Hopp et al. 2004). ERβ has been associated with good prognostic factors like lymph node negative status in addition to low proliferation status in some studies (Jarvinen et al. 2000a), while others have found ERβ to be a negative prognostic factor correlated with PgR negative and lymph node positive tumours (Speirs et al. 1999b). In conclusion, the recently identified ERβ has a potential role as both a prognostic and predictive factor in breast cancer, but needs to be further investigated.

However, lack of ER cannot be the only cause of de novo resistance, which occur despite the presence of ER expression, but 17-28% of women developing resistance to tamoxifen presented a concurrent loss of ER expression (Gutierrez et al. 2005; Johnston et al. 1995). Furthermore, there are data demonstrating a discordant hormone receptor expression between the primary and secondary tumours, why it is important to verify receptor status in the current metastasis. Moreover, the fact that overall response rates of 16-21% were demonstrated in tamoxifen resistant women, receiving second-line therapy with aromatase inhibitors or fulvestrant, indicate that the ER is still expressed despite acquired tamoxifen resistance (Howell et al. 2002; Osborne et al. 2002).

Down regulation of the PgR, caused by aberrant growth factor receptor signalling, has been suggested to interact with tamoxifen response (Cui et al. 2003). Both the PgR and ER expression change during endocrine therapy, and the majority of tumour cells demonstrate loss of the PgR at tamoxifen resistance (Gross et al. 1984; Hull et al. 1983).

5.2.2. Changed metabolism of tamoxifen

Genetic polymorphisms generating a changed metabolism of tamoxifen, may cause resistance to tamoxifen therapy (Nowell et al. 2002; Wegman et al. 2005).

5.2.3. Available co-regulators

Different nuclear-receptor co-regulators bind to the AF-2 domain of the ER and enhance or decrease the ER activity, respectively. Amplified in breast caner 1 (AIB1), also named SCR-3, is one of the most important and frequently discussed co-activators that is amplified in some oestrogen- and progesterone receptor positive breast cancers, enhancing transcription and growth mediated by the oestrogen receptor (Bautista et al. 1998). On the other hand, AIB1 is associated with high tumour grade, HER2 positivity, and TP53 mutated status, which suggests an inverse correlation per se to the hormone receptors (Bouras et al. 2001; Hudelist et al. 2003a). Furthermore, the prognostic and predictive values of AIB1 were different in women treated with adjuvant tamoxifen compared with in untreated women. Patients receiving adjuvant tamoxifen, displayed that high levels of AIB1, were associated with worse RFS compared with women whose tumours contained low levels of AIB1 (Osborne et al. 2003). On the contrary, the same
study presented high levels of AIB1 to prognosticate a better RFS in women not receiving adjuvant therapy. Moreover, AIB1 is restricted to the uterus, mammary gland and testis, while other co-regulators have a more general distribution in several organs (Suen et al. 1998).

ERα has been associated with the co-repressor NCoR, while ERβ has demonstrated correlations with the co-activators SRC-1 and TIF2 in addition to NCoR (Hudelist et al. 2003a). Nevertheless, even though the lack of benefit from endocrine therapy seems unclear, the cell and tissue specific balance between co-repressors and co-activators probably is important, deciding whether the anti-oestrogen or the oestrogen agonistic effects mediated by tamoxifen will rule (Jordan et al. 1987; Osborne et al. 2003).

5.2.4. Increased growth factor receptor signalling

Reduced ERα transcription and/or function with subsequent tamoxifen resistance might be due to increased growth factor receptor signalling. ERα is able to interact with and/or activate as well as to be activated by several kinases including the Insulin like growth factor 1 receptor (IGF-1R), the phosphatidylinositol-3-kinase (PI3K)/protein-serin/threonine kinase Akt (also called protein kinase B), the mitogen activated protein kinases (MAPK)/ extra cellular regulated kinases 1 and 2 (ERK1/2), EGFR, and HER2 (Bunone et al. 1996; Campbell et al. 2001; Kahlert et al. 2000; Kato et al. 1995; Migliaccio et al. 1996; Schiff et al. 2004; Stoica et al. 2003b). Ligand independent cross-talk between the ER, IGF and EGFR, respectively, allowing signalling in both directions, display great biologic complexity (Gutierrez et al. 2005; Lee et al. 1999; Schiff et al. 2003). AF-1 can be phosphorylated in a ligand independent manner at serine 118 by activated ERK1/2 (Bunone et al. 1996; Chen et al. 2002; Kato et al. 1995). Growth factors affecting tyrosine kinase membrane receptors such as EGF, IGF-1, heregulin and tumour growth factor α (TGFα) and β, are the major regulators of the ERK1/2 pathway (Pearson et al. 2001).

The hypothesis that ERα may repress growth inhibitory genes in addition to its ability to repress other growth signalling pathways, respectively, could explain the conflicting biological effects induced by oestrogen (Gross and Yee 2002; Soto and Sonnenschein 2001). Conflicting results can probably be explained at least in part by the fact that ERα is unable to induce transcription on its own but is dependent on the cell and tissue specific available co-regulators, why the transcriptional activity is dependent on the cellular pool of ER-regulators (Gross and Yee 2002; Horwitz et al. 1996; McKenna et al. 1999).

These cross-talks are sometimes unpredictable, and ambiguous, especially if we are not able to characterise the tissue and cell specific context. Better understanding of the interaction between these diverse elements including signalling in both directions will, hopefully, help us in the search for new prognostic, predictive, and therapeutic tools.

5.3. Mechanisms of cross-talk between the ER and EGFR pathways

The EGFR family mediates signalling possibly involved in endocrine resistance by cross-talk with the ER (Nicholson et al. 1999; Shou et al. 2004). The Ras-Raf-MAPK-, and the PI3K/Akt pathway, have been described as the two major down-stream signalling pathways for HER2 (Figure 7) (Alroy and Yarden 1997; Vivanco and Sawyers 2002). The Ras/Raf/MAPK pathway and the PI3K/Akt
pathway principally stimulate proliferation and survival, respectively, following EGFR signalling (Ben-Levy et al. 1994; Peles et al. 1992). Ligand availability and the composition of heterodimers will engage specific signalling pathways, to various degrees depending on the intensity and duration of the mediated signals (Abd El-Rehim et al. 2004; Citri et al. 2003). PI3K/Akt and ERK1/2 kinases are both able to phosphorylate the ER itself or some of its co-regulators, which may in turn affect the response to anti-oestrogen therapy (Font de Mora and Brown 2000; Hong and Privalsky 2000; Kato et al. 1995; Shou et al. 2004).

**Figure 7.** Cross-talk between the EGFR and ER through the ERK1/2 and the PI3K/Akt Pathways. Different drugs (in italic) and their targets are included. e=oestrogen, GPCR=G-protein coupled receptor, PI3K=Phosphatidylinositol 3-OH kinase, AKT=protein kinase B (PKB), MMP: matrix metalloproteinase, HB-EGF=Heparan-bound epidermal growth factor, Shc=SRC homology containing, ERK1/2=extracellular-regulated kinase, PDK-1= protein serine/threonine kinase 3'-phosphoinositide-dependent kinase 1, Mdm-2=murine double minute 2, p21: CDKN1A cycline dependent kinase inhibitor 1A (Cip-1, Waf-1), AIB1=Amplified in breast cancer 1, EGFR-TK: Epidermal growth factor receptor-tyrosin kinase.
5.3.1. The MAPK/ERK1/2 pathway

There are three major and well-known mitogen activated protein kinases (MAPK) in human. These are the Jun NH2-terminal protein kinase (JNK), p38 MAPK, and the extra cellular regulated kinases 1 and 2 (ERK1/2). The ERK1/2 pathway is so far considered to be the most relevant cascade of these three with regard to breast cancer (Santen et al. 2002). Growth factors and other soluble ligands together with their receptors are activators of the ERK1/2 pathway (Pearson et al. 2001).

Oestradiol can mediate cell proliferation via the ERK1/2 kinase cascade due to activation of Elk-1 and other factors involved in cell proliferation (Figure 7) (Pearson et al. 2001). Likewise, oestadiol-induced genomic activation may lead to ERK1/2 phosphorylation of ERα at Ser-118, which also enhances transcription (Kato et al. 1995). Probably as a security measure ERK1/2 activation usually stimulates apoptosis in the absence of certain necessary survival factors (Kauffmann-Zeh et al. 1997; Pearson et al. 2001).

Furthermore, oestradiol also induces rapid non-genomic ERK1/2 activation, most probably via a membrane-bound form of ER. Indeed, Castoria and colleagues suggest that such non-genomic ERK1/2 activating events, conduct the major proliferation effect by oestradiol (Castoria et al. 1999). Moreover, Filardo et al. claim that ER-independent, oestrogen-mediated transactivation of the EGFR family can occur through a G-protein-coupled receptor (GPCR) called GPR30 (Filardo 2002). However this oestrogen bound GPR30 can also elicit a cyclic AMP response that in turn inhibits the EGFR/ERK1/2 pathway (Filardo et al. 2002).

HER2 has been demonstrated to inhibit the apoptotic effect of tamoxifen through interaction with membrane bound ER (Chung et al. 2002). Tamoxifen will only inhibit the nuclear ER signalling, while HER2 activation induced by membrane-bound ER may continue. On the other hand, aromatase inhibitors can abrogate both nuclear and membrane bound ER signalling due to oestrogen deprivation (Osborne et al. 2005). Many possible interactions between the genomic and non-genomic actions of ER have been proposed.

The favourable effect of AIs compared with tamoxifen in postmenopausal women with ER and HER2 positive breast cancer, may be explained by their impact on both the genomic and non-genomic effects (Dowsett 2003; Ellis et al. 2001; Shou et al. 2004) (Smith et al. 2005).

5.3.2. The PI3K/Akt pathway

The HER2-PI3K/Akt signalling and subsequent interaction with nuclear ERα, affect the response to anti-oestrogen therapy (Stoica et al. 2003a). Growth factor signalling through the PI3K/Akt, can induce phosphorylation of ERα at Ser-167, and thereby stimulate oestrogen like effects (Figure 7) (Franke et al. 1995). Furthermore, activated Akt has been demonstrated to predict worse outcome in premenopausal women, treated with adjuvant tamoxifen and/or goserelin (Perez-Tenorio and Stal 2002). In addition, breast cancer metastases (both loco-regional and distant) with activated Akt, showed less endocrine therapy response compared with Akt negative metastases, in a recent publication (Tokunaga E 2006a). On the other hand, HER2 has been demonstrated to not adversely, but rather favourably, influence response to oophorectomy in women with ER positive breast cancer (Love et al. 2003).
Whether the HER2-ERK1/2 and the HER2-PI3K/Akt pathways act in parallel or interact with each other is unclear. Phosphorylation of Raf by Akt provides a molecular basis for cross-talk between the two pathways (Zimmermann and Moelling 1999), and it is also hypothesised that Ras can activate both the ERK1/2 and the PI3K/Akt kinases (Pearson et al. 2001). Consequently, future studies of endocrine resistance should include both the ERK1/2 and the PI3K/Akt pathways.
AIMS OF THE THESIS

• Investigate the responsiveness to tamoxifen and CMF in relation to TP53 status.

• Analyse the first Swedish clinical experiences with the HER2 monoclonal antibody trastuzumab.

• Study factors involved in tamoxifen responsiveness including HER2.

• Examine two new and potentially more optimal methods for HER2 determination.
PATIENTS AND METHODS

7. PATIENTS

7.1. Gothenburg cohort

In Paper I, we used a population-based procedure to include all women with a node-positive primary breast cancer diagnosed in the Gothenburg county 1984-1989, who received adjuvant endocrine treatment and/or chemotherapy. The resulting 376 women with node-positive primary invasive breast cancer were included in the study. In order to study the responsiveness to CMF and tamoxifen in relation to TP53 status, we designed three subgroups of patients according to given adjuvant therapy. There were 125 women who had received adjuvant polychemotherapy with “classical” CMF (CMF with cyclophosphamide administered per os). The other two subgroups consisted of 183 patients receiving tamoxifen therapy alone, and 68 who had received CMF and tamoxifen.

7.2. Trastuzumab cohort

The 48 first women with breast cancer receiving the HER2 monoclonal antibody trastuzumab in Sweden are described in Paper II. All women but two had metastatic breast cancer and a history of previous palliative chemo and/or endocrine therapy. Two patients had a primary advanced breast cancer that had failed prior neoadjuvant therapy. To be included in this analysis of the first clinical experience of trastuzumab in Sweden, the women had to have a HER2 protein overexpressing breast cancer, which had failed previous conventional therapies. The 48 women started trastuzumab therapy from December 1998 through April 2000. Trastuzumab was not commercially available during this time period why all women were treated on a named patient basis. The follow-up time was 6-24 months.

7.3. Stockholm cohort

Patients in Paper III and IV are derived from a population-based cohort of women receiving breast cancer therapy in the Stockholm County, Sweden. There were 524 women who were operated at the Karolinska Hospital during the years 1994 through 1996 due to primary invasive breast cancer, according to the Regional Cancer Registry. The medical records could not confirm that 11 of these 524 patients had not been diagnosed with primary invasive breast cancer, 4 were not operated at the Karolinska Hospital, and 3 patients had metastases at time of diagnosis. In addition we omitted 33 women who had received neoadjuvant therapy, in order to reduce the heterogeneity with regard to treatment strategies in the population. Furthermore, we had to exclude 84 patients because the formalin-fixed and paraffin-embedded tissue samples could not be found in the archive. In addition, we could not retrieve any remaining invasive breast cancer tissue in new tissue sections of tumour material from 110 women, which left us with 279 patients, described in Paper III. The substantial number of tumour samples not available, can probably be explained with that many different studies were performed on samples from the years 1994 through 1996 at the Karolinska Institutet.
In Paper IV we used a Test set of 40 breast cancer tumours derived from the 279 tumours used in Paper III. We designed the Test set by collecting the first 20 HER2 positive, and the first HER2 negative, respectively, of the 279 women with primary breast cancer in the consecutive list.

7.4. Uppsala cohort

To investigate and validate new techniques for HER2 determinations in Paper IV, we used a previously rather extensively examined population-based cohort from the Uppsala County, Sweden. This study population of 315 women with primary breast cancer was the result of a population-based approach derived from a total number of 484 women diagnosed within the region during 1987-1989. No invasive breast cancer tissue was visible upon re-examination in new or old tissue sections from 9 of the tumours, which left us with 306 patients. We used quantitative real time PCR (QRT-PCR) and RNA expression profiles (RNA-EP) in order to address HER2 status in the study population of 306 women and generated 244 QRT-PCR and 232 RNA-EP successful analyses, respectively. These sub-sets of patients are referred to as the QRT-PCR and the RNA-EP set in Paper IV.

8. METHODS

8.1. Sequenced-based TP53 analyses

The procedure for the used analysis is described in a previous publication by Sjögren and colleagues (Sjogren et al. 1996). Briefly, total RNA was extracted from frozen tumour samples using the QuickPrep™, Total RNA Extraction Kit, (Amersham Pharmacia Biotech, Uppsala, Sweden). This was followed by the enzymatic synthesis of cDNA by PCR, using four overlapping primer pairs covering the entire coding region of all the exons of the TP53 gene.

The sequencing products generated, were analysed using an automated laser fluorescence sequencer (ALFexpress™, Amersham-Pharmacia Biotech, Uppsala, Sweden). The obtained sequence was compared with the sequence of wild-type TP53 using a prototype software program, TP53 SBDecipher, version 1.00. Only nucleotide changes resulting in amino acid shifts were considered as mutations. Each identified mutation was verified by re-amplification and sequencing, from the corresponding cDNA.

8.2. Immunohistochemistry

Details with regard to immunohistochemical staining can be found in Paper III and IV. In brief, we used 4 μm sections of paraffin-embedded primary tumours, which were deparaffinised and rehydrated. We used the monoclonal antibody CB11 for HER2 determination (NovoCastra Laboratories Ltd, Newcastle, UK). For investigation of activated ERK1/2 (pERK1/2) and the ERα phosphorylated on Ser 118 (ERαS118), we used the phospho-p44/42 MAPK (Thr202/Thr204) (E10) monoclonal antibody, and the phospho-estrogen receptor α (Ser118) 16J4 monoclonal antibody, respectively, (Cell Signalling Technology, Inc., Beverly, MA). Examples of positive staining for HER2, ERαS118, and pERK1/2 are shown in Figure 1, Paper III.
8.2.1. HER2

Heat-induced epitope retrieval was performed in a microwave oven with the tissue sections covered with a citric acid buffer. The automatic immunostaining was performed in a DAKO Tech Mate instrument (DAKO, Glostrup, Denmark), using the recommended DAKO ChemMate Detection Kit (Peroxidase/DAB Rabbit/Mouse). The slides were counterstained with haematoxylin and dehydrated before evaluated. The evaluation was done as recommended by DAKO (Glostrup, Denmark): Partial or incomplete, weak to moderate, and moderate to strong membranous staining in more than 10% of the tumour cells were scored as 1+, 2+, and 3+, respectively. In order to obtain positive and negative controls in every run, we used sections from a multiple cell line block made of four human breast cancer cell lines with known HER2 intensity scores of 0 (DU 145), 1+ (RT 4), 2+ (MDA 453), and 3+ (BT 474), respectively. J Bergqvist and the pathologist G Elmberger read all slides.

8.2.2. pERK1/2

We used the same procedure for heat-induced epitope retrieval and automatic immunostaining as previously described for HER2. In order to evaluate the pERK1/2 stainings we used a classification system including both the proportion of tumour cells stained, and the intensity of the stainings, a slightly modified version of a classification system previously described by Harvey and colleagues (Harvey et al. 1999). In heterogeneous tumours, hot spots were examined to approximate the number of cells that were stained. Tissue sections of a tonsil with and without the primary antibody were used as negative and positive controls in each immunohistochemical experiment (personal recommendations from Cell Signalling, manufacturer of the antibody). J Bergqvist and the pathologist G Elmberger analysed all slides. In order to establish the pERK1/2 antibody specificity we made two additional experiments. First, we added a blocking peptide (Sc-16982, Santa Cruz Biotechnology Inc.) to our antibody solution 1 hour before staining procedures. Secondly, we placed a phosphatase (P 9614, Sigma, Sigma-Aldrich, Inc.) on the tissue sections one hour before the automatic staining was performed. Previously demonstrated activated ERK1/2 staining was not visible in re-staining preceded by either blocking peptide or phosphatase procedures (Figure 8). Therefore, we consider the pERK1/2 antibody immunohistochemical staining to be representative and specific.

8.2.3. ERα

For analysis of ERα, heat-induced epitope retrieval was done using a TRIS/EDTA buffer followed by automatic immunostaining in a DAKO Tech Mate instrument (DAKO, Glostrup, Denmark), using the DAKO ChemMate EnVision Detection Kit (Peroxidase/DAB, Rabbit/Mouse). The slides were counterstained with haematoxylin and dehydrated before evaluated. The immunostained slides were scored in the same manner as pERK1/2. Breast cancer tissue samples that were known to show ERα staining with the primary antibody were used as positive and negative controls (i.e., with and without the primary antibody) in every run. J Bergqvist and the pathologist G Elmberger read all slides.
Figure 8. Supplementary experiment to establish the specificity of the pERK1/2 antibody used in Paper, III. A. Primary pERK 1/2 antibody, 40X. Primary pERK 1/2 antibody and blocking peptide, 40X. C. Primary pERK1/2 antibody and phosphatase, 40X.
8.3. Fluorescence *in situ* hybridisation of HER2

The procedure behind performed FISH analysis is described in detail in a recent publication (Carlsson et al. 2004). We used the PathVysion HER2 DNA Probe Kit (Vysis Inc., Downers Grove, IL, USA). In brief, DNA in the formalin-fixed paraffin-embedded tissue specimens was denatured and allowed to hybridise with two fluorescent signals. The cell nuclei were stained with DAPI. The tissue specimens were mounted on slides and evaluated for HER2 gene copy number using a Leica DMLB microscope. The ratio of HER2 to chromosome 17 is expected to be 2.0 for normal or unamplified breast tissue specimens. The scoring conditions used were those recommended by Vysis Inc., and a ratio greater than 2.0 was considered amplified. The pathologist H Nordgren analysed all slides. A FISH analysis of a HER2 amplified breast cancer is displayed in Figure 5.

![Image](image.png)

**Figure 9.** Chromogenic in situ hybridisation (CISH) analysis of a:
A. Breast cancer without HER2 amplification. B. Breast cancer with HER2 amplification.
By courtesy of Jorma Isola, Tampere University, Finland.
8.4. Chromogenic in situ Hybridization of HER2

The CISH analysis was performed according to a detailed description by Isola and colleagues (Isola et al. 2004). The slides were deparaffinised prior to heat-induced epitope retrieval, and enzymatic digestion (Digest-All III solution; Zymed INC., South San Francisco, CA). The slides were washed and dehydrated. Then the HER2 DNA probe (Zymed; consisting of two contiguous BAC clones) was applied to the slides and hybridisation was carried out overnight. The HER2 probe was detected by sequential incubations with mouse anti-digoxigenin (diluted 1:300, Roche Biochemicals, Mannheim, Germany), antimouse-peroxidase polymer (Powervision+; Immunovision Inc., Daly City, CA) and diaminobenzidine chromogen according to the manufacturer’s protocol. Tissue sections were counterstained with haematoxylin. A positive and a negative control sample (tumours with and without HER2 amplifications using the FISH technique) were included in every hybridisation batch. Amplification was defined as ≥6 signals per nucleus in more than 50% of cancer cells, or when a large gene copy cluster was seen. In tumours with borderline copy number count, CISH of an adjacent section was hybridised with a chromosome 17 centromer probe (Zymed) for comparison. All slides were analysed by the pathologist J Isola. CISH analyses of one HER2 amplified breast cancer and one breast cancer without HER2 amplification, respectively, are displayed in Figure 9.

8.5. RNA and DNA extraction procedures

We used the RNeasy Mini protocol (Qiagen, Valencia, CA, USA) for RNA extraction from the previously deep frozen pieces of primary breast cancer, followed by homogenisation and addition of proteinase K (Egyhazy et al. 2004). RNA was isolated using the Qiagen kit’s microspin affinity columns, and the quality of the RNA was estimated with the Agilent 2100 bioanalyzer (Agilent Technologies, Rockville, MD, USA). After application of the initial lysate to the RNeasy microcartridge, the first centrifugate was stored in -70°C for later genomic DNA extraction. Genomic DNA was purified from the above-mentioned centrifugate by use of the QIamp Mini Kit protocol (Qiagen), according to the manufacturer’s instructions. A NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to assess DNA quality and quantity for each sample.

8.6. Quantitative real time PCR

HER2 amplification was quantified from extracted DNA, using the LightCycler-HER2 DNA Quantification Kit, according to the manufacturer’s instructions (discontinued kit, Roche Applied Biosciences, Penzberg, Germany). Briefly, the 112 bp HER2 and 133 bp GAPDH templates were amplified in a Roche LightCycler by specific primers in the same glass capillary. The amplicons of the genes are detected by fluorescence resonance emission technology (FRET). Subsequently, the obtained fluorescence from HER2 and GAPDH, respectively, were used in the RelQuant quantification software (Roche) to analyse the amount of HER2 relative to the reference gene. The final results were expressed as a ratio of HER2: reference gene copies in the sample, normalised with the ratio of HER2: reference gene copies in the Calibrator DNA. The manufacturer specifies that a ratio of < 2.0 is assumed to be negative for HER2 over-amplification and that a ratio of ≥ 2.0 is assumed to be positive. Typical reaction curves for the here presented PCR reactions are displayed in Figure 4, Paper IV.
8.7. RNA expression profiles

Preparation for the hybridisation of the biotinylated and fragmented cRNA to Affymetrix high-density oligonucleotide array human HG-U133, A and B chip, respectively, was performed using the Affymetrix protocol (Santa Clara, CA, USA). The used chips contain approximately 45,000 probe sets, corresponding to more than 39,000 transcripts representing about 33,000 human genes. The included probe sets for HER2 analysis were X216836_s_at, X210930_s_at, and X234354_x_at. We only used the results generated by X210930_s_at in our Test set, due to its favourable association with FISH, compared with the other two probes.

The arrays were stained with streptavidin-phycoerythrin (SAPE) after being washed and signal amplification was performed with a biotinylated anti-streptavidin antibody. The scanning process was performed according to the manufacturer's instructions (Affymetrix Genechip® Technical Manual, 2001). For normalisation of the expression data, the global mean method was used (Ploner et al. 2005). Criteria used to exclude samples from additional analysis were (a) a scaling factor >4 times the average scaling factor across all chips, (b) “Present” calls <30% and (c) R-squared value of the Pearson correlation coefficient of the expression data compared with all other arrays <0.6.

8.8. Statistical methods

Statistical analyses were performed using the SAS 8.2, SAS Institute Inc., USA (Paper I), JMP 3.1 software SAS Institute Inc., USA (Paper II), SPSS version 12.0 (Paper III), and 13.0 (Paper IV) SPSS Inc., USA.

The comparison of variables between groups was generated using the student’s T-test or the Chi-square tests, respectively. The Pearson correlation coefficient was used for correlations between variables that follow the normal distribution, while the Spearman correlation coefficient was used for associations between variables that do not follow the normal distribution. For associations between variables at nominal level and variables at interval scale we used the Eta value.

End points in the survival analyses were RFS, BCCS, and OAS survival. Contra-lateral and/or new primary breast cancer was not considered as a relapse, but patients were censored at the time of contralateral cancer. The time was calculated from date of surgery to date for relapse, death or latest follow-up, respectively. All survival curves were generated by the Kaplan-Meier method and statistical significance was determined by the log-rank test (p ≤0.05).

All factors significant in the univariate survival analyses according to Kaplan-Meier or Cox Regression were included in the following multivariate Cox Regression models. We used the forward step-wise selection using the Cox Regression models, and the level of significance to enter or stay in the model was p ≤0.05.

The statistical calculations were run by J Bergqvist with support from J Nilsson (Paper I), Trialformsupport, and Henrik Hellborg (Paper III and Paper IV), Oncology Centre, Stockholm.
RESULTS AND DISCUSSION

9. PAPER I

We analysed the primary tumours of 376 women with node-positive breast cancer, operated during 1987 through 1989, all receiving adjuvant chemotherapy and or endocrine treatment. Sequenced-based analysis of all exons of the TP53 gene generated 110 TP53 mutations, distributed in 105 (28%) of the 376 tumours. The more frequently analysed exons 5-8 contained the majority of the detected mutations (90/110), while 20/110 were found in exons 3-4 and 9-10, respectively. Our data indicate that the frequency of TP53 mutations might be underestimated if only exons 5-8 are analysed. In addition, other studies have demonstrated that approximately one third of TP53 mutations detected by sequence-based techniques cannot be detected by immunohistochemistry, due to lack of protein expression (Geisler et al. 2001; Sjogren et al. 1996; Soussi and Beroud 2001; Williams et al. 1998).

Furthermore, different types of TP53 mutations may be of greater importance than others as well as the location of the TP53 aberrations may be important (Bergh et al. 1995; Olivier et al. 2006). This imply the importance of a TP53 detection method being able to reveal all mutations and their sites. This was recently also found in a publication demonstrating a higher frequency of breast cancer mortality in patients with TP53 mutated tumours, compared with patients with wild type TP53 tumours (HR 2.9, 1.2-7.1), which was markedly increased in the subgroup of tumours containing nonsense mutations (HR 9.4, 1.3-70) (Lai et al. 2004).

In our study, TP53 status was investigated with regard to RFS, BCCS, and OAS. These analyses were performed on all patients, but also in three subgroups based on actually given adjuvant therapies. The majority of the women (n=125), received adjuvant polychemotherapy with “classical” CMF (CMF with oral cyclophosphamide). Another 183 women were given systemic endocrine therapy with tamoxifen. A minority of 68 patients were administered both “classical” CMF and tamoxifen. Out of the 376 women, 141 were treated in clinical randomised study protocols (International Breast Cancer Study Group V-VII). The treatment strategies for women treated outside randomised studies, were based on patient and tumour characteristics.

Mutated TP53 was associated with worse RFS, BCCS, and OAS in all patients, supporting a prognostic value of TP53. A similar statistically significant impact of TP53 status was found in the CMF treated patients. Furthermore, mutated TP53 in ER positive tumours was a negative prognostic factor with regard to BCCS for the complete study population, and for the tamoxifen treated subgroup, respectively. One explanation why TP53 status was not statistically significant for RFS, BCCS, and OAS in the tamoxifen treated women, might be explained by that tamoxifen was received based on postmenopausal status, rather than the hormone receptor status. Previously published data with regard to the therapy predictive value of TP53 status are conflicting (Bergh 1999; Bergh et al. 1995; Berns et al. 1998). We present survival after adjuvant CMF and tamoxifen, which only are indirect estimations of the therapy response. Prospective and randomised trials are needed to address the true predictive value of TP53, with time to relapse as the primary end-point, while therapies at time of relapse may work differently in relation to the TP53 status.
In conclusion, we confirm previous reports demonstrating a negative prognostic value of TP53 mutations (Bergh et al. 1995; Blaszyk et al. 2000; Overgaard et al. 2000; Tetu et al. 1998). Our data indicate that women with TP53 mutated breast cancer potentially might benefit more from chemotherapeutic regimes other than CMF, and a borderline-significant (p=0.05) trend for worse outcome after adjuvant tamoxifen for women with ER positive, TP53 mutated tumours.

10. PAPER II

This descriptive study is based on the first 48 women in Sweden receiving the monoclonal antibody trastuzumab. We summarised the experience from December 1998 when the first patient in Sweden received trastuzumab until April 2000. We identified 48 women with advanced breast cancer who were treated with trastuzumab. During this time period trastuzumab was not yet approved by EMEA, why all patients were treated on a named patient basis by individual permission from the Swedish Medical Products Agency, allowing the identification of all patients treated in Sweden during this time period. All women had advanced and HER2 protein overexpressing breast cancer failing on prior conventional therapies. Forty-six of the women had metastatic breast cancer and they had received several prior lines of chemotherapy and/or endocrine treatment. Two women had primary, locally advanced breast cancer that had failed on standard neo-adjuvant therapy.

We found a trend towards better survival from start of trastuzumab therapy for those who had tumours with a strong (3+) HER2 protein overexpression compared with those whose tumours had a moderate (2+) protein overexpression. One shortcoming with our retrospective analysis is that no reliable response evaluation could be carried out, due to the nature of the material, where the physicians had no standardised protocol for the clinical follow-up. However, the trend seen towards better survival for tumours with a strong HER2 protein expression was in line with previous reports demonstrating response rates of 18-35% for women with 3+ HER2 positive tumours compared with 0-6% for those with 2+ HER2 positive tumours (Cobleigh et al. 1999; Vogel et al. 2002). Today only patients with a strong immunohistochemical staining pattern (3+) or HER2 amplified tumours are recommended trastuzumab therapy.

The overall toxicity seemed manageable and acceptable. But two patients, however, suffered serious cardiac events. Both these women had received anthracyclines doses of more than 1000mg/m². They recovered from cardiac symptoms when trastuzumab was discontinued. Fever and chills occurred in 7 patients (15%) and were the most common side effects registered. It must be considered, however, that the adverse events, which were obtained from the medical records, are probably underestimated due to the retrospective setting. Nevertheless, our results confirm previous reports demonstrating a favourable overall toxicity profile (Baselga et al. 1996; Cobleigh et al. 1999; Slamon et al. 2001).

In conclusion, our population-based study indicated that trastuzumab therapy could be clinically useful, with a moderate efficacy and reasonable toxicity, in patients with an advanced breast cancer. This was previously only demonstrated for randomised clinical studies, most likely including highly selected patients aiming at demonstrating positive effects.
A population-based cohort of 279 women with primary breast cancer was investigated with regard to the (proto-) oncogene HER2, the activated extra cellular signal-regulated kinases 1 and 2 (pERK1/2), and oestrogen receptor α phosphorylated at Ser 118 (ERα\textsubscript{118}). We determined the protein expression of pERK1/2 and ERα\textsubscript{118}. In order to investigate HER2 gene amplification, tumours with HER2 protein overexpression (2+ and 3+) were re-tested with FISH. We analysed the results of all patients, and of a subgroup of 108 tamoxifen treated women with ERα positive primary breast cancer, in relation to other tumour- and patient characteristics and relapse and survival patterns.

HER2 was associated with other prognostic markers (high Elston score, larger tumour size) and correlated to poor prognosis, while pERK1/2 and ERα\textsubscript{118} were associated with factors indicating good prognosis (low Elston score, smaller tumour size). Furthermore, we found a statistically significant inverse relation between HER2 and pERK1/2.

Our frequency of 31% tumours with activated ERK1/2 staining was within the wide range of 24-72% previously reported (Adeyinka et al. 2002; Gee et al. 2001; Mueller et al. 2000). We used two additional experiments in our study, including a blocking peptide towards the used pERK1/2 antibody and a phosphatase, respectively, in order to establish the pERK1/2 antibody specificity.

HER2 was the only statistically significant independent factor in multivariate analysis, prognosticating worse RFS, BCCS and OAS. The tamoxifen treated women with pERK1/2 positive tumours demonstrated a trend towards better RFS and BCCS compared with those without, especially those with an intense pERK1/2 staining. We found, on the contrary to previous reports (Alblas et al. 1998; Gee et al. 2001), activated ERK1/2 to indicate good prognosis. However, conflicting results with regard to pERK1/2 status can be explained by the different methods used for detection, in addition to lack of standard cut-off levels, for positive and negative results, respectively. Furthermore, the analysed patient materials in the literature, including this one, are very heterogeneous, including various numbers of different treatments. Moreover, the possibility of different cellular responses to various degrees of pERK1/2 staining further increases the complexity (Rul et al. 2002).

In addition to the ERK1/2 pathway, HER2 can activate another important pathway, the PI3K/Akt cascade mediating cell survival, which can both activate (Chaudhary et al. 2000) and inhibit (Zimmermann and Moelling 1999) the ERK1/2 pathway. Recently, the PI3K downstream second messenger Akt, has been associated with worse outcome on endocrine therapy in both the adjuvant and metastatic situation (Stal et al. 2003; Tokunaga E 2006a; Tokunaga E 2006b). Moreover, we did not investigate the ER co-activator AIB1, which can be phosphorylated through the HER2 activated ERK1/2 pathway and be involved in endocrine resistance (Shou et al. 2004). Taken altogether the ideal study should have included both the HER2-ERK1/2 and the HER2-PI3K/Akt pathways, respectively, in addition to available co-regulators of the ER.

In conclusion, the hypothesis that HER2/ER cross-talk, via the ERK1/2 pathway, is the major contributor to tamoxifen resistance could not be confirmed by our data.
12. PAPER IV

We determined HER2 status with Quantitative real time PCR (QRT-PCR) and RNA expression profiles (RNA-EP), respectively, in both a test set of 40, and a validation set of 306 breast cancers. The results were compared with HER2 evaluations generated with IHC in combination with fluorescence in situ hybridisation (FISH) or chromogenic in situ hybridisation (CISH).

Both QRT-PCR and RNA-EP evaluating HER2 on the DNA and mRNA level, respectively, generated equivalent HER2 determinations compared with IHC in combination with FISH or CISH. The receiver operating characteristic (ROC) curves for QRT-PCR and RNA-EP showed good agreement compared with the IHC-CISH procedure. Furthermore, obtained HR for the prognostic value of HER2 in a 5 and 10-year perspective were similar for all used methods.

Trastuzumab is today routinely used in HER2 positive metastatic breast cancer based on two randomised trials demonstrating clinically important survival prolongations compared with chemotherapy only (Marty et al. 2005; Slamon et al. 2001). Adjuvant trastuzumab has been demonstrated to reduce the risk of breast cancer relapse with around 50% at a short median follow-up of 1 and 2 years, respectively. Recently, marked improvement in RFS was reported for trastuzumab in the adjuvant setting (Piccart-Gebhart et al. 2005; Romond et al. 2005). Altogether, this will increase the need for cost-effective and accurate assessments of HER2 status.

The response rate of trastuzumab therapy is dependent on either a strong HER2 protein overexpression (3+) or HER2 gene amplification (Vogel et al. 2002). A strong (3+) HER2 protein overexpression has been demonstrated to correlate well with gene amplification, and with high response rates on trastuzumab therapy (Harries and Smith 2002; Jacobs et al. 1999; Jimenez et al. 2000). Today the most common procedure to establish HER2 status is screening with IHC, followed by additional testing with FISH in tumours with a moderate HER2 protein overexpression, where only about 25% show HER2 amplification (Fornier et al. 2002; Mass 2000a).

Immunohistochemical staining is easily performed at routine diagnostic laboratories and additionally relatively inexpensive. Recent improvements have been accomplished with automated image analysis for improved scoring and intra-assay controls consisting of cell line standards (Andersson J 2004; Rhodes et al. 2004; Wang et al. 2001). However, drawbacks include the various antibodies used, lack of standardisations, and a scoring system with interobserver error (Mass 2000b; Thomson et al. 2001).

The chromogenic in situ hybridisation (CISH) technique is an emerging alternative to FISH generating equivalent results (Isola et al. 2004). CISH offers the advantages of being faster, and not requiring a fluorescent dye and the additional epifluoroscense microscope (Isola et al. 2004). Furthermore, the slides can be stored without fading (van de Vijver 2002). Nevertheless, dual probe FISH (Vysis) contain an intra-assay control distinguishing between HER2 and chromosome 17 amplifications, which CISH does not (Tanner et al. 2000).

Enzyme-linked immunosorbert assay (ELISA) is a relatively simple technique, which offers quantitative HER2 evaluation well suited for automated procedures (van de Vijver 2002; van de Vijver 2001). However, the method cannot be used on formalin-fixed paraffin-embedded tissue, which constitutes most of the tumour archives of today.
We performed the QRT-PCR and RNA-EP analyses on frozen tumour samples, but others have demonstrated the feasibility of using the same techniques on formalin-fixed paraffin-embedded samples, generating quality HER2 determinations (Benohr et al. 2005; Cronin et al. 2004; Gjerdrum et al. 2004; Specht et al. 2001; Vinatzer et al. 2005). Quantitative real time (reverse transcriptase) PCR enables rapid, reproducible and relatively inexpensive HER2 assays (Cronin et al. 2004; Ginestier et al. 2004). In addition, the automated procedure makes detection possible even when the sample volumes or sections are small (van de Vijver 2002). Furthermore, the results do not fade.

The limitations of QRT-PCR and RNA-EP include dilution of amplified DNA and RNA from non-malignant tissue (van de Vijver 2002). This can be overcome, however, by tissue extraction procedures like micro-dissection (Specht et al. 2001), and others report that the microdissection step is not a prerequisite for RNA target assays (Williams et al. 1998). However, it is important to correct for differences in DNA/RNA quality and quantity. RNA in particular, is sensitive to fragmentation, which has been reported to continue in paraffin-embedded tissue (Cronin et al. 2004). We used the Agilent chromatographic methodology that control for RNA quality as well as quantity (Egyhazi et al. 2004; Pawitan Y 2005), and the global mean method was used for normalisation of the expression data (Ploner et al. 2005). We used a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) in order to assess DNA quality and quantity.

We found that QRT-PCR and RNA-EP generate HER2 evaluations equivalent to those obtained by IHC in combination with CISH. Furthermore, our data indicate that analysis of HER2 based on these two new techniques is superior to the combination of IHC and CISH, in determining prognosis in breast cancer. It is also to be noted that we used the monoclonal antibody CB11 for the HER2 immunohistochemical assays, which was proved to be inferior to other antibodies according to a recent study (Press et al. 2002). However, no antibody has consistently been demonstrated to be superior with regard to sensitivity and specificity (Ellis et al. 2000; Fornier et al. 2002; Ginestier et al. 2004), why we consider it appropriate to compare our IHC data with the ones obtained by QRT-PCR and RNA-EP.

In conclusion, we suggest adjustments of QRT-PCR and RNA-EP for the development of accurate and high throughput HER2 clinical diagnostic assays.
GENERAL CONCLUSIONS

Present strategies to identify breast cancer patients at risk for relapse after surgery are insufficient. In addition, we have to date only two established therapy predictive factors, hormone receptors and HER2. A substantial cohort of women with invasive breast cancer receives non-optimal therapies, both in the adjuvant and metastatic setting, due to lack of sufficient prognostic and therapy predictive markers. Under-, and overtreatment are major problems. Patients receive therapy based on group statistics of risk and benefit (Bergh 2005). Identification of risk per se, by use of prognostic markers does not, unfortunately, fully correlate with a positive effect of selected adjuvant therapies. The relative effects of different adjuvant therapies have been similar in high versus low-risk patients, however, thus motivating the use of more aggressive adjuvant strategies for those patients with higher absolute risks.

This thesis is based on TP53 and HER2 analysed in cancer tissue. We have demonstrated TP53 and HER2 to prognosticate worse RFS, BCCS, and OAS, and focused especially on their associations with outcome after tamoxifen treatment. In addition, we discuss how to best analyse TP53 and HER2.

HER2 status is not only a prognostic factor, but is also used as a therapy predictive factor to select patients for therapy with the humanised anti-HER2 monoclonal antibody trastuzumab, both in metastatic disease and more recently in the adjuvant setting. Whether HER2 and TP53 in addition to their prognostic value may be used as predictive markers for chemotherapy and endocrine treatment, is still uncertain. TP53 is not used in clinical routine, but its ability to predict response to taxane regimens is presently explored in a randomised p53 study (Rutgers et al. 2004).

Accurate and cost-effective technology is required in order to optimise the use of available and future prognostic and predictive factors, including HER2 and TP53. The most frequently used method for TP53 evaluation is immunohistochemistry, which detect TP53 protein accumulation. Mutated TP53 protein has a longer half-life compared with wild type, which is not detectable with IHC do to its rapid degradation (Bartek et al. 1990; Crawford et al. 1984). However, about 20% of the TP53 mutations are not followed by protein accumulation, due to a truncated protein or no protein product at all, which will result in false negative results (Berns et al. 2000; Geisler et al. 2001; Sjogren et al. 1996; Soussi and Beroud 2001). On the other hand, accumulated TP53 protein may occur without concurrent mutation, which generate false positive results (Berns et al. 2000; Geisler et al. 2001; Soussi and Beroud 2001). In addition, certain types and cites of TP53 mutations may prognosticate worse survival compared with other mutations, why the actual sequence might be of interest (Bergh et al. 1995; Lai et al. 2004). Furthermore, sequenced-based TP53 assessments have been demonstrated to generate better prognostic information compared with immunohistochemical determination of TP53 (Sjogren et al. 1996). Consequently, we used cDNA based sequencing of all exons of the TP53 gene in Paper II.

The recommendations for HER2 evaluation include screening with IHC, followed by validation with fluorescence in situ hybridisation (FISH) of borderline cases (Ellis et al. 2004; Fornier et al. 2002). Chromogenic in situ hybridisation (CISH) is an alternative methodology to FISH, requiring less advanced equipment, but generates equivalent results (Isola et al. 2004). Moreover, the CISH results are possible to store, while FISH results based on fluorescent dye fade over time (van de
Vijver 2002). Nevertheless, IHC, FISH, and CISH are all relatively time consuming and labour-intensive techniques. In addition, especially IHC is limited by inter-observer variability and lack of standardised procedures (Hanna et al. 1999; Mass 2000b; Thomson et al. 2001).

However, rapid, high-quality, and relatively inexpensive assays can be obtained with quantitative real time PCR (Heid et al. 1996). The technique is possible to use on small tumour sections or volumes, but the procedure must include control of both the RNA/DNA quantity and quality, respectively (van de Vijver 2002). Furthermore, both tumour homogenates and formalin-fixed paraffin-embedded tissues can be used as templates (Benohr et al. 2005; Cronin et al. 2004; Gjerdrum et al. 2004; Specht et al. 2001; Vinatzer et al. 2005). We used this technique together with the emerging RNA expression profile methodology for investigation of the HER2 status. Our results show that QRT-PCR and RNA based gene expression chip methodology generate HER2 determinations with similar prognostic implication, compared with those obtained by the commonly used techniques IHC and FISH/CISH. Our data, including the advantages and limitations of both techniques, indicate that refined QRT-PCR and micro array based assessments of HER2 might be clinically useful in the future.

Optimised technology is a prerequisite in the investigation of the potentially therapy predictive value of TP53 and HER2. In order to investigate these and other potentially important predictive factors, we suggest that both DNA and mRNA expression profiles are used, supplemented with protein data, using immunohistochemical technique, in combination with FISH or CISH, whenever appropriate.

All women with hormone receptor positive breast cancer are eligible for endocrine therapy, both in the adjuvant and metastatic setting. Unfortunately, not all patients respond, even though they have tumours expressing both the oestrogen and progesterone receptor. Previously published and discussed data indicate that both TP53 and HER2 status might become clinically useful for the selection of endocrine treatment. The signalling, in both directions, between the ER and growth factors, including HER2, seem to be one of the major causes of tamoxifen resistance.

The ideal study for addressing tamoxifen resistance should include TP53, HER2, the two hormone receptors and their up-stream regulators, in addition to their down-stream mediators, and the ERK1/2, and PI3K/Akt pathways, respectively. New methodologies, including micro arrays and proteomics, which mirror biomarker-pathways and patterns, will hopefully generate a better understanding of not only the gene- and protein patterns, but lead the way towards a more individualised breast cancer therapy.
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