Chemosensitivity in Breast Cancer

KENNETH VILLMAN
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Abstract


Breast cancer mortality in Sweden is now in decline, thanks to early detection and the wide use of adjuvant endocrine therapy and chemotherapy.

While hormone receptor status is predictive of response to endocrine treatment, there is no clinically useful predictive marker of a patient’s response to chemotherapy. Consequently, patients receive chemotherapy with considerable toxicity but minimal benefit. The aim of this thesis was to investigate a number of methods with the potential to predict response to chemotherapy and thus enhance treatment efficacy in breast cancer patients.

We found that topo IIA, the key target enzyme of topo II inhibitors, is significantly expressed in nonproliferating breast cancer cells. This finding may explain why topo II inhibitors are effective in patients with slow growing tumors and a low proliferation rate.

Topo IIA gene amplification was suggestive of increased response to anthracyclines in advanced breast cancer, whereas the oncogene HER2 had no predictive value by itself. These findings are in accordance with current knowledge.

Cyclin A, a marker of cell proliferation, showed good prognostic value but did not predict response to chemotherapy in advanced breast cancer.

In vitro chemosensitivity testing with FMCA predicted tumor response in patients with advanced breast cancer with a sensitivity of 89% and a specificity of 53%. Our results are consistent with the results from similar assays, which predict drug resistance with good accuracy while clinical drug sensitivity is less reliably predicted. The use of FMCA and similar assays is not yet recommended outside clinical trials; their main utility is in preclinical testing of new anti-cancer drugs, including targeted therapies.

The combination of epirubicin, capecitabine, and cisplatin (EXC) demonstrated high clinical response rate (74%) and pathological complete response rate (22%) in locally advanced breast cancer, but with cumbersome toxicity. The fluoropyrimidine biomarkers TS, TP, and DPD did not predict response to the EXC regimen.

Keywords: breast cancer, chemotherapy, anthracycline, predictive, topoisomerase, TOP2A, HER2, cyclin A, in vitro

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List of papers

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


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<tr>
<td>cCR</td>
<td>Clinical complete response</td>
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<td>CISH</td>
<td>Chromogenic in situ hybridization</td>
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<tr>
<td>cRR</td>
<td>Clinical response rate</td>
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<tr>
<td>DFS</td>
<td>Disease-free survival</td>
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<td>EFS</td>
<td>Event-free survival</td>
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<td>ER</td>
<td>Estrogen receptor</td>
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<td>FISH</td>
<td>Fluorescence <em>in situ</em> hybridization</td>
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<td>FMCA</td>
<td>Fluorometric microculture cytotoxicity assay</td>
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<td>GEP</td>
<td>Gene expression profile</td>
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<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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<td>HR</td>
<td>Hormone receptor</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>LABC</td>
<td>Locally advanced breast cancer</td>
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<td>MBC</td>
<td>Metastatic breast cancer</td>
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<td>OS</td>
<td>Overall survival</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>pCR</td>
<td>Pathological complete response</td>
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<td>PR</td>
<td>Progesterone receptor</td>
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<td>RFS</td>
<td>Recurrence-free survival</td>
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<td>RR</td>
<td>Risk ratio</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<td>SPF</td>
<td>S-phase fraction</td>
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<tr>
<td>TNM</td>
<td>Tumor, node, metastasis</td>
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<tr>
<td>TOP2A</td>
<td>Topoisomerase IIα gene</td>
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<tr>
<td>Topo IIα</td>
<td>Topoisomerase IIα protein</td>
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Introduction

Breast cancer is the most common cancer among women in the Western world; in Sweden, the disease is fatal to nearly one in four breast cancer patients despite efforts for early detection and wide use of adjuvant systemic therapy (1, 2). Breast cancer accounts for 12% of mortalities in women aged between 45 and 64 (1). The objective response rate achievable with chemotherapy in advanced breast cancer is mostly in the range of 40–60% (3), with anthracyclines and taxanes being among the most active drugs (3, 4). The limited efficacy of chemotherapy is due to intrinsic and/or acquired drug resistance. Many patients are treated with toxic drugs with considerable side effects and very limited gain. The value of a test that could reliably predict the efficacy of chemotherapy in this intermediate chemotherapy-sensitive tumor type is obvious.

Incidence and mortality

Breast cancer is a major global public health problem. According to estimates, in 2002 there were 1.15 million new diagnoses of breast cancer and 410 712 deaths caused by breast cancer worldwide (5). Incidence rates are high in most developed countries (the exception is Japan), with the highest age-standardized incidence being found in North America (99.4 per 100 000) (5). This high incidence in the wealthier parts of the world is likely due in part to screening programs that detect early invasive cancers, some of which would otherwise have been diagnosed later or not at all. The marked differences in breast cancer incidence and mortality rate between different parts of the world are illustrated in Figure 1. Because of its high incidence and relatively good prognosis, breast cancer is the most prevalent cancer today, with an estimated 4.4 million living women who have been diagnosed with breast cancer in the past five years (5). In Sweden, the incidence of breast cancer is increasing, with an annual increase of 1.5% over the past decade (2).
Figure 1. Age-standardized incidence and mortality rates for breast cancer in different parts of the world. Data shown per 100 000. Figure from Parkin et al., CA Cancer J Clin, 2005 (5).

A recent analysis of time trends of breast cancer survival in relation to incidence and mortality in ten European countries showed striking differences in relative survival (6). Relative survival can be interpreted as the proportion of patients surviving at a given time after cancer diagnosis, after adjusting for the general mortality of the population of the same age and sex. The five-year relative survival was highest in Sweden, at 83.1%, with a pattern showing increasing survival with increasing incidence and declining mortality. The survival rates for Finland, Denmark, England, and Estonia were 81.9%, 75.6%, 74.2%, and 60.6%, respectively (6). According to the US SEER program of 1995–2000, the five-year relative survival rate in the United States was 89% (5).

Genetic factors, such as the two major susceptibility genes (BRCA1 and BRCA2), may account for up to 10% of breast cancer cases in developed countries (7), but their prevalence in the population is too low to explain
much of the international variation in risk. The majority of cases are therefore likely to be a consequence of different environmental factors. This theory is supported by studies on migrants, which clearly show that incidence rises following migration from low to high incidence countries, particularly if migration takes place at a young age (8). The major risk factors associated with breast cancer are reproductive factors (age at menarche, age at menopause, age at first full pregnancy), body size/obesity, alcohol, physical activity, exogenous hormones (oral contraceptives, hormone replacement therapy), diet (high intake of saturated fat), and exposure to ionizing radiation (7).

Between 1950 and about 1990, breast cancer mortality rates rose slowly but steadily, but then fell rapidly in most European countries. The improvement in breast cancer survival was most obvious in the UK and USA, as illustrated in Figure 2 (9). The abruptness of this improvement strongly suggests that it was mainly due to changes in the way breast cancer was diagnosed and treated, particularly the now widespread use of mammography screening and the increased numbers of women receiving the best available adjuvant hormonal therapy and chemotherapy. In Sweden, the improvement in survival was not as marked; but, on the other hand, it was already in progress by the mid 1970s, probably due to good compliance with early recommendations for systemic adjuvant therapy, mainly tamoxifen.

![Figure 2. Decrease in UK and USA breast cancer mortality at ages 50–69 years. Figure from Peto et al., Lancet, 2000 (9).](image)
Diagnosis

Advances in diagnostic imaging during the past 20 years have greatly changed detection and diagnostic strategies. Organized mammography screening, education programs, and improved consciousness in the female population have changed the type of breast cancer patient seen today compared with those seen a few decades ago.

If a patient presents with clinical signs of a tumor in the breast, the diagnostic work-up consists of clinical examination, mammography and/or ultrasonography, and fine-needle aspiration for cytology or core-needle biopsy for histopathological examination.

Today, a large proportion of the breast cancer patients in Sweden are detected with screening mammography. In the Uppsala-Örebro health care region, with 1.92 million residents, 40.6% of invasive breast cancers diagnosed between 1996 and 2005 were detected with screening mammography (10). Results from several randomized trials support the effectiveness of mammography screening, suggesting that it can reduce mortality from breast cancer by about 30% (11). Service screening in two Swedish counties was associated with an even greater reduction in breast cancer mortality, of about 40–50% (12).

Staging

Breast cancer stage is determined according to the TNM (tumor, node, metastasis) classification, which comprises tumor size, lymph node status, and the presence or absence of distant metastasis (13). Clinical staging (cTNM) is based on tumor characteristics prior to surgery, whereas pathological staging (pTNM) is based on information from the histopathological examination of the resected primary tumor in the breast and axillary lymph nodes.

Definition of pTNM (13):

Primary tumor (T): Tx = primary tumor cannot be assessed; T0 = no evidence of primary tumor; Tis = carcinoma in situ or Paget’s disease of the nipple; T1 = tumor of 20 mm or less; T2 = tumor of more than 20 mm but not more than 50 mm; T3 = tumor of more than 50 mm; T4 = tumor of any size with direct extension to chest wall or skin, or inflammatory breast cancer.

Regional lymph nodes (N): N0 = no node metastasis (includes cases with only isolated tumor cells, or small clusters of cells, not more than 0.2 mm); N1mi = micrometastasis (larger than 0.2 mm, but none larger than 2 mm); N1 = metastasis in 1–3 ipsilateral axillary node(s), and/or in ipsilateral internal mammary nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent; N2 = metastasis in 4–9 ipsilateral
axillary lymph nodes or clinically apparent in internal mammary lymph node(s); N3 = metastasis in 10 or more ipsilateral axillary lymph nodes, or in infraclavicular or supraclavicular lymph nodes, or in both ipsilateral axillary lymph nodes and clinically apparent in ipsilateral internal mammary lymph nodes.

Distant metastasis (M): M0 = no distant metastasis; M1 = presence of distant metastasis.

Based on the combination of the three parameters, patients are divided into different prognostic stage groups (Table 1).

Table 1. *Stage grouping of breast cancer according to pTNM* (13).

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
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<tr>
<td>Stage 0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
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<tr>
<td>Stage I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
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<td>Stage IIA</td>
<td>T0</td>
<td>N1</td>
<td>M0</td>
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<td></td>
<td>T1</td>
<td>N1</td>
<td>M0</td>
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<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
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<tr>
<td>Stage IIB</td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
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<tr>
<td></td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
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<tr>
<td>Stage IIIA</td>
<td>T0</td>
<td>N2</td>
<td>M0</td>
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<td></td>
<td>T1</td>
<td>N2</td>
<td>M0</td>
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<td></td>
<td>T2</td>
<td>N2</td>
<td>M0</td>
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<tr>
<td></td>
<td>T3</td>
<td>N1, N2</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>T4</td>
<td>N0, N1, N2</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>Any</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any</td>
<td>Any</td>
<td>M1</td>
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**Prognostic factors**

A prognostic factor ideally gives information about the risk of disease recurrence in the absence of any adjuvant therapy. A combination of prognostic factors is used to estimate the risk for disease recurrence and the need for adjuvant loco-regional and systemic therapy.

The most recent (2005) St Gallen guidelines for selection of adjuvant systemic therapy for early breast cancer give the established prognostic factors as: lymph node status, tumor size, histological grade, peritumoral vascular invasion, HER2 (Human Epidermal Growth Factor Receptor 2) status, age, and endocrine responsiveness (14). This list includes two new features not previously accepted as sufficiently reliable to define risk category. The first is HER2 positivity, defined as overexpression (excess production of protein) or amplification (an excess number of gene copies) of the HER2 gene. Despite issues related to the reproducibility of testing, HER2 status is regarded as useful for patient care, with overexpression indicating worse prognosis (15). The second new adverse prognostic feature, and a somewhat controver-
sial one, is peritumoral vessel invasion, particularly lymphovascular invasion (16).

The prognostic factors recommended for clinical use according to the Swedish Breast Cancer Group are: lymph node status, tumor grade, tumor size, HER2 status, and S-phase fraction (17).

Lymph node status remains the most important feature for defining risk category; and node-negative status, including negative sentinel node status, is the major condition defining low-risk breast cancer (18, 19).

Other potential prognostic factors, which have not yet been accepted by the St Gallen guidelines as being useful for either risk allocation or treatment choice, are: quantitative Ki67 expression, micrometastasis in bone marrow, uPA (urokinase-type plasminogen activator) and PAI-1 (plasminogen activator inhibitor type 1) expression, and gene profiling using cDNA microarrays (14).

Treatment

Surgery
Randomized studies have shown identical survival rates for breast conserving surgery and mastectomy (20, 21). Today, breast conserving surgery is the treatment of choice when tumor size allows for radical surgery with a good cosmetic result. Mastectomy is recommended for multicentric invasive carcinomas, extensive intraductal carcinomas, and cases where it is desirable to avoid radiotherapy. Mastectomy should also be considered in young patients, as it is now known that in these patients there is a significant risk of local failure after breast conserving therapy with radiotherapy, even when an additional radiation boost is administered to the tumor bed (22).

As lymph node status is an important prognostic factor, axillary surgery is still mandatory. Due to complications associated with traditional axillary dissection, this procedure is now replaced in most cases by the less extensive procedure of sentinel node biopsy. Identical five-year survival rates were recorded in patients treated with axillary dissection and those treated with axillary dissection only if the sentinel node contained tumor cells (23).

Adjuvant loco-regional therapy
Adjuvant radiotherapy after breast cancer surgery, whether breast conserving or mastectomy, is delivered to minimize the risk of loco-regional relapse and, ideally, to improve survival. In developed countries, the current standard of care for patients with early stage breast cancer consists of breast conserving surgery followed by 5–6 weeks of radiotherapy. In this situation,
radiotherapy reduces the risk of local recurrence by about 2/3 in all women, irrespective of patient age or tumor characteristics. Large absolute reductions in local recurrence are seen only if the risk of recurrence is substantial when radiotherapy is omitted (24). Radiotherapy in patients with a high risk of local recurrence (>10% absolute reduction in 5-year local recurrence rate with radiotherapy) has resulted in a 5.3% reduction in 15-year overall mortality (24). On the other hand, if the absolute reduction in 5-year local recurrence rate is less than 10%, there is no significant difference in 15-year mortality risk (24), and so radiotherapy might be avoided in a subgroup of low-risk patients. According to the Guidelines of the American Society for Clinical Oncology (ASCO), radiotherapy to the regional lymph nodes is recommended to patients with four or more positive axillary lymph nodes, but not to patients with one to three positive nodes (25). This recommendation is somewhat controversial, as some studies have shown a survival benefit from radiotherapy in patients with fewer than four positive axillary nodes (26, 27).

Adjuvant systemic therapy
In women with early breast cancer, all detectable cancer is, by definition, restricted to the breast and local axillary lymph nodes, and can be removed surgically. However, undetected micrometastatic deposits of the disease may remain and develop into a clinically detectable recurrence that eventually causes death. The aim of adjuvant systemic therapy is to eradicate these micrometastases.

Hormonal therapy
In patients with estrogen receptor-positive breast cancer, tamoxifen treatment for five years is highly effective. It reduces breast cancer mortality by a third, with an absolute gain at 15 years of 9.2% (28). Today, treatment with an aromatase inhibitor is an alternative to tamoxifen for postmenopausal women with endocrine responsive disease. Aromatase inhibitors have been shown to result in improved treatment outcome in comparison to tamoxifen, both as initial treatment (29, 30) and after 2–3 years of tamoxifen for a total treatment duration of five years (31, 32). Standard treatment duration for adjuvant endocrine therapy is five years; however, a recent study showed that extended therapy with the aromatase inhibitor letrozole for five years after five years of tamoxifen treatment produced improvement in DFS for all patients included in the study, and in OS for node-positive patients (33).

Chemotherapy
Present recommendations for adjuvant chemotherapy are mainly derived from the Oxford overviews and the St Gallen consensus on prognostic factors (14, 28). The 2000 Oxford overview showed that a regimen of cyclophosphamide, methotrexate, and fluorouracil (CMF) decreased the annual
breast cancer death rate by around 34% in women aged under 50 and by 10% in women aged 50–69, corresponding to absolute improvements in 15-year survival of 10% and 3%, respectively (28). Inclusion of an anthracycline further decreases the annual breast cancer death rate by around 16% compared to CMF (28). The gain in absolute survival rate at ten years is approximately 4% if CMF is replaced by an anthracycline-based regimen such as FEC (fluorouracil, epirubicin, cyclophosphamide) (28, 34).

This improvement, however, comes at a price. In comparison with the CMF regimen, FEC is associated with both a higher rate of acute adverse events, and, more importantly, late adverse effects, specifically a small but clinically important increase in the risks of cardiotoxicity and secondary leukemia (35, 36). In the Canadian MA5 trial, which compared CMF with dose-intensive FEC, the rates of congestive heart failure and acute leukemia in FEC-treated patients were 1.1% and 1.4%, respectively, at the ten-year follow-up (34). However, doxorubicin and cyclophosphamide are the mainstay of current adjuvant chemotherapy regimens. Adding a taxane, preferably docetaxel, to an anthracycline-based regimen will probably further reduce the breast cancer mortality rate, but this will be accompanied by a further increase in adverse events (37, 38). While docetaxel is generally well tolerated as a single agent, combination therapy is often complicated and limited by hematological tolerance, fatigue, nail toxicity, and fluid retention.

One much-debated question today regarding chemotherapy in both early and metastatic breast cancer is whether combination therapy offers a survival advantage over sequential single-agent therapy. This is an important issue, as combination therapy is associated with greater toxicity than single-agent therapy. Assessment of tumor response is also more difficult when combination therapy has been administered, as response to one agent can mask resistance to another. Two recently published studies in metastatic disease, the first assessing capecitabine and docetaxel over docetaxel alone, and the second paclitaxel and gemcitabine over paclitaxel alone, showed a survival benefit for the combination regimens (39, 40). A problem common to both studies is that only a small minority of patients in the single-agent arms, 17% in the docetaxel study and 14% in the paclitaxel study, received capecitabine and gemcitabine, respectively, after disease progression (39, 40). These results are in line with a review by Fossati et al. on systemic treatment for metastatic breast cancer, which showed both a superior response rate and a better overall survival with polychemotherapy compared to single agent therapy (3). The only study in metastatic disease that has truly compared sequential therapy with combination therapy is the ECOG 1193 study, which compared single agent doxorubicin (60 mg/m2), single agent paclitaxel (175 mg/m2), and combination therapy (doxorubicin 50 mg/m2 and paclitaxel 150 mg/m2) (41). Response rates in first-line therapy favored the combination, but there was no difference in overall survival (41).
Regarding the combination of different treatment modalities, the results from a large randomized trial, comparing sequential and simultaneous administration of adjuvant chemotherapy and tamoxifen, demonstrate an increase in recurrence with simultaneous administration (42).

The absolute survival gain from adjuvant therapy with tamoxifen and polychemotherapy is modest (5–10% with each modality), and each drug has its own pattern of mild to severe toxicity (28). Since the vast majority of women do not benefit from therapy, some relapsing despite therapy and others never destined to relapse, there is still uncertainty about who should be treated and with which therapy. Since the first patient was treated with a cytotoxic drug, nitrogen mustard in 1946, the majority of patients with the same malignant disease have been treated with the same combination of drugs — the “one shoe fits all” strategy. No molecular marker has been shown to reliably predict sensitivity or resistance to any individual chemotherapeutic agent. The question has been, and still is today, how do we treat this patient risk-group, and not how do we treat this patient with this tumor type.

Adjuvant trastuzumab

Trastuzumab is a humanized mouse monoclonal antibody with high affinity for the extracellular domain of the HER2 transmembrane protein. It has been widely studied in metastatic breast cancer and now has an established role in combination with chemotherapy, mainly taxanes, in patients with advanced disease (43, 44). Adjuvant trastuzumab in combination with or following chemotherapy has been studied in five randomized trials including more than 13,000 patients with HER2-positive tumors. Interim analyses from these studies, with follow-up times ranging from one to three years, have shown that the risk of relapse, and particularly of distant relapse, is half that found with chemotherapy alone; and one study has also shown improved overall survival (reviewed in (45)). Trastuzumab has a very favorable toxicity profile with one exception, namely risk of congestive heart failure (CHF), which increases with prior treatment with anthracyclines. The NSABP B31 study showed a 4.1% incidence of class III or IV CHF, and the overall incidence of cardiac dysfunction was as high as 19% (46). Although the trastuzumab cardiotoxicity was usually reversible after cessation of therapy, and was responsive to standard treatment (46), caution is necessary and longer follow-up is certainly needed.

In Sweden today, according to national guidelines (17), one year of treatment with trastuzumab is recommended for patients with HER2-positive breast cancer treated with adjuvant chemotherapy.
Predictive factors in clinical use

A predictive factor provides information about the probability of response to a specific treatment modality. The only established predictive factors in breast cancer today are hormone receptors, which predict response to endocrine therapy, and HER2, which predicts response to trastuzumab.

Hormone receptor status

In contrast to normal breast epithelial cells, which have very low levels of steroid hormone receptor (HR) expression, the majority of breast cancer cells express estrogen (ER) and progesterone (PR) receptors (reviewed in (47)). When analyzed with immunohistochemistry (IHC), the currently preferred method, approximately 70% of all breast cancers overexpress ER, and 50% overexpress PR (48). In earlier studies, HR-positivity was reported to correlate with better prognosis, however studies with longer follow-up suggest that the prognostic importance of HR is very limited (48-50).

The main utility of ER and PR is as predictive factors for response to endocrine therapy with both tamoxifen and aromatase inhibitors (28-30, 50). Steroid hormone receptors are strong predictors of endocrine responsiveness, though not all patients with tumors expressing hormone receptors will have a clinically useful response. Response rates to endocrine therapy range from 80% in ER+/PR+ patients, through 30% in ER+/PR- patients, to <10% in ER-/PR- breast cancers (47). The cut-off point used with IHC to define a tumor as HR+ in most studies and treatment recommendations, including those of the Swedish Society of Pathology (Svensk Förening för Patologi), is ≥10% positive tumor cells (51). However, there are data suggesting that as few as 1% positive tumor cells may be associated with significant clinical benefit from endocrine therapy with either tamoxifen or the aromatase inhibitor letrozole (50, 52). The St Gallen guidelines classify this group of patients, with some expression of HR, as “endocrine response uncertain”, implying that there may be some benefit from endocrine therapy (14).

HER2

The HER2 oncogene encodes a 185-kDa transmembrane glycoprotein, which belongs to the family of epidermal growth factor (EGF) receptor tyrosine kinases (RTK). This family has four members: HER1 (EGFR), HER2, HER3, and HER4 — see Figure 3 (15). After a ligand binds to a receptor, that receptor must interact with another receptor of identical or related structure in a process known as dimerization, in order to trigger phosphorylation and activation of a signaling cascade, which affects cell proliferation and cell survival. While no known ligand for the HER2 receptor has been identified, it is the preferred dimerization partner of the other family members (re-
viewed in (53)). HER2 positivity (overexpression of the HER2 protein or amplification of the HER2 gene) is seen in approximately 20–25% of primary breast cancers (54). A review analyzing HER2 status in primary tumors and metastasis showed that metastases, both lymph node and distant metastases, generally overexpress HER2 to the same extent as the primary tumor (55). This stability of HER2 expression is of utmost importance when treating patients with metastatic disease. In the same study it was noted that the primary tumors and corresponding lymph node metastasis were HER2-positive in 55% of patients developing distant metastasis (55).

HER2 protein overexpression can be measured using IHC. Data from a randomized trial suggest that the beneficial treatment effects are largely limited to patients with the highest expression (3+) (43). Measurement of the number of HER2 gene copies using fluorescence in situ hybridization (FISH) may be used as a surrogate for protein overexpression. FISH is probably a superior method for selection of patients for trastuzumab therapy; in a study of patients with metastatic disease, the beneficial effect of trastuzumab in combination with chemotherapy was seen solely in FISH-positive patients (56). In this study, HER2 gene amplification was detected in 89% of patients with IHC 3+ and 31% of those with IHC 2+ (56). According to the Swedish national treatment guidelines, only patients with FISH-verified gene amplification are candidates for trastuzumab therapy (17).

HER2 is the molecular target for the humanized monoclonal antibody trastuzumab, and there is clear evidence from randomized trials that HER2 overexpression or HER2 gene amplification predicts efficacy of trastuzumab (43, 45). HER2 overexpression has also been associated with worse outcome when using tamoxifen (57), while aromatase inhibitors in a small number of patients with HR-positive/HER2-positive LABC resulted in higher response rates compared to tamoxifen (52). According to a recent review, despite some suggestive evidence for the predictive effects of HER2, the overall consensus was that there is insufficient evidence to develop guidelines on the use of HER2 to select specific treatments for individual patients, other than in the case of treatment with trastuzumab (58).
Figure 3. The HER (erbB) family. Note that HER2 has no known ligand and HER3 has no intrinsic tyrosine kinase activity. Figure from Ross et al., The Oncologist, 2003 (15).

Prediction of chemosensitivity

Topoisomerase IIα

Topoisomerases are enzymes which regulate topological changes in DNA. They are vital for many cellular processes, including replication, transcription, and chromosome condensation and segregation. They perform their function by introducing transient protein-bridged DNA breaks on one (topoisomerase I) or both (topoisomerase II) DNA strands (59) (60). A model for topoisomerase II action, taken from “Molecular Biology of the Cell” (61), is illustrated in Figure 4.

Topoisomerase II has two isoenzymes, with genetically and biochemically distinct features. Topoisomerase IIα (topo IIα) has a molecular weight of 170 kDa, and is located on chromosome 17q21–22 (62), while topoisomerase IIβ has a molecular weight of 180 kDa, and is located on chromosome 3 (63).

Topo IIα is the primary drug target for a group of anticancer drugs known as topo II inhibitors; this group includes the anthracyclines and the epipodophyllotoxins. Through binding to topo IIα, the topo II inhibitors stabilize the so-called cleavable complex which leads to DNA double-strand breaks (60, 64, 65). Though the pathway from DNA breaks to cell death has not been completely explored (66), it is known that when the burden of genomic insult is simply too large to be effectively met by the DNA-repair machinery, cells are able to initiate programmed cell death (apoptosis), thereby eliminating themselves (67). Interaction with topo IIα is today regarded as the main mechanism of action of anthracyclines (68).

Being the primary drug target for anthracyclines, topo IIα is a potential predictive factor for anthracycline efficacy in breast cancer. In vitro studies on breast cancer cell lines have shown that TOP2A gene amplification is associated with increased topo IIα protein expression and increased sensitivity to topo II inhibitors (69-71).

While overexpression of topo IIα analyzed by IHC has failed to predict response to epirubicin in metastatic breast cancer (72), it has been shown to be predictive of clinical response in operable and locally advanced breast cancer treated with neoadjuvant epirubicin (73). In a study of 59 patients with locally advanced breast cancer (LABC) or metastatic breast cancer (MBC), both TOP2A gene amplification and topo IIα protein overexpression seemed to correlate with response to anthracycline (74). Two studies on LABC have shown co-amplification of HER2 and TOP2A to be associated with increased response to neoadjuvant anthracycline-containing chemotherapy (75, 76). In contrast to these findings, a study of 119 patients with operable breast cancer treated with neoadjuvant anthracycline chemotherapy found that HER2 and TOP2A gene amplification evaluated by real-time polymerase chain reaction (PCR) were not predictive of response (77). Knowledge regarding gene amplification of TOP2A as predictor of response to anthracycline in advanced breast cancer is thus still limited, and the results are contradictory.

In the adjuvant setting, the influence of TOP2A gene aberrations on the outcome of anthracycline-based adjuvant treatment has been analyzed in three published studies (78-80). All of these studies, including one study using IHC (81), reported evidence of a predictive value of TOP2A. In the study by Di Leo et al (79), the advantage in DFS was seen only in the subgroup of patients with co-amplification of HER2 and TOP2A; this result, however, was based on very small numbers. The most convincing evidence of an interaction between TOP2A and treatment outcome has been demonstrated in two recent studies by Tanner et al. and Knoop et al. (78, 80). Tan-
ner et al. noted better recurrence-free survival (RFS) in patients with co-amplification of HER2 and TOP2A after treatment with nine cycles of tailored dose-escalated anthracycline therapy compared with only three cycles of standard dose anthracycline treatment followed by treatment with high-dose chemotherapy with cyclophosphamide, thiotepa, and carboplatin (78). Of note in this study is that all patients were treated with anthracycline, and probably had some benefit from this treatment, thereby reducing the observed difference. In accordance with Tanner et al. (78), Knoop et al. (80) also noted better RFS in co-amplified tumors, but, unexpectedly, noted in addition that deletion of TOP2A had a predictive value almost equal to that of amplification, albeit with broad confidence intervals (probably due to the low number of patients in this group). This finding was not expected, as the hypothesis is that deletions predict anthracycline resistance (69). The molecular mechanisms underlying the adjacent HER2 amplification and TOP2A deletion are unknown.

None of the studies exploring the benefit of TOP2A gene aberrations in the adjuvant setting have been able to prove a statistically significant difference in overall survival. Part of the reason for this may be that patients in the “control arms” of the studies had most likely received anthracyclines as first-line treatment for metastatic disease. The fact that many controls in randomized studies receive the study drug is a rather general phenomenon today, and has caused RFS to replace OS as primary outcome in many adjuvant trials.

The abovementioned studies, our own study in metastatic breast cancer (82), and the BCIRG 006 study presented at the 2005 San Antonio Breast Cancer Symposium (83) are summarized in Table 2.
Table 2. TOP2A status and efficacy of anthracycline-based chemotherapy.

<table>
<thead>
<tr>
<th>Reference</th>
<th>N</th>
<th>Tr</th>
<th>Method</th>
<th>Cut-off</th>
<th>TOP2A Amp %</th>
<th>Resp</th>
<th>RFS</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tanner 2006</td>
<td>391</td>
<td>A</td>
<td>CISH</td>
<td>≥6 or cluster</td>
<td>12 (37% of HER2+)</td>
<td>P=0.049</td>
<td>Predictive for RFS</td>
<td></td>
</tr>
<tr>
<td>Knoop 2005</td>
<td>773</td>
<td>A</td>
<td>FISH</td>
<td>≥2</td>
<td>12</td>
<td>P=0.01</td>
<td>Predictive for RFS</td>
<td></td>
</tr>
<tr>
<td>Slamon 2005</td>
<td>2120</td>
<td>A</td>
<td>FISH</td>
<td>≥2</td>
<td>35 (all HER2+)</td>
<td>P&lt;0.001</td>
<td>Predictive for DFS</td>
<td></td>
</tr>
<tr>
<td>Di Leo 2002</td>
<td>354</td>
<td>A</td>
<td>FISH</td>
<td>≥1.5</td>
<td>6 (38% of HER2+)</td>
<td></td>
<td>Suggestive</td>
<td></td>
</tr>
<tr>
<td>Di Leo 2001</td>
<td>481</td>
<td>A</td>
<td>IHC</td>
<td>&gt;10%</td>
<td>ND</td>
<td>NS</td>
<td>NS</td>
<td>Suggestive</td>
</tr>
<tr>
<td>Petit 2004</td>
<td>119</td>
<td>O</td>
<td>PCR, L</td>
<td>≥2</td>
<td>24</td>
<td>NS</td>
<td>NS</td>
<td>Negative</td>
</tr>
<tr>
<td>Cardoso 2004</td>
<td>31</td>
<td>L</td>
<td>IHC</td>
<td>≥1.5</td>
<td>13</td>
<td>NS</td>
<td>Suggestive</td>
<td></td>
</tr>
<tr>
<td>Park 2003</td>
<td>67</td>
<td>L</td>
<td>CISH</td>
<td>&gt;4 or cluster</td>
<td>28</td>
<td>P=0.038</td>
<td>Predictive</td>
<td></td>
</tr>
<tr>
<td>MacGrogan 2003</td>
<td>125</td>
<td>O</td>
<td>IHC</td>
<td>&gt;15%</td>
<td>ND</td>
<td>P=0.006</td>
<td>Predictive</td>
<td></td>
</tr>
<tr>
<td>Coon 2002</td>
<td>35</td>
<td>L</td>
<td>FISH</td>
<td>&gt;2.5</td>
<td>18</td>
<td>P=0.034</td>
<td>Predictive</td>
<td></td>
</tr>
<tr>
<td>Villman 2006</td>
<td>85</td>
<td>M</td>
<td>CISH</td>
<td>≥6 or cluster</td>
<td>16</td>
<td>NS</td>
<td>Suggestive</td>
<td></td>
</tr>
</tbody>
</table>

N, number of participants; Tr, treatment; Resp, response; A, adjuvant treatment; O, operable breast cancer; L, locally advanced breast cancer; M, metastatic breast cancer; IHC, immunohistochemistry; PCR, polymerase chain reaction; FISH, fluorescence in situ hybridization; CISH, chromogenic in situ hybridization; Amp, amplified; Del, deleted; RFS, recurrence-free survival; DFS, disease-free survival; cRR, clinical response rate; cCR, clinical complete response; ND, not done; NS, non-significant.

Both a high pretreatment level of topo IIα expression and a decline in this level after chemotherapy may predict response to anthracycline-based treatment (84). This association of falling levels with sensitivity may be accounted for by the selective chemotherapy killing of cells within the tumor that preferentially express topo IIα (84).

Gene amplification of TOP2A, though a surrogate marker for the actual target protein overexpression, is today regarded as a more solid marker for favorable response to anthracycline treatment than IHC analysis of topo IIα. A basic assumption behind the theory that TOP2A gene status is related to the efficacy of topo II inhibitors is the connection between TOP2A gene copy number and topo IIα protein expression. It has generally been postulated in most studies that TOP2A gene amplification leads to increased levels of topo IIα mRNA and protein overexpression, which leads in turn to increased sensitivity to anthracyclines. There are studies both supporting (69, 85-87) and questioning (77, 88) this assumption. The number of gene copies
is not the only factor regulating the level of active protein; topo IIα expression is regulated at the level of transcription and translation, and coordinated with the different phases of the cell cycle (89), with higher expression in proliferating cells (88, 90, 91). One factor known to regulate TOP2A gene expression is the tumor suppressor protein p53. *In vitro* studies have shown that wild-type p53 can downregulate TOP2A expression via transcriptional regulation (92, 93), and modulate the activity of the enzyme via direct protein interaction (94). This mechanism is probably designed to halt replication in the case of DNA damage. p53 mutation may reduce normal regulatory suppression of TOP2A and contribute to abortive cell cycle checkpoints, accelerated cell proliferation, and alterations in genomic stability associated with neoplasia (93). Regulation of the level of transcription can be a critical factor in drug-induced downregulation of topo IIα mRNA, which may have implications during chemotherapy for cancer. Topo IIα also undergoes post-translational modifications that regulate the activity of the enzyme (89). Overexpression of topo IIα measured by IHC in the absence of gene amplification has been demonstrated, and may be biologically meaningful and predictive of anthracycline response (75). Gene amplification or protein overexpression used in isolation might be useful to identify patients who will not benefit from anthracyclines, while their combined use could increase the likelihood of identifying those patients most likely to benefit (74). Taken together, this means that gene amplification may not dramatically alter either the level of topo IIα in tumor cells or their sensitivity to anthracyclines.

At the 2005 San Antonio Breast Cancer Symposium, Dennis Slamon presented results from the BCIRG 006 study showing impressive superiority for HER2-positive patients when treated with a combination of adjuvant trastuzumab and chemotherapy compared with chemotherapy alone (83). Of the 2,120 patients analyzed, 744 (35%) displayed TOP2A amplification, while 91 (4%) displayed TOP2A deletion. An efficacy analysis according to the different status of HER2 and TOP2A amplification suggests that anthracycline-based regimens are better than other regimens in women treated with trastuzumab only if there is amplification of TOP2A (83). If this result is confirmed, patients with HER2-positive but TOP2A-negative breast cancer could be spared treatment with anthracyclines, and thus minimize their risk of developing treatment-related congestive heart failure and acute leukemia.

**HER2**

While HER2 is an established prognostic factor, and a predictive factor for trastuzumab, its role as a predictive factor for response to chemotherapy is still a controversial issue. HER2 has been extensively studied in recent years, and in the vast majority of these studies overexpression of HER2 has been determined by IHC. The conflicting results seen in the studies using IHC may be due to differences in the choice of antibodies and scoring systems,
resulting in very different proportions of tumors being classified as overexpressive of HER2.

The correlation between overexpression of the HER2 protein or amplification of the HER2 gene and benefit from adjuvant anthracycline-based chemotherapy has been studied in several published studies. The results of these studies have varied; the correlation between HER2 overexpression and the outcome of chemotherapy has been shown to be negative (78, 80), suggestive (79, 81, 95), of borderline significance (96), positive for DFS (97), and positive for both DFS and OS (98-100). It should be noted that the studies by Thor et al. (99) and Dressler et al. (100) are from the same clinical trial, CALGB 8541. The results of these studies are summarized in Table 3.

Table 3. HER2 status and efficacy of adjuvant anthracycline-based chemotherapy.

<table>
<thead>
<tr>
<th>Reference</th>
<th>N</th>
<th>Method</th>
<th>HER2+ %</th>
<th>RFS/DFS</th>
<th>OS</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pritchard 2006</td>
<td>639</td>
<td>FISH, (IHC, PCR)</td>
<td>26</td>
<td>P=0.003</td>
<td>P=0.06</td>
<td>Dose-dense anthracycline</td>
</tr>
<tr>
<td>Tanner 2006</td>
<td>391</td>
<td>CISH</td>
<td>33</td>
<td>NS</td>
<td>NS</td>
<td>Negative</td>
</tr>
<tr>
<td>Knoop 2005</td>
<td>805</td>
<td>FISH</td>
<td>33</td>
<td>NS</td>
<td>NS</td>
<td>Negative</td>
</tr>
<tr>
<td>Dressler 2005</td>
<td>524</td>
<td>FISH, (IHC, PCR)</td>
<td>17</td>
<td>P=0.033</td>
<td></td>
<td>Dose-escalated anthracycline</td>
</tr>
<tr>
<td>Moliterni 2003</td>
<td>506</td>
<td>IHC</td>
<td>19</td>
<td>HR=0.83</td>
<td>HR=0.61</td>
<td>Borderline significance</td>
</tr>
<tr>
<td>Di Leo 2002</td>
<td>354</td>
<td>FISH</td>
<td>21</td>
<td>NS</td>
<td>ND</td>
<td>Suggestive</td>
</tr>
<tr>
<td>Di Leo 2001</td>
<td>481</td>
<td>IHC</td>
<td>12</td>
<td>NS</td>
<td>ND</td>
<td>Suggestive</td>
</tr>
<tr>
<td>Paik 2000</td>
<td>2034</td>
<td>IHC</td>
<td>29</td>
<td>NS</td>
<td>NS</td>
<td>Suggestive</td>
</tr>
<tr>
<td>Paik 1998</td>
<td>638</td>
<td>IHC</td>
<td>37.5</td>
<td>P=0.002</td>
<td>P=0.01</td>
<td>Predictive for DFS</td>
</tr>
<tr>
<td>Thor 1998</td>
<td>992</td>
<td>PCR, (IHC)</td>
<td>17</td>
<td>P=0.001</td>
<td>P&lt;0.001</td>
<td>Dose-escalated anthracycline</td>
</tr>
</tbody>
</table>

N, number of participants; RFS, recurrence-free survival; OS, overall survival; IHC, immunohistochemistry; PCR, polymerase chain reaction; FISH, fluorescence in situ hybridization; CISH, chromogenic in situ hybridization; HER2+, HER2-positive; ND, not done; NS, non-significant.

The most convincing interaction between the outcome of treatment and HER2 overexpression was found with the dose-intensive CEF combination used in the Canadian trial (98). An interaction between HER2 status and anthracycline dose intensity was also suggested in two other studies (99, 100), but was not observed in the study by Tanner et al. exploring tailored and dose-escalated anthracycline-based chemotherapy (78).

These conflicting results regarding the predictive role of HER2 are not surprising, as there is no obvious molecular rationale for a relationship between HER2 status and anthracycline efficacy. Pegram et al. found no consistent relationship between HER2 overexpression and the response to a large panel of chemotherapeutic agents using HER2 transfected breast can-
cer cell lines (101). The association observed in some studies between HER2 and anthracycline efficacy may in fact be explained at a molecular level by concomitant amplification of TOP2A, as these two genes are located next to each other on chromosome 17q12–q21 (69, 102). Due to this proximity, it was originally thought that both HER2 and TOP2A are present on the same amplicon. However, it has since been shown that they are actually on separate amplicons (102). This finding is supported by the fact that tumors with HER2 and TOP2A co-amplification often have different copy numbers, with TOP2A amplification often seen at a lower level than HER2 (103, 104), and some HER2-amplified tumors displaying deletion of the TOP2A gene (80).

Proliferation rate
Tumor cell proliferation has been extensively studied, both as a possible prognostic factor and as a potential treatment predictive factor. In the earliest studies, tumor proliferation was measured by tumor cell uptake of tritiated thymidine or bromodeoxyuridine. Routine use of these methods is limited, however, by its requirement for fresh tumor material with metabolically intact cells. Consequently, more feasible methods have been developed. The two most widely used are determination of S-phase fraction (SPF) by flow cytometry and measurement of the fraction of Ki67-positive cells with IHC. Although neither SPF nor Ki67 is regarded as an established prognostic factor according to the St Gallen guidelines (14), their prognostic and predictive potential have been studied extensively.

DNA analysis: SPF
Flow cytometric measurement of the number of cells synthesizing DNA makes it possible to assess the intratumoral percentage of cells in the S phase of the cell cycle, that is, the S-phase fraction (SPF), which provides information on the proliferation rate (18). A review by Wenger and Clark on the prognostic value of SPF concluded that high SPF is generally associated with worse prognosis, but standardization and quality control must be improved before SPF can be recommended for general clinical use (105). SPF is often dichotomized in investigations of its prognostic significance, using either a single cut-off point for all tumors or separate cut-off points for diploid and aneuploid tumors. The percentage of tumors classified as having high SPF depends largely on whether one or two cut-off points are used, and the level chosen. The predictive value of SPF in advanced breast cancer has been reviewed by Sjöström, who found a clear association between high SPF and response to neoadjuvant chemotherapy in locally advanced breast cancer, but concluded that the role of SPF in metastatic breast cancer is less clear (106). In the adjuvant setting, a significantly higher benefit from CMF was observed in patients with a high SPF (≥ 10%) (107). However, the clinical utility of this information is poor, since many patients with slowly prolif-
erating tumors also benefit from chemotherapy. Flow cytometry also has its drawbacks, specifically its requirement for fresh or frozen tumor material with a large number of tumor cells, and the lack of morphological control of the analyzed cells.

**Ki67**

The rationale behind the use of Ki67 analysis is the fact that it can be performed on paraffin-embedded tissue with very small amounts of tissue with good morphological control. The shortfall of the method is mainly related to the subjectivity of the evaluation. MIB-1 is an antibody that recognizes an epitope on Ki67 in paraffin-embedded tissue (108). Ki67 is a specific nuclear antigen expressed only in proliferating cells (109, 110). A high level of Ki67 in breast cancer has a statistically significant correlation with poor clinical outcome (reviewed in (111)).

Results regarding the predictive value of Ki67 in the neoadjuvant setting are inconsistent, with some studies supporting a better response in tumors with high Ki67 levels (112) (77, 113, 114), and others showing no difference (115-117). A decrease in Ki67 during neoadjuvant chemotherapy has been associated with a favorable response in some studies (112, 113, 118, 119). The decreased proliferation found following chemotherapy supports the hypothesis that clones of slowly proliferating cells are selectively preserved during therapy.

In a study by MacGrogan et al., Ki67 emerged as an independent predictive factor for the therapy response in the multivariate analysis (114), but its statistical significance vanished when KiS7 (topo IIα) was included in the logistic regression model (73). This suggests that topo IIα expression may have an impact on the responsiveness of tumors to cytotoxic therapy beyond its association with tumor cell proliferation.

**KiS2**

KiS2 is an antibody that recognizes a 100-kDa proliferation-specific nuclear protein expressed exclusively in the cell cycle phases S, G2, and M (120, 121). The KiS2/Ki67 ratio represents the relative cell fraction in the cell cycle compartments after the critical G1/S transition, and is known as the cycling ratio. The cycling ratio may vary widely in neoplastic cell populations (120). The complementary value corresponds to the relative percentage of cells in G1. Cycling ratio has shown independent predictive value for survival and may be a powerful indicator of the biological behavior of cancer (120). Tumors that simultaneously overexpress HER2 and p53 have a high cycling ratio, probably explained by the increased number of cells passing through the G1/S checkpoint due to impaired p53 function (122).
Cyclin A

Cyclins are a family of proteins involved in the progression of cells through the cell cycle. A cyclin forms a complex with a cyclin-dependent kinase (Cdk), which activates the protein kinase function of the latter. The concentration of each cyclin varies cyclically during the cell cycle. Cyclin A is synthesized during the S phase; it is required for both S phase progression and for passage from G2 into mitosis, and is therefore a useful marker for proliferating cells (123). Overexpression of cyclin A has been associated with worse prognosis in breast cancer (124). Lack of prognostic significance of cyclin A is mainly seen in patients with lymph node negative breast cancer, indicating that cyclin A plays a more important role in aggressive tumors and metastatic disease (125). In patients with soft tissue sarcomas, a high cyclin A score predicted a better chemotherapy response and longer progression-free survival (126).

Hormone receptor status

Paradoxically, tumor features commonly associated with a more favorable prognosis, such as low tumor grade, low proliferation rate, and expression of HR, are unfavorable predictors of response to neoadjuvant chemotherapy. In particular, HR status seems to be predictive of relative chemoresistance. Multiple trials have shown that the probability of achieving pCR is significantly inferior in tumors expressing HR (115, 127-130). The value of HR has also been shown in the adjuvant setting. A retrospective analysis of three adjuvant trials including over 6 000 patients showed that the absolute benefit due to chemotherapy was greater for patients with ER-negative compared with ER-positive tumors: 22.8% more ER-negative patients survived to 5 years disease-free if receiving chemotherapy vs 7.0% for ER-positive patients; corresponding improvements for overall survival were 16.7% vs 4.0% (131).

Tumors completely lacking hormone receptors are particularly sensitive to neoadjuvant chemotherapy, but despite a pCR rate exceeding 30%, survival of patients with this phenotype was shorter than that of patients with receptor-positive tumors, who obtained pCR significantly less frequently (115). This difference in long-term outcome is probably due to the responsiveness of HR-expressing tumors to endocrine therapies such as tamoxifen, aromatase inhibitors, and ovarian function suppression. Colleoni et al. also demonstrated that the pattern of response to chemotherapy varies in relation to HR status, with a pCR rate of 33.3% in the HR-absent cohort compared with 7.4% and 7.6% in the low-HR (ER and/or PR ≥ 1% <10%) and HR-positive (ER/PR ≥10%) cohorts, respectively (115).

Studies using gene expression profiling have confirmed the importance of ER biology in breast carcinogenesis (132), and expression of ER-related
genes has shown predictive value in LABC patients receiving neoadjuvant anthracyclines and paclitaxel chemotherapy (133).

**Fluoropyrimidine biomarkers**

Fluoropyrimidines are cytotoxic agents commonly used in breast cancer therapy. Traditionally, 5-fluorouracil (5-FU) has been used in combination with anthracyclines and cyclophosphamide, as in the FEC and CAF regimens.

Capecitabine is an oral fluoropyrimidine carbamate that was developed in an effort to improve tumor selectivity. After oral administration, capecitabine is metabolized to FU through three enzymatic steps (134). The final step involves the conversion of 5-DFUR to 5-FU by thymidine phosphorylase (TP). Because TP has a high level of expression in tumor tissue, capecitabine metabolism results in the preferential activation of 5-FU in tumors (134). Capecitabine is today indicated as single therapy for patients with advanced breast cancer progressing after anthracycline and taxane therapy.

Thymidylate synthase (TS) is a rate-limiting enzyme in DNA synthesis, and represents the cellular target of 5-FU. 5-FU is metabolized intracellularly to FdUMP, which binds to TS in the presence of reduced folate cofactor. This binding inhibits the formation of thymidylate from uracil, which is required for DNA synthesis (135). Dihydropyrimidine dehydrogenase (DPD) is the first, and rate-limiting, enzyme for the catabolism of 5-FU. The interaction in 5-FU pathways means that the enzymes TP, TS, and DPD are potential predictive markers for 5-FU and capecitabine efficacy. DPD quantification is also useful in predicting or explaining exaggerated toxicity reactions to 5-FU based treatment. Polymorphism in the DPD coding gene gives rise to varying degrees of defective 5-FU metabolism.

Since TS is the molecular target for 5-FU, its predictive value has been analyzed in several studies. These studies rather consistently demonstrate an inverse relationship between the level of TS expression and clinical response to 5-FU (136). However, 30–50% of patients fail to obtain an objective response despite a low TS level (136), indicating that TS is not the only factor regulating sensitivity to 5-FU. Patients with colorectal cancer and low gene expression levels of TP, TS, and DPD, measured by quantitative reverse transcription-PCR, have shown increased response rate to 5-FU (137). In this study there was no correlation among TP, TS, and DPD values, indicating that the expressions of these genes are independent values (137). In contrast to the low level of TP expression predicting response to 5-FU, a recent phase II study in metastatic colorectal cancer showed that a high level of TP expression seems to predict response to capecitabine (138). The same study also showed that TP in primary tumors, measured by IHC, was significantly associated with response to treatment with a combination of capecitabine and irinotecan (138) A similar trend was observed with TP expression in
metastases, although this was not statistically significant. TS and DPD expression as measured by IHC was not associated with response to irinotecan and capecitabine. The sensitivity, specificity, positive predictive value, and negative predictive value for TP expression in primary tumors were 85%, 96%, 48%, and 30%, respectively (138). The relationship between a high level of TP expression and enhanced efficacy of capecitabine is unsurprising, as TP is the key enzyme in the activation pathway of capecitabine. TP expression may become useful for the selection of a particular fluoropyrimidine, 5-FU or capecitabine.

There is only limited knowledge regarding the predictive value of TP, TS, and DPD in breast cancer.

p53

p53 is a tumor suppressor gene with a central role in cell cycle control in response to DNA damage (122); it senses DNA damage and sets into motion a series of events that leads to either G1 arrest or apoptosis (139). Nearly a third of breast cancers have mutations of the p53 gene, mainly point mutations, leading to translation of a stable, malfunctional protein with prolonged half-life that accumulates in the cell, and is therefore detectable by IHC (140). A higher rate of p53 positivity has been observed in patients with more advanced breast cancer stages (T3 and T4 tumors) (141). The association between p53 mutation and unfavorable tumor characteristics may well reflect a loss of the checkpoint control function of p53, resulting in an accumulation of genetic defects that promote an aggressive phenotype (122). The prognostic value of p53 has been extensively studied and reviewed, with somewhat mixed results; however, there is evidence to support an association between alterations of the p53 gene and poor prognosis (reviewed in (58)). With IHC, there is a risk of false negatives in relation to the type of p53 mutations, and p53 gene sequencing has shown superior prognostic value over IHC (142).

Since p53 is involved in control of the cell cycle, in repair after DNA damage, and in apoptosis, there is a biological rationale for its being a predictive factor for response to DNA-damaging agents. Alkylating agents such as cyclophosphamide, as well as anthracyclines, intercalate with DNA to induce cross-links and strand breaks considered to produce a p53-dependent induction of apoptosis.

The role of p53 as a predictive factor for response to chemotherapy has been evaluated in two reviews, with the common conclusion that p53 status is not predictive of response (143, 144). In the majority of the studies included in these reviews, p53 was evaluated with IHC, which may be inferior to gene sequencing for the predictive value just as with the prognostic value. The DNA-damaging combination FEC (fluorouracil, cyclophosphamide, epirubicin) was compared to a microtubule stabilizing therapy with pacli-
taxel in 67 patients with LABC (141). In this study, p53 was determined by IHC and sequencing of the entire p53 gene, and a strong correlation between lack of response and presence of a p53 mutation was observed in the FEC group but not in the paclitaxel group (141). Another finding of the study was that IHC was positive in 23% of patients without p53 gene mutations, and this positive IHC staining was of functional importance and correlated with decreased clinical response in patients treated with FEC (141). A study by Aas et al. suggests that only specific p53 mutations confer resistance to anthracycline (145). The predictive value of p53 is currently being evaluated in a phase III trial.

In vitro assays

The first publication describing the use of an in vitro assay using human tumor material was by Black & Speer in 1953 (146). Since then, several in vitro techniques have been developed with the aim of providing prognostic and, more importantly, predictive information to increase the effectiveness of chemotherapy (147). Taken as a whole, these assays show rather impressive data on the correlation between the activity of cytotoxic drugs in vitro and known drug activity at the tumor type level as well as for individual patients, and also seem to predict long-term survival (148).

A review of published in vitro assay results, covering 4 263 patients for whom correlations with treatment response were available, indicated that clinical response rates were significantly associated with in vitro results, with an overall sensitivity of 85% and an overall specificity of 80% (147). Of note was the finding that prediction of drug resistance was >90% accurate, compared to 72% accuracy for prediction of chemosensitivity.

A number of prospective randomized trials have aimed to actually prove that assay-guided therapy is more effective than empirically-based therapy, but they all suffer from major insufficiencies, for example, poor patient recruitment and technical difficulties, making it difficult to draw firm conclusions. However, a review of such trials did show a tendency for assay-based therapy to be better than empirically-based regimens (149).

Some recent studies have shown promising results. A randomized trial including 180 patients with platinum-resistant ovarian cancer, presented at the 2005 ASCO meeting, showed a trend towards improved response rate (40.5% vs. 31.5%) and PFS (104 days vs. 93 days) for assay-directed therapy compared to physician’s choice (150). Of the 53 evaluable patients treated with individualized chemotherapy based on in vitro chemosensitivity testing in a phase II study on metastatic malignant melanoma, a superior response was found among those whose tumor samples were classified as chemosensitive (151); the response rate in chemosensitive patients was 36.4% compared with 16.1% in chemoresistant patients (p=0.014). These preliminary results will be evaluated further in a phase III trial.
The fluorometric microculture cytotoxicity assay (FMCA) used in our study in advanced breast cancer is a short-term in vitro drug sensitivity assay based on the concept of total cell kill. It is based on the measurement of fluorescence generated from hydrolysis of non-fluorescent fluorescein diacetate (FDA) to its fluorescent derivative, fluorescein, by cells with intact plasma membranes (152). The technical success rate of FMCA in solid tumors is around 70% (153, 154). FMCA has been shown to report clinically relevant drug sensitivity data in individual patients with leukemia, non-Hodgkin’s lymphoma, and ovarian carcinoma (153, 155, 156).

Gene expression profiling
A defining moment for DNA research was the discovery of the double helical structure of DNA by James Watson and Francis Crick, published in Nature in April 1953 (157). Two features of the DNA structure account for much of its remarkable impact on science: its digital nature and its complementarity, whereby one strand of the helix binds perfectly with its partner. DNA contains two types of digital information; the genes that encode proteins, and the gene regulatory networks that specify the behavior of the genes. The discovery of the structure of DNA established the basic framework that would develop into the field of molecular genetics.

The Human Genome project (HGP) was an international collaborative research program whose goal was the complete mapping and understanding of all the genes of human beings (the genome). Completed in 2004, the HGP revealed that the genome sequence contains 2.85 billion nucleotides and seems to encode 20 000–25 000 protein coding genes, a number significantly lower than previously estimated (158). The accuracy and completeness of the currently near-complete human genome sequence has important consequences for biomedical research. It allows systematic searches for the causes of disease, such as the somatic mutations underlying breast cancer, and it facilitates the development of experimental tools for recognizing cellular components.

Gene expression profiling (GEP), or microarray analysis, is the process of determining which genes are active in a specific cell or group of cells; it is accomplished by measuring mRNA, the intermediary between genes and proteins. GEP enables the measurement of thousands of genes in a single RNA sample. With the advent of GEP, it became possible to divide breast cancers into clinically relevant molecular subgroups, and to identify molecular signatures that can predict the success or failure of treatment.

GEP of breast cancer has led to new insights into the heterogeneity of this disease (132). In particular, HER2-negative status includes at least three different forms of breast cancer: “basal-like”, which is hormone receptor-negative and very aggressive; “luminal B”, which is hormone receptor-positive and has a poor prognosis; and “luminal A”, which is also hormone
receptor-positive, but indolent and with very limited benefit from chemotherapy. GEP has been shown to be a more powerful predictor of outcome than standard systems based on clinical and pathological risk assessment in both node-negative and node-positive primary breast cancer patients (159, 160). The prognostic value of the 70-gene signature used in the studies by the Amsterdam group has recently been validated in an independent group of patients from five European centers (161).

Gene profiles that relate to prognosis may also help define new therapeutic targets. A study by Sotiriou et al. found that cell cycle regulation was clearly important, suggesting the continued use of antiproliferatives as a rational approach (132).

The experience gained from using GEP as a tool to predict response to chemotherapy is still very limited and the findings somewhat contradictory. Chang et al. studied response to neoadjuvant docetaxel monotherapy in 24 patients, and found a correlation between response and the expression of 92 genes (162). Ayers et al. used GEP to predict response to neoadjuvant paclitaxel followed by fluorouracil, doxorubicin, and cyclophosphamide (163). A 74-gene predictor was devised from a group of 24 patients and then tested in a cohort of 18 patients, showing an accuracy of 78%, a sensitivity of 43%, and a specificity of 100% (163). In a study by Gianni et al., paraffin-embedded biopsy material from 89 patients was analyzed by RT-PCR to identify which of 384 candidate genes were associated with pCR (133). Univariate analysis revealed 86 genes that were associated with response to sequential doxorubicin and paclitaxel-based chemotherapy (133). Hannemann et al. could not identify any gene expression profile that predicted response to neoadjuvant chemotherapy with either doxorubicin-cyclophosphamide or doxorubicin-docetaxel in 48 patients with primary breast carcinomas; however, they did demonstrate that tumors that respond to neoadjuvant treatment show dramatic changes in their expression profiles (164).

Although some of the results of using GEP in predicting response to chemotherapy are promising, further studies are needed before these gene signatures can be applied in patient management.

**In vivo chemosensitivity testing**

Neoadjuvant therapy has advantages over adjuvant therapy: (1) it provides higher rates of breast conservation (165, 166), (2) response to therapy can be assessed in real time, so that ineffective therapies can be abandoned in favor of alternative, non-cross-resistant drugs, and (3) prognosis can be refined according to degree of residual disease after therapy. Pathological complete response (pCR) in patients receiving neoadjuvant chemotherapy is a powerful prognostic marker for both DFS and OS, and has been confirmed as such in several studies (129, 167-169). Achievement of pCR leads to a better outcome, probably due to eradication of micrometastatic disease. Surprisingly,
the doubling of the pCR rate observed in patients in the NSABP B27 study when docetaxel was added to an anthracycline-containing regimen did not translate into an improvement in survival (129). This result challenges the hypothesis that a difference in the rate of pCR with neoadjuvant chemotherapy is a valid surrogate predictor for long-term outcome (129). Interpretation of the pCR rate is further complicated by the tumor proliferation rate and HR status. pCR in a patient with a rapidly proliferating breast tumor does not necessarily rule out the existence of a disseminated micrometastatic disease that may become resistant to the chosen treatment. This may explain why young breast cancer patients and highly proliferative tumors (high SPF) show inferior long-term outcome despite high response rates to neoadjuvant chemotherapy (170). Similarly, the absence of HR predicts better response rates, but not necessarily better survival (115). Despite these limitations, the importance of pCR justifies the continued search for new regimens yielding a high pCR rate.

The concept of in vivo chemosensitivity-guided chemotherapy has been tested in two randomized studies in the neoadjuvant setting. In the Aberdeen study, 159 patients were initially treated with a doxorubicin-containing regimen for four cycles (169). Patients showing a clinical response (68%) were randomized to either four more cycles of the same regimen or four cycles of docetaxel. The change to docetaxel increased the clinical response rate after eight cycles from 68% to 94% and the pCR rate from 16% to 34%. In patients who failed to respond to the initial treatment, four cycles of docetaxel resulted in a pCR rate of only 2% (169). The German GEPARTRIO study provides a clinical model for testing new treatment approaches in primary chemoresistant breast cancer (127). In the GEPARTRIO trial, the value of the non-cross-resistant regimen NX (vinorelbine, capecitabine) was evaluated in patients not responding to two cycles of neoadjuvant TAC (docetaxel, doxorubicin, cyclophosphamide). Preliminary data, after 286 patients had been enrolled, failed to demonstrate any benefit from NX. The pCR rate was 7.3% in 40 patients treated with four additional cycles of TAC, and 3.1% in 32 patients treated with four cycles of NX (127). Updated results including 2 106 patients, presented at the 2005 San Antonio Breast Cancer Symposium, demonstrated almost identical pCR rates; 5.3% in 324 patients randomized to TAC, and 5.9% in 303 patients randomized to NX (171). Both these studies suggest that patients with nonresponding tumors derive minimal benefit from converting to alternative chemotherapy (169, 171).

If lack of response to initial anthracycline-based treatment indicates generalized chemotherapy resistance, then the long-term outcome will not improve with additional cytotoxic therapy. In the NSABP B27 study, patients who had a clinical partial response to anthracycline-based treatment, indicating chemosensitivity, showed improved DFS when neoadjuvant docetaxel was added (129). The lack of demonstrable benefit from postoperative do-
cetaxel, in contrast to preoperative administration, suggests that it may be important to proceed to the alternative chemotherapy without delay for surgery (129).

The problem of tumor heterogeneity

Tumor heterogeneity is cumbersome in several aspects with regard to prediction of treatment efficacy. The Goldie-Coldman model was the first major attempt to place the theory of the evolution of drug resistance by clonal selection on a mathematical basis (172). One of the assumptions of this model is that at each cell division of a nonmutant tumor cell, there exists a fixed probability that any new daughter cell will be a resistant mutant. By the time a tumor is detected, that is, by the time it reaches $10^9$ cells, the Goldie-Coldman model estimates that drug-resistant mutants are already present. According to the Skipper hypothesis, metastatic clones of tumor cells respond to treatment differently from the primary tumor (173). If the Skipper hypothesis is true, it will blunt the predictive relationship between response to neoadjuvant chemotherapy and long-term outcome, and bring into question the value of evaluating primary tumor material when treating metastatic disease. The NSABP studies B18 and B27 have come to different conclusions regarding the Skipper hypothesis. The significant association between clinical and pathological tumor response to preoperative chemotherapy and long-term outcome in the B18 study does not support the Skipper proposal (168), whereas the lack of survival benefit despite increased response rate in the B27 study is in agreement with the proposal (129).

An intriguing finding with regard to tumor heterogeneity and response to topo II inhibitors has been demonstrated in a study by Järvinen et al. (69), which showed intratumoral heterogeneity in primary breast cancer taking place at the TOP2A locus, with several HER2-amplified tumors containing both TOP2A-amplified and TOP2A-deleted cancer cells adjacent to each other in the same tumor. The presence of two different populations of cells with opposite gene copy number aberrations of TOP2A may have important therapeutic implications. It is possible that cells in the primary tumor with TOP2A deletion are highly resistant to topo II inhibitors and can survive and proliferate despite treatment with these drugs, as illustrated in Figure 5. This may explain the gradual loss of efficacy of topo II inhibitors observed when treating a patient with advanced breast cancer. Depending on the proportion of sensitive and resistant cells, the initial partial or complete clinical response will ultimately lead to clinical tumor progression. This is probably a general phenomenon which also concerns other types of chemotherapeutic agents and targeted therapies with different mechanisms of action. This preclinical model is supported by findings from a clinical study in 33 patients with LABC treated with anthracycline monotherapy, which showed that topo IIα levels declined in responsive tumors (84). However, this hypothesis is
not supported by findings from the study by Knoop et al. showing increased sensitivity to anthracycline also in patients with TOP2A-deleted tumors (80), a finding with no obvious explanation.

![Figure 5: Schematic presentation of the effects of the TOP2A amplification and deletion on the sensitivity to anthracyclines (topoisomerase II inhibitors). TOP2A-amplified tumor cells are sensitive, while TOP2A-deleted tumor cells are resistant to anthracycline chemotherapy. TOP2A-amplified and TOP2A-deleted tumor cells can be found adjacent to each other in the same tumor. Figure from Jarvinen et al., Breast Cancer Research and Treatment, 2003 (174).](image)

The importance of tumor heterogeneity is also demonstrated by the decrease in proliferation found following neoadjuvant chemotherapy, which supports the hypothesis that clones of slowly proliferating cells are selectively preserved during therapy (112, 113, 118).

Tumor heterogeneity also has consequences for the evaluation of predictive markers for neoadjuvant chemotherapy. Breast cancers are known to be heterogeneous with regard to proliferation (175, 176). A relative increase in proliferation has been demonstrated in the tumor periphery (176). The use of true-cut biopsies to analyze predictive markers provides a relatively small tumor specimen, raising the possibility of sampling error as a result of tumor heterogeneity. A considerable difference in tumor proliferation rate has been observed in studies measuring Ki67 on true-cut biopsies from different parts
of primary breast tumors (177, 178). Changes produced by therapy must therefore be profound if they are to overcome this inherent heterogeneity in proliferation in individual breast cancers.

It is still unclear whether metastases are derived from distinct subpopulations of tumor cells within the primary site with higher metastatic potential, or whether they originate from a random fraction of tumor cells. GEP has shown that primary breast tumors are strikingly similar to both distant metastasis (179) and lymph nodes (180) in the same patient. A good concordance between primary tumor and metastasis, both lymph node and distant metastasis, has also been observed regarding HER2 status (reviewed in (55)). The data regarding TOP2A is more limited, but good concordance between the primary tumor and the corresponding metastases has been demonstrated (104, 181). In the study by Tanner et al. (181), TOP2A amplification and deletion was identical in ten of 13 paired tumors studied; however, in the remaining three cases the predominant cell population in metastatic tissue was present only as a subpopulation in the primary tumor.

The analysis of predictive factors in relation to tumor heterogeneity is further obscured by the cancer stem cell hypothesis (182). There is a growing body of evidence that supports the idea that malignant tumors are initiated and maintained by a population of tumor cells that share similar biological properties to normal adult stem cells. This model, the cancer stem cell hypothesis, is based on the observation that tumors, like adult tissue, arise from cells that exhibit the ability to self-renew as well as to give rise to differentiated tissue cells. In the stem cell hypothesis for cancer, the key event in tumorigenesis is the disruption of genes involved in the regulation of stem cell renewal (183). Cancer stem cells have been identified for hematological malignancies (184), brain tumors (185), and breast cancer (186).

In a study using a model in which human breast cancer cells were grown in immunocompromised mice, Al-Hajj et al. found that the tumorigenic cells gave rise to both additional tumorigenic cells and phenotypically diverse non-tumorigenic cells that recapitulated the complexity of the primary tumors from which the tumorigenic cells had been derived (186). This finding is in line with studies which have used GEP to demonstrate genetic similarity between a primary breast tumor and its distant metastasis (179).

Cancer stem cells generally represent only a minority of the neoplastic cells, but it has been hypothesized that they contribute to most aspects of tumor malignancy; if true, this hypothesis suggests that these cells may also be important therapeutic targets. Current therapeutic strategies fail to account for potential differences in drug sensitivity between the cancer stem cells and the more frequent differentiated cells. If the cancer stem cells are inherently resistant to the therapeutic agents, and if these cells comprise only a minority of the tumor cell population, then shrinkage of tumors may reflect the effects of these agents on the differentiated cells in a tumor rather than the cancer stem cell compartment (183).
In summary, current knowledge suggests that metastatic capability is an inherent feature and is not based on clonal selection, and that predictive factors related to response in primary tumors are also relevant when treating metastatic disease.
Aims of the study

The general aim of this thesis was to investigate a number of methods with the potential to predict response to chemotherapy in breast cancer.

The specific aims were as follows:

**Paper I:**
To establish an assay for analysis of topoisomerase IIα in different cell cycle phases with two-parameter flow cytometry in fresh tumor specimens from 50 patients with primary breast cancer.

**Paper II:**
To evaluate topoisomerase IIα and HER2 gene amplification analyzed with chromogenic *in situ* hybridization (CISH) as predictive markers for response to anthracycline-based chemotherapy in advanced breast cancer.

**Paper III:**
To investigate the ability of *in vitro* drug sensitivity analyzed with the fluorometric microculture cytotoxicity assay (FMCA) to predict clinical response and long-term survival in advanced breast cancer.

**Paper IV:**
To evaluate cyclin A as a predictive marker for tumor aggressiveness and chemotherapy response in patients with advanced breast cancer.

**Paper V:**
To evaluate the efficacy and safety of the combination of epirubicin, capecitabine, and cisplatin (EXC), and the predictive value of TP, TS, and DPD analyzed with real-time PCR in the treatment of locally advanced breast cancer.
Materials and methods

Paper I:

Patients
Fresh tumor specimens were taken from 50 tumors from 49 women undergoing surgery for primary breast cancer at Örebro University Hospital. The inclusion criterion for patients was having a tumor large enough to provide adequate material for routine histology, DNA analysis, estrogen receptor and progesterone receptor determinations, and flow cytometry (FCM) for topo IIα. Patient age at operation ranged from 33 to 94 years, with a median age of 61 years. All tumors were invasive breast carcinomas.

Determination of S-phase fraction and ploidy
A regular DNA staining was performed, and the stained nuclei were analyzed in a FACSscan equipped with a 488 nm argon laser (Becton Dickinson, Immunocytometry Systems, CA, USA) with CellQuest (Becton Dickinson) software. The DNA analysis was based on 15 000 events. Tumors were defined as either diploid or non-diploid.

Flow cytometry topo IIα analysis
Simultaneous measurement of topo IIα and DNA content was performed using two-parameter FCM. 1 × 10^6 cells were added to each of two tubes. Topo IIα clone SWT3D1 IgG1k (DAKO, Glostrup, Denmark) (1:100) was added to one of the tubes. After incubation, a FITC-conjugated rabbit anti-mouse (RAM) antibody (DAKO) (1:50) was added to both tubes and incubated. The stained nuclei were analyzed in a FACSscan. The two-parameter analysis was based on 5 000 events. In both the analysis of the total topo IIα expression and that of the expression in the separate cell cycle phases, the cut-off limits were set individually on the negative control samples according to the appearance of the scatterplot. The separation of the different cell cycle phases was performed using the DNA histogram.
Statistical methods

Statistical software (StatSoft, OK, USA) was used for all calculations. The difference in proportion of topo IIα-positive cells between diploid and non-diploid tumors was tested using the Mann-Whitney test. The correlation between the proportion of topo IIα-positive cells and SPF was determined by Spearman’s rank correlation. The difference in expression of topo IIα between cells in G0/G1 phase and S/G2/M phase in diploid tumors was assessed with the Wilcoxon matched pairs test. The three-group analysis in non-diploid tumors was performed with the Friedman ANOVA test and post-hoc testing with the Wilcoxon signed rank sum test with Bonferroni correction. A level of P less than 0.05 was accepted as statistically significant.

Paper II:

Patients

The patient population consisted of 85 patients treated with anthracycline-based chemotherapy as first line treatment for advanced breast cancer, with paraffin-embedded tumor blocks available, from seven different centers. This was a subgroup of the 283 patients included in a randomized multicenter trial comparing docetaxel (T) to sequential methotrexate and 5-fluorouracil (MF) in advanced breast cancer (4).

Preparation of tissue arrays

Multitissue array blocks were produced as described by Kononen et al. (187). In short, representative tumor regions were selected from hematoxylin and eosin-stained sections for implantation in the multitumor array blocks. One tissue cylinder with a diameter of 0.6 mm was punched through the selected tumor area from each donor tissue block. One tissue core from each donor block was then inserted into four recipient tissue array paraffin blocks.

CISH

Chromogenic *in situ* hybridization (CISH) was performed according to Rummukainen et al. (188). Briefly, before hybridization, the tissue sections were deparaffinized, washed and dried. They were then incubated in buffer at a high temperature in an autoclave, and after cooling and rinsing the sections were treated with pepsin, washed, dehydrated, and dried. A digoxigenin-labeled HER2 probe (Zymed) was added, and the slides were sealed and denaturated and hybridization was allowed to take place. The slides
were then opened and washed, and the HER2 probe was detected using anti-digoxigenin, goat anti-mouse HRP, and DAB. The slides were incubated with DAB enhancer, counterstained with hematoxylin, and then analyzed in a light microscope. CISH with topo IIa probe (Zymed) was performed using the same method.

At least 50 non-overlapping nuclei in every tumor sample were scored to determine the number of HER2 and TOP2A signals. The results were expressed as the actual copy numbers per cell in each sample. Amplification was defined to be present when six or more gene copies were detected in at least 20% of the screened malignant cells, or when a gene copy cluster was found to be present.

Statistical methods
The association between response rate and gene amplification for HER2 and topo IIa was tested by the chi-square test with clinical response divided into two categories: response, defined as complete response (CR) or partial response (PR), and non-response, defined as no change (NC) or progressive disease (PD).

Time to progression (TTP) was measured from the date of the first course of chemotherapy until disease progression, and overall survival (OS) from start of chemotherapy until death. The univariate analysis of TTP and OS was performed using the Cox proportional hazards model.

Paper III:
Patients
Between 1992 and 2000, fresh tumor specimens from 200 patients with breast cancer were successfully tested with the fluorometric microculture cytotoxicity assay (FMCA). Of these patients, 37 individuals treated with anthracycline-containing chemotherapy as first line treatment for advanced breast cancer were included in our study. The eligibility criteria were having specimens successfully analyzed by the FMCA in close connection to the administration of chemotherapy, and having tumors evaluable for response according to WHO recommendations.

FMCA
FMCA is based on the concept of total cell kill; it uses fluorescein diacetate (FDA) for assessment of cell survival after 72 h of continuous drug exposure in vitro. Viable tumor cells were in most cases collected by surgery or ultrasound-guided biopsy as part of the routine diagnostic or treatment proce-
Tumor cell preparation and the FMCA procedure were performed as previously described (189). Briefly, on day one, 180 μl/well of the tumor cell preparation (10 000 to 30 000 cells/well) were seeded in triplicates into V-shaped 96-well microtiter plates (Nunc, Roskilde, Denmark), prepared in advance with cytotoxic drugs as previously described (190). After 72 h incubation, the culture medium was washed away, and FDA was added to control, experimental, and blank wells. After incubation, the fluorescence from each well was read in a Fluoroscan 2 (Labsystems OY, Helsinki, Finland). Only successfully analyzed samples were reported in this study.

The results obtained by the indicator FDA are presented as survival index (SI), defined as the fluorescence of the test as a percentage of control cultures, with blank values subtracted. Based on the median and standard deviation (SD) SI values, each tumor sample was characterized for each drug tested in terms of low drug resistance (LDR; SI < median), intermediate drug resistance (IDR; SI ≥ median but < median + 1 SD), or extreme drug resistance (EDR; SI ≥ median + 1 SD), as described previously (190). This categorization formed the basis for the analysis of correlation between the in vitro and in vivo data.

Clinical evaluation and in vitro/in vivo correlation

Patients were treated according to clinical protocols with no guidance from the assay results. Clinical response evaluation was performed according to WHO recommendations. The in vitro/in vivo correlation was based on the most active drug in vitro, in terms of LDR, IDR, and EDR, that was actually given to the patient. Clinical response was divided into clinical responders and non-responders. Correlation between in vitro drug activity and clinical response was calculated using the chi-square test for trend. Assay sensitivity was calculated as the percentage of clinical responders having at least one drug scoring LDR in the FMCA, and specificity as the percentage of clinical non-responders scoring IDR or EDR in the FMCA to the most active drug included in the treatment. The predictive values for clinical response for samples having a best score of LDR, IDR, and EDR, respectively, were calculated as the percentage of samples in each category deriving from patients with a clinical response.

Paper IV:

Patients

The patient population consisted of 96 patients with advanced breast cancer included in the same randomized study as patients in paper II (4).
Immunohistochemical assays
Immunohistochemical (IHC) determination of cyclin A was performed using a mouse monoclonal antibody from Novocastra (Newcastle, UK) and determination of Ki67 using the mouse monoclonal Mib-1 antibody (Immunotech, Marseille, France). The tumor area with highest density of positive nuclear staining was chosen for quantification of the immunostaining.

Statistical methods
The association of TNM stage and tumor characteristics with cyclin A was tested with the Spearman correlation coefficient for ordinal variables and Student’s t-test for dichotomous variables. The association between different proliferation markers was also tested with the Spearman correlation coefficient. The association between treatment response and proliferation markers was measured by computing the Pearson correlation coefficient. Overall survival and time to first relapse curves were prepared by the Kaplan-Meier method, and prognostic variables were tested using Cox regression analysis with proliferation markers as continuous variables. Groups with high and low proliferation rate were divided using cut-off points corresponding to Ki67 of 25%. Thus, the cut-off point for cyclin A was 10.5%, which placed about 65% of the patients in the high-risk group.

Paper V
Patients
This phase II study included 48 women with locally advanced breast cancer (LABC). Eight patients (17%) had inflammatory breast carcinoma (IBC) and 40 (83%) had non-inflammatory LABC. Median patient age was 48 years (range 33–69).

Treatment and response evaluation
Patients received four three-week cycles of EXC (epirubicin 60 mg/m² i.v., day 1; cisplatin 60 mg/m² i.v., day 1; oral capecitabine 1 000 mg/m² twice daily, days 1–14). Patients then underwent modified radical mastectomy followed by 2–4 cycles of post-operative EXC and radiotherapy according to local guidelines. Patients with HR-positive tumors received tamoxifen 20 mg/day for five years. Clinical response was evaluated after cycles 2 and 4 according to WHO criteria. pCR was defined as the absence of both invasive and in situ cancer in the breast and axillary lymph nodes.
Analysis of predictive markers

Total RNA was isolated from snap-frozen needle biopsies. LightCycler® mRNA Quantification Kits from Roche Diagnostics GmbH (Mannheim, Germany) were used to measure TP, TS, and DPD. The results were expressed as ratios of target:reference gene copies in the sample.

HER2 status was also determined using the LightCycler®. HER2 and TOP2A were in addition analyzed by fluorescence in situ hybridization (FISH) using the PathVysion HER2 DNA Probe Kit (Vysis Inc., Downers Grove, IL, USA). The fluorescent signal for HER2 and TOP2A was related to the signal for chromosome 17, and a ratio > 2.0 was considered as gene amplification.

Statistics

Sample size calculation was based on a Simon two-stage design (191). The primary endpoint was clinical response rate, and the secondary endpoints were safety, pCR, time to relapse, survival, and evaluation of predictive markers.
Results

Paper I

Expression of topo IIα in the total cell population
The median proportion of topo IIα-positive cells in the total cell population was 25%, and the interquartile range (IQR) was 23%. There was no statistically significant difference between the proportions of topo IIα-positive cells in the 21 diploid and 29 non-diploid tumors (p = 0.8), and no statistically significant correlation between SPF and the proportion of topo IIα-positive cells (p = 0.1).

Topo IIα in relation to cell cycle phase in diploid tumors
The expression of topo IIα in G0/G1 cells and S/G2/M cells from the 21 diploid tumors is shown in Figure 6. The median proportion of positive cells among those in G0/G1 phase was 26% (IQR 31%) compared to 41% (IQR 29%) among those in S/G2/M phase (p = 0.002). Of all topo IIα-positive cells, a mean of 65% (SD 18%) were in G0/G1 phase (Figure 8). In 18 cases, more than 50% of the positive cells were in G0/G1 phase.
Figure 6. Percentage of topo IIα-positive cells in G0/G1 phase and S/G2/M phase in 21 diploid breast tumors. The upper and lower quartiles and the median values are depicted as box plots. Whiskers indicate maximum and minimum values.

Topo IIα in relation to cell cycle phase in non-diploid tumors

Figure 7 presents topo IIα expression in G0/G1 diploid cells, G0/G1 non-diploid cells, and S/G2/M non-diploid cells from the 29 non-diploid tumors. The median expression of positive cells was 9% (IQR 9%) in G0/G1 diploid cells, 34% (IQR 30%) in G0/G1 non-diploid cells, and 60% (IQR 22%) in S/G2/M non-diploid cells. These differences were statistically significant (p < 0.001).

In these non-diploid tumors, the expression in diploid cells in G0/G1 phase was significantly lower than the expression in cells in G0/G1 phase in the diploid tumors (p = 0.003). Of all topo IIα-positive cells, a mean of 56% (SD 15%) were non-diploid cells in G0/G1 phase, 32% (SD 11%) were non-diploid cells in S/G2/M phase, and 12% (SD 13%) were diploid cells in G0/G1 phase (Figure 8). In 25 cases, more than 50% of the positive cells were in G0/G1 phase.
Figure 7. Percentage of topo IIα-positive cells in diploid cells in G0/G1 phase, nondiploid cells in G0/G1 phase, and non-diploid cells in S/G2/M phase in 29 nondiploid breast tumors. The upper and lower quartiles and the median values are depicted as box plots. Whiskers indicate maximum and minimum values.

Figure 8. Mean distribution of the topo IIα-positive cells in different cell cycle phases in 21 diploid and 29 non-diploid breast tumors.
Paper II

Among the 85 patients treated with anthracycline as first line therapy for advanced breast cancer, there were 8 CRs (9%), 31 PRs (36%), 18 patients with NC (21%), and 28 patients with PD (33%). TOP2A gene amplification was present in 14 (16%) and HER2 gene amplification in 38 (45%) of the primary tumors. Two of the 14 cases with TOP2A amplification were amplified without concurrent HER2 amplification. Neither TOP2A nor HER2 gene amplification was significantly associated with response to chemotherapy (p = 0.35 and p = 0.49, respectively). Only one of the eight patients showing CR had TOP2A gene amplification. The response rate was significantly higher in patients treated every three weeks than in those treated weekly (51% vs. 15%, p = 0.02). In the multivariate analysis, weekly treatment remained significant, with a risk ratio (RR) of 7.29 (95% CI: 1.39–38.17), while TOP2A amplification was associated with a non-significant increase in response rate, RR 2.52 (95% CI: 0.68–9.31).

TTP and OS were similar irrespective of both TOP2A status (p = 0.73 and p = 0.09, respectively) and HER2 status (p = 0.25 and p = 0.11, respectively).

TTP was significantly worse for patients treated weekly than for those treated every three weeks (p = 0.01), and also for patients treated with anthracycline monotherapy compared to those treated with an anthracycline-containing combination regimen (p = 0.03).

Among patients with HER2 gene amplification, response rate, TTP, and OS did not differ between those with simultaneous amplification of TOP2A and those with normal TOP2A (p = 0.49, p = 0.13, and p = 0.66, respectively). Among the 47 patients with normal HER2 status, there were two patients with TOP2A amplification and a median TTP of 6 months, while the remaining 45 patients, with normal TOP2A status, had a median TTP of 22 months (p = 0.001).

Paper III

The overall response rate (CR + PR) among the 37 patients was 59% (22 PR, 11 NC, 4 PD). In the 17 patients with LABC the response rate was 71% (12 PR, 5 NC), whereas in the 20 patients with metastatic disease it was 50% (10 PR, 6 NC, 4 PD). This difference in response rate between LABC and MBC was not statistically significant (p = 0.32). For drugs considered important in breast cancer treatment and tested in the FMCA, that is, doxorubicin, 4-hydroperoxycyclophosphamide, 5-fluorouracil, and docetaxel, the SI values tended to be lower, indicating lower drug resistance, in samples from LABC than in MBC, but none of these individual differences were statistically significant (Figure 9).
Figure 9. Activity of Dox (doxorubicin), 4-HC (4-hydroperoxy-cyclophosphamide), 5-FU (5-fluorouracil), and Taxe (docetaxel) in 20 patients with MBC (metastatic breast cancer) and 17 patients with LABC (locally advanced breast cancer). The results are presented as mean SI values at EDCC (empirically derived cut-off concentrations).

In vitro data could be correlated to tumor response in all but five cases, which experienced clinical response but lacked FMCA data on one or two drugs to allow for correlation (192). Fifteen of the 17 patients with tumor response scored LDR in the FMCA, while 8 of the 15 non-responding patients scored IDR or EDR (Table 4). This distribution was statistically significant (p = 0.0092). The assay sensitivity was 89% (15/17), and the specificity 53% (8/15). The probabilities of tumor response for samples scoring LDR, IDR, and EDR were 68% (15/22), 22% (2/9), and 0% (0/1), respectively.

Table 4. In vitro drug activity determined by FMCA, and clinical response, in 32 patients.

<table>
<thead>
<tr>
<th>FMCA</th>
<th>Responders</th>
<th>Non-responders</th>
<th>Predictive value for response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDR</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>IDR</td>
<td>2</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>LDR</td>
<td>15</td>
<td>7</td>
<td>68</td>
</tr>
</tbody>
</table>

EDR (extreme drug resistance), SI (survival index) above the median + 1 SD
IDR (intermediate drug resistance), SI between the median and the median + 1 SD
LDR (low drug resistance), SI value below the median
Responders include CR (complete response) and PR (partial response)
There was a significant association between in vitro sensitivity and response to chemotherapy (p=0.0092).
As all patients were treated with epirubicin, and FMCA results for an anthracycline were present in all cases, we made a separate analysis of *in vitro* activity for anthracycline and clinical response. There was no significant association between *in vitro* anthracycline sensitivity and clinical response ($p = 0.65$).

Ten of the 18 FMCA tests performed before treatment (prospective), scored LDR for an anthracycline, compared to 4 of the 19 tests performed after treatment (retrospective) ($p=0.045$).

Median TTP was 11.6 months among the 37 patients as a whole; it was 15.6 months in the patients with LABC and 10.4 months in the patients with MBC ($p = 0.02$), with an indication of long-term disease control in LABC only. In patients with a drug sensitivity index below the median, the TTP was 11.2 months, compared to 11.0 months in those with an index at or above the median ($p = 0.17$). When making this comparison within each subgroup of patients, a low sensitivity index was correlated with prolonged TTP in patients with LABC ($p = 0.035$), but not in patients with MBC ($p = 0.33$) (*Figure 10*).
Figure 10. A low drug sensitivity index was correlated with prolonged TTP in patients with LABC (p=0.035) (A), but not in patients with MBC (p=0.33) (B).
Paper IV

The median cyclin A positivity of tumor cells was 14.5% (range 1.2–45.0). The frequency distributions of all tested proliferation markers (mitotic count, tumor grade, Ki-67, and cyclin A) are presented in Table 5.

Table 5. Characteristics of the primary tumors at the time of diagnosis and the time to first relapse (TFR) of the 96 investigated patients.

<table>
<thead>
<tr>
<th>Factor</th>
<th>No. of patients (%)</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>94 (98)</td>
<td></td>
</tr>
<tr>
<td>Lobular</td>
<td>2 (2)</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>48 (50)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>39 (41)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>9 (9)</td>
<td></td>
</tr>
<tr>
<td>Mitotic count</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12 (13)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>29 (30)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>55 (57)</td>
<td></td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>32 (33)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>63 (66)</td>
<td></td>
</tr>
<tr>
<td>Ki67</td>
<td></td>
<td>38 (10–90)</td>
</tr>
<tr>
<td>&lt;25%</td>
<td>29 (30)</td>
<td></td>
</tr>
<tr>
<td>≥25%</td>
<td>67 (70)</td>
<td></td>
</tr>
<tr>
<td>Cyclin A</td>
<td></td>
<td>14.5 (1.2–45)</td>
</tr>
<tr>
<td>&lt;10.5%</td>
<td>33 (34)</td>
<td></td>
</tr>
<tr>
<td>≥10.5%</td>
<td>63 (66)</td>
<td></td>
</tr>
<tr>
<td>TFR (years)</td>
<td></td>
<td>1.57 (0–22.8)</td>
</tr>
</tbody>
</table>

All proliferation markers were correlated with each other, with high statistical significance (P<0.0001). The strongest correlation was between Ki-67 and cyclin A (Spearman correlation coefficient: 0.74). Spearman correlation coefficients between tumor proliferation markers are presented in Table 6.

Table 6. Spearman correlation coefficient and significance between the investigated tumor biological factors.

<table>
<thead>
<tr>
<th></th>
<th>Grade</th>
<th>Ki67</th>
<th>Cyclin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotic count</td>
<td>0.82</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>Grade</td>
<td>0.36</td>
<td></td>
<td>0.40</td>
</tr>
<tr>
<td>Ki67</td>
<td></td>
<td>0.74</td>
<td></td>
</tr>
</tbody>
</table>

P = 0.001 for all comparisons
Of the 96 patients, 58 were evaluable for response after first line anthracycline treatment and 96 after second line docetaxel or MF treatment (50 in the docetaxel group and 46 in the MF group). The overall response rate (CR+PR) was 47% after first line anthracycline therapy. The response rates were 46% after docetaxel treatment and 26% after MF treatment. In the parent study (n=283), the corresponding response rates for docetaxel and MF treatment were 42% and 21%, respectively (4). There was no significant correlation between chemotherapy response and any proliferation marker after anthracycline, docetaxel, or MF treatment.

There was a significant association between a high cyclin A score and time to first relapse (RR: 1.031, 95% CI: 1.01–1.05, P=0.001 as continuous variable; RR: 1.94, 95% CI: 1.24–3.03, P=0.004 as discrete variable), while the other markers showed only non-significant trends in the same direction. There was also a highly significant association between a high cyclin A score and overall survival from diagnosis (RR: 1.05, 95% CI: 1.02–1.07, P<0.001 as continuous variable; RR: 2.49, 95% CI: 1.45–4.29, P=0.004 as discrete variable), but not for survival from start of first or second line chemotherapy. Since the prognostic value of cyclin A seemed to depend on chemotherapy, we separately analyzed the association between cyclin A and whether or not the patient had received adjuvant chemotherapy. While the prognostic impact of cyclin A on survival from diagnosis was significant for both groups, the risk ratio was somewhat higher in patients who did not receive adjuvant chemotherapy (RR: 1.07, 95% CI: 1.03–1.10) compared to patients who were given adjuvant chemotherapy (RR: 1.03, 95% CI: 1.00–1.06).

Paper V

Treatment administered

In total, 263 cycles of EXC were administered: 175 neoadjuvant cycles (median number per patient: 4, range: 1–6) and 88 adjuvant cycles (median: 2, range: 2–4). Clinical response data were available for 46 patients (96%) who received at least two cycles of treatment at > 50% of the recommended doses. Only 38 of the 48 patients (79%) received the planned four cycles of neoadjuvant EXC. Treatment administration is illustrated in Figure 11.
Figure 11. Design and treatment administration in the EXC study.

The mean planned and delivered doses for each drug in each neoadjuvant cycle are shown in Figure 12. The mean dose of cisplatin was maintained at 99% (SD 5%) for all four cycles, whereas mean epirubicin and capecitabine doses gradually decreased to 90% (SD 15%) and 91% (SD 22%), respectively, by the fourth cycle.
Anti-tumor activity and survival

The RR among the 46 patients completing at least two cycles of therapy was 74% (95% CI: 59–86%), including complete responses in 13% (95% CI: 5–26%). Forty-one EXC-treated patients underwent breast surgery. Five were withdrawn due to toxicity. Nine patients achieved a pCR, giving a pCR rate in the intent-to-treat population of 19% (95% CI: 9–33%), and 22% (95% CI: 11–38%) in the EXC-treated patients who underwent surgery. In two patients without pCR, residual cancer cells were found only in lymph nodes.

With a median follow-up of 35 months (range 0–44), nine patients experienced disease recurrence and seven died. Although the study was not designed to formally compare efficacy according to disease type, OS and DFS were significantly shorter in patients with IBC versus non-inflammatory disease (P ≤ 0.00002). Disease relapsed in only one of nine patients achieving pCR (P = 0.39).

Safety

Apart from alopecia, the most common treatment-related adverse events were nausea, fatigue, vomiting, hand-foot syndrome, and stomatitis. Despite dose reductions (of capecitabine and epirubicin, but rarely cisplatin) and treatment interruptions, grade 3/4 adverse events were frequent throughout the treatment period. There were two treatment-related deaths. A 68-year-old
died from septic shock caused by intestinal necrosis and grade 4 neutropenia after the first cycle. The second death was a 53-year-old woman who had a venous sinus thrombosis after her second post-operative cycle: a fall during anti-coagulant therapy caused head injury leading to lethal intracerebral hemorrhage. Four additional patients experienced possible hypercoagulative disorders: a second patient was diagnosed with a venous sinus thrombosis after her fourth post-operative cycle, which was not lethal but resulted in neurological sequelae; two patients had deep vein thrombosis; and one had splenic infarction after cycle 2.

Predictive markers
Tumor specimens were available from 43 patients. The technical success rate for the PCR analysis of TS, TP, and DPD was 84% (36/43). TS, TP, and DPD concentrations and TP:DPD ratios were not significantly associated with response.

Due to a low success rate (28/43, 65%) with the initial HER2 and TOP2A FISH assessment due to suboptimal tumor sampling and shortage of evaluable tumor tissue, these data are not presented. We re-evaluated all 43 samples by quantitative real-time PCR for HER2 as described above. This produced a 91% (39/43) sample success rate and HER2 amplification rate of 25%, which is concordant with published data (193). HER2 amplification analyzed by PCR was not predictive of treatment benefit.
General discussion

Papers I–II

There is still no established factor for clinical use in the prediction of chemotherapy efficacy in breast cancer. The selection of chemotherapeutic drugs is still focused on identifying the most effective regimen for all patients in whom chemotherapy is indicated. This approach is empirically intended to benefit most patients, but it does not take into account the possibility that some regimens may work better for some individuals than for others. The diverse response to chemotherapy in breast cancer patients with apparently identical tumors proves the existence of as yet unrecognized tumor characteristics determining sensitivity to chemotherapy.

Until recently, research on predicting response to chemotherapy has mostly, with the exception of *in vitro* assays, been focused on single markers, despite the fact that most patients are treated with combination chemotherapy.

Negative HR is rather consistently associated with greater chemotherapy sensitivity and an increased rate of pCR after neoadjuvant chemotherapy (115, 127-130). However, the strength of this association is not sufficient to differentiate patients at different degrees of risk, and does not allow for an individualized therapeutic choice. Results regarding the predictive value of different methods for measuring tumor proliferation rate are ambiguous, and these methods are not yet ready for clinical use (reviewed in (194)).

HER2 and topo IIα are two factors that have shown predictive potential with regard to anthracycline-based chemotherapy. While HER2 is well-established as a prognostic factor (14), and as a predictive factor for trastuzumab (45), its role as a predictive factor for response to chemotherapy is still uncertain. The results of studies evaluating HER2 status and the efficacy of adjuvant anthracycline-based chemotherapy are summarized in Table 3. These conflicting results may reflect the fact that HER2 merely serves as a surrogate marker for topo IIα, the most important molecular target for anthracyclines, as these two genes are located adjacent to each other on chromosome 17q, and gene copy number aberrations of the two genes are often noted in the same tumor (69, 102). Two recently published studies showed a predictive value for topo IIα with regard to RFS in patients treated with anthracycline-based adjuvant chemotherapy (78, 80). The experience in advanced breast cancer is still limited, and the results contradictory (76, 77).
In Paper I we analyzed the expression of topo IIα in different cell cycle phases, and found a significantly higher expression of topo IIα in S/G2/M cells than in G0/G1 cells, both in diploid and in non-diploid tumors. This finding is not surprising, since topo IIα is a marker of proliferation (65, 90, 195). We also demonstrated that in clinical breast cancer samples, topo IIα is also expressed in G0/G1 phase. Although this expression is lower than that in S/G2/M phase, it might be high enough to have implications for the efficacy of topo II inhibitors. In fact, in 18 of 21 diploid tumors and 25 of 29 non-diploid tumors, more than 50% of the topo IIα-positive cells were in G0/G1 phase. These results on fresh breast cancer tissue are in accordance with the findings from in vitro studies where malignant transformation was accompanied by qualitative changes in the expression of topo IIα (196, 197). However, the level of topo IIα in individual tumor cells in G0/G1 phase may be too low to be detected with IHC; this may be the reason why analysis of topo IIα with IHC has failed to predict response to epirubicin in metastatic breast cancer (72). This potential drawback of IHC may favor FCM as a method for analysis of topo IIα expression. The assumption that topo IIα may have an impact on the response to chemotherapy beyond its association with tumor cell proliferation is supported by findings from a study on patients treated with anthracycline-based neoadjuvant chemotherapy (73). In this study, topo IIα emerged as an independent predictor of tumor regression, while Ki67 lost its significance after inclusion of topo IIα in the logistic regression model (73).

Given this significant expression of topo IIα in G0/G1 phase, it can be postulated that topo II inhibitors exert their action by the same mechanism in non-proliferating as in proliferating tumor cells. These findings may explain why some human neoplasms can be cured with chemotherapy even though the majority of the tumor cells are in a non-proliferative cell cycle phase. It may be the case that tumor cells with no or low expression of topo IIα in G0/G1 phase, like normal cells, can evade the topo II inhibitors and repair the DNA damage, whereas tumor cells with persistent expression of topo IIα throughout the cell cycle cannot.

In Paper II, gene amplification of TOP2A and HER2 was analyzed with CISH. TOP2A amplification was present in 16% of the primary tumors. This frequency is higher than the 6% found in patients with stage II disease (79), but is comparable with the proportions of 17% (75) and 28% (76) found in patients with locally advanced disease. A higher frequency of TOP2A amplification in patients with more advanced disease is expected, as topo IIα expression is related to HER2 positivity and worse prognosis in primary breast cancer (90). We found no significant association between TOP2A amplification and response to chemotherapy, TTP, or OS. The observed trend for increased response rate in TOP2A-amplified tumors is in accordance with the accumulating evidence supporting the weak but rather consistent predictive value of TOP2A amplification noted in anthracycline-based adjuvant chemo-
therapy (Table 3). The lack of significant correlation may partly be due to the limited number of patients treated, and the fact that 13 patients (15%) were treated according to a weekly schedule with low response rate.

Tumor heterogeneity also complicates studies on predictive factors. The Skipper hypothesis (173) proposes that metastatic clones of tumor cells behave differently from the primary tumor. If the Skipper hypothesis is true, it implies that evaluation of primary tumor tissue is not valid when treating metastatic disease. In Paper II, HER2 and TOP2A were evaluated on primary tumor samples. Regarding HER2, there seems to be good concordance between HER2 status in the primary tumor and HER2 status in the metastasis (reviewed in (55)). The data regarding concordance of TOP2A in the primary tumor and corresponding metastasis is limited and based on few studies, but the level of concordance has been good (104, 181). In the study by Tanner et al. (181), TOP2A amplification and deletion were identical in 10 of 13 paired tumors studied; however, in the remaining three cases, the predominant cell population in metastatic tissue was present only as a subpopulation in the primary tumor. The same degree of discordance in the present study could have influenced the results, as only a small number of patients were evaluated.

The picture is also obscured by intratumoral heterogeneity at the TOP2A locus, with both TOP2A-amplified and TOP2A-deleted breast cancer cells adjacent to each other in the same tumor (102, 174). The presence of different cell populations with different chemosensitivity could partly explain the variable clinical responses seen in otherwise similar patients with advanced breast cancer. The significance of TOP2A deletion could not be analyzed in Paper II, as the CISH method used did not allow simultaneous analyses of TOP2A and the chromosome 17 centromere. This problem cannot be solved by using GEP instead of CISH, as the tumor cell population responsible for the heterogeneity will not change the overall GEP profile.

Previous reports have indicated that TOP2A gene aberrations, whether deletions or amplifications, are only seen in connection with HER2 amplification (75, 102). However, the present study identified two tumors with TOP2A amplification but no HER2 amplification, a finding also supported by other studies (80, 198, 199).

The lack of predictive value of HER2 seen in Paper II is not unexpected, as there is no molecular rationale for such a relationship. Pegram et al. (101) investigated the relationship between HER2 and sensitivity to doxorubicin in four breast cancer cell lines that were transfected with the HER2 gene and then exposed to doxorubicin. No alteration in chemosensitivity was observed in any of the cell lines.
Paper III

In breast cancer, as well as in most other cancer types, there are now several medical alternatives at each stage, notably cytotoxic drugs. A great number of new ‘targeted drugs’ are currently in clinical trials, and may well be used in the future to augment the arsenal of treatment options (200). Such an expansion of treatment alternatives would make the choice of the most appropriate treatment for the individual patient a critical task, and hence robust and simple predictive tests would certainly be useful in the selection of the optimal medical treatment for each individual patient. This idea forms the basis for the various in vitro drug sensitivity tests that have emerged. The more recent versions of these tests are based on the concept of measurement of cell death in the total population of tumor cells from patient tumors after short-term incubation with cytotoxic drugs. Taken as a whole, these tests seem to adequately reflect the clinical situation in terms of drug activity profiles and prediction of response in individual patients (147, 148).

One such test is the FMCA used in Paper III. FMCA has been found to adequately report individual cytotoxic drug sensitivity data in leukemia, non-Hodgkin’s lymphoma, and ovarian carcinoma (155, 156, 189), and to predict long-term outcome in childhood leukemia (201). The data obtained in Paper III extends our knowledge regarding FMCA to breast cancer. The results on prediction of response to chemotherapy are close to those reported for other tumor types, with an overall assay sensitivity of 89% and specificity of 53%, and tumor response probabilities of 68%, 22%, and 0% for patients with samples scoring LDR, IDR, and EDR, respectively, in the FMCA, which could be compared with the overall response rate of 59% among the patients included. These data show that in breast cancer, as in other tumor types, the test is better at prediction of drug resistance than prediction of response, which is not surprising considering the additional drug resistance mechanisms which are operative in vivo. As indicated above, it is inherent in these types of tests that the specificity will be much lower than the sensitivity, and from a clinical point of view false positives are more acceptable than false negatives. It might be possible to increase the test specificity while retaining sensitivity, for example, via improvements in cell preparation and culture, but it may also be necessary to incorporate information on other potential resistance factors, such as pharmacokinetics and cell growth, in order to more exactly predict chemotherapy sensitivity in individual patients.

As expected, the LABC group had longer TTP. Using the drug sensitivity index as an overall measurement of cytotoxic drug sensitivity, a favorable index significantly predicted longer TTP in LABC but not in MBC. The reason for this difference is unclear. The number of patients in each group was small, giving low statistical power to detect differences. Furthermore, it is only to be expected that in vitro assays like the FMCA will correlate better to tumor response than to long-term outcome. Earlier findings in metastatic
breast cancer are in accordance with our findings; that is, tumors which were sensitive \textit{in vitro} showed a higher response rate that did not translate into prolonged survival (202). On the other hand, in primary ovarian cancer, improved PFS and OS were observed for patients with \textit{in vitro}-sensitive tumors tested with the ATP (203) or MTT (204) assays. The FMCA and similar assays have also been found to provide prognostic information on long-term outcome in some tumor types (201, 205).

There was a significant association between prospective FMCA for anthracycline and \textit{in vitro} drug sensitivity \((p = 0.045)\). This finding could be explained by patient selection; patients sampled for drug sensitivity testing after a number of treatment cycles are likely to be resistant to the treatment and/or have acquired drug resistance. A tendency for samples from patients previously treated with chemotherapy to be less sensitive in FMCA has also been observed in ovarian carcinoma (189). Ideally, \textit{in vitro/in vivo} correlations should be studied in patients in which the treatment to be correlated is delivered immediately after the \textit{in vitro} assay.

Much of the current interest in this field is now focused on GEP and proteomics (206). GEP prediction of response to neoadjuvant chemotherapy in breast cancer has been reported with a sensitivity and specificity of 43% and 100\%, respectively (163). In comparison to this, the data from \textit{in vitro} drug sensitivity testing based on cell culture seem not to be inferior.

The FMCA and similar tests also have, according to the experience so far, the advantage of reporting adequate data on most cytotoxic drugs, whereas it seems reasonable to believe that a specific gene array fingerprint would have to be designed for each drug or drug combination. Furthermore, preliminary data show that the FMCA adequately reflects the antitumor activity of ‘targeted drugs’, such as antitumor antibodies and growth factor tyrosine kinase inhibitors, indicating that it may be relatively easy to adapt these assays to developmental drugs.

Management of cancer patients on an empirical basis using a trial and error approach for second and third line chemotherapy often leads to toxicity without benefit, a result both patients and clinicians would like to avoid if possible. \textit{In vitro} resistance assays can provide some guidance in this regard. However, the ASCO guidelines from 2004 do yet not recommend the use of chemotherapy sensitivity and resistance assays to select chemotherapeutic agents for individual patients outside the clinical trial setting (207).

Paper IV

The main finding in Paper IV was the significant association of a high cyclin A expression with a short time to first relapse (TFR) and poor overall survival (OS). The difference in survival from diagnosis of breast cancer was quite remarkable despite the fact that all patients developed metastatic dis-
ease, since the patient material was recruited from a study in metastatic disease. The cut-off point for the high and low cyclin A positive groups was 10.5%, which is in line with three other studies where the median value, varying between 8% and 11%, was used (125, 208, 209).

We found a strong correlation between cyclin A, mitotic count, tumor grade, and Ki-67, a result in concordance with previous studies in breast cancer (208, 209). Cyclin A was the only marker that showed a statistically significant correlation with both TFR and OS, and so it seems to be the most useful marker of proliferation. There are a number of well-known problems with the use of traditional markers for proliferation in breast cancer. The duration of mitotic phase can vary, and the mitotic count is not linearly correlated to proliferating cells, particularly in aneuploid tumors. Although histological grade is a well-established prognostic factor, the reproducibility of the method has been questioned (210). The flow cytometric determination of S-phase fraction (SPF) requires larger tumor volumes than IHC, and there is also pronounced intratumoral heterogeneity in SPF (175). Moreover, fresh frozen tissue is needed for S-phase analysis, and the costs for flow cytometry are higher than for IHC. Cyclin A is expressed during the late S, G2, and M phases, and is therefore a useful marker of proliferation (211). Cyclin A can be analyzed on paraffin embedded tissue with IHC, which is an advantage for both practical and economical reasons. An association between a high cyclin A expression and poor prognosis has previously been described (124) (208); however, a study including solely node-negative patients showed no prognostic value of cyclin A (125).

Regarding the predictive value of tumor proliferation rate, the findings are inconclusive (reviewed in (194)). A clear association between high SPF and response to neoadjuvant chemotherapy has been shown in LABC, while the value of SPF in metastatic breast cancer is less clear (106). We are aware of no clinical data on cyclin A as a predictive factor for chemotherapy in breast cancer. High cyclin A score has been shown to predict better response to chemotherapy and longer progression-free survival in both soft tissue sarcoma and advanced head and neck cancer (126, 212).

Anthracyclines are active throughout the cell cycle, but the effects are most pronounced for cells in S or G2 phase. The antimetabolites methotrexate and 5-fluorouracil mainly inhibit the cell proliferation in the S phase, while docetaxel, which is a microtubulin stabilizer, exerts its cytotoxic effect in the G2/M phase. Since cyclin A is active and detectable from the beginning of the S phase to the beginning of mitosis, it should theoretically label the proportion of cells that are sensitive to the chemotherapeutic drugs used in our study. Despite this, we found no correlation between cyclin A activity and response rate, TTP, and OS calculated from the start of chemotherapy.
Our findings add further doubts to the assumption that a high proliferation rate implies increased general sensitivity to chemotherapy, but show that cyclin A is a strong prognostic factor.

Paper V

Neoadjuvant chemotherapy is the standard of care in reasonably fit patients with LABC. Besides the primary goal of rendering inoperable tumors resectable, neoadjuvant chemotherapy enables in vivo assessment of chemosensitivity and provides an opportunity to study predictors of response. pCR is predictive of long-term survival in both LABC and operable breast cancer (129, 167), and therefore the continued search for new regimens yielding high pCR rate is important.

We demonstrated in Paper V that neoadjuvant EXC is highly effective in LABC, with a clinical RR of 74% and a pCR rate of 22% (19% in the intent-to-treat population). Despite a diagnosis of LABC in all patients, rigorous criteria for pCR, and administration of only four cycles of EXC, the pCR rate was similar to that seen with sequential anthracycline-/taxane-based primary chemotherapy (19–34% (129, 169)). It also compares favorably with the pCR rates of 3–14% seen with dose-intensified anthracycline-based chemotherapy in LABC (213, 214). Furthermore, only one of the nine patients achieving a pCR had relapsed after a median follow-up of 35 months. Consistent with previous findings (215), survival was shorter in patients with IBC than in those with non-inflammatory LABC. High efficacy was demonstrated in this study despite inclusion of a high percentage (17%) of patients with IBC.

None of the biomarkers evaluated was predictive for outcome. Both HER2 and the enzymes we investigated relate to only one component of the triplet regimen.

Two aspects of the safety profile of EXC in this study are of particular interest: chemotherapy-induced nausea and vomiting (CINV), and thrombogenic events. Although the incidence of grade 3/4 nausea and vomiting was initially of little concern, investigators reported that episodes of nausea were unusually protracted and posed a major problem. Consequently the protocol was amended, reducing the total number of cycles from eight to six. A more aggressive anti-emetic scheme including dixyrazin and metoclopramide as well as 5HT3 receptor antagonists and steroids was also recommended.

CINV was substantially less frequent in trials of EXC in esophago-gastric and biliary tract carcinoma, including a randomized phase III trial (REAL-2) in patients with gastric cancer (216-218). The all-female population in the present study compared with a predominance of males (63–88%) in the esophago-gastric and biliary tract cancer studies may have contributed to the
higher incidence of CINV, since female gender is an established risk factor for CINV (219). The difference may also be due to the higher epirubicin dose and higher dose intensity delivered for all three drugs in the present study. In the present study, the dose of cisplatin, one of the most emetogenic cytotoxic compounds known, was maintained at almost 100% through all four neoadjuvant cycles, despite the high incidence of CINV. This suggests that, in some cases, cisplatin dose was not reduced appropriately. According to the recently published American Society of Clinical Oncology (ASCO) guideline on anti-emetic therapy in oncology, the two-drug combination of dexamethasone and aprepitant is recommended for the prevention of delayed emesis in all patients receiving cisplatin and other agents of high emetic risk (220). Therefore, future studies evaluating the EXC regimen should include dexamethasone and aprepitant prophylaxis in the protocol.

The second side effect meriting further discussion is the possible thrombogenic properties of the EXC regimen, based on four cases of venous thrombosis in this study. Increased risk of thromboembolism has not previously been attributed to capecitabine monotherapy (221), whereas 5-FU, the active metabolite of capecitabine, has been reported to increase the risk of thrombosis (222). There is also some evidence of thrombogenic effects of both cisplatin (223) and epirubicin (224), and the possibility that EXC induces hypercoagulability cannot be eliminated. Chemotherapy-induced dehydration may also have contributed. It is notable that increased incidence of thromboembolic events was reported with cisplatin-containing therapy compared with oxaliplatin-containing treatments in the large, randomized, phase III REAL-2 trial. However, this was unrelated to use of i.v. 5-FU or capecitabine (218).

In conclusion, the EXC regimen showed high efficacy in LABC in terms of both RR and pCR rate. None of the biomarkers was predictive of outcome. Nausea and vomiting were unexpectedly frequent, and more aggressive prophylaxis and management of these side effects is recommended in future studies of this combination. Close monitoring for potential thrombogenic effects is advisable.
General conclusions

- Topo IIα, the key target enzyme of topo II inhibitors, is significantly expressed in the G0/G1 phase of breast cancer cells. This finding may have clinically important implications for treatment efficacy of topo II inhibitors.
- TOP2A gene amplification was suggestive of increased response to anthracyclines in advanced breast cancer, whereas HER2 had no predictive value in isolation. These findings are in accordance with current knowledge.
- FMCA predicts drug resistance with good accuracy, while clinical drug sensitivity is less reliably predicted. The use of FMCA and similar assays is not recommended outside clinical trials. Their main utility is in preclinical testing of new anti-cancer drugs, including targeted therapies.
- Cyclin A is a marker of cell proliferation with good prognostic value, but did not predict response to chemotherapy in advanced breast cancer.
- The combination of epirubicin, capecitabine, and cisplatin (EXC) is an active regimen in locally advanced breast cancer, but with cumbersome toxicity. The fluoropyrimidine biomarkers TS, TP, and DPD did not predict response to the EXC regimen.
- Tumor heterogeneity complicates the evaluation of predictive factors due to inherent or acquired diverse chemosensitivity and due to the risk of sampling error when analyzing only fragments of the tumor, as with core biopsies and cytology.
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References


106. Sjostrom J. Predictive factors for response to chemotherapy in advanced breast
phase fraction and survival benefit from adjuvant chemotherapy or radiother-
clonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and
MIB 3) detect proliferating cells in microwave-processed formalin-fixed
al. Growth fractions in breast cancers determined in situ with monoclonal anti-
110. Endl E, Gerdes J. The Ki-67 protein: fascinating forms and an unknown func-
111. Brown DC, Gatter KC. Ki67 protein: the immaculate deception? Histopathol-
tion of response to primary chemotherapy for operable breast cancer. Eur J
113. Chang J, Ormerod M, Powles TJ, Allred DC, Ashley SE, Dowsett M. Apop-
tosis and proliferation as predictors of chemotherapy response in patients with
114. MacGrogan G, Mauriac L, Durand M, Bonichon F, Trojani M, de Mascarel I,
et al. Primary chemotherapy in breast invasive carcinoma: predictive value of
the immunohistochemical detection of hormonal receptors, p53, c-erbB-2, MiB1,
Chemotherapy is more effective in patients with breast cancer not expressing
steroid hormone receptors: a study of preoperative treatment. Clin Cancer Res
A, et al. No significant predictive value of c-erbB-2 or p53 expression regard-
ing sensitivity to primary chemotherapy or radiotherapy in breast cancer. Int J
Combined sequential approach in locally advanced breast cancer. Ann Oncol
logic markers as predictors of clinical outcome from systemic therapy for pri-
Studies of the potential utility of Ki67 as a predictive molecular marker of
clinical response in primary breast cancer. Breast Cancer Res Treat
2003;82(2):113-23.
The immunohistochemical marker Ki-S2: cell cycle kinetics and tissue distri-
121. Heidebrecht HJ, Buck F, Steinnann J, Sprenger R, Wacker HH, Parwaresch R.
p100: a novel proliferation-associated nuclear protein specifically restricted to


153. Csoka K, Tholander B, Gerdin E, de la Torre M, Larsson R, Nygren P. In vitro determination of cytotoxic drug response in ovarian carcinoma using the


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