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PROGNOSIS AND PREDICTIVE FACTORS IN HUMAN BREAST CANCER DURING TUMOR PROGRESSION

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Never measure the height of a mountain
until you have reached the top. Then
you will see how low it was.

Dag Hammarskjöld

To Håkan, Agnes and Jesper

ABSTRACT

In 2010, 1.6 million women contracted breast cancer globally, almost three times the number in 1980, making breast cancer the most common malignancy among women. In Sweden, approximately one out of nine women is expected to develop breast cancer during their lifetime.

Traditionally, therapy decisions have been based on primary tumor predictive markers such as the estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) assuming these are unchanged in the relapse site.

The overall aim of this thesis was to investigate if prognostic and predictive factors such as ER, progesterone receptor (PR) and HER2 status change during breast cancer progression.

In our first cohort from Stockholm region consisting of patients with recurrent breast cancer, HER2 (n=151) status from both primary- and relapse tumor were assessed. We found a worse survival for patients with changed HER2 (n=15) status primary tumor and relapse compared to patients with a stable HER2 (n=35) positive disease.

In our second cohort from Stockholm region, consisting of breast cancer patients with biopsy (mostly by cytology) verified recurrences, ER (n=459), PR (n=430) and HER2 (n=104) status in both the primary tumor and the corresponding relapse were determined. The discordance of receptor status was 32.4%, 40.7% and 14.5%, respectively. Loss of ER in the relapse resulted in a statistically significantly increased risk of dying (HR 1.48; 95% CI, 1.08-2.05) compared with patients with stable ER-positive tumors.

A further population based cohort was established; inclusion of 2102 patients with a primary breast cancer diagnosis during the years 2000 through 2011 from the county of Värmland, at a mean follow-up time of 4.8 years. 1060 out of 2102 patients have had a biopsy taken after the initial breast cancer diagnosis demonstrating that 8.4% (n=177) of the patients had developed a recurrence, 4.4% (n=93) secondary cancers (colorectal-, lung-, skin cancer), 1.9% (n=40) cancer in situ (skin, breast) and 40.8% (n=857) were found to have benign lesions. For patients with recurrence, discordance in ER, PR and HER2 status between the primary- and metastatic tumor occurred in 14.2% (n=18), 39.6% (n=40) and 9.6% (n=7), respectively. Loss of ER or PR at relapse, resulted in statistically significantly increased risk of death (HR 3.62; 95% CI, 1.65-7.94) and (HR 2.34; 95% CI, 1.01-5.47) compared with patients with stable ER or PR positive tumors. The proportion patients with loss of ER was highest among the patients treated with adjuvant endocrine therapy.

In a cohort of patient with a primary ductal cancer *in situ* (DCIS), ER (n=112), PR (n=113) and HER2 (n=114) status from both the primary DCIS and the corresponding local events were assessed, revealing a conversion for ER, PR and HER2 status in 10-30% of instances. However, no general pattern for the conversion was seen, not even when stratified for either *in situ* or invasive relapse. Nevertheless, this study could not support the premise that HER2 overexpression had any major impact on tumor progression to invasive cancer.

In conclusion, the best approach of suspected breast cancer recurrence is to re-biopsy since this may change management of a substantial proportion of them.

LIST OF PUBLICATIONS

I. Wilking, U. **Karlsson, E**. Skoog, L. Hatschek, T. Lidbrink, E. Elmberger, G. Johansson, H. Lindström, L. Bergh, J.
HER2 status in a population derived breast cancer cohort – discordances during tumour progression.
Breast Cancer Res Treat, 125(2): 553-561, 2011.

II. Lindström, LS *. **Karlsson, E** *. Wilking, U. Johansson, U. Hartman, J. Lidbrink, E. Hatschek, T. Skoog, L. Bergh, J.
Clinically used breast cancer markers such as estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 are unstable throughout tumor progression.
*** Shared first authorship**
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III. **Karlsson, E**. Appelgren, J. Solterbeck, A. Bergenheim, M. Alvariza, V. Bergh, J.
Breast cancer during follow-up and progression – Data from a complete population based cohort on new cancers and changed biology.
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IV. **Karlsson, E**. Sandelin, K. Appelgren, J. Zhou, W. Jirström, K. Bergh, J. Wärnberg, F.
Clonal alteration of breast cancer receptors between primary ductal carcinoma *in situ* (DCIS) and corresponding local events.
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CONTENTS

LIST OF ABBREVIATIONS

AI	Aromatase Inhibitor
BRCA1, BRCA2	Breast Cancer susceptibility gene 1, 2
BCS	Breast Conserving Surgery
CI	Confidence Interval
CNB	Core Needle Biopsy
CT	Chemotherapy
DCIS	Ductal Cancer *In Situ*
EBCTCG	Early Breast Cancer Trialists´ Collaborative Group
EMA	European Medicines Agency
ER	Estrogen Receptor
ESMO	European Society For Medical Oncology
ET	Endocrine Therapy
FISH	Fluorescence In Situ Hybridization
FNA	Fine Needle Aspirate
HR	Hazard Ratio
HRT	Hormone Replacement Therapy
HER2	Human Epidermal Growth Factor Receptor 2
ICC	Immunocytochemistry
IHC	Immunohistochemistry
ISH	In Situ Hybridization
NCCN	National Comprehensive Cancer Network
OS	Overall Survival
PR	Progesterone Receptor
RT	Radiotherapy
SNB	Sentinel Node Biopsy
TNM	Tumor Size, Node, Metastasis
TMA	Tissue Microarray

1 INTRODUCTION BREAST CANCER

1.1 BACKGROUND

In 2010, 1.6 million women contracted breast cancer globally, almost three times the number in 1980, making breast cancer the most common overall malignancy among women. Breast cancer is the most common malignancy among Swedish women. Approximately one out of nine women is expected to develop breast cancer during their lifetime.

Primary breast cancer is generally treated with surgery. Adjuvant treatment is often offered to reduce the risk for breast cancer recurrence and eradicate disseminated micro-metastases. Today, the most important treatments in the adjuvant settings are radiotherapy, chemotherapy, monoclonal antibody (trastuzumab) and endocrine therapy (1-4). Preoperative chemotherapy/endocrine/trastuzumab are offered to patients with locally advanced breast cancer for down staging in order to make surgery possible, but it is standard care for patients with a primary operable breast cancer (4). The latter case has an advantage compared to adjuvant treatment since it enables response guided therapy. The Cochrane overview from 2007 demonstrated no significant difference in overall survival (OS) for patients receiving either pre- or postoperative chemotherapy (5).

For “early detection” mammography screening was started with several Swedish prospective and randomized studies resulting in a recommendation of population based screening in 1986 for females between the age of 40 and 74 years by the National Board of Health and Welfare (6, 7). The long-term follow-up of Swedish randomized controlled studies on mammography screening have shown the advantageous effect that screening has on breast cancer survival rates (8).

The 5- and 10-year relative survival rates for breast cancer has increased from approximately 65% and 53% respectively for those diagnosed in the 1960´s to 84% and 74% respectively for those diagnosed in the 1980´s (9). The 10-year relative survival was 83.5% and the 5-year relative survival was 90% during 2011 (10). The most important factor(s) behind the increasing survival rate is very unlikely due to improved surgical techniques since present recommendations are for more limited excisions but it is rather achieved by earlier detection through mammography screening programs and more extensive usage of adjuvant therapies with the latter seeming to be the most important factor (3, 11-15).

Adjuvant treatments are offered based on the individual patient´s assumed prognosis and predictive breast cancer markers analyzed and calculated from the primary breast cancer tissue together with clinical features like age and co-morbidities. A number of prognostic and predictive markers are currently in clinical use. Tumor size, lymph node involvement, metastases (TNM classification), tumor histological grade, age, progesterone receptor (PR), estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) status and the proliferation rate using Ki67 are in use according to the National guidelines (4).

The Oxford overviews by the Early Breast Cancer Trialists´ Collaborative Group (EBCTCG) are published with quinquennial intervals based on meta-analyses of individual patient data from all prospective and randomized studies within each therapy area (1-3, 16-18). EBCTCG data from 2011, has shown that postoperative radiotherapy both reduces the 10 year risk of local recurrence from 35% to 19.3% as well as reduces the 15 year risk of breast cancer mortality from 25.2% to 21.4% comparing the group receiving radiotherapy with the group not receiving radiotherapy (2). Furthermore, 10-year risk of breast cancer mortality was reduced by approximately 30%, comparing the group receiving chemotherapy with the group not receiving chemotherapy (3). In addition, the risk reduction was independent of age, stage, grade, ER status or tamoxifen use (3), contradictory to some previous reports (19-21). Moreover, in ER positive patients, five years of tamoxifen will reduce breast cancer mortality by approximately one third after 15 years follow-up. The advantageous effect of tamoxifen has been shown independent of PR status (1) and most recent data reveals that 10 years of postoperative tamoxifen is superior compared with a shorter duration, five years (22, 23).

Still, despite achievements in the use of different adjuvant therapy approaches approximately 20 % of all primary breast cancer patients will suffer a recurrence and most of them will die due to metastatic disease (1, 3, 10, 14, 24).
One can speculate whether this likely is due to insufficient biomarker analysis (25-27), less optimal selection of adjuvant therapies (under treatment) or altered tumor biology throughout tumor progression (28).

The diagnosis of breast cancer recurrence was previously frequently based on a combination of clinical and/or radiological signs of relapse and not always by morphological confirmation of the lesion. Still today in many institutions, the treatment following on from the diagnosis of the primary cancer and “metastases” and/or local relapses is based on the primary tumor marker assessment. ER and HER2 are of particular interest since they are both important predictors of the likelihood of response to endocrine therapy and efficacy of anti-HER2 therapies, respectively. However, emerging data (including our data) indicate discordance of ER, PR and HER2 status between the primary tumor and the corresponding recurrence (28-51), and indeed, a few studies have reported a prognostic value of such a change in receptor status (28, 29, 32, 33).

Biopsy of suspected recurrences in patients with previous breast cancer is now recommended, whenever feasible, by both the National and International guidelines (ESMO-guidelines, National Comprehensive Cancer Network (NCCN), and by the 1st international consensus conference for Advanced Breast Cancer (ABC 1)) (4, 52-54). Despite these recommendations (based on two prospective clinical studies and a long list of retrospective studies) (55) the biopsy verification procedure is still not performed by many institutions as part of the clinical routine. Biopsies may confirm diagnosis, reveal secondary malignancies or benign conditions as well as express clinically used biomarkers such as ER, PR and HER2 which may change the management of one in six/seven patients (29-31, 55, 56).

1.2 BREAST CANCER EPIDEMIOLOGY

Worldwide – incidence and mortality

Worldwide, breast cancer is the most common cancer among women with approximately 1.6 million women diagnosed with the disease in the year 2010. Breast cancer incidence has increased from about 600 000 in 1980 to 1.6 million in 2010 (corresponds to an annual rate of increase of 3.1%). During the same time of period, breast cancer mortality has increased at an annual rate of 1.8% (57). Breast cancer is the most common cancer in females both in developed and developing regions. The range of mortality rates, however, is markedly variable approximately 6 to 19 per 100 000. Nevertheless, breast cancer is one of the leading causes of cancer death in women in developing as well as in developed regions.

Sweden – incidence and mortality

Breast cancer is the most common cancer among Swedish women (Fig 1). In 2011, 8382 new breast cancers were diagnosed in Swedish women. This corresponds to almost a third of all female cancers diagnosed in Sweden during the same time period. Statistically one out of nine women is expected to develop breast cancer during their lifetime. The incidence has increased by approximately 1.2% annually the last 20 years. The mortality rate has slowly decreased and presently around 1400 women die from breast cancer in Sweden every year (Fig 2). One should, however, remember that the incidence in 1960 was around 2500 of whom 1200 died making the incidence/mortality ratio alteration over time quite impressive. Breast cancer is most common in women aged 60-64 years (Fig 3) (10).

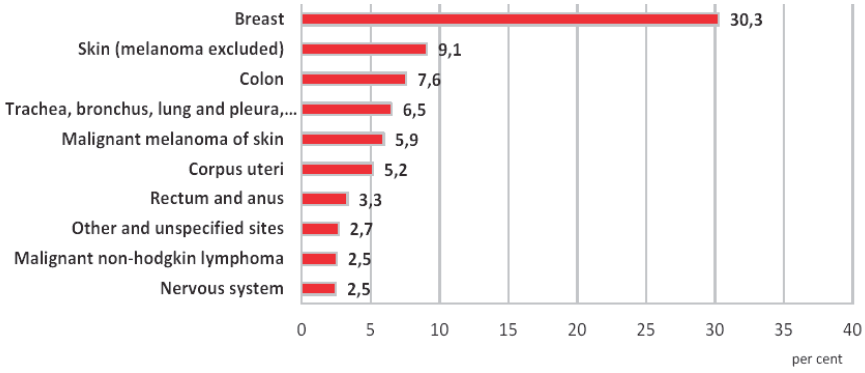


Figure 1. Most common cancers, females, Sweden (The National Board of Health and Welfare).

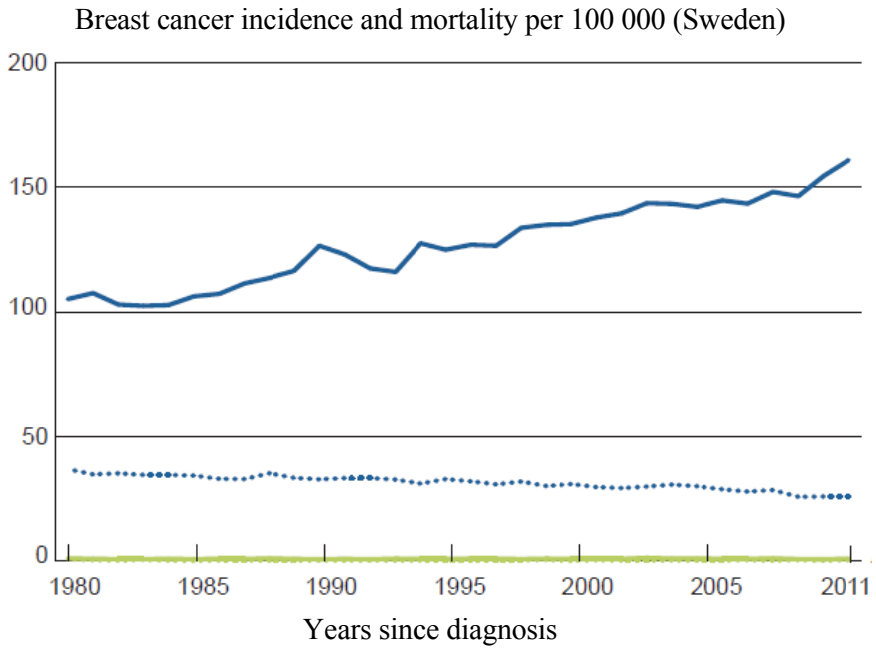


Figure 2. Breast cancer incidence (continuous line) and mortality (dotted line) for women (blue line) and male (green line) in Sweden, per 100 000 (The National Board of Health and Welfare).

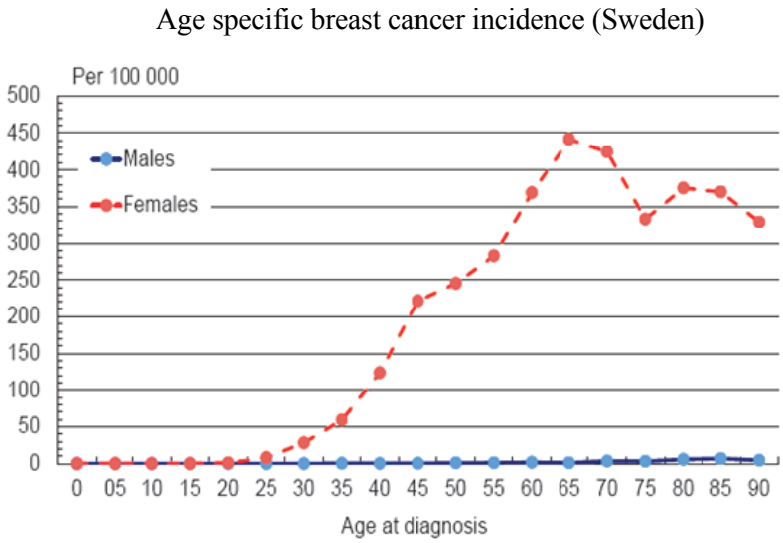


Figure 3. Age specific breast cancer incidence, Sweden (The National Board of Health and Welfare).

1.3 DIAGNOSIS

Approximately half of all breast cancer diagnoses in Sweden are detected by screening mammography, whereas the others are clinically detected (58, 59). As expected the proportion of tumors detected by screening was higher among the small tumors. For tumors < 10 mm, 73% were detected by screening, for tumors 11-20 mm, 71% and tumors > 20 mm 43 %, respectively (59). The diagnostic procedures usually denoted "triple diagnostic procedure"; clinical examination, mammography and fine needle aspiration for cytological examination or core needle biopsy for histopathological investigation.

1.4 ETIOLOGY

According to present knowledge, approximately 5-10% of all breast cancers are strongly related to hereditary factors, namely mutations BRCA1 and BRCA2 genes. Women with mutations in those genes have a 30-80% life-time risk to develop breast cancer and ovarian cancer (50-80% life-time penetration risk to develop breast cancer for BRCA1 or BRCA2 carriers) (4, 60-62). Special follow-up programs for women with known hereditary breast cancer have been established (4). However, the majority of all breast cancers are sporadic without any family history of the disease. The etiology for the vast majority, called sporadic breast cancer, is multifactorial involving hormonal-parity status, environmental-, socioeconomic- and yet poorly defined genetic factors (62-65). For instance, young age at first childbirth, multiple pregnancies and breast feeding have been associated with a reduced risk of breast cancer whereas an early menarche and late menopause is associated with an increased risk, possibly related to a high number of ovulatory menstrual cycles (63, 64, 66). A list of established risk factors for breast cancer is shown in table 1. Nevertheless, the most important risk factor for breast cancer is increasing age (10). Finally, age is a risk factor, younger women with breast cancer have a worse prognosis (24, 67-72).

Table 1. Risk factors in breast cancer. Adapted from Ref. (64) with permission.

Factor	Relative Risk	High-risk group
Age	> 10	Elderly individuals
Geographical location	5	Developed countries
Breast density	> 5	Extensive dense breast tissue visible on mammogram
Age at menarche	3	Before age 11 years
Age at menopaus	2	After age 54 years
Age at first full pregnancy	3	First child after 40 years
Family history	≥ 2	Breast cancer in first-degree relative
Previous benign breast disease	4-5	Atypical hyperplasia
Cancer in the other breast	> 4	
Body-mass index		
Premenopaus	0.7	High body-mass index
Postmenopaus	2	High body-mass index
Alcohol consumption	1.07	7% increase with every daily drink
Exposure to ionising radiation	3	Abnormal exposure to young girls after age 10 years
Breastfeeding and parity	Relative risk falls by 4.3% for every 12 months of breastfeeding in addition to a 7% reduction for every birth	Women who do not breastfeed
Use of exogenous hormones		
Oral contraceptives	1.2	Current users
Hormone-replacement therapy	1.66	Current users

1.5 PROGNOSTIC AND PREDICTIVE FACTORS

Breast cancer is a heterogeneous disease with different biological profiles such as various expressions of prognostic and predictive markers. Accordingly the prognosis for each patient differs widely. Ideally a prognostic marker in breast cancer would predict the risk of developing recurrences in an untreated patient whereas a predictive marker would predict the likeliness of response to a certain treatment.

With the goal of "personalized medicine" the treatment offered is based on the individual patient´s calculated breast cancer prognosis, predictive breast cancer markers but indeed, also guided of the benefit by the proposed therapy versus risk of recurrence, side effects and adverse events related to treatment (73-75). Obviously, also the patient´s preference need to be taken into consideration (76).

What makes a biomarker useful in the clinical practice? Well, in the first place one has to find out whether there is high evidence of *analytical validity*, i.e. how precise and reliable (extent of consistency) the test is that measures the biomarker of interest (25, 77). Second, to ensure that there is an occurrence of *clinical validity*, which means an established correlation between the test and clinical outcome (e.g. survival, response to therapy etc.) (25, 77). Finally, to find out whether the biomarker has *clinical utility* i.e. the test of the biomarker must provide information that contributes to the current management of the patient (25, 26, 77). During the years a huge amount of biomarkers have been reported. In spite of this only a few tumor markers have been shown sufficient evidence to guide treatment of breast cancer patients (78).

In Sweden, the prognostic- and therapy predictive markers in clinical use are the TNM classification; (see below), histological grade, age, ER status, PR status, HER2 status and the proliferation marker (Ki67). At present, the predictive markers in clinical use are ER status, which predicts response to endocrine therapy and HER2 status which predicts efficacy of trastuzumab therapy (4).

Stage and TNM classification

Breast cancer stage is based on TNM classification, tumor size (T), presence of regional lymph node metastasis (N) and/or distant metastasis (M). Different combination of those parameters divides patient in groups with prognostic impact (Table 2) (79, 80).

Tumor size and in particular lymph node involvement have mostly been considered as the most important prognostic factors (81-85).

Table 2. TNM classification and Tumor stage.
Adapted from the America Joint Committee on cancer (7th edition) with permission.

Stage	Tumor size (T)	Lymph node status (N)	Distant metastais (M)
Stage 0	Tis	N0	M0
Stage I A	T1	N0	M0
Stage I B	T0	N1	M0
	T1	N1	M0
Stage II A	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage II B	T2	N1	M0
	T3	N0	M0
Stage III A	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage III B	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage III C	Any T	N3	M0
Stage IV	Any T	Any N	M1

Primary tumor size (T)
T0 = No evidence of primary tumor, Tis = Carcinoma in situ, T1 ≤ 20mm, T2 > 20-50mm, T3 >50mm,
T4 = Tumor of any size with direct extension to the chest wall and/or to the skin

Lymph node status (N)
N0 = No regional lymph node metastasis, N1 = Movable ipsilateral axillary metastasis
N2 = Fixed axillary or internal mammary node metastasis
N3 = Metastasis in supra/infraclavicular nodes or internal mammary together with axillary metastasis

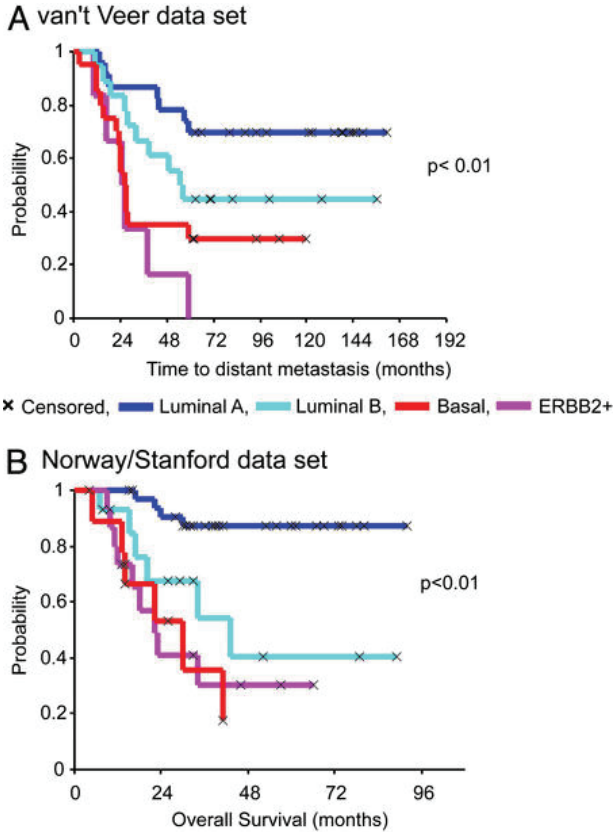
Distant metastasis (M)
M0 = No distant metastasis, M1 = Distant metastasis

Classification based on gene expression profiles

More recent subgroup classification of breast cancer based on gene expression profiles with different impact of prognosis has been shown clinically relevant when considering the therapy management of the patients (86-103). The subtypes are:

- **Luminal A:** Hormone receptor positive, low proliferation index.
- **Luminal B:** Hormone receptor positive, high proliferation index.
- **HER2 positive:** Overexpression of HER2.
- **Basal-like/"Triple negative":** Hormone receptor negative and HER2 negative.
- **Normal like:** Expression of genes seen in adipose and other non-epithelial tissue.

Figure 4. Kaplan-Meier curves of disease outcome in two breast cancer patient cohorts. (A). Time to development of distant metastasis. (B). Overall survival for patients from primary diagnosis (Sorlie.T et al. PNAS 2003, Copyright National Academy of Sciences, USA). From Ref. (90) with permission.



As can be seen, the “Luminal A” subgroup has a good prognosis while the “Basal like” and the HER2 positive (ERBB2+) subgroups have worse prognosis (87, 90).

Estrogen Receptor (ER)

ER was first identified in the 1960s when the development of radiolabelled hormones made it possible to demonstrate the binding of estrogen to its receptor (104-106). ER is a nuclear transcription factor and normally involved in pathways controlling cell proliferation (107, 108). Approximately 80% of all breast cancers have estrogen receptor positive (ER+) tumor cells (58, 109). Estrogen stimulates growth of ER+ normal- and tumor cells.

ER status, the protein expression, is a strong predictive marker for the response to endocrine therapies i.e. tamoxifen and aromatase inhibitors (AIs) (1, 110-113). Treatment with tamoxifen will reduce the effect of estrogen by blocking the ER. Tamoxifen, the anti-estrogen, has been used for treatment of breast cancer for about 40 years. Aromatase is an enzyme that naturally converts the androgens testosterone and androstenedione to estrone and estradiol in the peripheral tissue. In postmenopausal women estrogens are mostly synthesized this way in contrast to the premenopausal women where most of estrogen is produced by the ovaries (114). AI will thereby reduce the level of estrogen by inhibition of the aromatase enzyme. In addition, the AIs have been used since early 2000s. However, only about 50% of breast cancer patients with an ER+ expressing cancer will respond to endocrine therapy.

The tissue distribution of ER is generally visualized by immunohistochemistry (IHC) /immunocytochemistry (ICC) using a monoclonal antibody based biochemical method where the antibody binds to the DNA located estrogen receptor in the nucleus. The cut-off point used for classification of ER+ is often ≥10% positive tumor cell, which is recommended by the National and European guidelines (1, 4, 115). However, St Gallen and ASCO guidelines have recommended a cut-off > 1% ER+ tumor cells for the likelihood of response to endocrine therapy (116, 117). Currently, there are conflicting opinions of whether endocrine therapy should be the treatment of choice or not in patients with tumors with an ER expression in the range of 1-9% (1, 118, 119). Furthermore a previous study from Karolinska showed that only few tumors had an ER expression in the spectrum between 1-9% (118). Earlier the content of ER was determined by using biochemical methods such as the isoelectric focusing on a polyacrylamide gel or an enzyme immunoassay and finally by use of monoclonal antibodies (120, 121). The present IHC methods have been run since around the 2000s. The reliability of the evaluating of ER by IHC or cytosol assay has been documented (118, 122).

Estrogen Receptors

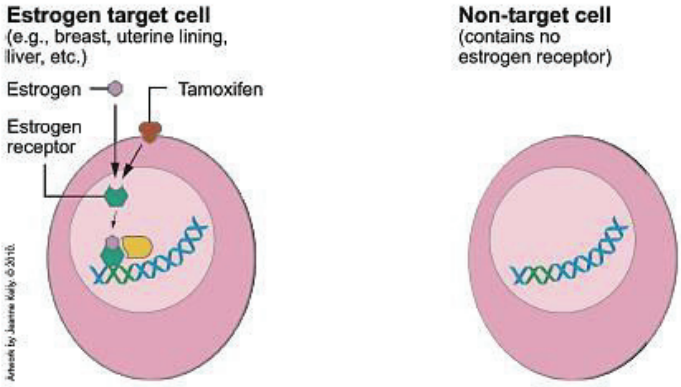


Figure 4. ER positive and ER negative cell. Estrogen receptor (ER) in the nucleus is also target for Tamoxifen. Artwork originally created for The National Cancer Institute. Reprinted with permission of the artist, Jeanne Kelly.

Progesterone Receptor (PR)

PR is a nuclear receptor. PR expression is induced by ER activation (123). The activity of progesterone in breast tissue is not clarified, however it is assumed that it induces lobular development (124). Diverging results about proliferative activity of progesterone have been reported (125). However, it has been clearly demonstrated that hormone replacement therapy (HRT) estrogen and in particular estrogen and gestagen combinations are associated with an increased risk of breast cancer (126-128). Additionally, PR negativity has been demonstrated to be an independent negative prognostic factor for breast cancer survival (100, 116, 129, 130). High S-phase, PR negativity in tumors larger than 20mm was described as an independent marker signature already in 1990 (131) recently confirmed in a prospective study (132). Furthermore, the advantageous effect of tamoxifen in ER+ patients is independent of PR status (1).

Proliferation index – (Ki67)

The Ki67 is a nuclear protein expressed by all proliferating cells. Ki67 is used as a marker for proliferation. Previous reports have indicated the potential usefulness of high Ki67 proliferation index as a prognostic marker in primary breast cancer, resulting in a worse outcome (133-135). In addition, other prognostic factors such as high grade, ER negativity and younger age in primary breast cancer patients are suggested to be associated with a high Ki67 value (136-138). Furthermore, high proliferation index has been shown proposed to be related to a higher degree of sensitivity to chemotherapy (134, 139).

However, the claimed lack of robust consistency across laboratories and lack of consensus about definition of cut-off value for high versus low proliferation index have limited the clinical utility of Ki67. Today there is no international consensus on the assessment of Ki67. Indeed, big efforts are made to standardize Ki67 analysis by the International Ki67 Working Group. They reported from an international Ki67 reproducibility study a substantial variability in Ki67 scoring among laboratories. Consequently, Ki67 values in the clinical practice cannot be transferred between laboratories due to limited analytical validity (27). The latter statement has recently been challenged by a quality assurance program for breast cancer markers, (SweQA , Swedish Qality Assurance) (140) including Ki67 run by Swedish pathologists together with Prof Giuseppi Viale, Milan, Italy under the guidance of Professor Mårten Fernö and the Swedish Breast Cancer Group demonstrating excellent concordance values for the studied markers including Ki67 in this comparative study (Mårten Fernö and Jonas Bergh, personal communication).

Human Epidermal Growth Factor Receptor 2 (HER2)

HER2, also known as HER2/neu or ErbB-2 is a protein encoded by the ERBB2 gene located on the long arm of chromosome 17(17q21-q22). HER2 belongs to the Epidermal Growth Factor (EGF) Receptor Tyrosine Kinases (RTK) family. Breast cancer patients with cancers with protein overexpression and/or gene amplification of HER2 have been demonstrated to have worse survival (141-144).

HER2 content is analyzed either by; HER2 protein quantity, using IHC, a semi quantitative method (0, 1+, 2+ or 3+) or with measurements of HER2 gene copies, using an in situ hybridization method (ISH). In the latter case, a single-probe (detection of HER2 gene expression) or a dual-probe (detection of HER2 gene expression and chromosome 17) are used (145). According to the National guidelines, HER2 positivity (HER2+) is defined as overexpression of HER2 protein (3+) or gene amplification (HER2 copy number ≥5 or HER2/CEP17 ratio ≥ 2.0) (146). Approximately 10-30% of all primary breast cancers is HER2 positive (142, 147, 148), particularly, the early studies reported HER2 overexpression in the range of approximately 30% or more, likely for highly selected cohorts. Additionally, HER2 positivity in breast cancer is used for selection of patients sensitive for anti-HER2 directed therapies, e.g. trastuzumab, lapatinib, pertuzumab and trastuzumab emtansine (149-154).

In Sweden, trastuzumab therapy has been available in the adjuvant therapy armory since 2005, in some regions in Sweden, including Stockholm in the adjuvant situation and on name patient basis since 1998 (155-157) in the metastatic situation, since 2000 based on the European Medicines Agency (EMA) approval.

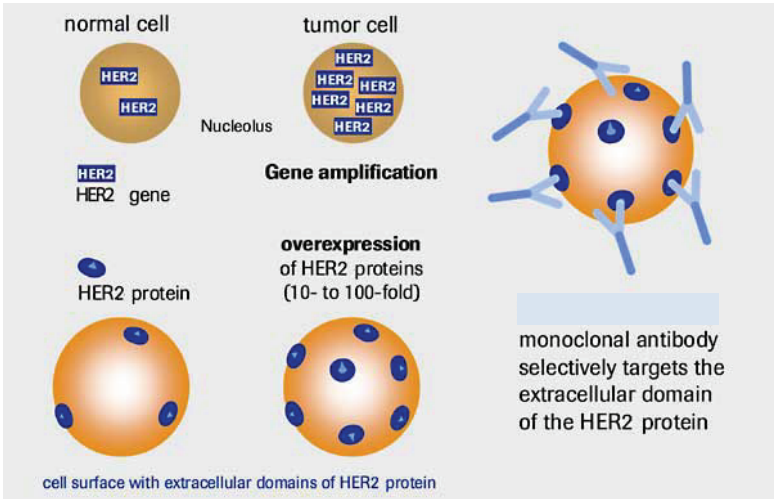


Figure 5. Normal cell and tumor cell. Tumor cell with overexpression of HER2. The HER2 is also target for trastuzumab therapy.
Image reprinted with permission from Roche Pharmaceutical.

1.6 TREATMENT

Surgery

The general treatment today for primary invasive breast cancer involves surgical removal of the tumor. The surgical techniques consists of breast conserving surgery (BCS) or mastectomy. The type of surgery depends on size and location of the tumor and patients preference. BCS in patients with invasive breast cancer is followed by radiotherapy to reduce the risk of loco-regional recurrences (2, 158-160). Several studies with long term follow-up have shown that BCS followed by radiotherapy is as effective as radical mastectomy for patients with stage I and II breast cancer (160, 161). Surgery of the axilla is performed for staging, (i.e. define the extent of the disease) for further therapy decisions. It involves either sampling i.e. sentinel node biopsy (SNB) or removal of axillary lymph nodes (162-168). The sentinel node is the assumed first lymph node to receive lymphatic drainage from the breast tumor. Several reports have shown that SNB is the preferable approach in terms of reliability in predicting axillary lymph node status as well as decreasing the morbidity related to axillary surgery (162, 163, 169-173). If the SNB is negative no further surgery in the axilla is necessary.

Radiotherapy

Postoperative radiotherapy is given to eliminate possible micro-metastases in the breast parenchyma, chest wall and/or in the axilla. The treatment should be offered to all patients who have undergone BCS (158-160). The Oxford overview from 2011, limited to women irradiated after BCS has shown that postoperative radiotherapy both reduces the 10 year risk of local recurrence from 35% to 19.3% as well as reduces the 15 year risk of breast cancer death from 25.2% to 21.4% comparing the group receiving radiotherapy with the group with not receiving radiotherapy (2).

Furthermore, in the Oxford overview from 2005 including women with mastectomy, axillary clearance and node positive disease, the 5-year local recurrence risk decreased from 23.0% to 6.0% (absolute reduction 17%) and the 15-year breast cancer mortality decreased from 60.1% to 54.7% (absolute reduction 5.4%), in the group receiving radiotherapy versus the group not receiving radiotherapy (17).

Systemic adjuvant therapy

Systemic adjuvant therapy is given to eliminate possible micro-metastases remaining in any part of the body. Adjuvant therapy in primary invasive breast cancer consists of chemotherapy, endocrine therapy and/or monoclonal antibody therapy.

Adjuvant chemotherapy was introduced around the1970s. The landmark clinical trials of Bernard Fisher (174) and Gianni Bonadonna (175) showed that adjuvant chemotherapy after surgical resection of the breast cancer significantly improved survival. Gianni Bonadonna initiated the first randomized clinical trial comparing poly-chemotherapy versus no chemotherapy in node positive breast cancer patients (175).

The long term result for the combination of cyclophosphamide, methotrexate and fluorouracil (CMF) showed survival benefit compared to no chemotherapy (176). In the 1990s the anthracyclins were added to the therapy armory. The Oxford overview 2005 demonstrated that anthracycline-based polychemotherapy compared to CMF-based polychemotherapy further produced a moderate survival gain (16). Next, contributions to the anthracycline based polychemothrapy were the taxans in the 2000s and survival was further slightly increased (177). The Oxford overview from 2012, shows that standard CMF reduces breast cancer mortality around 20-25% (3). Regimens with substantially more chemotherapy i.e. the modern anthracyline polychemotherapy or the combination of taxan-anthracycline based regimes versus CMF produced a further proportional reduction of 15-20% in breast cancer mortality (3). Adding taxanes to a fixed anthracykine-based control regimen breast cancer mortality was reduced by 14%. Although not statistically significant when compared versus anthracycline containing combination given with longer duration/higher dose intensities (3).

To sum up, the estimated benefit is a relative reduction of the breast cancer mortality by a third for treatment with chemotherapy versus no treatment with chemotherapy, after10 years of follow-up, independently of age, nodal status, tumor size, grade, ER status or tamoxifen use, (3) the latter in contrast to earlier reports (19-21).

Preoperative treatment in breast cancer patients enables monitoring of treatment response. No significant difference in OS for patients receiving either pre- or postoperative chemotherapy for operable breast cancers have been shown (5).

However, results from the Gepar Trio trial shows that preoperative response guided therapy produce a statistically significantly longer disease free survival than conventional therapy, (178, 179). Moreover, results from a meta-analysis from the Collaborative Trials in Neoadjuvant Breast Cancer (CTNeoBC) show that patients who obtain pathological compete response in the breast as well as in lymph nodes had better OS compared to patients with remaining tumor (HR 0.36; 95% CI, 0.31-0.42). This impact on survival was particularly seen among patients with a more aggressive tumor (180).

Endocrine therapy is offered to patients with an ER positive breast cancer. Patients with an ER negative breast cancer do not have benefit of adjuvant endocrine therapy (16). It has been shown that in patients with an ER positive breast cancer five years of tamoxifen will reduce breast cancer mortality by approximately one third after 15 years of follow-up. The advantageous effect of tamoxifen is independent of PR status (1).

In addition, recent published data have shown amongst those with an ER positive breast cancer that continuing tamoxifen to ten years compared with five years of treatment further reduces breast cancer mortality from 15.0% to 12.2% during follow-up 5-14 years (absolute mortality reduction 2.8%) (22).

In the early 2000s, the AIs were introduced in the adjuvant settings for postmenopausal patients with an ER positive cancer (181-183). The AIs are used either as monotherapy for five years or as a sequential treatment with tamoxifen for 2-3 years. AIs in comparison to tamoxifen have marginal effect on survival (111).

Adjuvant trastuzumab is offered to patients with overexpression of the HER2. Trastuzumab reduces recurrences by 50% and mortality by 30% in the adjuvant setting (151, 184). Since 2005, 1 year of treatment with trastuzumab is offered to patients with HER2 positive breast cancer treated with adjuvant chemotherapy (4, 185-188). The optimum duration of trastuzumab treatment has been an issue in several studies. The HERA trial showed no benefit for 2 years of trastuzumab treatment versus 1 year of

treatment (185). However, patients receiving 1 year of trastuzumab treatment in combination with chemotherapy (CT) compared only receiving CT showed a significantly better overall survival (HR 0.76; 95% CI, 0.65-0.88, p=0.0005) (185). The PHARE trial, failed to demonstrate that 6 months of treatment with trastuzumab in combination with CT was non-inferior to 1 year of treatment with trastuzumab (187).

Finally, the smaller FinnHER trial with a short treatment of only 9 weeks of trastuzumab in combination with CT versus only CT showed benefit for trastuzumab treatment, however, not statistically significant (HR 0.65; 95% CI, 0.38-1.12; p=0.12) for disease-free survival (188). Still, 1 year of treatment with trastuzumab in combination with CT remains the standard treatment.

More recent, perstuzumab another anti-HER2 drug has been approved by FDA, (U.S. Food and Drug Administration) for use in the preoperative treatment of breast cancer patients with an HER2 positive tumor. It has been shown that patients receiving a combination of pertuzumab, trastuzumab and doceaxel have a significantly improved pathological complete response rate compared to those who only receive trastuzumab and docetaxel, which will likely be associated with a long term survival advantage (180, 189).

2 DIAGNOSIS OF BREAST CANCER RECURRENCE

Still, despite united efforts in the use of different adjuvant therapy approach, about 20% of women with primary breast cancer will later develop metastatic disease and the majority of these women remain incurable and will ultimately succumb (1, 3, 10, 14, 24).

The diagnosis of breast cancer recurrence has often been based on a combination of clinical and/or radiological signs of relapse and not always by morphological confirmation of the suspicious relapse. In many institutions, the treatment following on from the diagnosis of the primary cancer and possible metastases and/or local relapses is based on the primary tumor marker assessments. ER and HER2 are of particular interest since they are both important predictors of the likeliness of response to endocrine therapy and efficacy of anti-HER2 based therapies, respectively.
However, emerging data (including data in this thesis) indicate discordance of biomarkers between the primary tumor and the corresponding recurrence (28-49), and indeed a few studies have reported a prognostic value of such a change in receptor status (28, 29, 32, 33).

Clinical suspicion of recurrence should include a routine staging work-up including clinical examination, imaging and routine biochemical tests. The imaging should include chest, abdomen and bone. A biopsy of a metastatic lesion should be performed to confirm diagnosis whenever feasible and particularly when recurrence is diagnosed for the first time (4, 52-54). In addition, biopsies may reveal unsuspected secondary malignancies or benign lesion as well as expression of biomarkers (ER, PR and HER2 status) which may affect therapy decisions.

Most metastatic site is available with minimal invasive methods. Biopsies of palpable and superficial lesion can be performed without image guidance (190, 191). Other lesion can be assessed by percutaneous image guide technique relatively easily with few complications (55, 56, 192-194).

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Fine Needle Aspirate (FNA)

The FNAs is an established method used since decades in preoperative diagnosis of suspected lesions in the breast (195, 196). It is easy to perform, causing very little inconvenience for the patient and with insignificant risk of a complication both in the primary as well as in the metastatic situation (55). In addition, the development of monoclonal antibodies used for assessment of ER, PR and HER2 status etc. have made it possible to characterizes the biologic feature of the cytological samples (191) (Fig 5).

Core Needle Biopsy (CNB)

CNB has the major advantage of allowing both analysis of the surrounding stroma as well as obtaining a piece of tissue for further analysis (Fig 6). Recently also reports show that contributors of the stroma tissue can harbor important breast cancer functions which may have the capacity to influence tumor progression (197-201).

Comparison of the two techniques CNB and FNA in the primary breast cancer have demonstrated improved sensitivity and equivalent/or better specificity with CNB compared with FNA (202-205). The reliability of evaluating FNAs in the assessment of ER has been documented in numerous reports (122, 206-209). Several studies have revealed a high concordance value, in the assessment of ER, PR and HER2 between the findings obtained by ICC and IHC (210, 211). In addition, other studies have shown a good correlation between CNB and excisional biopsy for both ER (98.2%) and HER2 (98.8%) (212).

However, both FNAs and CNBs have the potential limitation that they may result in a false negative result. The best results are obtained when an experienced pathologist provides on-site assessment of the sample adequacy (190). Moreover, for appropriate interpretation of the core/cytological stains, a negative and positive control should preferably always be used to minimize the risk of false positive as well as false negative immunostaining. In addition, the use of an optimal fixation of the FNAs is of importance since it will influence the visualization of certain antigens (191, 208). The strategy is that one should introduce handling of the cells allowing penetration of the antibodies to cytoplasmic and nuclear antigens. Nuclear antigens such as ER and PR are best visualized using fixation of air-dried specimens then in buffered formalin (191).

In addition, special attention is needed in the evaluation of bone biopsies since bone decalcification procedure might influence and reduce the staining for ER, PR and HER2 (47, 213, 214).

In a previous prospective study evaluating tissue confirmation of metastatic breast cancer patients using both FNA and CNB (121 biopsies) demonstrated treatment delay (approximately 15 days) and one serious adverse event (bleeding, from a biopsy of the skin resolving conservative measures) (31). However, the majority of patients who have been subjected for a re-biopsy recommended the same course of action to other breast cancer patients (31).

Tissue Microarray (TMA)

The TMA technique allows a large numbers of tumors to be analyzed together on the same microscopic slide and is tissue saving. The technique was developed in the 1990s (215). From tumor blocks, CNBs with a diameter of 1mm are mounted into the recipient TMA block (see also Paper IV). The documentation of reliability of using TMA technique in the assessment of ER, PR and HER2 in tumor samples has been documented (215-219). The described concordance between TMA and corresponding whole sections slides for ER, PR and HER2 was approximately 85-95%, 81-88% and 90-100%, respectively (216, 220). A lower correlation for PR was seen and a possible explanation for this might be a more heterogeneous expression of PR within the tumor compared to ER (216, 220-222).

Figure 5. Photos illustrating FNAs. (A). Tumor cells in liver (breast cancer recurrence). (B). ER positive tumor cells in liver (breast cancer recurrence).
(Photo, Anja Solterbeck).

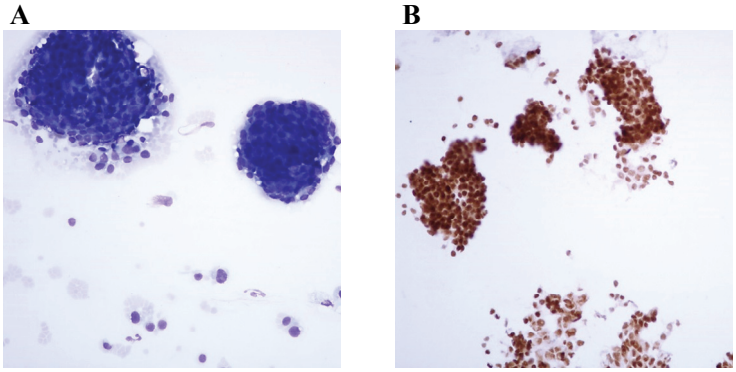
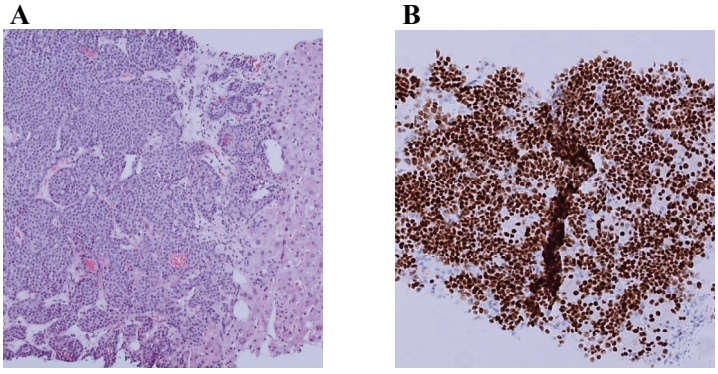


Figure 6. Photos illustrating CNBs. (A). Tumor cells in liver (breast cancer recurrence). (B). ER positive tumor cells in liver (breast cancer recurrence).
(Photo, Anja Solterbeck).



3 AIMS OF THE THESIS

The overall aim of this thesis was to investigate if prognostic and predictive factors such as ER, PR and HER2 status change during breast cancer tumor progression.

The specific aims were:

I. To investigate possible changes of intra-patient HER2 status between the primary breast cancer and the corresponding recurrence and analyze these data in relation to outcome.

II. To investigate possible changes of intra-patient hormone receptor status and HER2 status between the primary breast cancer and the corresponding recurrence and to investigate these findings in relation to outcome.

III. To investigate tumor related events (e.g. relapse, other malignancies, and benign conditions) after primary breast cancer. For patients with confirmed recurrence perform a comparative analysis of ER, PR, HER2 and Ki67 between primary tumor and corresponding relapse and analyze these data in relation to outcome.

IV. To investigate possible changes of intra-patient hormone receptor status and HER2 status between the primary ductal cancer *in situ* (DCIS) and the corresponding ipsilateral event.

4 PATIENT AND METHODS

In paper I, the cohort was identified from the population-based Stockholm and Gotland Breast Cancer registry and included all breast cancer patients (n= 1181) who suffered a breast cancer recurrence during 1997-2007 (patients diagnosed at the Karolinska University hospital and St Göran hospital, both located in the Stockholm region). Data on HER2 status was collected from pathology reports. Information of HER2 status in both the primary tumor and corresponding relapse was available in 151 patients. The information on trastuzumab treatment for these patients was collected from individual patient files. HER2 status was assessed by IHC/ICC analysis and the staining was set at; 0, 1+, 2+ and 3+ protein levels. According to the Swedish Breast Cancer Group recommendation confirmation by FISH was performed, if IHC/ICC protein level was 2+ and 3+. The cut-off level for HER2 amplification was set at HER2/CEP17 ratio > 2.0, according to the manufacturer's instructions. If HER2 status by IHC/ICC was 3+, but FISH analysis showed no amplification this was interpreted as a negative result. HER2 status was assessed by IHC in 144 primary tumors and 86 was verified by FISH whereas in the relapse setting 84 cases were assessed by ICC and 102 verified by FISH. The first available recurrence with an assessment of HER2 status was used. This study was approved by the Ethical committee at Karolinska Institutet, Stockholm.

In paper II, the cohort was identified from the population-based Stockholm and Gotland Breast Cancer registry and included all breast cancer patients (n=1092) who suffered a breast cancer recurrence during 1997-2007 (patients diagnosed at the Karolinska University hospital, St Göran hospital and Sophiahemmet all located in the Stockholm Region). The predefined exclusion criterions were advanced disease at the time of primary diagnosis or patients with synchronous bilateral breast cancer. The information on ER, PR and HER2 status was collected from pathology reports. ER, PR and HER2 status in both the primary breast cancer and one (or more) corresponding relapses had been assessed in 459, 430 and104 patients respectively. ER and PR status was assessed either by monoclonal antibody based biochemical methods (with cut-off ≥ 0.05 fmol/μg DNA as positive) or by IHC/ICC (with cut-off ≥ 10% as positive). Priority was given for results based on IHC (1) or ICC (2) and if not available, the results from biochemical methods were used. HER2 status was assessed by IHC/ICC (or directly by FISH) and the protein staining was set at four levels according to the manufacturer´s instructions, 0, 1+, 2+ and 3+. IHC/ICC was positive at 3+ protein level. According to the Swedish Breast Cancer Group; confirmation by FISH was performed for samples if the protein level was 2+ and 3+. The cut-off level for HER2 amplification was set at HER2/CEP17 ratio > 2.0, according to the manufacturer's instructions. The first available recurrence with assessment of biomarker was used. This study was approved by the Ethical committee at Karolinska Institutet, Stockholm.

In paper III, the population based cohort includes all women (n=2102) diagnosed with a primary invasive breast cancer during 2000-2011 in Värmland County, Sweden. The cohort was identified from the population-based Breast Cancer registry for Uppsala-Örebro region. The information on different diagnosis, ER, PR, HER2 and Ki67 status was collected from pathology reports. ER and PR status was assessed by IHC/ICC (with cut-off ≥ 10% as positive). Proliferation index, Ki67 was assessed by IHC/ICC (with cut-off > 10% as high proliferation). HER2 status was assessed by IHC/ICC and staining of the membrane was set at four levels, according to the manufacturer´s instructions, 0, 1+, 2+ and 3+. IHC/ICC was classified as positive at the 3+ protein level. According to National guidelines confirmation by FISH was carried out for samples if the protein level was 2+ or 3+. Determination of HER2 status by FISH assay is based on gene copy number and ratio between numbers of HER2 and chromosome 17 (CEP 17)-sequences. The cut off-level for HER2 amplification was set at HER2/CEP17 ratio ≥ 2.0. Priority was given for FISH and if not available IHC/ICC was used for evaluation of HER2 status. Data on adjuvant therapy was obtained from the Breast Cancer Registry for Uppsala-Örebro region. This study was approved by the Central ethical committee at the Swedish Research Council in Stockholm.

In paper IV, the source population includes 1504 patients, from two separate cohorts, diagnosed with a primary DCIS between 1986 and 2004. Of the total 1504 patients, 458 were identified from a population based cohort diagnosed during 1986-2004. The remaining 1046 women were identified from the randomized SweDCIS trial of patients diagnosed during 1987-1999. A total of 274 patients suffered a relapse/new cancer up to follow-up 31st of December 2011. TMA-blocks were constructed from both primary tumor and relapse. ER and PR were assessed by IHC (> 10% positive). Using Hercept-kit, tumors were classified as positive at the 3+ protein level. HER2 SISH was performed on an automated instrument (Ventana Benchmark) according to manufacturer´s protocol. The cut-off level for HER2 amplification was set at HER2/CEP17 ratio ≥ 2.2. HER2 status was relying on SISH and if not available IHC was used. Biomarkers were scored by one single observer and thus, there was no problem with inter-laboratory differences or intra-observer variability. This study was approved by the Ethical committee at Uppsala University Hospital and Umeå University.

4.1 STATISTICAL METHODS

In paper I, an intra-patients comparison between the primary tumor and relapse for HER2 status was performed. Survival after both the primary breast cancer and the relapse was estimated with the Kaplan-Meier method for three groups based on intra-patient HER2 status in primary tumor and relapse. Any difference between these groups and the risk of death was tested with the log rank test and cox proportional hazards regression models respectively. Potential confounders such as age and calendar year of primary breast cancer diagnosis, PR and ER status were adjusted for.

In paper II, any change in ER, PR and HER2 status between primary tumor and relapse was assessed using McNemar´s test. The association between intra-patient ER status and adjuvant therapy was investigated by Fisher´s test. Survival after both the primary breast cancer and the relapse was estimated with the Kaplan-Meier method for four groups based on intra-patient ER status in primary tumor and relapse. The risk of dying in relation to ER status in primary tumor and relapse was tested with the log rank test and cox proportional hazards regression models respectively. Potential confounders such as age, year of primary diagnosis, PR status, and tumor stage and adjuvant therapy were adjusted for.

In paper III, any change in ER, PR, HER2 and Ki67 status between primary tumor and relapse was assessed using McNemar´s test. Survival after both primary breast cancer and the relapse was estimated with the Kaplan-Meier method for four groups based on intra-patients ER and PR status in primary tumor and relapse respectively. Any difference between these four groups and the risk of death, was tested with the log rank test and Cox proportional hazards regression models respectively. Potential confounders such as age, year of primary diagnosis and relapse diagnosis, PR and ER status, tumor stage, adjuvant therapy were adjusted for.

In paper IV, Fisher´s test was applied for comparison of ER, PR and HER2 status between baseline primary tumor characteristics and in situ and invasive recurrence groups.

5 RESULTS

Paper I.

For clinicians knowledge of HER2 status in the tumor is required for the correct management of breast cancer patients.

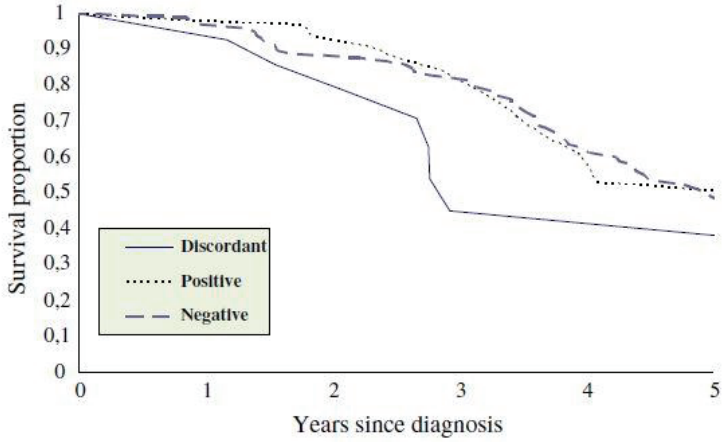
This study retrospectively investigates the relationship of intra-patient HER2 status between primary breast cancer and corresponding recurrences in a recurrence breast cancer cohort.

HER2 positive breast cancer was seen in 43 (28%) patients out of the 151 patients with a recurrence. In 15 (10%) out of 151 patients HER2 status changed between primary tumor and relapse. 8 patients changed from HER2 positive to negative and 7 patients changed from HER2 negative to positive respectively.

Figure 7A and B, shows Kaplan-Meier survival analysis from both the primary breast cancer diagnosis and from the time of recurrence for three groups based on HER2 status in primary tumor and relapse (i.e. HER2 stable positive, HER2 stable negative and discordant HER2 status). The analysis indicated possible difference between the three curves (log rank test OS since primary diagnosis, $p = 0.55$ and OS since relapse diagnosis, $p = 0.04$). The risk of death for the same three groups was calculated using a multivariable Cox proportional regression model. Patients with discordant HER2 status had an increased risk of death both from the time of primary breast cancer diagnosis (HR 5.47; 95% CI, 2.01-14.91) and from the time of the relapse diagnosis (HR 3.22; 95% CI, 1.18-8.77) compared with patients with HER2 positive stable disease (Table 3).

Figure 7. Kaplan-Meier survival curves in patients diagnosed with breast cancer. (A). Overall survival after primary diagnosis for the different HER2 groups (i.e. stable and changed HER2 status between primary tumor and recurrence). (B). Overall survival after relapse diagnosis for the different HER2 groups. Adapted from Ref. (32) with permission.

A



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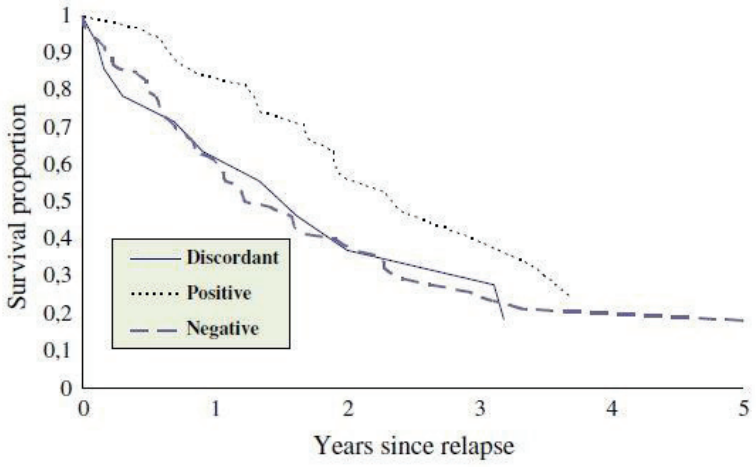

Table 3. Risk of death depending on HER2 status in primary breast cancer and corresponding relapse. Adapted from Ref. (32) with permission.

HER2 status		Overall survival - Primary diagnosis	Overall survival- Relapse diagnosis
Primary tumor and relapse	Number	HR* (95% CI)	HR* (95% CI)
Positive	34	1.0 (ref)	1.0 (ref)
Discordant	14	5.47 (2.01-14.91)	3.22 (1.18-8.77)
Negative	99	1.85 (0.99-3.45)	2.38 (1.27-4.43)
Total	147		

* Adjusted for age and year of diagnosis, estrogen receptor status, progesterone receptor status and stage

Paper II.

This study retrospectively investigates whether hormonal receptor- and HER2 status change throughout tumor progression in a relapse breast cancer cohort (n=1092).

In this study, ER (n=459), PR (n=430) and HER2 (n=104) status in both primary and the corresponding relapse were assessed. In total, discordance in ER, PR and HER2 status from primary tumor to relapse was 32.4%, 40.7% and 14.5%, respectively. ER, PR or HER2 changed from positive in primary tumor to negative in relapse in 24.6%, 33% and 8.7% (n=9) of the patients, whereas gain of ER, PR and HER2 between primary tumor and relapse was seen in 7.8%, 7.7% and 5.8% (n=6) of the patients respectively (Table 4).

Figure 8, shows Kaplan-Meier survival analysis from both the primary breast cancer diagnosis (A) and from the relapse diagnosis (B) of the four groups based on different ER status in primary tumor and relapse (i.e. ER primary positive/relapse positive, ER primary positive/relapse negative, ER primary negative/relapse positive and ER primary negative/relapse negative). A statistically significant different OS was observed for the four groups (log rank test OS since primary diagnosis, $p < 0.001$ and OS since relapse diagnosis, $p = 0.014$). The risk of death for the same four groups was calculated, using a multivariable Cox proportional regression model. Patients with loss of ER to the relapse had a statistically significant increased risk of dying from primary diagnosis (HR 1.48; 95% CI, 1.08-2.05) and from diagnosis of the recurrence (HR 1.46; 95% CI 1.06-2.01) compared with patients with an ER positive stable tumor (Table 5).

The effect of adjuvant therapy on change in ER receptor status is described for four therapy groups separately, i.e. adjuvant endocrine therapy (ET)/adjuvant chemotherapy (CT), adjuvant ET alone, adjuvant CT alone and no adjuvant systemic therapy. The proportion of patients losing ER was larger in the group treated with ET/CT or ET alone, 34.3% and 29.0% respectively, compared with the group treated with CT alone or that which received no treatment 19.8% and 11.5% respectively ($p < 0.001$ for Fisher´s test between ET, CT and the no therapy groups respectively).

We had information of ER, PR and HER2 status from patients with multiple consecutive relapses (from two to six) available in 119, 116 and 32 patients respectively. Discordance in ER, PR and HER2 status between different relapses was seen in 33.3%, 32% and 15.7% respectively. As can be seen, all these markers were unstable in approximately the similar proportions in the repeated relapse setting as in primary relapse situation.

Table 4. Intra-patient ER, PR and HER2 status throughout tumor progression

Intra-individual	ER* status		PR* status		HER2[£] status	
Hormonal and HER2 status	Number	Percent	Number	Percent	Number	Percent
Primary tumor and relapse						
Local and systemic relapse						
Prim(+)/Rel(+)	216	47.1	109	25.4	20	19.2
Prim(+)/Rel(-)	113	24.6	142	33.0	9	8.7
Prim(-)/Rel(+)	36	7.8	33	7.7	6	5.8
Prim(-)/Rel(-)	94	20.5	146	33.9	69	66.3
Total number	459	100.0	430	100.0	104	100.0
Systemic relapse						
Prim(+)/Rel(+)	134	43.0	59	19.9	16	18.8
Prim(+)/Rel(-)	89	28.5	106	35.7	6	7.1
Prim(-)/Rel(+)	26	8.3	26	8.7	4	4.7
Prim(-)/Rel(-)	63	20.2	106	35.7	59	69.4
Total number	312	100.0	297	100.0	85	100.0
Multiple relapses						
Local and systemic relapse						
Rel(+)/Rel(+)	43	36.1	15	12.9	3	9.4
Rel(+)/Rel(-)	19	16.0	25	21.6	3	9.4
Rel(-)/Rel(+)	15	12.6	8	6.9	2	6.2
Rel(-)/Rel(-)	36	30.3	64	55.1	24	75.0
Heterogeneity[$]	6	5.0	4	3.5	0	0.0
Total number	119	100.0	116	100.0	32	100.0

*Cut-off value of 0.05 fmol/μg DNA and 10%, for monoclonal antibody based biochemical and IHC/ICC methods, respectively

[£]Analyzed using IHC/ICC (or by FISH directly) and a majority confirmed by FISH for IHC/ICC 2+ and 3+

[$]Tumor marker status altering between positive and negative throughout tumor progression (different relapse sites) were labeled heterogeneity

Adapted from Ref. (28) with permission.

Figure 8. Kaplan-Meier survival curves in women diagnosed with breast cancer. (A). Overall survival after primary diagnosis for the different ER groups (intra-patient ER status primary tumor and relapse). (B). Overall survival after relapse diagnosis for the different ER groups (both local and systemic relapses included). Adapted from Ref. (28) with permission.

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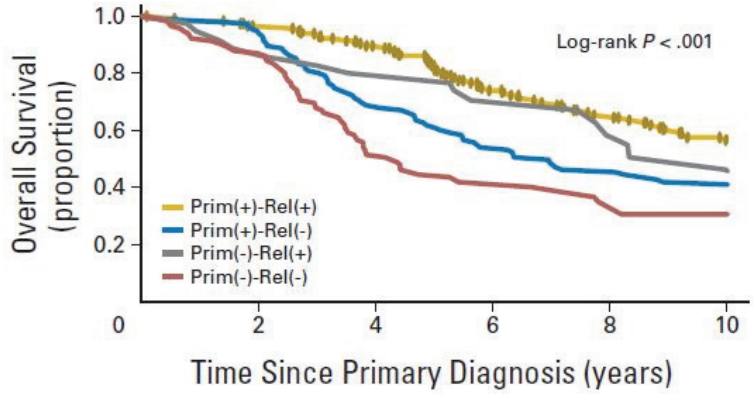

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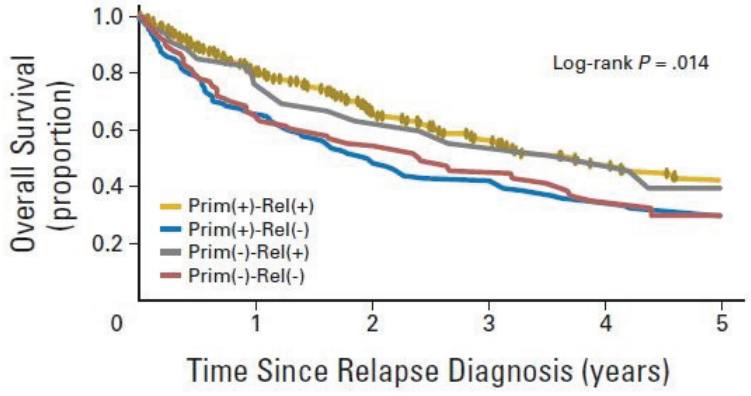


Table 5. Risk of death in breast cancer patients depending on intra-patient ER status in primary tumor and relapse. Adapted from Ref. (28) with permission.

Intra-individual primary tumor and relapse	Patients	Deaths	Overall survival from breast cancer diagnosis	Overall survival from breast cancer relapse diagnosis
			Adjusted*	Adjusted*
ER status	Number	Overall	HR (95% CI)	HR (95% CI)
Local and systemic relapse				
Prim(+)/Rel(+)	216	109	1.0 ref.	1.0 ref.
Prim(+)/Rel(-)	113	75	1.48 (1.08-2.05)	1.46 (1.06-2.01)
Prim(-)/Rel(+)	36	17	1.07 (0.61-1.89)	0.99 (0.56-1.76)
Prim(-)/Rel(-)	94	54	1.14 (0.74-1.76)	1.00 (0.65-1.55)
Total	459	255		
Systemic relapse				
Prim(+)/Rel(+)	134	73	1.0 ref.	1.0 ref.
Prim(+)/Rel(-)	89	67	1.62 (1.12-2.34)	1.51 (1.05-2.17)
Prim(-)/Rel(+)	26	14	1.12 (0.59-2.13)	1.01 (0.53-1.93)
Prim(-)/Rel(-)	63	42	1.30 (0.77-2.21)	1.16 (0.68-1.97)
Total	312	196		

*Adjusted for age and calendar year of diagnosis, progesterone receptor, tumor stage, endocrine therapy and chemotherapy

Paper III.

The unique feature of this study is that all the patients (n = 2102) came from a defined geographical region. They were identified using the regional Breast Cancer registry for Uppsala-Örebro region. These data were matched to pathology reports using the personal 12-digit id-number, given to all individuals living in Sweden.

In this study we retrospectively investigated all tumor related events (e.g. relapses, other malignancies, benign conditions) after a primary breast cancer diagnosis in a population based cohort.

Figure 9 presents a flow chart of the total cohort of patients. With a mean follow-up time of 4.8 years approximately 50% out of all 2102 patients have had a biopsy taken after the initial breast cancer demonstrating 177 (8.4%) recurrences, 93 (4.4%) other malignancies (colorectal-, lung-, skin cancer), 40 (1.9%) cancer *in situ* (skin-, breast cancer) and 857 (40.8%) benign lesions. It might be worth to clarify that these biopsies were not necessarily related to their previous cancer.

ER, PR, HER2 and Ki67 status in both the primary breast cancer and the corresponding relapses were determined in 127, 101, 73 and 55 patients respectively. The discordance of receptor status for ER, PR, HER2 and Ki67 was 14.2%, 39.6%, 9.6% and 36.3% respectively. ER, PR, HER2 and Ki67 status changed from positive in primary tumor to negative in relapse in 11.8%, 29.7%, 5.5% and 12.7% of the patients (Table 6). Loss of ER or PR between primary tumor and relapse was bigger in systemic relapse site compared to local, 16.2% versus 6.8% (p=0.028) and 42.2% versus 19.6% (p=0.069) respectively.

Figure 10A and B, show Kaplan-Meier survival analysis from both the primary breast cancer diagnosis (A) and from the relapse diagnosis of the four groups based on different ER status in primary tumor and relapse (i.e. ER primary positive/relapse positive, ER primary positive/relapse negative, ER primary negative/relapse positive and ER primary negative/relapse negative). A statistical significant differential OS between the four groups was seen (log-rank p=0.003 from time of primary diagnosis and log-rank p=0.053 from time of relapse diagnosis). The risk of death for the same four groups was calculated using a multivariable Cox proportional regression model. Patients with loss of ER in the relapse biopsy had a statistically significant increased risk of death from both the time of breast cancer diagnosis (HR 3.68; 95% CI, 1.66-8.13) and from the time of relapse diagnosis (HR 3.62; 95% CI, 1.65-7.94) compared with patients with an ER positive stable tumor (Table 7).

Figure 9. Flow chart

Table 6. Potential discordances in ER, PR, HER2 and Ki67 status between primary tumor and relapse, presented for all-, local- and systemic relapses separately. Statistical analysis with McNemar´s test.

	All relapses			Local relapse		Systemic relapse	
	Number	Percent	*P value*	Number	Percent	Number	Percent
Primary tumor and relapse							
ER*			0.008				
primary positive/relapse positive	87	68.5		38	64.4	49	72.1
primary positive/relapse negative	15	11.8		4	6.8	11	16.2
primary negative/relapse positive	3	2.4		2	3.4	1	1.5
primary negative/relapse negative	22	17.3		15	25.4	7	10.3
Total	127	100.0		59	100.0	68	100.0
PR*			0.002				
primary positive/relapse positive	30	29.7		21	37.5	9	20.0
primary positive/relapse negative	30	29.7		11	19.6	19	42.2
primary negative/relapse positive	10	9.9		6	10.7	4	8.7
primary negative/relapse negative	31	30.7		18	32.1	13	28.9
Total	101	100.0		56	100.0	45	100.0
HER2♣			1.00				
primary positive/relapse positive	14	19.2		6	13.6	8	27.6
primary positive/relapse negative	4	5.5		2	4.5	2	6.9
primary negative/relapse positive	3	4.1		3	6.8	0	0.0
primary negative/relapse negative	52	71.2		33	75.0	19	65.5
Total	73	100.0		44	100.0	29	100.0
Ki67€			0.263				
primary positive/relapse positive	26	47.3		15	36.6	11	78.6
primary positive/relapse negative	7	12.7		6	14.6	1	7.1
primary negative/relapse positive	13	23.6		11	26.8	2	14.3
primary negative/relapse negative	9	16.4		9	22.0	0	0.0
Total	55	100.0		41	100.0	14	100.0

Abbreviations: ER= estrogen receptor, PR= progesterone receptor, HER2= human epidermal growth factor receptor 2, Ki67= proliferation

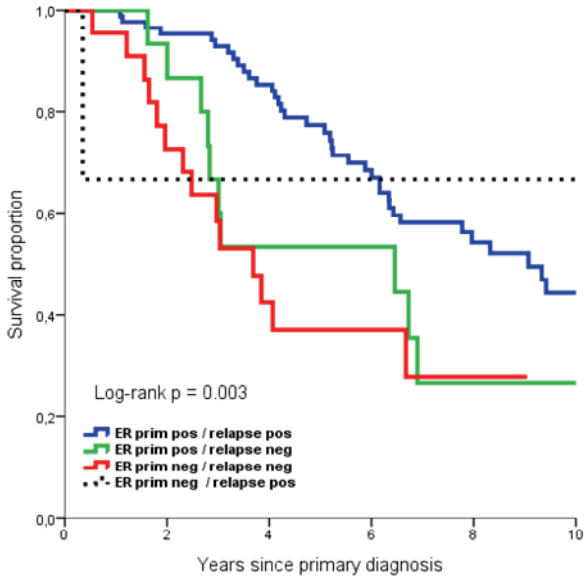
* Cut-off value ≥ 10% for IHC/ICC methods

♣ Analysed using IHC/ICC or by FISH directly, IHC/ICC 2+ and 3+ confirmed by FISH

€ = Cut-off value > 10% for IHC/ICC methods

Figure 10. Kaplan Meier survival curves in women diagnosed with breast cancer. (A). Overall survival after primary diagnosis for the different ER groups (intra-patient ER status primary tumor and relapse). (B). Overall survival after relapse diagnosis for the different ER groups (both local and systemic relapses included).

A



B

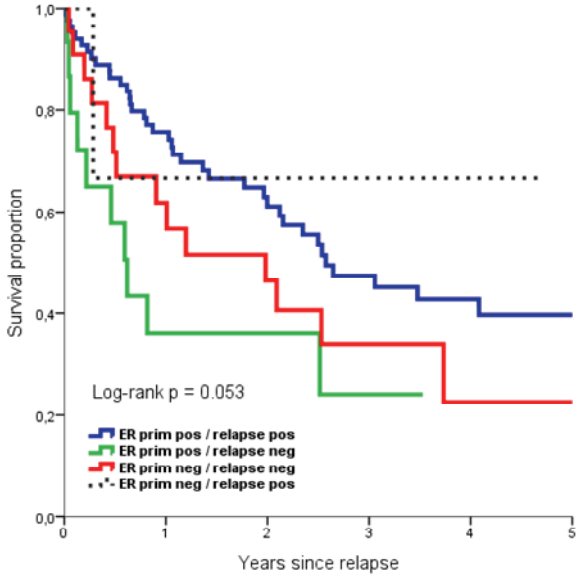


Table 7. Risk of death depending on ER status in primary breast cancer and corresponding relapse.

		Overall survival -Primary diagnosis♣			Overall survival - Relapse diagnosis€		
Intraindividual ER status	Number	HR*	95% CI		Number	HR*	95% CI
Local and systemic relapse							
primary positive/relapse positive	86	1.0 (ref)			84	1.0 (ref)	
primary positive/relapse negative	14	3.68	1.66-8.13		14	3.62	1.65-7.94
primary negative/relapse negative	21	2.19	0.80-5.99		21	1.88	0.70-5.05
primary negative/relapse positive	3	0.54	0.061-4.79		3	0.51	0.06-4.45
Total	124				122		

Abbreviations: ER= estrogen receptor, HR = hazard ratio,

♣ = From breast cancer diagnosis to death or censoring, € = From breast cancer relapse diagnosis to death or sensoring,

* Adjusted for age, calender year of primary diagnosis and relapse diagnosis, progesterone receptor status, tumor stage, adjuvant hormonal treatment and chemotherapy.

In addition patients with loss of PR to the relapse had a statistically significant increased risk of dying from the time of relapse diagnosis (HR 2.34; 95% CI, 1.01-5.47) compared with stable PR positive patients.

In table 8, we describe the effect of adjuvant therapy on change in ER status in primary tumor and corresponding relapse. As can be seen, the proportion of patients losing ER was higher in the group treated with endocrine therapy (ET) alone or in combination with chemotherapy (CT), (16.7% and 13.3% respectively), compared with the group treated with CT alone or the group which received no treatment (4.3% and 7.7% respectively) ($p < 0.001$ for Fisher´s test between treatment groups).

Table 8. Potential discordances in ER status in primary tumor and relapse stratified for four therapy groups separately.

	ET (only)		CT + ET		CT (only)		No therapy	
ER* status	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Primary positive/relapse positive	40	83.3	25	83.3	5	21.7	17	65.4
Primary positive/relapse negative	8	16.7	4	13.3	1	4.3	2	7.7
Primary negative/relapse positive	0	0	0	0	3	13.0	0	0
Primary negative/relapse negative	0	0	1	3.3	14	60.9	7	26.9
Total	48	100.0	30	100.0	23	100.0	26	100.0

Abbreviations: ER= estrogen receptor, ET= endocrine therapy, CT= chemotherapy

* Cut-off value ≥ 10% for IHC/ICC methods

P < 0.001 for Fischers test between treatment groups and ER status

Furthermore, in our cohort of 2102 patients with previous breast cancer, 93 patients (4.4%) had been subjected to a biopsy showing secondary cancer. In summary, the most common secondary cancers were: colorectal- (22.4%), lung- (14.3%), skin- (no melanoma) (12.2%) and corpus uteri cancer (9.2%).

Paper IV.

Primary DCIS is a pre-invasive disease of the breast with a heterogeneous presentation with different malignant potential (223-226). For women having BCS with radiation, the risk of local recurrence is about 1-2% (227-231). For women having mastectomy, the risk of local recurrence ranges about 0-5% (232-237). However, approximately half of the relapses developed after a primary DCIS will be invasive cancer (228, 230, 238).

In the present study we investigate the relationship between ER, PR and HER2 status in the primary lesion and the corresponding ipsilateral event in a cohort with primary DCIS and a known relapse.

In this cohort, no patient received adjuvant endocrine therapy or chemotherapy. However, approximately a third of all patients received postoperative radiotherapy to the remaining breast.

ER (n=112), PR (n=113) and HER2 (n=114) status in both the primary DCIS and the corresponding relapse were determined.

Discordance in ER, PR and HER2 from primary DCIS to relapse was 15.1%, 29.2% and 10.5% respectively (both in situ and invasive relapses included). The receptor conversion was both from positive to negative and from negative to positive with no general pattern being seen in spite of sub-dividing into *in situ* relapse and invasive relapse. Primary DCIS was HER2 positive in 40.3% whereas in situ and invasive relapses were HER2 positive in 42.9% and 34.5% respectively (Table 9).

Table 9. Intra-patient discordances in ER, PR and HER2 status in primary DCIS and corresponding new event presented for in situ- and invasive relapses separately. Adapted from Ref. (239) with permission.

	Primary DCIS/All relapses		Primary DCIS/In situ relapse		Primary DCIS/Invasive relapse	
	Number	Percent	Number	Percent	Number	Percent
ER^ status primary tumor and relapse						
primary pos/relapse pos	77	68.8	34	63	43	74.1
primary pos/relapse neg	9	8	4	7.4	5	8.6
primary neg/relapse pos	8	7.1	4	7.4	4	6.9
primary neg/relapse neg	18	16.1	12	22.2	6	10.3
	112	100	54	100	58	100
PR^ status primary tumor and relapse						
primary pos/relapse pos	49	43.4	20	36.4	29	50
primary pos/relapse neg	19	16.8	13	23.6	6	10.3
primary neg/relapse pos	14	12.4	8	14.5	6	10.3
primary neg/relapse neg	31	27.4	14	25.5	17	29.3
	113	100	55	100	58	100
HER2♣ status primary tumor and relapse						
primary pos/relapse pos	39	34.2	22	39.3	17	29.3
primary pos/relapse neg	7	6.1	5	8.9	2	3.4
primary neg/relapse pos	5	4.4	2	3.6	3	5.2
primary neg/relapse neg	63	55.3	27	48.2	36	62.1
	114	100	56	100	58	100

Abbreviations: ER= estrogen receptor, PR= progesterone receptor, HER2= human epidermal growth factor receptor 2

^ Cut-off value >10% for IHC methods

♣ Analysed using SISH directly, if not available IHC 3+ was used as positive

In addition HER2 positivity was seen more frequently in the primary DCSI group that later developed an *in situ* relapse compared to invasive relapse, 48.3% versus 29.8% (p=0.014) (Table 10).

Table 10. Tumor marker characteristics in 274 primary DCIS with a subsequent ipsilateral event. Adapted from Ref. (239) with permission.

	Primary DCIS (n=274)		Primary DCIS with a subsequent in situ event (N=135)		Primary DCIS with a subsequent invasive cancer (n=139)		**P value ***
	Number	Percent	Number	Percent	Number	Percent	
Primary ER^ status							0.023
positive	152	78.8	69	71.9	83	85.6	
negative	41	21.2	27	28.1	14	14.4	
Primary PR^ status							0.562
positive	113	58.5	53	56.4	60	60.6	
negative	80	41.5	41	43.6	39	39.4	
Primary HER2♣ status							0.014
positive	70	38.7	42	48.3	28	29.8	
negative	111	61.3	45	51.7	66	70.2	

*Comparison between in situ and invasive relapse groups using Fishers test

Abbreviations: ER= estrogen receptor, PR= progesterone receptor, HER2= human epidermal growth factor receptor 2

^ Cut-off value >10% for IHC methods

♣ Analysed using SISH directly, if not available IHC 3+ was used as positive

The intra-patient biomarker status in primary tumor and relapse was stratified into either primary DCIS nuclear grade 1, 2 or 3. The proportion of patients with discordant biomarkers was bigger among the primary DCIS with nuclear grade 3 compared with those with nuclear grade 1 and 2.

Moreover, ER, PR and HER2 discordances were described for two groups separately, i.e. postoperative radiotherapy or no therapy. A trend of a lower proportion of patients with altered ER and HER2 status was seen in the group that did receive radiotherapy compared with the group that received no postoperative radiotherapy, 11.1% (n=3) versus 16.5% (n=14) (p=0.8) and 3.4% (n=1) versus 13% (n=11) (p=0.3) respectively. However, as can be seen, no statistically significant difference was seen between the groups.

6 DISCUSSION

Our studies demonstrate that clinically used biomarkers such as ER, PR and HER2 are unstable during breast cancer tumor progression. Importantly, our results show a significant impact on overall survival by the changes in ER and PR status (paper II and III) and HER2 status (paper I) between the primary and relapse tumors. In addition, the results indicate that adjuvant therapy might be related to the loss of hormonal receptors; and in particular the use of endocrine therapy (paper II and III), although other studies have not been able to demonstrate that (30, 31). These findings strongly indicate an important role for biopsies of tumor related events in the management of patients with a previous breast cancer diagnosis.

Traditionally, ER and HER2 have been assessed in the primary tumor, used to direct therapy decisions in the primary as well as in the relapse situation, assuming these markers are unchanged (52-54, 240). However this approach is no longer adequate. In other words "Put simply, failure to biopsy recurrent or metastatic breast cancer carries a significant risk that our management is inadequately informed and may be inappropriate" from Sharma et al. (240).

Discordance of hormone receptor status between the primary breast cancer and corresponding metastasis was already reported around 30 years ago (37, 241), but did not in general influence management since such discordance has been considered unreliable (242). Today, emerging data (including our data) have demonstrated that discordance of ER, PR and HER2 status occur between the primary tumor and corresponding relapse (28-51). Most studies in this field have been retrospective, however three prospective studies have reported considerable discordances of ER (10-16%), PR (24-40%) and HER2 (3-10%) between the primary tumor and corresponding relapse (30, 31, 48).

To our knowledge, Paper II is the largest study of change in tumor markers between the primary breast cancer and the corresponding recurrence. In this study almost a third of the patients alter hormone receptor status and 15% HER2 status, respectively, during tumor progression.

However, paper II shows a loss of ER between primary tumor and relapse for 113 patients out of 459, (24.6%; 95% CI, 17.1-32.1%), while paper III shows a loss of ER for 15 out of 127 patients, (11.8%; 95% CI, 6.2-17.4). A possible explanation for such discrepancies might be a slight difference between the two cohorts, i.e. all the patients in study II were identified from the Breast Cancer registry at the Oncologic Centre in Stockholm having a breast cancer *relapse* during 1997-2007, (71.9 % ER positive primary tumor) versus all the patients in a geographical region presenting a primary breast cancer during 2000-2011, (85.7% ER positive primary tumor) (paper III). The latter cohort might mirror a more "true" picture of discordance rate between the primary and the metastatic breast cancer since it consists of only primary breast cancer patients unlike the relapse cohort in study II.

Importantly, the results from paper II and III show a significant impact on overall survival by the changes in ER and PR status between the primary and corresponding

recurrence. In other words, it seems that patients with cancers losing hormonal receptors during tumor progression have poorer survival compared to those with stable expression of the hormonal receptors.

Furthermore, paper I shows that HER2 status changed from the primary tumor to relapse in 10% of the total study population. This corresponds to a change in 19% of the primary HER2 positive tumors and 6% of the primary negative cancers to the relapse sites. In addition, impact on survival was seen with a significantly increased risk of dying for patients with discordant HER2 status primary tumor and relapse compared to those with stable HER2 positive disease.

This finding is in line with another study showing that 24% (43/182) of the patients with a primary HER2 positive tumor change to HER2 negative in the relapse site. In addition patients with concordant HER2 status in primary tumor and corresponding relapse had significantly better overall survival than patients with discordant HER2 status (HR 0.47, p=0.003) (29).

In contrast to our study, none of the prospective studies has presented any prognostic influence of biomarker change (30, 31, 48), possibly related to a limited follow-up time. However, a change in patient management in about 15-20% of cases due to biomarker change was reported.

Impact on survival as a result of biomarker change is only shown in few studies including our data (28, 29, 32, 33).

Interestingly, paper II and III show that the proportion of patients losing ER from the primary breast cancer to the relapse was larger among the patients who had received adjuvant endocrine therapy alone or in combination with chemotherapy compared with the group that had received chemotherapy alone or the group that received no therapy. This finding is in line with other studies demonstrating that preoperative therapy such as chemotherapy, trastuzumab and endocrine therapy seems to affect the hormonal and HER2 receptor status of the primary tumor (243-246). In addition, loss of ER has been observed following treatment with endocrine therapy in the advanced settings (51, 247). Also experimental data using a long-term estrogen deprivation model to imitate the clinical situation of breast cancer patients treated with endocrine therapy have demonstrated instability of ER and PR expression on both protein as well as on gene level (248).

In a study from Niikura et al. included patients with paired primary- and metastatic tumors, loss of HER2 overexpression was significantly higher among those who received chemotherapy compared to the patients who did not (p=0.022) irrespectively of whether the patients received trastuzumab therapy (29).

However, the authors of a recent review article identifying several studies (28-34, 39, 40, 48, 50, 51, 249-259) reporting HER2 discordance between primary and metastatic breast cancer suggest that there are several limitations in the majority of these studies (260). The majority of the studies had a retrospective nature limiting their reliability. No clear factor promoting HER2 alteration had been identified and the worse outcome for patients with loss of HER2 might be confounded by lack of targeted therapy in these cases (260).

In contrast, a recent meta-analysis of mostly retrospective studies concluded that it is not likely that technical issues alone explain the discordance of ER, PR and HER2 status between primary breast cancer and relapse (261). If there were only methodological problems one could expect discordance rate to be about the same for

different antigens but this is not the case and furthermore one could expect that there would be similar positive and negative conversion for the biomarkers. However it is much more common with the occurrence of negative conversion between primary- and relapse tumor (261).

ER and HER2 are of particular interest since they are both prognostic markers and predictive factors of response to endocrine therapy and efficacy of trastuzumab therapy respectively in the management of breast cancer patients. Loss of ER and HER2 positivity generally indicates a resistance to endocrine therapy and trastuzumab therapy, whereas gain of ER expression or HER2 positivity may expand the therapy opportunities with endocrine- and/or anti-HER2 directed therapies respectively. Previous data have shown, that patients with an ER negative breast cancer do not benefit from endocrine therapy (16, 262, 263). Moreover, another group has reported that endocrine therapy in patients with an ER negative tumor may be harmful and even worsen survival (264).

Additionally, tumor instability is not seen only between the primary and relapse tumor, but throughout tumor progression (paper II). This indicates the need to considerate retest of any relapse also in the advanced setting for optimal management (i.e. targeted therapy) of the patients.

In paper IV, we addressed the issue of biomarker alteration after primary DCIS to corresponding ipsilateral event. This study demonstrates receptor conversion for ER, PR and HER2 status between primary DCIS and corresponding local relapse in 10 to 30% of instances. However no general pattern for the conversion was seen, not even when stratified for either *in situ* or invasive relapse.

As earlier described, HER2 is of particular interest since it is both a negative prognostic marker and a molecular target for trastuzumab therapy in the management of primary invasive breast cancer. However, the main track of HER2 in DCIS has not been clarified. Some studies have shown a more frequent overexpression of HER2 in DCIS compared to invasive breast cancer and particularly in high grade DCIS (265, 266). In addition, it has been suggested that HER2 overexpression in DCIS is of major importance for tumor progression towards invasive cancer (267-269). The findings from paper IV do not support such an influence regarding tumor progression. Our result shows that HER2 overexpression was more frequent in the group of patients with a primary DCIS that later developed an *in situ* relapse compared to those who developed an invasive relapse, 48.3% versus 29.8% (p=0.014). In addition, in this cohort of DCIS with a known recurrence HER2 status in the primary DCIS was positive in 40.3% of instances whereas *in situ* and invasive relapses were HER2 positive in 42.9% and 34.5% of instances respectively.

This finding is in line with previous studies demonstrating a higher risk of developing a new *in situ* relapse amongst patients with a HER2 positive and Ki67 positive primary DCIS, whereas these patients did not show a higher risk of developing an invasive relapse (270, 271).

There are several possible explanations for the findings of biomarker change during tumor progression (Figure 11). Both technical/methodological issues and pure biological explanation have been proposed (55, 242). For instance, the retrospective study design has often been criticized for relying on pathological reports, reflecting a

variety of tissue processing and sampling techniques which might lead to false discordances in biomarkers (242). However, in the assessment of ER several studies have revealed a high concordance value, around 90% or more between the findings obtained by different methods (i.e. ICC, IHC and biochemical receptor determinations) (118, 122, 210, 211) or by different sampling techniques (212).

The results from paper II and III are both based on different sampling techniques (FNAs, CNBs and surgical excisions) as well as different methods for determination of receptor status. Nevertheless, both studies showed similar biomarker discordance proportions irrespectively of used sampling techniques/methods (Table 11, Paper III). In addition, a subsample of 58 tumors from (Paper II) was reanalyzed for ER status. All the samples with exception of four samples corresponded to the original ER status.

Therefore our findings strongly indicate that the observed receptor changes are reflections of true "biological changes". However, we cannot exclude that methodological issues to some extent have influenced our results.

Table 11. Potential discordances in ER, PR, HER2 and Ki67 status between the primary tumor and relapse.
Presented for different methods; FNA, CNB, excision biopsy. From Paper III.

	All relapses		FNA		Core/excision biopsy	
	Number	Percent	Number	Percent	Number	Percent
Primary tumor and relapse						
ER*						
primary positive/relapse positive	87	68.5	26	74.3	61	66.3
primary positive/relapse negative	15	11.8	4	11.4	11	12.0
primary negative/relapse positive	3	2.4	0	0	3	3.3
primary negative/relapse negative	22	17.3	5	14.3	17	18.5
Total	127	100.0	35	100.0	92	100.0
PR*						
primary positive/relapse positive	30	29.7	7	33.3	23	28.8
primary positive/relapse negative	30	29.7	7	33.3	23	28.8
primary negative/relapse positive	10	9.9	2	9.5	8	10.0
primary negative/relapse negative	31	30.7	5	23.8	26	32.5
Total	101	100.0	21	100.0	80	100.0
HER2♣						
primary positive/relapse positive	14	19.2	3	25.0	11	18.0
primary positive/relapse negative	4	5.5	1	8.3	3	4.9
primary negative/relapse positive	3	4.1	0	0	3	4.9
primary negative/relapse negative	52	71.2	8	66.7	44	72.1
Total	73	100.0	12	100.0	61	100.0
Ki67€						
primary positive/relapse positive	26	47.3	5	55.6	21	45.7
primary positive/relapse negative	7	12.7	1	11.1	6	13.0
primary negative/relapse positive	13	23.6	2	22.2	11	23.9
primary negative/relapse negative	9	16.4	1	11.1	8	17.4
Total	55	100.0	9	100.0	46	100.0

Abbreviations: ER= estrogen receptor, PR= progesterone receptor, HER2= human epidermal growth factor receptor 2, Ki67= proliferation
* Cut-off value ≥ 10% for IHC/ICC methods
♣ Analysed using IHC/ICC or by FISH directly, IHC/ICC 2+ and 3+ confirmed by FISH
€ = Cut-off value > 10% for IHC/ICC methods

Interestingly, several biological explanations have been discussed concerning receptor change such as tumor heterogeneity or clonal selection (272-275). The observed change of hormonal receptor status over time may partly represent tumor progression and clonal selection. In the heterogenic metastatic process cells need to separate from the primary tumor, invade surrounding tissue and basement membranes, proceed to the blood vessel or lymphatic system and survive in the circulation to be able to initiate growth or become dormant in distant/other organs (276).

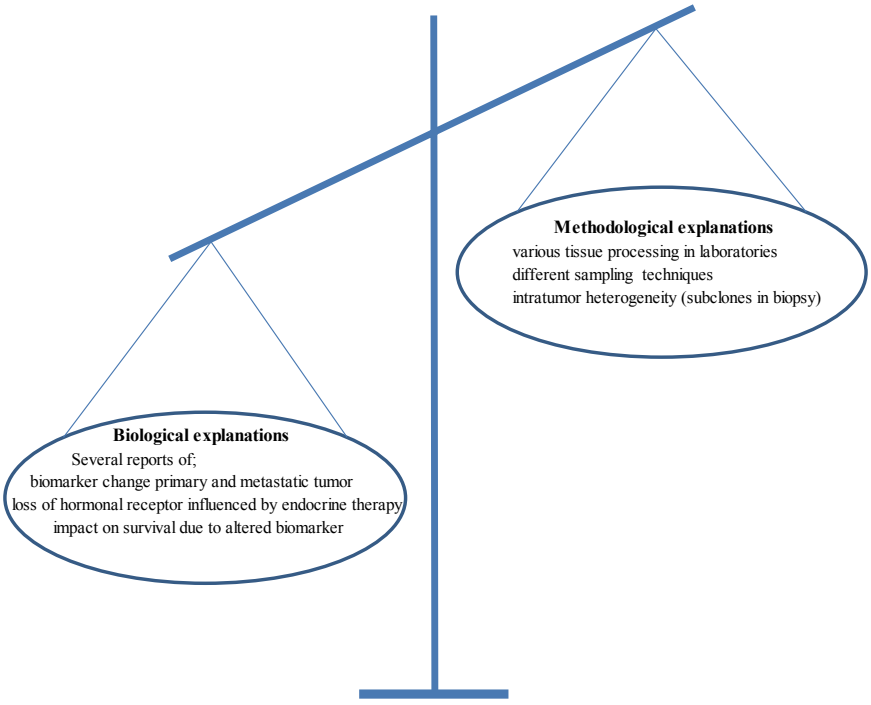
Intratumor heterogeneity of the breast cancer between different patients with different biological/prognostic profiles has been known for decades first recognized by pathologists and more recently by gene expression profiles that have clearly visualized the different patterns of breast cancers (86, 87, 90, 102, 277-279).
Moreover, reports have shown that intratumor heterogeneity can exist both within the tumor, between the primary- and metastatic site as well as between different metastasis (35, 273, 280-288). In the first case varying metastatic capacity between the tumor clones has been described (272, 274, 289). In the second case, gene expression studies have reported heterogeneity between the primary tumor and the corresponding metastasis i.e. gene loss and or increased prevalence of mutation (273, 280, 284, 287, 288). Indeed, new data from our group demonstrate that the overall mutation pressure was larger in the metastatic site compared to the corresponding primary tumor when exome sequencing in 10 paired breast cancer samples was performed. Also loss of heterozygosity and the mutation allele frequencies suggest that there might be multiple clones already up-front and throughout the evolution of new metastases (Källquist.U et al. personal communication). In a previous study from Wu et al. they report heterogeneity between primary and paired metastatic breast cancer as well as among multiple metastases from the same patient (n=10) (35). However, concerning ER and PR status they appeared to be similarly down-regulated in the different metastases (35).
Finally, a report from Gerlinger et al. has demonstrated occurrence of genetic heterogeneity in the primary tumor as well as between different metastases in a patient with renal cell cancer (273).

Additionally, adjuvant therapies may also influence clonal selection resulting in different phenotype of the metastatic- and primary tumor (28, 29, 51, 290). Indeed our data (paper II and III) support such an influence although others have not been able to substantiate that (30, 31).

Other reports have shown that contributors of the stroma and white adipose tissue may harbor important breast cancer functions (199, 201, 291, 292). Bone-marrow-derive mesenchymal stem cells and endothelial progenitor cells may have the capacity to influence tumor progression (197-199, 201).

Alteration in biomarkers have also been shown in studies measuring circulating tumor cells, in which patients with relapse of breast cancer may shift biomarker expression compared to primary tumor (43, 293-295). All these findings support true biological change during tumor progression (Figure 11).

Figure 11. Possible explanations for the findings of biomarker change during tumor progression.



Furthermore, paper III describes what happens during a ten-year-period for diagnosed breast cancer patients with reference to recurrence, new malignancies, benign lesions and tumor marker changes occurring in recurrences in relation to outcome. The unique feature with paper III, is that it consists of more than 2000 primary breast cancer patients from a defined geographical region using the Swedish registers using the unique 12-digits ID-number. Therefore, we will have a "complete picture" of the time range, something that is not easily available in many regions. There was an incidence of approximately 4 % secondary cancers (colorectal-, lung-, skin cancer) and 2 % cancer in situ (skin, breast).

In comparison, a previous study included more than 500 000 women identified from population based breast cancer registries in Europe, Canada, Australia and Singapore in their study and followed them for secondary cancer during 1943-2000. For women followed for up to nine years a secondary cancer of approx. 3.9% was demonstrated. Indeed, women with a primary breast cancer had a 25% increased risk for all secondary cancers compared to women without cancer (296). The reason for the elevated risk of a secondary cancer after primary breast cancer is not clear and both genetic predispositions, shared etiological risk factors and treatment related explanation have been discussed (297).

In another prospective study 205 breast cancer patients had been subjected to a biopsy, 18 patients (8.8%) did not have a recurrence or another malignancy despite clinical diagnosis of recurrence (30).

Radiological assessments by bone scan computerized tomography (CT scan), magnetic resonance imaging (MRI) and positron emission tomography (PET) or PET-CT have clearly increased the sensitivity in the diagnostic procedure. However, they all lack the capacity for reliable distinction of discriminate among metastasis, a new malignancy or benign lesions (55). Some have wrongly taken a positive radiological examination as a proof of certain malignancy (298), which is incorrect. Nevertheless, maybe with the exception making use of the promising specific tumor specific PET tracers used for imaging diagnostics as well as for identification of targeted therapy and therapy resistance (for breast ER and HER2 targeted tracers) (299-305).

Finally, the best management today of suspected breast cancer recurrence is to re-biopsy since that can reveal if the lesion represents a benign condition, another malignancy or a breast cancer relapse, with or without changes of the studied markers.

Clinical implications

The clinical implications of the results from the present studies are important.
Firstly, it seem as if breast cancer patients who have lost either the ER or PR or have discordant HER2 status from primary tumor to relapse have poorer survival rates compared with patients with stable positive biomarkers. Secondly, adjuvant therapy may potentially affect the loss of hormonal receptors.
Thus it seems clear that biopsies of all tumor related events are of major importance as outcome might change management of these patients i.e. targeted therapy, reveal unexpected diagnosis like secondary cancers or indeed, exclude a serious condition.

7 CONCLUSION

- Discordance of receptor status for ER, PR and HER2 occurred between primary tumor and corresponding relapse in 10-40% of the studied patients.

- An increased risk of dying was seen in patients losing ER or PR in the relapses compared with the patients who had stable positive expression of ER or PR during tumor progression.

- Increased risk of dying was seen in patients with discordant HER2 status between primary and recurrent tumor compared with patients who had a stable HER2 positive expression/amplification during tumor progression.

- Adjuvant therapy seems to influence the loss of hormonal receptors between the primary cancer and corresponding recurrence, in particular for those patients who had received endocrine adjuvant therapy.

- Biopsies during follow-up breast cancer patients from a population based cohort revealed development of recurrence (8.4%), cancer *in situ* (skin, breast) (1.9%) secondary malignancies (colorectal-, lung- and skin cancer) (4.4%) as well as benign conditions (40.8%).

- Our studies support that one should strongly consider to re-biopsy all tumor related events, while this strategy may enable the use of targeted therapies, to discover other malignancies or benign lesions requiring a completely different management.

8 FUTURE PERSPECTIVES

Our studies demonstrated instability of tumor markers during tumor progression. In addition impact on survival was shown.

Ideally ”the changeable nature” of a tumor would preferably be monitored throughout tumor progression. Taken together, we expect future clinical trials to address the issue of heterogeneity and obtain tissue from the metastatic setting as well as from the adjuvant setting (i.e. preoperative studies) to assess relevant markers which seems to be essential for therapy resistance, importance in the development of new drugs and therefore open into a better overall survival for the patients (26, 306-308).

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