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Predicting Prognosis and Tamoxifen Response in Breast Cancer

With a special focus on contralateral breast cancer

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To all patients at the Clinic of Oncology

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Papers I-IV

List of papers

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

I AIB1 is a predictive factor for tamoxifen response in premenopausal women.

Sara Alkner, Pär-Ola Bendahl, Dorthe Grabau, Kristina Lövgren, Olle Stål, Lisa Rydén, Mårten Fernö. On behalf of the South Swedish and South-East Swedish Breast Cancer Groups.

Annals of Oncology 2010; 21(2): 238-244

II The role of AIB1 and PAX2 in primary breast cancer; validation of AIB1 as a negative prognostic factor

Sara Alkner, Pär-Ola Bendahl, Dorthe Grabau, Per Malmström, Mårten Fernö, Lisa Rydén. On behalf of the South Swedish Breast Cancer Group.

Manuscript 2012

III Tamoxifen reduces the risk of contralateral breast cancer in premenopausal women: Results from a controlled randomized trial

Sara Alkner, Pär-Ola Bendahl, Mårten Fernö, Bo Nordenskjöld, Lisa Rydén. On behalf of the South Swedish and South-East Swedish Breast Cancer Groups.

European Journal of Cancer 2009; 45(14):2496-502

IV Prediction of outcome after diagnosis of metachronous contralateral breast cancer

Sara Alkner, Pär-Ola Bendahl, Mårten Fernö, Jonas Manjer, Lisa Rydén.

BMC Cancer 2011; 11: 114

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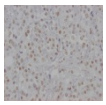
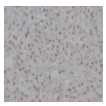
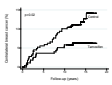
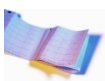
Dissertation at a glance

Study	Question	Methods	Results and Conclusion
I 	Does AIB1 affect prognosis and tamoxifen response?	AIB1 was investigated with IHC in premenopausal patients randomized to tamoxifen for two years *vs.* control.	High AIB1 corresponds to a worse prognosis. On the other hand, patients with high AIB1 respond very well to tamoxifen, increasing RFS and OS to the same levels as in patients with low AIB1.
II 	Can the prognostic effect of AIB1 be confirmed in an independent cohort, and is this effect modified by PAX2?	AIB1 and PAX2 were investigated by IHC in two independent cohorts, one with and the other without adjuvant tamoxifen.	AIB1 was confirmed as a negative prognostic factor in patients not receiving tamoxifen. PAX2 does not seem to modify this effect.
III 	Does tamoxifen reduce the risk of CBC in premenopausal women?	Controlled trial of premenopausal patients randomized to tamoxifen for two years *vs.* control.	12% of all patients and 20% of the women <40 years old developed CBC. Tamoxifen reduced the risk by 50% in all women, and by 90% in women <40 years.
IV 	Is prognosis following CBC affected by the time interval between tumours and the mode of detection?	Cohort study including 723 patients with CBC diagnosed from 1977 to 2007 within the Southern Healthcare Region of Sweden.	Patients with a short time interval between tumours have a worse prognosis. In addition, patients symptomatic at diagnosis of CBC have a higher risk of developing distant metastases than those diagnosed by mammography or clinical examination.

Abbreviations: AIB1 amplified in breast cancer 1, CBC contralateral breast cancer, IHC immunohistochemistry, OS overall survival, PAX2 paired box 2 gene product, RFS recurrence-free survival.

Abbreviations

AD	activation domain
AF	activation function
AI	aromatase inhibitor
AIB1	amplified in breast cancer 1
Ap1	activation protein 1
ATAC	Arimidex, Tamoxifen, Alone or in Combination
BC1	breast cancer number 1
BC2	breast cancer number 2
Bcl-2	B-cell lymphoma 2
BIG	Breast International Group
CA	coactivator
CBC	contralateral breast cancer
CI	confidence interval
CISH	chromogenic *in situ* hybridization
CMF	cyclophosphamide, methotrexate, fluorouracil
DDFS	distant disease-free survival
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
ER	oestrogen receptor
ERE	oestrogen response element
FISH	fluorescent *in situ* hybridization
GF	growth factor
GPR30	G-protein-coupled oestrogen receptor 30
GTF	general transcription factor
HER2	human epidermal growth factor receptor 2
HR	hazard ratio
IHC	immunohistochemistry
LHRH	luteinizing hormone-releasing hormone
MRI	magnetic resonance imaging
N	number
N0	lymph-node-negative
NHG	Nottingham histological grade
NR	nuclear hormone receptor
OS	overall survival
PAX2	paired box 2 gene product
PgR	progesterone receptor
RFS	recurrence-free survival
RTK	receptor tyrosine kinase
SERM	selective oestrogen receptor modulator
SOFT	Suppression of Ovarian Function Trial
Sp1	specificity protein 1
SRC	steroid receptor coactivator
TAnDEM	Trastuzumab and Anastrozole Directed against ER-positive HER2-positive Mammary carcinoma
TEAM	adjuvant tamoxifen and exemestane in early breast cancer
TF	transcription factor
TMA	tissue microarray

Abstract

One of the great challenges in breast cancer treatment today is to customize adjuvant treatment to each patient's individual needs. To do this it is necessary to learn more about the prognostic and treatment predictive factors that determine the risk of relapse and response to a certain mode of treatment. This thesis describes studies on the effect of amplified in breast cancer 1 (AIB1), a coactivator of the oestrogen receptor, on prognosis and tamoxifen response through a controlled trial on premenopausal patients randomized to tamoxifen or a control group. AIB1 was found to be a negative prognostic factor, although patients with high AIB1 responded very well to tamoxifen. The findings were validated in two independent cohorts, one consisting of premenopausal patients not receiving tamoxifen, and the other of pre- and postmenopausal patients receiving tamoxifen.

It has recently been suggested that the effect of AIB1 is modified by paired box 2 gene product (PAX2). PAX2 is a transcription factor important during embryogenesis, and may also play a role in carcinogenesis. This is the first time PAX2 has been investigated in well-defined cohorts of patients receiving or not receiving tamoxifen. PAX2 was not found to affect prognosis on its own, or to modify the effect of AIB1.

The second part of this thesis focuses on contralateral breast cancer (CBC). Within their lifetime, previous breast cancer patients have a 2-20% risk of developing a second tumour in the contralateral breast. From the trial on premenopausal patients randomized to tamoxifen or control, it was found that without tamoxifen 12% developed CBC within a median follow-up period of 14 years. This risk was even higher in the youngest women (<40 years), in which 20% developed CBC. Treatment with tamoxifen reduced the risk by 50% in all patients, and by 90% in the youngest women.

Since CBC is still a rather rare event, previous studies are often small or based only on register data. Detailed patient, tumour and treatment information has been collected for a large cohort (>700) of patients with CBC in the Southern Healthcare Region of Sweden. From these data it was found that a short time interval between tumours was associated with a poorer prognosis, especially in young patients. This could indicate that some of these CBCs are in fact metastases of the first tumour, and would thus require different treatment. It could also be that tumours that develop soon after previous treatment have developed resistance to treatment and are of a more aggressive phenotype.

Finally, it was found that patients who first noticed symptoms of their CBC themselves had a higher risk of developing metastases than patients diagnosed by mammography or clinical examination in a follow-up programme. The difference in prognosis in relation to mode of detection remained even when the time interval between tumours was ≥10 years, indicating that a long follow-up period is valuable.

Background

Epidemiology, risk factors and diagnosis

In Sweden, almost 8000 women are diagnosed with breast cancer every year, and 90,000 women are currently living with the disease[1, 2]. Breast cancer is the most common female cancer worldwide, and constitutes almost 30% of all malignant disease in women in Sweden. The incidence is higher in industrialized countries than in most developing countries of the world. In 2008, the incidence varied from almost 90 per 100,000 women in Western Europe to less than 20 per 100,000 women in eastern Africa[3]. The differences can be explained in part by access to medical care, the quality of cancer registers and differences in life expectancy. However, additional explanations could be differences in hereditary and life-style factors[4]. During the past 20 years, the incidence of breast cancer in Sweden has increased by 1.3% annually, although during the past 10 years the increase has been somewhat less, 0.9%[1]. At the same time mortality has decreased, and the 5-year survival rate today is almost 90%[1]. This improved outcome is probably due to early detection by mammographic screening and modern systemic adjuvant treatment. The increased incidence, on the other hand, is more difficult to explain. Possible explanations could be changes in lifestyle and increasing exposure to female hormones. Examples of this are obesity, use of hormone replacement therapy, lower age at menarche and higher age at first pregnancy.

Risk factors for developing breast cancer

The risk of developing breast cancer increases with age. Although the disease is quite rare among women younger than 45 years, by 75 years of age 11% of Swedish women will have developed breast cancer[1]. The most important risk factor is life-time exposure to female sex hormones. Age at menarche, menopause and first pregnancy therefore affect the risk. Increased risk is also correlated to hormone replacement therapy, oral contraceptives, obesity and height[5]. Breast feeding, on the other hand, reduces the risk[5]. Apart from risk factors associated with exposure to sex hormones, an increased risk has been related to previous benign breast disease, alcohol intake and prior exposure to radiation[5, 6].

Another important risk factor is a family history of breast cancer. The most well-known high-risk genetic predisposition arises from mutations in the BRCA1 or BRCA2 gene. The prevalence of these mutations among breast cancer patients varies with geographic location, the patient's age and family history. Among young breast cancer patients (<41

years) in southern Sweden as many as 10% seem to carry mutations in the BRCA1 gene[7]. Inherited mutations in p53 and PTEN are also associated with syndromes that include a high risk of breast cancer, although these syndromes are rare. Many cases of familial predisposition can still not be explained. Studies on twins suggest that 20-30% of all breast cancer is due to genetic predisposition[8], and a woman's risk of developing breast cancer is increased by a factor of two if she has a first-degree relative with the disease[9]. The more cases in the family and the younger the relatives were when they developed breast cancer, the higher the risk.

Diagnosis

Today about 50% of breast cancer patients in the Southern Healthcare Region of Sweden are diagnosed by screening mammography. For a screening programme to be meaningful, cost-efficient and ethically acceptable, a number of criteria should be met[4, 10]:

1. The disease should be common, and when not treated have serious consequences for the individual and/or society.
2. The screening method should be safe, simple, not too expensive, and have a high sensitivity and specificity of discovering the disease.
3. Effective treatment should be available, so that early detection leads to reduced mortality and/or increased quality of life for those diagnosed.
4. The screening method should be cost-efficient and possible to repeat at a certain time interval.

Several studies since the 1970s have investigated the effect of screening mammography. The first results from Swedish trials were published in the 1980s, showing a significant reduction in breast cancer mortality among the women invited[11-14]. This led to common guidelines being issued by the Swedish National Health Board in 1986[15], and since 1997 all regions of Sweden have established mammographic screening programmes[10]. In the Southern Healthcare Region of Sweden mammographic screening has been available since 1989. Several recent reviews and meta-analyses suggest the relative risk reduction of breast cancer mortality to be around 15%[16-18]. However, the results are not undisputed. Some claim that the increased rate of survival is questionable, while there is a substantial risk of overdiagnosis[19].

When a suspicious mass is detected by screening or by palpation, a combination of three diagnostic modalities is used: Clinical examination of the breast and loco-regional lymph nodes, radiologic examination combined with ultrasound, and in some cases magnetic resonance imaging (MRI), and histological examination of fine-needle aspirates, often supplemented with a core biopsy. Complete diagnostic work-up is mandatory, and for diagnosis a confirmative biopsy is required.

Treatment of primary breast cancer

Surgery

Although there is evidence that breast cancer was treated surgically in ancient Greece, the procedure was quite challenging and often considered more harmful than beneficial. It was not until the middle of the 19th century, after the introduction of inhalation anaesthesia and aseptic techniques, that surgical treatment for breast cancer became a standardized procedure. Radical mastectomy involving removal of the entire breast, axillary lymph nodes, and the minor and major pectoral muscles was introduced in 1882 by Halstead[20]. This was replaced by the modified radical mastectomy described by Patey in 1948[21]. With this method, the pectoral muscle is spared, and this remained the standard procedure until the 1970s. Several prospective randomized studies then compared radical mastectomy with partial mastectomy followed by radiotherapy, showing no difference in survival between the two techniques[22-26]. In the 1980s, breast-conserving therapy therefore became the treatment of choice for patients with early breast cancer.

The primary location of metastases arising from breast cancer is the lymph nodes in the axilla. Today, these nodes are often investigated with the sentinel node technique. This technique is based on the knowledge that lymphatic drainage to the axilla from the breast passes through a single or a few lymph nodes. It should therefore be sufficient to analyse only this node (or these nodes) as an indicator of axillary node status. A frozen section of the sentinel node is investigated peroperatively, and only when malignant cells are present is further dissection of the axilla performed. A recent randomized trial showed no differences in overall survival (OS), disease-free survival or regional control when comparing sentinel node with conventional axillary dissection[27]. The sentinel node technique is also associated with fewer complications[28-30].

Radiotherapy

Postoperative radiotherapy is administered in order to eradicate possible residual microscopic disease. A meta-analysis by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) in 2011 showed postoperative radiotherapy after breast-conserving therapy to reduce the relative risk of recurrence by 50%, the largest effect being seen on local recurrences[31]. Overall, about one breast cancer death by year 15 was avoided for every four recurrences avoided[26, 31] The proportional benefit was similar regardless of other prognostic factors such as lymph node status, tumour size or the patient's age[31]. The benefit of radiotherapy is thus dependent on the patient's inherent risk of recurrence. According to international guidelines, radiotherapy is indicated if the risk of developing a

local recurrence within the next 20 years is higher than 20% (Eusoma, ASCO, Swedish Breast Cancer Group). This includes women undergoing partial mastectomy, women with a tumour larger than 50 mm and women with more than three lymph node metastases in the axilla[32].

Acute side effects of radiotherapy are erythema of the skin and pneumonitis. Late side effects include brachial plexus neuropathy and lymphoedema. Previous studies have also shown an excess mortality from cardiac disease and lung cancer among patients treated with radiotherapy, although these risks seem to be reduced with the more modern techniques used today[5, 26, 32].

Systemic treatment

Systemic treatment includes chemotherapy, endocrine treatment and targeted drugs, such as antibodies. Treatment can be given neoadjuvantly, adjuvantly or for palliation. In the neoadjuvant setting the primary goal is to reduce the size of the tumour, making it operable. Adjuvant therapy is given postoperatively to eradicate micro-metastases and to reduce the risk of recurrence. When treatment is given as a preventive measure the possible side effects must be related to the risk of recurrence. Generally, adjuvant therapy is considered if the risk of recurrence is higher than 20-30%. However, endocrine treatment with few side effects can also be considered for patients with a lower risk[32]. In the palliative setting, the purpose of treatment is to shrink the tumour and metastases, reduce symptoms and prolong life. The following sections describe adjuvant systemic treatment.

Chemotherapy

The idea of treating cancer with chemotherapy arose from research on poisonous gases in the period between the two World Wars. Laboratory personnel accidentally exposed to nitrogen mustard were found to develop leucopoenia. This substance was therefore investigated in the treatment of lymphoma. The first patients were treated in 1942 at Yale University in the USA[33]. Therapy was initially given as small daily doses. However, experiments soon showed intermittent treatment to be more effective, exploiting the difference in resilience between normal and cancer cells[33]. Poly-chemotherapy has also proven to be more effective than single-agent regimes in neoadjuvant and adjuvant settings[34]. The reasons for this are the potential synergetic effects and the different toxicity profiles, allowing more intense treatment.

Adjuvant treatment with poly-chemotherapy reduces breast cancer mortality by about one third[35, 36]. Although the relative reduction in breast cancer mortality is similar for patients

with and without lymph node metastases, the absolute benefit is greater in the group with lymph node metastases due to the higher initial risk. Comparisons between different regimes have shown anthracycline-based therapy to be more effective than CMF (cyclophosphamide, methotrexate, fluorouracil), and the addition of a taxane to anthracycline-based therapy to be more effective than anthracyclines alone[32, 35, 36]. Adjuvant chemotherapy is today recommended for most patients with lymph-node-positive disease, and for patients with lymph-node-negative disease if the tumour exhibits a low sensitivity to endocrine treatment, or if other risk factors are present[32].

Endocrine treatment

One of the most validated risk factors for developing breast cancer is exposure to oestrogen. Most invasive breast cancers (70-80%) express the oestrogen receptor (ER), and are dependent on oestrogen for their survival. It was discovered early on that blocking the ER pathway could be used as a treatment strategy. In 1896 it was shown that oophorectomy had a good clinical effect on locally advanced breast cancer in premenopausal women[20]. The ER pathway can be targeted by inhibiting the ER (tamoxifen), or by removing the ligand oestrogen (oophorectomy or aromatase inhibitors (AIs)). In premenopausal patients the ovaries are the main source of oestrogen, while in postmenopausal women oestrogen is predominantly produced by aromatization of adrenal and ovarian androgens in the liver, muscle and fat tissue[37]. AIs block this aromatization in peripheral tissue, although they do not affect the production of oestrogen in the ovaries. Hence, AIs are only effective in postmenopausal patients, while tamoxifen can be used for all women regardless of menopausal status.

While tamoxifen, AIs and ovarian suppression are the methods of treatment currently used in the adjuvant setting, additional endocrine therapies are used in metastatic disease. Examples are fulvestrant, a pure ER antagonist that also downregulates the ER, and synthetic progestogens such as megestrol acetate. In addition, high doses of oestrogen can be effective in patients resistant to previous endocrine therapy[38]. This thesis focuses mainly on adjuvant treatment; ovarian suppression and AIs are discussed below, and tamoxifen in a later chapter.

Ovarian suppression

In premenopausal women oestrogen levels can be dramatically reduced by ovarian ablation. This can be achieved surgically by oophorectomy, by radiotherapy or by using LHRH (luteinizing hormone-releasing hormone) agonists to suppress ovarian function. Ovarian ablation has been found to significantly reduce recurrences and breast cancer mortality[39]. However, the results of more recent trials are not as convincing as those from

earlier trials, in which ovarian ablation was not tested against a background of other effective systemic treatments[35]. The 2005 EBCTCG overview showed an absolute improvement in OS of 3.2% after 15 years[35]. Another recent meta-analysis showed LHRH agonists to significantly reduce the relative risk of recurrence by 12.7%, and death by 13.6%, when given in combination with tamoxifen, chemotherapy or both[40]. However, the major advantage seems to be in patients not receiving tamoxifen, while the combination of a LHRH agonist and tamoxifen was not shown to significantly improve the prognosis compared to tamoxifen alone[40, 41].

When comparing a LHRH agonist alone or in combination with tamoxifen with CMF-based chemotherapy alone, the regimes seemed to be equally efficient in ER-positive premenopausal patients[40]. Treatment with a LHRH analogue could hence be considered when there are contradictions for chemotherapy. LHRH agonists also show a small additional benefit when used together with chemotherapy, but only in the youngest premenopausal patients, <40 years old[40]. This could be due to the fact that chemotherapy is more likely to induce permanent amenorrhoea the closer the woman is to her natural menopause. However, the possible advantage of including LHRH agonists in adjuvant treatment of premenopausal patients must be further evaluated. A large study currently investigating this is the SOFT study (Suppression of Ovarian Function Trial), in which several Swedish hospitals are taking part. Treatment with tamoxifen is being compared with the combination of a LHRH agonist and tamoxifen or the AI exemestane.

Aromatase inhibitors

Several recent studies comparing five years adjuvant treatment with tamoxifen *vs.* five years of AIs have found AIs to be significantly more effective. A recent meta-analysis by Dowsett et al. showed an absolute reduction of recurrences of 2.9% after five years and 3.9% after eight years[42]. The proportional decrease was, however, greater for local recurrences and contralateral breast cancer (CBC) than for distant metastases. After 5 years there was a 1.1% absolute reduction in breast-cancer-free mortality. Neither this nor OS was significantly different between the groups, although this might change with a longer follow-up.

Another approach has been sequential treatment starting with either tamoxifen or AIs, and then changing to the other after 2-3 years of treatment. For patients receiving sequential treatment starting with tamoxifen compared to tamoxifen alone, the meta-analysis showed a 3.1% absolute reduction in recurrences after five years. Switching treatment also significantly reduced breast-cancer-free mortality, with an absolute gain of 0.7%[42]. The advantage of switching to AIs is not lost post-treatment, although there does not seem to be any additional benefit once treatment has ceased[43]. In the BIG 1-98 trial (Breast International Group) and the TEAM trial (adjuvant tamoxifen and exemestane in early

breast cancer) sequential treatment with tamoxifen and an AI was compared with treatment with an AI alone. No significant differences were seen between the treatment groups[44, 45]. When comparing sequential treatment starting with the AI letrozole and sequential treatment starting with tamoxifen in BIG 1-98, patients starting with letrozole seemed to have a slightly, though not significantly, better recurrence-free survival (RFS)[45].

Although prolonged treatment with tamoxifen beyond five years is not recommended, continued treatment with AIs after five years of tamoxifen treatment seems to improve both RFS, distant disease-free survival (DDFS) and OS[46-49]. According to data presented at the San Antonio Breast Cancer Symposium in 2009, the benefit of continued treatment is highest in premenopausal women who have become postmenopausal during treatment[50].

Endocrine treatment is today recommended to, in principle, all patients with ER-positive breast cancer. Although monotherapy with tamoxifen is still offered to postmenopausal patients with a low risk of recurrence, AIs alone or sequentially with tamoxifen are recommended for patients with an intermediate or high risk[32]. Since AIs are not effective in pre- or perimenopausal women, special care must be taken to ensure that the patient is truly postmenopausal before commencing treatment with AIs. Premenopausal women should instead be given tamoxifen.

Targeted therapy

The term “targeted therapy” is generally used to describe medications disrupting specific molecules involved in carcinogenesis and tumour growth, rather than generally affecting rapidly dividing cells, as is the case in most traditional forms of chemotherapy. The monoclonal antibody trastuzumab is directed against the human epidermal growth factor receptor 2 (HER2) oncogene, which is expressed in 15-30% of breast cancers[4, 51]. Patients overexpressing HER2 have a poorer prognosis and an increased risk of metastasis. However, the use of trastuzumab in addition to chemotherapy and endocrine treatment in these patients significantly improves RFS and OS[52-55]. Today, trastuzumab is recommended for most tumours overexpressing HER2. One year of treatment is the current standard, although the optimal treatment time must be further investigated.

Other drugs currently being investigated and used in targeted treatment of breast cancer include the tyrosine kinase inhibitor lapatinib and the angiogenesis-inhibiting antibody bevacizumab.

Prognostic and treatment predictive factors

Prognostic and treatment predictive factors are of great importance in clinical practice regarding the choice of adjuvant therapy after breast cancer surgery. Several of them, such as the ER and HER2 are also used as therapeutic targets. The prognostic and treatment predictive factors used in clinical routine today are presented below.

Patient-related characteristics

Two important patient-related risk factors are age and menopausal status. Breast cancer at a very young age (<35) seems to be particularly aggressive and is associated with an unfavourable prognosis[56, 57]. These tumours are often ER-negative, of a higher grade, and have a higher proliferation index[57]. Likewise, poorer prognosis has also been seen in the oldest women (>70)[56], which could be partly explained by comorbidity and a lower tolerance to therapy. In addition, menopausal status has an impact on the choice of treatment. For example, AIs are not effective as long as the ovaries are producing oestrogen. Several other patient-related factors have also been suggested to affect prognosis, such as race, weight, physical activity, diet, alcohol consumption and socioeconomic factors[6, 58-60].

TNM classification

The clinical stage of the disease is determined by the TNM-classification, based on tumour size (T), lymph node involvement (N) and the presence of distant metastasis (M). These are three important risk factors that together provide prognostic information on which decisions regarding treatment are based.

Histological classification

The dominant morphological subtypes are ductal carcinomas (75%) and lobular carcinomas (5-15%). Examples of other rarer subtypes are mucinous, medullary, apocrine and tubular carcinomas[61]. Histological subtype has a relatively small prognostic impact. However, mucinous, tubular and medullar carcinomas have a better prognosis than ductal and lobular subtypes[62].

Nottingham Histological Grade

The Nottingham histological grade (NHG) is determined by the method described by Elston and Ellis[63]. Tubule formations, degree of nuclear polymorphism and mitotic count are graded separately, and the combined score determines the histological grade: grade 1 (total score 3-5), grade 2 (total score 6-7) and grade 3 (total score 8-9). The NHG was

constructed to express the aggressiveness of the tumour, and is strongly correlated to prognosis.

Oestrogen and progesterone receptors

The most important role of the ER is in predicting the tumour's responsiveness to endocrine treatment. Expression of the progesterone receptor (PgR) may provide some additional information in the ER-positive subgroup[64, 65], but its value has been questioned, and recent meta-analyses failed to show that PgR was of any additional value in predicting tamoxifen response[35, 66]. Although response to endocrine treatment seems to increase gradually with increased ER expression[65], a cut-off of 10% positive cells determined by immunohistochemistry (IHC) is currently used in Sweden to classify tumours as ER-positive. However, internationally a cut-off of 1% is often used[67, 68].

HER2

HER2 belongs to the human epidermal growth factor receptor family, consisting of four different receptor tyrosine kinases: HER1 (EGFR), HER2, HER3 and HER4. The HER family plays an important role in cell proliferation, differentiation, adhesion, survival and migration[69, 70]. Upon ligand binding the receptors dimerize and the kinase region is activated. This leads to signalling through multiple pathways including RAS/MAPK and PI3K. Unlike the other family members, HER2 has no ligand-binding site of its own. Instead it forms heterodimers with the other HER receptors, thereby extending ligand interaction and prolonging pathway activation[69, 70]. Overexpression of HER2 is seen in about 90% of all ductal carcinomas *in situ*, and in 15-30% of invasive breast cancers[51, 70]. In invasive cancers overexpression is associated with a more aggressive phenotype and poorer prognosis[71, 72]. Treatment specifically targeting HER2 is available, such as the monoclonal antibody trastuzumab and the tyrosine kinase inhibitor lapatinib[69, 73]. The level of expression of HER2 is determined by IHC, resulting in a score of 0, 1+, 2+ or 3+. In the case of moderate to strong staining (2+ or 3+) gene amplification is evaluated using *in situ* hybridization techniques. This is described below in the Methods section.

Ki67

Ki67 is a proliferation marker universally expressed in proliferating tissue, but absent in quiescent cells[74]. Despite its presence during all phases of the cell cycle, its function is still relatively unknown. Ki67 has been found to have some prognostic value, especially in node-negative breast cancer[74, 75]. In addition, changes in Ki67 are correlated to the response to both endocrine treatment and chemotherapy[74, 76]. Another marker of proliferation is the S-phase fraction.

Molecular subclasses

Gene expression profiling has allowed subgroups of breast cancers to be correlated to prognosis and treatment response[77-80]. Eventually, four subgroups have emerged: luminal A, luminal B, HER2 and triple negative. The concordance between molecular subtypes and IHC phenotypes is relatively high (75-90%)[80]. The use of the following IHC markers to determine molecular subtype has been suggested according to the St Gallen International Expert Consensus 2011[68, 81]:

1. Luminal A (ER+ and/or PgR+, HER2-, low Ki67)
2. Luminal B (ER+ and/or PgR+, HER2- with high Ki67 or HER2+ with any Ki67)
3. HER2 (ER-, PgR-, HER2+)
4. Triple negative (ER-, PgR-, HER2-)

However, the added value of PgR has been questioned[80]. If the expression of Ki67 is not available, histological grade can be used to separate the luminal A from the luminal B (HER2-negative) subtype[68, 80]. One major advantage with this system is the ability to subdivide the large group of ER-positive tumours into a low- and high-risk group[80, 82]. Other prognostic gene signatures available are the Oncotype DX® and the MammaPrint®. Trials are ongoing to clarify their roles in clinical practice[68].

The oestrogen receptor

In normal breast tissue about 15-25% of the epithelial cells express the ER. These cells are largely non-dividing, while oestrogen-stimulated proliferation instead occurs in the surrounding ER-negative cells. It has therefore been suggested that ER-positive cells induce growth of surrounding cells through paracrine secretion[83]. On the other hand, 70-80% of breast cancers express the ER and depend on oestrogen for survival and proliferation[4, 83]. The ER is an intracellular receptor belonging to the steroid nuclear receptor super-family of transcription factors[83]. The receptor contains two activation domains: activation function (AF) 1, which is regulated by phosphorylation, and AF2, which is regulated by oestrogen binding[83-86]. AF1 and AF2 can activate transcription individually and/or synergistically.

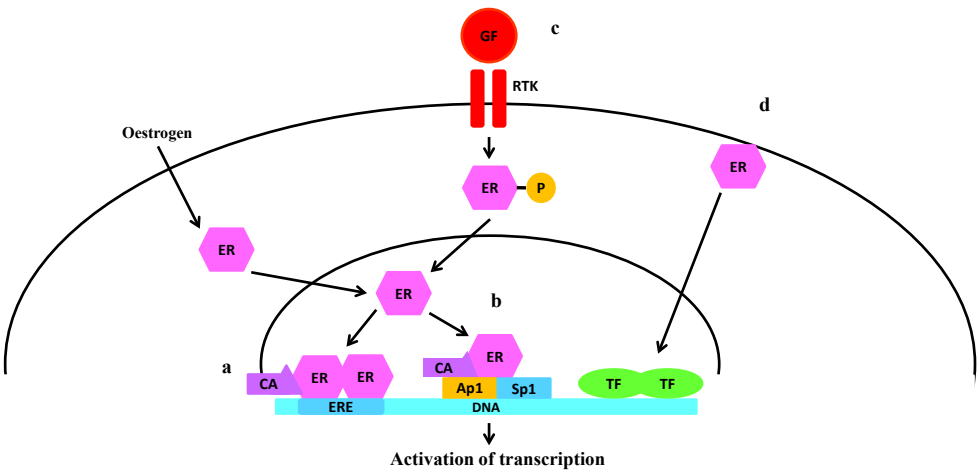


Figure 1. ER activation.

a Dimeric binding of ER to EREs.
b Protein-protein interaction with TFs such as Ap1 and Sp1.
c Activation by RTKs.
d Non-genomic events mediated by the ER, activating intracellular signalling cascades and TFs.

Abbreviations: Ap1 activation protein 1, CA coactivator, ER oestrogen receptor, ERE oestrogen response element, GF growth factor, P phosphorylation, RTK receptor tyrosine kinase, Sp1 specificity protein 1, TF transcription factor.

In classical oestrogen signalling ligand binding of oestrogen to the ER leads to a conformational change in AF2, facilitating interaction with coactivators and histone acetyltransferases[83, 87]. In complex with these factors the ER then activates gene expression on a nuclear level. This can be done by direct dimeric binding of the ER to specific DNA response elements, so-called oestrogen response elements (EREs) (Figure 1, step a). It can also take place through interaction with other transcription factors, such as activation protein 1 (Ap1) and specificity protein 1 (Sp1) (Figure 1, step b)[84, 86, 87]. Apart from the classical ligand-bound pathway, the ER can be activated by signalling events downstream of receptor tyrosine kinases, such as HER2 (Figure 1, step c). Additionally, signalling can be mediated by non-genomic events by ERs localized at the cell membrane or in the cytoplasm (Figure 1, step d)[87, 88].

Oestrogen receptor beta

The previous section refers to the classical ER, ER-α. However, in 1996 a new class of oestrogen receptors was discovered, ER-β[89]. The ERs can form homo-dimers (ER-α:ER-α, ER-β:ER-β) or hetero-dimers (ER-α:ER-β) on EREs, leading to different transcriptional activity[90]. Although expression of ER-α is essential for mammary development in mice, the mammary glands of ER-β-null mice develop normally[91].

The role of ER-β in breast cancer is still not fully understood. Generally, ER-β is considered to oppose the effect of ER-α. The addition of ER-β to ER-α-positive cell lines impairs the ability of oestrogen to stimulate proliferation[86, 92, 93], and while the expression of ER-α is generally induced during carcinogenesis, the expression of ER-β is reduced, suggesting a role for ER-β as a tumour suppressor[86, 93, 94]. Due to the lack of well-characterized ER-β-specific antibodies, early trials on the role of ER-β as a prognostic and treatment predictive marker were carried out by correlating survival to ER-β mRNA. The results of these studies were inconsistent. In some, ER-β mRNA was found to be a negative prognostic factor and related to endocrine resistance, while in others ER-β mRNA was correlated with a good prognosis[86, 93, 94]. One problem associated with this method is that mRNA levels are not exact predictors of protein levels. mRNA is also measured in pieces of tissue that could include several different cell types, not only tumour cells[86, 94]. Since ER-β-specific antibodies have become available, several studies have been carried out on ER-β using IHC. The vast majority of these studies support the role of ER-β as a positive prognostic marker, especially in relation to tamoxifen treatment[86, 94]. ER-β is thus today generally considered a predictor of good response to endocrine therapy[86]. The presence of ER-β may also explain why some ER-α-negative tumours still respond to tamoxifen[86].

Due to the still relatively unclear function of ER-β, the investigations described in this thesis were limited to ER-α. Hence, in the following sections, as well as in the papers, the term ER refers to ER-α.

Nuclear Hormone Receptor Coregulators

Transcriptional regulation of nuclear hormone receptors (NRs) involves protein-protein interactions between the receptor, coregulators and the transcriptional machinery at the chromatin of target genes. Coregulators are divided into two classes: coactivators, which enhance transcription, and corepressors, which inhibit it. They function in large complexes of approximately six to seven coregulators. Most are enzymes that participate in remodelling the local chromatin structure at the target promoter, initiating transcription, regulating RNA synthesis and splicing, and finally destroying the active transcription factors[95, 96]. These stages of transcription are controlled by sequential occupation of the promoter by specific coregulators complexes that direct the reaction[95, 97].

So far, the vast majority of known coregulators are coactivators[95]. Most influence the activity of multiple NRs and occur in the majority of tissues. However, each tissue has a specific relative concentration of different factors[95]. Their activity is regulated by cellular concentration and by post-translational modifications, predominantly phosphorylation and monoubiquitylation[95, 98].

The p160/SRC gene family

The p160/SRC (steroid receptor coactivator) genes were among the first characterized NR coactivators, and include SRC-1/NCOA1, SRC-2/TIF2/GRIP1, and AIB1/ACTR/RAC3/SRC-3/TRAM1/NCOA3. These proteins serve as a platform for the assembly of coactivator complexes on the regulatory region of NR-targeted genes, acting as bridging factors between the receptor and histone-modifying regulators[96]. The amino acid sequence and the function of SRC proteins are relatively well conserved between species[97]. There is also a sequence similarity of 50-55% and a sequence identity of 43-48% between the three members.

Although SRC-1 and amplified in breast cancer 1 (AIB1) exhibit intrinsic histone acetyltransferase activity, this is weak and does not seem to be essential for NR-directed initiation of transcription[97]. Instead, SRCs mainly play their role in chromatin remodelling through recruitment of other coactivators. This is done by two intrinsic transcriptional activation domains (ADs): AD1, which is responsible for interactions with the acetyltransferases CBP/p300 and p/CAF, and AD2, responsible for interactions with histone metyltransferases CARM1 and PRMT1[97 99]. These interactions induce transcription in a step-by-step cyclic fashion, in which each step initiates the next, as illustrated in Figure 2[95, 97].

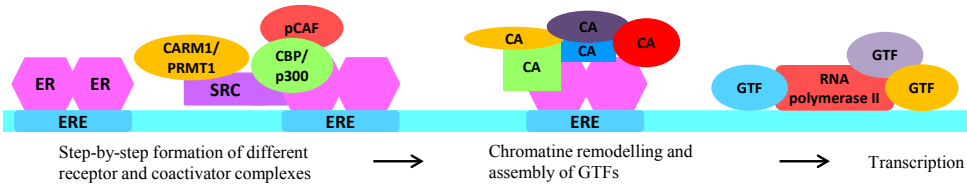


Figure 2. Transcription by NRs mediated by coactivator complexes.

Coactivator complexes follow each other in a step-by-step cyclic fashion, leading to chromatin remodelling, assembly of GTFs, and finally initiation of transcription.

Abbreviations: CA coactivators, ER oestrogen receptor, ERE oestrogen response elements, GTF general transcription factor, NR nuclear receptor, SRC steroid receptor coactivator.

The SRC family members can partially compensate for some of each other's functions, while other functions are specific to each member[97]. Knockout mice lacking any one of the factors exhibit different phenotypes. While SRC-1 seems to be important in brain development and response to steroid and thyroid hormones[100-102], SRC-2 knockout mice show testicular degeneration/placental hypoplasia and resistance to obesity[103, 104].

Deregulation of coregulators has been suggested in several diseases, specifically in malignancies. Among the SRC family AIB1 seems to be of special importance in several types of human cancer, particularly in breast cancer. This is further described in the section below, "Novel biomarkers investigated in this thesis".

Endocrine treatment with tamoxifen

History

Tamoxifen was discovered during research on contraceptive drugs[105]. Although inefficient for that purpose, it was found to be useful in breast cancer treatment. Tamoxifen was first introduced in the metastatic setting in the 1970s, where it was shown to be as efficient as oestrogen therapy, but with fewer side effects[106]. Due to its efficiency and tolerability tamoxifen was soon also evaluated in the adjuvant setting[107, 108], where subsequent results from the EBCTCG have shown an improvement in both RFS and OS in patients with ER-positive tumours[66].

Mechanism of action

Tamoxifen belongs to the SERM (selective oestrogen receptor modulator) group of drugs, which mimic the effect of oestrogen in some tissues, while opposing it in others. This group also includes drugs such as raloxifene and toremifene. When oestrogen binds to the AF2 region of the ER a conformational change is induced in the receptor, such that helix 12 orients itself to form a lid over the ligand-binding cavity[90, 109]. This position of helix 12 is essential for coactivator recruitment and ligand-activated transcription via AF2. In contrast, the bulky side chain of SERMs, such as that of tamoxifen, prevents helix 12 from taking its position. Instead, it will overlap the coactivator docking surface, preventing interaction with coactivators while making the receptor more accessible for interaction with corepressors[90, 109]. Binding of the pure antagonist ICI 164.384, on the other hand, completely destabilizes helix 12[110].

Tamoxifen inhibits ER activation in the breast, while stimulating it in the endometrium. The reason why the effect of tamoxifen differs between tissues is not completely understood, however, several mechanisms have been proposed. Since SERMs block AF2 activity, they may act as oestrogen antagonists in cells where AF2 plays a major role in ER transcriptional activation. In cells where AF1 is more important, however, they may have oestrogen-like effects[90]. Agonist/antagonist effects are also dependent on the type and ratio of coactivators and corepressors expressed, and the gene promoter specific recruitment of these factors[90]. Another influencing factor may be the distribution of ER-α and ER-β in the tissue. When adding tamoxifen to cell lines exclusively expressing ER-α or ER-β, only 27% of the tamoxifen regulated genes were the same in cells exclusively expressing ER-α as in those exclusively expressing ER-β[111].

Clinical use

Tamoxifen is only effective in patients with an ER-positive breast cancer, and should thus only be given to these patients. Five years' adjuvant treatment with tamoxifen reduces the relative risk of recurrence in patients with an ER-positive tumour by about 50%, and mortality by 30%[35, 66, 112]. Five years' treatment has been found to be significantly more effective than one or two years' treatment[112]. However, treatment for longer than five years has not yet been proven to further improve prognosis and may even be detrimental due to a somewhat higher non-breast cancer mortality[35]. Most of the effect on recurrences is seen during the first five years, when tamoxifen is still being given. There is a carry-over effect during the first few years after the discontinuation of treatment, but after ten years the recurrence rate is the same as in untreated patients. On the other hand, the reduction in mortality continues to increase even after more than ten years follow-up[35, 66]. The proportional mortality reduction seems to be similar regardless of age, node status, or whether chemotherapy has been administered. However, the absolute reduction in mortality is greater in node-positive women due to their higher original risk (the absolute improvement in ten-year survival is 12.6% for node-positive women *vs*. 5.3% for node-negative women)[35].

Tamoxifen significantly reduces the risk of CBC by about 30-50%[35, 112]. The risk of uterine cancer, thromboembolic disease and stroke is somewhat increased, while the risk of heart disease is reduced[35, 113]. Overall this leads to a similar non-breast-cancer mortality rate for tamoxifen-treated and untreated patients[35].

Resistance to tamoxifen treatment

Despite treatment with tamoxifen, several patients receiving adjuvant treatment, and practically all with metastatic disease, eventually relapse and die from their disease. In some cases, the effect of tamoxifen on cancer cells even seems to switch from inhibition to stimulation of the ER[114]. Resistance to endocrine treatment can be divided into intrinsic and acquired resistance. The primary mechanism for intrinsic resistance is a lack of expression of ER-α. Another intrinsic mechanism, present in 6-10% of European women, is reduced CYP2D6 enzyme activity, leading to an inability to convert tamoxifen into its most active metabolite endoxifen[115]. However, in a recent study the CYP2D6 genotype was not found to be of importance for prognosis[116].

Acquired resistance can be induced by several mechanisms. While lack of ER-α is the most common mechanism behind intrinsic resistance, loss of the ER or mutations in the receptor do not seem to be common causes of acquired resistance[83, 87, 117-119]. About 20% of patients on endocrine treatment lose expression of the ER over time[119], while mutations in the ER are estimated to be present in only 1% of breast tumours[120]. There is also little

evidence that changes in tamoxifen metabolism or the intracellular influx/efflux of the drug are of any great importance[114, 118]. Several studies have shown second-line therapy with AIs or pure ER antagonists (fulvestrant) to be effective in tamoxifen-resistant cases, although response to second-line endocrine therapy is often shorter. This indicates that the ER continues to regulate tumour proliferation[83, 117, 119], with a gradual shift from oestrogen dependence to alternative pathways.

The ER signalling network is complex, with multiple levels of regulation, allowing it to adapt to different circumstances. Disturbances in these pathways could thus be of importance regarding the response to endocrine treatment. Examples of this are an increase in activity in transcription factors mediating ER expression (Ap1, Sp1, NF-κB), a change in the balance between coactivators and corepressors, as well as deregulation of post-translational modifications of ER or its coregulators[83, 87, 119]. A new G-protein-coupled membrane-bound oestrogen receptor (GPR30) has recently been suggested to be of importance for tamoxifen resistance, possibly since tamoxifen acts as an agonist on GPR30[121, 122].

Apart from changes in the classical ligand-dependent ER pathway, the effect of tamoxifen could be inhibited through increased expression of receptor tyrosine kinases (EGFR, HER2, IGF-1), or increased signalling in their downstream pathways. Activation of the RAS-RAF-ERK and the PI3K-AKT pathways has been shown to be of special importance[83, 87, 119]. Exactly how resistance is mediated is unclear, but several contributing factors have been suggested: decreased ER-α expression; loss of ER-mediated repression of EGFR and HER2 leading to the activation of mitogenic pathways; ligand-independent activation of the ER and its coactivators through phosphorylation; upregulation of cell cycle regulators such as MYC, cyclin D1 and cyclin E1; and inhibition of apoptosis[87]. Patients with metastatic ER-positive breast cancer respond less well to tamoxifen if they overexpress HER2[123], and several trials have begun to test the combination of endocrine treatment with HER2- or EGFR-targeted therapies. Results so far suggest a positive effect in ER-positive HER2-positive patients, as well as in a subgroup of HER2-negative patients[119, 124]. It has been suggested that ER-positive HER2-positive patients might show a better response to AIs than to tamoxifen. However, this is not supported by recent data from the BIG 1-98 and ATAC (Arimidex, Tamoxifen, Alone or in Combination) trials, where an impaired prognosis was found for all HER2-positive patients, regardless of whether they were treated with tamoxifen or AIs. Hence, HER2 has not been found to be a selection criterion for the most appropriate endocrine treatment[124-126].

Since endocrine therapy induces cell cycle arrest and apoptosis, all the genes that control these events could have an impact on drug sensitivity and resistance. Examples of deregulations are: overexpression of cell cycle regulators (MYC, cyclin E1, cyclin D1); inactivation of tumour suppressor genes (Rb, IRF-1); decreased expression of cell cycle inhibitors (p21, p27); increased expression of anti-apoptotic molecules (Bcl-2); and

decreased expression of pro-apoptotic molecules (Bak, Bax, caspase 9)[87, 117]. It has also recently been suggested that breast cancer stem cells might play a role in endocrine resistance[127].

Apart from factors within the tumour cell, there is accumulating evidence that the microenvironment is of importance in several malignant processes including response to endocrine therapy. This includes stromal cells, structural elements of the extracellular matrix, growth factors and cytokines, as well as conditions such as hypoxia and acidity[119].

Gene expression analyses have led to the development of gene signatures intended to predict response to endocrine therapies. These signatures contain a substantial proportion of ER target genes, as well as genes involved in cell proliferation, survival, apoptosis, invasion and metastasis[87]. These studies have broadened our knowledge concerning the potential mechanisms of endocrine resistance. However, many of the signatures and the genes within them also predict outcome in women not treated with tamoxifen, and could thus be markers of a generally poor prognosis rather than specific treatment resistance[87].

The factors suggested to be of importance for tamoxifen resistance are summarized in Table 1.

Table 1. Factors suggested to be of importance in tamoxifen resistance.

TAMOXIFEN RESISTANCE		
Intrinsic	**Aquired**	**Microenvironment**
Lack of ER-α	Loss of ER-α or ER-α mutations	Stroma
Reduced CYP2D6	Changes in drug metabolism, influx/efflux	• Cells
	Changes in the ER-signalling network	• Structural elements
	• Increased activity of transcription factors	Growth factors and cytokines
	• Changed balance between coregulators	Local conditions
	• GPR30	
	Increased signalling in tyrosine kinase receptor pathways	
	• HER2, EGFR, IGF-1	
	Deregulation of cell cycle control and apoptosis	
	Breast cancer stem cells?	

Treatment of tamoxifen-resistant breast cancer

Since the ER frequently continues to regulate tumour proliferation after the development of tamoxifen resistance, second-line treatment with an AI or a pure ER antagonist, such as fulvestrant, can often be used[128]. Although not widely used in the clinic today, high doses of oestrogen have also been shown to be effective[38, 129]. Once the tumour fails to respond to further endocrine therapy, chemotherapy is another therapeutic option.

Given the interaction between the ER and growth-factor-signalling pathways, research is ongoing to investigate the combination of endocrine therapy with inhibitors of receptor tyrosine kinases and their down-stream targets. Previous trials have often been disappointing[130]. However, this could be due to the fact that the studies were performed on unselected patient cohorts, whereas these drugs are probably only efficient in a subset of tumours, dependent on the pathway targeted[130]. The TAnDEM trial (Trastuzumab and Anastrozole Directed against ER-positive HER2-positive Mammary carcinoma) showed the combination of trastuzumab and anastrozole to be superior to anastrozole alone in HER2-positive metastatic disease[131], although both treatment arms performed poorly and more adverse events were seen with the combination treatment. Adding lapatinib to letrozole when treating ER-positive metastatic breast cancer increased the response rate and progression-free survival in HER2-positive patients[132]. Interestingly, an effect was also seen in HER2-negative patients with low ER levels discontinuing tamoxifen therapy within a six-month period prior to entering the trial. Other substances currently being investigated are EGFR inhibitors (gefitinib, erlotinib) and mTOR inhibitors (temsirolimus, everolimus)[119, 130].

Novel biomarkers investigated in this thesis

The choice of adjuvant treatment is decided by prognostic and treatment predictive factors as discussed above. However, some patients will still not respond to treatment and suffer relapse. Others will receive unnecessary treatment with possible side effects. It is therefore important to learn more about prognostic and treatment predictive markers, to better be able to customize treatment according to individual patient's needs. Two interesting new possible biomarkers are AIB1 and paired box 2 gene product (PAX2).

Amplified in breast cancer 1

The *AIB1 gene*, also known as *SRC-3, NCOA3, p/CIP, RAC3, ACTR* and *TRAM1*, was discovered upon microdissection of region 20q[133], a region that is often amplified in breast cancer[134, 135]. AIB1 belongs to the SRC family and interacts with the ER in a ligand-dependent manner to enhance transcription[136-140]. Upon binding to the ER, AIB1 recruits a histone acetyltransferase complex containing CBP/300 and p/CAF, and a histone methyltransferase complex with CARM1 and PRMT1, to the promoter[141]. This results in modification of the local chromatin structure, facilitating transcription. In the oestrogen-dependent breast cancer cell line MCF-7, AIB1 is rate-limiting for hormone-dependent growth[142].

Regulation of AIB1

AIB1 seems to have oncogenic potential in breast cancer and plays an important role during development. Levels of AIB1 are higher in breast cancer than in normal breast tissue[137, 139]. Overexpression has been found in 30-60% of human breast tumours and gene amplification in 5-10%[133, 136-138]. Hence, gene amplification is not the only way through which AIB1 levels can be increased. Also, although AIB1 mRNA and protein levels seem to be well correlated[143], this has not been confirmed in all studies[137, 144]. This is probably due to the fact that total AIB1 protein expression can be regulated at the DNA (gene amplification, transcription), RNA (translation) and protein (stability) levels[143]. AIB1's promoter contains binding sites for the transcription factors E2F1 and Sp1, and since AIB1 is a transcriptional coactivator of E2F1 a certain self-regulating function has been suggested[145, 146]. AIB1 mRNA is also repressed by oestrogen, possibly through a feed-back mechanism reducing hormone sensitivity[147]. This repression is reversed by anti-oestrogens[147, 148]. Translation of AIB1 mRNA to DNA is controlled by microRNAs, and protein levels through ubiquitination, methylation and phosphorylation, influencing proteasomal degradation pathways[143]. Phosphorylation is also of importance for the binding of AIB1 to other transcription factors and for its functions as a coactivator[143].

Apart from changing the levels of AIB1 in the cell, its activity can be controlled by redistribution from the nucleus to the cytoplasm[141]. Finally, a splice variant, AIB1-Δ4 (previously called AIB1-Δ3), has been found to be even more efficient than regular AIB1 in enhancing oestrogen-dependent transcription[149, 150]. Hence, the level and activity of AIB1 are regulated through several different, complex mechanisms.

AIB1 not only functions as a coactivator of the ER

Apart from acting as a coactivator of the ER, several studies have shown AIB1 to interact with other transcription factors and signalling pathways including IGF-1/AKT, NF-κB, E2F1, C/EBPβ and HER2/MAPK[151-160], inducing hormone-independent proliferation and survival. There is evidence that AIB1 is involved in cell cycle regulation, where deregulation of AIB1 may promote cancer initiation and progression[161]. It also appears to be important in the regulation of apoptosis and autophagy[162, 163]. AIB1 suppresses apoptosis in several cancer cell lines, however, in the tamoxifen-resistant breast cancer cell line MCF-7:5C, it is also required for induction of apoptosis in response to oestrogen[162].

Recent evidence implies that AIB1 promotes tumour invasion and distant metastasis[164]. AIB1 is essential for both epithelial-mesenchymal transition and proteolytic breakdown of the extracellular matrix by matrix-metalloproteinase complexes; processes that endow the cancer cell with motile characteristics, enabling them to invade the surrounding stroma. At the cell membrane, the truncated isoform of AIB1, AIB1-Δ4, serves as a signalling adaptor for EGF-dependent cell migration and invasion[149].

In humans amplification and/or overexpression of AIB1 has been found not only in endometrial and ovarian carcinomas, but also in urothelial, colorectal, gastric, pancreatic, hepatocellular and oesophageal carcinomas[136, 165-171]. In several of these cancers, a high level of AIB1 is correlated with a more advanced clinical stage and/or a poorer prognosis[165-167, 170, 171]. In conclusion, although AIB1 was initially described as a coactivator of the ER, recent studies have shown it to have several other important functions.

Low AIB1 protects against tumourigenesis in mice, while high AIB1 induces tumours

AIB1 knockout mice display growth retardation, delayed puberty, reduced female reproductive function and disturbed mammary gland development[172]. Deletion of one allele of AIB1 delays HER2-induced tumour formation, while homozygous deletion completely prevents it[173]. Loss or lowering of AIB1 has been suggested to reduce phosphorylation and thereby activation of HER2. AIB1 deficiency also protects mice against mammary carcinogenesis induced by DMBA (a chemical carcinogen) or the v-Ha-ras oncogene[158, 174]. Reduced carcinogenesis is accompanied by inhibition of the

PI3K/AKT, ERK1/2, JNK, and IGF-1 signalling pathways, as well as reduced expression of cyclin D1[173, 174].

Transgenic mice overexpressing AIB1, on the other hand, show increased activation of PI3K/AKT and increased levels of cyclin D1 and E-cadherin[153, 175]. These mice develop mammary hypertrophy, hyperplasia, and subsequently malignant mammary tumours. Increased tumour formation is also seen in other organs such as the pituitary and uterus. Ovariectomy or lack of ER-α prevents the development of invasive mammary and uterine tumours[176]. However, these mice still develop tumours in hormone-independent tissue, such as the lungs, skin, pituitary and bone, and at a higher frequency than when oestrogen is present. Torres-Arzayus et al. therefore speculated that AIB1 might be the limiting factor for several pathways (including the ER), deciding which one is activated[176]. When one pathway is blocked, the other takes over.

Although the initial development of AIB1-induced mammary tumours seems to be oestrogen-dependent, a significant proportion of these tumours do not later express ER-α. Also, cell lines derived from these tumours grow equally well in ovariectomized, wild-type male and female mice[176]. This indicates that although AIB1-induced tumours may initially depend on oestrogen, this dependence can be quickly lost. In fact, oestrogen actually seems to provide some protection against metastasis in mice injected with cell lines from AIB1-induced tumours[176].

AIB1 in breast cancer

In breast cancer, AIB1 correlates with factors indicating a more aggressive phenotype (a high S-phase fraction, overexpression of HER2, DNA-nondiploidy, high tumour grade, high Ki67)[156, 177-183]. However, the correlation between AIB1 and the ER and PgR differ. Some studies show high AIB1 to be associated with ER- and/or PgR-positive disease[138, 178, 184], while others report an association with ER- and/or PgR-negativity[144, 156, 179], or no correlation with receptor status at all[136, 177, 180].

AIB1 as a prognostic and tamoxifen treatment predictive factor

Deregulation of coactivators, including AIB, has been suggested to be of importance in tamoxifen resistance[156, 185]. However, results from preclinical as well as from clinical trials are ambiguous. A few studies have shown increased levels of AIB1 in tamoxifen-resistant cell lines, and that a decrease in AIB1 in these cells can restore the inhibitory effect of tamoxifen[186-190]. Others have reported no difference in AIB1 levels in resistant *vs.* nonresistant cells[191], and have found cell lines from AIB1-dependent tumours or with increased ER/AIB1 association to respond well to tamoxifen[192, 193]. Tamoxifen also completely abolishes the effect of oestrogen and AIB1 on cyclin D1 transcription[194], even when the dose of AIB1 is successively increased.

In an unselected cohort Osborne et al. found overexpression of AIB1 to be associated with a lower disease-free survival rate in patients receiving tamoxifen[156], while others have shown a correlation between high AIB1 and recurrences during the first two *vs.* five years of tamoxifen[177, 195]. AIB1 has also been found to induce transcriptional changes associated with a poorer prognosis in tamoxifen-treated patients[196]. On the other hand, Iwase et al. found patients with high AIB1 to respond well to endocrine therapy[184]. In addition, several studies have found no correlation between AIB1 and prognosis in ER-positive patients receiving tamoxifen[144, 178, 190, 197, 198]. Regarding AIs, high AIB1 was found to be correlated to better response to neoadjuvant treatment with exemestane[199], while a recent study instead found an association between high AIB1 and recurrences during treatment with an AI[198]. The predictive value of AIB1 for endocrine treatment must thus be further evaluated.

Regarding prognosis, most studies show AIB1 to be a negative factor[144, 180, 183, 195, 200, 201 202]. However, the results are not unanimous. In some studies, AIB1 was not found to predict prognosis[181, 190, 203, 204], and it has even been suggested to be a positive prognostic factor[156]. The negative effect of AIB1 was found to be more pronounced in ER-negative patients in several recent studies[144, 180, 200]. This could mean that the effect of AIB1 is not solely dependent on its interaction with the ER. However, in these studies, ER-positive patients had either received tamoxifen, or information on their treatment was missing. Hence, another explanation could be that ER-positive patients with high AIB1 in fact respond well to the treatment given, overriding the negative effect of AIB1.

It has been suggested that there is an interaction between HER2 and AIB1, and that tamoxifen-treated patients overexpressing both AIB1 and HER2 have a worse prognosis[144, 156, 178]. Flemming et al., on the other hand, found no correlation between AIB1 and recurrences in HER2-positive patients on endocrine treatment[205]. In the studies carried out to date, AIB1+HER2+ patients represent only small subgroups, making further and larger studies necessary.

In the present study on premenopausal patients randomized within a controlled trial to receive tamoxifen for two years or to a control group, high AIB1 was found to be a negative prognostic factor in the control group. On the other hand, ER-positive patients with high AIB1 responded very well to tamoxifen. This indicates that high AIB1 is a predictive factor for improved response to tamoxifen, and not tamoxifen resistance, as has previously been discussed. These results were confirmed in a second study investigating AIB1 in combination with PAX2, including both pre- and postmenopausal women. These studies are described and discussed in Papers I and II.

Treatment directed against AIB1

Due to its role in cancer initiation and progression, AIB1 is an interesting target for new anti-cancer therapies. SRCs are large unstructured proteins with no high-affinity ligand-binding sites, making the production of drugs directed against them challenging. However, research on peptides or small-molecule inhibitors interfering with the interaction between SRCs and nuclear receptors is ongoing[206, 207]. Recently gossypol, a natural polyphenol, was shown to selectively reduce levels of AIB1 and SRC-1 in several cancer cell lines, including breast cancer[208]. Further studies showed hepatocellular carcinoma cells to be more sensitive to treatment than normal hepatocytes, reflecting the selective cytotoxic effect on cancer cells. In addition, treatment with gossypol sensitized breast cancer cell lines to treatment with inhibitors of growth factor signalling pathway (MEK, EGFR, and IGFR inhibitors)[208].

Gossypol was initially considered as a male antifertility agent[209, 210], but was deemed unsuitable due to the risk of permanent infertility and hypokalaemia. It is currently being investigated in clinical trials in prostate cancer, lung cancer and leukaemia, but then as a Bcl-2 (B-cell lymphoma 2) inhibitor[211]. However, part of gossypol's ability to reduce Bcl-2 might be due to upstream inhibition of AIB1[208].

Hence, AIB1 is an attractive target for new anti-cancer therapies and there is hope that such treatments could be available in the near future.

Paired box 2 gene product

PAX2 belongs to the paired box gene family of nine tissue-specific transcription factors, which play an important role in determining cell differentiation and boundaries between tissues during development[212]. PAX2 itself is involved in the development of the central nervous system, sense organs such as the eye and ear, the urogenital system and the pancreas[212, 213]. Mice lacking PAX2 die within a few hours of birth due to exencephaly and kidney failure[213]. Expression of PAX2 protects against apoptosis, and persistent expression is associated with a blockage of tissue differentiation and hyperplasia[214-216]. Overexpression can lead to transformation of cells *in vitro,* giving them the ability to form tumours in nude mice[217]. Deregulation of PAX2, as well as other PAX transcription factors, has therefore been proposed to be important in cancer development[215, 218]. PAX2 is frequently expressed in Wilms' tumour and in renal cell carcinomas[219-221]. Expression has also been found in several other tumour types, including brain, lung, colon, breast, lymphoma, thyroid, uterine, urothelial, pancreatic and ovarian cancer[215, 222]. In endometrial carcinomas it is activated by oestrogen and tamoxifen, and promotes cell growth[223]. Furthermore, PAX2 is silenced in normal endometrium and reactivated in endometrial cancer by hypomethylation of its promoter[223]. Expression of PAX2 may thus contribute to proliferation and cell survival early in tumour progression, by preventing differentiation and apoptosis.

Breast cancer cell line studies

Hurtado et al. found ER-binding sites within the *ERBB2-gene* and the *PAX2-gene* in human breast cancer cell lines[224]. Upon treatment with tamoxifen or oestrogen ER and PAX2 in complex bound to a cis-regulatory element within the *ERBB2 gene*, reducing transcription. Treatment with PAX2 siRNA abrogated this inhibition, and HER2 levels were then increased by both oestrogen and tamoxifen. This in turn led to increased proliferation of tamoxifen-treated cells, an effect that was blocked by trastuzumab. Further studies showed that PAX2 seemed to compete with AIB1 for binding to *ERBB2*, thereby regulating transcription and tamoxifen response. The authors therefore suggested that the relationship between AIB1 and PAX2 could be of importance in determining response to endocrine treatment.

Another cell line study showed levels of phosphorylated PAX2 to be higher in luminal ER-positive cell lines than in non-luminal cell lines[225], with maximum levels in the least invasive cell lines. Activation of PAX2 by oestrogen was selectively seen in luminal cell lines, a process that was inhibited by ICI 182.780 (fulvestrant) or IGF-1. Furthermore, PAX2 seemed to be of importance for the oestrogen-induced downregulation of *ERBB2* and decrease in cell invasion.

In vivo *studies of PAX2 in breast cancer*

In vivo studies regarding PAX2 in the normal breast and in breast cancer are sparse. In normal breast tissue, Silberstein et al. found expression of PAX2 in some myoepithelial, luminal and ductal cells[226]. PAX2-negative and -positive cells were often arranged side by side, which could indicate a function in paracrine signalling. Grafting of PAX2 null mammary *anlagen* to immune-compromised mice showed PAX2 to be necessary for side-branching and lobular development in response to progesterone[226], a process in which paracrine signalling is known to be of importance.

A few studies have investigated PAX2 in breast cancer patients[215, 224, 226, 227]. Although IHC was used in all these studies, techniques and cut-off levels differed. Despite this, PAX2 positivity was found in 40-60% of the breast cancers investigated. A high expression of PAX2 correlated to hormone receptor positivity and a low histological grade[226, 227]. No association was found with the patient's age, the histological classification, the size of the tumour, lymph node metastasis, HER2 or p53[226, 227]. This is similar to findings in ovarian and renal cell carcinomas, where PAX2 seems to be associated with tumours of a lower grade and an earlier stage[228, 229].

Only two studies have investigated PAX2 as a prognostic or a tamoxifen treatment predictive factor in clinical breast cancer trials. Hurtado et al. found a high expression of PAX2 to correlate to a better RFS in 109 ER-positive tamoxifen-treated patients with primary breast cancer[224]. Risk of recurrence was modified by expression of AIB1, with PAX2+AIB1- patients having the best prognosis, and PAX2-AIB1+ patients the worst. A lower risk of recurrence with high PAX2 has also been found in another study of 74 patients[227]. Treatment information was not available in the latter study.

In summary, deregulation of PAX2 could promote cell proliferation and dedifferentiation. PAX2 may, therefore, be of importance in the early stages of tumour progression. In endometrial carcinoma it is activated by oestrogen or tamoxifen and increases cell growth[223]. However, the opposite effect has been suggested in breast cancer[224]. Paper II of this thesis presents a study on the prognostic and tamoxifen treatment predictive value of PAX2 in relation to AIB1. Although AIB1 was found to be both a prognostic and a treatment predictive factor, PAX2 did not seem to provide additional prognostic information; alone, or in combination with AIB1.

Contralateral breast cancer

Today, most women are cured of breast cancer. However, apart from the risk of relapse, breast cancer patients have a 0.5-1% annual risk of developing a tumour in the contralateral breast, with a lifetime risk of 2-20%[230-232]. This is 2-6 times higher than the risk of primary breast cancer in the general population[232]. The CBC is called synchronous if the second breast tumour (BC2) develops within a short time of the first tumour (BC1), and metachronous if the interval between tumours is longer. No clear cut-off time has been defined, and varies in the literature from 0-12 months. In the present work metachronous tumours are defined as CBC diagnosed at least three months after BC1, in line with several previous studies[233-236]. Lobular breast carcinoma, young age at diagnosis of BC1 and mutations in breast cancer associated genes are known indicators of increased risk[234, 237-240]. Lymph node involvement of BC1, prior radiotherapy, inflammatory breast cancer, overexpression of HER2 in BC1, a high BMI, alcohol consumption, smoking and diabetes are other risk factors that have been discussed[241-246]. Treatment with chemotherapy, tamoxifen or AIs, on the other hand, reduce the risk of a second breast cancer[34, 35, 231, 247, 248], and bisphosphonates have also been suggested to have a protective effect in a recent study[249].

Impact on prognosis

Several studies have compared survival after unilateral and bilateral breast cancer, but the results are inconsistent[250, 251]. Some found no difference in survival between women with unilateral and bilateral tumours, others an impaired prognosis for women with synchronous tumours, or an impaired prognosis for women with metachronous tumours. One explanation of the differences seen could be that CBC is a relatively rare event, leading to underpowered studies. Also, the mortality risks were not always adjusted for prognostic factors such as age, stage at diagnosis or treatment[250]. Thus, the impact of a second tumour on prognosis remains unclear. However, many studies suggest that a short time interval between tumours is associated with a poorer prognosis, once diagnosed with metachronous CBC[233, 251-255]. There are also indications that synchronous breast cancer (defined as CBC diagnosed within 3-12 months after BC1) might have a worse prognosis than metachronous breast cancer[236, 250, 256, 257].

Biological relationship between the first and the second tumour

A question that arises when studying CBC is whether the second tumour is truly a new breast cancer, or the result of metastatic spread of the primary breast cancer. The most widely used criteria to discriminate between an independent primary lesion and a

metastasis in the contralateral breast have been summarized by Chaudrey et al[238]. Bilateral carcinomas are considered independent if: 1) the subsequent tumour has an *in situ* component; or 2) the lesions are of distinct histological subtypes; or 3) the subsequent cancer has a better degree of differentiation; or 4) there is no evidence of local, regional or distant metastases from the ipsilateral lesion. A long time interval between tumours may also be regarded as proof of true bilaterality. Furthermore, metastatic cancers are usually located in the fat, while primary neoplasms affect the parenchyma[258].

More recent studies have used X-chromosome inactivation status, p53 mutations, allelotyping, microsatellite instability, and comparative genomic hybridization analysis to assess the clonal origin of the contralateral tumour[259-268]. These analyses have shown the majority of CBCs to be clonally independent from the first tumour. However, in some cases, the CBC is so similar to the primary tumour that it could represent metastatic spread[258, 262, 264-266]. A recent study also shows lymph node involvement of BC1 to be a risk factor for CBC, indicating that some CBCs are indeed metastases from the primary tumour[241]. In a few studies high genetic and morphological similarities were found between the first and the second tumour when the time between them was short[263, 269, 270]. This could suggest a higher prevalence of metastatic spread, but could also reflect the fact that these tumours have developed in a similar biological environment. In the study by Imyanitov et al. the highest similarity score was found in women who developed both tumours while premenopausal, and the lowest when tumours were separated by menopause[263].

Effect of prior adjuvant treatment on contralateral breast cancer

Adjuvant treatment with chemotherapy, tamoxifen or AIs reduces the risk of CBC[35, 112, 237, 247]. In a few trials tamoxifen has been administered to women at high risk of developing breast cancer in order to study its preventive effect[271-275]. These trials showed a reduction in incidence of ER-positive breast cancers by about half, while the incidence of ER-negative breast cancers was not affected[271]. Similar results have been seen with regard to the development of CBC[276, 277]. Hence, tamoxifen seems to reduce the incidence of ER-positive tumours, while having less, if any, effect on the development of ER-negative tumours. In fact, there have even been indications of an increased risk of ER-negative CBC after tamoxifen use[276, 278].

Another modifying factor could be age. In a study by Bertelsen et al. the most pronounced effect of tamoxifen on CBC was observed in women <45 years of age[180], although this was not confirmed in a recent EBCTCG overview[66]. Age might also be significant with regard to the protective effect of chemotherapy. In an overview by the EBCTCG, chemotherapy reduced the incidence of CBC significantly only in women younger than 50 years[35].

The preventive effect of adjuvant tamoxifen has been reported to be independent of ER status of the primary tumour[112], although some studies have found the effect to be restricted to, or more pronounced in, women with an ER-positive primary tumour[35, 180, 279]. If so, it is unclear why tamoxifen would be effective only in women with ER-positive primary breast cancers. One explanation could be that the ER status of the primary tumour is associated with the ER status of the secondary tumour. Some studies have found such a correlation[279-281], while others have not[282]. Also, even if there is a correlation, some women with an ER-negative BC1 will still develop an ER-positive BC2, and tamoxifen should be effective in preventing these[279]. However, several large studies have shown tamoxifen to have no effect on ER-negative primary breast cancers, and there have even been indications that tamoxifen could be detrimental in these patients[283, 284]. The idea that tamoxifen could also be used to prevent a secondary tumour in patients with an ER-negative primary tumour is thus controversial.

Using population-based register data, Hartman et al. found that the incidence of CBC in Sweden has decreased since the 1970s[233], and a similar decrease has been seen in the USA[285]. One explanation of this could be the increased use of adjuvant treatment, preventing the development of a secondary tumour. Although fewer patients were diagnosed with CBC, the prognosis compared to that for unilateral breast cancer, deteriorated during the same time period[233]. This might reflect a treatment escape phenomenon and the development of a more aggressive phenotype once treatment has failed to prevent a second cancer. Indeed, Hartman et al. showed that the survival of patients with early metachronous CBC was poorer if they had received adjuvant chemotherapy for their primary cancer, than if this kind of treatment had not been given[233]. This is similar to findings in the metastatic setting, where having received prior adjuvant chemotherapy was found to be a negative prognostic factor for patients diagnosed with distant metastases[286]. In addition, a recent study showed that endocrine treatment for BC1 worsened the prognosis after an ER-positive BC2[281]. Interestingly, the study of CBC with and without prior adjuvant treatment could hence serve as an "*in vivo*" model for advanced analysis of adjuvant treatment resistance.

Mode of detection

The follow-up of breast cancer patients after the completion of treatment is debated. In the Southern Healthcare Region of Sweden clinical surveillance has been reduced from ten years up to the mid-1990s, to five years, or even one year, followed by regular mammographic surveillance within a screening programme. Previous studies have shown that intense regular follow-up with x-ray examinations in an attempt to detect distant metastasis early does not improve prognosis or quality of life[287, 288]. However, several recent studies show local recurrences or CBC detected by mammography or clinical

examination to be found at an earlier stage and have a better prognosis[289-292]. When comparing asymptomatic diagnosis of CBC with mammography to diagnosis by other means, estimated hazard ratios for breast cancer death are between 0.10 and 0.86[293]. However, these results are not undisputed, and several of the previous studies are small, heterogeneous observational studies, vulnerable to bias[4, 294]. The optimal method of investigating the effect of follow-up would be a randomized trial. However, such a trial might be difficult to construct due to ethical reasons.

The sensitivity of mammography for detecting CBC seems to be about 60%, while the reported specificity varies between 50 and 99%[289, 295]. In women compliant with annual mammography, the sensitivity increases to 70%[289]. In total, 45-90% of CBCs are detected by mammography[293]. The sensitivity of mammography seems to be higher in older women[296], although a recent study shows it also to be useful in young women[297], especially when MRI resources are limited. When comparing detection by mammography and clinical examination, mammography is more sensitive and is associated with better survival[290, 291]. Interestingly, however, a previous study has shown clinical examination to be the only positive finding in 10% of patients diagnosed with CBC[291].

Although there are indications that follow-up programmes could lead to earlier detection of CBC, several issues require further investigation. One is the matter of compliance, and another that some patients with symptoms might wait for their planned appointment to see a doctor. One study found this number to be as high as 18% of patients with symptomatic locoregional recurrences[298]. Finally, even if follow-up programmes can improve survival, there is as yet no standard limit on how long they should continue.

Aims

- To study AIB1, a coactivator of the oestrogen receptor, as a prognostic and a treatment predictive factor for tamoxifen response in premenopausal women using a controlled randomized trial of tamoxifen for two years *vs.* control (Paper I).

- To validate AIB1 as a prognostic factor, and to study the effect of PAX2 in relation to AIB1 on prognosis and tamoxifen response (Paper II).

- To study the effect of adjuvant tamoxifen on the development of contralateral breast cancer in premenopausal patients included in a randomized trial of tamoxifen for two years *vs.* control (Paper III).

- To investigate the prognosis of metachronous contralateral breast cancer by epidemiological studies in a population-based cohort from the Southern Healthcare Region of Sweden (Paper IV).

Patients

Papers I and III

The studies described in Papers I and III include 564 premenopausal women participating in a randomized trial. Premenopausal patients with unifocal stage II breast cancer during the period 1986 to 1991 were enrolled and randomized to two years of adjuvant tamoxifen or a control group without tamoxifen treatment. Randomization was performed independent of ER and PgR status, and stratification for tumour size or lymph node status was not included in the protocol. The median age of the patients was 45 years (range 25-57 years). All patients over 50 years had documented ongoing menstruation, and in dubious cases pituitary hormones (FSH and LH) were checked to confirm menopausal status. Patients undergoing breast-conserving surgery or with node-positive disease received radiotherapy according to clinical standards. Less than 2% received additional adjuvant treatment. The median duration of follow-up for patients without a subsequent breast cancer event was 14 years.

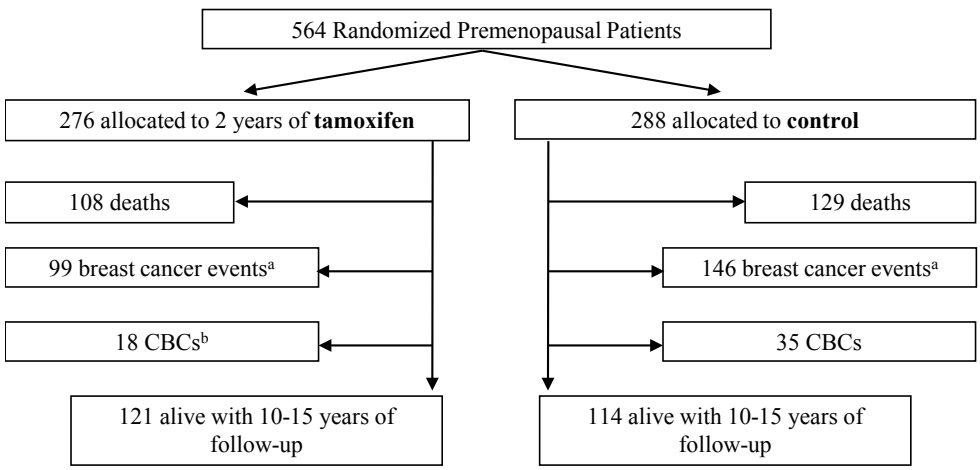


Figure 3. Flow-chart of the premenopausal patients included in the randomized trial of tamoxifen for two years *vs*. control (Papers I and III).

[a]Breast cancer events include local/regional/distant recurrences or breast-cancer-related death as primary event.

[b]One synchronous CBC was excluded from further analysis, leaving 17 cases of metachronous CBC.

Randomization was performed by the South Swedish (N=427) and the South-East Swedish Oncological Centres (N=137). At randomization a central secretariat was called, in which a closed envelope with a pre-randomized allocation was selected. Patient identity, date, and allocated treatment were documented by the secretariat, and also on case report forms by each institution. The study was not blinded, thus the control group did not receive placebo instead of tamoxifen. Clinical and pathological characteristics were evenly distributed between the treatment arms, with the exception of a higher proportion of larger tumours in the group receiving tamoxifen. Oral informed consent was obtained for all patients and the study was approved by the Ethics Committees of the Universities of Lund and Linköping. The study is registered as "SBII:2" in accordance with the criteria outlined by the International Committee of Medical Journal Editors, at the Regional Oncological Centers in Lund and Linköping. Study design and patient flow are described in more detail in Figure 3, and in Papers I and III.

Patients for whom paraffin-embedded tumour blocks were available were selected for further analysis (Paper I). AIB1 scores were obtained from 349 of the 564 patients initially included in the study. Reasons for lack of AIB1 scores were loss of the tumour blocks, missing tissue microarray (TMA) cores, the TMA including only cancer *in situ*, or ≤10 cancer cells. Statistical analysis showed no selection bias when comparing patients with IHC scores with those originally included in the trial.

Paper II

Two different patient cohorts were used in this study in order to obtain information on both tamoxifen-treated and untreated patients. The tamoxifen-treated cohort included both post- and premenopausal women, while the cohort not given tamoxifen included only premenopausal women.

Patients not receiving adjuvant tamoxifen

The initial patient cohort consisted of 237 premenopausal, lymph-node-negative women included from 1991-1994 in a prospective study of the prognostic value of the S-phase fraction[299]. All patients underwent surgery in the form of radical mastectomy or breast-conserving sector resection, together with dissection of levels I and II of the axilla. A median of nine lymph nodes were removed. Some of the patients undergoing breast-conserving surgery were then randomized to receive either postoperative radiotherapy (50 Gy in 25 fractions) to the remaining breast, or no further treatment, in a Swedish multicentre trial evaluating postoperative radiotherapy. In total, 172 patients underwent breast-conserving therapy; 110 with radiotherapy and 62 without. Sixty-five patients underwent radical mastectomy, of which seven also received radiotherapy due to narrow

margins. Seven patients received adjuvant tamoxifen, 21 chemotherapy and one underwent oophorectomy. The eight patients receiving tamoxifen and the one that underwent oophorectomy were excluded from further analysis. A flow-chart of the patients included in the study is shown in Figure 4. The study was approved by the Ethics Committee of Lund University.

In 14 cases the paraffin-embedded tumour blocks could not be retrieved. In the remaining 215 patients PAX2 scores were evaluable for 208 and AIB1 scores for 205. The non-evaluable cases were due to loss of the TMA core, the TMA containing only cancer *in situ*, or ≤10 cancer cells. Both AIB1 and PAX scores were available for 205 patients.

Tamoxifen-treated patients

The initial cohort consisted of 86 pre- and 359 postmenopausal women diagnosed with stage II breast cancer in the Southern Healthcare Region of Sweden from 1985-1994. The patients were initially included in two randomized clinical trials investigating the effect of adjuvant tamoxifen[300, 301]. These patients all received tamoxifen for two years, regardless of ER status, and had previously been selected from the original trials in order to investigate concordance between different laboratory methods for evaluation of hormonal receptor status[302]. AIB1 scores had already been evaluated in this cohort, and were available for 297 out of the 445 patients[177]. Unfortunately, the TMA used for AIB1 scoring was no longer available, but a new TMA had been constructed including 277 of the patients. This was used to evaluate PAX2 scores, which were obtained from 264 patients. Again, non-evaluable cases were due to loss of the TMA core, the TMA containing only cancer *in situ*, or ≤10 cancer cells. Scores for both AIB1 and PAX2 were available for 192 patients. A flow-chart of the study is shown in Figure 5.

All patients underwent surgery in the form of mastectomy or breast-conserving surgery, both in combination with axillary lymph node dissection. Patients with breast-conserving surgery or node-positive disease received radiotherapy according to clinical standards. None of the patients received any systemic adjuvant treatment other than tamoxifen. The study was approved by the Ethics Committee of Lund University.

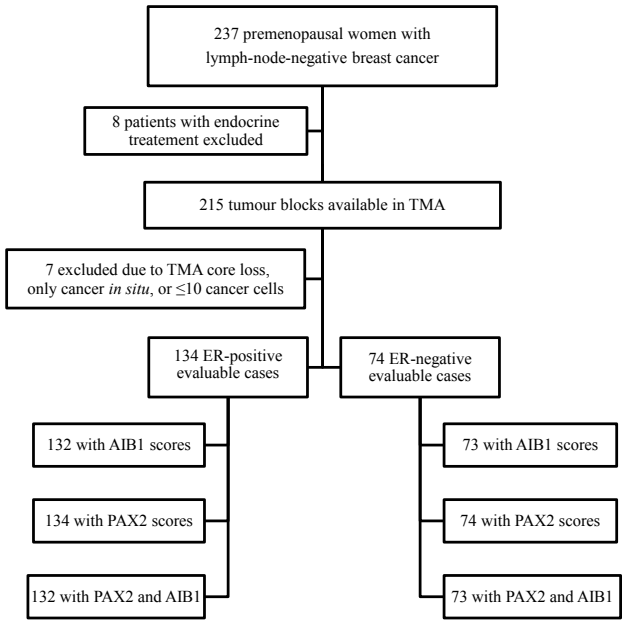


Figure 4. Flow-chart of patients not receiving tamoxifen (Paper II).

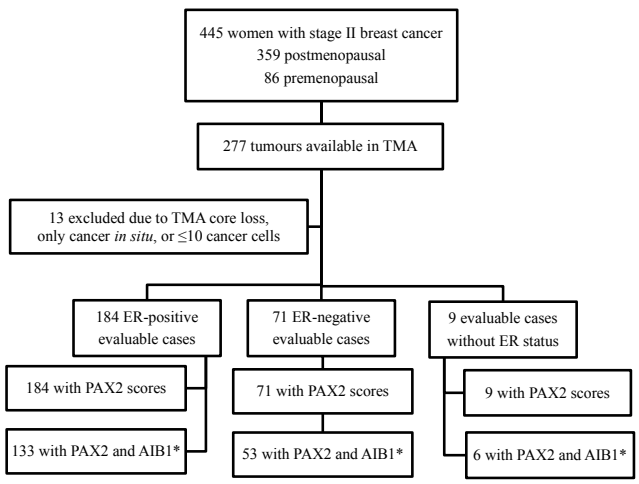


Figure 5. Flow-chart of patients receiving tamoxifen (Paper II).

*AIB1 scores available from previous TMA for 297 patients; 201 ER-positive, 84 ER-negative, and 12 without ER status.

Paper IV

The study described in Paper IV includes 723 patients with metachronous CBC as primary event. In this study an attempt was made to include all patients with metachronous CBC within the Southern Healthcare Region of Sweden. This region has 1.7 million inhabitants, and 14 hospitals (Lund, Malmö, Helsingborg, Ängelholm, Landskrona, Ystad, Trelleborg, Hässleholm, Kristianstad, Växjö, Ljungby, Halmstad, Karlshamn, and Karlskrona). The South Swedish Breast Cancer Group was established in 1977, and all 14 hospitals within the region have been active members since then. This means that they have used the same guidelines for diagnosis, treatment and follow-up. Data were obtained from the Swedish Cancer Register on all women within the Southern Healthcare Region with two breast cancer diagnoses, whose second tumour was diagnosed in 1977 or later. The Swedish Cancer Register is a nationwide database including the International Classification of Diseases code and date of diagnosis. An initial cohort was obtained from the register including 1970 patients. A flow-chart of the study is shown in Figure 6.

In 651 of the patients the time interval between tumours was less than three months. These tumours were considered synchronous and were therefore not included in the metachronous cohort. Medical records could not be found for 150 patients, and in 204 patients the second diagnosis represented a local recurrence or a new ipsilateral primary tumour. Of the patients who later developed CBC, 105 patients developed distant metastases, 37 patients a local or regional recurrence of BC1, and 93 another malignancy before diagnosis of BC2. Metastasis status was ambiguous in three patients, BC2 was only found in the axilla in three patients, and one patient was diagnosed at autopsy. All these patients were excluded according to predefined exclusion criteria, leaving 723 patients with metachronous CBC as primary event. For patients with multiple exclusion criteria, the first criterion mentioned in the chart is given above.

Data were abstracted from individual medical records (clinical notes, pathology, and X-ray records) from September 2007 to November 2009 in a systematic manner, using a predefined protocol. The protocol was designed at the Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, for collecting data on patients with CBC. Individual patient records at the Departments of Surgery and Oncology (Lund, Malmö and Växjö) were retrieved, in order to optimize data abstraction and minimize patients lost to follow-up. The protocol used included data on epidemiological factors, prior medical history, mode of detection, surgical and oncological treatment, tumour biology for BC1 and BC2, and outcome.

Clinical facts and considerations

Due to the long follow-up period information on some of the factors that are of importance in the choice of treatment today, such as ER status and histological grade, was not

available for some of the patients. ER status for both tumours was available for less than half of the patients, and histological grade for less than one third. Additionally, methods of determining the histological grade differed during the follow-up period and between various pathology departments.

One aim of this study was to investigate the effect of time interval between BC1 and BC2 on prognosis. Previous studies have used different time intervals to distinguish early from late metachronous CBC, and it was not entirely clear which time interval should be used. However, several previous studies used an interval of three years[239, 252, 253], and this was also chosen in the present study. In line with previous studies, the age of the patients was defined as the age at diagnosis of BC1[233, 253, 255, 303]. To be able to adjust for the time of diagnosis of CBC the material was divided into three calendar periods: 1977 to 1986, 1987 to 1996, and 1997 to 2007.

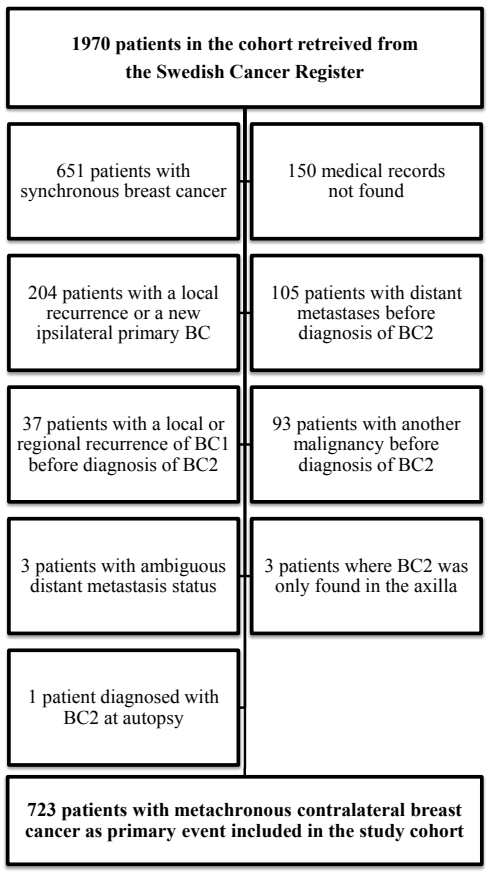


Figure 6. Flow-chart of patients included in the study of CBC (Paper IV).

Methods

Tissue microarray

The introduction of tissue microarrays in the late 1990s provided a new method of evaluating large numbers of tumour markers[304], saving tissue, time and money compared to conventional molecular pathology techniques. TMAs are today a well-established and frequently used method for IHC and *in situ* hybridization, e.g. fluorescent *in situ* hybridization (FISH). To create a TMA, tissue core biopsies from representative areas of paraffin-embedded blocks are punched out and mounted on the recipient block. The biopsies are taken from an area of interest of the original specimen, and are arranged in a coordinate system in the donor block, so that each biopsy can be easily identified.

Since only a small part of the tumour is examined, there have been concerns that biomarkers that exhibit heterogeneity in the tumour would not be adequately assessed. Several groups have investigated this and demonstrated a strong correlation between results from TMA cores and whole tissue sections. In most instances, two 0.6 mm "histospots" seem to adequately represent the staining seen on an entire section[304-307]. However, for biomarkers that exhibit significant heterogeneity or location-dependent expression, multiple and larger TMA cores are probably required to ensure reliable results[304, 308, 309]. It is also recommended that the cores be taken at nonadjacent locations, to include different tumour areas[304, 310]. The size of the core biopsy varies from 0.6 mm to 2.0 mm. Since a smaller biopsy saves tissue in the original block, 0.6 mm biopsies are generally favoured[310]. In the present studies cores of either 0.6 mm or 1.0 mm were used.

The tissue microarrays used in Studies I, II and III were constructed using a manual arrayer (Beecher Instruments, Sun Prairie, WI, USA). Two biopsies from each specimen, 0.6 mm or 1.0 mm in size, were punched from marked areas of invasive cancer and mounted on a paraffin block. Each recipient block contained approximately 60-120 biopsies, corresponding to 30-60 patients. When no tumour was visible in the TMA, or biopsies were lost during preparation, a second round of biopsies was taken.

Immunohistochemistry and *in situ* hybridization

IHC is a widely used method for the analysis of protein expression. A primary antibody specific to the antigen of interest is first added to the sample. Thereafter, a second labelled antibody, which reacts with the primary antibody, is added, labelling the antigen. The expression of protein in the tissue can be assessed by investigating the staining intensity and frequency. For the analysis of intracellular location, haematoxylin is used to stain the cell nuclei.

In situ hybridization involves the use of labelled complementary strands of DNA or RNA to localize a specific DNA or RNA sequence in the tissue. In fluorescent *in situ* hybridization the probe is fluorescent, and is detected by fluorescence microscopy. In chromogenic *in situ* hybridization (CISH), the probe is detected using a peroxidase reaction, making it visible with ordinary light microscopy[311]. These methods can be used to evaluate gene amplification, gene deletion, chromosome translocation and chromosome number.

AIB1

AIB1 was evaluated with IHC, using an automatic IHC stainer (Autostainer Plus Dako, Sweden or TechMate™ 500 Plus Dako, Denmark). Thin sections (4µm) were cut from the TMA, mounted on capillary microscope slides, and dried overnight at room temperature followed by 1-2 h at 60°C. Sections were then deparaffinized in xylene and rehydrated in a graded series of ethanol. Antigen retrieval was performed by microwaving the slides in Tris-EDTA, pH 9, at 800 W for 7 min followed by 15 min at 350 W, after which the slides were allowed to cool to room temperature for 20 min.

As primary antibody for AIB1 detection a mouse-monoclonal IgG antibody was used at 1:100 dilution (Cat. no. #611105 BD Bioscience, San José, CA, USA). Slides were then counterstained with haematoxylin, dehydrated in ethanol, cleared in xylene and coverslipped.

Results for AIB1 were estimated semi-quantitatively as the percentage of stained nuclei (proportion score) and the intensity of positive tumour cells (intensity score). The proportion of stained tumour cell nuclei was scored 0-3: score 0 representing no stained cell nuclei, score 1, 1-10%, score 2, 11-50%, and score 3, 51-100% stained tumour cell nuclei. The staining intensity of positive nuclei was also scored 0-3, where 0 represents negative, 1 weak, 2 moderate, and 3 intense staining (Figure 7). Proportion and intensity scores were then added to a give a total score from 0 to 6. In cases of discrepancies in the staining results from the two cores from the same patient, the core with the highest score

was used for further analyses. Missing TMA cores, cores with only cancer *in situ*, or ≤10 cancer cells were excluded.

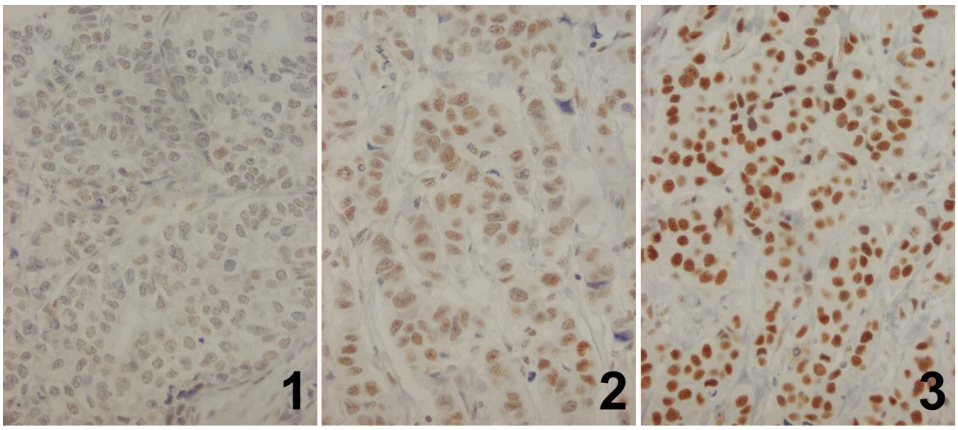


Figure 7. Staining intensity for AIB1 in breast cancer, score 1 to 3.
Dilution 1:100, 200x magnification.

In accordance with a previous study by our group, a total score of 5 or higher was considered high AIB1 and a total score below 5 low AIB1[177]. In both studies described in Papers I and II scoring was done by two independent viewers blinded to the clinical and tumour characteristics data (Sara Alkner and Kristina Lövgren or Sara Alkner and Dorthe Grabau). In the first study (Paper I) divergent results were reexamined by a pathologist (Dorthe Grabau). In the second study (Paper II) cases where the results differed by more than one step between the viewers (8%), were reexamined to reach a mutual consensus. In the remaining cases the mean value was used.

PAX2

For IHC evaluation of PAX2, 3-4 µm sections were taken from each TMA block, transferred to glass slides, and heated for 2 h at 60°C. Deparaffinizing and antigen retrieval were performed in a PT-Link module (Dako, Sweden) with a high-pH buffer. Staining was carried out in a Dako Autostainer Plus, according to standard procedures. PAX2 primary antibody (Cat. no. #2549-1 Epitomics Inc., Burlingame, CA, USA. Diluted 1:500) was applied for 60 min at room temperature, and staining was detected using a Dako K8010 EnVision kit.

To make scoring as similar as possible to previous studies[215, 224, 226, 227, 312], the proportion score was determined as: score 0 no stained tumour cell nuclei, score 1, 1-10%, score 2, 11-80%, and score 3, 81-100% stained tumour cell nuclei. Staining intensity 0 represents no staining, 1 weak, 2 moderate and 3 intense staining. The proportion and intensity scores were then added to give a total score of 0-6. Scoring was performed by two independent viewers (Sara Alkner and Dorthe Grabau) as described for AIB1 above. In cases with discrepant staining results between the two cores from the same patient, the core with the highest score was used for further analyses.

No consensus regarding the choice of cut-off value for PAX2 was found in the literature. However, previous studies investigating PAX2 in breast cancer have found 40-60% of the tumours to be PAX2-positive[215, 224, 226, 227]. A cut-off of ≥5 gave 43% PAX2-positive patients in the cohort not receiving tamoxifen, and 56% in the tamoxifen-treated cohort. This cut-off was thus used for further analysis.

ER and PgR

ER and PgR were analysed with IHC using the Ventana Benchmark system with prediluted antibodies (anti-ER clone 6F11 and anti-PgR clone 1A6) or the Techmate 500 system (anti-ER ID5, Dako, 1:100 and anti-PgR, polyclonal, Dako, 1:50) (Papers I, III, and for the tamoxifen-treated cohort described in Paper II). In line with clinical guidelines a cut-off of 10% positive cells was used to classify tumours as receptor-positive.

For the cohort not receiving tamoxifen (Paper II) ER and PgR were measured in the cytosol by enzyme immunoassay (Abbott Laboratories, Diagnostic Division, Chicago, IL, USA). These analyses were previously performed every week, within 8 days of the primary surgery. Samples with receptor content ≥25 fmol/mg protein were classified as ER- or PgR-positive. Several studies have shown good concordance between ER and PgR status measured with IHC and in cytosol[302, 313-315].

HER2

HER2 was measured by both IHC and FISH or CISH (Papers I, II and III). The Ventana Benchmark system with a prediluted antibody (Pathway CB-11, 760-2694) was used for the IHC measurements[51]. The tumours were categorized into four groups according to the standard protocol of the HercepTest: grade 0 representing total lack of staining or membrane staining in less than 10% of the tumour cells, grade 1+ weak, not circumferential membrane staining in more than 10% of the tumour cells, grade 2+ intermediate, circumferential membrane staining in more than 10% of the tumour cells, and grade 3+ intense and circumferential staining in more than 10% of the tumour cells.

HER2 gene amplification was determined with FISH or CISH as previously described[51, 75, 316]. Briefly, paraffin-embedded tumour sections were deparaffinized, pretreated, denatured, incubated overnight with the hybridization probe, and finally counterstained. Tumours were considered amplified when displaying six or more signals per tumour cell. All patients with amplified tumours and all patients with an IHC score of 3+ where FISH/CISH could not be evaluated were considered HER2-positive.

Endpoints

The primary endpoint in Study III was the development of contralateral metachronous breast cancer. In Studies I, II and IV the prognosis of breast cancer patients in relation to the presence of AIB1 and PAX2, or after the development of CBC, was investigated. In Study I RFS and OS were chosen as primary and secondary endpoints. RFS includes local, regional and distant recurrences, and breast-cancer-specific death, but not CBC, as primary event. OS includes death from any cause. In Studies II and IV, distant disease-free survival (DDFS) was chosen as primary endpoint. This includes the development of distant metastases (visceral, skeletal, brain, or cutaneous metastases) as primary event.

Using RFS, DDFS, OS, and breast-cancer-specific death provides different ways of analysing prognosis. The endpoint used is important for the interpretation of the results. For example, the rate of survival after the development of distant metastasis is much worse than after a local recurrence. In contrast to breast-cancer-specific death, OS includes death by any cause. One form of treatment could, for example, be very effective in reducing breast-cancer-specific death, but the patients die from the side effects, and OS could then be similar to that for untreated patients.

In Study IV, event-free survival was measured from the diagnosis of CBC. Survival from BC1 was not considered since this would automatically prolong the follow-up until event for patients with a long time interval between tumours, and hence bias the results. If no prior event was recorded, DDFS was calculated to the last follow-up date in the patient's medical records. For patients who developed a malignancy other than breast cancer after the diagnosis of CBC, the diagnosis date of this malignancy was considered to be the last follow-up date.

Statistical analysis

The software packages Stata 10.1 or 11.1 were used for statistical calculations (StataCorp., College Station, TX, USA). Correlations between different prognostic factors, and comparisons between treatment groups were evaluated by t-test, the χ^2-test, or, where appropriate, the χ^2-test for trend.

Kaplan-Meier plots were used to describe the development of CBC, RFS, OS and DDFS in Papers I-IV, respectively, and the log-rank test was used to evaluate the hypothesis of equal survival. In Papers I and IV the Kaplan-Meier curves were curtailed when less than five individuals remained at risk. Hazard ratios (HRs) and confidence intervals (CIs) were estimated using Cox regression. Univariate Cox regression was used to compare different subgroups, and multivariate Cox regression to adjust for the effects of other prognostic markers. To assess whether the effect of a factor differed in different subgroups, Cox models with an interaction term were used. Assumptions of proportional hazards were checked using Schoenfeld's test[317]. All p-values correspond to two-sided tests, and values of less than 0.05 were considered to indicate statistical significance. In Studies I, II and III all analyses were performed using the intention to treat rule, meaning that data from a patient were analysed in the treatment group to which the patient was randomized, regardless of whether that treatment was given as planned or not.

In Study IV prognosis after CBC was studied in relation to mode of detection. When doing this retrospectively there is a risk of lead time bias, meaning that earlier detection could lead to a longer follow-up until event, even if disease progression is the same. Instead of studying DDFS, the risk of distant metastasis was therefore studied using logistic regression, thereby excluding the time factor.

In multivariate analyses patients with missing values for any of the variables included are generally excluded from the analysis. To ensure that this did not affect the results, all multivariate analyses in Study IV were repeated including the patients with missing values. This was done by treating the missing category for discrete variables as a separate category and by imputing the sample mean over all patients for continuous variables.

Potential Bias

The strengths, limitations, and possible bias of the studies are summarized in Table 2. A controlled randomized trial was used in Studies I and III. This is generally considered the best method to study the effect of treatment, and prognostic and treatment predictive factors. Bias may arise if the randomization process does not create equivalent groups, or if there is a large loss of patients during the study, especially if the loss is unequally distributed between the treatment arms. Preferably the trial should be blinded for all parts (patients, physicians, and the researchers), since expectations could otherwise affect the results. However, this is not always possible in clinical practice. It is also important to consider the power of the trial to detect a certain difference between study groups. This is dependent on the size of the trial and the expected effect of treatment.

In Study III, patient and tumour characteristics were similar in both arms, with the exception of larger tumours among patients who received tamoxifen. The trial was, however, not blinded. In Study I, AIB1 scores were obtained from 349 of the 564 women included in the initial trial. No difference was seen between groups when comparing patients with and without AIB1 scores. In patients with an AIB1 score, those receiving tamoxifen had a higher proportion of larger tumours, while the control group a higher proportion of HER2-positive patients.

In Study II, PAX2 and AIB1 were investigated in two large breast-cancer cohorts, in patients receiving tamoxifen and in those not receiving this treatment. Although the tamoxifen-treated cohort included both pre- and postmenopausal women, the cohort not receiving tamoxifen only included premenopausal women. This cohort also included only node-negative patients, while the tamoxifen-treated cohort included women with stage II breast cancer. This difference in tumour stage between cohorts could be considered a limitation or an advantage, and may well to some extent reflect clinical reality. In addition, if the prognostic value of a certain factor is significant in a low-risk group with few events, this indicates that it is probably quite a strong risk factor.

Although previous studies of PAX2 in breast cancer have also used IHC[215, 224, 226, 227], techniques and cut-off levels differ. However, PAX2 positivity was found in 40-60% of the breast cancers investigated, similar to the values found in the present study (43% and 56%). A recent study indicated that the level of phosphorylated PAX2 could be of importance, and not the total protein level[225]. However, phosphorylated and non-phosphorylated PAX2 were not measured separately in our study or in previous *in vivo* studies in which PAX2 was found to influence prognosis[224, 227]. AIB1 scores in the tamoxifen-treated cohort were already available from a previous trial. Since then a new TMA from this cohort has been constructed, which was used to determine PAX2 scores. Although the two TMAs were similar, the second TMA did not include all cases included in the first. In addition, the TMA cores that could not be evaluated were not always the

same in the two TMAs. Hence, cases with AIB1 and PAX2 scores did not completely overlap.

Paper IV is based on a cohort study, intending to include all women with CBC within the Southern Healthcare Region of Sweden. Inclusion was based on data obtained from the National Swedish Cancer Register. However, some cases of CBC may be missing or misclassified in the register. Among the patients identified from the register, 150 medical records could not be found. At one centre, many of the older files had intentionally been destroyed by incineration. Another problem was that the amount of information that could be abstracted from the clinical notes and pathology records varied widely between different physicians and over time. In addition, standard surgical methods, routine histopathological analysis and adjuvant treatment had changed during the study period, due to the long follow-up time. However, information abstracted from the patients' medical records is probably still likely to be more accurate than information based only on register data. Moreover, a long follow-up period was necessary to obtain a large cohort due to the low annual incidence of CBC. Hence, some of these sources of bias were unavoidable.

Another possible bias could arise from the fact that patients were included in the cohort based on the date of diagnosis of CBC. Hence, for women initially treated before 1977, there might be a selection bias towards patients with a longer time interval between BC1 and BC2. To investigate this, analyses were adjusted for the date of diagnosis of BC1. Similar results were obtained, indicating that this was not a major source of bias.

In all retrospective studies concerning mode of detection, lead time and length time may cause bias. Lead time bias arises when earlier detection results in a longer follow-up until event, even if disease progression is the same. Length time bias, on the other hand, arises if a certain mode of detection is more prone to detect tumours with a benign phenotype. For example, slower-growing tumours could be more easily detected since they are detectable over a longer period of time. To avoid lead time bias the risk of metastasis was studied using logistic regression instead of DDFS, with regard to mode of detection. Length time bias may still be a problem in this study, although previous studies have not shown this to be a major source of concern[291, 318-320]. Another limitation is that the study did not include data on how long and how often each individual patient was followed by mammography and clinical examinations, if they were compliant with the follow-up programme or not.

In Study IV the effect of prior treatment on prognosis after the development of CBC was investigated. When doing this it must be borne in mind that the choice of treatment is strongly related to tumour stage and biology. Patients selected to receive chemotherapy are those with the worst predicted prognosis, and this could bias the results when investigating prognosis in relation to treatment. To avoid this problem, the analyses were adjusted for several prognostic factors including tumour stage and all treatment given. However, due to facts discussed above this study did not include information on some of the factors affecting prognosis and choice of treatment (hormone receptor status, histological grade).

In addition, there could be other, unknown, factors which also affect prognosis in relation to treatment.

Finally, it is important to constantly reflect on the number of patients and events included in each analysis. Even with a large initial cohort of patients, division into subgroups can quickly lead to small groups with few events, reducing the statistical power.

Table 2. Strengths, limitations and possible bias in these studies.

	Strengths	Limitations and possible bias
Study I	Randomized trial. Detailed patient and tumour information. Similar level of AIB1-high tumours as in previous studies. Multivariate analyses adjusted for other prognostic factors.	AIB1 scores not available for all patients. No clear cut-off value in the literature. Differences in methodology between studies. Only premenopausal patients.
Study II	Two large patient cohorts with detailed patient and tumour information. Patients receiving and not receiving tamoxifen. Cohort 1 included only premenopausal patients, making confirmation of findings in Study I possible. Both pre- and postmenopausal patients in the tamoxifen-treated cohort. Similar levels of PAX2- and AIB1-high tumours as in previous studies. AIB1 a negative prognostic factor both when treated as a continuous and a dichotomized variable. Log-rank test for trend confirmed ≥5 to be a reasonable cut-off value for AIB1. Multivariate analyses adjusted for other prognostic factors.	Not a randomized trial. No access to cohort of postmenopausal patients not receiving tamoxifen. No clear cut-off values for PAX2 and AIB1 in the literature. Differences in methodology between studies. ER, PgR and HER2 measured by different methods in the two cohorts. AIB1 and PAX2 scores not available for all patients, TMAs not completely overlapping in the tamoxifen-treated cohort.
Study III	Randomized trial. Detailed patient and tumour information for BC1. Multivariate analyses adjusted for other prognostic factors.	ER status available for BC2 in only a minority of patients.
Study IV	Large patient cohort. Detailed patient, tumour and treatment information collected from clinical records. Multivariate analyses adjusted for other prognostic factors. Multivariate analyses repeated to include patients with missing values. Analyses adjusted for date of diagnosis of BC1 giving similar results, suggesting inclusion based on date of diagnosis of BC2 not to be a major problem. Logistic regression was used to avoid lead time bias in relation to mode of detection.	Patients included over a long time period - changes in treatment, follow-up, and pathology reports. Several patients excluded due to missing data. Patients included based on date of diagnosis of BC2 - risk of selection of patients with a longer time interval between tumours if BC1 before 1977. Risk of lead time and length time bias in relation to mode of detection. Data on how long and how often individual patients were followed by mammography and clinical examination not available. Choice of treatment dependent on prognostic facts - must be considered when investigating effect of treatment on prognosis.

Results and discussion

AIB1 and PAX2 as prognostic and tamoxifen treatment predictive factors - Papers I and II

AIB1 was investigated as a prognostic and a treatment predictive factor for tamoxifen response in premenopausal women (Paper I). A controlled trial was used in which premenopausal women were randomized to two groups, one given tamoxifen for two years and the other no tamoxifen, independent of ER or PgR. The results of the study were confirmed in a second study on pre- and postmenopausal women receiving tamoxifen and premenopausal women not receiving tamoxifen (Paper II). In the second study data on PAX2 were included (Paper II).

Correlation between AIB1, PAX2 and other prognostic factors

A high level of AIB1 was found to correlate with ER and PgR negativity. It was also correlated with other negative prognostic factors such as overexpression of HER2, high NHG, high Ki67 and the presence of lymph node metastases. No association was found between AIB1 and age or tumour size. PAX2, on the other hand, was significantly associated to ER negativity and high Ki67. In addition, a correlation was found between AIB1 and PAX2.

That AIB1 correlates to other negative prognostic factors has previously been reported[156, 177-179], while the correlation of AIB1 to ER and PgR varies between studies. In contrast to the present results, PAX2 has previously been suggested to be correlated with ER positivity[225-227]. However, studies on PAX2 in breast cancer are rare and include few patients. Although PAX2 has been suggested to reduce levels of HER2, no correlation was observed between HER2 and PAX2 in the present work. In fact, there appeared to be a higher proportion of HER2-positive patients among those that were PAX2-postive, although this was not statistically significant.

AIB1 as a prognostic factor

To investigate the prognostic effect of AIB1 two large independent cohorts of premenopausal breast cancer patients not receiving tamoxifen were studied. The first cohort was the control arm of the randomized trial presented in paper I and III, including patients with stage II breast cancer. The second cohort included low-risk lymph-node-negative patients. AIB1 was found to be a significant negative prognostic factor in both

these cohorts, as can be seen in Figure 8. The results remained significant when adjusting for other prognostic factors. In subgroup analysis in both cohorts, the prognostic effect was only significant in ER-positive patients. A similar trend was seen in ER-negative patients, although it did not reach statistical significance. However, this might be due to the smaller size of these subgroups.

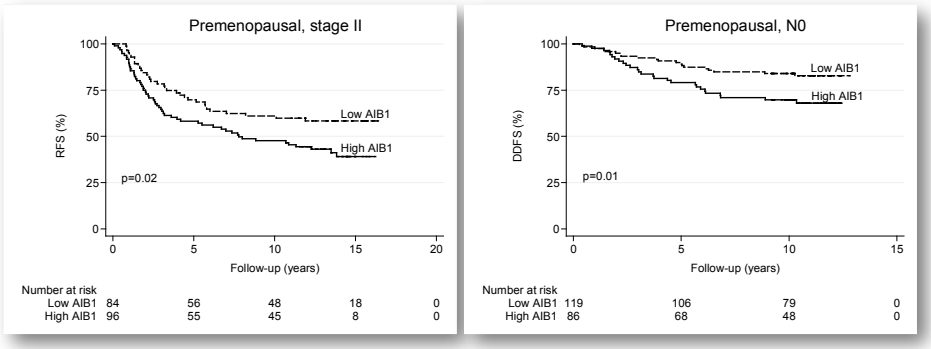


Figure 8. Prognostic effect of AIB1 in premenopausal patients (Papers I and II).
Abbreviations: N0 lymph-node-negative.

Preclinical trials suggest that AIB1 is associated with tumour progression and a more aggressive phenotype. In breast cancer it is also correlated to several other negative prognostic factors. However, its effect on prognosis in the clinical setting is still under debate. Although a number of studies have indicated AIB1 to be a negative prognostic factor[144, 195, 200, 201, 321], others have reported AIB1 not to be related to prognosis[181, 190, 203, 204], and one study has even found it to be a positive prognostic factor[156]. The results of the present work confirm AIB1 to be a negative prognostic factor. In addition, the fact that it remains a significant prognostic factor in the cohort of low-risk patients with few events, suggests its negative effect to be quite strong.

AIB1 as a predictive factor for tamoxifen response

The controlled randomized trial described in Papers I and III was used to investigate AIB1 as a predictive marker of response to tamoxifen treatment (Paper I). In ER-positive premenopausal patients with high AIB1, treatment with tamoxifen significantly increased both RFS (HR=0.4, p=0.002, 95% CI 0.2–0.7) and OS (HR=0.4, p=0.003, 95% CI 0.2-0.8) as shown in Figure 9. On the other hand, no difference in prognosis was seen in relation to

tamoxifen treatment in ER-positive patients with low AIB1. As expected, tamoxifen had no effect in the ER-negative group, regardless of the level of AIB1. Interaction analysis between AIB1 and treatment showed a borderline significant difference using univariate analysis and a significant difference using multivariate analysis. This indicates that AIB1 could be a predictive marker of response to tamoxifen treatment.

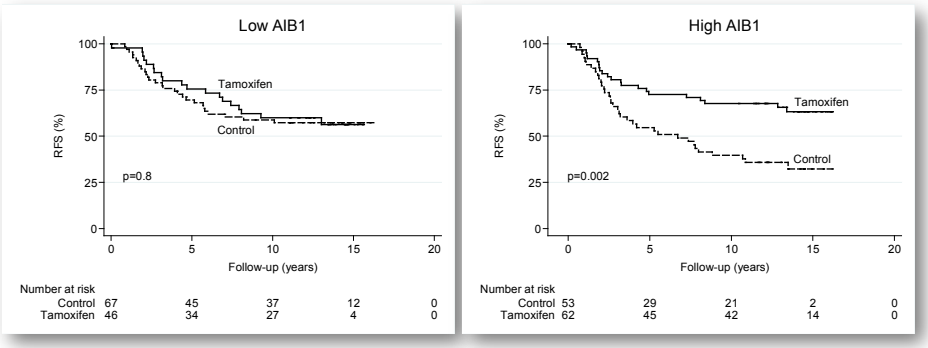


Figure 9. RFS in ER-positive patients in relation to tamoxifen treatment (Paper I).

These results are in contrast to some previous studies indicating AIB1 to be involved in tamoxifen resistance[156, 195]. The reasons for these contradictory results may be differences in study design (randomized trial *vs.* unselected cohort) and differences in methodology. For example, we used IHC to determine levels of AIB1, while Osborne et al. used western blot[156]. No general cut-off level for AIB1 has been defined in the literature. In the present work AIB1 was analysed in accordance with a previous study by our group, and it was found that 56% of tumours expressed high levels of AIB1, in line with several previous studies[136, 137, 177, 200]. In addition, a log-rank test for trend (Paper II) showed a turning point from fewer to more events than expected with an AIB1 score of 5 and higher, confirming this to be a reasonable cut-off. However, high AIB1 has been defined as values in the top quartile of AIB1 expression in some previous studies[156, 178]. When comparing the highest 18% with the remaining 38% within the AIB1-positive tumours no significant difference was seen between the groups (Paper I).

Study I includes only premenopausal patients, while previous studies mainly postmenopausal patients. One explanation of the discrepancies could hence be that AIB1 has a different effect in post- and premenopausal women. Previous studies have shown that, apart from acting as a coactivator of the ER, AIB1 can interact with several other transcription factors and signalling pathways[151, 153-155], leading to hormone-independent cell proliferation. Hence, AIB1 could act mainly as a coactivator of the ER in the presence

of oestrogen, while in the postmenopausal setting, with low oestrogen levels, it could act mainly through other pathways. In Study II AIB1 was investigated in a cohort of pre- and postmenopausal tamoxifen-treated women. No difference was seen in the prognostic value of AIB1 in pre- or postmenopausal ER-positive women treated with tamoxifen. This may be due to the good response to tamoxifen in patients with a high AIB1, eliminating prognostic differences between high and low AIB1. In that case, no difference in tamoxifen response was seen in regard to menopausal status.

Recent studies have shown AIB1 to be a negative prognostic factor mainly in ER-negative patients[144, 200, 321] This could reflect that the effect of AIB1 is not solely dependent on its interaction with ER. However, in these previous studies ER-positive patients either received tamoxifen or information on treatment was missing. Hence, another explanation, as discussed above, could be that ER-positive patients with high AIB1 in fact respond well to the treatment given, overriding the negative effect of AIB1.

PAX as a prognostic or treatment predictive factor

In a previous study it was suggested that the relationship between AIB1 and PAX2 affected levels of HER2, and thus the response to tamoxifen[200]. In addition, PAX2 has been associated with a better prognosis in breast cancer patients[200, 227]. However, previous studies have been carried out on small groups and none has investigated PAX2 in a large cohort including both women receiving and not receiving tamoxifen. In the present work, PAX2 was not found to be a prognostic factor on its own, as can be seen in Figure 10. Neither does it seem to modify the prognostic effect of AIB1.

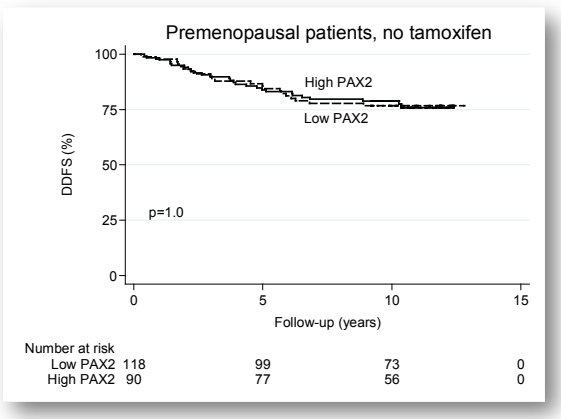


Figure 10. PAX2 in premenopausal lymph-node-negative patients (Paper II).

However, an exception was seen in the interaction between menopausal status and prognostic effect of PAX2 in ER-positive tamoxifen-treated patients. In postmenopausal patients PAX2 seemed to be a negative prognostic factor, while in premenopausal patients it was a positive factor. Again, this could be explained by differences in the levels of circulating oestrogen, affecting which proliferation pathway the tumour mainly depends on. However, although this result is interesting and deserves to be investigated further, the present study contained only a few premenopausal tamoxifen-treated patients with both AIB1 and PAX2 scores (39), and it is difficult to draw any reliable conclusions in regard to this from this study.

Incidence and prognosis of CBC - Papers III and IV

In Study III the effect of adjuvant tamoxifen treatment on the development of CBC in premenopausal patients was studied using the controlled trial presented in Papers I and III, where 564 premenopausal women were randomized to tamoxifen for two years or a control group not receiving tamoxifen. In Study IV the prognosis after development of CBC was investigated in 723 patients with metachronous CBC, diagnosed within the Southern Healthcare Region of Sweden.

Incidence of CBC in relation to tamoxifen treatment (Paper III)

Of the 564 women included in the trial, 52 (9%) developed a metachronous CBC; 17 in the tamoxifen group and 35 in the control group. In the control group, 20% of the women <40 years at diagnosis of BC1 developed CBC, 9% of the women 40-49 years, and 14% of those ≥50 years. There thus appeared to be a trend towards a higher incidence in younger women, although a significant difference was only found between women <40 years and 40-49 years old. The results are summarized in Table 3.

Table 3. Development of CBC in relation to age and tamoxifen treatment.

	Control N=288		**Tamoxifen N=276**		**p-value[a]**
Contralateral breast cancer	**No**	**Yes**	**No**	**Yes**	
All ages	253 (88%)	35 (12%)	258 (94%)	17 (6%)	0.02
<40 years	49 (80%)	12 (20%)	51 (98%)	1 (2%)	0.02
40-49 years	167 (91%)	17 (9%)	165 (93%)	12 (7%)	0.4
≥50 years	37 (86%)	6 (14%)	42 (91%)	4 (9%)	0.5

[a]Univariate Cox regression

In the tamoxifen arm, 2% of the women aged <40 years developed CBC, 7% of those aged 40-49 years, and 9% of those ≥50 years. Tamoxifen significantly reduced the risk of developing metachronous CBC for all women, regardless of age (HR=0.50, p=0.02, 95% CI 0.28-0.88). This statistical significance remained in multivariate analysis adjusted for age, ER, HER2, NHG, tumour size, and lymph node status.

Tamoxifen reduced the risk of CBC in patients with ER-positive and ER-negative primary tumours. However, the reduction in risk only reached statistical significance in the

ER-positive subgroup. No interaction was found between ER status and the effect of tamoxifen, indicating that there was no difference in the effect of treatment with regard to ER status.

In the age-related analysis, the highest reduction in risk of CBC with tamoxifen was found in the youngest women, i.e. those aged <40 years at diagnosis of BC1 (HR=0.09, p=0.02, 95% CI 0.01-0.68). Although risk reduction was also seen in women aged 40-49 years and those ≥50 years, it did not reach statistical significance in these subgroups.

In the present work, tamoxifen was found to significantly reduce the risk of CBC in premenopausal women, by 50%. To the best of the author's knowledge, this is the first time the effect of tamoxifen on CBC in premenopausal patients has been investigated in a randomized trial. Previous studies have shown tamoxifen or AIs to reduce the risk of CBC in postmenopausal women[34, 231, 247, 248]. Although a similar effect has been suggested in premenopausal women[112, 180], there have also been indications of no risk reduction or even a marginally increased risk in these patients[322]. The risk reduction of 50% found in the present work is similar to, or somewhat greater than, that previously reported in postmenopausal patients[112, 277].

In the age-related analysis the greatest effect was seen in the youngest women (<40 years old), where the risk of CBC was reduced by 90%. Bertelsen et al. also found the effect of tamoxifen on CBC to be modified by age, with the largest effect in women <45 years old[180]. Hence, age might be of importance regarding the effect of treatment with tamoxifen on the development of CBC, and perhaps also for other types of therapy. In an overview by the EBCTCG, published in 2005, chemotherapy was found to reduce the incidence of CBC significantly only in women <50 years of age[35]. However, in another more recent EBCTCG overview, the effect of tamoxifen on the development of CBC was not found to be dependent on age[66].

Although the greatest effect of tamoxifen was seen in the youngest women, they also had the highest incidence of CBC. Without adjuvant tamoxifen, 12% of all premenopausal patients and 20% of the women <40 years old developed CBC within a median follow-up of 14 years. It has been shown previously that young age is a risk factor for the development of CBC[234, 237]. There have also been indications that the prognosis after CBC could be worse in young women[233]. It is unclear why CBC seems to be more common in young patients. One explanation could be that these women are more susceptible to breast cancer in general, due to hereditary mutations, or other genetic and environmental factors. Another explanation could be an increased time at risk of developing CBC, since younger women have a lower risk of dying from other causes. However, since the present study included only premenopausal women, with 475 out of 564 patients being <50 years old

when their first breast cancer was diagnosed, it seems unlikely that time at risk could explain the differences observed.

It has been discussed whether the preventive effect of tamoxifen on the development of CBC is limited to, or more pronounced in, patients with an ER-positive primary tumour[35, 112, 180, 279]. If so, the reasons for this are unclear. One explanation could be that the ER status of BC2 is related to that of BC1. Some studies have found such a correlation[279-281], while others have not[282]. Also, some women with an ER-negative primary tumour will still develop an ER-positive secondary tumour, regardless if there is such a correlation, and tamoxifen should be effective in preventing these[279]. In the present study, tamoxifen reduced the risk of CBC from 13% to 6% in patients with an ER-positive BC1, and from 11% to 4% in patients with an ER-negative BC1. Although the risk reduction was statistically significant only in the ER-positive patients, we believe this to be due to the smaller number of patients with ER-negative tumours, rather than a true difference in the effect of treatment. One implication of this could be to consider giving tamoxifen to ER-negative patients, in order to prevent the development of CBC. However, several large studies have shown tamoxifen to have no effect on prognosis after ER-negative breast cancer, and there have even been indications that tamoxifen could be detrimental to these patients[283, 284]. Hence, this would be very controversial.

Do previous treatment and time interval between tumours affect the biology and prognosis of CBC? (Paper IV)

The median time interval between BC1 and BC2 for the 723 patients included in Study IV was 6.7 years (range 0.30-36). A time interval between diagnoses of less than five years was most common (42%), with a decline in the percentage of patients diagnosed with BC2 with increasing time between tumours.

To establish whether the time interval between tumours affected prognosis, the time interval between BC1 and BC2 was first used as a continuous variable. A significant improvement in DDFS per year was seen with increasing time interval, both in univariate (HR=0.97, p=0.002, 95% CI 0.94-0.99) and multivariate analysis (HR=0.94, p<0.001, 95% CI 0.91-0.97) (adjusted for age, calendar period, mode of detection of BC2, tumour size, lymph node status and treatment for BC1 and BC2). When the patients were divided into two groups, those with a short time interval between tumours (<3 years) showed a significantly impaired DDFS compared with those with a longer time interval (≥3 years), as can be seen in Table 4. When further dividing the longer time interval into the categories 3-9 years and ≥10 years, DDFS was found to improve with increasing time interval between tumours.

Table 4. DDFS in relation to time interval between BC1 and BC2 (Paper IV).

Age at BC1	Time to BC2	Cases N	Metastasis N (%)	Metastasis/100,000 person-years	HR (95% CI)	HR* (95% CI)	HR** (95% CI)
All	<3 years	200	74 (37)	5800	1.4 (1.1-1.9)	1.6 (1.1-2.2)	1.6 (1.1-2.3)
N=723	≥3 years	523	136 (26)	4100	1.0	1.0	1.0
					p=0.01	p=0.009	p=0.01
<50 years	<3 years	55	34 (62)	11000	2.2 (1.4-3.4)	2.0 (1.2-3.4)	2.2 (1.2-3.8)
N=217	≥3 years	162	60 (37)	4900	1.0	1.0	1.0
					p<0.0001	p=0.01	p=0.006
≥50 years	<3 years	145	40 (28)	4100	1.2 (0.78-1.7)	1.5 (0.92-2.3)	1.3 (0.77-2.2)
N=506	≥3 years	361	76 (21)	3700	1.0	1.0	1.0
					p=0.5	p=0.1	p=0.3

**Adjusted for calendar period, age, mode of detection of BC2, and size and node status of BC1 and BC2.*

***Adjusted for factors listed under * and in addition radiotherapy, chemotherapy and endocrine treatment for BC1 and BC2.*

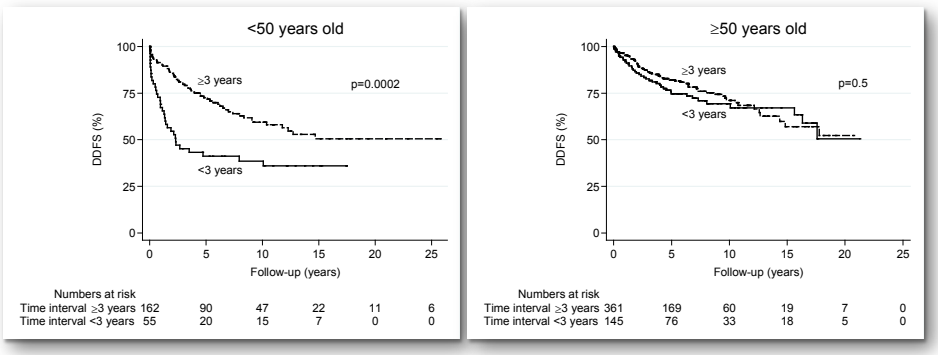


Figure 11. DDFS in relation to age and time interval between tumours (Paper IV).

Age-related analyses showed the time interval between tumours to be quite important in young women (<50 years), though not so in older women (≥50 years) (Figure 11, Table 4). A Cox model with an interaction term showed a significant interaction between age and the prognostic effect of time interval between tumours, although this significance did not remain after adjustment for other prognostic factors. Further analyses showed that a cut-off of 50 years effectively separated pre- and postmenopausal women, indicating that differences in hormonal status could be a possible explanation of the differences seen in relation to age.

To investigate the relationship between treatment given and prognosis after BC2, a multivariate Cox regression analysis was used. This was adjusted for several prognostic factors including all treatment given, as above. The analysis showed chemotherapy given for BC1 to be an independent negative prognostic factor for DDFS, whereas chemotherapy given for BC2 had no effect on DDFS. Radiotherapy for BC1 seemed to be associated with a worse prognosis, although this did not reach statistical significance. Adjuvant radiotherapy and endocrine treatment for BC2, on the other hand, were positive prognostic factors, while endocrine treatment for BC1 did not affect DDFS.

A correlation between a short time interval between tumours and poorer prognosis after CBC has been reported in previous studies[233, 252-254]. However, it has not previously been shown that this effect is predominantly seen in younger women. The reason why time interval between tumours affects prognosis is not known. One explanation could be that a higher percentage of the CBCs actually represent a metastatic spread of the primary tumour with a short time interval. A recent study by Vichapat et al. showed extensive lymph node involvement of BC1 to be a risk factor for the development of CBC[241], implying that some CBCs are metastases of BC1. Comparisons of genetic characteristics in bilateral breast cancer also indicate that contralateral spreading from BC1 does occur[258, 259, 262, 266]. Indeed, three studies have shown a short time interval between tumours to correlate with increased genetic and morphological similarities between bilateral tumours[263, 269, 270]. Although this could reflect a higher percentage of metastatic spread, another explanation could be that these tumours have developed in a similar biological environment. Vichapat et al. found young age to be a risk factor for CBC particularly in women with a recurrence of BC1 prior to CBC, while Imyanitov et al. found the greatest similarities between tumours in women who developed both tumours while premenopausal[263]. This could indicate that metastatic spread of BC1 to the contralateral breast is more common in young women with a short time interval between tumours. However, in the study by Imyanitov et al. the lowest correlation was observed in bilateral tumours separated by menopause, making hormonal environment at time of development another likely cause of similarities *vs.* differences seen[263].

Another explanation of why the time interval between tumours affects prognosis, could be that when CBC develops during or shortly after treatment of BC1, BC2 develops treatment resistance and is of a more aggressive phenotype. Since younger women generally receive more adjuvant therapy, this might explain the differences seen with regard to age. The incidence of CBC has declined since the 1980s, probably due to increased use of adjuvant treatment[233, 285]. However, the rate of mortality of those women who did develop CBC increased during the same time period[233], possibly reflecting a treatment escape phenomenon. Indeed, Hartman et al. show that patients with early metachronous breast cancer have a worse prognosis if they received adjuvant chemotherapy for their primary tumour, compared to no such treatment having been given[233]. In the present work, it was found that treatment of BC1 with chemotherapy was an independent negative prognostic factor, although not chemotherapy of BC2. Radiotherapy and endocrine treatment are positive prognostic factors when used to treat BC2, but not when used for BC1. These findings could support the hypothesis that CBC developed despite prior treatment is of a more aggressive phenotype. However, the choice of treatment is strongly correlated to tumour stage and biology, and patients selected to receive chemotherapy are those with the worst predicted prognosis. The present analyses were adjusted for several prognostic factors including tumour stage and all treatment given. However, other factors affect prognosis and choice of treatment (hormone receptor status, histological grade, etc.), information on which was not available for all the patients in this study. Hence, the results should be interpreted with caution and further studies are required for confirmation.

Does mode of detection of CBC affect prognosis? (Paper IV)

Mode of detection was known for 692 patients. Of these, 250 first noted symptoms of their CBC themselves. These patients were referred to as symptomatic. Ninety-seven patients were diagnosed at clinical examination, 257 by mammography during follow-up, and 70 by screening mammography after re-admittance to the screening programme. These 424 patients were denoted asymptomatic. Eighteen patients were diagnosed by other means (such as prophylactic mastectomy or during examination for other symptoms). These, together with the 31 patients with missing data were excluded from further analyses.

Patients who were symptomatic at diagnosis had larger CBCs, and a higher frequency of lymph node involvement. In addition, they were younger and had a longer time interval between tumours than asymptomatic patients. Logistic regression showed symptomatic patients to have a significantly higher risk of later developing metastases, in both univariate and multivariate analysis. Mode of detection remained a significant risk factor for the development of distant metastasis, even with a long time interval between tumours (0-3 years between tumours: reference group. 3-9 years: HR=2.2, p=0.005 95% CI 1.3-4.0. ≥10 years: HR=3.0, p=0.001, 95% CI 1.5-5.8).

When comparing those diagnosed by clinical examination with those diagnosed by mammography in the group of asymptomatic patients, those diagnosed by mammography were younger, had smaller tumours, and more seldom lymph node metastases associated with BC2. Additionally, the risk of later metastasis was higher for those diagnosed by clinical examination, although this did not reach statistical significance.

Previous studies have shown that regular follow-up with x-ray examinations in order to detect distant metastasis early does not improve prognosis or quality of life[287, 288]. However, recent studies suggest early detection of local recurrences or CBC to improve prognosis[289-291], although conflicting results have also been found[294, 298, 323]. A problem when studying mode of detection in relation to prognosis is that the results are easily affected by lead time and length time bias. To avoid lead time bias, the risk of metastasis was used as an endpoint instead of DDFS, thus excluding the time factor. Patients diagnosed with CBC by mammography or clinical examination were found to have a significantly lower risk of developing metastasis than patients who first noted symptoms of CBC themselves. This is probably mainly due to earlier detection. However, mode of detection remained a significant prognostic factor after adjusting for tumour size, node status and treatment for both tumours. This is in line with results in unilateral breast cancer, where diagnosis by screening mammography has been shown to be an independent prognostic factor even after adjustment for disease stage[324-327].

Assuming that surveillance after breast cancer treatment is effective, the question is how long it should be continued. In the Southern Healthcare Region of Sweden, clinical surveillance was reduced from ten years up to the mid-1990s, to five years, or even one year, followed by regular mammographic surveillance within a screening programme. For patients who developed CBC this study shows mode of detection to be associated with risk of metastasis, even when BC2 was diagnosed more than ten years after BC1, suggesting that a long follow-up period is of value.

Conclusions

AIB1 and PAX2 as prognostic and tamoxifen treatment predictive factors

A high level of AIB1 was found to be a negative prognostic factor in premenopausal breast cancer patients. This was seen both in low-risk, lymph-node-negative patients, and in patients with stage II disease. On the other hand, tumours with a high AIB1 responded very well to treatment with tamoxifen. Although patients with a low AIB1 already had a better prognosis, this was not further increased by treatment with tamoxifen. The present findings indicate AIB1 to be an independent predictive factor of improved response to tamoxifen, and not tamoxifen resistance, as has previously been suggested in unselected cohorts.

PAX2 was not found to be a prognostic or treatment predictive factor for tamoxifen on its own. Neither did it modify the effect of AIB1.

Effect of treatment with tamoxifen on the development of CBC

Women not receiving tamoxifen were found to have a 12% risk of developing CBC within a median follow-up of 14 years. This risk was even higher in the youngest women, where 20% of women <40 years developed CBC. Adjuvant treatment with tamoxifen for two years reduced the risk by 50% in all women, and by 90% in women <40 years of age.

Impact of previous treatment, time interval between tumours and mode of detection on prognosis of CBC

In the study based on clinical data from a large cohort of patients with CBC (N=723), time interval between BC1 and BC2 was found to be a strong prognostic factor in patients <50 years old at diagnosis of BC1. Additionally, mode of detection was found to be closely related to the risk of developing metastases. Indeed, among symptomatic patients diagnosed with CBC within three years of BC1, more than 50% later developed metastases.

Future perspectives

Further evaluation of AIB1 as a prognostic and treatment predictive factor

The results of these studies indicate AIB1 to be a negative prognostic factor, making it an interesting possible target for future anti-cancer drugs. However, further large studies are required to confirm these results. For example, it would be interesting to investigate AIB1 in a randomized trial of tamoxifen including postmenopausal women, or in relation to treatment with AIs. The present studies relate levels of AIB1 at the time of surgery to response to adjuvant tamoxifen. However, it would also be interesting to study changes in AIB1 levels during treatment, for example, to compare primary tumours with metastases, or BC1 with BC2 in CBC. A previous study investigating changes during treatment with AIs found an increase in coactivators and HER2, although no significant change in AIB1 was seen[328].

Additionally, preclinical studies have shown AIB1 to have many other functions apart from acting as a coactivator of the ER. This complex network of functions of AIB1 must be better understood in order to explain the effects of changes in AIB1 levels under different conditions.

Although PAX2 did not provide additional prognostic information in Study II, its prognostic effect was found to vary in relation to menopausal status in tamoxifen-treated patients. This is an interesting finding which should be further evaluated.

Further evaluation of the relationship between BC1 and BC2 in contralateral breast cancer

For the patients included in the contralateral cohort presented in Paper IV, frozen material is available in the South Swedish Breast Cancer Group's tumour bank for both tumours in 70 cases, and for only BC2 in 214 cases. In order to determine whether some CBCs actually represent metastasis of BC1, how common this is, and how these patients can be identified, we plan to investigate cases for which material is available for both tumours. This will be done by whole genome DNA sequencing, studying rearrangements and breaks. Each tumour will then have its specific "fingerprint", which will help determine the relationship between BC1 and BC2. Analysis will commence with patients exhibiting a short time interval between tumours (26 patients, <3 years) since we suspect that contralateral metastasis could be more common among these women.

The study of tumour markers and biology of CBC in relation to previous treatment and time interval between tumours

Paraffin-embedded tissue from BC1 and BC2, and when available also from lymph node metastases, local recurrences and distant metastases, have been collected from the respective pathology departments for the cohort described in Paper IV. This will be constructed into a TMA, which can be used for IHC, FISH and possibly also comparative genomic hybridization. This will allow us to compare BC1 and BC2 in relation to factors such as previous treatment, time interval between tumours and prognosis. Interesting factors include ER, PgR, HER2, AIB1, Ki67, GPR30 and cyclin D1. It will also be possible to compare lymph node metastases, local recurrences and distant metastases with the primary tumours.

The task of adapting adjuvant treatment to each patient's specific needs is not easy. The signalling networks controlling cancer proliferation and treatment response are complex, probably including several as yet unknown factors. However, with each new study we learn a little more and come one step closer to our goal. I believe that the studies presented in this thesis have contributed to this knowledge, and hope that my future work will continue to increase our understanding of the mechanisms of breast cancer.

Populärvetenskaplig sammanfattning

Varje år drabbas cirka 8000 svenska kvinnor av bröstcancer, och antalet nyinsjuknade ökar årligen med någon procent. I Sverige idag finns fler än 90 000 kvinnor som har avslutat eller är under behandling för sjukdomen. Med hjälp av nya återfallsförebyggande läkemedel har prognosen kraftigt förbättrats och 5-årsöverlevnaden är nästan 90 %. Den stora utmaningen i dagens bröstcancervård är dock att veta vilken patient som kommer att ha nytta av vilken typ av behandling. För att förhindra återfall hos några få behandlas idag många kvinnor ”i onödan”, eftersom de även utan behandling aldrig hade fått något återfall. Andra får återfall trots behandling och hade således varit bättre hjälpta av andra åtgärder. Genom att på förhand kunnat säga vilken patient som har nytta av vilken typ av behandling hade vi kunnat spara mycket onödigt lidande, tid, pengar och även liv.

Av samtliga bröstcancrar uttrycker cirka tre fjärdedelar receptorer för det kvinnliga könshormonet östrogen, vilket betyder att de är beroende av östrogen för att tillväxa och överleva. Blockering av östrogentillförseln har då visat sig vara en mycket effektiv behandlingsmetod. Detta kan göras antingen genom att blockera tumörcellernas receptorer eller genom att minska östrogennivåerna i kroppen. Tamoxifen är ett läkemedel som binder till receptorn istället för östrogen och förhindrar östrogenets effekter i bröstvävnaden. Behandling med tamoxifen minskar återfallsrisken med 50 % för opererade kvinnor och kan även hålla tillbaka sjukdomen hos dem med spridd bröstcancer. Ändå finns det de som inte svarar på behandlingen och vid spridd sjukdom utvecklar i stort sett alla med tiden resistens (det vill säga att behandlingen inte längre är verksam mot cancern). Vi har undersökt två faktorer, som man tror kan påverka prognosen vid bröstcancer, samt hur man kommer att svara på tamoxifen. AIB1 är en faktor som hjälper till att aktivera östrogenreceptorn och man har tidigare trott att den eventuellt kunde vara involverad i resistensutveckling mot tamoxifen. Vi har för första gången sett att även om kvinnor som har högt uttryck av AIB1 i sina tumörer har en sämre prognos, så har de stor nytta av tamoxifen-behandling. Att ett högt AIB1 ger en ökad risk för återfall och en kortare livslängd har vi kunnat visa i två oberoende stora patientgrupper. Det gör AIB1 till en mycket intressant faktor att studera vidare och i framtiden eventuellt kunna rikta nya läkemedel mot.

Nyligen föreslogs i cellinjer att effekten av AIB1 påverkas av en annan faktor, PAX2, och att de gemensamt avgör hur tumören kommer svara på tamoxifen. PAX2 är viktig under fosterlivet för utveckling av bland annat hjärna och njurar. Man har också sett att överuttryck av PAX2 kan förhindra celldöd och leda till cancerutveckling. Detta är första gången som PAX2 studeras i en större grupp av kvinnor med bröstcancer, som antingen fått eller inte fått tamoxifen. Vi kan visa att för bröstcancerpatienter verkar inte PAX2 ensamt påverka prognosen. Inte heller förändrar PAX2 den prognostiska effekten av AIB1.

Den andra delen av denna avhandling handlar om kvinnor som först fått en tumör i ena bröstet och sedan en i andra bröstet, så kallad kontralateral bröstcancer (CBC). För dem som botas från sin första bröstcancer är risken att drabbas av CBC 2-6 ggr högre jämfört med risken för befolkningen i övrigt att drabbas av bröstcancer. Faktorer som ökar risken är ung ålder, ärftlighet samt en viss form av bröstcancer som kallas lobulär.

I en studie där unga bröstcancerpatienter (före klimakteriet) lottats till att få behandling med tamoxifen eller inte, kunde vi se att utan tamoxifen utvecklade 12 % av kvinnorna CBC inom 14 år. Bland de yngsta kvinnorna (<40 år) var dock risken så hög som 20 %. Behandling med tamoxifen minskade risken med 50 % hos samtliga patienter, och med så mycket som 90 % hos de allra yngsta. Riskminskningen sågs oberoende av om den första bröstcancern uttryckte östrogenreceptorer eller ej. Normalt används inte tamoxifen till kvinnor vars tumörer inte uttrycker östrogenreceptorer, eftersom man vet att läkemedlet inte hjälper mot den typen av tumör. Av denna studie kan man dock dra slutsatsen att vissa av dessa kvinnor, som har en hög risk för CBC, kanske ändå hade haft nytta av tamoxifen för att förebygga att de får en ny tumör. Att överväga att ge även dem läkemedlet är dock kontroversiellt, eftersom det inte hjälper mot den tumör de för tillfället behandlas för, samt då det trots allt finns en risk för biverkningar.

Även om risken för CBC är relativt hög för kvinnor som tidigare behandlats för bröstcancer, är sjukdomen totalt sett i befolkningen inte så vanlig. Tidigare studier är därför ofta små eller enbart baserade på registerdata. Vi har samlat in journalmaterial från över 700 kvinnor med CBC i södra Sverige. Där finns information om bakgrundshistoria, hur tumörerna upptäckts, hur de behandlats och hur det sedan gått. Från detta har vi kunnat se att kvinnor som fått sin andra tumör nära inpå den första har en sämre prognos. Orsaken till detta är oklar. En förklaring kan vara att vissa av dessa tumörer inte är en ny cancer utan en spridning av den första. Detta hade i så fall krävt en helt annan typ av behandling. En annan förklaring kan vara att om en ny tumör lyckas uppstå trots att man nyligen genomgått behandling för den första, är den nya tumören mer aggressiv och resistent mot den behandling som redan givits.

Slutligen såg vi även att kvinnor vars CBC diagnosticerades inom ett uppföljningsprogram (med mammografi-röntgen eller vid undersökning av en läkare) hade en lägre risk att senare utveckla spridd sjukdom jämfört med de kvinnor som själva upptäckte tumör nr 2. Detta gällde även om lång tid (≥10 år) förflutit mellan tumörerna och tyder på att regelbunden uppföljning efter bröstcancerbehandling kan vara av värde.

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