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PO Box 117
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+46 46-222 00 00

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Modern Staging in Primary Breast Cancer

Aspects of micrometastatic disease in bone marrow and molecular profiles in lymph node metastases

ANNA-KARIN FALCK, MD

DEPARTMENT OF SURGERY | CLINICAL SCIENCES LUND | LUND UNIVERSITY

MODERN STAGING IN PRIMARY BREAST CANCER

Modern Staging in Primary Breast Cancer

Aspects of micrometastatic disease in bone marrow and molecular profiles in lymph node metastases

Anna-Karin Falck, MD

Doctoral dissertation
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Faculty opponent:
Professor Jan Frisell
Department of Molecular Medicine and Surgery
Karolinska Institutet, Karolinska Universitetssjukhuset,
Solna, P9:03 SE 171 76 Stockholm

LUND UNIVERSITY
Faculty of Medicine

Lund 2013
Division of Surgery
Department of Clinical Sciences Lund

Anna-Karin Falck 2013
e-mail: anna-karin.falck@med.lu.se

Supervisor: Associate professor Lisa Rydén
Division of Surgery, Department of Clinical Sciences Lund
Lund University, Sweden

Co-supervisor: Professor Mårten Fernö
Department of Oncology, Clinical Sciences Lund
Lund University, Sweden

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Abstract
Breast cancer is a heterogenous disease with variations in the biological profile and subsequent clinical prognosis. Molecular mechanisms for tumour progression are currently being explored in scientific settings, suggesting parallel evolution of tumour cells at primary and metastatic locations. The molecular profiles could be altered affecting individual patient's treatment and prognosis. In the work presented in this thesis the main focus was on evaluating tumour tissue and disseminated cells from different metastatic locations, to analyse the distribution of biomarkers and relate them to the prognosis for individual patients.

A relationship has been recognised between the presence of disseminated tumour cells (DTCs) in bone marrow and poor prognosis in several studies, but the method of DTC detection has yet to be validated. It is at present not known whether DTCs will grow into overt metastases or which biologic events are involved in the metastatic process. In paper I, bone marrow biopsies were performed at the time of primary surgery and the presence of DTCs analysed. After 5 years of follow-up, all reports of events were abstracted from the patient's records and a database was constructed for all the patients included in the study. The presence of DTCs in the bone marrow was found not to have any prognostic influence and it was concluded that it is too early for clinical implementation due to discrepancies in methods between studies, and the invasive nature of bone marrow biopsies.

Oestrogen receptors (ER), progesterone receptors (PR), Ki67 and human epidermal growth factor receptor 2 (HER2) were compared in primary breast cancer tumours and the synchronous lymph node metastases (Papers II and IV) and primary tumours and relapses (Paper IV). High concordance was found between primary tumours and lymph node metastases regarding separate biomarker expression in both papers, but a significant skewness was observed when biomarkers in primary tumours were compared to those in relapses. Classification into molecular subtype according to the St Gallen guidelines (Papers III and IV) identified a shift in molecular subtype in individual patients that affected prognosis, suggesting that the molecular subtype of the lymph node metastasis has a prognostic influence. Prognostic information for the individual patient can thus be obtained by analysing biomarker expression in synchronous metastatic lymph nodes. The findings presented in Paper IV support the recommendation that biomarker analysis is performed in loco-regional and distant relapses.

Key words: Breast cancer, biomarker expression, lymph node metastasis, relapse, molecular subtype classification, disseminated tumour cells, bone marrow, prognosis, mode of detection

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"If you want to win a race try the 100 meter.
If you want to win an experience try the marathon."
Emil Zatopek

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Included Papers

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

I **Anna-Karin Falck**, Pär-Ola Bendahl, Christian Ingvar, Jorma Isola, Per-Ebbe Jönsson, Pia Lindblom, Kristina Lövgren, Karin Rennstam, Mårten Fernö, Lisa Rydén
Analysis of and prognostic information from disseminated tumour cells in bone marrow in primary breast cancer: a prospective observational study
BMC Cancer 2012, 12:403, denoted "highly accessed".

II **Anna-Karin Falck**, Mårten Fernö, Pär-Ola Bendahl, Lisa Rydén
Does analysis of biomarkers in tumor cells in lymph node metastases give additional prognostic information in primary breast cancer?
World Journal of Surgery 2010, 34(7):1434-41

III **Anna-Karin Falck**, Mårten Fernö, Pär-Ola Bendahl, Lisa Rydén
St Gallen molecular subtypes in primary breast cancer tumour and matched lymph node metastases – aspects on distribution and prognosis for patients with luminal A tumours
Submitted

IV **Anna-Karin Falck**, Pär-Ola Bendahl, Gunilla Chebil, Hans Olsson, Mårten Fernö, Lisa Rydén
Biomarker expression and St Gallen molecular subtype classification in primary tumour, synchronous lymph node metastasis and asynchronous relapse in primary breast cancer patients with 10 years´ follow-up.
Breast Cancer Research and Treatment 2013, 140:93–104

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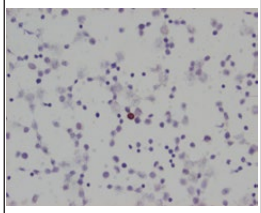
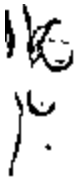
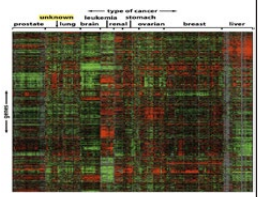
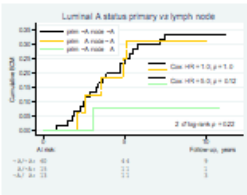
Related paper

Strand C, Bak M, Borgquist S, Chebil G, Falck AK, Fjällskog ML, Grabau D, Hedenfalk I, Jirström K, Klintman M, Malmström P, Olsson H, Rydén L, Stål O, Bendahl PO, Fernö M. The combination of Ki67, histological grade and oestrogen receptor status identifies a low-risk group among 1,854 chemo-naïve women with N0/N1 primary breast cancer. *Springerplus*. 2013 Dec;2(1):111. Epub 2013 Mar 14

Thesis at a glance

Study	Aim	Methods	Results and Conclusions
I 	To determine whether prognostic information can be gained from the presence of disseminated tumour cells in bone marrow in primary breast cancer.	The presence of tumour cells in bone marrow was investigated prospectively in samples collected from patients with primary breast cancer, and related to prognosis.	Cytokeratin-positive cells were identified in 38% of the patients. No correlation was found between the presence of tumour cells in bone marrow and poor prognosis.
II 	To investigate whether there is a difference in the expression of ER, PR, HER2 or Ki67 in primary breast tumour and the corresponding lymph node metastases.	ER, PR, Ki67 and HER2 were analysed in breast carcinoma and metastatic lymph node samples using immunohistochemistry. The expression of tumour characteristics was related to prognosis.	High concordance was found of the analysed biomarkers between primary breast tumour and metastatic lymph nodes. Prognostic information was obtained from biomarker analyses in both primary tumours and lymph node metastases.
III 	To determine whether there is a difference in molecular subtype when comparing primary breast tumours with the corresponding lymph node metastases.	Combination of biomarker expression (Paper II) to classify St Gallen molecular subtypes in both primary tumour and corresponding lymph node, in relation to prognosis.	7/45 (16%) shifted from luminal A in the primary tumour to a molecular subtype with a worse prognosis in the corresponding lymph node metastasis. Prognostic information was obtained from biomarker analyses in both primary tumours and lymph node metastases.
IV 	To investigate whether there is a difference in biomarker expression or molecular subtype, between the primary tumour, lymph node metastasis and relapse from the same patient.	Re-analysis of tumour tissue from primary tumours, lymph node metastases and relapses regarding ER, PR, HER2 and Ki67 separately and in combination in St Gallen molecular subtypes. Results were related to prognosis.	Prognostic information for the individual patient can be obtained by analysing biomarker expression in synchronous metastatic lymph nodes. The findings support the use of biomarker analysis in loco-regional and distant relapses.

Abbreviations

AIs	aromatase inhibitors
BCM	breast cancer mortality
BCSS	breast cancer-specific survival
CI	confidence interval
CKs	cytokeratins
CTC	circulating tumour cell
CMF	cyclophosphamide, methotrexate, 5-fluorouracil
DDFS	distant-disease-free survival
DFS	disease-free survival
DTC	disseminated tumour cell
EGFR	epidermal growth factor receptor
ER	oestrogen receptor
FNA	fine-needle aspiration
GnRH	gonadotropin-releasing hormone analogues
HER2	human epidermal growth factor receptor 2
HR	hazard ratio
ICC	immunocytochemistry
IF	immunofluorescence
IHC	immunohistochemistry
ISH	*in situ* hybridization
MRI	magnetic resonance imaging
NHG	Nottingham histological grade
PR	progesterone receptor
SISH	silver-*in situ* hybridization
SN	sentinel node
TNM	tumour size, lymph node metastasis, distant metastasis
TMA	tissue microarray

Introduction

Breast cancer is the most common form of cancer among women in Sweden, 8,382 new tumours being diagnosed in 2011 [1]. Early detection by mammography [2] and a high awareness among women together with effective adjuvant treatment [3] have improved the prognosis for those affected, and approximately 87% survive 5 years after diagnosis [1]. Depending on the patient and tumour characteristics adjuvant treatment is often recommended, such as endocrine, chemo-, anti-human epidermal growth factor receptor 2 (HER2) or radiation therapy, alone, or in combination. Prognostic information for the individual patient is based on the analysis of the biological characteristics of the primary tumour including the presence of oestrogen receptors (ER), progesterone receptors (PR), HER2 and the antigen Ki67 which, together with age, tumour size, histological grade and lymph node engagement gives a prognostic profile as a basis for clinical recommendations on adjuvant treatment. However, the clinical outcome varies despite identical biomarker profiles and stages: 20% of patients with node-negative disease will have a recurrence, and 20–30% of patients with lymph node metastases will remain disease-free [3, 4]. A more precise prognostic tool is thus needed to identify patients who would have benefited from adjuvant therapy, as well as patients in whom adjuvant therapy can be safely omitted. Recently, microarray-based gene expression studies [5, 6] and subsequent immunohistochemical (IHC) studies [7–10] have shown that further prognostic and predictive information can be gained by combining the biological characteristics in the primary tumour, rather than determining them separately [7–9]. At least four molecular subtypes have been identified: luminal A, luminal B (HER2– and HER2+), HER2 type and triple negative [11] illustrating the heterogeneity of breast cancer. The St Gallen molecular subtype definitions [11] were recently validated in a single hospital cohort of primary operable breast cancer patients [12].

Two different models have been proposed for tumour progression (Figure 1) [13]. Clinical management today is based on the linear progression model, in which it is assumed that the tumour cell characteristics are fully developed at the site of the primary tumour [13, 14]. Dissemination of these cells at a later stage of the disease leads to metastases with a biological profile identical to that of the primary tumour [13]. The second model, the parallel progression model, is based on the assumption that cancer cells can spread early in the development of the disease, from different clones of the same primary tumour [13]. The disseminated tumour cells can evolve in their new microenvironment, and exhibit a different biological profile from the primary tumour [15]. The identification of disseminated tumour cells (DTCs) and biomarker classification of malignant cells in the metastatic lymph node or distant metastasis could, therefore, be of importance for the prognosis and choice of adjuvant treatment for the individual. The work presented in this thesis was focused on evaluating tumour tissue and disseminated cells from different locations, formed during tumour progression, to analyse and evaluate the distribution and changes in biomarkers, separately and classified according to surrogate molecular subtypes, and the possible relation to prognosis for individual patients.

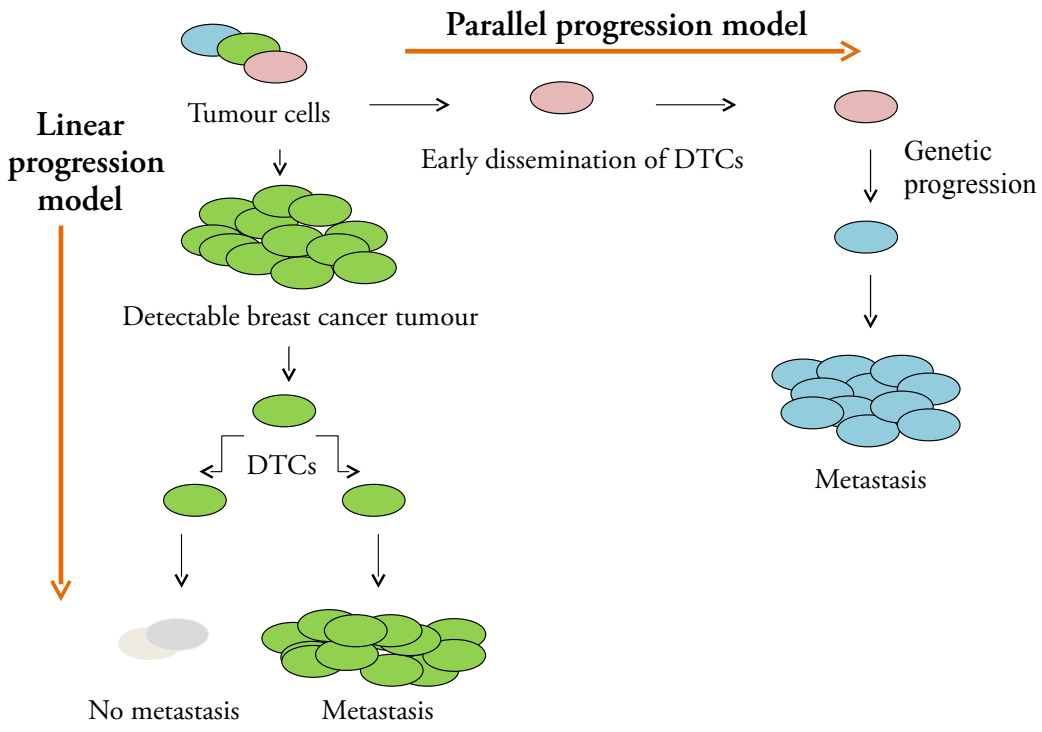


Figure 1.

Background

The breast

The breast is composed mainly of fatty tissue and connective tissue. The glandular tissue constitutes a minor part of the breast but the proportion between fat and glandular tissue varies individually [16]. The mammary glands are made up of alveoli lined by milk-secreting, luminal cells, surrounded by myoepithelial cells responsible for contraction when stimulated by the hormone oxytocin. The alveoli join to form lobules that are connected to ducts terminating in the nipple. With the onset of puberty, hormonal changes trigger a cascade of molecular activity that affects the mesenchymal cells located in the connective tissue and the multipotent cells in the premature glandule. The multipotent cells mature into luminal epithelial cells and myoepithelial cells with the ability to contract. In menopause, the glandular tissue is gradually replaced by fat as a result of the decrease in the level of oestrogens [17, 18].

The lymphatic drainage of the breast closely follows the blood vessels, and is mainly directed to lymph nodes in the axilla on the same side, but also in the medial direction to internal mammary nodes and, occasionally in the connective tissue (stroma) of the breast [16]. The physiological function of the lymphatic system is to return interstitial fluid to the blood and to expose the immune system to foreign intruders (antigens). The lymph nodes are located in the lymphatic flow to capture the antigens and organize the immune response in a dynamic way. The presence of lymph nodes is the major difference between

the blood and lymphatic circulatory systems, and the physiological connection between them could provide conditions for circulating tumour cells to transit from one system the other [15, 19]. In breast cancer, the most common metastatic location is the axillary lymph nodes, and the presence of such metastases is an important prognostic factor, suggesting that adjuvant systemic treatment should be recommended in the clinical setting [20]. One suggested route for the formation of lymph node metastases is that tumour cells could be selectively attracted to transit to the lymph nodes by chemokines produced by lymphatic vessels [21]. The lymphatic system may act on these tumour cells to promote the formation of metastases and to provide them with the ability to induce the formation of distant metastases [22, 23].

Epidemiology and risk factors

Almost 8,400 breast tumours are diagnosed in Sweden every year [1], accounting for 30% of all female malignancies [1]. It is not known why a normal breast cell mutates and becomes a malignant cell, but some risk factors have been identified in the development of breast cancer. The association between hormonal activity and the risk of breast cancer is thoroughly investigated. Early menarche, late menopause, low parity and high age at first full-term pregnancy [24] [25], together with hormone replacement therapy consisting of a combination of gestagen and oestrogen [26], have been shown to increase the risk, as have oral contraceptives to a moderate degree [27]. Life style factors such as obesity [28] and a high intake of alcohol [29] are both associated with a higher incidence of breast cancer in postmenopausal women while regular physical activity seems to decrease the risk of breast cancer [30]. Exposure to radiation at a young age, for example in the treatment of lymphoma, is a confirmed risk factor for developing breast cancer later in life [18]. High breast density, i.e. a breast rich in connective and epithelial tissue, is also an established independent risk factor [31]. The incidence of breast cancer increases with socioeconomic status, a fact that is at least partially, explained by the higher participation in mammography screening programmes [32].

Previous diagnosis among family members and genetic inheritance are specific factors associated with an increased risk of developing breast cancer for the individual patient. Genetic testing, risk prediction and counselling are offered to those with an accumulation of breast cancer in the families in Sweden. To date, two genes have been identified as being related to breast cancer: BRCA1 (chromosome 17, 1994) and BRCA2 (chromosome 13, 1995), which are associated with a life time risk of developing breast cancer of up to 80%, usually with a tendency to occur at an earlier age [33, 34]. The BRCA-1 associated breast cancer is more often negative for ER and PR and shows no HER2 amplification [35]. Microarray-based gene expression analysis has identified BRCA1 as a basal-like molecular subtype – triple negative, with a special cytokeratin profile [36]. The characteristics of BRCA2-associated breast cancer are more heterogeneous, but ER are more often expressed [35]. Patients with known mutations of these genes are given advise according to explicit guidelines [37]. The majority of patients with a familial predisposition have genes of low penetrance [38]. Statistical models for estimating the risk for individual patients have been developed and are currently used in oncogenetic counselling [39].

Diagnostics

Triple assessment is the golden standard for breast cancer diagnosis, when a woman presents with clinical symptoms in the breast, or when breast cancer is suspected as a result of screening mammography. The assessment includes radiological imaging, clinical examination of the breast and loco-regional lymph nodes and cytological examination of cell samples obtained with fine-needle aspiration (FNA) or histopathological examination of core biopsies. Triple

assessment have almost100% sensitivity [40]. In Sweden approximately 50% of all breast cancers are diagnosed by mammography screening, varying between 30 and 80% in different geographical regions, according to a report by the Swedish Cancer Society [41]. Mammographic screening in Sweden started in 1986 when several studies showed a significant reduction in breast cancer mortality among women attending [42, 43]. According to updated data from meta-analyses and reviews, the relative reduction in the risk of mortality due to breast cancer is approximately 15% [44, 45], and according to current Swedish guidelines, all women between the ages of 40 and 74 are offered mammographic examination at regular intervals [37]. The benefit of mammography screening has been intensely debated during the past twenty years [46], and it has been suggested that the suggested 15% screening-related reduction in breast cancer mortality [44] is cancelled out by an inherent risk of overdiagnosis [47]. The tumours detected by screening are smaller, are more often node negative, generally have a lower histological grade, and are associated with a better prognosis than clinically detected tumours [48, 49]. The prognostic disparity is not fully explained by biological differences [49, 50].

Magnetic resonance imaging (MRI) is recommended as a complement to mammography and ultrasound in screening of women with the BRCA 1 and 2 genes [51] as it has been reported that MRI has a higher sensitivity than other radiological imaging modalities [52]. Ultrasound and MRI are complementary to radiological imaging of the breast, and ultrasound has been suggested to have a higher sensitivity in diagnosing breast cancer, especially in women with high density breasts [53]. Ultrasound is also preferable method in women younger than 35 years, and in women who are pregnant or breast-feeding [54].

FNA is complemented with a core needle biopsy for histological analysis when cytological examination is inconclusive. Core needle biopsy has become the method of choice for tissue examination at some breast units in Sweden and in other countries, and when neoadjuvant treatment is planned in order to confirm the invasiveness of the disease and evaluate the biological characteristics to ensure optimal treatment. The sensitivity and specificity of the two methods are comparable regarding the detection of malignancy [55, 56], although core needle biopsy is superior for the analysis of biomarker expression [57].

Prognostic and predictive factors

Prudent selection of patients for adjuvant treatment requires consideration of clinical, pathological and biological factors [3]. The risk associated with recurrence and death have traditionally been based on a combination of tumour size and lymph node status, which is integrated in the tumour node metastasis (TNM) staging system [58], histological grade and age. In addition, biomarkers expressed by tumour cells are included in prognostic and/or predictive information. Prognostic information is related to the outcome in the systemically untreated patient, while predictive factors can be used to predict the response to a specific treatment. Sometimes, information on a biomarker can provide both prognostic and predictive information, as is the case for the expression of ER and HER2.

Age

Breast cancer incidence increases with age [59], 50% of all new breast cancer patients in Sweden are 63 years of age or older [1]. In women older than 80 years of age 1,195 new cases were diagnosed, and in the youngest group of patients, women < 40 years, the incidence was 115 new cases in 2011 [1]. Diagnosis of breast cancer before the age of 40 is a prognostic factor and associated with worse outcome than in older women [59, 60]. In young women with breast cancer, tumours are more often ER negative, have a higher proliferation index and are of higher histological grade [61, 62].

Histopathological subtypes

Breast carcinomas exhibits different morphological characteristics and are classified according to the World Health Organization (WHO)[63]. Invasive ductal carcinomas comprise the largest group (50–80%), followed by invasive lobular cancer (5–15%), and more rare tumour types (1–7%), which are classified as: tubular carcinomas (2–7%), medullary (1–7%), invasive cribiform (0.8–3.5%), mucinous (2%) and papillary (< 1–2%)[63]. Inflammatory breast cancer has special pathological and clinical features which are caused by lymphatic tumour obstruction. The clinical signs include redness and oedema of the skin, warmth and tenderness, all signs of inflammation although it is not an inflammatory condition. The reported frequency varies between 1 and 10% depending on the inclusion criteria [63–65].

The TNM staging system

The TNM classification system is based on tumour size and invasiveness (T), number, location and fixation of lymph node metastases (N), and the presence of distant metastases (M)[66]. Tumour size is related to the frequency of nodal metastases, such that 50% of patients with a tumour size > 20 mm have lymph node metastases, whereas only 10–20% with tumour size < 10 mm have a metastatic node [67, 68]. The prognostic information obtained from T is well established. Approximately 90% of women with a tumour < 10 mm will not suffer recurrence. The corresponding proportion for patients with tumours 20–50 mm in size is 60% [62, 69, 70]. The presence of lymph node metastases is a well-known, important prognostic factor for relapse and death due to breast cancer disease [70–73]. It has also been found that cancer-related mortality increases with increasing number of lymph node metastases for patients with tumours of equivalent size, irrespective of systemic therapy with up to 15 years follow-up [74, 75]. With the introduction of the sentinel node (SN) technique for staging axillary lymph node metastases, concerns were raised about stage migration as the result of increased detection of micrometastases (< 0.2 mm) [76, 77]. Micrometastatic disease in associated lymph nodes is being evaluated in clinical studies in terms of recurrence and survival, and recent data suggest impaired prognosis, similar to that when macrometastases are present [78–82]. The need for axillary clearance when micrometastases are present requires further investigation [83]. The TNM system may be further refined by taking the ratio of the number of metastatic lymph nodes to the number of resected lymph nodes into account [84], but this also needs further evaluation. In clinically detected breast cancer approximately one-third of the operable patients presents with metastatic lymph nodes, and 10–15% with > 3 lymph node metastases [67]. Early detection of breast cancer by mammographic screening and awareness of breast cancer have resulted in an increasing number of patients with smaller tumours and a smaller number of positive lymph nodes [85, 86].

The presence of distant metastasis defines the point at which the disease becomes incurable although survival can be prolonged with varying systemic treatment [87–89].

The Nottingham Histological Grade

The Nottingham histological grade (NHG) of tumours is a microscopic evaluation, by a pathologist, of tubule formation, mitotic count in a defined field area, and nuclear polymorphism [90]. Each of these features is assigned 1–3 points which are then summed: 3–5 points = grade 1, 6–7 points = grade 2, and 8–9 points = grade 3. The combined score is a measure of the degree of differentiation or, in other words, how closely the tumour cell resembles a normal breast epithelial cell. Cancerous cells lose their differentiation and thus their normal function. Grade 1 represents highly differentiated tumours, grade 2 tumours are intermediately differentiated, and grade 3 have a low degree of differentiation and are associated with the worst

prognosis [91]. Studies on the reproducibility of grading have been performed revealing some inter-observer variability [92, 93].

Oestrogen and progesterone receptors

The connection between hormones and breast cancer was established in 1896, when Beatson presented the results of oophorectomy for advanced breast cancer, reporting that some of the patients showed remarkable regression of their metastatic lesions [94]. The identified link was further investigated with the ablation of different hormone-influencing organs, and later by the administrations of substances affecting hormone levels in breast cancer patients. ER was identified in the 1970s, and could be linked to the response of a patient's tumour to endocrine ablation [95]. ER is an intracellular nuclear receptor in the steroid transcription factor family [96]. ER is regulated by ligand-binding of oestrogens to the activation function (AF) 2 domain and/or by phosphorylation of AF1 [96, 97]. Gene expression by activated ER may be direct, on specific DNA response elements, or through interactions with other transcription factors [98]. Apart from this classical signalling pathway, the action of ER located in the cell membrane or cytoplasm can be mediated by non-genomic events or by signalling events of tyrosine kinase receptors such as HER2 [99]. ER is expressed in two forms, ERα and ERβ of which ERα is the most studied. ERβ is not routinely measured in the clinical setting, and the descriptions of ER in this thesis refer to ERα. ER is expressed in over three-quarters of breast cancer patients [100], and can be evaluated by IHC. ER expression is known to be associated with a better prognosis for the first five years after diagnosis [101], but seems to predict late relapses, as ER-negative patients and ER-positive patients with no adjuvant endocrine treatment have similar recurrence rates after 15 years [3, 72].

Tamoxifen, a partial oestrogen receptor antagonist that prevents AF2 activation [96], was initially developed in a contraceptive research programme, but proved to be useless for this purpose [102]. Instead, it was found to be effective in breast cancer patients, producing few side effects [102]. The benefits of tamoxifen for the treatment of advanced disease have been demonstrated since the 1970s [103] and is also a thoroughly investigated therapy in the adjuvant setting. Tamoxifen reduces mortality by approximately one-third in ER-positive patients treated for about five years [101], thus ER is a strong predictive factor for adjuvant endocrine therapy with tamoxifen. The relationship between ER expression levels and sensitivity to different forms of endocrine treatment has not been established, although some studies have indicated a reduction in recurrence and mortality in patients with tumours showing higher levels of ER [3, 104]. The recommended level for ER-positivity according to Swedish national guidelines, is 10% [37], based on the results of a meta-analysis in which patients with tumours expressing low levels of ER showed only a weak response to endocrine treatment, and those with ER-negative tumours showed no response at all, at long-term follow-up [101].

The method of assessing ER expression in the majority of the studies included in the meta-analysis was, however, a ligand-binding ER assay method in cytosol, which has now been replaced by IHC methods. Retrospective studies have shown IHC analysis to be superior for prognostic and predictive purposes [105–107], and is cheaper, less labour-intensive, more reliable since it enables morphological assessment, and can be used on archived formalin-fixed, paraffin-embedded tissue [108]. The two methods have been compared, showing reasonably good concordance [106, 107, 109], although the lack of standardised protocols for pre-analytical and analytical variables is the cause of discrepancies between laboratories concerning ER assessment by IHC [104, 110, 111]. The recommended level for ER-positivity applied internationally is 1% to minimize the risk of excluding patients who may benefit from endocrine treatment [20, 111].

PR is simultaneously expressed in > 50% of

the ER-positive tumours [112] but has been less studied than ER. PR is a nuclear receptor that influences normal breast development [100] and, like ER, it can be assessed by IHC. No ER-independent mechanisms of action have been established, and no benefit of endocrine therapy was detected in the treatment of ER-negative / PR-positive tumours in a recent long-term meta-analysis [101]. PR expression has also been used for the differentiation of luminal A and luminal B molecular subtypes. ER-positive tumours simultaneously expressing PR > 20% exhibited a better prognosis [113]. The recommendations of the St Gallen International Expert Consensus Conference (2009) are that in the absence of ER, PR-positive cases should be submitted for further pathological review [20].

Human epidermal growth factor receptor 2

HER2 is a protein, a receptor tyrosine kinase, located on the cell surface, as a transmembrane protein, belonging to the human epidermal growth factor receptor family (HER) which includes HER1 (epidermal growth factor receptor, EGFR), HER2, HER3 and HER4 which affect cell proliferation, differentiation, adhesion and cell survival [114]. HER2 is encoded by the proto-oncogene *erbb2,* which was identified and reported to be amplified in women with breast cancer in the late 1980s. Its presence has been correlated to a shorter time to relapse and lower survival rates, irrespective of nodal status, tumour size, histological grade or ER/PR expression [115–118]. HER2 can be activated by ligand-independent fusion with other tyrosine kinase receptors in the HER family or with another HER2 molecule, which results in downstream signalling leading to stimulation of cell growth [114, 119]. A monoclonal antibody against the extracellular domain of the HER2 was developed, and the first clinical studies of anti-HER2 therapy (trastuzumab) for metastatic disease started in the early 1990s. HER2 is overexpressed/amplified in approximately 15% of breast tumours [120, 121], half of which are ER negative [122]. About 10% of ER-positive patients show simultaneous expression of HER2 [123]. The level of expression of HER2 protein can be assessed by IHC, and is reported according to a standard protocol using the HercepTest (DAKO, Copenhagen, Denmark). The protocol categorises tumours on a four-level scale (0; lack of staining in all tumour cells or membrane staining in less than 10% of the tumour cells; 1+: weak, non-circumferential membrane staining in less than 10% of the tumour cells; 2+: intermediate, circumferential membrane staining in more than 10% of the tumour cells; 3+: intense and circumferential staining in more than 10% of the tumour cells. If the level is 3+ or 2+, *in situ* hybridization (ISH) should be used as a complement to assess the amplification of the gene [124, 125]. The predictive value of HER2 has been further evaluated in various clinical settings, and treatment with trastuzumab in combination with chemotherapy has shown efficacy in metastatic disease [126, 127], in primary operable breast cancer [128, 129], and in the neoadjuvant setting [130, 131].

Epidermal growth factor receptor

EGFR is a cell-surface protein in the same receptor tyrosine kinas HER family as HER2. EGFR can be activated by ligand binding or by dimerization with other receptors in the HER family to send signals to the cell nucleus resulting in DNA synthesis and cell proliferation [132]. Overexpression of EGFR has been found in different forms of cancers, especially lung and anal cancer [133, 134]. In breast cancer, EGFR is reported to be overexpressed in 14–92% of patients [135–137], and has been associated with poor prognosis [135], although the prognostic value has not been established [138]. Expression of either EGFR or basal cytokeratins (CK 5/6) has been suggested to identify the basal-like molecular subtype within the subset of triple-negative molecular subtype [9], but the definition of the basal-like subtype remains to be established [11].

Ki67

Tumour cells are characterised by high proliferation which is associated with a poor prognosis [139, 140]. Proliferation can be evaluated by various techniques including the mitotic count, expressed as the mitotic activity index, measurement of S-phase fraction, cyclins/cyclin-dependent kinases and thymidine-labelling index [140]. IHC staining of antigens associated with proliferation, includes the proliferation marker Ki67, a nuclear protein present in all phases of the cell cycle except G0, and reaches a maximum at mitosis [141]. The exact function of Ki67 is not known, but blocking Ki67 prevents cell proliferation [142]. High expression is associated with poor prognosis [143, 144], and has been shown to identify patients with poor prognosis among grade II, ER-positive tumours [145]. However, values defining high and low proliferation have not been established, and no systematic comparison of the assessment of Ki67 on tissue microarrays (TMA) *vs* whole slides have been performed, which restricts the clinical utility [146]. Staining of 10–20% of the nuclei in invasive cancer cells, is the most commonly used level to differentiate high and low proliferation [147] The recommendation in the St Gallen guidelines from 2011 is to use a cut-off of 14% to distinguish between luminal A and B molecular subtypes [7, 11]. Further work is needed to validate the method and demonstrate the reliability of Ki67 staining before it can be used as a predictive factor in patients.

Disseminated tumour cells

The term DTC is defined as any tumour cell that has left the primary site and disseminated to a secondary location. The importance of DTCs is based on the hypothesis that persistent tumour cells are the origin of metastatic disease. At present, it is not known which DTC will form overt metastasis or which mechanisms govern the metastatic process [13].

One way for tumour cells to disseminate is through the vascular system and thereafter return to the primary tumour [148] or extravasate into distant organs [149] promoted by hitherto unknown environmental and/or internal factors [150]. DTCs can be found in bone marrow in several tumour types, suggesting bone marrow to be a reservoir for the haematogenous spread of tumour cells [151]. CKs form part of the intermediate filaments, or the cytoskeleton, in mammalian cells that keep cells together [152]. Twenty CKs have been identified, and different forms are located in the epithelial and myoepithelial cells lining the glandular ducts in the breast [153]. The identification of CKs by IHC has been suggested to indicate cells of epithelial origin in lymph nodes and bone marrow of patients with breast cancers [151] [154], as haematopoietic and lymphoid cells do not express CKs [155]. The detection of CK positive cells in bone marrow is considered to classify these cells as being of epithelial origin, indicating subclinical tumour dissemination, denoted micrometastases [151, 154–156]. The prognostic relevance of presence of the micrometastases in bone marrow has been evaluated in a number of studies showing varying association with prognosis and clinicopathological characteristics [157–162]. However, level I evidence for poor prognosis was established in 2005 when a pooled analysis of bone marrow micrometastases was reported [163]. The presence of DTCs was reported in approximately 30% of women with primary breast cancer, and a 10-year follow-up showed a lower disease-free and overall survival than in patients with no DTCs in the bone marrow. However, sampling of bone marrow for the detection of DTCs is not included in clinical guidelines, as it is an invasive procedure and there is no validated method for the IHC detection of the very small numbers of DTCs, although protocols for standardisation have been published [164]. DTCs can be further characterised according to their expression of specific biomarkers (ER, HER2) or genetic changes [165–169], analyses that has been reported to show discordances when compared with prima-

ry tumours [165, 167]. Genetic analyses suggest early dissemination of DTCs from the primary tumour, in line with the parallel progression model [13], showing less discordances in genetic aberrations of cells from primary tumours *vs* DTCs from bone marrow in patients with no evidence of metastatic disease, than *vs* tumour cells from identified metastases [166, 170]. Micrometastatic disease in bone marrow is detected in 30% (median) of primary breast cancer patients, with a range from 16%–60% [163]. The disparity in detection rate is partly attributed to differences in the method of detection, however, it has also been suggested that it indicates that DTCs can be quiescent or dormant, a phenomenon in the progression of cancer that is not yet fully understood [171, 172]. Cells in the dormant state are viable but not proliferating [173], this could be a reflection of a latency period needed for mutation, selection and inheritance, as DTCs from the same individual often display genetic heterogeneity years before a metastasis is clinically detected [174]. Fairly recent animal studies suggest that the seeding of cancer cells from a primary tumour may not only be directed from primary site, but also is suggested to return to the primary tumour and promote growth and further maturation in the form of DTC or circulating tumour cells (CTC), in a self-seeding way [148]. However the mechanisms for induction and reentering of tumour cells in the metastatic process are unknown.

The spread of tumour cells to the lymph nodes has been suggested to follow a different route [175]. Comparisons of micrometastatic disease in bone marrow and lymph node metastases show that dissemination rarely occurs simultaneously at both sites [176–179]. No correlation was found between the micrometastatic disease in sentinel lymph node and bone marrow in a prospective trial including 5,210 patients with primary operable breast cancer with no clinical signs of lymph node metastases. No association was found between survival and the presence of occult disease in sentinel lymph nodes; occult bone marrow metastases were associated with decreased overall survival only when clinicopathological variables were not considered [179].

Circulating tumour cells

CTCs can be detected in the peripheral blood circulation by highly sensitive techniques [180]. As with DTCs, it has been suggested that CTCs are progenitors of distant metastasis, and their presence in peripheral blood has been associated with reduced recurrence-free and overall survival in primary breast cancer [181–183]. Baseline levels of CTCs and treatment-induced changes have been related to progression-free survival and overall survival in metastatic disease [184–186]. When characterising the CTCs with respect to the expression of HER2, discordances have been found compared with the primary tumour [187, 188]. Several studies have investigated the rate of concordance between DTCs and CTCs in recent years, and found no significant correlation between them [189, 190].The detection of CTCs in peripheral blood provides a more convenient method than bone marrow sampling for the detection of DTCs, and is associated with less pain to the patient.

Molecular profiling

Individual biomarkers (ER, PR and HER2) expressed in breast tumour tissue have been shown to be of prognostic and predictive value in clinical patient management [20]. However, there is an awareness that these traditional biomarkers are insufficient to reflect the heterogeneity of breast cancer, and that it may be possible to improve the stratification of patient risk. Global gene expression profiling describes the activity or level of expression of a particular gene by counting the number of mRNA instead of the protein for which the gene encodes. The technique was pioneered at the beginning of the 21st century by Perou and Sorlie, who used it to identify molecular classes with distinct combinations of genes that better reflect the biological variation and heterogeneity of breast cancer disease [5, 6]. The

molecular classes are described according to the degree of gene expression of ER, PR, HER2 and rate of proliferation, and prognostic differences and/or response to therapy between the molecular subtypes have also been reported [191–193]. ER positive tumours can be further divided into two groups, luminal A and luminal B, where luminal A tumours do not express HER2, have a low proliferation rate and are related to the best prognosis [6]. The luminal A molecular subtype is most common, 30–50% of all tumours [194–196], while luminal B tumours constitute 10–25%, HER2 type 5–10% and triple negative 10–15% [195, 196]. Proliferation-associated genes have been studied since it has been suggested that they divide ER-positive and histological grade 2 breast cancer tumours into groups with different prognoses [197, 198]. The technique has been developed, and commercially available multi-gene assays include *Oncotype DX* (21-gene profile) and *Mamma Print* (70-gene profile), which are approved by the St Gallen guidelines and the American Society of Clinical Oncology (ASCO) for determining prognosis [11, 199]. However, experience in these assays is limited, and the prognostic value of gene-based assays has been questioned [200, 201]. Swedish national guidelines are awaiting further evaluation and more conclusive data regarding the prognostic and predictive value from the clinical trials MINDACT, TAILORx and RxPONDER [51, 202, 203]. These genetic techniques have limitations in routine clinical use due to their high costs, which has led to the development of surrogate molecular subtypes based on IHC analyses of the routine clinical biomarkers ER, PR, HER2 and proliferation markers such as Ki67 and NHG as expressed by gene expression arrays [10]. A number of studies have been performed and shown better prognostic and predictive value of these surrogate molecular subtypes than analysis of individual biomarker expression [7, 8, 204]. The St Gallen guidelines (2011) recommend the use of surrogate molecular subtypes as a useful substitute for subtypes defined by gene expression [11]. However, the identification of molecular subtypes could be further improved as the subtypes currently used may be a simplification of the underlying cancer biology. The triple negative molecular subtype could, for example, be further classified into basal-like subtype according to gene expression profiling. Furthermore, the addition of proliferation levels in ER-positive breast cancer with histological grade 2 tumours has been suggested to further differentiate this group of patients regarding prognosis [145, 205, 206]

The surrogate molecular subtype definitions currently recommended by the St Gallen 2011 guidelines [11] are:

- **luminal A:** ER+ and/or PR+, Ki67 low and HER2–
- **luminal B HER2–:** ER+ and/or PR+, Ki67 high and HER2–
- **luminal B HER2+:** ER+ and/or PR+, any Ki67 and HER2+
- **HER2 type:** ER–, PR– and HER2+
- **triple negative:** ER–, PR– and HER2–

Treatment

Pre- and postoperative multidisciplinary conferences are required for all breast cancer patients according to Swedish guidelines [51]. In the case of neoadjuvant treatment, a full pathological report is obtained by histological analysis of a core needle biopsy in order to optimise treatment and shrink the tumour to make it operable, or to be able to perform breast-conserving surgery.

Surgery

Surgery of the breast is performed either as a mastectomy or breast-conserving surgery. The decision regarding the surgical procedure is made together with the patient, and is based on: tumour size, whether there are multiple cancer regions within the breast, tumour size in relation to the size of the breast and the patient's own wishes. No difference in survival has been found between mastectomy and breast-

conserving therapy followed by radiotherapy of the breast, in several prospective randomised studies with long-term follow-up [207–209].

Historically, breast cancer was considered a systemic disease, then a local disease. Today, the risk of systemic spread is the main issue considered in adjuvant treatment and risk stratification. In the era when breast cancer was treated as a local disease, surgery developed as a possible curable treatment [102]. Breast cancer surgery included removal of the breast and the associated lymph nodes in the axilla and clavicula, together with the pectoralis muscles covering the thorax (radical mastectomy, introduced by Halstead in the late 19th century), sometimes even a rib was extracted if overgrowth was suspected [102]. In 1907, Halstead presented a study in which he had divided his patients into three groups, based on whether the breast cancer had spread to the axillary nodes, the clavicular nodes or was limited to the breast. From this study, it became apparent that the prognosis was dependent on the extent to which the cancer had spread before surgery [102] and not the extent of the surgical procedure. Extended radical mastectomy was then replaced by modified radical mastectomy, as described by Patey in 1948 [210], in which the pectoralis muscle is spared. With the adoption of radiotherapy and improving knowledge on the outcomes of extensive surgery, alternative surgical procedures such as breast-conserving surgery and mastectomy with or without radiotherapy were introduced in clinical studies [207, 211, 212]. Gradually, a modern form of surgery developed, associated with less deformity, but with the same prognosis [3, 207, 212]. Axillary lymph node clearance was a standard surgical procedure that extended into the 1990s, when the SN technique was developed in breast cancer surgery. The sentinel lymph node is the hypothetical first lymph node or group of nodes draining a cancer. Axillary dissection is associated with arm morbidity while the SN technique is a less invasive surgical procedure to modify treatment according to the stage of the disease [213, 214]. The SN is identified by injecting a radioactive isotope intradermally, close to the tumour prior to the operation. Blue dye is thereafter injected in the same area when the patient is on the operating table. It is assumed that the lymphatic drainage of the area carries the isotope, and with it the blue dye, to the lymph nodes of the axilla, in the same way as tumour cells are drained. The assimilation of the isotope and the blue dye helps the surgeon to identify the affected lymph node or nodes, which are resected and sent for immediate histological analysis by a pathologist. If this peroperative analysis show metastases (≥ 0.2–2 mm), complementary axillary dissection is performed. If no cancer cells or isolated tumour cells (< 200 cells) are identified, no further dissection is performed in the axilla. The assessment of tumour invasion in the SN technique is important in decisions regarding further surgery and therapy [215–218]. The SN technique has been validated in several studies, and has shown a high sensitivity [217, 218] and an identical prognosis regarding regional control, disease-free survival and overall survival to complete axillary dissection [219, 220].

Reconstruction of the breast can be performed using an implant [221] or autologous tissue [222–224] or a combination of both [225]. Breast reconstruction can be performed at the time of mastectomy (primary reconstruction) or at a later date (delayed reconstruction).

Radiotherapy

Postoperative radiotherapy is recommended in order to eradicate microscopic residual disease and to potentially improve overall survival and reduce loco-regional recurrences. According to Swedish and International guidelines, radiotherapy of the breast or thoracic wall is indicated if the risk of a local recurrence within the next 10 years is > 20%, and is thus recommended for all patients who have undergone breast-conserving surgery and those in which the tumour was > 50 mm [51]. The most recent meta-analysis reported that local and distant recurrences in patients treated with breast-conserving surgery,

with the addition of radiation were reduced by 50% [226], as well as positive effects on overall survival [226, 227]. The benefit of loco-regional radiotherapy of the axilla and supraclavicular area in patients with tumours < 50 mm, but with 1–3 metastatic lymph nodes has not been proven. Radiotherapy may be considered when 1–3 axillary lymph node metastases are detected, in the presence of other risk factors, such as age ≤ 40 years, NHG 3 or if > 20% of the examined lymph nodes are found to have metastases, or in cases of lymphovascular invasion, in which the ten-year cumulative incidence has been shown to exceed 20% [209, 228]. Loco-regional radiotherapy is recommended to all patients with metastases in four or more lymph nodes since it has been shown to provide an absolute reduction in local relapses and an increase in breast-cancer-specific survival; the extent of the benefit depending on the presence of other risk factors [51, 209]. Side effects of radiotherapy include erythema of the skin, pneumonitis, neuropathy of the affected plexus, lymphoedema of the upper extremity and cardiac effects [209, 229].

Systemic therapy

Adjuvant systemic therapy is recommended to remove micrometastatic disease and includes endocrine therapy, chemotherapy and anti-HER2 therapy, either alone or in combination. Risk reduction is based on large groups of patients, and is partially assessed based on predictive biomarkers expressed by individual tumours.

Endocrine therapy

Oestrogens are mainly produced by the ovaries in premenopausal women, while in postmenopausal women they are synthesized from adrenal and ovarian androgens in the liver, muscles and fatty tissue. Approximately 70–80% of all breast tumours express ER [100, 230], which is the main driver of tumour development in these patients. The ER pathway can be targeted by blocking ER with tamoxifen, by the degradation of ER with fulvestrant, or by preventing the synthesis of oestrogens by aromatase inhibitors (AI). The production of oestrogens can also be blocked irreversibly by oophorectomy or radiation, or reversibly by treatment with gonadotropin-releasing hormone (GnRH) analogues.

ER-negative patients receive no benefit from endocrine treatment [3, 101].The relationship between ER expression levels and sensitivity to different forms of endocrine treatment has not been established. It is recommended in the European and American guidelines that patients with > 1% ER-positive cell nuclei be offered endocrine treatment [20, 111], although the biological relevance is questioned by other reports [101, 231, 232].

Tamoxifen inhibits ER activation in the breast, as described in previous section, by antagonistic effects on AF2 region of ER, while it can have agonistic effects in other tissues, such as endometrium and bone [233]. The use of tamoxifen in the neoadjuvant setting should only be considered in frail patients with limited life expectancy according to a review of early studies mainly performed in the 1980s and 1990s. On average, objective response was seen in about 50% of the patients treated preoperatively with tamoxifen, irrespective of ER expression in the tumour [234]. In a 15-year follow-up study of adjuvant tamoxifen, it was shown that ER-dependent tumour growth and mortality could be reduced by approximately one-third when ER-positive patients were treated with tamoxifen for about five years, regardless of PR expression, age, presence of lymph node metastases or the use of chemotherapy [101]. Tamoxifen can be used for all breast cancer patients irrespective of menopausal status.

The effect of AIs is restricted to postmenopausal patients since they have no effect on the ovarian production of oestrogens. They exert their effect by blocking the conversion of androgens to oestrogen by enzyme-inhibition in the peripheral tissues. AIs include letrozol, anastrozole (non-steroidal, reversible AIs) and exemestane (steroi-

dal, irreversible AI). Five years' treatment with AIs or sequential treatment for 2–3 years with tamoxifen followed by 2–3 years with AIs, has been found to significantly reduce the risk of relapse and indicated a survival benefit in postmenopausal women with breast cancer in two large studies [235, 236]. However, a meta-analysis of the data in these two studies revealed no statistically significant decrease in breast cancer mortality (1.1% absolute decrease), and the absolute risk of recurrences was reduced by 2.9% when comparing 5 years of treatment with tamoxifen to 5 years with AIs [237]. This may be explained by the pattern of recurrences, which showed a greater decrease in local relapses and cancer of the contralateral breast than distant relapses following treatment with AIs. Tamoxifen has been compared with AIs in the neoadjuvant setting, showing a higher response rate in the AI-treated patients [238, 239]. AIs have also been shown to extend the time to progression in metastatic disease compared with tamoxifen [89], and are considered the first line treatment in metastatic disease. The side effects of tamoxifen treated patients include thromboembolic events and endometrial cancer while the side effects for AIs include osteoporosis with fractures, arthralgia and hypercholesterolemia [89, 240].

Patients who are premenopausal at the time of diagnosis but who, after treatment with tamoxifen for five years has become postmenopausal, can be offered an additional five years of treatment with AIs since this has been shown to further reduce the risk of recurrence and death in this group of patients [241]. Prolonged treatment with tamoxifen for an additional 5 years to, a total of, 10 years, has recently been reported to further reduce the risk of recurrences and death, especially after 10 years [242]. In an ongoing International study in which Sweden is participating, patients are randomised to treatment with AIs for another 5 years continuously or in sequence, after initial treatment with tamoxifen or AIs (SOLE study).

Degradation of ER by fulvestrant has a similar effect to tamoxifen and AIs regarding the response of ER-positive patients with metastases [243]. However the benefit of combinations of tamoxifen and AIs with fulvestrant remains to be determined.

Oestrogen depletion by medical suppression, radiation or surgical resection of the ovaries can be considered in premenopausal women. Suppression by surgery or radiation is not included in the clinical routine because of the irreversible nature of the intervention. Systemic treatment with a GnRH analogue provides a reversible alternative acting on the pituitary gland, which results in a temporary suppression of ovarian function. The results of a meta-analysis suggest that the addition of a GnRH analogue to other endocrine treatment and/or chemotherapy improves survival for premenopausal patients with ER-positive disease, especially those younger than 40 years [244]. GnRH analogues alone or in combination with tamoxifen have been shown to be just as effective in risk reduction as treatment with chemotherapy based on cyclophosphamide, methotrexate, fluorouracil (CMF) [245]. Although this chemotherapy is considered out of date, it offers an alternative when chemotherapy is contradicted in premenopausal women. The aim of an ongoing study, the Suppression of Ovarian Function Study (SOFT), is to further investigate the benefit of the addition of GnRH analogues to other endocrine treatment with different oestrogen blocking effects (tamoxifen and AIs) in the context of modern chemotherapy. A recent Cochrane analysis concluded that too few studies have been conducted for a reliable recommendation of GnRH analogues over other treatments options [245].

Chemotherapy

Systemic chemotherapy targets to cells with accelerated proliferation, and inhibits cell reproduction unselectively. Clinical trials have been carried out since the 1950s to evaluate the additional value of adjuvant chemotherapy for breast cancer patients, and the first reports of a sur-

vival benefit for premenopausal, lymph-node-positive patients was presented in the 1960s [246]. Polychemotherapy with CMF was found to decrease the rate of recurrence in a study by Bonnadonna and colleagues [247], and at a 20-year follow-up the beneficial effect included overall survival [248]. Adjuvant treatment with anthracycline-based chemotherapy has been shown to be more effective than CMF-based chemotherapy, and the addition of taxanes to anthracyclines further reduces the 10- year overall mortality by about one third, independent of age, nodal status, tumour size, histological grade, ER status or tamoxifen use [249]. These studies have provided the basis for modern treatment with chemotherapy, and chemotherapy is currently recommended for patients with lymph-node-positive disease and those whith tumours > 10 mm who do not express ER, and thus have a low sensitivity to endocrine treatment [20, 51, 101]. The presence of other risk factors (low age, high proliferation rate, node positivity and HER2 positivity) can also indicate beneficial effects of chemotherapy [51]. However, research is also aimed at identifying and selecting patients who could be spared chemotherapy, due to the little benefit in recurrence and survival, to avoid side effects associated with impaired quality of life [250].

Other targeted therapies

Trastuzumab is a monoclonal antibody directed against the extracellular domain of the HER2, which is expressed in approximately 15% of breast cancers [120, 121], and is associated with an increased risk of recurrence and worse prognosis [117, 118]. Trastuzumab was developed after the detection of the HER2, and has been shown to reduce mortality by 30% and recurrences by 50% [122, 128, 129] in the adjuvant setting. Furthermore, trastuzumab increases progression-free survival in patients with metastatic disease, when administered alone [251] or together with endocrine therapy [252] or chemotherapy [126]. In locally advanced disease in HER2 positive patients, optimal treatment in the neoadjuvant setting is not determined, data indicates improved remission when trastuzumab and chemotherapy is given in combination compared with chemotherapy alone [131]. Trastuzumab is recommended for treatment if the patient is considered HER2 positive, which at present includes patients with gene amplification and/or a score of 3+ assessed by IHC [11, 253, 254]. The effect of trastuzumab for patients expressing HER2 levels of 2+ and 1+, but with no amplification, is being evaluated since a benefit has been indicated in a previous study [255].

Pertuzumab is a humanised monoclonal HER2 antibody that binds to a different part of the HER2 from trastuzumab, preventing the receptor from fusing with other receptors in the HER family, thereby inhibiting the effect of HER2 [256]. Pertuzumab has been shown to increase progression-free survival by 6 months in combination with chemotherapy and trastuzumab in patients with metastatic disease [257].

Lapatinib is a small tyrosine kinase inhibitor directed against both HER2 and EGFR. Its mechanism in the treatment of metastatic disease has not yet been fully elucidated, but it has been shown to prolong the time to progression in combination with chemotherapy as second- or third-line treatment [258].

Trastuzumab emtansine (T-DM1) combines the inhibition of HER2 signaling with a cytotoxic tubulin inhibitor, selectively delivered to HER2-expressing cells. In a recent study, patients with advanced disease who previously had been treated with trastuzumab and chemotherapy, were randomly assigned to receive T-DM1 or lapatinib plus chemotherapy. The reported progression-free survival for the T-DM1 treated patients was 9.6 months compared to 6.5 months in the group of patients treated with lapatinib plus chemotherapy. Lower frequencies of adverse events were observed in the T-DM1 treated patients [259].

Tumour Progression

Linear and parallel progression models

The term tumour progression can be confusing since it refers to both the progress of the disease in the individual, and the development and maturation of cancer cells. Tumour invasion and the formation of metastases are the major causes of treatment failure and death in cancer patients. The traditional explanation of cancer progress has been the successive accumulation of mutated cells that have acquired the ability to invade the primary and secondary sites sequentially. The theory is that the early detection and treatment of primary tumours should prevent the occurrence of distant metastasis, by removing the tumour before it develops the ability to invade other tissue. Clinical management is currently based on this traditional *linear progression model* (Figure 1)[13], advocating stepwise progression in which cancer cells develop genetically in the context of the primary tumour. The mature cell clones expand, and individual cancer cells subsequently disseminate and form metastases at secondary sites. The linear progression model assumes the molecular characteristics of the metastatic cells to be highly similar to those of the primary tumour. The dissemination of tumour cells is restricted to advanced stages of the disease, and when the DTCs have formed metastases at a secondary location, they can spread further to other organs [260]. The association between tumour size and increased risk of metastasis [62, 67, 69] has been used as evidence supporting the linear progression model. However, the traditional sequential progression theory is currently being challenged by new concepts of the formation and development of metastases. The *parallel progression model* (Figure 1) [13] is based on early dissemination of immature cancer cells, with different molecular characteristics, derived from different cell clones. Somatic progression is assumed to occur at a metastatic site, and the disseminated tumor cells evolve further in their new microenvironment, and exhibit a different biological profile [15]. The model of parallel dissemination was initially proposed in the 1950s, when it was concluded that some metastases were too large to be a late event in tumour progression, according to observations of growth rate [261].The tumour volume doubling time measured in radiographic studies varies considerably between patients, but less so between the primary tumour and metastases in the same patient [262], where metastatic growth rates are well correlated to that of the primary tumour [262, 263]. According to another studie on tumour growth rates, a tumour takes, on average approximately12 years to reach 1 cm, and 3 years to grow from 1.4 to 7 cm [264]. The median time from surgical resection of a primary tumour < 1 cm to distant metastasis has been determined to be 35 months, compared with 20 months for a tumour > 5 cm [265]. It has been suggested that the observed growth rate and time to systemic disease are inconsistent with a linear progression of metastatic disease, as the growth rates required in metastases would have to be much higher than those observed [13]. Additionally, breast cancer patients with tumours < 2 cm and no lymph node metastases can present with distant metastases at diagnosis [218], and this is not compatible with the linear progression model. The stepwise seeding of metastases is also contradicted by observations in breast cancer patients, where lymph node metastases are frequently observed, although their removal does not affect the formation of distant metastases or survival [208, 218, 266].

Lymphatic tumour progression

Despite the lack of survival benefit from the removal of lymph node metastases, their presence is still an important prognostic factor [267, 268]. Complete axillary lymph node dissection was routinely conducted in the patients found to have positive lymph nodes until the early 1990s [269]. The less invasive SN procedure identifies

a positive sentinel node or nodes in 20–35% of breast cancer patients [162, 220, 270] and further dissemination to non-sentinel lymph nodes is observed in approximately 30% of patients with a positive sentinel node [271]. Increasing size and increasing numbers of lymph node metastases have been shown to affect prognosis [268, 272]. Taken together, these observations indicate that breast tumour cells prefer lymphatic routes from the primary site. Tumour cell migration into lymph nodes is facilitated by the process of lymphangiogenesis, which generates new lymphatic vessels, further facilitating tumour migration [273]. The results of previous studies suggest that the lymph node microenvironment is involved in conditioning tumour cells, providing them with metastatic competence through chemokine signalling [274, 275]. Chemokine signalling may emanate from the primary tumour and influence early DTC and/or the new stromal environment to promote metastatic development [276]. The conditioning of tumour cells in the lymph node microenvironment would render them more metastatically competent and their transit in the closely related vascular system would provide conditions for both lymph node metastases and haematogenous spread of distant metastases [277].

CTCs, DTCs and tumour progression

DTCs have the potential to disseminate in many organs in the body, although some organs are more frequently targeted. Characteristic metastatic patterns for different types of tumours was demonstrated in autopsies conducted during the 1970s and 1980s [278, 279], showing that the average number of metastatic locations was 2–3 organs. These observations led to the conclusion that direct dissemination from the primary tumour could not explain the findings, and that the metastatic process must take place from one metastasis to the next, in an orderly way [280]. More recent reports suggest a number of mechanisms whereby DTCs and CTCs may target specific organs. For example, chemokines expressed in particular organs are recognised by CTCs and DTCs in the vascular system, leading to increased invasive and chemotactic behaviour [22]. Another recent explanation of preferred metastatic locations is the formation of a pre-metastatic niche, a microenvironment with optimal conditions for the formation of metastases [281]. Soluble factors from the primary tumour appear to determine the organs in which these niches form, and the pattern of metastases [276]. The considerable time that can elapse between initial diagnosis and the recurrence of a tumour is thought to be due to the activation of DTCs that have remained dormant [282]. Regulation of extracellular matrix proteins, tumour suppressor genes and immune-related cells has been associated with the activation of dormant DTCs, and DTCs may be able to form their own metastatic milieu [175]. The dormancy concept is also applicable in lymph nodes where it has been suggested that it explains why micrometastases in lymph nodes can give rise to loco-regional relapses many years later [283].

Clinical data and progression models

Comparisons of primary tumours and lymph node metastases based on tumour characteristics have been performed, generally with high concordance rates [284–286], but the opposite has also been found [287, 288]. It has recently been suggested that lymph node metastases are derived from the most aggressive cell clone in the primary tumour [277]. If this is the case, analyses of tumour characteristics in lymph node metastases should be highly interesting in the prognosis of individual patients, since the cell clones in the lymph node metastases are a selected population with inherent properties allowing them to evolve further and spread to secondary locations. Changes in biomarkers between the primary tumour and metastases could be biologically explained by independent evolution of early disseminated tumour cells, as proposed in the parallel progression model [13]. Clonal

expansion, related to intra-tumour heterogeneity, during tumour progression, and therapy-induced changes in genetic composition have also been proposed as explanations of the divergence [289].

Discordances in biomarker expression between primary breast tumours and asynchronous recurrences at various locations have also been reported. The majority of the studies describing discordances are retrospective, and different pathological or laboratory techniques have been used, which makes the clinical impact difficult to interpret. Some of these retrospective studies also related discordances between primary tumours and relapses to clinical outcome [290, 291], and the prognosis seemed to be related to the expression of biomarkers in the recurrence [290, 292]. Stable ER-positive patients had the same outcome as primary ER-negative breast cancer patients with ER-positive recurrence, and patients who exhibited ER-negative recurrence had a shorter survival regardless of the ER status of the primary tumour [292]. Lindström *et al.* found that ER-positive patients in relapse had a better prognosis than ER-negative patients, regardless of the expression of ER in the primary tumour. Two prospective studies on tissue biomarker expression were conducted; the Breast Recurrence In Tissues Study (BRITS) [293], in which 205 patients were enrolled, of whom 168 underwent biopsy of the recurrence, and DESTINY [294], which included 137 patients, 121 of which underwent biopsy of the recurrent lesion. A change in therapy in approximately 14% of the included patients was observed in both studies. The discordance rates were 13% for ER, 28% for PR and 5% for HER2 in a pooled analysis [295]. A change of therapy was seen more often when biomarker expression was gained, especially the expression of ER and HER2. Suggestions that discordances between primary tumours and relapses could be due to inter-laboratory and interobserver variability, variations in tissue processing or intratumour heterogeneity, cannot be excluded [291, 296]. Confirmation of new metastases and verification of receptor status in newly diagnosed metastases have had effects on clinical decision making as discussed above, but whether this improves the outcome for individual patients is a question connected with ethical and economical aspects. It would require a randomised trial in a large patient cohort, in which the treatment decision was based either on the characteristics of the primary tumour or the relapse.

However, no standard methods are available for monitoring DTCs, and clinical decisions concerning treatment rely on the prognostic and treatment predictive factors associated with the primary tumour. Regardless of the model of tumour progression, the disparity in genotype of the primary tumour and metastatic cancer cells [15, 297] and the molecular profile [293, 298] could have implications for the systemic treatment in the individual patient.

Aims

I To identify disseminated tumour cells in prospectively retrieved samples of bone marrow in primary breast cancer patients, and relate the prognostic significance of DTCs to distant disease-free survival (DDFS) during five years of follow-up.

II To compare the expression of biomarkers ER, PR, Ki67 and HER2 in primary breast cancer tumours and matched lymph node metastases, and relate these to DDFS during five years of follow-up.

III To combine biomarker expression (ER, PR, Ki67 and HER2) into molecular subtypes according to the St Gallen classification, and compare the inherence in primary breast cancer tumours and corresponding lymph node metastases, and to relate this to DDFS and breast cancer-specific survival during five years of follow-up.

IV To assess biomarker expression (ER, PR, Ki67 and HER2) and evaluate changes in separate biomarkers as well as St Gallen molecular subtypes, in primary tumours, corresponding lymph node metastases and relapses, and relate to breast cancer mortality during 10 year of follow-up.

Patients

Papers I and IV

569 women diagnosed with primary breast cancer in the South Swedish Health Care Region (Helsingborg, Landskrona, Lund) between 1999 and 2003, were included in a prospective, observational study. All patients had unifocal primary invasive breast cancer and underwent surgery of the breast and the axillary lymph nodes based on preoperatively identified characteristics and staging with no signs of distant metastasis. Neoadjuvant endocrine and chemotherapy were administered to less than 1% of the patients. Adjuvant systemic therapy and postoperative radiotherapy were administrated according to National Guidelines. Trastuzumab was administered to 6 patients (1%) in the adjuvant setting.

Patients were followed by clinical examination and mammography annually. Further examinations were performed when clinical signs indicated recurrence of the disease. After 5 years of follow-up, all reports of events were abstracted from the individual patient's records. The median follow-up period for patients without any breast-cancer-related events was 61 months. Information on death related to breast cancer was retrieved from the Swedish Register of Causes of Death (Central Statistics Office).

In the first study (Paper I), the patients underwent bone marrow aspiration from the sternal crest under general anaesthesia at the time of primary surgery. Neoadjuvantly treated patients (n = 11), patients with local recurrence when included (n = 3) and those not receiving standardised surgical treatment (laser treatment, n = 1) were excluded from further follow-up.

In the fourth study (Paper IV), fourteen patients were excluded from further follow-up due to: non-standardised surgical treatment, n = 1; bilateral breast cancer, n = 5; previous history of breast cancer, n = 5 and local recurrence at primary visit; n = 3. The final cohort included 555 patients (Figure 2, flowchart).

Papers II and III

The studies are based on a cohort of patients previously selected from two prospective randomised clinical trials of adjuvant tamoxifen treatment, and included patients from the hospitals in Simrishamn, Ystad, Trelleborg, Malmö, Lund, Landskrona, Hässleholm, Ängelholm, Kristianstad, Halmstad, Ljungby, Växjö, Karlskrona and Karlshamn in the Southern Swedish Health Care Region. The original studies took place during 1985–1994, and included patients with stage II, unifocal, radically operated primary breast cancer. In the postmenopausal study, the patients were allocated to 2 years (n = 496) or 5 years (n = 469) of adjuvant tamoxifen treatment irrespective of hormonal receptor status [299]. Identical inclusion and exclusion criteria were used for premenopausal patients, except for menopausal status, and the patients were randomly allocated to two years of tamoxifen treatment (n = 213) or no adjuvant treatment (n = 214) [300]. An original cohort was comprised from these two randomised trials to investigate the compatibility of different laboratory methods for the evaluation of hormonal receptor status [109]. The quality assurance study included 425 patients treated with adjuvant tamoxifen for two years, 297 of whom had lymph node metastases (Figure 2, flowchart). All the patients underwent surgical treatment of the breast and axilla. Radiotherapy was given to the breast in the cases of breast-conserving surgery, and loco-regionally if lymph node metastases were present. Adjuvant systemic treatment was given as 2 years of tamoxifen irrespective of hormone receptor status.

Biomarker expression was analysed separately (Paper II) and in combinations according to the St Gallen molecular subtype recommendations (Paper III) in the primary tumour and corresponding lymph node metastases. Some data were lost due to one or more missing assessments of one or more biomarkers from either primary tumours or lymph node metastases. Thus, 85 patients from the original cohort were catego-

rised into four molecular subtypes: luminal A, luminal B, HER2 type and triple negative, according to ER, PR, HER2 and Ki67 expression (Figure 3, flowchart, Paper III). Information on clinical outcome, as well as patient and tumour characteristics, was already available.

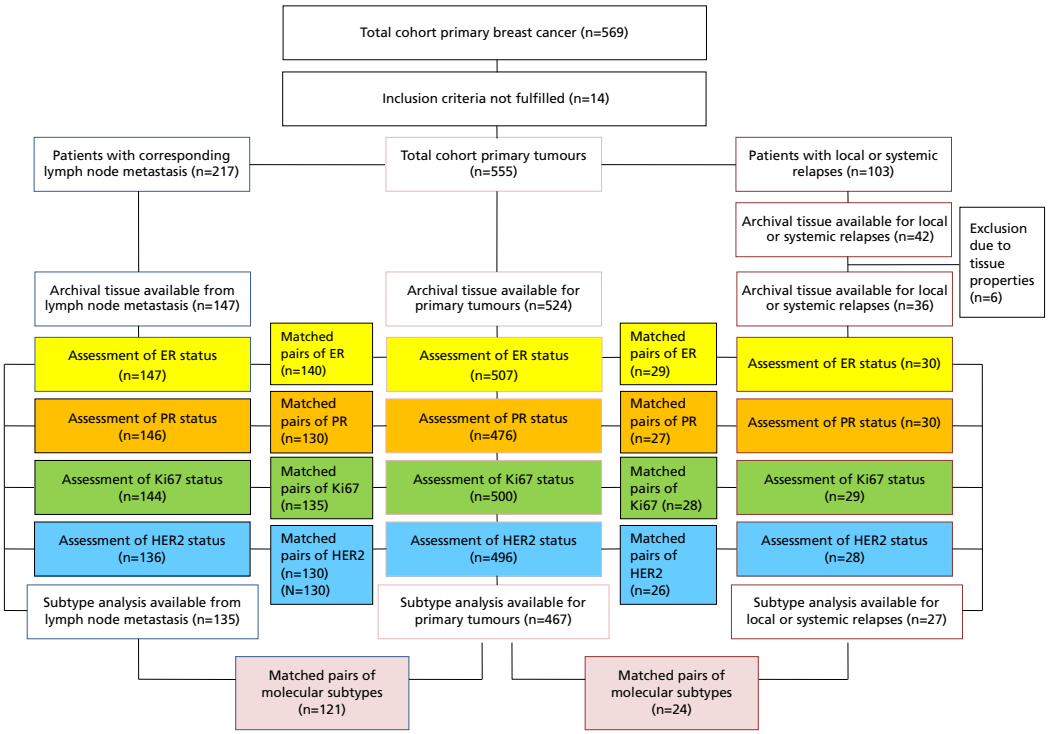


Figure 2. Flowchart Paper IV.

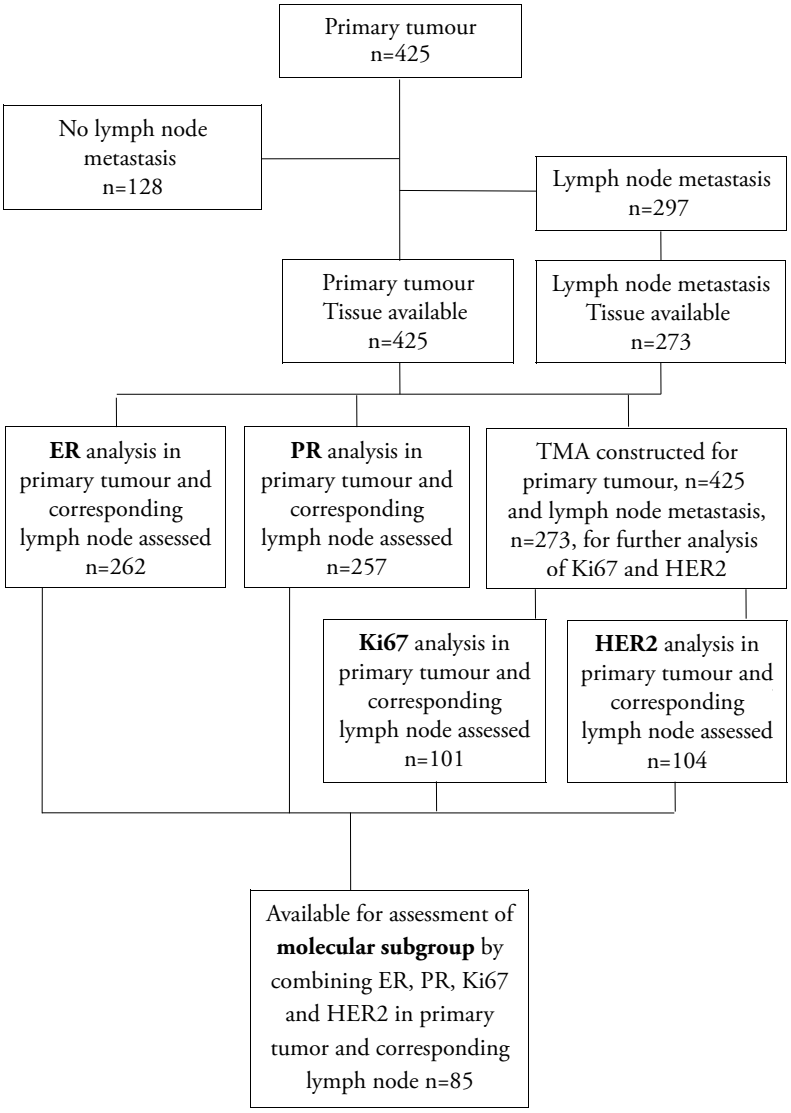


Figure 3. Flow chart Papers II and III

Methods

Assessment of DTCs in bone marrow

The detection of very few cells requires processing to reduce background cells, labelling of the desired cells for identification, and evaluation of the cell morphology. The methods used for these steps vary in different studies, as do the target CKs and the membrane antibodies used to detect epithelial cells [163]. A concept for the standardised detection of DTCs including the use of immunocytochemistry (ICC), a strict protocol for negative controls, and morphological evaluation of stained mononuclear cells was eventually published in 2006 [164]. The importance of morphological classification has since been validated [301]. The study presented in Paper I was performed before this standardised protocol was published, and DTCs were mainly detected using immunofluorescence (IF) staining procedure.

Bone marrow aspirates were obtained from the sternum at two sites by needle aspiration while the patient was under general anaesthesia at the time of primary surgery. The samples were transported to the research laboratory at room temperature and prepared within 1 h. Mononuclear cells were separated by density gradient centrifugation (Ficoll-Paque™PLUS, Cat. No. 17-1440-03, Amersham Biosciences AB, Uppsala, Sweden) and then washed and counted, before 1.5–2.0 x 10^6 cells were placed on each glass slide. Two microscope slides were prepared for each patient, one from each site in the sternum.

Immunofluorescence (IF) and immunocytochemical (ICC) analysis

An IF staining procedure, including staining with antibodies against the CKs 4,5,6,7,8,10,13,14,15,16,18 and 19 (CK Pan Ab-2, Neomarkers, Union City, CA, USA) was used for the detection of DTCs in 327 patients. Visualization was achieved by IF using an IF microscope (Zeiss Axioplan II, Jena, Germany). The cytospins were incubated with the pan-cytokeratin antibody and a secondary FITC (isothiocyanate)-conjugated goat antimouse antibody (Zymed Laboratories Inc. Labora, San Francisco, CA, USA), and finally counterstained with DAPI (4,6-diamidino-2-phenylindol) in mounting medium using Vectashield (Vector Laboratories, Burlingame, CA, USA). The procedure was changed when a new cytokeratin antibody kit (AE1/AE3, Daco, Glostrup, Denmark) was introduced with antibodies against the CKs 1,2,3,4,5,6,7,8,10,13,14,15,16 and 19. The EnVision™ system (Dako, Glostrup, Denmark) was used for visualization together with a secondary antibody, NovaRed™ (Vector Laboratories, Immunkemi AB, Sollentuna Sweden), and Mayers haematoxylin for nuclear staining. This enabled direct ICC evaluation of the cells and analysis by light microscopy (Olympus CX41, Tokyo, Japan) in 74 patients.

The presence of DTCs was defined as CK-positive cells with DTC morphology (irregular staining of the cytoplasm) with an enlarged nucleus, irregularity of the nucleus, a high nuclear-to-cytoplasmic ratio, CK staining of the cytoplasm at the periphery of the cell causing a ring-like appearance, and fluorescence-positive intact cells (IF technique) according to Fehm [165]. For the ICC evaluation, the criteria proposed by Borgen, using the same antibody, were followed [302]. These criteria include moderate to strong staining intensity for the entire cytoplasm in mononuclear cells lacking haematopoietic characteristics [302]. Evaluation was performed by two observers independently. All specimens were considered either positive or negative when one or more cytokeratin-positive cells was diagnosed. The number of stained cells was also determined in quantitative analyses. DTCs detected with IF are illustrated in Figure 4 and with ICC in Figure 5.

Positive controls for CK immunostaining were obtained by spiking blood from healthy

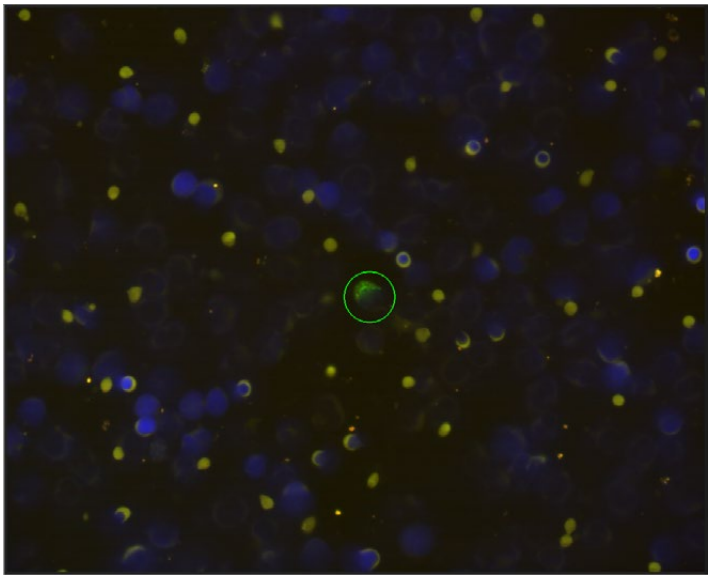


Figure 4. Cytokeratin staining of DTCs isolated from bone marrow by immunofluorescence.

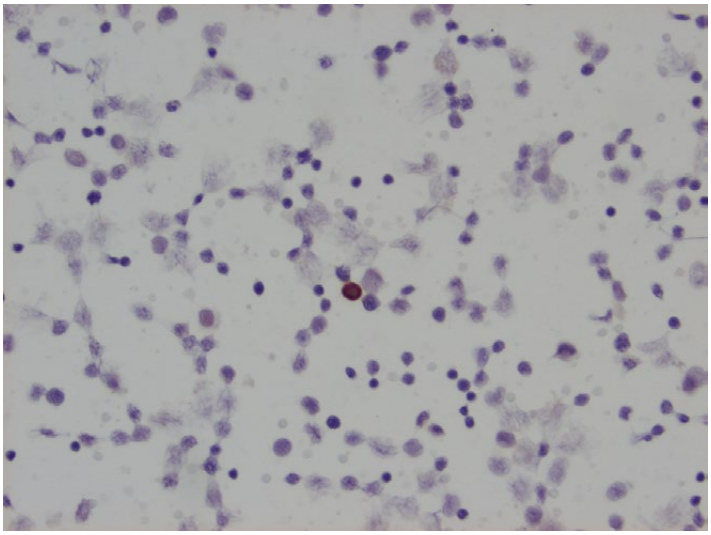


Figure 5. Cytokeratin staining of DTCs isolated from bone marrow by immunocytochemistry.

volunteers with the breast cancer cell line MCF7. Negative controls were prepared in parallel with those stained with anti-cytokeratin by omitting the primary antibody, and thus contained the same number of cells 1.5–2 x 10^6. There were no positive results in the negative controls. Bone marrow from 76 healthy bone marrow donors was analysed separately.

Tissue microarray

The tissue microarray (TMA) technique has become a standard since the introduction of the automated method in the late 1990s [303] and has been validated for IHC analysis of ER, PR and HER2 in breast carcinoma [304]. The advantage of TMA technology is the possibility to arrange hundreds of patients' tissue samples on one microscopic slide, in contrast to whole sections, where only one patient's tumour sample is mounted on one slide. The technique also offers the possibility of using small amounts of tissue for studies, and saves laboratory time and money compared to conventional pathology, which involves molecular analysis of tumour markers using IHC and ISH [305]. It is also considered an additional advantage that all tumour specimens on a single slide are stained consistently, under the same uniform conditions [305]. One of the drawbacks of TMA technology is associated with tissue heterogeneity, and concerns have been raised as to whether biomarker expression in small tumour samples is adequately representative of the whole section. This question has been addressed in several studies, and a strong correlation has been demonstrated between TMA and whole tissue sections regarding ER, PR and HER2, using two 0.6 mm cores [304–306].

In the TMA technique, two or more cores, 0.6–2.0 mm in diameter, are punched out from a donor block of paraffin-embedded tumour tissue sample and mounted in a recipient block, arranged in a coordinate system to enable correct identification. The cores are selected by a laboratory technician and/or a pathologist, from predefined areas of invasive tumour identified with haematoxylin-eosin-stained glass slides of tissue sections. After construction, 4 µm tissue sections are cut and glass slides are prepared for microscopy or scanned into a digital pathology platform allowing scanned slides to be reviewed remotely. In the present work, TMA was constructed for the analysis of Ki67 and HER2 (Paper II) and for ER, PR, Ki67, HER2, EGFR and CK 5/6 (Paper IV). In the study presented in Paper II, two 0.6 mm cores from the primary tumour and one 0.6 mm core from the corresponding lymph node were obtained, and in the other study (Paper IV), two 1.0 mm cores were punched out from each patient's paraffin embedded tumour sample and mounted in the recipient block using a tissue array machine, in accordance with the manufacturer's instructions (Beecher Instruments, MD, USA). After construction, 4 µm tissue sections were cut, and glass slides were prepared for microscopy (Paper II) and finally scanned (Aperio Scan Scope CS, CA, USA with Aperio Spectrum TMA lab software, CA, USA, Paper IV).

Immunohistochemistry and in situ *hybridization*

Microscopic analysis has been used for the diagnosis of tumours since the 19th century. With the additional use of dye it was possible to distinguish intra- and extracellular processes, and the diagnosis of malignant tumours could be made with high accuracy [102]. In the 1950s, a complement to staining was introduced, IHC, which is a method of detecting antigens (proteins) in cells in tissue sections using a specific antibody. IHC is a widely used method for the detection of biomarkers in cancer tissue. The antigen-antibody complex is visualized by the addition of a marker or a second antibody that renders the antigen visible, and the intensity and frequency of staining are evaluated for the detection of the protein of interest. IHC staining from primary tumour and different metastatic locations is shown in Figure 6.

ISH can be used to detect and evaluate gene amplification, gene deletion, chromosome number and translocation. A labeled probe (DNA or RNA) is used to localise a specific sequence of DNA in a tissue section. Gene amplification or deletion is detected and evaluated using a chromogen reaction together with light microscopy (chromogenic ISH, CISH), or a fluorescent label that is visualized in a fluorescence microscope (fluorescent ISH, FISH). Recently, a label employing silver and a chromogen (Silver ISH, SISH) has been developed for the detection of HER2 amplification on chromosome 17 using light microscopy, which is considered less time consuming, less technically demanding and thus offers an alternative to the more demanding FISH in the clinical setting [307]. The tumour is considered amplified when the ratio between the signals from the HER2 amplified gene signals and that from chromosome 17 ≥ 2.0 [254].

ER and PR

In the studies described in Papers II and III in this thesis, ER and PR had been analysed previously by two pathologists independently (G Chebil, I Idvall) using IHC on formalin-fixed, paraffin-embedded breast carcinoma on whole slides. Samples were considered positive when ≥ 10% of the nuclei were stained. The study was carried out to compare different laboratory methods for the evaluation of hormonal receptor status [109].

In the study presented in Paper IV, ER and PR status were assessed using the Ventana Benchmark system, using the anti-ER clone SP1 and the anti-PgR Clone 1E2 as primary antibodies [111] at a central clinical laboratory (Skåne University Hospital, Malmö). At least 100 invasive tumour cells were visually scored and evaluated as a semi-quantitative, grouped variable, where tumours with ≥ 10% stained nuclei were considered positive. The assessment was made by two pathologists independently (G Chebil, H Olsson).

Ki67

The proliferation marker Ki67 was assessed using the Ki67 antibody MIB1 (DAKO, Glostrup, Denmark) diluted 1:50, incubated for 32 min and visualized with 3,3´-diaminobenzidine (Papers II–IV). Areas with increased numbers of Ki67-positive cells within the cancerous regions (hot spots) were identified, and at least 200 cells were analysed 10 cells at a time. Cells were visually scored according to the percentage of positive immunostaining above the background level. The cut-point for separating high and low proliferation was set such that one-third of the study population with the highest percentage was separated from the two-thirds with the lowest percentage, as advocated by the Swedish National Guidelines of Pathology [308], and was consistent with a cut-off for high proliferation of > 20% in the present cohorts.

HER2

HER2 was evaluated by means of IHC using the anti-HER2 clone 4B5, and categorised into four groups (0, 1+, 2+, 3+) using the previously described HercepTest (Paper II). The same procedure was performed in Paper IV together with ISH (Inform HER2 dual ISH DNA, Product no. 800-4422, with visualization using kit product no. 780-001 and 800-504, Ventana Benchmark Ultra). *HER2* gene amplification was not performed in the study described in Paper II due to lack of nodal tissue preventing further analyses. All patients with HER2 score 3+ were denoted positive in Paper II, and those with HER2 score 0, 1+ and 2+ were denoted negative. In Paper IV, all patients with amplified tumours according to SISH (i.e., the ratio of the signals from stained HER2 genes: chromosome 17 ≥ 2.0) were considered HER2 positive [253].

EGFR and CK 5/6

EGFR was evaluated using the Ventana Benchmark system clone 3C6 [309], and for the assess-

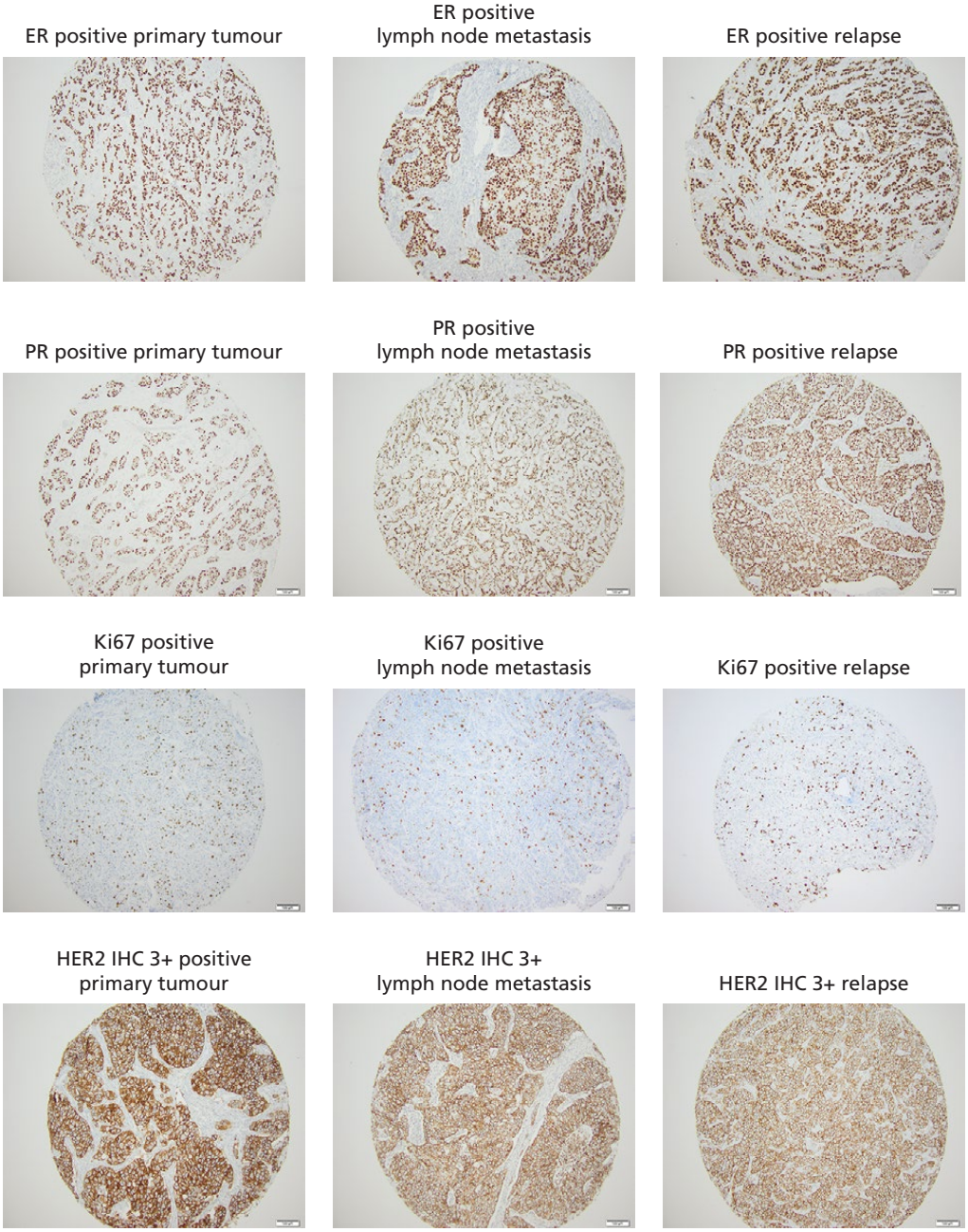


Figure 6. Microscopy of biomarker expression in primary tumour, lymph node metastasis and relapse. Abbreviations: ER=oestrogen receptor, PR=progesterone receptor, HER2=human epidermal growth factor receptor 2, IHC 3+=immunohistochemistry positive

ment of CK 5/6 antibodies, the clone D5/16B4 (DAKO, CA, USA) diluted 1:100 [310] was defined as positive if any cytoplasmic and/or membranous invasive carcinoma cell staining was positive.

Molecular subtype definitions

Classification according to the St Gallen [11] recommendations was based on the IHC analysis of ER, PR, Ki67 and HER2.

The following definitions were used in Paper III:

- **luminal A** (ER+ and/or PR+, Ki67 low and HER2–)
- **luminal B** (ER+ and/or PR+, Ki67 high and/or HER2+)
- **HER2 type** (ER–, PR– and HER2+)
- **triple negative** (ER–, PR– and HER2–)

In Paper IV, classification was also based on the St Gallen recommendations, IHC analysis of ER, PR, Ki67 and amplification of HER2 (SISH) but a distinction was made regarding HER2, separating luminal B into two groups due to the predictive information obtained:

- **luminal A** (ER+ and/or PR+, low Ki67 and HER2–)
- **luminal B** HER2– (ER+ and/or PR+, high Ki67 and HER2–)
- **luminal B** HER2+ (ER+ and/or PR+, any Ki67 and HER2+)
- **HER2 type** (ER–, PR– and HER2+)
- **triple negative** (ER–, PR– and HER2–)

In addition, EGFR and CK 5/6 identified a basal-like subgroup of patients within the triple negative subtype, but this subgroup was not considered in the descriptive or survival analysis since the St Gallen recommendations advise against using the markers to discriminate the basal-like subtype as they are considered insufficiently reproducible [11].

Statistical analysis

Statistical analysis is used to analyse data from well-defined populations, to test specific hypotheses. The intention may be, for example, to determine whether observed distributional differences between groups or associations between measured factors and outcome, are extreme under the null hypothesis, i.e. significant at a specific level of uncertainty, usually 5%. If the study is well-conducted, the results will be generalisable to the underlying population.

Differences in the distributions of categorical or categorised clinical variables and tumour characteristics between groups of patients were evaluated by the χ^2 test, while concordance in biomarker expression, dichotomised into positive or negative using cut-off values, between matched pairs of primary tumours and synchronous lymph node metastases/asynchronous relapses were evaluated by the McNemar test. For ordinal variables, e.g. biomarker expression in three or more categories, the Wilcoxon matched-pair signed-rank test was used. The McNemar-Bowker test of symmetry was used to compare molecular subtypes in primary tumours and lymph node metastases or relapses. This test evaluates whether the pattern in a cross tabulation matrix is symmetric or not. Hence, it can be used to detect non-random shifts of molecular subtype between the primary tumour and metastatic sites. This test is a generalisation of the McNemar test to more than two categories with an assumed order. Differences between three or more groups according to number of lymph node metastases were evaluated by the Kruskal-Wallis test.

Survival analysis and endpoints

Different endpoints were used to evaluate the prognosis for breast cancer patients in relation to the variables studied. BCSS (Paper I) is based on time from surgery to breast cancer-related death. The survival times for patients who either died from other causes or were alive at the end of the study were censored. The time variable is the same for overall survival (OS), but in analyses with this endpoint all causes of death are regarded as events. Cumulative BCM (Paper IV) is an alternative to BCSS that is more appropriate in the presence of so-called competing risks. If breast cancer is the only cause of death registered, BCM = 1-BCSS. DDFS includes any distant relapse (lung, liver, skeleton, brain, visceral) or death due to breast cancer as an event, and was calculated from the day of operation until the first event or the last review of the patient's records (Papers I, II and III). Kaplan-Meier analysis was used to estimate DDFS and BCSS while BCM was estimated as described by Marubini & Valsecchi [311]. The log-rank test was used to evaluate the null-hypothesis of equal survival in the different groups. The Cox proportional hazards model was used to calculate hazard ratios for the effects of the study variables on DDFS or BCSS with and without adjustment for other prognostic factors. Assumptions of proportional hazards were checked with Schoenfeld's test (Papers II and III) or graphically (Papers I and IV); p values < 0.05 were considered significant.

The software packages Stata 10.1, 11.1 and 12.1 (Stata Corp., College Station, TX, USA) and SPSS Statistics v 19 (IBM Svenska AB, Stockholm, Sweden) were used for all statistical calculations.

Strengths, limitations and potential bias

Patients in the two cohorts examined in the works of this thesis, were prospectively recruited for an observational study of the presence of DTCs in bone marrow and for a randomised study of tamoxifen treatment. Blocks of tumour tissue were collected for reanalysis of biological characteristics from patients included in the bone marrow cohort, by two pathologists independently with regard to ER, PR HER2 and Ki67. Methods of assessing ER, PR and HER2 are validated and the analysis of ER and PR in-

cluded in Paper II, were previously assessed by two pathologists independently in a validation study of IHC compared to cytosol [109]. The lack of consensus regarding the optimal cut-off for Ki67, and difficulties associated with reproducibility has been addressed earlier in this thesis. The use of TMAs for Ki67 analysis has not been validated for individual treatment decisions, however, in the analysis of groups of patients for research purposes it is sustained [146].

Prospective studies are generally ranked higher in scientific settings than retrospective studies, and allow patients to be followed over time to study the development of the disease [312, 313]. The main difference between prospective and retrospective studies is that the patient cohort of a prospective study is chosen in the present, and then followed and assessed according to variables intended to be observed in the future. In retrospective studies the cohort of patients is assembled from the past and the outcome is assessed in the present. Prospective studies have the advantage of accuracy in data retrieval regarding the defined endpoints and exposures to different risk factors. This kind of study is, however, time-consuming and expensive. Retrospective studies can answer new questions with known data already retrieved, which is effective in terms of both time and money. However, answers can only be found if the collected data contain the variables needed to address the question [314]. Cohort studies in general can be used to study multiple variables separately or in combination, and to relate them to multiple outcomes. There is, however, always a risk that an association may be explained by other variables, known or unknown, so-called confounders [314]. Confounders can be adjusted for in statistical analysis if they are known and measured. In all cohort studies there is a potential risk of selection bias. In Study I, for example, patients were asked to undergo a biopsy of the sternum at the time of surgery. This method of sample collection is invasive and could have affected on the patient's willingness to be included in the study as well as subconscious exclusion of elderly or fragile patients by the physician through not even asking them if they wanted to participate.

The method chosen to evaluate the presence of DTCs in bone marrow was IF staining in the majority of the patients. This method was used at the time for the identification of tumour cells [315], and several studies in the pooled analysis, which gave strong support for the prognostic value of the presence of DTC [163], used an IF staining protocol for detection. The disadvantage of the method is that it is difficult to evaluate the morphology, and the method is generally considered to result in a high false-positive rate [315]. During the period of the study, the method of analysing DTCs changed and ICC was introduced. This is considered a more validated method as morphology can be evaluated in greater detail using a bright-field microscopy. The ICC method is preferred in the consensus protocol of standardisation [164]. Although thoroughly adjusted for in the analysis of the results, the change in method is a drawback of this study.

When evaluating the results for biomarker expression and molecular subtypes (Papers II–IV), the subgroups tended to be small, despite the fact that the patient cohort was large. The power of the statistical tests (the probability of the test finding a statistically significant difference between groups as a function of the size of a true difference between the groups) depends on the size of the group in which the association or difference is measured. Patients with discordant biomarker expression or molecular subtype are few, and the subgroups used for comparisons will thus be too small for high-powered tests of prognosis, this is true for all studies with the same aim. Nevertheless, such analysis could be of importance for the individual patient and for further evaluation of the results in our quest to understand the molecular progression in breast cancer disease [313].

One of the discriminating variables between the groups of patients described in Paper IV was the mode of detection, i.e. screening detected *vs* clinically detected lesions. The groups were fur-

ther divided into molecular subtype and discordances between the primary tumour and lymph node metastases and shifts in inherence were related to outcome. In all studies concerning mode of detection a bias for lead time and length time must be accounted for [316]. Lead time bias arises when earlier detection results in longer follow-up to the event, i.e., the difference in time from when a tumour was detected with screening mammography to the time when it would have been detected clinically, without screening mammography. Length time bias is related to the susceptibility of screening mammography to diagnose more slowly growing tumours since they are detectable over a longer time [316]. The endpoint of the calculations presented in Paper IV was BCM, and data were retrieved from the Swedish Register of Causes of death (Central statistics office) on breast-cancer-related deaths up until 31 December 2011. The implication that screening led to the detection of tumours results in a longer time to event, and the fact that the event has not yet occurred is partly satisfied by the long observation time, but can nevertheless be a source of bias.

The strengths and limitations of the studies included in this thesis are summarised in Table 1.

Table 1. Strengths and limitations

	Strengths	Limitations
Study I	Study design: Prospective observational study. Methods: Controls for DTCs were available from healthy volunteers. Patient, tumour and treatment information collected from patients' records during 5 year follow-up period. No differences were identified in detection rate, DDFS or BCSS between the two methods used. Statistical method: Distinct endpoint, DDFS, BCSS. Multivariate analysis adjusted for other prognostic factors.	Methods: No standardised method for the detection of DTCs in bone marrow was available at the time. Two different methods were used to detect DTC. High percentage of DTC-positive samples in healthy volunteers (25%). Limited follow-up time.
Study II	Study design: Study based on prospective, randomised trial. Detailed patient and tumour characteristics available. Methods: Validated methods used for the analysis of ER, PR and HER2. Statistical methods: Distinct endpoint, DDFS. Comparison of biomarker expression between tumour locations with the McNemar test. Multivariate analysis adjusted for other prognostic factors.	Methods: HER2 only assessed by IHC. No validation of Ki67 cut-off value available. Limited follow-up time. Statistical methods: Small subgroups when analysing shift in biomarker expression in matched pairs of primary tumours and lymph node metastasis (low statistical power).
Study III	Study design: Study based on prospective, randomised trial. Detailed patient and tumour characteristics available. Molecular subtype classification according to the St Gallen recommendations. Methods: Validated methods for the analysis of ER, PR and HER2. Statistical methods: Distinct endpoint, DDFS. Comparison of biomarker expression between tumour locations with the McNemar-Bowker test. Multivariate analysis adjusted for other prognostic factors.	Methods: No validation of Ki67 cut-off value available. Limited follow-up time. Statistical methods: Small cohort of patients assessed regarding molecular subtype.
Study IV	Study design: Study based on prospective observational patient cohort. Re-analysis of biomarker expression of all tumour samples available, by two pathologists independently. Methods: Validated methods for analysis of ER, PR and HER2. Molecular subtype classification according to the St Gallen recommendations. Statistical methods: Distinct endpoint, 10 years BCM available through Swedish Register of Causes of Death. Comparison of biomarker expression separately and in combination, between tumour locations using the McNemar/McNemar-Bowker test. Molecular subtype cohort analysed and no differences observed regarding patient or tumour characteristics compared to total cohort.	Study design: Risk of patient selection in molecular subtype cohort due to limited availability of all biomarkers for subtype classification in primary tumour and lymph node metastases. Relapse tissue available only for a minority of the patients, reinforced by combination of different biomarkers. Methods: No validation of Ki67 cut-off value available.

Results

Tumour samples

Bone marrow

The bone marrow sample was excluded in 154 patients: in 37 patients the sample volume was inadequate, and in 117 analyses was not performed due to a change in research strategy at the analysing laboratory. The final cohort included 401 patients (Paper I).

Primary tumours and lymph node metastases

ER and PR had been evaluated on whole sections in a previous study, both in the primary tumour and lymph node metastasis: thus, 262 and 257 matched pairs were assessed for ER and PR, respectively. TMAs were constructed using samples from the original cohort of patients for the evaluation of Ki67 and HER2. Non-evaluable cases were due to loss of TMA core, more frequently in the lymph node metastasis than in the primary tumour samples, and unsuccessful staining. Assessment of Ki67 in both primary tumour and lymph node metastasis was possible in 101 matched pairs, and assessment of HER2 in 104 matched pairs (Paper II).

Molecular subtypes were classified according to the assessment of ER, PR, Ki67 and HER2 (Paper II). Eighty five patients had known expression of all the included markers in the primary tumour and corresponding lymph node metastases and could thus be classified according to the St Gallen guidelines [11] (Paper III).

Primary tumours, lymph node metastases and relapses

Formalin-fixed paraffin-embedded archival blocks of study samples were obtained in 524/555, 147/217 and 42/97 of the primary tumour, synchronous lymph node and asynchronous relapse, respectively, for the study cohort (Figure 3, flowchart, Paper IV). Of the 147 re-analysed lymph node metastases, 142 were macrometastes- and 5 micrometastases. The distribution of lymph node metastases in the node positive group of patients was: 35 patients (24%) with 1; 42 patients (28%) with 2–3 and 70 patients (48%) with ≥ 4 lymph node metastases.

In 9 of the 42 re-analysed cases of relapse, recurrences were identified during the process of block retrieval, after the completion of data abstraction from the patient's records, and were not registered as relapses in the database. Four of these cases had cancer of the contralateral breast, one sample was benign and one showed a new cancer (cholangiocarcinoma) and these six cases were therefore excluded from further analyses. The locations of the included relapses were: local (skin or tissue in breast area), regional (ipsilateral lymph nodes in the axilla or infraclavicular area) or distant metastasis (supraclavicular lymph nodes, bone, visceral, lung, liver and cerebral) and are further addressed in Table 2. Non-evaluable samples resulted from unsuccessful staining and loss of individual tumour sections in the TMA preparation in 14 samples. Paraffin-embedded tumour material could not be retrieved in more than half of the patients, partly because biopsy from the relapse location was not included in national guidelines at the time.

Disseminated tumour cells in bone marrow

DTCs were analysed in 401 patients, CK-positive cells being found in 152 of these (38%). The IF-based method resulted in 131/327 (40%) DTC-positive cases, whereas the ICC method resulted in 22/74 (30%) positive cases. However, there was no statistically significant difference between the detection rates of the two methods ($p = 0.11$). The detection of DTCs in bone marrow was not related to either DDFS ($p = 0.60$, log-rank test, Figure 7) or BCSS ($p = 0.37$, log-rank test). Stratifying the cohort according to the method used for the detection of DTCs re-

Table 2. Location of relapse and biopsy, Paper IV.

			Distribution according to subtype				
Site of relapse	Total N	Successful analysis of molecular subtype in relapse N	luminal A N	luminal B HER2 – N	luminal B HER2+ N	HER2 type N	triple negative N
Local (chest or chestwall)	29	18	4	3	8	2	1
Regional (axilla, fossa infra-clavicularis)	4	1		1			
Fossa supraclavicularis	8	2				1	1
Bone	23	3	1	1	1		
Lung	10	2		1	1		
Liver	20	0					
CNS	3	1				1	
Total	97	27			27		

sulted in similar results using Cox univariable analysis (Table 3). When quantitative data for the number of DTCs present in bone marrow were analysed, no further differences in DDFS were observed.

The presence of DTCs in bone marrow was not significantly related to patient or tumour characteristics. Significant prognostic factors were lymph node metastases, ER-positivity, NHG, and tumour size according to Cox univariable analysis (Table 3).

Subgroup analysis

When the cohort was stratified according to lymph node status, 157 patients were identified as having lymph node metastases (N+) and 233 without metastatic lymph nodes (N0). Cox univariable analysis showed that the presence of DTCs had no statistically significant effect on prognosis in terms of DDFS in either subgroup (**N0:** DTC+ *vs* DTC–: HR = 2.7; 95% CI = 0.72–9.1; p = 0.14 and **N+:** DTC+ *vs* DTC–: HR = 0.84; 95 CI = 0.42–1.72; p = 0.6, Figure 7). There were 4 registered events in the N0 patient group and 20 events in the N+ group. The results were independent of the detection method used (IF or ICC).

Table 3. Cox univariable and multivariable analysis of distant disease-free survival, Paper I.

	Univariable analysis (n ≤401)			Multivariable analysis (n=377)		
Variable	HR*	95% CI	*p*	HR**	95% CI	*p*
DTC status (IF and ICC) (n=401)						
DTC+ *vs* DTC-	1.2	0.66–2.2	0.55			
DTC status (IF) (n=327)						
DTC+ *vs* DTC-	1.2	0.63–2.2	0.60			
DTC statuc (ICC) (n=74)						
DTC+ *vs* DTC-	0.84	0.09–8.1	0.88			
Age						
per year	0.99	0.97–1.02	0.61			
Node status						
N+ *vs* N0	5.5	2.7–11	<0.001	3.4	1.6–7.2	0.001
Tumour size						
>20 mm *vs* ≤ 20 mm	4.9	2.6–9.4	<0.001	2.5	1.2–5.2	0.01
NHG status						
NHG 2 *vs* NHG 1	6.9	0.92–52	0.06	4.9	0.65–37	0.12
NHG 3 *vs* NHG 1	20	2.7–147	0.004	8.7	1.1–70	0.04
ER status						
ER+ *vs* ER-	0.39	0.21–0.72	0.003	0.85	0.38–1.9	0.7
PR status						
PR+ *vs* PR-	0.43	0.24–0.79	0.007	0.67	0.33–1.4	0.3

* No significant deviations from proportional hazards (Schoenfeld's test)

* **p=0.05 in Schoenfeld's global six degree-of-freedom test of proportional hazards

Abbreviations: IF= immunofluorescence, ICC= immunocytochemistry, HR= hazard ratio, CI= confidence interval, DTC= disseminated tumour cells, N0= node negative, N+= node positive, NHG= Nottingham histological grade, ER= oestrogen receptor, PR= progesterone receptor.

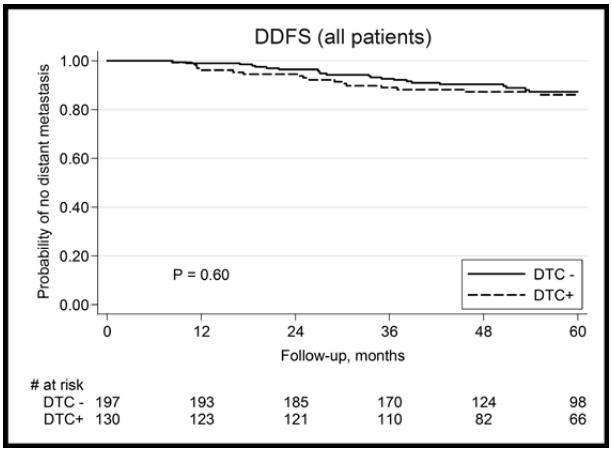


Figure 7a. Distant disease-free survival by presence of DTC in total cohort.

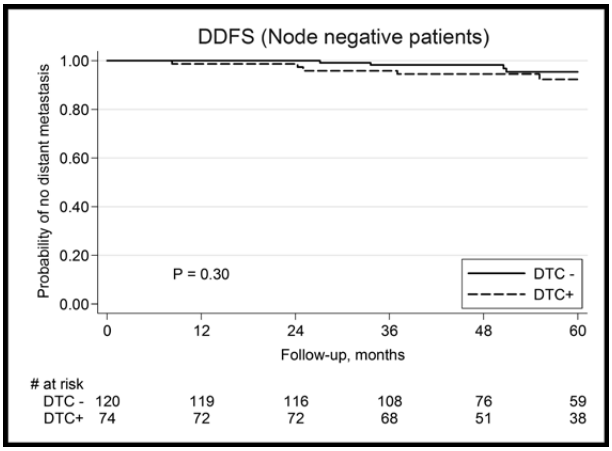


Figure 7b. Distant disease-free survival by presence of DTC in node negative patients.

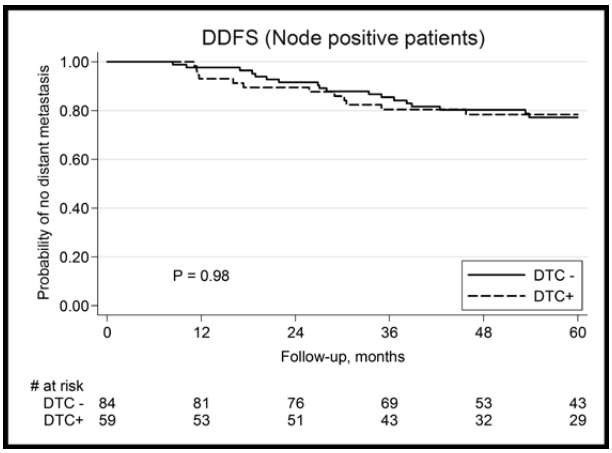


Figure 7c. Distant disease-free survival by presence of DTC in node positive patients.

Table 4. Distribution of biomarkers in primary tumour, lymph node metastases and relapses, Paper IV.

	Primary tumour		Lymph node			
	N	% (95 % CI)*	N	% (95 % CI)*	N	% (95 % CI)*
ER ≥10%	442/507	87 (84–90)	117/147	79 (72–86)	23/30	77 (58–90)
PR ≥10%	321/476	67 (63–70)	85/146	58 (50–66)	14/30	47 (28–66)
Ki67 >20%	165/500	33 (29–37)	66/144	46 (37–54)	17/29	59 (39–76)
HER2+ (SISH)	100/496	20 (17–24)	43/136	32 (24–40)	14/28	50 (31–69)

Abbreviations: ER= oestrogen receptor, PR= progesterone receptor, HER2= human epidermal growth factor receptor 2, SISH= silver *in situ* hybridization, CI= confidence interval
* Exact binominal confidence interval

Expression of separate biomarkers

Descriptive data for the distribution of all biomarkers found to be positive in primary tumours and at the different metastatic locations, describes a regress of the expression of ER and PR, with a corresponding increase in proliferation (Ki67) and amplification of HER2, from primary tumour to synchronous lymph node metastases and asynchronous relapses, when matched pairs were not taken into account (Paper IV, Table 4).

However, comparison of ER, PR, Ki67 and HER2 between primary tumours and matched lymph node metastases in two patient cohorts (Papers II and IV) showed high concordance in all the markers analysed with no significant shifts in biomarker expression in either direction (Table 5).

When biomarker expression in primary tumours and asynchronous relapses was evaluated as a semi-quantitative, grouped variable (Paper IV), a significant shift in all biomarkers was observed (ER: $p = 0.006$, PR: $p = 0.04$ and Ki67: $p = 0.02$, Wilcoxon signed-rank test). No significant skewness of the changes was observed in the biomarkers when positive and negative expression were compared using a cut-off value of ≥ 10% for ER and PR, and > 20% for Ki67 (ER: $p = 1.0$, PR: $p = 0.5$ and Ki67: $p = 0.4$, McNemar test) except for HER2, where 7/26 patients (27%) gained amplification in relapses, while no patients lost their HER2 expression in the relapse ($p = 0.02$, McNemar test , Table 5b).

It is noteworthy that the three cases showing discordance in HER2 expression between the primary tumour and lymph node meta-stases, (Paper II), all gained HER2 expression. HER2 expression in the primary tumour was not correlated to shorter DDFS in the five-years follow-up, where HER2 expression in the lymph node metastases was indicative of adding prognostic information (primary tumour: HR = 1.7, $p = 0.23$; lymph node metastases: HR = 2.0, $p = 0.06$).

Molecular subtype according to the St Gallen classification

No significant discordance in terms of molecular subtype could be detected between primary tumours and synchronous lymph node metastases (Paper III: $p = 0.06$, Paper IV: $p = 0.3$, McNemar-Bowker test of symmetry, Table 6). A tendency towards a shift between primary tumour and relapse was noted, although it was not strictly significant ($p = 0.07$, McNemar-Bowker test of symmetry, Paper IV). However, shifts were seen in subtype inherence in individual patients. In

Table 5a. Biomarker discordance in matched pairs of primary tumours and corresponding lymph node metastases (Paper II)

Primary tumour *vs* lymph node metastasis					
Biomarker	N	+/–	–/+	Discordant N (%)	*p* *
ER	262	12	7	19 (7)	0.36
PR	257	27	15	42 (16)	0.09
Ki67	101	4	10	14 (14)	0.18
HER2	104	0	3	3 (3)	0.25

Table 5b. Biomarker discordance in matched pairs of primary tumour, corresponding lymph node metastasis and relapse (Paper IV)

	Primary tumour *vs* lymph node metastasis					Primary tumour *vs* relapse				
Biomarker	N	+/–	–/+	Discordant N (%)	*p* *	N	+/–	–/+	Discordant N (%)	*p**
ER	140	1	1	2 (1)	1.0	29	3	1	4 (13)	0.6
PR	130	9	12	21 (16)	0.7	27	6	3	9 (33)	0.5
Ki67	135	13	18	31 (23)	0.5	28	1	4	5 (18)	0.4
HER2	130	7	15	22 (16)	0.1	26	0	7	7 (27)	**0.02**

Abbreviations: *ER*= oestrogen receptor, *PR*= progesterone receptor, *HER2*= human epidermal growth factor receptor 2. *N*= number of analysed matched pairs. +/- = positive expression in the primary tumour and negative in the corresponding lymph node metastasis/relapse. -/+ = negative expression in the primary tumour and positive in the corresponding lymph node metastasis/relapse.
*McNemar test

the tamoxifen-treated patient cohort (Paper III, Table 6a), the luminal A subtype in the primary tumour shifted to a subtype with a worse prognosis in lymph node metastases in 7 of 45 cases (16%), whereas no shift in the opposite direction was observed (0/38, $p = 0.02$, McNemar-Bowker test of symmetry). In the comparison of luminal A and non-luminal A subtypes in primary tumours and lymph node metastases (Paper IV) a shift from luminal A in the primary tumour to non-luminal A subtype in the lymph node metastases was observed in 15/48 (31%) patients, and primary tumours of the non-luminal A molecular subtype shifted to luminal A subtype in 13/73 (18%) in the lymph node metastases (Table 6b).

St Gallen molecular subtypes primary tumours and lymph node metastases and prognostic information

Patients with luminal A subtype had a favourable prognosis compared with all other molecular subtypes in both patient cohorts. For both primary breast tumour and synchronous lymph node metastases, all other subgroups showed an increased hazard of developing distant metastases or of dying from breast cancer within five

Table 6a. Comparison of St Gallen molecular subtype distribution in primary tumour with matched lymph node metastases (Paper III)

Subtype in primary tumours N	Subtype in lymph node metastases (n=85) N					
	luminal A	luminal B	HER2 type	triple negative	*p**	Total
luminal A	38	5	0	2		45
luminal B	0	17	0	0		17
HER2 type	0	1	10	0	0.06	11
triple negative	0	0	1	11		12
Total	38	23	11	13		85

*McNemar-Bowker test of symmetry for all subclasses
p= 0.02 McNemar-Bowker test of symmetry for Luminal A subclass *vs* non-Luminal A subclasses

Table 6b. Comparison of St Gallen molecular subtype distribution in primary tumours with matched lymph node metastases and relapses (Paper IV)

Subtype in primary tumours	Subtype in lymph node metastases (n=121) N					
	luminal A	luminal B HER2-	luminal B HER2+	HER2 type	triple negative	*p**
luminal A	33	7	8	0	0	
luminal B HER2-	9	18	3	0	0	
luminal B HER2+	3	3	15	1	0	0.3
HER2 type	1	0	1	7	0	
triple negative	0	0	0	3	9	

Subtype in primary tumours	Subtype in relapses (n=24) N					
	luminal A	luminal B HER2-	luminal B HER2+	HER2 type	triple negative	*p**
luminal A	3	1	5	0	0	
luminal B HER2-	1	3	1	0	0	
luminal B HER2+	0	0	4	2	0	0.07
HER2 type	0	0	0	0	0	
triple negative	0	1	0	1	2	

Abbreviations: HER2= human epidermal growth factor receptor 2
* McNemar-Bowker test of symmetry

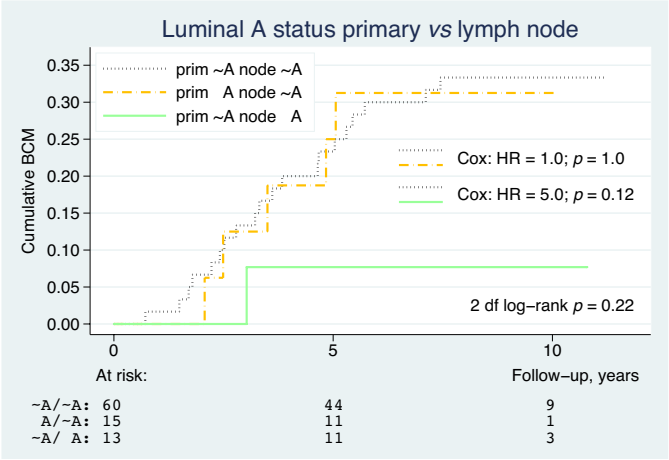


Figure 8. Cumulative breast cancer mortality according to shifts in molecular subtype inherence between primary tumour and synchronous lymph node metastasis.
Abbreviations: *Prim ~A node ~A*= stable non-luminal A in primary tumour and synchronous lymph node metastasis. *Prim A node ~A*= shift from luminal A in primary tumour to non-luminal A in synchronous lymph node metastasis. *Prim ~A node A*= shift from non-luminal A in primary tumour to luminal A in synchronous lymph node metastasis. *HR*= Hazard ratio. *2 df log-rank*=two degrees of freedom log-rank test.

years, compared with the luminal A subtype (primary tumour: DDFS: $p = 0.002$, log-rank test, lymph node metastases: DDFS: $p = 0.003$, log-rank test, Paper III). The HER2 type and triple-negative subtype were associated with the shortest survival time. Patients with luminal A primary tumours had significantly lower 10-year BCM than patients with all other molecular subtypes (Paper IV) ($p = 0.002$, log-rank test): the highest BCM was noted in patients with triple negative primary tumours compared to luminal A (HR = 4.0; 95% CI = 2.0–8.2, $p < 0.001$, Cox proportional hazard model). The difference in BCM between the molecular subtypes remained significant ($p < 0.001$) using a Cox proportional hazard model adjusted for age (continuous), tumour size (> 20 mm *vs* ≤ 20 mm), presence of lymph node metastases (N+ *vs* N0), and mode of detection (clinically detected *vs* screening detected). When comparing the relation between BCM and the St Gallen molecular subtypes in the synchronous lymph node metastases, a similar pattern to that in primary tumours was observed: luminal A had a favourable prognosis whereas the triple negative subtype was associated with the worst prognosis, but the null hypothesis of equal BCM in the five groups was not significant.

Prognostic information of a shift in molecular subtype from primary tumour to synchronous lymph node metastases

Patients showing a shift in molecular subtype from luminal A in the primary tumour to non-luminal A (n = 15) in the metastatic lymph node had equally poor prognosis as patients with stable non-luminal A subtype (n = 60) inherence in both the primary tumour and the synchronous lymph node metastases (HR = 1.0, 95% CI = 0.4–2.6, $p = 1.0$, Cox proportional hazard model, Figure 8), suggesting a prognostic influence of the molecular subtype in synchronous lymph node metastases.

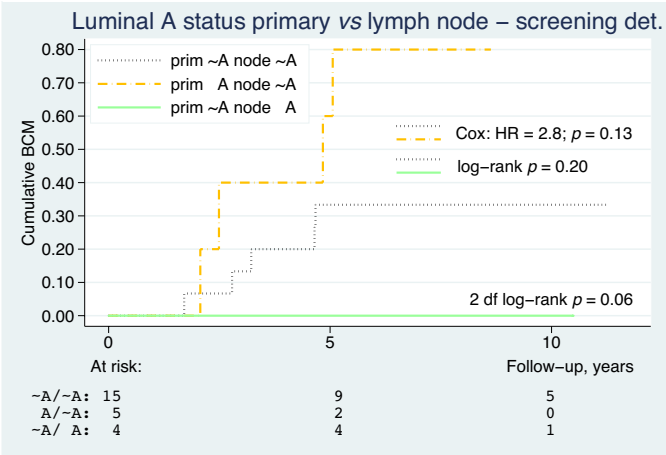


Figure 9. Cumulative breast cancer mortality according to shifts in molecular subtype between primary tumour and synchronous lymph node metastasis in screening-detected patients.
Abbreviations: *Prim ~A node ~A*= stable non-luminal A in primary tumour and synchronous lymph node metastasis. *Prim A node ~A*= shift from luminal A in primary tumour to non-luminal A in synchronous lymph node metastasis. *Prim ~A node A*= shift from non-luminal A in primary tumour to luminal A in synchronous lymph node metastasis. *HR*= Hazard ratio. *2 df log-rank*=two degrees of freedom log-rank test.

When analysing the subgroup of patients who showed a change in subtype inherence from non-luminal A in the primary tumour to luminal A in lymph node metastases (n = 13) the BCM was five times higher in the non-luminal A group (n = 60) at both locations (HR = 5.0, 95% CI = 0.7–37.0, p = 0.12, Cox proportional hazard model, Figure 8). Twelve of the 13 patients (92%) who changed to luminal A subtype in the lymph node survived 10 years, compared with 20/60 (33%) of the patients with stable non-luminal A subtype in both the primary tumour and the lymph node metastases who died from breast cancer.

Prognostic information of a shift in molecular subtype from primary tumour to synchronous lymph node metastases, stratified according to mode of detection

Patients with screening detected luminal A primary tumours (n = 124, 48%) had an improved prognosis compared to those with clinically detected luminal A primary tumours (n = 136, 52%): 10-year BCM 6% compared to 13% (p = 0.02, log rank test), and were thus identified as a subgroup with excellent prognosis. In both screening detected and clinically detected patients with luminal A tumours, lymph node metastases were a significant negative prognostic factor (p = 0.003 and p < 0.001, log rank test, respectively). We therefore investigated whether prognosis according to nodal molecular subtype could explain the prognostic influence of lymph node metastases in this subgroup. All patients with screening detected primary tumours and luminal A subtype in the lymph node metastases (n = 17) had an excellent outcome compared to non-luminal A subtypes (n = 20), regardless of the subtype of the primary tumour (p = 0.001, log rank test, Figure 9).

Discussion

The formation of new tumours at local or distant sites of the body after the diagnosis and treatment of a primary tumour is referred to as a metastatic process. Traditionally, this process has been described as a stepwise procedure in which tumour cells develop and expand at the primary site and eventually leave as fully mature cell clones to form metastases at a secondary site. According to this model, metastases share the same biological properties as the primary tumour. This theory of the metastatic process has been questioned during recent decades, and evidence supports the theory that the seeding of cancer cells from the primary site is an on-going process, starting before the primary tumour is even discovered, with independent modification of genetic aberrations [13, 277]. One aim of the work included in this thesis, was to compare the phenotype in primary tumours and metastatic sites, in order to study the metastatic process in breast cancer.

Early dissemination of tumour cells may occur through two different pathways: lymphatic or haematogenous spread. It is still not clear which factors determines whether tumour cell clones from primary tumours are more likely to disseminate through the lymphatic vessels or enter the haematogenous pathway, whether they are two independent processes or are connected. A second aim of the work included in this thesis was to study DTCs at the time of diagnosis in primary breast cancer as a prognostic marker in all patients, as well as in node-negative patients, in order to reveal if the metastatic process in node-negative patients is different from the metastatic process in node-positive patients. In a recent study, DTCs in bone marrow and the sentinel node in primary breast cancer patients were compared, showing no correlation in the presence of DTCs from the different locations. The authors concluded that lymphatic and haematogenous spread were independent routes of dissemination [317]. The findings in Paper I could not clearly give any support for this hypothesis, since there was no correlation of the presence of DTCs and prognosis, although indicative of importance in terms of survival in node-negative patients.

The evaluation of concordance between biological characteristics in primary tumours and lymph node metastases has been less well studied than that between primary tumours and relapses in the same patient. Table 7 gives a brief summary of the studies in the area and, generally, a high concordance has been found in ER, PR, and HER2 between primary tumours and lymph node metastases [284, 285, 287, 318–321]. This was also confirmed in the present work (Papers II and IV). However, no studies have been performed to compare the rate of concordance in biomarker expression in molecular subtypes using IHC, a classification system that has been recognised as providing further prognostic information [11, 12] when assessed in the primary tumour. Genetic microarray analysis has been used to evaluate the concordance between primary tumour and lymph node metastases [288, 322] showing a discordant expression where Feng *et al.* also found a subgroup with favourable prognosis. The information obtained from the lymph node metastases in Studies III and IV suggests that the cancer cell clone that has evolved in the lymph node metastasis has a prognostic influence and not the molecular information obtained from the primary tumour. Similar results were found in the explorative analysis of screening detected breast cancer patients with lymph node metastases, namely the observation that patients with luminal A subtype in the lymph node metastases had an excellent prognosis regardless of the subtype in the primary tumour; no BCM was recorded in this group where 4/20 patients had > 4 lymph node metastases. The finding of a low risk-group in a subset of patients with lymph-node-positive disease, as previously described [288], could identify a subgroup of patients in which endocrine adjuvant treatment could be sufficient. Although the differences are small and not significant, some au-

thors have hypothesized that the cells in lymph node metastases could provide more information in terms of aggressiveness than the analysis of the primary tumour [321, 323].

Tumour cell migration into the lymph nodes is facilitated by the process of lymphangiogenesis [273], which has been suggested to be associated with the development of sentinel node metastases [324, 325]. The SN technique is an important part of staging and provides guidance for recommendations on further surgery and adjuvant treatment. The scientific basis for SN assumes an orderly flow of cancer cells to lymph nodes in a sequential way; if the sentinel lymph node is free of disease there is strong evidence that no other lymph nodes in the axilla are affected by disease [326]. If the axillary contents are free from lymph node metastases, the prognosis should consequently be favourable. However, distant metastases can occur without prior lymph node metastases and, conversely, lymph node involvement does not necessarily predict systemic spread. A large fraction of lymph-node-positive patients will remain disease-free over a long period, while being treated by locoregional therapy alone [4]. Moreover, the presence of lymph node metastases at the time of diagnosis has been associated with poorer survival after relapse than in node-negative patients [327]. The identification of lymphatic-specific molecular factors that promote the growth of lymphatic vessels has recently increased our knowledge about the process involved. Overexpression of vascular endothelial growth factors (VEGF) C and D, which binds to the VEGF receptor (VEGFR) expressed on the lymphatic endothelium, has been shown to significantly increase tumour-associated lymphatic vessel growth [328]. Increased levels of VEGFR-3 have been found to be correlated with an increased number of metastatic lymph nodes and reduced disease-free and overall survival [329]. The active attraction of CTCs to the lymphatic vasculature by tumour-derived VEGFs may drive chemokinetic processes with the ability to alter the lymphoid microenvironment and the immune system, creating a 'tumour friendly' environment. CTCs may be conditioned in this altered microenvironment and further disseminate to distant organs, before the development of overt lymph node metastasis. The process could be part of the explanation of the prognostic value of lymph node metastases, although their removal does not affect outcome [277].

The complex interaction between malignant cells at different locations, and at different degrees of development, together with molecular changes in the microenvironment at these locations may explain the relatively higher discordance in the biological characteristics of tumours when primary tumours are compared with distant metastases, than in comparisons with lymph node metastases that have been reported in clinical evaluations. In study IV, this was confirmed, showing discordances between primary tumours and relapses, but not in primary tumours compared with lymph node metastases.

A recent meta-analysis comparing HER2 expression in primary tumours with that in lymph node metastases and/or relapse in 2,520 cases confirmed a significant difference in discordance rate between the metastatic sites, showing a higher discordance rate when primary tumours were compared to relapses [286]. The site of relapses analysed in the present work (Paper IV) is given in Table 2, and reveals that most biopsies available for the assessment of biomarkers, are local relapses in the breast area. The selection of relapse site reflects the higher availability of biopsies from local relapses and the fact that biopsy of relapses was not included in the Swedish National Guidelines at the time of study inclusion. Nevertheless, it may affect the results regarding concordance rate, as the meta-analysis included studies with varying criteria for relapse. When distant metastases only were compared with the primary tumour, the discordance rate was found to be significant, which was not the case when lymph nodes and local relapses were included and distant metastases were present in ≤ 25% of the cohort[286]. Despite the low number of assessable samples in Study IV, significant shifts

were identified between primary tumours and relapses, most notably in HER2, where all the patients gained HER2 expression/amplification in the relapse and no shifts in the other direction were observed. The same trend was noted when comparing primary tumours and synchronous lymph node metastases (Paper II) where three patients expressed HER2 in the lymph node but not in the primary tumour, and no change in the other direction was observed. In an exploratory analysis, the HER2 positive lymph node metastases seemed to be linked to a shortened DDFS during 3-year follow-up, implying a possible influence on prognosis for patients with discordant HER2 expression. Discordance rates may be attributed to various factors, including the processes involved in tumour progression, factors related to technical analysis and intratumoural heterogeneity. The contributions of these factors are not known, and caution has been proclaimed in the interpretation of data [330]. Intratumoural heterogeneity could result in sampling bias as small samples from core biopsies or FNA can result in different histological and biologic features. This has been addressed with regard to HER2, showing an intratumoural heterogeneity of HER2 in < 1% of women participating in the trial [331] A similar study on microarray-based assays concluded that although heterogeneity was present in breast cancer, it does not preclude precise predictions of tumour behaviour or clinical breast cancer [332]. The clinical implication of discordance rates between primary tumours and relapse has been evaluated in terms of a change in therapy with a reported change of the individual patient management in 14–20% of cases [293, 294, 333]. Biopsy of relapses for the analysis of biomarker expression is now performed in the clinical settings and recommendations for this have been included in the Swedish National Guidelines since 2012 [51].

Although the subset of patients with discordant molecular subtype is small, the prognostic information could be relevant for the individual patient. The implication is that large patient series are required for high-powered statistical comparisons of prognosis for the subgroup of patients showing discordances in biomarker expression. The choice of statistical method also has implications for the interpretation of shifts when comparing matched pairs of tumour samples from different locations in the same patient. Table 7 shows the statistical methods chosen to compare matched pairs of tumour tissue from primary tumours and lymph node metastases and/or relapse from the same patient. In the author's opinion, the χ^2 test is applicable when comparing distribution in groups, not taking matched pairs into account. The aim of the work presented in this thesis was not only to compare the differences in biomarker expression or molecular subtype, but to investigate the effects of a shift in metastatic sites compared to the primary tumour in the same patient. To do so correctly, the McNemar test was used when evaluating shifts from positive to negative or negative to positive expression, and the McNemar-Bowker test of symmetry was used when assuming mutual distribution between groups (molecular subtype according to the St Gallen guidelines). In line with a previous study [293], the Wilcoxon matched-pairs signed-rank test was used in the present work when analysing the biomarker expression without cut-off. This test is comparable to the McNemar test, but with a slightly higher power. The Wilcoxon matched-pairs signed-rank test showed a significant discordance between biomarker expression in the primary tumour and relapse. However, significance was reached only for HER2 when applying the McNemar test for concordance analysis (Paper IV).

The kappa-value (κ) is often used to determine the consistency of two observers in intra- or inter-laboratory quality assurance tests. When used for the comparison of matched pairs of tumour samples it does not take into account the direction of the observed discordances.

The cut-off values used for biomarker expression are based on accepted guidelines [111, 124]. The predefined cut-off value for ER responsiveness in clinical practice is traditionally 10%, although there is support for a lower cut-

off value of 1% for endocrine treatment, and thus the detection of any ER-positive cell in the tumour will define it as an ER-responsive tumour [11]. The ASCO/PAP guidelines support the 1% cut-off [111], but these guidelines has been questioned in a recent study [253]. The prognostic value of Ki67 has been investigated in several recent publications [7, 145, 334] but the assessment of the cut-off value of Ki67 has not been established and the reliability of the measures varies between laboratories [11]. In the studies included in this thesis, a Ki67 cut-off based on population sectioning, distinguishing the one-third of the patients in the population with the highest proliferation from the remaining two-thirds was used [254] resulting in 20% cut-off separating low from high proliferation. Values close to the cut-off were seen in < 5% for ER and PR at all locations, while values of Ki67 were assessed as being close to the cut-off in 10% (66/500) of the primary tumours, in 18% (26/144) of lymph node metastases, and in 17% (5/29) of recurrences (Paper IV).

The detection of DTCs in bone marrow in women with primary breast cancer at the time of diagnosis had no prognostic impact (Paper I). Although most publications report that the detection of DTCs in primary breast cancer is an independent prognostic factor for recurrence and death, the clinical significance of micrometastases in bone marrow remains controversial, and the American Society of Clinical Oncology has not advocated it as a prognostic marker for clinical use, due to insufficient data [11]. Furthermore, concerns have been raised regarding the standardisation of detection using monoclonal antibodies against CKs. The detection of DTCs in bone marrow has been identified in several publications as an independent predictor of poor outcome in patients with non-metastatic breast cancer disease [159, 160, 335]. The level of evidence increased when a pooled analysis of 4,703 breast cancer patients was published, showing poor prognostic significance of the presence of DTCs in the bone marrow at 10 years follow-up [163]. Aspects that should be considered when evaluating the results of these early reports are the heterogeneity of the patients included and the variation in methods and techniques used for determining bone marrow dissemination. However, more recent studies performed with standardised methods of detection, also suggest that DTCs in bone marrow have a prognostic value of DTCs in bone marrow [176, 179, 336]. Molloy *et al.* (2011) reported a clinical significance of DTCs in bone marrow in terms of BCSS (HR = 2.1, p = 0.003) but not in metastasis-free survival (HR = 1.5, p = 0.127). Giluiano *et al.* reported that DTCs were present in 104/3413 (3.0%) patients, and were associated with decreased overall survival according to univariable analysis, but did not reach clinical validity in multivariable analysis. They concluded that bone marrow aspiration should not be recommended as part of routine clinical practice for patients with early breast cancer, without improved techniques for the isolation and detection of occult tumour cells in bone marrow [179]. Solá *et al.* found a higher frequency of DTCs in the subgroup of patients who experienced breast-cancer-related events (13%), but the results did not reach statistical significance due to a low power with few events [176]. Future evaluation of the prognostic and predictive value of CTCs may offer more unambiguous data since the method of detection is standardised. CTC sampling is also a less invasive procedure, associated with less pain for the patients.

The development and maturation of disseminated tumour cells can occur at a secondary site, implying the possibility of a different phenotype from that of the primary tumour. The impact on clinical management and recommendations to patients with metastatic disease concerning therapy will be affected since there may be differences in biomarker profile between the primary tumour, lymph node metastases and relapses in the same patient. The prognostic heterogeneity of lymph node positive breast cancer may be explained by molecular changes in lymph node metastases.

Table 7. Brief summary of reported discordances in ER, PR, HER2 in lymph node metastases and distant metastasis

Author	Method	ER discordance N (%)	PR discordance N (%)	HER2 discordance N (%)	Survival data	Location	Statistics
Tsutsui S *et al.,* 2002	IHC	20/104 (19) p=<0.0001	22/104 (21) p=<0.0001	5/76 (7) p=<0.0001	No	LN	χ2 test
De la Haba-Rodrigue JR *et al.,* 2004	IHC	4/60 (7) p=1.01 p=0.352	9/60 (15) p=0.041 p=0.022	17/60 (28) p=0.211 p=0.072	No	LN	McNemar1 Wilcoxon2
D'Andrea M *et al.,* 2006	IHC	3/88 (3) r=0.9 p<0.000	3/87 (3) r=1.0 p<0.000	3/76 (4) r=0.9 p<0.000	No	LN	Pearson
Guarneri V *et al.,* 2008	IHC/ FISH	17/75 (22) κ=0.4	27/75 (36) κ=0.3	7/45 (16) κ=0.6	No	DM	κ value
Aitken S J *et al.,* 2009	IHC/ FISH	55/194 (28) p=0.66	45/192 (24) p=0.84	17/190 (9) p<0.00001	No	LN	Student's t-test
Simmons C *et al.,* 2009	IHC/ FISH	3/26 (12) ER+PR: p=0.026	7/26 (27)	2/26 (8) p=not reported	No	DM	McNemar
Strien L *et al.,* 2009	IHC/ CISH	7/56 (13)	9/56 (16)	4/65 (6)	No	LN (SN)	NS
Hoefnagel LD *et al.,* 2010	IHC/ SISH	24/233 (10) p=not reported	70/233 (30) p<0.001	12/233 (5) p=not reported	No	DM	χ2 test
Idrisinghe PK *et al.,* 2010	IHC	13/72 (18) p=0.017 κ=0.20	30/72 (42) p=0.000 κ=0.759	5/72 (7) p=not reported	Yes	DM	χ2 test κ value
Thompson A *et al.,* 2010 (BRITS)	IHC/ FISH	5/49 (10) not significant	12/49 (24) not significant	2/49 (2) not significant	No	DM	Wilcoxon signed-rank test
Bogina G *et al.,* 2011	IHC/ CISH	4/50 (8) 5/90 (5.5)	16/50 (32) 14/90 (15.5)	0/48 1/88 (1)	Yes	DM LR	McNemar χ2 test
Curigliano G *et al.,* 2011 [341]	IHC/ FISH	37/255 (14) p=0.001	124/255 (49) p<0.0001	24/172 (14) p≤0.0001	No	DM	Fisher's exact test
Houssami N *et al.,* 2011 [286]				Meta analysis			
Jensen JD *et al.,*2011 [284]	IHC/ FISH	2/50 (4) Not significant 14/118 (12) p=0.2	– –	0/52 – 10/114 (9) p=0.1	No	LN DM	McNemar
Wilking U *et al.,*2011 [292]	IHC/ FISH			15/156 (10) p=not reported	Yes	LR+DM	κ value
Amir E *et al.,* 2012 [294]	IHC/ FISH	117 (16)	117 (40)	117 (10)	Yes	Prospective study DM	
Amir E *et al.,* 2012 [295]				Pooled analysis			
Jabbour M N *et al.,* 2012 [342]				Review			
Lindström LS *et al.,* 2012 [290]	IHC/ ICC/ FISH	149/459 (32) p<0.001	175/430 (41) p<0.001	15/104 (14) p=0.44	Yes	local & systemic relapse	McNemar
Markiewicz A *et al.,* 2012 [321]	IHC	7/40 (18) κ=0.6	12/41 (29) κ=0.3	1/34 (1) κ=0.9	Yes	LN	κ value

Abbreviations: ER= oestrogen receptor, PR= progesterone receptor, HER2= human epidermal growth factor receptor 2, IHC= immunohistochemistry, ICC= immunocytochemistry, FISH= fluoescent *in situ* hybridization, SISH =silver in situ hybridization, DM= distant metastasis, LR= local recurrecence, LN= lymph node metastasis, SN= sentinel node, NS= not specified

Conclusions

The main conclusions of the work presented in this thesis can be summarised as follows.

- The presence of DTCs in bone marrow does not identify patients with a poor prognosis, nor did it correspond to accepted prognostic markers. Previous reports have shown a correlation between DTCs in bone marrow and poor prognosis, but reported varying results according to correlation to standard prognostic markers. The technical challenges associated with the detection method, together with the invasive nature of sampling makes it unsuitable for clinical implementation (Paper I).

- Concordance between biomarkers in primary tumours and synchronous lymph node metastases was high in two cohorts of patients when ER, PR, Ki67 and HER2 were analysed separately (Papers II and IV).

- Significant discordance in ER, PR, Ki67 and HER2 was seen between primary tumours and relapses when analysed separately. Observations of discordances in ER, PR and HER2 have been reported previously, but the finding of discordances in the expression of Ki67 has not been reported previously (Paper IV).

- Classification into molecular subtypes according to the St Gallen recommendations revealed shifts in subtype in individual patients when comparing the primary tumour to lymph node metastases and relapses (Papers III and IV).

- The prognostic information obtained from the comparison of biomarkers in synchronous lymph node metastases and the primary tumour, could be of importance for the individual patient when classified into molecular subtypes. The shift in the subtype of metastatic lymph nodes may affect the choice of treatment based on the subtype of the primary tumour. The expression of HER2 and/or Ki67 in the metastatic node suggests treatment with chemotherapy, together with endocrine treatment for the affected patients.

- The finding of a low-risk subgroup of screening detected patients with a favourable prognosis based on molecular subtype of the metastatic node, could identify patients who would not gain any benefit from adjuvant treatment. Future studies including larger cohorts of patients are necessary to confirm these results. The finding that the molecular subtype of the synchronous lymph node metastases could have a prognostic influence could also provide more information on the molecular mechanisms involved in disease progression.

Future perspectives

Evaluation of tumour progression

Meta-analysis

The use of different statistical methods to analyse the concordance in the biomarker characteristics of primary tumours and corresponding lymph node metastases in published reports, leads to uncertainty in the interpretation of the results. A meta-analysis of the available and published data could clarify whether the discordances have any implications on patient outcome, and the retrieval of raw data and bundled statistical analysis could further improve our knowledge on the biological role of lymph node metastases.

Prospective trial – primary tumour and lymph node metastases...

The analysis of lymph node metastases and their role in tumour progression should be included in large prospective patient trials to provide insights into the biology of metastases, and potentially provide important clinical information with implications for patient treatment. The hypothesis that lymph node metastases develop from the most aggressive cell clone of the primary tumour and provide signals to DTCs for the development of distant metastases at different locations should be further evaluated, both clinically and molecularly. Prospectively recruiting breast cancer patients in Sweden for the analysis of HER2, ER, PR and Ki67 in both primary tumours and lymph node metastases would allow reliable prognosis based on the discordances for expression/amplification in four groups; concordance in expression/amplification, concordance in no expression/amplification, and a shift from primary tumour to lymph node metastases or the reverse, during five years´ follow-up. Randomisation to treatment with anti-HER2 therapy and/or endocrine treatment could follow if prognostic implications were found, suggesting a biological change in HER2 amplification or expression of ER. Simultaneous use of next-generation sequencing for genetic information from both locations could provide further knowledge on the relationship between primary tumours and lymph node metastases.

...and relapses, DTCs and CTCs

If patients develop distant metastasis, core-needle biopsies should be performed and assessed regarding both biological characteristics and next-generation sequencing.

The proposed influential model of cancer progression, suggesting that circulating cells with different degrees of maturity and characteristics have an impact on both the development of the metastases and the characteristics of primary tumours is challenging. Further evaluation of the importance of the presence of DTCs/CTCs in relation to molecular subtype in systemically spread disease could provide more information on prognostic information in individual cases. The inherence of HER2 and further characterisation of the DTCs/CTCs by next-generation sequencing in the primary tumour and lymph node metastases, may suggest interactions in tumour progression, and interactions between DTCs and their environment may provide knowledge on the molecular mechanisms behind the induction and maintenance of dormancy. Gene expression analysis of DTCs may provide further knowledge on the heterogeneity of metastatic potential inherent in DTCs and/or in their interplay with the microenvironment. The factors involved in the complex process of cancer cell dissemination are currently being evaluated in intense laboratory research, and may be of relevance not only for the dissemination process, but also for regulating the balance between tumour cells and the immune system of the patient.

Summary in Swedish

Bröstcancer är den vanligaste cancerdiagnosen hos svenska kvinnor, ca 8000 tumörer diagnosticeras årligen. Sjukdomen har god prognos, den totala femårsöverlevnaden är ca 90 %. Vid rekommendation om tilläggsbehandling till patienter med nyupptäckt bröstcancer utgår man från ett antal prognostiska (risk för återfall och död utan behandling) och prediktiva (möjlighet till bot med specifik tilläggsbehandling) faktorer. Patientens ålder, tumörstorlek, cancercellens grad av avvikelse från den normala bröstcellen, och spridning till lokala lymfkörtlar utgör tillsammans med mikroskopisk analys av tumörens enskilda uttryck av biomarkörer (östrogen- och progesteronreceptorer, proliferationsmarkör Ki67 och tillväxtproteinet HER2) sådana faktorer. Nyligen genomförda genetiska analyser av bröstcancerceller har indikerat att bröstcancersjukdomen kan delas in i olika grupper baserat på geners låga/höga uttryck av en kombination av biomarkörer. Genuttrycksanalys är tekniskt krävande och ekonomiskt kostsamt och används idag inte i kliniskt arbete. Man har istället valt att analysera de i klinisk rutin använda biomarkörerna då dessa har visat sig kunna bidra med likvärdig prognostisk information som genanalyser, när de bedöms i kombination. Nyligen rekommenderade en högt ansedd expertgrupp vilken kombination av biomarkörer som hade bäst vetenskapligt stöd, i den presenterade avhandlingen följer vi därför denna rekommendation - St Gallens molekylära subtypklassificering.

Återfall, fjärrmetastaser, kan uppkomma till följd av spridning av cancerceller från den primära tumören genom bildandet av nya tumörer i andra organ i kroppen. Dessa kan vid bröstcancer uppkomma flera år efter det primära insjuknandet trots att inga tecken på spridd sjukdom fanns från början. Återfall verkar vara en ”gömd” process i kroppen om vilken vi vet väldigt lite. Traditionellt har man utgått från antagandet att metastasers uppkomst är en stegvis process där primärtumören sprider cancerceller till andra delar av kroppen där de bildar metastaser. Därför är det viktigt att identifiera primärtumören tidigt, innan dessa celler sprids, såsom vid tidig diagnostik genom t ex mammografiscreening. Systembehandling av fjärrmetastaser har rekommenderats utifrån primärtumörens uttryck av prognostiska och prediktiva biomarkörer då man utgått från att dessa varit desamma som i metastaserna vid spridd sjukdom. På senare tid har denna teori ifrågasatts och man beskriver nu en parallell process där tumörceller tidigt, innan tumören är upptäckt, sprids via blodbanan och lymfsystemet ut i kroppen. De flesta av dessa spridda celler överlever inte men i en del fall utvecklas tumörcellen i andra organ och bildar då en metastas. Detta skulle betyda att de spridda omogna tumörcellerna genomgår en omvandling i andra organ och kanske uttrycker andra prognostiska och prediktiva biomarkörer vilket i sin tur skulle medföra att annan behandling skulle kunna vara aktuell vid lymfkörtelpositiv sjukdom, eller vid senare uppkomna återfall av sjukdomen.

Den tidiga spridningen av tumörceller går inte att se genom någon idag känd diagnostisk metod, t ex på röntgenbilder. Det har föreslagits att dessa spridda tumörceller vilar i benmärgen, som skulle fungera som en reservoar. Genom analys av benmärgsbiopsi för identifiering av förmodade tumörceller, har man i studier funnit att förekomst av dessa celler identifierar patienter med större risk för återfall. Analysmetoden för identifiering av tumörceller har dock inte säkert fastställts, även om det idag finns ett standardiserat protokoll. Vår undersökning av ca 500 patienter påvisade ingen prognostisk information av förekomsten av tumörceller i benmärg hos patienter som genomgick benmärgsbiopsi i samband med operation av bröstcancer. Metoden för identifiering och fastställande att det är tumörceller man analyserar, måste standardiseras och provtagningen är desssutom smärtsam för patienterna. Utifrån detta kan man sammanfatta att det är för tidigt att använda benmärgsbiopsi för prognostisk information i kliniskt arbete. Pågående studier där man letar efter cirkulerande tumörceller i blod och relaterar till prognos och

behandlingseffekt kan sannolikt bidra till mer kunskap om dessa enstaka spridda tumörcellers betydelse, till mindre obehag för patienterna då det är ett blodprov i armen som ligger till grund för analysen.

En av de enskilt starkaste prognostiska faktorerna är förekomst av spridning av tumörceller till armhålans lymfkörtlar, finns det tumörväxt (lymfkörtelmetastaser) i dessa vid den primära kirurgin opereras lymfkörtlarna i armhålan bort samtidigt med brösttumören. Sjukdomen ses då fortfarande som botad men med högre risk för återfall i bröstcancersjukdomen. Vi analyserade uttryck av biomarkörer i tumörceller från primärtumörer och tumörceller från samtidiga lymfkörtelmetastaser hos samma patient med primär bröstcancer, i syfte att identifiera eventuella skillnader och relatera detta till prognos. Vi fann hög samstämmighet vid analys av enskilda biomarkörer mellan primärtumör och lymfkörtelmetastas i två olika patientgrupper omfattande ca 400 patienter.

Vid analys av en kombination av biomarkörer enligt St Gallens molekylära subtypklassificering visade det sig dock, att för de enskilda patienter som klassificerades till olika molekylära subtyper i primärtumör och lymfkörtelmetastas, hade uttrycket i lymfkörteln, och inte i primärtumören, den mest avgörande prognostisk informationen. Rekommendation om behandling baserat på lymfkörtelns molekylära subtypklassificering skulle för dessa patienter innebära tillägg av kemoterapi utöver endokrin behandling. Vi identifierade också en grupp av patienter som det gick mycket bra för, som kanske inte skulle ha nytta av tilläggsbehandling, trots förekomst av riskfaktorer, vars tumörer diagnosticerats inom ramen för hälsokontroller med mammografi (screening), där den molekylära subtypen i lymfkörtelmetastasen gav prognostisk information. Dessa patienter hade en mycket gynnsam biomarkörprofil i lymfkörteln, oberoende av uttrycket i primärtumören vilket tycks ha medfört en gynnsam prognos Det är få patienter och resultaten behöver bekräftas i större studier för att avgöra om man i framtiden bör analysera biomarkörutryck i lymfkörteln för prognostisk information men det är svårt då endast en tredjedel av patienterna drabbas av metastasering till lymfkörtlar och ännu färre drabbas av fjärrmetastasering i ett senare skede av sjukdomen.

Skillnader i biomarkörutryck mellan brösttumören och, hos samma patient, senare uppkommet återfall i sjukdomen har påvisats i tidigare studier. Vi kunde också identifiera förändrad biologi då vi jämförde uttryck av enskilda biomarkörer mellan brösttumör och återfall i form av fjärrmetastas till lunga, lever, skelett och lokalt i bröstområdet, med signifikant förändrad biomarkörprofil i fjärrmetastasen indikerande att återfallet är av en mer aggressiv typ än primärtumören. Patienter med återfall i bröstcancersjukdomen kan således ha mer nytta av behandling som är rekommenderad utifrån analys av vävnadsprov från återfallet, än analys av uttrycket av biomarkörer i brösttumören, för sjukdomskontroll. Fynden i vår studie ger stöd för den rekommendation om vävnadsprovtagning från återfall som är inkluderat i de Nationella riktlinjerna för behandling av bröstcancer sedan 2012.

Med utökad kunskap om cancercellens utveckling och vilka molekylära processer som föregår återfall i sjukdomen, tillsammans med analys av biomarkörer i såväl primärtumör som lymfkörtelmetastas kan vi förhoppningsvis i större utsträckning skräddarsy tilläggsbehandling för enskilda patienter i framtiden.

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RESEARCH ARTICLE Open Access

Analysis of and prognostic information from disseminated tumour cells in bone marrow in primary breast cancer: a prospective observational study

Anna-Karin Falck[1,2], Pär-Ola Bendahl[3], Christian Ingvar[1,4], Jorma Isola[5], Per-Ebbe Jönsson[2], Pia Lindblom[1,4], Kristina Lövgren[3], Karin Rennstam[3], Mårten Fernö[3] and Lisa Rydén[1,4*]

Abstract

Background: Disseminated tumour cells (DTCs) in the bone marrow of patients with breast cancer have been identified as an independent predictor of poor prognosis in patients with non-metastatic disease. This prospective study aimed to evaluate the presence and prognostic value of DTCs in the bone marrow of female patients with primary breast cancer.

Methods: Between 1999 and 2003, bone marrow aspirates were obtained from patients at the time of surgery for primary invasive breast cancer. DTCs in bone marrow were identified using monoclonal antibodies against cytokeratins for detection of epithelial cells. The detection of DTCs was related to clinical follow-up with distant disease-free survival (DDFS) and breast cancer-specific survival as endpoints. Bone marrow aspirates from adult healthy bone marrow donors were analysed separately.

Results: DTCs were analysed in 401 patients, and cytokeratin-positive cells were found in 152 of these (38%). An immunofluorescence (IF) staining procedure was used in 327 patients, and immunocytochemistry (IC) was performed in 74 patients. The IF-based method resulted in 40% DTC-positive cases, whereas 30% were positive using IC ($p = 0.11$). The presence of DTCs in bone marrow was not significantly related to patient or tumour characteristics. The presence of DTCs was not a prognostic factor for DDFS (IF: hazards ratio [HR], 2.2; 95% confidence interval [CI], 0.63–2.2; $p = 0.60$; IC: HR, 0.84; 95% CI, 0.09–8.1; $p = 0.88$). Significant prognostic factors were lymph node metastases, oestrogen receptor positivity, Nottingham histological grade, and tumour size using Cox univariate analysis. The analyses were positive for epithelial cells in bone marrow from adult healthy donors in 19 (25%) samples.

Conclusions: The detection of DTCs in bone marrow in primary breast cancer was previously shown to be a predictor of poor prognosis. We were not able to confirm these results in a prospective cohort including unselected patients before the standard procedure was established. Future studies with a standardised patient protocol and improved technique for isolating and detecting DTCs may reveal the clinical applications of DTC detection in patients with micrometastases in the bone marrow.

Keywords: Breast cancer, Disseminated tumour cells, Cytokeratin-positive cells, Micrometastases, Prognosis

* Correspondence: lisa.ryden@med.lu.se
[1]Department of Surgery, Clinical Sciences, Lund University, Lund SE-22185, Sweden
[4]Department of Surgery, Skåne University Hospital, SE-22185, Lund, Sweden
Full list of author information is available at the end of the article

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Background

Breast cancer remains the most common cancer diagnosis among women in Sweden today, with an incidence of 7400 patients per year. It is generally associated with a good prognosis; more than 85% of Swedish patients are free from recurrence of the disease at the 5-year follow-up because of early detection combined with extended adjuvant therapy [1].

Adjuvant treatment is delivered to eradicate micrometastatic spread at the time of diagnosis and thus minimise the risk of subsequent clinically overt metastasis from the micrometastatic stage. Adjuvant treatment is tailored to a prognostic profile including validated prognostic factors (age, nodal status, tumour size, Nottingham histological grade [NHG], and human epidermal growth factor receptor 2 [HER2] amplification) and predictive factors (hormone receptor status, HER2) [2]. Metastatic lymph node involvement (N+) is still considered to have the strongest impact of all accepted prognostic factors. However, approximately 30% of patients with no sign of metastatic involvement of the lymph nodes (N0) relapse and suffer from metastatic disease [3,4]. In contrast, 40% of N+ patients survive 10 years or more without recurrence [5,6]. The heterogeneity of breast cancer challenges the search for further prognostic markers that will provide a more direct measure of the disease's metastatic potential. Recognition and understanding of the metastatic process includes investigation of the molecular mechanism of early spread of tumour cells.

Micrometastatic spread to bone marrow by disseminated tumour cells (DTCs), defined as cytokeratin (CK)-positive cells, occurs in up to 40% of patients with primary breast cancer at the time of diagnosis [7-10]. DTCs seem to be unrelated to lymphatic spread and occur in both N0 and N+ disease [7-9], and no distinct pattern is found in relation to standard prognostic factors [8,9,11,12]. The prognostic influence of DTCs in bone marrow has been evaluated by several groups over the last 30 years with the aim of finding a tool to detect micrometastatic disease at the time of diagnosis [10,13,14]. There has been an increasing acceptance of DTCs as an independent marker of a poor prognosis in breast cancer after the publication of a pooled analysis [7], and DTCs are now included in the new American Joint Committee on Cancer classification as a diagnostic criteria for micrometastatic spread. Early detection of these epithelial cells may help to identify patients with micrometastatic disease who would benefit from adjuvant treatment to prevent further metastatic disease. However, aspiration and analysis of bone marrow for detection of DTCs as a prognostic tool is not yet a routine clinical procedure [15] and is not recommended by the American Society of Clinical Oncology [16]. Aspiration may be associated with pain and discomfort for the patient, particularly if performed repeatedly to monitor treatment. Furthermore, the increasing acceptance of DTCs as a prognostic factor is based on studies using different CKs as well as membrane antibodies to detect epithelial cells [7,15]. Comparisons among different detection methods were performed [17] before standardised guidelines were published [15]. These comparisons showed difficulties in interpreting CK-positive cells as tumour cells and recommended the use of markers that allow discrimination between CK-positive cells of haematopoietic and non-haematopoietic origin. To ensure validation of the method used, it is important to have tumour cell samples as positive controls, specific negative controls, and bone marrow samples from healthy individuals. Results from clinical studies will vary until the technique has been standardised and the optimal dilution with antibodies has been identified.

The aim of the present study was to detect the presence of DTCs and analyse the prognostic implications of DTCs in bone marrow at the time of diagnosis in a prospective cohort of patients with primary breast cancer. An additional aim was to further stratify the cohort according to lymph node status to enable the clinical information obtained in N+ and N0 patients to be studied separately.

Methods

Patients

This study included patients (median age, 58 years) diagnosed with primary breast cancer in the South Swedish Health Care Region between June 1999 and May 2003 as well as patients diagnosed in Lund, Landskrona and Helsingborg. The patients underwent bone marrow aspiration from the sternal crest under anaesthesia at the time of primary surgery. The study was approved by the ethics committee at Lund University, and written informed consent was obtained from all included patients (LU699-09, LU75-02).

Patient and tumour characteristics are given in Table 1. Patients underwent either mastectomy or breast-conserving therapy based on preoperatively identified characteristics and staging. A sentinel node biopsy was performed in patients with no sign of axillary node engagement before surgery, followed by axillary lymph node dissection (level I and II) at the time of either the primary operation or a second operation if histopathological analysis showed metastatic involvement in the sentinel node biopsy. If node involvement was known preoperatively, axillary lymph node dissection was performed at the time of the primary surgery.

Adjuvant therapy was recommended according to clinical standards following Regional Guidelines, and included chemotherapy for N+ premenopausal women and N+ postmenopausal women with oestrogen receptor-negative tumours (ER-) (n = 65, 16%). Endocrine therapy

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Table 1 Patient- and tumor characteristics

Characteristics	No of patients (%)
All patients	401 (100)
Age	
Median (range)	58 (29–91)
< 50 years	80 (20)
≥ 50 years	321 (80)
Mode of detection	
Screening detected	167 (42)
Clinical signs	232 (58)
Unknown	2
Tumor size	
≤ 20mm	263 (66)
> 20mm	136 (34)
Unknown	2
Node status	
N0	233 (60)
N+	157 (40)
Unknown	11
NHG	
1	77 (20)
2	221 (56)
3	94 (24)
Unknown	9
ER status	
Positive	312 (80)
Negative	77 (20)
Unknown	12
PR status	
Positive	242 (62)
Negative	147 (38)
Unknown	12
Surgery breast	
Mastectomy	157 (39)
Breast concerving surgery	243 (61)
Unknown	1
Surgery axilla	
Axillary dissection	247 (63)
Sentinel node biopsy	142 (37)
No Surgery (incl in clinical trial)	12
Adjuvant treatment	
No adjuvant treatment	137 (34)
Only Chemotherapy	65 (16)
Only Endocrine therapy	197 (49)
Missing	2
Radiotherapy	
Breast	198 (49)
Locoregional lymph nodes	35 (9)
Breast + locoregional	39 (10)

Abbreviations: N0= node negative, N+= node positive, NHG= Nottingham histological grade, ER= oestrogen receptor, PR= progesterone receptor.

was delivered to 197 patients (49%); premenopausal patients with oestrogen receptor-positive (ER+) tumours and no nodal engagement received tamoxifen, and postmenopausal women with ER + tumours received tamoxifen or aromatase inhibitors regardless of nodal status. Chemoendocrine therapy was given to 22 patients; 137 patients had no adjuvant therapy. Radiotherapy to the breast was given after breast-conserving surgery (50 Gy) (n = 198, 49%) and locoregional radiotherapy was delivered if four or more axillary lymph nodes were metastatic (n = 35, 9%). A combination of radiotherapy to the breast and to the locoregional lymph nodes was delivered in 39 patients (10%).

The patients were followed by annual clinical examination and mammography. Further clinical and radiological examinations were performed when clinical signs indicated recurrence of the disease. After 5 years of follow-up, all clinical and histopathological results concerning tumour grading and staging, as well as reports of events, were abstracted from individual patient's charts. The median follow-up for patients without any breast cancer-related event was 61 months. For patients for whom no cause of death was registered, we received information from the Swedish Register of Causes of Death (Central Statistics Office). The inclusion criteria were re-evaluated.

The original cohort included 569 patients, 544 of whom were followed according to the schedule. The exclusion criteria were no standardised surgical treatment (laser, n = 1), neoadjuvant treatment (n = 11), and local recurrence (n = 3) and the sample volume was inadequate in 36 patients. The analysis was not performed in 117 patients due to change in research strategy at our laboratory. The final cohort thus included 401 patients.

Bone marrow

Bone marrow aspirates were obtained from the sternum at two sites by needle aspiration while the patient was under general anaesthesia at the time of primary surgery. The samples were transported to the research laboratory at room temperature and prepared within 1 h. Mononuclear cells were separated by Ficoll density gradient centrifugation (Ficoll-Paque™ PLUS, Cat. no. 17-1440-03; Amersham Biosciences AB, Uppsala, Sweden) and then washed and counted before 1.5 to 2.0 × 10^6 cells were

placed on each glass slide. Two microscope slides were prepared for each patient, one from each site at the sternum.

Immunofluorescence and immunocytochemical analysis

In 327 patients, an immunofluorescence (IF) staining procedure was used for detection of DTCs, including staining with antibodies against CKs (Pan-CK Ab-2; Neomarkers, Union City, CA, USA) 4, 5, 6, 7, 8, 10, 13, 14, 15, 16, 18, and 19 and visualised by IF using an IF microscope (Axioplan 2; Zeiss, Jena, Germany). The cytospins were incubated with the pan-CK antibody and a secondary fluorescein isothiocyanate-conjugated goat antimouse antibody (Zymed Laboratories Inc., San Francisco, CA, USA) and finally counterstained with 4,6-diamidino-2-phenylindol in mounting media using Vectashield (Vector Laboratories, Burlingame, CA, USA). The procedure changed when a new CK antibody kit (AE1/AE3; Daco, Glostrup, Denmark) was introduced with antibodies against CKs 1, 2, 3, 4, 5, 6, 7, 8, 10, 13, 14, 15, 16, and 19. For the IC method, the cells were fixed in buffered formaldehyde (4%) and thereafter pre-treated in citrate buffer (pH 6) in a microwave oven for 20 minutes. The cytokeratin antibody kit (AE1/AE3, Daco, Glostrup, Denmark) was used as primary antibodies against CK1,2,3,4,5,6,7,8,10,13,14,15,16 and 19. The EnVision™ (Dako, Glostrup, Denmark) was used as detection system, NovaRed™ (Vector Laboratories, Immunkemi AB, Sollentuna Sweden) for the visualisation and Mayers hematoxylin for nuclear staining. This enables direct immunocytochemical evaluation (IC) of the cells and analysis by light microscope (Olympus CX41, Tokyo, Japan) in 74 patients.

The presence of DTCs was defined as CK-positive cells with DTC morphology (irregular staining of the cytoplasm) with an enlarged nucleus, irregularity of the nucleus, a high nuclear-to-cytoplasmic ratio, CK staining of the cytoplasm at the periphery of the cell causing a ring-like appearance, and fluorescence-positive intact cells (IF technique) according to Fehm [8]. For the IC evaluation, we followed the criteria proposed by Borgen, who used the same antibody [18,19]. The criteria include moderate to strong staining intensity for the entire cytoplasm in mononuclear cells lacking haematopoietic characteristics [18,19]. The evaluation was performed by two observers independently. All specimens were considered either positive or negative when one or more CK-positive cell was diagnosed. DTCs detected by IF are illustrated in Figure 1 and by IC in Figure 2.

As a positive control for CK immunostaining, we used the breast cancer cell line MCF7 spiked into blood from healthy volunteers. The cell line was kindly provided by Prof. Stina Oredsson at Lund University. The slides for negative controls were prepared in parallel with those stained with anti-CK by omitting the primary antibody, and thus contained the same number of cells (1.5 to 2 × 10^6). No positive results were observed in the negative controls.

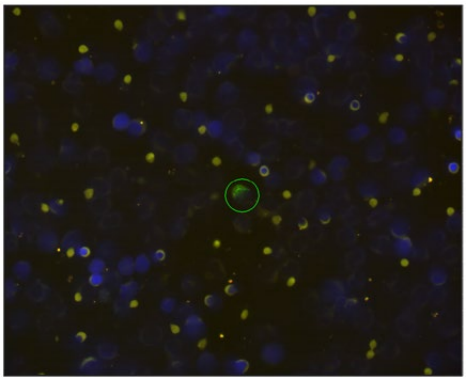

Figure 1 Cytokeratin staining of DTCs isolated from bone marrow by immunofluorescence.

Bone marrow aspirates from 76 adult healthy bone marrow donors were analysed separately.

Statistical analysis

Distant disease-free survival (DDFS) and breast cancer-specific survival (BCSS), measured from the month of surgery to the last clinical follow-up or any breast cancer-related event, were used as endpoints. DDFS

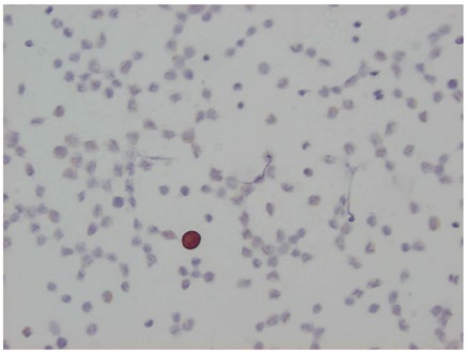

Figure 2 Cytokeratin staining of DTCs isolated from bone marrow by immunocytochemistry.

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included distant metastases to the skeleton, brain, lung, and liver.

Differences in the distribution of clinical data and tumour characteristics between the DTC+ and DTC- patients were evaluated using the X^2 test. DDFS and BCSS were estimated according to the Kaplan-Meier method, and the log-rank test was used to compare survival in different subgroups. The Cox proportional hazards model was fitted to explore the effects of tumour size, lymph node status, ER and progesterone receptor content, age, and NHG on BCSS and DDFS. Proportional hazards assumptions were checked graphically.

P-values of <0.05 were considered statistically significant. The statistical software package Stata 11.1 (Stata Corp., College Station, TX, USA) was used for all statistical calculations.

Results

Detection of micrometastases

Analysis of DTCs was performed in 401 patients, and CK-positive cells were found in 152 of these (38%). The IF-based method resulted in 40% DTC-positive cases, whereas 30% were positive using the IC method. However, there was no statistically significant difference between the detection rates of the two methods (p = 0.11). The number of positive cells was not taken into account.

Characteristics of DTCs and patients

Patient and tumour characteristics are listed in Table 1. The relationship between DTC detection in bone marrow and clinicopathological variables in the study cohort is presented in Table 2. There was no statistically significant correlation between the presence of DTCs and the characteristics, regardless of the method used for DTC detection.

For survival analysis, we initially included all 401 patients for whom DTC analysis was performed. In the cohort of patients analysed with the IF staining procedure, the detection of DTCs in bone marrow was not related to either DDFS (log-rank test, p = 0.60) (Figure 3) or BCSS (log-rank test, p = 0.37) (Figure 4). Stratifying the cohort according to the method used for the detection of DTCs resulted in similar results using Cox univariate analysis (Table 3). In Cox univariate analysis of DDFS, the following clinicopathological variables were related to prognostic information: lymph node metastases (+ *vs.* -: hazard ratio [HR], 5.5; 95% confidence interval [CI], 2.7–11), tumour size (>20 *vs.* ≤20 mm: HR, 4.9; 95% CI, 2.6–9.4), NHG (3 *vs.* 1: HR, 20; 95% CI, 2.7–147), ER (+ *vs.* -: HR, 0.39; 95% CI, 0.21–0.72), and PR, progesterone receptor (+ *vs.* -: HR, 0.43; 95% CI, 0.24–0.79). In a Cox proportional hazards model for DDFS, lymph node metastases (+ *vs.* -: HR, 3.6; 95% CI, 1.7–7.4), tumour size (>20 *vs.* ≤20 mm: HR, 2.5; 95% CI, 1.1–5.1), and NHG (3 *vs.* 1: HR, 8.7; 95% CI,

Table 2 Patient`s and tumor characteristics in relation to presence of DTC in bone marrow

Characteristics	Patients analyzed by IF	No DTC in bone marrow N (%)	DTC in bone marrow N (%)	p-value*	Patients analyzed by IC	No DTC in bone marrow N (%)	DTC in bone marrow N (%)	p-value*
Tumor size								
≤ 20 mm	203	118 (58)	85 (42)	0.3	60	42 (70)	18 (30)	1.0
> 20 mm	123	78 (63)	45 (37)		13	9 (69)	4 (31)	
Node status								
N0	184	113 (61)	71 (39)	0.5	49	35 (71)	14 (29)	0.7
N+	133	77 (58)	56 (42)		24	16 (67)	8 (33)	
NHG								
1	60	30 (50)	30 (50)		17	13 (76)	4 (24)	
2	181	110 (61)	71 (39)	0.12	40	24 (60)	16 (40)	0.7
3	80	51 (64)	29 (36)		14	12 (86)	2 (14)	
ER status								
Positive	252	145 (58)	107 (42)	0.12	60	41 (68)	19 (32)	0.8
Negative	66	45 (68)	21 (32)		11	8 (73)	3 (27)	
PR status								
Positive	189	110 (58)	79 (42)	0.5	53	37 (70)	16 (30)	0.8
Negative	129	80 (62)	49 (38)		18	12 (67)	6 (33)	

* X^2-test for two categories and X^2-test for trend for three ordered categories.
Abbreviations: IF= immunofluorescence, IC=immunocytochemistry, DTC= disseminated tumor cells, N0= node negative, N+=node positive, NHG= Nottingham histological grade, ER= oestrogen receptor, PR= progesterone receptor.

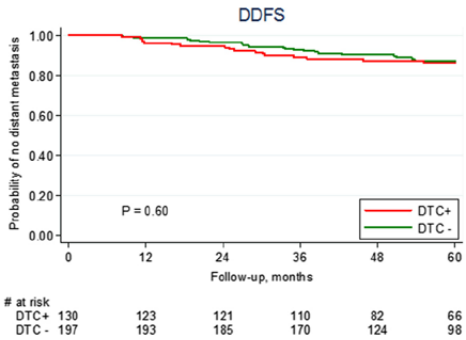

Figure 3 Distant disease-free survival (DDFS) in relation to presence of DTC.

1.1–69) remained independent prognostic factors (Table 3). The results for BCSS were similar (data not shown).

Subgroup analysis

When the cohort was stratified according to lymph node status, Cox univariate analysis of N0 patients showed that the presence of DTCs had no statistically significant effect on prognosis in terms of DDFS (DTC+ *vs.* DTC-: HR, 2.7; 95% CI, 0.72–9.1; p = 0.14). In the N+ group of patients, the presence of DTCs had no significant effect on DDFS (DTC+ *vs.* DTC-: HR, 0.84; 95% CI, 0.42–1.72; p = 0.6). Although the presence of DTCs seemed to have a more pronounced effect in the N0 subgroup, the interaction between lymph node status and the presence of DTCs was not significant (p = 0.13). The results were similar in the subgroups of patients in whom DTCs were detected by IF and IC (data not shown).

The bone marrow from healthy adult bone marrow donors was analysed using both methods. The analyses were positive for epithelial cells in bone marrow in 19 (25%) samples, negative in 53 (70%), and inadequate or ambiguous in 4 (5%).

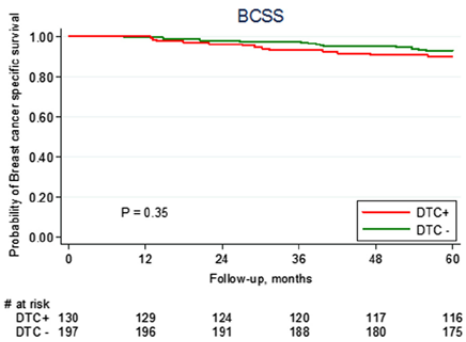

Figure 4 Breast cancer-specific survival (BCSS) in relation to presence of DTC.

Discussion

In the present study, the detection of DTCs in bone marrow in female patients with primary breast cancer at the time of diagnosis had no prognostic impact. Although most publications report that detection of DTCs in primary breast cancer is an independent prognostic factor for recurrence and death, the clinical significance of micrometastases in bone marrow remains controversial. The American Society of Clinical Oncology did not advocate it as a prognostic marker for clinical use because of insufficient data [16], and several concerns have been raised regarding the standardisation of detection using monoclonal antibodies against CKs. The standardisation of the detection method is based on IC using a strict protocol for negative controls and morphological evaluation of stained mononuclear cells. The present study included patients before the standard protocol was published [15], and the data are mainly derived from detection by an IF staining procedure that was not included in the published meta-analysis and is not advocated by the consortium [7,15].

The detection of DTCs in bone marrow has been identified in several publications as an independent predictor of poor outcome in patients with non-metastatic breast cancer disease [14,20,21]. The level of evidence increased when a pooled analysis of 4703 patients with breast cancer was published, assessing the poor prognostic significance of the presence of DTCs in the bone marrow at the 10-year follow-up [7]. The pooled analysis, which included a large patient cohort, also enabled the analysis of subgroups with statistical power. Interestingly, the largest difference in outcome for patients with DTC+ *vs.* DTC- disease was in the subgroup in which all patients received adjuvant systemic therapy. Although there was a significantly higher risk of relapse in patients with DTC+ disease compared with patients with DTC- disease in the subset of patients who did not receive adjuvant systemic therapy (n = 1036, 22%), the effect was relatively small, but still significant (5-year follow-up: incidence ratio, 2.0; 95% CI, 1.2–3.4). Aspects to consider when estimating the results of these early reports are the heterogeneity of the patients included and different methods and techniques used to determine bone marrow dissemination. However, more recent studies performed with standardised methods of detection also propose a prognostic value of DTCs in bone marrow [22-24]. Molloy et al. found clinical significance of DTCs in bone marrow in terms of BCSS (HR, 2.1; p = 0.003), but not in metastasis-free survival (HR, 1.5; p = 0.127) [23]. Giluiano et al. reported that DTCs were present in 104/3413 (3.0%) patients and was associated with

Table 3 Cox univariate and multivariate analysis of distant disease-free survival

	Univariate analysis (n ≤ 401)			Multivariate analysis (n = 377)		
Variable	**HR***	**95% CI**	**p-value**	**HR****	**95% CI**	**p-value**
DTC status (IF and IC) (n=401) DTC+ *vs* DTC-	1.2	0.66-2.2	0.55			
DTC status (IF) (n=327) DTC+ *vs* DTC-	1.2	0.63- 2.2	0.60			
DTC statuc (IC) (n=74) DTC+ *vs* DTC-	0.84	0.09-8.1	0.88			
Age per year	0.99	0.97-1.02	0.61			
Node status N+ *vs* N0	5.5	2.7- 11	< 0.001	3.4	1.6- 7.2	0.001
Tumor size > 20 mm *vs* ≤ 20 mm	4.9	2.6-9.4	< 0.001	2.5	1.2- 5.2	0.01
NHG status NHG 2 *vs* NHG 1	6.9	0.92-52	0.06	4.9	0.65-37	0.12
NHG 3 *vs* NHG 1	20	2.7- 147	0.004	8.7	1.1- 70	0.04
ER status ER+ *vs* ER-	0.39	0.21-0.72	0.003	0.85	0.38- 1.9	0.7
PR status PR+ *vs* PR-	0.43	0.24- 0.79	0.007	0.67	0.33- 1.4	0.3

* No significant deviations from proportional hazards (Schoenfeld's test).
* *P=0.05 in Schoenfeld's global six degree-of-freedom test of proportional hazards.
Abbreviations: IF= immunofluorescence, IC=immunocytochemistry, HR= hazard ratio, CI= confidence interval, DTC= disseminated tumor cells, N0= node negative, N+= node positive, NHG= Nottingham histological grade, ER= oestrogen receptor, PR= progesterone receptor.

decreased overall survival in univariate analysis, but this did not reach clinical validity in multivariate analysis. They concluded that bone marrow aspiration was not recommended in routine clinical practice for patients with early breast cancer without an improved technique for isolation and detection of occult tumour cells in bone marrow [22]. Solá et al. found a higher frequency of DTCs in a subgroup of patients who experienced breast cancer-related events (13%), but the results did not reach statistical significance because of low power with few events [24]. Future studies will show whether there is a predictive value in the choice of chemotherapy regarding repeated sampling of DTCs during progression of the disease. The persistent presence of DTCs during follow-up was shown to be associated with a significantly increased risk for relapse and death in breast cancer disease in a recently published pooled analysis [25].

The biological tumour characteristics and the clinical stage and follow-up data in the present study are in accordance with those of previous publications in the field [8,9,11,12,26]. The presence of DTCs in 152 of 401 (38%) patients in this study is in line with what previous authors have reported, with a diagnostic rate of 20% to 40% regardless of nodal status [7-9]. Although most authors report a positive association with pathological stage and tumour grade, other investigators have failed to detect any correlation of DTCs with standard prognostic markers [8,9,11,12,27]. The lack of correspondence between accepted prognostic markers and the presence of DTCs in bone marrow spans T1, T2, and T3 disease, and it shows no correlation with nodal involvement [12].

The present study involved a prospective cohort of women diagnosed with early breast cancer from the era of screening mammography. This may have had an effect on the present cohort, which was weighted toward a 'good' prognostic profile with a favourable prognosis and few events: small tumours (T1) in 66% of included patients, N0 disease in 61%, and ER-positive tumours in 80%. However, the DTC detection rate is not necessarily dependent on the clinical stage or tumour profile and is still reported to be around 30% to 40% of included patients in recently published studies [8,9,12]. Adjuvant treatment with chemotherapy was given to only 65 of 401 patients. The subgroup analysis of N0 patients in the present cohort gives the impression that DTCs may be of some importance for these patients compared with N+ patients. However, the few events reported to date indicate that the study lacks the power to allow the detection of any significant effect of DTCs in the N0 subgroup. A longer follow-up period will be necessary to fully elucidate this issue.

Standardisation of the detection of DTCs has been widely discussed, and the main limitation of the present study is that it was launched before a standardised method was established. Using antibodies against different CKs to detect epithelial cells in mesenchymal bone marrow is considered to classify these cells as being of tumour origin and thus micrometastases [28]. However, conclusive studies comparing techniques and optimal antibody dilution are not yet available from the same cohort of patients. In the present study, a switch was made from analysing the samples with IF (n = 327) to IC (n = 74), a method with growing acceptance at the time, and with the use of published standards for handling of the samples, enabling a more strict morphological evaluation [18,19]. No differences were found between the subset of patients analysed using the different methods when comparing tumour and patient characteristics or survival. Although there was no statistically significant difference in detection rate, DTCs were diagnosed in 40% of the patients using the IF technique compared

with 30% of the patients with IC. We used negative controls for all analysed samples irrespective of the method used. Furthermore, we included bone marrow from 76 healthy adults, and the analyses were positive for epithelial cells in the bone marrow of 19 (25%) of the samples. This illustrates the lack of standardisation of the assays used. Because CK-positive samples were found among the healthy donors in the present study, we cannot exclude the possibility that some of the DTC+ breast cancer patients were incorrectly classified. Although it was not our initial intention, we also tested higher cut-offs for defining DTC positivity without finding any association with prognosis (data not shown).

Not all studies included in the meta-analysis reported whether samples from healthy donors were analysed, and in several of them, control samples were sparse (<50 samples) [7]. Furthermore, false-positive controls have been observed in the use of epithelial-specific markers (CKs), incorrectly classifying haematopoietic cells (HCs) as tumour cells [18,29]. Epithelial-positive rates in bone marrow have been reported in patients without cancer, even after morphological criteria were applied (5% and 30% in two different cohorts) [18,29]. However, the findings in the present study, including 25% positive cases among adult healthy bone marrow donors, raise concerns about the specificity of the method used. One possible mechanism for false-positive staining of HCs in bone marrow is a direct reaction between specific HCs and alkaline phosphatase (AP) [18] using the chromogenic visualisation technique. Staining bone marrow with AP alone gave a strong positive reaction, and further analysis identified these cells as possible plasma cells/pre-B cells [18]. In addition, HCs can express CKs illegitimately and thus stain with anti-CK, but strict morphological evaluation often reveals the characteristics of true DTCs [18]. Morphological evaluation of CK-positive cells is thus crucial for the correct diagnosis of DTCs, and in the present study, we applied the morphological criteria for diagnosis of DTCs analysed by IF and IC [8,18].

A future topic of interest in the field of DTCs is the investigation of the molecular characteristics in individual DTCs with the purpose of finding a marker to monitor treatment susceptibility [8,30]. Molecular characteristics are often diagnosed by the IF-based technique, which is less standardised than the preferred IC-based method. Furthermore, molecular investigation of matched pairs of primary tumours, metastasis, and DTCs may give information about the progression of cancer disease [28]. Of interest is that molecular subclasses of breast cancer tend to express different families of CKs, which can be translated into different detection rates throughout the molecular subclasses when only one antibody against CKs is used [31]. Diagnosis of DTCs during tumour progression must thus consider using multiple antibodies against different CKs to correctly diagnose DTCs with a molecular profile other than that of the primary tumour.

Conclusions

The present study did not confirm the results of previous publications that suggested that DTCs in bone marrow are an independent prognostic marker of poor prognosis in primary breast cancer. A more standardised detection method of DTC in bone marrow has been proposed since the start of the present study. The invasive nature of the diagnostic procedure of DTCs and technical challenges linked to the method makes the technique unsuitable for inclusion in the standard care of primary breast cancer.

Abbreviations

AP: Alkaline phosphatase; BCSS: Breast cancer-specific survival; CI: Confidence interval; CK: Cytokeratin; DDFS: Distant disease-free survival; DTC: Disseminated tumour cell; ER: Oestrogen receptor; HC: Haematopoietic cell; HER2: Human epidermal growth factor receptor 2; HR: Hazard ratio; IC: Immunocytochemistry; IF: Immunofluorescence; N+: Metastatic lymph node involvement; N0: No sign of metastatic lymph node involvement; NHG: Nottingham histological grade; PR: Progesterone receptor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AKF was responsible for data acquisition and drafting of the manuscript. POB constructed the database and performed the statistical analysis, CI initiated and designed the study, JI set up the immunofluorescence technique, PEJ was responsible for including patients, PL took part in data acquisition, KL carried out the preparation of the samples and the analysis of them, KR carried out the preparation of the samples and the analysis of them, MF initiated and designed the study, LR was responsible for patients follow-up, data acquisition, survival analysis and drafting of the manuscript. All authors read and approved the final version of the manuscript.

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Author details

[1]Department of Surgery, Clinical Sciences, Lund University, Lund SE-22185, Sweden. [2]Department of Surgery, Hospital of Helsingborg, Helsingborg SE-25187, Sweden. [3]Department of Oncology, Clinical Sciences, Lund University, Lund SE-22185, Sweden. [4]Department of Surgery, Skåne University Hospital, SE-22185, Lund, Sweden. [5]Institute of Medical Technology, University of Tampere, Tampere FI-33014, Finland.

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Paper II

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Does Analysis of Biomarkers in Tumor Cells in Lymph Node Metastases Give Additional Prognostic Information in Primary Breast Cancer?

Anna-Karin Falck · Mårten Fernö · Pär-Ola Bendahl · Lisa Rydén

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Abstract

Background Prognostic and treatment-predictive biomarkers in primary breast cancer are routinely analyzed in the primary tumor, whereas metastatic tumor cells in lymph node metastases are not characterized. The present study aimed to define the concordance between biomarkers in matched pairs of breast cancers and lymph node metastases and to relate their expression to clinical outcome.

Methods Patients with primary breast cancer treated with adjuvant tamoxifen for 2 years were included. A tissue microarray of primary tumors and lymph node metastases was constructed, and estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki67 were analyzed immunohistochemically in 262, 257, 104, and 101 patients, respectively. Five years' distant disease-free survival (DDFS) was used as the primary end point.

Results The concordance for biomarker expression in primary tumors and corresponding lymph node metastases was 93% for ER, 84% for PR, 97% for HER2, and 85% for Ki67. The discordant cases for HER2 status were all negative in the tumor but positive in the node. ER positivity was a significant independent predictor of improved 5-year DDFS when analyzed in the primary tumor as well as in the lymph node metastases. Ki67 positivity analyzed in both locations correlated with shortened DDFS. HER2 positivity at both locations was an indicator of early relapse.

Conclusions The concordance for the biomarkers analyzed in matched pairs of primary tumors and lymph node metastases was high. Moreover, survival analyses showed that the expression of biomarkers in lymph node metastases can provide prognostic information when no analyses of the primary tumor can be done. Treatment selection based on biomarkers in the lymph node is a topic for further studies.

A.-K. Falck · L. Rydén (✉)
Department of Surgery, Institution of Clinical Sciences Lund, Lund University Hospital, SE-221 85 Lund, Sweden
e-mail: lisa.ryden@med.lu.se

M. Fernö · P.-O. Bendahl
Department of Oncology, Institution of Clinical Sciences Lund, Lund University Hospital, SE-221 85 Lund, Sweden

Introduction

Breast cancer, the most frequently diagnosed cancer among females in the Western world today, is a heterogeneous disease. It is generally associated with a good prognosis, and in Sweden more than 80% of patients are disease-free at 5 years following surgery alone or in conjunction with medical systemic adjuvant treatment. The prediction of clinical outcome and the selection of patients for medical adjuvant therapy are based on prognostic factors [age, hormone receptor status, human epidermal growth factor receptor 2 (HER2) tumor size, lymph node involvement] according to international guidelines [1]. Metastatic lymph node involvement is still the most powerful prognostic factor for relapse and death in breast cancer [2]. Moreover, survival after relapse is poorer in node-positive patients than in patients with node-negative disease, an indication that nodal metastasis may also be a marker of aggressive phenotype and not simply a marker of disease recurrence at a later point [3].

Treatment-predictive biomarkers analyzed in the primary tumor [i.e., estrogen receptor (ER), progesterone receptor (PR), HER2, Ki67] are of great importance in

clinical practice with respect to the choice of adjuvant therapy after breast cancer surgery [1]. ER, PR, and HER2 are also used as therapeutic targets for endocrine treatment and trastuzumab [1, 4, 5]. Tamoxifen, a selective estrogen receptor modulator, is still commonly used as an adjuvant endocrine therapy in patients with hormone-responsive tumors, and it reduces the risk by 50% of breast cancer recurrence after 5 years of treatment in women with early-stage breast cancer [4]. Despite the administration of adjuvant tamoxifen therapy to patients with hormone receptor-positive disease, 15–20% of these women will have a relapse of their disease [4, 6], and metastatic disease is one of the major causes of death in middle-aged women in Sweden. Much effort has been put into the research field of endocrine treatment resistance in order to clarify the issue [7]. One hypothesis for explaining treatment resistance is that micrometastatic spread of the disease at the time of diagnosis is not targeted by adjuvant therapy with tamoxifen despite the selection of treatment based on the specific hormone receptor target in the primary tumor [8]. Disseminated micrometastases have different biological properties compared with the primary tumor and have developed from separate cell clones [8].

An alternative approach to the study of adjuvant treatment resistance is to compare the biomarker status in the primary tumors with that of the corresponding metastases. However, there are few detailed studies that compare biomarkers of the primary tumor and metastases from the same patient [8]. Until now, only data from small retrospective studies have been available, generally showing a high concordance between matched pairs of primary tumors and lymph node metastases using immunohistochemical (IHC) analyses of standard biomarkers (ER, PR, HER2, Ki67) [9–11]. The clinical outcome in relation to the biomarker status in lymph nodes has not yet been reported and no information on adjuvant treatment has been provided [9–11].

The aim of the present study was to determine the molecular characteristics of the primary tumor and corresponding lymph node metastases using a cohort of patients treated with adjuvant tamoxifen for 2 years. The results are based on the largest tumor material, analyzing standard biomarkers (ER, PR, HER2, and Ki67). Furthermore, survival data at 5 years of follow-up is provided. There was no significant discordance for any of the analyzed biomarkers between the primary tumor and lymph node metastases. At 5 years of distant disease-free survival (DDFS), both ER positivity and the Ki67 labeling index were independent predictors of survival when analyzed at any location. HER2 positivity was not a predictor of 5-years DDFS at any location. However, at 3-year DDFS, both HER2 positivity in the primary tumor and lymph node metastases were significant predictors of recurrence. The study adds to the knowledge of biomarker status in lymph node metastases and supports the contention that analyses of them can be informative when no analyses of the primary tumor can be performed.

Materials and methods

Patients

The patients (median age = 63 years, range = 26-81) included in the study had stage II (pT2pN0pM0, pT1-2pN1pM0) breast carcinoma and were diagnosed in the South Sweden Health Care Region (1985-1994). All patients were treated with adjuvant tamoxifen for 2 years, irrespective of ER status, and were previously selected from two prospective randomized clinical trials to investigate the compatibility of different laboratory methods for the evaluation of hormonal receptor status [12]. The original cohort included 425 patients, 297 of whom had lymph node-positive disease. All patients underwent surgery in the form of a modified radical mastectomy or breast-conserving surgery with axillary lymph node dissection (levels I and II). After breast-conserving surgery, radiotherapy (50 Gy) was given to the breast, and patients with axillary lymph node metastases received locoregional radiotherapy. The patients were followed for 5 years with annual mammogram and physical investigations. None of the patients received any systemic adjuvant therapy other than tamoxifen.

Effects on distant disease-free survival (DDFS) during a maximum follow-up time of 5 years were studied. Information on clinical outcome and patient- and tumor-related factors was already available. These factors included age, tumor size, lymph node status, and ER, PR, and HER2 status [12, 13]. In the present study, tissue microarrays from paraffin-embedded tumor samples were used with approval from the Ethics Committee at Lund University.

Tissue microarrays

Tissue microarrays from the primary tumors and corresponding lymph node metastases had been constructed using specimens from all 425 patients included in the previous study [12]. Representative areas of invasive breast cancer, embedded in paraffin blocks, were marked. Two 0.6-mm-diameter tissue core biopsies from tumor blocks of the primary tumor were punched out, and one biopsy specimen was taken from the corresponding lymph node metastases using a manual arrayer (Beecher Instruments, Sun Prairie, WI, USA) and positioned into a recipient paraffin array block. Biopsies from corresponding lymph node metastases were obtained from patients with lymph

node-positive disease. Staining with hematoxylin and cytokeratin (AE1/AE3) was carried out for morphological overview and localization of invasive breast cancer cells. This series of primary breast cancer specimens had previously been used to study various other potential prognostic factors and markers in breast cancer [12, 13].

Ki67 staining and evaluation

The Ki67 labeling index was determined using the antibody MIB-1 (M7240) (DAKO, Glostrup, Denmark). Sections of 4 μm were cut, mounted onto capillary microscope slides (DAKO), and dried overnight at room temperature followed by 1–2 h at 60°C. The sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Antigen retrieval was performed in a microwave oven, pH 9 buffer (S2367, DAKO). Staining was performed using an automatic immunostainer (TechMate™ 500 Plus, DAKO) with an incubation time of 30 min at room temperature and with MIB-1 diluted 1:1000. DAKO Envision™ (DAKO) was used as the visualization system. Diaminobenzidene was used as the chromogen.

The immunohistochemical staining for the Ki67 labeling index was examined by light microscopy by two independent observers (JK, KL) who were blinded to clinical and tumor characteristics data. Results for Ki67 were estimated quantitatively as the percentage of stained nuclei (proportion score). At least 200 tumor cells were evaluated using 40× magnification and the percentage of labeled nuclei was calculated. For statistical analysis, a cutoff point of 20% labeled nuclei was used to demarcate Ki67-positive, as advocated in previous studies [14].

Analysis of other tumor characteristics

ER and PR were analyzed immunohistochemically on formalin-fixed, paraffin-embedded breast carcinoma [8]. Tumors with more than 10% positive nuclei staining were considered ER- and PgR-positive.

HER2 scoring was determined semiquantitatively according to a standard protocol (HercepTest) after staining with a primary antibody (A0485, DAKO). The protocol categorizes tumors into four groups: grade 0: lack of staining in all tumor cells or membrane staining in less than 10% of the tumor cells; grade 1+: weak, not circumferential membrane staining in more than 10% of the tumor cells; grade 2+: intermediate, circumferential membrane staining in more than 10% of the tumor cells; grade 3+: intense and circumferential staining in more than 10% of the tumor cells. HER2 scoring was denoted as HER2-positive for all 3+ tumors and HER2-negative for 0, 1+, and 2+.

Statistical methods

For each of the dichotomized variables measured in the present study, McNemar's test was used to evaluate whether differences in both directions (+/− and −/+) were equally common when comparing primary tumor and lymph node. Distant disease-free survival (DDFS), including distant metastases in the skeleton, brain, liver, or lung, was used as the primary end point. The Kaplan-Meier method was used to estimate survival, and the log-rank test was used to evaluate null hypotheses of equal survival in two patient strata. Uni- and multivariate Cox regression were used to calculate hazard ratios for the factors in relation to DDFS. Assumptions of proportional hazards were checked using Schoenfeld's test.

P values less than 0.05 derived from two-sided tests were considered significant. The statistical software package Stata 10.1 (StataCorp., College Station, TX, USA) was used for all the statistical calculations.

Results

Distribution of ER, PR, HER2, and Ki67 in matched pairs of primary tumors and lymph node metastases

ER and PR were evaluable in 262 and 257 matched pairs of primary tumors and lymph node metastases, respectively. The fraction of discordant pairs was 7% (ER) and 16% (PR), and no statistically significant skewness was calculated for any of them (Table 1). The distribution of patients converting from hormone receptor-positive to negative and negative to positive is given in Table 1.

HER2 status and the Ki67 labeling index were scored in 104 and 101 matched pairs of primary tumors and lymph node metastases, respectively, using cores in the tissue

Table 1 Biomarker distribution in primary tumor and corresponding lymph node metastases

Variable	*N*	+/−	−/+	+/− or −/+	% Discordant	*P* Skewness
ER	262	12	7	19	7	0.36
PR	257	27	15	42	16	0.09
HER2	104	0	3	3	3	0.25
Ki67	101	4	10	14	14	0.18

ER estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor 2, *Ki67* Ki67 labeling index, *N* number of analyzed matched pairs of primary tumors and lymph node metastases, +/− = positive in primary tumor and negative in the lymph node, −/+ = negative in primary tumor and positive in the lymph node, % discordant = proportion of analyzed matched pairs with discordant results, *P* value calculated by McNemar's test for skewness

microarray. Only cases that could be analyzed at both locations were considered. Tissue microarrays either torn or displayed mostly with lymphatic tissue or stroma were excluded from further analysis, leaving a smaller number of evaluable cases that included lymph node metastases compared with ER and PR evaluation using whole slides. The reduced area of metastatic infiltration in the lymph node after previous sectioning performed in earlier studies explains the redundant number of evaluable cases. The discordant cases are given in detail in Table 1, showing that only 3% of evaluated matched pairs had discordant HER2 status, although all of them converted from HER2-negative in the primary tumor to HER2-positive in the lymph node metastases. For Ki67, the corresponding fraction of discordant pairs was 15%. None of the biomarkers had statistically significant skewness.

Association of biomarker status in the primary tumor and lymph node metastases with DDFS: univariate analyses

Estrogen receptor status

When the whole follow-up period of 5 years was considered, ER status added predictive information for the prognosis after tamoxifen treatment and was associated with identical reduction in terms of 5-year DDFS when scored in the primary tumor or in the lymph node metastases (hazard ratio [HR] = 0.39; $P < 0.001$) (Fig. 1). When cases with ER-positive status at any location were compared with cases with ER-negative status at both locations (with +/+, −/+, and +/− vs. −/−), a significant prediction for DDFS was noted (HR = 0.34; $P < 0.001$), suggesting that being ER-positive at any location is linked to responsiveness to tamoxifen treatment (Fig. 2). Of further interest is the indication that patients who are ER-negative in their primary but ER-positive in the lymph node may still have a better prognosis than patients who are double negative (−/+ vs. −/−) (HR = 0.25; $P = 0.16$). Since the data set included only seven patients in this category, no definitive conclusion can be drawn due to the lack of statistical power.

PR status

PR positivity in the primary tumor was not correlated to 5-year DDFS (HR = 0.70; $P = 0.2$), and similar results were calculated for PR positivity in the lymph node (HR = 0.95; $P = 0.8$).

HER2 status

HER2 positivity in the primary tumor was not correlated with 5-year DDFS (HR = 1.7; $P = 0.23$). Although there were only three discordant cases, HER2 positivity in the lymph node was indicative of adding prognostic information at 5-year DDFS (HR = 2.0, $P = 0.06$), but it did not reach formal statistical significance (Fig. 3). When 3-year DDFS was analyzed, HER2 positivity in the primary tumor was linked to reduced survival (HR = 2.6; $P = 0.03$), as was HER2 positivity in the lymph node metastases (HR = 3.0; $P = 0.004$). Schoenfeld's test showed a significant violence by time for proportional hazard ratios for HER2 positivity in the lymph node ($P = 0.01$), which was not true for HER2 positivity in the primary tumor ($P = 0.07$), suggesting that HER2 positivity in lymph node

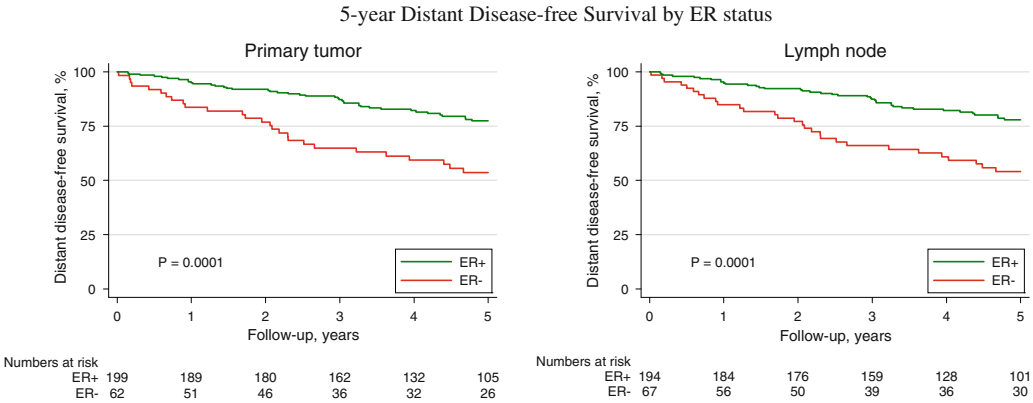

Fig. 1 Kaplan-Meier estimates of distant disease-free survival (DDFS) for ER status in the primary tumor and corresponding lymph node metastases. Patients with ER-positive tumors were compared with patients with ER-negative tumors. The P value was calculated using two-sided log-rank tests

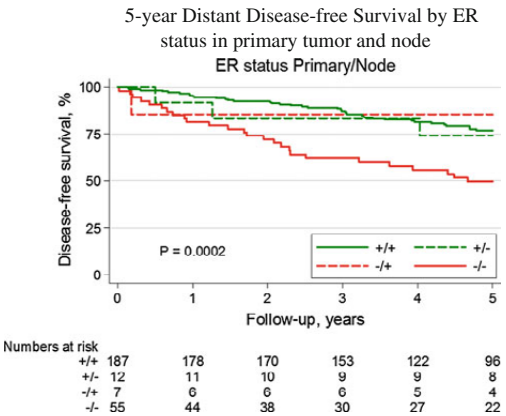

Fig. 2 Kaplan-Meier estimates of distant disease-free survival (DDFS) for ER status as a combined variable of the ER status in the primary tumor and corresponding lymph node metastases. Patients with ER-positive tumors and/or lymph node metastases were compared with patients with ER-negative tumors and lymph node metastases. The *p* value was calculated using two-sided log-rank tests

metastases can be a superior predictor of early recurrence compared with HER2 positivity in the primary tumor (Fig. 3).

Ki67

Ki67 positivity (labeling index with a cutoff at 20%) in the primary tumor was a significant predictor of prognosis for 5-year DDFS following tamoxifen treatment (HR = 3.6; $P = 0.003$), a finding consistent with Ki67 positivity in the corresponding lymph node metastases (HR = 2.2; $P = 0.06$) (Fig. 4).

Multivariate analysis

Factors significant in the univariate analysis throughout the follow-up of 5 years in both the primary tumor and the lymph node metastases, i.e., ER and Ki67, were included in the multivariate analysis, as were the well-established clinicopathological prognostic factors of tumor size and age. In a multivariate analysis, both ER positivity and Ki67 positivity remained significant predictors of 5-year DDFS in the primary tumor (Table 2). Identical results were achieved when the multivariate analyses for biomarker status in the lymph node metastases were calculated (Table 2). PR and HER2 were not included in the multivariate analysis because they were not significant factors in the univariate analysis when the whole follow-up period was considered.

Discussion

The present study provides information from the largest study published to date of biomarker analysis in matched pairs of primary tumors and lymph node metastases, including more than 250 pairs for analysis of ER and PR and 100 pairs for analysis of Ki67 and HER2. Furthermore, this is the first time information on survival analysis has been presented in relation to biomarker status at both locations. In line with previous publications, there are few discordant cases for all analyzed markers, with no significant calculated skewness for the distribution. It is

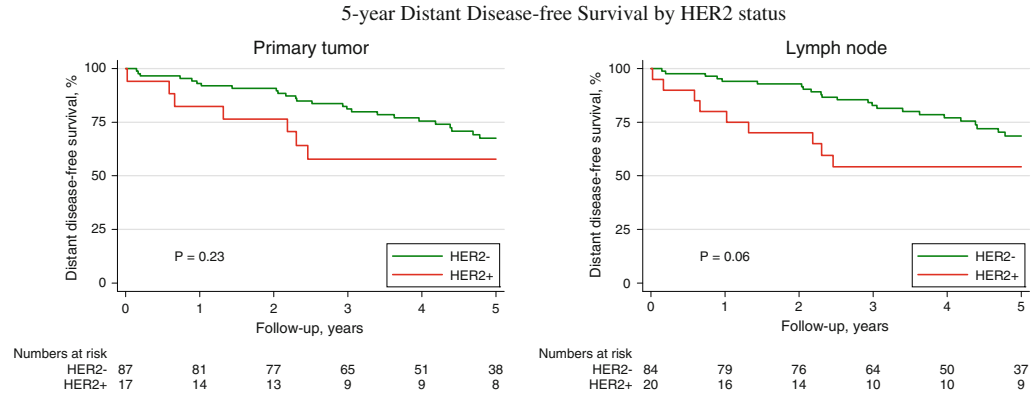

Fig. 3 Kaplan-Meier estimates of distant disease-free survival (DDFS) for HER2 status in the primary tumor and corresponding lymph node metastases. Patients with HER2-positive tumors were compared with patients with HER2-negative tumors. The *P* value was calculated using two-sided log-rank tests

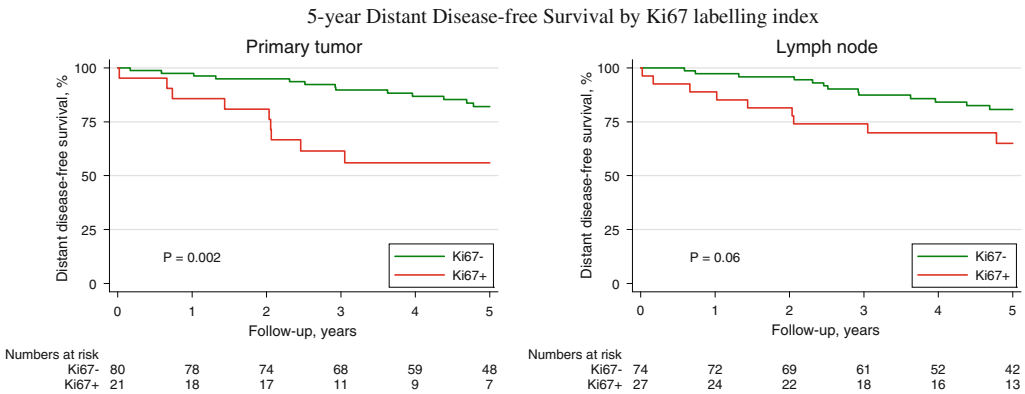

Fig. 4 Kaplan-Meier estimates of distant disease-free survival (DDFS) for the Ki67 labeling index in the primary tumor and corresponding lymph node metastases. Patients with Ki67-positive tumors were compared with patients with Ki67-negative tumors. The *P* value was calculated using two-sided log-rank tests

noteworthy that the three discordant cases for HER2 status all switched from HER2-negative in the tumor to HER2-positive in the lymph node. The survival data using 5-year DDFS as the end point for ER and Ki67 demonstrated that analyses of these biomarkers adds important prognostic information independent of the location of the cancer cells. HER2 positivity correlated with 3-year DDFS at both locations, although an interesting observation is that HER2 positivity in the lymph node was more indicative of earlier relapses than HER2 positivity in the primary tumor.

Adjuvant treatment selection is based on the characteristics of the primary tumor and on the linear progression model, where the metastatic cells are assumed to be derived from the primary site and to share most of the characteristics of the primary tumor [1, 8]. It is hypothesized that dissemination occurs after the selection of an advanced cancer cell population within the primary lesion. In contrast, the parallel progression model is based on the assumption of early dissemination of separate clones of tumor cells to different locations: peripheral blood, bone marrow, and ipsilateral lymph nodes [8]. The origin of micrometastases from separate clones explains why these micrometastases have different characteristics and thus different responses to adjuvant therapy. Classification of these malignant cells can therefore be of importance for adjuvant treatment selection by the diagnostic pathology of disseminated cancer cells in lymph node metastases [8].

There are few detailed studies comparing matched pairs of primary tumors and metastases from the same patient [9–11]. In small retrospective series, immunohistochemical (IHC) analysis of standard biomarkers has shown a high degree of concordance between matched pairs of primary tumors and lymph nodes, although the clinical outcome in relation to biomarker status in the lymph nodes has not been reported. Modern molecular techniques using comparative genomic hybridization (CGH) have shown multiple distinct genetic aberrations between primary tumors and lymph node metastases [15], supporting a parallel progression model, whereas few have found overlapping genotypes supporting the linear progression model [16].

Tamoxifen treatment is still the most commonly advocated adjuvant endocrine therapy for hormone-responsive primary breast cancer, although around 15-20% of patients treated with adjuvant tamoxifen will relapse despite having hormone receptor-positive tumors [4, 6]. Endocrine resistance is therefore a topic of clinical importance in understanding the underlying mechanisms, and research has been focused on biological characteristics within the primary tumor [7]. Crosstalk between receptor tyrosine kinase receptors like HER2, epidermal growth factor receptor (EGFR), and insulin growth factor receptor (IGFR) is one model for explaining how ER can be activated independently of a selective estrogen receptor modulator like tamoxifen [7]. In the present study, there were few discordant cases for ER status, but we found that tumors that are ER-positive at any location have a better prognosis after tamoxifen treatment than tumors that are ER-negative at both locations (HR = 0.34; $P < 0.001$). However, no definitive conclusion can be drawn from our study regarding the effect of tamoxifen treatment for patients who are ER-positive in the lymph node metastases and ER-negative in their primary tumor because of the lack of statistical power (cases with this phenotype = 7). Clarification of the issue is important because for elderly patients and patients with comorbidity who cannot tolerate adjuvant chemotherapy, their hormone-responsive disease makes them candidates for adjuvant endocrine therapy. The multivariate analyses provide evidence that ER positivity is

Table 2 Results of the multivariable analyses of distant disease-free survival in lymph node-positive patients with adjuvant tamoxifen therapy with a maximum follow-up of 5 years

Variable	HR (95% CI)	*P* value
A. Multivariate analyses according to biomarker status in the primary tumor		
ER status: Pos vs. Neg	0.3 (0.1-0.7)	0.009
Ki67 labeling index: Pos vs. Neg	2.6 (1.1-6.6)	0.04
Tumor size: >20 vs. ≤20	3.5 (1.2-10.9)	0.03
Age: continuous	0.96 (0.91-1.00)	0.06
B. Multivariate analyses according to biomarker status in the lymph node metastases		
ER status: Pos vs. Neg	0.2 (0.1-0.5)	0.001
Ki67 labeling index: Pos vs. Neg	2.9 (1.1-7.5)	0.03
Tumor size: >20 vs. ≤20	4.8 (1.5-15.6)	0.009
Age: continuous	0.95 (0.91-0.99)	0.03

Multivariate analysis was performed using Cox regression. Factors significant in the univariate analyses were included as well as the established clinicopathological prognostic factors tumor size and age

also an independent positive prognostic factor in tamoxifen-treated patients when analyzed in lymph node metastases.

HER2 is a key prognostic factor indicating a high risk of early recurrence and death in primary breast cancer [17], and, more importantly, a predictor of response to treatment with the monoclonal antibody trastuzumab, which has dramatically improved the prognosis for this subgroup of breast cancer patients [5]. In the context of endocrine treatment, HER2 positivity is correlated with shortened recurrence-free survival whatever the type of endocrine treatment administered, and all patients with HER2-positive tumors should be offered treatment with trastuzumab [7]. In line with previous studies on this marker, high concordance was noted with only three discordant cases, all of which shifted from HER2-negative in the primary tumor to HER2-positive in the lymph node. The relevance of biomarker discordance between primary tumors and synchronous metastases has been discussed by Santinelli et al. [18], because the metastatic lesion hosts cancer cells with metastatic capacity and thus the biomarker status at the metastatic location might give more relevant prognostic information. In this study, HER2 positivity was linked to shortened DDFS at 3-year follow-up when analyzed at both locations. HER2 positivity in the node was also of borderline significance for 5-year DDFS (HR = 2.0; $P = 0.06$) and there was a significant violence by time for the risk of recurrence, indicating that HER2 status in the node was informative for early recurrence. Although this observation is interesting, the result is preliminary due to the low number of observations. Analyses of *HER2* gene amplification by fluorescence in situ hybridization (FISH) would have been preferable because this method has greater reproducibility than IHC [19]. Unfortunately, *HER2* gene amplification was not performed because the tissue available from the nodes did not allow further analyses.

In this cohort of tamoxifen-treated patients we found few discordant cases in matched pairs of primary tumors and lymph node metastases. Biomarker analysis in lymph node metastases is feasible in clinical practice, and the study supports the analysis of lymph nodes if analysis of the primary tumor can be performed. Additional study will be required to determine if ER positivity and HER2 positivity in lymph node metastases are independent prognostic factors in tamoxifen-treated patients with early breast cancer.

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Paper IV

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CLINICAL TRIAL

Biomarker expression and St Gallen molecular subtype classification in primary tumours, synchronous lymph node metastases and asynchronous relapses in primary breast cancer patients with 10 years' follow-up

Anna-Karin Falck · Pär-Ola Bendahl · Gunilla Chebil · Hans Olsson · Mårten Fernö · Lisa Rydén

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Abstract Molecular profiles of asynchronous breast cancer metastases are of clinical relevance to individual patients' treatment, whereas the role of profiles in synchronous lymph node metastases is not defined. The present study aimed to assess individual biomarkers and molecular subtypes according to the St Gallen classification in primary breast tumours, synchronous lymph node metastases and asynchronous relapses and relate the results to 10-year breast cancer mortality (BCM). Tissue microarrays were constructed from archived tissue blocks of primary tumours ($N = 524$), synchronous lymph node metastases ($N = 147$) and asynchronous relapses ($N = 36$). The samples were evaluated by two independent pathologists according to oestrogen receptor (ER), progesterone receptor (PR), Ki67 and human epidermal growth factor receptor 2 (HER2) by immunohistochemistry and in situ hybridisation. The expression of biomarkers and molecular subtypes in the primary tumour was compared with that in the synchronous lymph node metastases and relapses, and related to 10-year BCM. Discordances were found between primary tumours and relapses (ER: $p = 0.006$, PR: $p = 0.04$, Ki67: $p = 0.02$, *HER2*: $p = 0.02$, St Gallen subtypes: $p = 0.07$) but not between primary tumours and metastatic lymph node. Prognostic information was gained by the molecular subtype classification in primary tumours and nodal metastases; triple negative subtype had the highest BCM compared with the luminal A subtype (primary tumours: HR 4.0; 95 % CI 2.0–8.2, $p < 0.001$, lymph node metastases: HR 3.5; 95 % CI 1.3–9.7, $p = 0.02$). When a shift in subtype inherence between primary tumour and metastatic lymph node was identified, the prognosis seemed to follow the subtype of the lymph node. Molecular profiles are not stable throughout tumour progression in breast cancer. Prognostic information for individual patients appears to be available from the analysis of biomarker expression in synchronous metastatic lymph nodes. The study supports biomarker analysis also in asynchronous relapses.

Keywords Breast cancer · Lymph node metastases · Relapse · Prognosis · Tumour progression · St Gallen molecular subtypes

A.-K. Falck · L. Rydén (✉)
Division of Surgery, Department of Clinical Sciences Lund, Skåne University Hospital, Lund University, 22185 Lund, Sweden
e-mail: lisa.ryden@med.lu.se

A.-K. Falck
Department of Surgery, Hospital of Helsingborg, 25187 Helsingborg, Sweden

P.-O. Bendahl · G. Chebil · M. Fernö
Department of Clinical Sciences Lund, Barngatan 2B, Lund University, 22185 Lund, Sweden

H. Olsson
Molecular and Immunological Pathology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Department of Clinical Pathology and Clinical Genetics, Östergötland County Council, 58183 Linköping, Sweden

L. Rydén
Department of Surgery, Skåne University Hospital, 22185 Lund, Sweden

Introduction

The treatment of patients with metastatic axillary lymph node involvement is based on the analysis of biomarker

expression in the primary tumour, and assumes the expression of predictive markers to be stable throughout tumour progression from primary tumours to lymph node metastases. The presence of synchronous lymph node metastases is still an important prognostic factor, although axillary lymph node dissection seems to have little effect on overall survival [1]. However, there has been increasing attention in recent years to the possibility that the evolution and progression of cancer disease have biological implications; different cancer clones giving rise to phenotypes of different origins in the metastatic setting may thus express different biological profiles compared with that of the primary tumour [2, 3].

Several studies have evaluated the concordance/discordance in biomarker expression between primary tumours and synchronous lymph node metastases [4–7] and between primary tumours and asynchronous relapses [8–10], showing lower discordance rates when primary tumours were compared with lymph node metastases than with distant metastases. A recent meta-analysis of human epidermal growth factor receptor 2 (HER2) expression, comparing primary tumours and lymph node metastases and/or relapses in 2,520 cases, confirmed the significant difference in discordance rate between the metastatic sites [11]. The observed difference in biomarker expression in the metastatic site could affect the treatment decision [8, 9] and thus makes the question of analysing biomarker expression in lymph node metastases and relapses clinically relevant. Biopsies of relapses for the analysis of biomarker expression are now performed in clinical settings, and recommendations have been included in the Swedish National Guidelines since 2012 [12].

Primary breast cancer tumours are routinely analysed for oestrogen receptor (ER), progesterone receptor (PR) and HER2, which, together with age, nodal status, tumour size and Nottingham histological grade (NHG), propose a prognostic and predictive profile that guides clinicians in tailoring adjuvant treatment. The benefit of adjuvant endocrine treatment for ER-positive tumours [13] and of treatment with humanised monoclonal antibody trastuzumab for HER2-positive disease has been shown repeatedly [14, 15].

Recently, a combination of the specific biomarkers into surrogate molecular subtypes has been proposed to give additional biologic and prognostic information. Microarray-based gene expression studies [16, 17] and subsequent immunohistochemical (IHC) studies [18–21] have revealed at least four major subtypes with additional prognostic and predictive information [18, 20, 21]. The proposed classification, by the St Gallen International Breast Cancer Conference 2011, into molecular subtypes when routine biomarker analysis by IHC is used as a surrogate for genetic analysis, includes: luminal A (ER+ and/or PR+, Ki67 low and HER2−), luminal B HER2− (ER+ and/or PR+, Ki67 high and HER2−), luminal B HER2+ (ER+ and/or PR+, any Ki67 and HER2+), HER2-type (ER-, PR- and HER2+) and triple negative (ER−, PR− and HER2−). Beyond the important prognostic and treatment-predictive biomarkers ER/PR and HER2, the molecular classification adds information on proliferation. The association of proliferation with poor prognosis is well established [22] where Ki67 is currently applied as an additional proliferation marker in the clinical setting. However, thresholds for determining a tumour as having a high proliferation rate are not established, and improvements in the reproducibility of assessment are warranted [23, 24]. Recently, the St Gallen criteria were validated retrospectively using tumour grade instead of Ki67, assessing luminal A as a molecular subtype with a favourable prognosis [25].

The aim of this study was to assess ER, PR, Ki67 and HER2 as individual biomarkers as well as the molecular subtype according to the St Gallen classification in primary tumours, synchronous lymph node metastases and asynchronous relapses, and to relate the results to 10-year breast cancer mortality (BCM).

Patients and methods

Patients

The study is based on a cohort of patients previously included in an observational prospective study aiming to evaluate the presence and prognostic value of disseminated tumour cells in bone marrow. The characteristics of this trial have been described in detail previously [26]. In brief, 569 women diagnosed with primary breast cancer in the South Swedish Health Care Region between 1999 and 2003 were included. All patients had unifocal primary invasive breast cancer and underwent surgery of the breast and the axillary lymph nodes based on preoperatively identified characteristics and staging. Adjuvant systemic therapy and postoperative radiotherapy were administrated according to National Guidelines. Neoadjuvant endocrine and chemotherapy were administered to less than 1 % of the patients, respectively, and trastuzumab was administered to 6 (1 %) patients in the adjuvant setting.

Patients were followed annually by clinical examination and mammography. After 5 years of follow-up, all reports of events were abstracted from individual patients' charts. Information on breast cancer-related death was retrieved from the Swedish Register of Causes of Death (Central Statistics Office) after 10 years. Data retrieval was performed 2012 and events until 31 of December 2011 was

included in the register. Fourteen patients did not fulfil the inclusion criteria and were excluded; the final cohort included 555 patients (Fig. 1, flow chart).

The study was approved by the ethics committee at Lund University and written informed consent was obtained from all included patients (LU699-09 and LU75-02).

Tumour samples and tissue microarray (TMA)

Formalin-fixed paraffin-embedded archival blocks of study samples were retrieved from the Departments of Clinical Pathology in Lund and Helsingborg. TMAs were constructed from defined areas of invasive tumour, and identified with haematoxylin and eosin-stained glass slides of tissue sections by a pathologist. Two 1.0 mm cores were punched from each donor paraffin block and mounted into the recipient block using a tissue array machine in accordance with the manufacturer's instructions (Beecher Instruments, MD, USA). After construction, 4 µm tissue sections were cut, and glass slides were prepared for microscopy and finally scanned (Aperio Scan Scope CS, CA, USA, with software Aperio Spectrum TMAlab, CA, USA).

Immunohistochemistry and in situ hybridisation

ER and PR status were assessed using the Ventana Benchmark system, using Anti-ER clone SP1 and Anti-PR Clone 1E2 as the primary antibodies [27], at a central

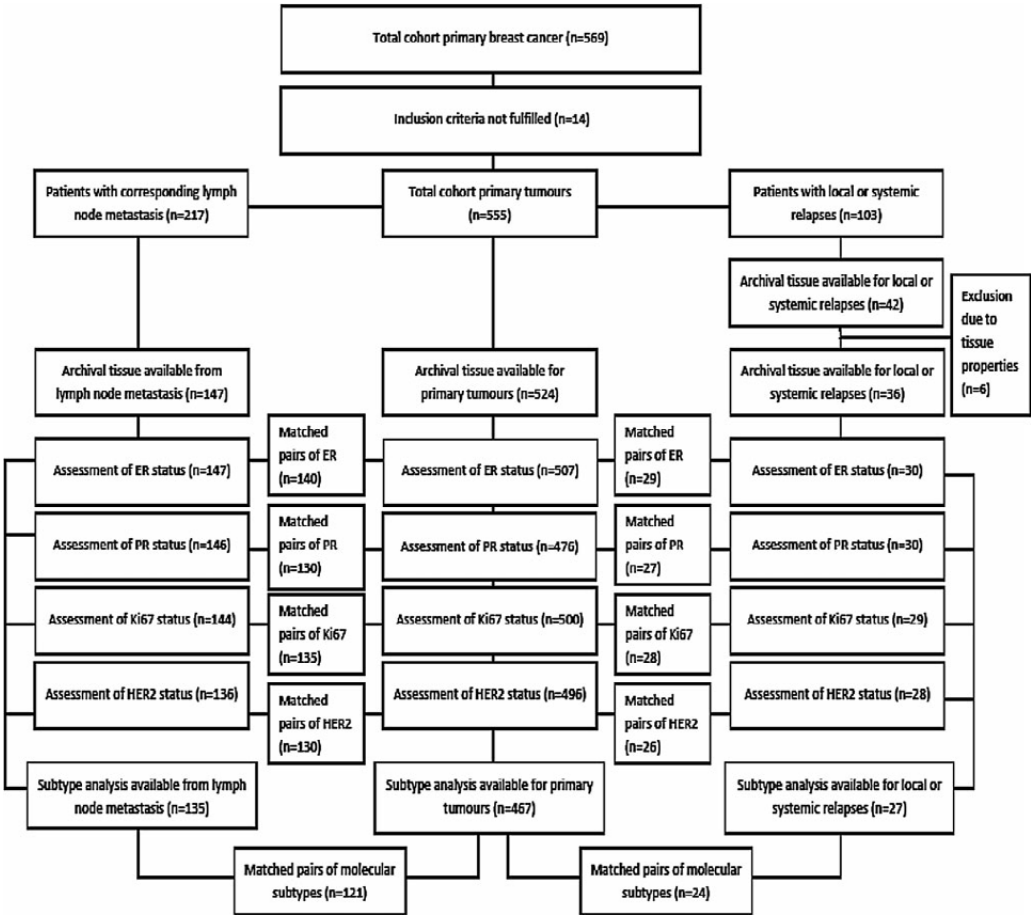

Fig. 1 Flow chart of patient cohort. *ER* oestrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2

clinical laboratory (Skåne University Hospital, Malmö). No fewer than 100 invasive tumour cells were visually scored and evaluated. Samples with more than 10 % stained nuclei were considered positive.

HER2 was evaluated by means of both IHC using Anti-HER2 clone 4B5 and in situ hybridisation (ISH) (Inform HER2 dual ISH DNA, Product no. 800-4422, with silver and chromogen (SISH) visualisation kit, product nos. 780-001 and 800-504, Ventana Benchmark Ultra). All patients with amplified tumours according to SISH (quota ≥2.0) were considered positive [28].

The proliferation marker Ki67 was assessed using the Ki67 antibody MIB1 (DAKO, Glostrup, Denmark) diluted 1:50, incubated for 32 min and visualised with DAB (3,3′-diaminobenzidine). Areas with increased numbers of Ki67-positive cells within the cancerous regions (hot spots) were identified, and at least 200 cells were analysed in sets of 10 cells at a time. Cells were visually scored for the percentage of positive immunostaining. The chosen cut-point for separating high and low proliferation was the third of the study population with the highest observed Ki67 percentages, which corresponded to >20 % in the present cohort.

Epidermal growth factor receptor (EGFR) was evaluated and CK 5/6 antibodies assessed using the Ventana Benchmark system (clone 3C6 for EGFR and clone D5/16B4 (DAKO, CA, USA) diluted 1:100 for CK 5/6), and scored positive if any cytoplasmatic and/or membranous invasive carcinoma cell staining was positive.

All biomarkers (ER, PR, Ki67, HER2, EGFR and CK 5/6) were scored independently by two pathologists (GC, HO).

Molecular subtype definitions

Classification according to the St Gallen recommendation [29] was based on the IHC analysis of ER, PR and Ki67 and SISH-analysis of *HER2*, and defined as:

Luminal A (ER+ and/or PR+, low Ki67 and HER2−), luminal B HER2− (ER+ and/or PR+, high Ki67 and HER2−), luminal B HER2+ (ER+ and/or PR+, any Ki67 and HER2+), HER2 type (ER−, PR− and HER2+) and triple-negative (ER−, PR− and HER2−).

In addition, EGFR and CK 5/6 identified a basal-like subgroup of patients within the triple-negative subgroup. However, this subgroup was not considered in any of the descriptive or survival analyses.

Statistical analysis

Biomarker expression was evaluated independently by two pathologists. Since the concordance in evaluation was close to 100 % for all markers, the analyses are based on the evaluation by one of the contributing pathologists (GC).

The primary end-point in this study was BCM with 10 years' follow-up, data was retrieved from the Swedish Register of Causes of Death (Central Statistics Office) 2012 and registered events until 31 of December 2011 were recorded.

Biomarker expression was summarised as frequencies and percentages. The Wilcoxon matched-pairs signed-rank test was used to compare biomarker expression at two locations, i.e. matched pairs of primary tumours and lymph node metastases/relapses, respectively. This test is equivalent to the McNemar test for binary data, but has higher power than the generalisation of the McNemar test to more than two categories.

Differences between matched pairs of primary tumours, synchronous lymph node metastases or asynchronous relapses regarding the inherence in molecular subtype were evaluated by the above-mentioned generalised McNemar test: the McNemar-Bowker test of symmetry. The use of this test is motivated by the ordered nature of the classification from best prognosis (luminal A) to worst prognosis (triple-negative). The null hypothesis, symmetry, will hence correspond to balanced subtype shifts and the alternative, skewness, to preferential shifts to groups with better or worse prognosis. In our view, it is not appropriate to test the null hypothesis of no association between the molecular subtypes at different locations using the Chi squared test or a Fisher's exact test. Another test, seen in the literature for comparisons of this kind, is based on the chance-adjusted agreement measure kappa (κ). This measure is valid, and a test of the null hypothesis $\kappa = 0$ versus $\kappa \neq 0$ has higher power than the McNemar–Bowker test. However, the drawback is that significant deviations from random group allocation conditioned on the observed marginal totals will also be picked up by the test for symmetric differences.

Differences between three or more groups according to the number of lymph node metastases were evaluated by the Kruskal–Wallis test.

Cumulative incidence curves were used to describe BCM, and log-rank tests were applied to evaluate the hypotheses of equal survival in subgroups of patients. Multivariable analysis by the Cox proportional hazard model was used to calculate hazard ratios for biomarkers and molecular subtype inherence with and without adjustment for other prognostic factors. Proportional hazard assumptions were checked graphically.

The statistical software packages Stata 12.1 (Stata Corp. 2012, College Station, TX, USA) and IBM SPSS Statistics v 19 (IBM Svenska AB, Stockholm, Sweden) were used for all statistical calculations.

Results

Tumour samples

Paraffin-embedded blocks were available for TMA construction in 524/555 primary tumours, 147/217 synchronous lymph node metastases and 42/103 asynchronous relapses (Fig. 1, flowchart). Of the 147 re-analysed lymph node metastases, 142 were macro- and 5 micro-metastases. In 6 of the 42 re-analysed suspected relapses, no loco-regional or distant relapse was found. Of these six, which were excluded from further analyses, four showed cancer of the contralateral breast, one was benign and one showed a new cancer (cholangiocarcinoma). Only eight patients had tumour samples available from all three locations.

ER and PR status

In the analysis of ER expression, the median of stained nuclei was 90 % in primary tumours and 90 % also in lymph node metastases ($p = 0.9$, Wilcoxon signed-rank test, Fig. 2). The median value for ER was 80 % in the relapses, and there was a significant shift in the fraction of ER-stained nuclei compared with that in the primary tumours ($p = 0.006$, Wilcoxon signed-rank test, Fig. 2). PR had median values of 60, 30 and 7.5 % of stained nuclei in primary tumours, lymph node metastases and relapses, respectively and, as for ER, a significant shift was seen when comparing primary tumour and asynchronous relapse but not when compared to the synchronous lymph node (primary tumour vs lymph node; $p = 0.9$ and primary tumour vs relapse; $p = 0.04$, Wilcoxon signed-rank test, Fig. 2). When ER and PR were analysed as grouped variables with a cut-off value at 10 %, no significant skewness in discordance could be detected when comparing ER and PR statuses in primary tumours with those in lymph node metastases (ER: $n = 140$, $p = 1.0$ and PR: $n = 130$, $p = 0.7$, McNemar test, Table 1) or in relapses (ER: $n = 29$, $p = 0.6$ and PR: $n = 27$, $p = 0.5$, McNemar test, Table 1).

Ki67

Ki67 was assessed in 500/524 primary tumours, 144/147 synchronous lymph node metastases and 29/36 asynchronous relapses. When analysed as a continuous variable, the median of stained nuclei was 10 % both in primary tumours and in lymph node metastases ($p = 0.8$, Wilcoxon signed-rank test, Fig. 2). In the asynchronous relapses, the median score for Ki67 was 20 %, which corresponds to a significant shift in the Ki67 distribution compared with that in primary tumours ($p = 0.02$, Wilcoxon signed-rank test, Fig. 2). When Ki67 was analysed as a grouped variable, with a cut-off >20 %, no significant skewness between primary tumours and lymph node metastases ($n = 135$, $p = 0.5$, McNemar test, Table 1) or relapses ($n = 28$, $p = 0.4$, McNemar test, Table 1) was found.

Her2

HER2 was determined in 496/524 primary tumours, 136/147 lymph node metastases and 28/36 relapses. The distribution of *HER2* showed a trend towards worsening characteristics throughout tumour progression with a higher proportion of *HER2*-positive cell clones in the synchronous lymph node metastases, 43/136 (32 %), and asynchronous relapses, 14/28 (50 %), compared with that in the primary tumour, 100/496 (20 %). In the synchronous lymph node metastases, 22/130 (16 %) patients altered their *HER2* status; 7/130 (5 %) patients lost and 15/130 (11 %) patients gained *HER2* amplification ($p = 0.1$, McNemar test, Table 1). Comparison of *HER2* amplification in primary tumours and relapses revealed a significant shift from the primary tumour to the asynchronous relapse; 7/26 patients (27 %) gained amplification in *HER2* non-amplified primary tumours, while no patients lost *HER2* amplification in the relapses ($p = 0.02$, McNemar test, Table 1).

Molecular subtypes

A molecular subtype classification according to St Gallen could be established in 467/524 primary tumours, 135/147 synchronous lymph node metastases and 27/36 asynchronous relapses (Fig. 1). In addition, analysis of EGFR and CK 5/6 distinguished a basal-like group of patients within the triple-negative molecular subtype; 25/38 of primary triple-negative tumours were classified as basal-like, expressing both EGFR and CK 5/6. Since the St Gallen recommendations advise against using the markers to discriminate the basal-like subtype, considering them insufficiently reproducible [29], the basal-like subtype is not further addressed in the study.

Patients' and tumour characteristics according to molecular subtype are presented in Table 2, showing that the luminal A molecular subtype was found in 56 % of primary tumours and was more often detected by screening ($p = 0.01$, χ^2 test) with smaller tumour size ($p = 0.002$, χ^2 test) and of low histological grade ($p < 0.001$, χ^2 test, Table 2) compared with the other molecular subtypes. No significant discordance in terms of molecular subtype could be detected between primary tumours and synchronous lymph node metastases ($p = 0.3$, McNemar–Bowker test of symmetry, Table 3) and there was no association between the number of metastatic lymph nodes and molecular subtype in the lymph node ($p = 0.1$, Kruskal–

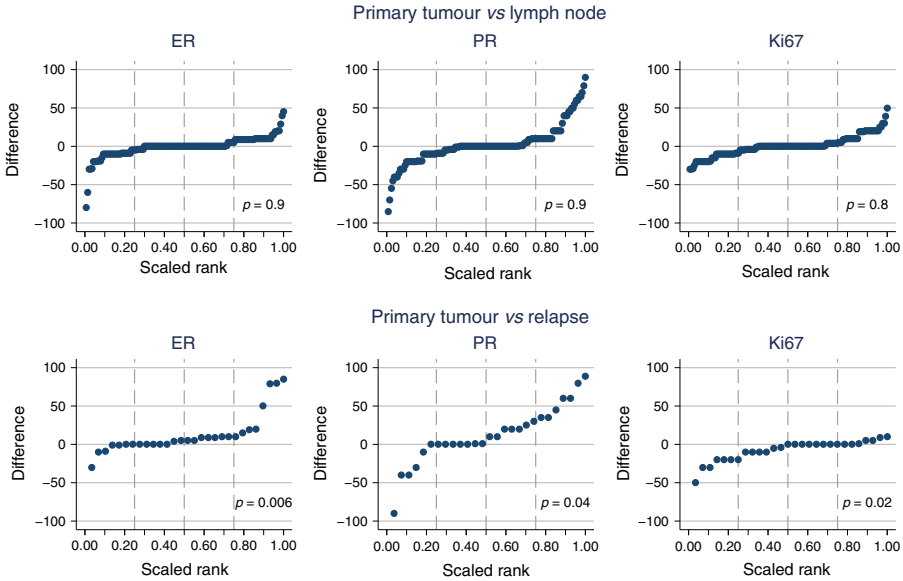

Fig. 2 Pairwise differences in biomarker expression between samples from different sites; primary tumour versus lymph node or primary tumour versus relapse. The ordered differences are plotted versus the scaled ranks which are defined as the ranks divided by the total number of pairs. The lower quartile, the median and the upper quartile will hence correspond to the scaled ranks 0.25, 0.50 and 0.75, respectively, which are highlighted with *dashed vertical lines*. *ER* oestrogen receptor, *PR* progesterone receptor. *p* value by Wilcoxon matched-pairs signed-rank test

Table 1 Comparison of biomarker status between primary tumours, lymph node metastases and relapses

Biomarker expression	ER			PR			Ki67			*HER2*		
	N	%	*p**	*N*	%	*p**	*N*	%	*p**	*N*	%	*p**
PT positive/LNM positive	112	80	1.0	66	51	0.7	44	33	0.5	28	22	0.1
PT positive/LNM negative	1	.5		9	7		13	10		7	5	
PT negative/LNM positive	1	.5		12	9		18	13		15	11	
PT negative/LNM negative	26	19		43	33		60	44		80	62	
Total	140	100		130	100		135	100		130	100	
PT positive/R positive	21	73	0.6	11	41	0.5	12	43	0.4	7	27	0.02
PT positive/R negative	3	10		6	22		1	4		0	0	
PT negative/R positive	1	3		3	11		4	14		7	27	
PT negative/R negative	4	14		7	26		11	39		12	46	
Total	29	100		27	100		28	100		26	100	

Cut-off for ER and PR >10 % of stained nuclei, Ki67 >20 % of stained nuclei and HER2 amplified by SISH

ER oestrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2, *PT* primary tumour, *LNM* lymph node metastases, *R* relapses

* McNemar test

Wallis test). A tendency towards a shift between primary tumour and relapse was noted, although not strictly significant ($p = 0.07$, McNemar–Bowker test of symmetry, Table 3). When luminal A versus non-luminal A subtypes in primary tumours and lymph node metastases were compared, a switch from luminal A in the primary tumour to non-luminal A subtype in the lymph node metastasis was detected in 15/48 (31 %) patients, and primary tumours of

the non-luminal A molecular subtype shifted to luminal A subtype in 13/73 (18 %) in the lymph node metastasis. However, no significant skewness for luminal A versus non-luminal A in primary tumours compared with lymph node metastases or relapses was noted (primary tumour vs lymph node metastasis: $p = 0.4$ and primary tumour vs relapse: $p = 0.1$, McNemar test).

Survival analysis

Survival according to the molecular subtypes in primary tumours and synchronous lymph node metastases

Patients with luminal A primary tumours had significantly lower 10-year BCM compared with luminal B HER2∓,

Table 2 Patient's and tumour characteristics according to St Gallen molecular subtype in the primary tumour ($n = 467$)

	Luminal A *N* (%)	Luminal B HER2− *N* (%)	Luminal B HER2+ *N* (%)	HER2 type *N* (%)	Triple negative *N* (%)
Number	260 (56)	80 (17)	74 (16)	18 (4)	35 (7)
Age at diagnosis (years)	58 (30–88)	52 (32–88)	58 (26–80)	58 (30–82)	53 (29–86)
Mode of detection					
Screening detected	124 (48)	34 (42)	22 (30)	5 (28)	14 (40)
Clinically detected	136 (52)	46 (58)	52 (70)	13 (72)	21 (60)
Tumour size					
≤20 mm	191 (74)	42 (52)	51 (69)	11 (61)	18 (51)
>20 mm	68 (26)	38 (48)	23 (31)	7 (39)	17 (49)
Missing	1	0	0	0	0
Nodal status					
N0	155 (61)	41 (53)	42 (58)	8 (44)	20 (57)
N+	99 (39)	36 (47)	31 (42)	10 (56)	15 (43)
N1	48 (19)	10 (13)	12 (16)	1 (6)	3 (9)
N2–3	29 (11)	7 (9)	10 (14)	3 (17)	5 (14)
N4+	22 (9)	19 (25)	9 (12)	6 (33)	7 (20)
Missing	6	3	1	0	0
NHG					
1	86 (34)	10 (12)	7 (10)	0	0
2	151 (59)	40 (50)	38 (52)	6 (33)	8 (24)
3	19 (7)	30 (38)	28 (38)	12 (67)	26 (76)
Missing	4	0	1	0	1
Adjuvant chemotherapy					
No	243 (94)	63 (80)	62 (84)	7 (39)	13 (37)
Yes	16 (6)	16 (20)	12 (16)	11 (61)	22 (63)
Missing	1	1	0	0	0
Adjuvant endocrine therapy					
No	93 (36)	18 (23)	20 (27)	16 (89)	34 (97)
Yes	167 (64)	62 (78)	54 (73)	2 (11)	1 (3)
Adjuvant radiotherapy					
Breast					
No	111 (43)	32 (40)	27 (36)	8 (44)	13 (37)
Yes	148 (57)	47 (60)	47 (64)	10 (56)	22 (63)
Missing	1	1	0	0	0
Locoregional					
No	222 (86)	56 (71)	59 (80)	13 (72)	26 (74)
Yes	36 (14)	23 (29)	15 (20)	5 (28)	9 (26)
Missing	2	1	0	0	0

N0 Lymph node negative, *N+* Lymph node metastasis, *NHG* Nottingham Histological grade, *HER2* human epidermal growth factor receptor 2

Table 3 Comparison of St Gallen molecular subtype distribution in primary tumours, lymph node metastases and relapses

Subtype in primary tumours	Subtype in lymph node metastases ($n = 121$) N					p*	Subtype in relapses ($n = 24$) N					p*
	Luminal A	Luminal B HER2−	Luminal B HER2+	HER2 type	Triple negative		Luminal A	Luminal B HER2−	Luminal B HER2+	HER2 type	Triple negative	
Luminal A	33	7	8	0	0	0.3	3	1	5	0	0	0.07
Luminal B HER2−	9	18	3	0	0		1	3	1	0	0	
Luminal B HER2+	3	3	15	1	0		0	0	4	2	0	
HER2 type	1	0	1	7	0		0	0	0	0	0	
Triple negative	0	0	0	3	9		0	1	0	1	2	

HER2 human epidermal growth factor receptor 2

* McNemar Bowker test of symmetry

HER2 type and triple-negative (Fig. 3; $p = 0.002$, log-rank test). The highest BCM was noted in patients with triple-negative primary tumours compared with luminal A (HR 4.0; 95 % CI 2.0–8.2, $p < 0.001$, Cox proportional hazard model). The difference in BCM between the molecular subtypes remained significant ($p < 0.001$) in a Cox proportional hazard model adjusting for age (continuous), tumour size (>20 mm vs ≤20 mm), presence of lymph node metastases (N+ vs N0) and mode of detection (screening vs clinical). When BCMs according to the St Gallen molecular subtypes in the synchronous lymph node metastases were compared, a similar pattern to that in primary tumours was observed (Fig. 3). For example, luminal A had a favourable prognosis, though the null hypothesis of equal BCMs in the five groups was not significant (Fig. 3, $p = 0.15$, log-rank test). However, the highest BCM was noted in patients with triple-negative lymph node metastases compared with luminal A in lymph node metastases (HR 3.5; 95 % CI 1.3–9.7, $p = 0.02$, Cox proportional hazard model).

Subgroup analysis in patients with discordant luminal A classification

The prognosis of patients with a switch in molecular subtype from luminal A in the primary tumour to non-luminal A ($n = 15$) in the metastatic lymph node was as bad as that of patients with stable non-luminal A subtype ($n = 60$) inherence in both the primary tumour and synchronous lymph node metastasis (HR 1.0, 95 % CI 0.4–2.6, $p = 1.0$, Cox proportional hazard model, Fig. 4), suggesting a prognostic influence of the molecular subtype in the synchronous lymph node metastases.

When the subgroups of patients who had a change in subtype inherence from non-luminal A in the primary tumours to luminal A in the lymph node metastases ($n = 13$) were analysed, the BCM was five times higher in the group of non-luminal A patients ($n = 60$) in both locations (HR 5.0, 95 % CI 0.7–37, $p = 0.12$, Cox proportional hazard model, Fig. 4). Twelve of the 13 patients (92 %) who switched to luminal A in the lymph node survived 10 years compared with 20/60 (33 %) patients with stable non-luminal A in both the primary tumour and the lymph node metastasis who died from breast cancer.

Luminal A molecular subtype and mode of detection

BCM according to the molecular subtypes in the primary tumour was further explored by analysing the effect on outcome by mode of detection, i.e. screening detected (SD) versus clinical detected (CD). Stratification for mode of detection showed that the St Gallen molecular subtypes added significant prognostic information in terms of BCM in both subsets (SD; $p = 0.04$ and CD; $p = 0.003$, log-rank test). Patients with luminal A primary tumours detected by screening had an improved prognosis compared with luminal A primary tumours detected clinically, with a 10-year BCM of 6 % compared with 13 % ($p = 0.02$, log-rank test), and identified a subgroup with an excellent prognosis. In patients with luminal A tumours, whether detected by screening or clinically, lymph node metastases were a significant negative prognostic factor ($p = 0.003$ and $p < 0.001$, log-rank test, respectively). We therefore explored whether prognosis according to inherence of nodal molecular subtype could explain the prognostic influence of lymph node metastases in this subgroup. All patients with primary tumours detected by screening and luminal A subtype in the lymph node metastases ($n = 17$) had an excellent outcome compared with non-luminal A subtypes ($n = 20$), regardless of subtype inherence in the primary tumour ($p = 0.001$, log-rank test). The prognosis of patients switching from luminal A in the primary tumour to nodal non-luminal A was worse than that of patients non-luminal A in both locations (HR 2.8, 95 % CI

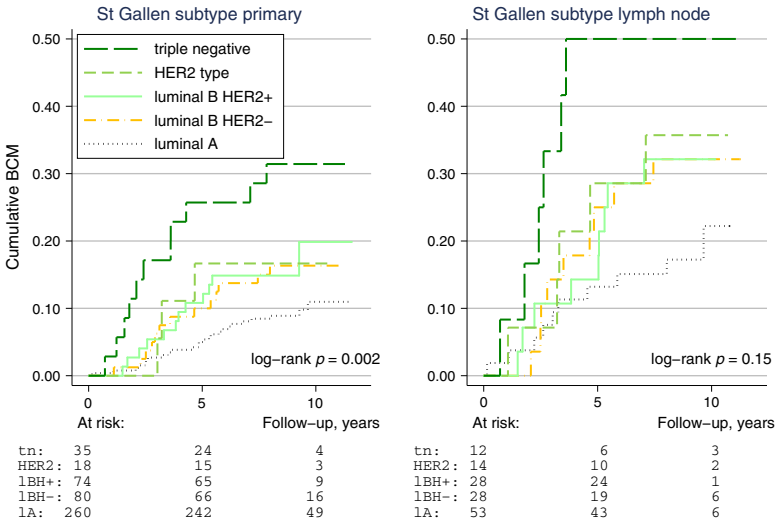

Fig. 3 Breast cancer mortality according to molecular subtype in primary tumours and lymph node metastases. *tn* triple negative, *HER2* HER2 type, *lBH* + luminal B HER2+, *lBH-* luminal B HER2−, *lA* luminal A

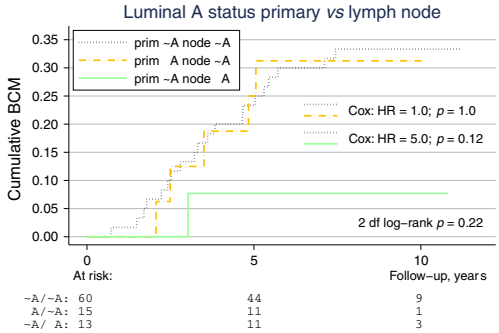

Fig. 4 Cumulative breast cancer mortality according to shifts in molecular subtype inherence between primary tumour and synchronous lymph node metastasis. *prim ~ A node ~ A* stable non-luminal A in primary tumour and synchronous lymph node metastasis, *Prim A node ~ A* shift from luminal A in primary tumour to non-luminal A in synchronous lymph node metastasis, *Prim ~ A node A* shift from non-luminal A in primary tumour to luminal A in synchronous lymph node metastasis, *HR* hazard ratio, *2 df log-rank* two degrees of freedom log-rank test

0.7–10.4, $p = 0.13$). No BCM was recorded among the 17 patients detected by screening with luminal A subtype in the lymph node metastases, including four patients with four or more metastatic nodes.

Discussion

Lymph node metastases are still an important negative prognostic factor for relapse and death in breast cancer, yet their removal seems to have little impact on patients' survival [1]. Moreover, the presence of lymph node metastases at the time of diagnosis has been associated with poorer survival after relapse compared with node-negative patients [30]. In spite of these findings, it is concluded that lymph node metastases are a sign of the extent of the disease rather than having any implications for tumour biology explaining the negative prognostic influence. In contrast, a large fraction of lymph node-positive patients will remain disease-free during the long-term follow-up of patients treated by loco-regional therapy alone [31]. In this study we have explored whether the prognostic heterogeneity of lymph node-positive breast cancer could be explained by molecular alterations in lymph node metastases.

The comparison of primary tumours and synchronous lymph node metastases in the present study showed a high concordance of biomarker expression with no statistically significant discordances. This is in line with what has been shown previously [7, 32] and further explored in a recent meta-analysis with respect to HER2 [11]. However, when a combination of biomarkers was applied and molecular subtypes defined according to St Gallen, a shift in subtype inherence was indicated. In this study, 15 patients shifted from luminal A in the primary tumour to non-luminal A in the synchronous lymph node metastases with a subsequent impact on prognosis, following the molecular subtype of the lymph node and not the primary tumour. In patients detected by screening with lymph node metastases, similar results were shown with the observation that patients with luminal A subtype in the lymph node metastases had an

excellent prognosis regardless of subtype inherence in the primary tumour; no BCM was recorded in this low-risk group in which 4/17 patients had four or more lymph node metastases. The finding in the present study of a low-risk group in a subset of patients with lymph node-positive disease has been described previously [33], and could identify a subgroup of patients where endocrine adjuvant treatment could be sufficient.

The assumption that the molecular characteristics of tumour cells are identical throughout tumour progression and that the expression of biomarkers in the primary tumour can guide clinicians' therapy decisions in relapses has been challenged repeatedly [9–11, 34]. The clinical implication of discordance rates between primary tumour and relapse has been evaluated in terms of therapy change with a reported change of the management of individual patients in 14–20 % of cases [8, 9, 35]. It is suggested that the mechanisms for biomarker conversion are explained by the heterogeneity of breast cancer tumours, with multiple different subclones existing side-by-side in the primary tumour [36] and by the clonal divergence described between primary tumours and metastasis [37–39]. The development of different subclones during tumour progression is suggested to be an early and ongoing process parallel with the development of the primary tumour with independent modification of genetic aberrations [2, 40].

The present study describes significant discordance between primary tumour and relapse for all assessed biomarkers and all patients with discordant *HER2* status gained *HER2* amplification in the relapse. Moreover, combining biomarker expression into molecular subtypes gave discordance rates showing a trend towards a molecular subtype with a worse prognostic profile being expressed in the relapse. Although the present study did not aim to record potential therapy changes, a cautious conclusion would be that the clinical implication of a re-biopsy would alter the management of particular patients who might benefit from targeted therapies according to their molecular subtype in the relapse.

In the present study, all TMAs were constructed, re-stained and re-evaluated by standardised methods for IHC and SISH. Samples were evaluated by two pathologists independently, with high concordance between them (data not shown). Several groups have compared biomarker expression on TMA and whole tissue sections, addressing the concern that the heterogeneity of breast cancer tumours would cause inadequately assessed expression when analysed on TMA. The concordance between TMA and whole sections for ER and HER2 has been demonstrated to be high. Generally, two 0.6 mm cores from predefined areas of invasive tumour cells seem to represent a whole tissue section [19, 41] thus, two or more 1.0 mm cores were used. In the present study, the cut-off values for biomarker expression are based on accepted guidelines [27, 42]. The cut-off value for ER responsiveness in clinical practice is traditionally 10 %, although there is support for a lower cut-off value of 1 % for endocrine treatment. The detection of any ER-positive cell in the tumour will therefore define it as an ER-responsive tumour [29]. While ASCO/PAP guidelines support the 1 % cut-off [27], the guidelines are questioned in a recent study [28]. The prognostic value of Ki67 has been investigated in several recent publications [20, 43, 44], but the assessment of the cut-off value for Ki67 is not settled, and the reliability of the measures varies in different geographic settings [29]. The present study used a predefined 20 % cut-off point based on the population sectioning, distinguishing the one-third of patients in the population having the highest proliferation from the remaining two thirds [45].

Patients with discordant biomarker expression, who exhibit a switch in molecular subtype during the progression from primary tumour via axillary lymph node metastasis to relapse, are assumed to represent a small subset of all breast cancer patients. Although the subset of patients with discordant molecular subtype is minor, the prognostic information may be relevant to individual patients. The implication is that large patient series are required for well-powered comparisons of prognoses for the discordant subgroups of patients. Like most previous studies of this topic, the present study is underpowered for comparisons of this kind, but it may nevertheless add an important piece to the puzzle. Biomarker or subtype shift may be of essential therapeutic significance for individual patients.

The present study shows that tumour biology is not stable throughout tumour progression in primary breast cancer. A shift towards a poor prognostic profile was noted in relapses, but no statistically significant shift was observed in lymph node metastases. Although there was no significant shift in molecular subtype in lymph node metastases, prognostic information for individual patients can be gained by analysing biomarker expression in synchronous metastatic lymph nodes. Including the analysis of lymph node metastases in research settings could provide more information about the molecular mechanisms involved in disease progression. Furthermore, the identified discordances in biomarker analyses in relapses supports biopsies in loco-regional and distant relapses.

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