Department of Pathology, Haartman Institute, and
Helsinki University Central Hospital, Helsinki, Finland
Municipal Authority for Medical and Social services in Kymenlaakso,
Kymenlaakso Hospital services, Pathology Department

TUMOR DORMANCY IN BREAST CANCER

Kristiina Joensuu

ACADEMIC DISSERTATION

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Supervised by
Docent Päivi Heikkilä
Departement of Pathology
University of Helsinki

Reviewed by
Docent Riikka Huovinen
Department of Oncology
University of Turku

and

Docent Pauliina Kronqvist
Department of Pathology
University of Turku

Opponent
Professor Ylermi Soini
Department of Pathology and Forensic Medicine,
Institute of Clinical Medicine, School of Medicine,
University of Eastern Finland, Cancer Center of Eastern Finland

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To Jyrki,
my husband of forty-two years
and my best friend

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LIST OF ORIGINAL PUBLICATIONS

I Joensuu K, Heikkilä P, Andersson LC. Tumor dormancy: Elevated expression of stanniocalcins in late relapsing breast cancer. Cancer Letters 2008; 265: 76-83.

II Joensuu K, Leidenius MH, Andersson LC, Heikkilä PS. High expression of maspin is associated with early tumor relapse in breast cancer. Human Pathology 2009; 40: 1143-1151.

III Joensuu K, Hagström J, Leidenius M, Haglund C, Andersson LC, Sariola H, Heikkilä P. Bmi-1, c-myc, and Snail expression in primary breast cancers and their metastases – elevated Bmi-1 expression in late breast cancer relapses. Virchows Arch 2011; 459:31-39.

IV Joensuu K, Leidenius M, Kero M, Andersson LC, Horwitz KB, Heikkilä PH. ER, PR, HER2, Ki67 and CK5 in early and late relapsing breast cancer - Reduced CK5 expression in metastases. Submitted.

ABBREVIATIONS

ADH	Atypical ductal hyperplasia
ALH	Atypical lobular hyperplasia
ASCO	American Society of Clinical Oncology
Bcl-2	B-cell lymphoma-2
BMI	Body mass index
Bmi-1	B-cell-specific Moloney murine leukemia virus Integration site I
CAP	College of American Pathologists
CGH	Comparative genomic hybridization
CISH	Chromogenic in situ hybridization
CK5	Cytokeratin 5
CSC	Cancer stem cell
DCIS	Ductal carcinoma in situ
DTCs	Disseminated tumor cells
CTCs	Circulating tumor cells
EMT	Epithelial-mesenchymal transition
EGF	Epidermal growth factor
ER	Estrogen receptor
HER2	(c-Erb-2) Human epidermal growth factor receptor 2
IHC	Immunohistochemistry
LN	Lobular neoplasia
LCIS	Lobular carcinoma in situ
Maspin	Mammary serine protease inhibitor
PR	Progesterone receptor
SCID	Severe combined immunodeficiency
STC-1	Stanniocalcin 1
STC-2	Stanniocalcin 2
TAAs	Tumor-associated antigens
TD	Terminal duct
TDLU	Terminal ductal lobular unit
TGF-beta	Transforming growth factor beta
UDH	Usual ductal hyperplasia
uPAR	Urokinase-type plasminogen activator receptor

ABSTRACT

BACKGROUND

Breast cancer is the most common cancer among women. 20–40% of breast cancers recur after initial treatment. Breast cancer has an unusual propensity of late recurrence even after decades of clinically undetectable disease. The biology behind this phenomenon of tumor silency, or tumor dormancy, is still poorly understood. There are currently no reliable tools to identify and therapeutically target a dormant breast cancer in its quiescent non-progressing state for preventing its recurrence.

To gain insight into the phenomen of tumor dormancy, various scientific approaches have been used, including animal models, cell culture studies, genetic and epidemiological investigations. Mathematical models have also been created to predict the progress of the disease.

The aim of this study was to analyze whether early and late relapsing breast cancers differ in their expression pattern of selected biomarkers, and whether the expression differs in relation to clinicopathological parameters.

This analysis is based on immunohistochemical (IHC) demonstration of the expression of different antigens in surgically removed breast cancer tissues. The expression levels of different biomarkers were compared between early- and late-relapsing breast cancers. Analysis of these differences may provide additional information for understanding the phenomenon of tumor dormancy in breast cancer.

METHODS

Metastatic breast cancers were identified from the database of the Department of Pathology, University of Helsinki (QPati database). The tumors were divided into three categories according to the time of recurrence after the initial intervention. Of 73 primary tumors, 19 had relapsed before 2 years, 33 after 5 to 10 years, and 21 after 10 years from the treatment of the primary cancer. The paraffin-embedded tissue or paraffin blocks from the metastatic tumors and their corresponding primary tumors were collected from the archives of the Department of Pathology/HUSLAB.

Nine antibodies against proteins known to have a prognostic role in breast cancer were evaluated by IHC. These nine markers included the glycoprotein hormones stanniocalcin 1 (STC-1) and stanniocalcin 2 (STC-2), the mammary serin protease

inhibitor maspin, the antiapoptotic factor Bcl-2, the mutated oncoprotein product p53, the transcription factors Bmi-1, c-myc, and Snail, and the basal-like cytokeratin CK5.

In addition the four established biomarkers used in clinical prognostication, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), and the proliferation marker Ki67, were studied by IHC. *HER2* gene amplification was further confirmed by chromogenic *in situ* hybridization (CISH), if the IHC result was positive (2+ or 3+).

For analysing the subtype approximations to the subtypes defined by genetic array testing, the tumors were divided to seven subtypes by using IHC: luminal A (ERorPR+HER2-), luminal B (ERorPR+HER2+), HER2 overexpressing (ER-PR-HER2+), triple negative (ER-PR-HER2-), basal-like (ER-PR-HER2-CK5+), unclassified (ER-PR-HER2-CK5-) and luminobasal (ERorPR+CK5+).

All statistical analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, ILL, USA).

RESULTS AND CONCLUSION

The proteins studied were divided into three categories according to their relation to tumor dormancy.

The expression of ER, STC-1, STC-2, Bcl-2, and Bmi-1 was associated with late relapse. The proteins associated with early tumor progression were: HER2, Ki67, p53, Maspin and CK5. The presence of PR, c-myc, and Snail did not correlate with the time of tumor recurrence.

INTRODUCTION

Regardless of the vast progress made in the diagnosis and treatment of breast cancer, distant metastases remain the main cause of breast cancer-related deaths. Even early detected breast cancers, with no detectable metastases, have the potential risk to recur later, years after the primary treatment. Breast cancer can remain dormant, not progressing for a long period of time and, for reasons not understood, can reactivate as a local relapse or distant metastasis. At present, the clinical data, the histopathological parameters, tumor size, histological type, grade, and axillary lymph node status, together with the classical biomarkers, ER, PR, HER2, and Ki67, determined from the primary tumors, are the main factors which constitute the guidelines for treating breast cancer patients. These parameters are powerful in predicting the time of cancer recurrence, but they do not give us tools for dealing with the dormant residual cancer cells.

Breast cancer is composed of a population of cells which are heterogenous in their molecular, genetic, and protein expression profiles, and so are the disseminated tumor cells (DTC) which escaped from the primary tumor. The heterogenous nature of the DTCs and the poorly understood mechanisms, which either keep these cells in a quiescuent state or awake them from dormancy are the main reasons why we can not therapeutically influence tumor dormancy and inhibit the cancer recurrence. This hampers the formulation of a tailored therapy.

The research on tumor dormancy has been mostly based on cell culturing and animal models, with focus on angiogenesis, immunology and cancer stem cell theory. The aim of investigations on tumor dormancy is to identify new biomarkers, *i.e* markers of tumor dormancy that would better predict the outcome of breast cancer patients and help to develop new therapeutic strategies.

In addition, there have been efforts to identify markers circulating in the blood of breast cancer patients in the remissive phase, which could be used for predicting an upcoming relapse. To be useful, such markers should be capable of foretelling whether the tumor is still dormant, or activated and starting to progress. If such markers were available, it could be possible to begin treatment already before metastatic dissemination of the tumor.

REVIEW OF THE LITERATURE

1. THE EPIDEMIOLOGY OF BREAST CANCER

INCIDENCE

Breast cancer is the most common cancer in Finland among women. Altogether, 4674 new breast cancers were diagnosed in Finland in 2010. The number of new breast cancer cases has steadily risen from 1953 to 2007 (Fig. 1). The annual number of breast cancer cases is predicted to rise from 4090 in the period 2005–2007 to 5119 in 2020, an increase of 25%.

The age-specific and age-adjusted incidence rate of breast cancer in Finland in 2006-2010 in all ages was 90.2 (www.finnishcancerregistry.fi) (Table 1). The incidence was highest in patients in the age category of 60-64 years, by 391.4 per 100,000. The youngest breast cancer patient in this time period was 15 years old. The highest rates concentrate to the menopausal and postmenopausal ages between the incidences 290.7 (50–54 years) and 389.8 (65–69 years) (Table 1).

Fig. 1 Annual numbers of cases of selected cancers in Finland in 1953–2007, and prediction until 2020, females, Finnish Cancer Registry 2010

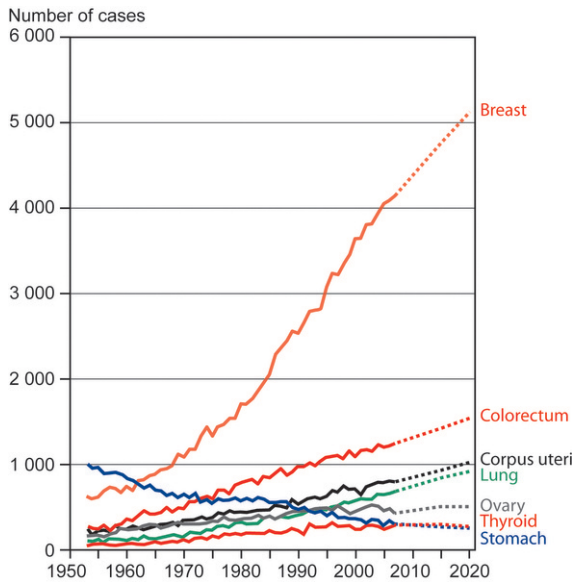


Table 1. Age-specific and age-adjusted incidence rates of female breast cancer in 2006–2010 per 100,000, Finland, Finnish Cancer Registry 2010

Age range (years)	15-25	30-39	40-49	50-54	55-59	60-64	65-69	70-84	all ages
Incidence	2.5	33.5	247.6	290.7	319.1	391.4	389.8	310.8	90.2

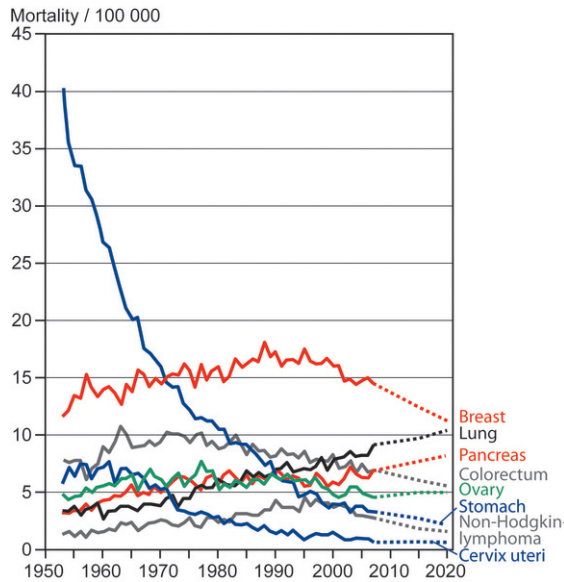
MORTALITY

The leading fatal cancer among women in Finland is breast cancer (Fig. 2). The number of breast cancer deaths in 2010 was 887, which comprises 16% of all 5543 female cancer deaths (mortality 14.7/100,000). The second highest cause of female cancer death is lung cancer, which is predicted to equal the mortality of breast cancer in 2020 (Fig. 2).

The annual number of cancer deaths in 2020 is predicted to increase by 13% in females and by 18% in males, compared with the recorded annual numbers of cancer deaths in 2005–2007. Breast cancer mortality has decreased slightly in the past few years, and the mortality has been estimated to decrease to the level of female lung cancer mortality by 2020 (Fig. 2).

The annual number of breast cancer deaths in females is predicted to rise from 846 in 2005–2007 to 894 in 2020 (+ 6%) and the age-adjusted rate/100,000 is predicted to decline from 14.8 in 2005–2007 to 11.3 in 2020 (-24%).

Fig. 2 Age-adjusted mortality from selected cancers in Finland in 1953–2007, and prediction until 2020, females. Finnish Cancer Registry 2010



SURVIVAL

The 5-year relative survival of breast cancer patients in Finland was 89% among patients diagnosed between 2002–2009, and newly observed in 2007–2009 (Finnish Cancer Registry 2010), Table 2.

Table 2. 1-year and 5-year relative survival ratios (%) of Finnish female cancer patients diagnosed in 2002–2009 and observed in 2007–2009 in the most frequent primary site, Finnish Cancer Registry 2010.

Primary site	1 year	5 years
All sites	79	65
Breast	97	89
Pancreas	19	3
Colon	78	61
Ovary	80	49
Non-Hodgkin's lymphoma	77	64
Stomach	48	25
Corpus uteri	93	82
Brain, central nervous system	78	69
Liver	18	3
Rectum, rectosigmoid, anus	84	65

The relative survival of women with breast cancer increased greatly from 1964 to 2003 in all five Nordic countries. The breast cancer incidence increased rapidly, but mortality nevertheless remained largely unchanged. The progress in 5-year relative survival was around 20 to 30%, and the greatest improvement was in Finland. The 10-year survival for breast cancer in the Nordic countries was highest during 1999–2003, with the highest rate in Finland, N: 172888, RS: 77% (CI 76–79) (Tryggvadottir *et al.* 2010).

The future scenario for breast cancer patients seems to be the following: there are consistently more women who live with primary and relapsed, treated breast cancers, and whose disease has taken a more chronic course. In Finland the number of prevalent breast cancers on January the first 2011 was 57,000 (Finnish Cancer Registry 2010).

2. ETIOLOGY OF BREAST CANCER

RISK FACTORS

Endogenous estrogen

Epidemiologic studies have shown that many risk factors increasing the probability of developing breast cancer are associated with life-long exposure to endogenous estrogen. Breast cancer occurs more frequently among women who have early menarche, who remain childless, or have only a few children, and the first delivery at a later age. Infertility, lack of breast feeding and a late age at menopause also increase the risk (Kesley *et al.* 1993). A lower risk for breast cancer has been reported in women who have had an early full-term pregnancy (Macmahon *et al.* 1970, Russo and Russo 1982). The protective effect of lactation appears to be limited to long-term cumulative breast feeding, preferably exceeding two years (Chang-Claude *et al.* 2000).

Kotsopoulos *et al.* analysed 4,655 ductal and 659 lobular cases of postmenopausal breast cancers from the Nurses Health Study (NHS) initiated in 1976 (Kotsopoulos *et al.* 2010). They found that the age at menarche, age at first birth, and postmenopausal hormone use was more strongly associated with lobular cancers than ductal cancers. These epidemiological analyses demonstrate that different types of breast cancer differ in their association with a number of risk factors, and indicate that breast cancers are heterogeneous diseases and may have different etiologies.

Exogenous hormones

The evidence points to a small increase in the relative risk associated with the use of combined oral contraceptives, especially among current and recent users. This is not related to the duration of use, or the type or dose of the preparation, and may partly be due to detection bias (IARC 1999).

Hormone replacement therapy (HRT) is linked to an increased risk of breast cancer according to the report from the Women's Health Initiative (Narod 2011). The risk of breast cancer increases after therapies with both estrogen and progesterone, when compared to estrogen alone. After cessation of HRT, the increased risk dissipates within two years. Factors associated with an increased risk of breast cancer include the initiation of hormone use immediately after menopause, a lean body mass, and high mammographic breast density (Narod 2011).

Familial risk of breast cancer

Familial risk for breast cancer has been known for over 100 years (Broca 1866). A review of 74 published studies (Pharoah *et al.* 1997) calculated a relative familial risk of 2.1 (95% CI 2.0 - 2.2) for breast cancer in any first degree relative, 2.3 for a sister affected, and 2.0 for an affected mother, and a relative risk of 3.6 if both an individual's mother and a sister were affected. The most important genes related to familial breast cancer are *BRCA1* and *BRCA2*, which explain about 20% of the overall familial risk of breast cancer with a higher risk at younger ages (Antoniou *et al.* 2001).

Nutrition

The report of the World Cancer Research Fund (WCRF 2007) has clearly established that overweight/obesity is a risk factor for developing breast cancer. Gaining weight during aging was associated with a statistically significant increase in the risk of breast cancer among both pre- and postmenopausal women (Cummings *et al.*2009).

A strong positive association with height was found among Norwegian women who were born during World War II. The study showed that early nutrition, during the period of gestation, may play a role in the etiology of breast cancer. (Nilsen *et* Vatten 2001).

To date, there is very limited information on the potential role of a specific dietary pattern in breast cancer survival (Hauner and Hauner 2010). Several prospective studies on fruit and vegetable consumption as well as on the intake of vitamins, minerals, and trace elements, have not revealed any significant association with breast cancer (Cummings 2009, WCRF 2007), and normal body weight is the only demonstrated protective factor to avoid breast cancer (Cummings *et al.* 2009).

Smoking and alcohol

In a study by Xue *et al.* (2011), breast cancer incidence was higher among current and past smokers, among those who had started smoking at a younger age, and those with a longer duration of smoking and more pack-years of smoking. Active smoking was associated with an increased risk of breast cancer among postmenopausal women. An association between passive smoking and an increased risk of breast cancer has also been suggested (Luo *et al.* 2011).

The most recent statistics on alcohol consumption and the risk of breast cancer were published in November 2011. Breslow *et al.* studied prospectively the association between the quantity and frequency of alcohol consumtion and cancer-specific mortality using the data of the National Health Interview Survey

(n=323,354). Women who drank more frequently tended to have an increased risk of breast cancer (Breslow *et al.* 2011). In the study of Chen *et al.,* 5.0–9.9 g of alcohol intake per day, equivalent to 3–6 drinks per week, significantly increased the risk of breast cancer (Chen *et al.* 2011).

Physical activity

The association between physical activity and reduced risk of breast cancer is independent of menopausal status. Among the most physically active women, the risk decreased by about 20–40%. Activity that is sustained throughout life, may be particularly beneficial (IARC 2002).

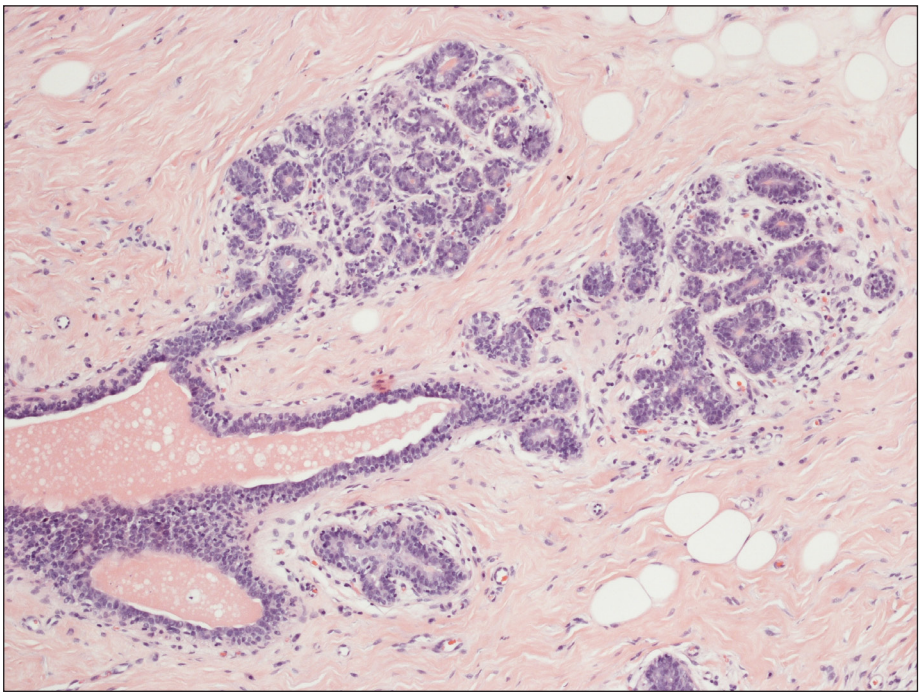
In their study, Zeng *et al.* determined the effect of physical activity on DNA methylation and predicted the consequence of this effect on gene expression and breast cancer survival. Their results suggested that increasing physical activity after a breast cancer diagnosis may affect epigenetic regulation of tumor suppressor genes, which has a favorable impact on the survival outcome of breast cancer patients (Zeng *et al.* 2011).

3. THE ORIGIN OF BREAST CANCER

Breast carcinoma is a malignant epithelial tumor characterized by the invasion of adjacent tissues and the ability to metastasize to distant sites. The vast majority of these tumors are adenocarcinomas believed to derive from the mammary gland parenchymal epithelium, particularly from cells in the terminal duct lobular units (TDLU, Fig 3), (Wellings *et al.* 1975). The hypothesis of ductal origin (Gallagher *et* Martin 1969, Levin *et al.* 1964, Sandison 1962) is based on the presence of preneoplastic epithelial hyperplasia in ducts. Sandison studied the histopathology of breasts in 800 autopsies, and found that the epithelial proliferations, both ductal and 'acinar' were more extensive in cancer breasts than in non-malignant breasts (Sandison 1962).

The human breast undergoes a complex series of changes from embryonic life to senescence. The developmental phase begins at puberty and includes the early stages of morphogenesis, from the formation of the nipple epithelium to lobule formation. Glandular development begins with growth and division of small bundles

Fig. 3 TDLU



of primary and secondary ducts, forming bulbous terminal end buds (TEB), which then progress to alveolar buds (Abs) that are smaller ductless structures clustered around the duct, forming a Type 1 lobule, also called TDLU, (Russo *et al.* 1990). Further gradual maturation from Type 1 lobules is a gradual process of sprouting of new Abs, called ductules. The full maturation of the breast ductules is attained during pregnancy and lactation, differentiating in this phase into ductules called alveoli and acini.

The mammary duct is composed of two layers of epithelial cells, the inner luminal epithelial and the outer myoepithelial compartment, and a basement membrane between the myoepithelial layer and the surrounding stroma (Gudjonsson *et al.* 2005).

Gudjonsson *et al.* have previously shown that a subset of the luminal epithelial cells could convert into myoepithelial cells in culture, signifying the possible existence of a progenitor cell. Later, they succeeded in identifying and isolating the putative precursor in the luminal epithelial compartment. By using cell surface markers and immunomagnetic sorting, they isolated two luminal epithelial cell populations from primary cultures of reduction mammoplasties (Gudjonsson *et al.* 2002). In clonal cultures, only the minor suprabasal derived epithelial cells were able to generate themselves as well as the major epithelial cells and myoepithelial cells, thus expressing stem cell properties with the ability to form entire TDLUs inside 3-D reconstituted basement membrane (Bartek *et al.* 1985, Gudjonsson *et al.* 2002). The first identification of cancer stem cells (CSCs) in solid tumors was demonstrated in breast cancer by Al-Hajj and colleagues in 2003. They used a model in which human breast cancer cells were grown in immunocompromised mice and were able to distinguish the tumor initiating from the nontumorigenic cancer cells based on cell surface marker expression (Al-Hajj *et al.* 2003).

GENE ALTERATIONS IN BREAST CANCER

Breast cancer is a genetically heterogeneous disease. As in malignancies in other anatomical organs, the development of breast cancer is a consequence of the accumulation of sequential genetic alterations, including activation of oncogenes (*e.g.* by gene amplification), such as the epidermal growth factor *EGFR, MYC, HER2* and *Cyclin D1* or inactivation tumor suppressor genes, such as *TP53*, the retinoblastoma gene *RB1*, the insulin-like growth factor II coding gene *IGF2R* and *CDH1* coding E-cadherin. (Ellis *et al.* 2003,b).

The variability in the gene expression of tumors can be measured by using microarray technology which quantifies the expression levels of thousands of genes (Perou *et al.* 2000). Despite the great variation in gene expression, there are

also striking similarities between tumors, providing new opportunities for tumor classification.

Metastatic activity presents the endpoint in a sequence of genomic changes underlying the progression of the epithelial cells into invasive tumor cells which cause a lethal disease. Metastases in general contain more genomic alterations than do their corresponding primary tumors (Hampl *et al.* 1999).

4. MORPHOLOGICAL CLASSIFICATION OF BREAST CANCER

INVASIVE BREAST CANCERS

The latest version of breast cancer classification dates from the year 2003 (Ellis *et al.* 2003,a). There are 19 histologically different subtypes of breast cancer. Invasive ductal carcinoma (Fig. 4 a, b,c) is the most frequent subtype, comprising 40–75% of all breast cancers. The next most common type is lobular carcinoma (Fig. 4 d) which represents 5–10% of breast cancers. The proportion of lobular carcinoma has increased during the past 20 years, probably due to HRT. Lobular carcinoma is found approximately 1–3 years later than ductal carcinoma (Li *et al.* 2000). The outcome of these usual types is often worse than that of the more rare subtypes. The histopathological grading is influenced by the amount of tubule formation, nuclear atypia and mitotic count (Elston and Ellis 1991).

NON-INVASIVE *IN SITU* CANCERS

The stage in malignant breast tumor development, at which the ductal luminal malignant epithelium does not yet invade through the basement membrane into the surrounding stroma, is called ductal carcinoma *in situ* (DCIS). DCIS was first recognized by Bloodgood in 1893 (Bloodgood 1934), and DCIS has been shown to arise within the TDLUs of the breast (Wellings *et al.* 1975).

DCIS is classified into three groups, i.e., low grade DCIS, intermediate grade DCIS, and high grade DCIS. The grading of DCIS is based on nuclear atypia, the polarity of cells and necrosis (Tavassoli *et. al* 2003a, Elston and Ellis 1998). The risk of DCIS to develop into invasive breast cancer has been estimated as 8–10-fold, compared to the risk for a reference population (Fitzgibbons *et al.* 2000).

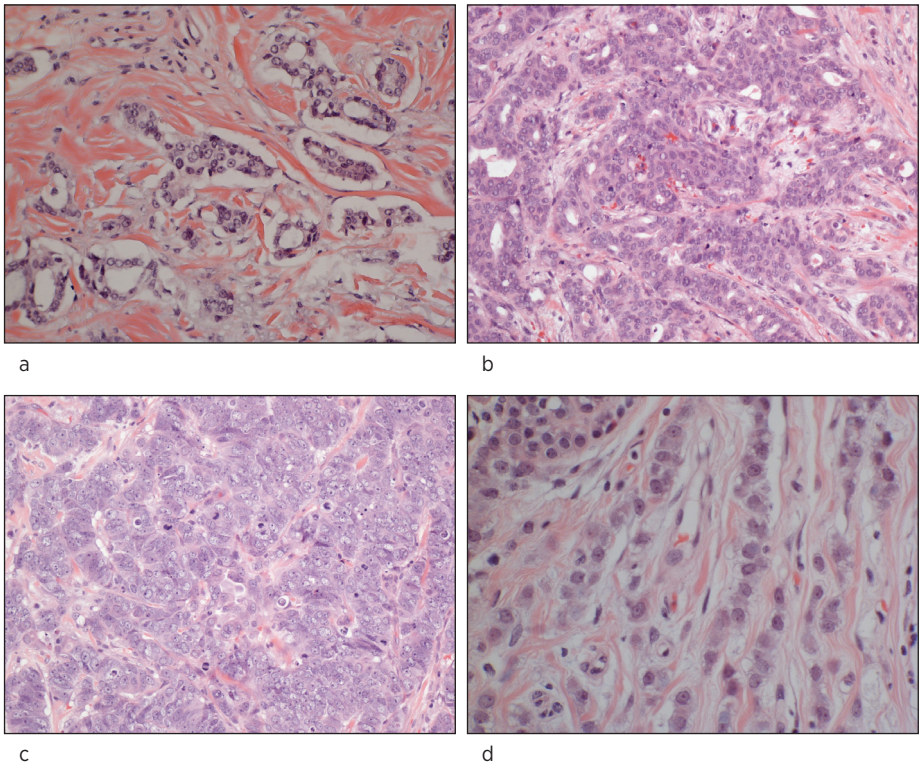


Fig. 4 Ductal carcinoma grade 1 (a), Ductal carcinoma grade 2 (b), Ductal carcinoma grade 3 (c), Lobular carcinoma (d)

LOBULAR NEOPLASIA

The histological features and the term lobular carcinoma *in situ* (LCIS) were characterized and assigned by Foote and Stewart in 1941 (Tavassoli *et al.* 2003b, Foote and Stewart 1941). The term lobular neoplasia (LN) includes the full spectrum of proliferation of the characteristic cell type within acini, ranging from atypical lobular hyperplasia (ALH) to LCIS. LN constitutes a risk factor and a non-obligatory precursor for the development of invasive breast cancer of either ductal or lobular type (Tavassoli *et al.* 2003b). The relative risk for subsequent development of invasive carcinoma among patients with LN ranges from 6.9 to about 12 times of that expected in women without LN (Anderson 1974).

5. PROGNOSTIC AND PREDICTIVE FACTORS

CLINICOPATHOLOGICAL PARAMETERS

Classical clinicopathological parameters guide the treatment of patients after initial surgery (Goldhirsch *et al.* 2009, Goldhirsch *et al.* 2011). In addition to clinical features, like age and menopausal status, the type of therapy is based on pathological reports derived from histological analysis of primary breast cancer samples, and include definition of tumor size, axillary lymph node status, histological grade, tumor type and lymphovascular invasion.

TRADITIONAL PROGNOSTIC AND PREDICTIVE BIOMARKERS ER, PR, HER2, KI67

Breast cancer treatment has undergone several changes in the past decades due to the discovery of specific predictive biomarkers that define different molecular subgroups of tumors that allow more individualized therapies. For over 30 years, the established molecular biomarkers, such as ER and PR, have been used to treat patients benefiting from endocrine therapy (Jordan 1993). HER2 has been validated not only as a prognostic factor, but also as a predictor of response to anti-HER2 therapy. The proliferation marker Ki67 has been used for over 10 years as a prognostic marker, and has recently emerged as an important marker having several applications in adjuvant and neoadjuvant therapy (Weigel and Dowsett 2010).

ER

The role of estrogen as the most important growth factor of breast cancer was first demonstrated by Beatson 1896, when regression of a metastatic tumor was induced by ovariectomy (Beatson 1896). ERs were first identified by Elwood V. Jensen in 1958 (Jensen and Jordan 2003). The gene for a second estrogen (ERbeta) was identified in 1996 by Kuiper et al. (1996). ERbeta and ERalpha share high sequence identity (Katzenellenbogen 1980), especially in the regions or domains responsible for specific binding to DNA and ligands (Kuiper *et al.* 1996). The current determination of ER positivity in breast cancer samples is based on IHC labeling of ERalfa.

Approximately 75% of breast cancers are ER-positive (Nadji *et al.* 2005). ER and PR belong to the steroid hormone receptor superfamily, including receptors for the steroid, retinoid, and thyroid hormones (Carcon-Jurica *et al.* 1990, Evans 1988). In

the classical estrogen-dependent pathway, estradiol (E2) binds ERalpha, and the resulting complex translocates into the nucleus, binds to estrogen response elements (ERE), resulting in the transcription of genes that promote cellular proliferation, for example those coding for cyclin-dependent kinases (CDK). Also non-genomic (i.e., not involving ERalpha-induced transcription) pathways of estrogen action have been described. E2 can stimulate phosphorylation of the adapter protein Shc and its interaction with ERalpha (Russo 2004; Tsai and O´Malley 1994).

Although 67% of breast cancers manifest during the postmenopausal period, the vast majority, 95%, are initially hormone-dependent (Hu *et al.* 2001). This indicates that estrogens play a crucial role in breast cancer development. However, it is still unclear whether estrogens are truly carcinogenic in breast tissue. Most of the current understanding of the carcinogenicity of estrogens is based on studies on experimental animals and on clinical observations (Hu *et al.* 2001).

Several studies have shown that the apparent effect of ER status on prognosis is limited to the early follow-up period (Lundin *et al.* 2006, Raemaerkers et al. 1985, Andry *et al.* 1989, Shek and Godolphin 1989, Crowe *et al.* 1991).

ER-positive tumors are more frequent among postmenopausal women than among younger women. Compared to patients with an ER-negative status, those with ER-positive tumors have a more indolent disease tending to display later recurrences, tumor dormancy, and a higher rate of bone metastases (Hess *et al.* 2003). The cellular and molecular mechanisms behind this distinct biological behavior are not known.

An estrogen receptor-positive status in breast cancer is associated with a positive response to hormonal therapy, a favorable prognosis, and a long disease-free life and overall survival (Vollenweider-Zerargui *et al.* 1986). Unfortunately, the correlation between an ER-positive status and endocrine dependence is not perfect. Approximately 40% of ER-positive tumors do not respond to endocrine therapy (Clark *et al.* 1983, Horwitz *et al.* 1975), suggesting that certain ER+ tumors do not regress with endocrine treatment due to a defect in the estrogen response pathway, distal to the binding step to EREs, thus leading to autonomous growth. Based on the finding that PR is induced by estrogen in normal reproductive tissues and in human breast cancer cells in culture, Horwitz et al. hypothesized that PR might be a better marker than ER for an intact estrogen pathway (Horwitz *et al.* 1975).

ER-positive breast cancer has the unfortunate propensity of recurring even after decades of remission. The long-term outcome for these patients is significantly worse than for patients with ER-negative tumors, regardless of menopausal status (Brewster *et al.* 2008).

PR

Approximately 55% of breast cancers are PR-positive (Nadji *et al.* 2005). Tumors expressing PR but not ER are uncommon, and represent < 1–1.5 % of all breast cancers (Viale et al. 2007, www.finnprog.fi). PR is also a member of the steroid/thyroid hormone nuclear receptor superfamily. It has two isoforms, PRalpha and PRbeta, which function as ligand-dependent nuclear transcription factors.

The prognostic and predictive value of PR expression has remained controversial. The results of randomized clinical trials in early breast cancer have shown that PR may add to the potency of ER for predicting the response to endocrine therapy in metastatic breast cancer (Early Breast Cancer Trialists 1998, 2005). Although ER and PR are members of different steroid hormone receptor subfamilies, there is evidence of crosstalk between ER and PR signalling pathways. In many cases, progestins suppress the stimulatory effects of estrogens in target cells. Estrogen has been shown to increase the expression of PR mRNA in uterine cells, and progestins suppress the stimulatory effects of estrogens in target cells. The molecular mechanisms underlying the antagonism of progestin on estrogen action are believed to be mediated via PR, but it is not known whether the ER protein or some other component of the ER signalling pathway is the target of repression (Kraus *et al.* 1995).

Liu *et al.* (2010) examined the value of PR for prognosis and the response to tamoxifen in a population-based series of 4,046 women with invasive early-stage breast cancer. Survival analyses for both the whole cohort and the ER-positive cases that were given tamoxifen therapy showed that patients with PR-positive tumors had better cancer-specific survival than PR-negative patients (Liu *et al.* 2010).

Cui et al. demonstrated that IGF-I (insulin-like growth factor-1) inhibits progesterone receptor expression in breast cancer cells *via* the phosphatidylinositol 3-kinase/akt/mammalian target of rapamycin pathway (Cui *et al.* 2003). Based on this observation, they suggested that low PR expression may indicate activated growth factor signalling in breast cancer cells, and therefore show an aggressive tumor phenotype and resistance to hormonal therapy. PR expression may define a subpopulation of breast cancer patients with a stronger dependence on hormone receptor-associated growth, and therefore a superior response to hormone therapy (Cui *et al.* 2005).

HER2

The epidermal growth factor (EGF) was discovered by Stanley Cohen and Rita Levi-Montalcini in the early 1970s. After grinding up submaxillary glands and applying the extract to the eyelids of newborn mice, the eyelids of the mice opened earlier than normally (Sliwkowski 2003). This work led to the discovery, not only of

epidermal growth factor (EGF), but also of its prototypical receptor tyrosine kinase, epidermal growth factor receptor (EGFR). The discovery of EGF by Stanley Cohen from Vanderbilt University led to the Nobel Prize in Physiology and Medicine in 1986. It was patented for cosmetic use by Greg Brown in 1989.

The Her2/neu gene (*c-erb-B2*) is a component of a four-member family of closely related growth factor receptors with tyrosine kinase activity, including epidermal growth factor receptors (EGFR or HER-1 (*erb-B1*), HER-2 (*erb-B2*), HER-3 (*erb-B3*), and HER-4 (*erb-B4*).

HER2, a new member of the tyrosine kinase gene family, was first described by King *et al.* (King *et al.*1985). The gene *erb-B2* was amplified in human mammary carcinoma and it encodes HER2, an integral plasma-membrane receptor tyrosine kinase that is normally involved in signal transduction pathways, leading to cell growth and differentiation.

Amplification of the (*c-erB2*) HER2 gene and RNA/protein over-expression correlate strongly (Pegram *et al.* 2000). Amplification of the HER-2/*neu* gene or over-expression of the HER2 protein has been identified in 10 to 34% of breast cancers, and HER2 over-expression is usually associated with an aggressive tumor phenotype and poor outcome (Ross and Fletcher 1998, Slamon *et al.* 1987). This group of patients benefits significantly from anti-HER2 therapies. In addition to the Herceptin treatment of patients with metastatic disease, adjuvant treatment of primary, HER2- positive breast cancers with trastuzumab has been shown to markedly improve the patient's outcome (Joensuu *et al.* 2009).

Ki67

Ki67 is a marker of cell proliferation first identified by Gerdes *et.al.* (1983), after immunization of mice with Hodgkin's lymphoma cells. Ki67 is a nuclear non-histone protein; it was named after the researcher's university. In this context, Ki stands for the Univercity of Kiel, Germany, and 67 refers to the number of the clone in the 96-well plate.

Ki67 expression varies during the cell cycle. Ki67 is expressed during G1, S, G2 phases and mitosis, but not during the resting phase G0 (Gerdes *et al.* 1984). Numerous studies have shown that Ki67 has prognostic value for many types of malignant tumors. In breast cancer, a strong correlation has been found between the proportion of cells positive for Ki67, nuclear grade, age, and mitotic rate (Sahin *et al.*1991, Keshgegian and Cnaan 1995). The advent of new genetic tests has emphasized the role of proliferative genes, including *Ki67*, as prognostic and predictive markers. There are, however, major financial issues that limit the use of multigene tests in daily practice. In addition to being more economical, immunohistochemical staining for Ki67 can be done in parallel with other immunohistochemical markers, and it can

be included in the initial pathology report of the core biopsy or surgical specimen (Yerushalmi *et al.* 2010).

Cheang *et al.* (2009) described an immunopanel of ER, PR, HER2, and Ki67 that can discriminate the hormone receptor-positive luminal A and B subtypes with an accuracy similar to that of a 50-gene expression profile. Luminal breast cancers in which at least 14% of all cells are Ki67-positive were assigned to the luminal B category with a worse prognosis. Both cancer recurrence and death were more common in patients having tumors with Ki67 positivity above 14% (Cheang *et al.* 2009, Yerushalmi *et al.* 2010).

GENE EXPRESSION PROFILING

Intrinsic breast cancer subtypes

Perou *et al.* (2000) showed that morphologically similar breast cancers can be divided into several groups, with different outcomes, based on their gene expression profiles. These studies revealed the existence of molecularly distinct neoplastic disorders which appear to originate from different cell types. Four molecular classes of breast cancer were distinguished with their 'intrinsic' classification: luminal A and B cancers, HER2-positive and basal-like cancers. Luminal cancers were almost all ER-positive, and they expressed typically cytokeratin (CK) 8 and 18. Luminal A tumors were histologically mostly low-grade, and luminal B represented high-grade tumors with a worse prognosis. The HER2-positive subtype of cancers that showed over-expression of HER2 protein and amplification of the *HER2* gene did not express hormone receptors, and had a poor prognosis. The basal-like breast cancers overlapped markedly with ER-, PR-, and HER2-negative (triple negative) tumors. They had a poor prognosis and expressed cytokeratins of the basal epithelial layer, CK 5/6 and CK17 (Sorlie *et al.* 2001, Sorlie et al 2003, Sotiriou *et al.* 2003).

Surrogate definitions of the intrincic subtypes by IHC

Gene expression profiling is not used in clinical practice to classify tumors because the methods are expensive, and there are no public algorithms available to define tumor classification based on gene expression profiling (Koscielny 2010). Instead of using gene expression profiles, clinicians use surrogates of the genetically defined subtypes. They divide the tumors into different subgroups, using IHC staining with specific antibodies to the hormone receptors, HER2, EGFR, and the basal cytokeratins.

The mRNA-based, 21-gene Genome Health Recurrence Score (GHI-RS) has been shown to be highly reproducible. It has become widely used in the United States, but not in many other countries due to its high cost. Cuzick *et al.* (2011) studied how much of the information of GHI-RS can be gained with the standard IHC markers ER, PR, Ki67, and HER2. They suggested that the amount of prognostic information obtained by these four widely used IHC assays is compatible to that obtained with the GHI-RS, and represents an inexpensive prognostic test battery for identifying women with a low risk of recurrence, who would therefore not benefit additionally from further chemotherapy.

The 12th St Gallen International Breast Cancer Conference (2011) Expert Panel adopted a new approach to classifying patients for therapeutic purposes. It is based on the recognition of intrinsic biological subtypes within the breast cancer spectrum. For practical purposes, the IHC surrogates to the intrinsic subtypes were defined as follows: luminal A= ERorPR+HER2-, luminal B= ERorPR+HER2+, HER2 overexpressing (ER-PR-HER2+), triple-negative (basal-like)=ER-PR-HER2- (Goldhirsch *et al.* 2011). A modified subtype definition was used by Cheang *et al.* and Carey *et al.* who further distinguished the triple negative basal-like phenotype subtype by EFGR and CK5 to a more specific definition of basal-like breast cancer which better predicts breast cancer survival. They defined the basal-like = ER-PR-HER2-CK5+ and unclassified= ER-PR-HER2-CK5- IHC subtypes (Cheang *et al.* 2008, Carey *et al.* 2006). Including EGFR and CK5 as positive IHC markers, has previously been shown to accurately identify basal-like tumors from gene microarray data with 100% specificity and 76% sensitivity (Nielsen *et al.* 2004). Later, a new entity between basal and luminal breast cancer, the basoluminal subtype (ERorPR+CK5+) was identified (Laakso *et al.* 2006).

6. METASTATIC DISSEMINATION

The understanding of events in the metastatic cascade of tumors is essential for finding factors that regulate the balance between tumor dormancy and escape from the dormant state (onset of metastases) (Pantel *et al.* 2009). Distant metastatic cascades of solid epithelial tumors are formed when individual cancer cells detach and lose their adhesion to adjacent cells in the primary tumors, and disseminate *via* hematogenous or lymphatic routes as circulating tumor cells (CTC). They begin to grow and form one or more recurrent/metastatic tumors, at distant sites and organs from the primary tumor.

The malignant metastatic capability of tumors must be an inherent propensity compared to benign neoplasms and, for example, basaliomas, which have the capacity to invade but not to metastasize. It is currently believed that the metastatic property is achieved at an early state of cancer development, and those DTCs and CTCs which have achieved this genetic stage possess this capability.

Solid epihelial tumors differ in their patterns to metastasize. For some tumor types, e.g. head and neck cancer, the correlation between lymph node metastases and distant metastases is strong. For breast cancer, this correlation is less evident. Approximately 20–30% of breast cancer patients who are free of axillary lymph node metastases develop distant metastases. So, breast tumor cells can bypass the lymph nodes and disseminate directly through the blood to distant organs. Gene expression profiling studies have shown that the molecular pathways for lymphatic dissemination differ from the hematogenous pathway (Wölfle *et al.* 2003). In breast cancer patients, hematogenous dissemination seems to be a very early event in tumor progression, so the disseminated tumor cells (DTC) found in the bone marrow might represent 'immature' tumor cells, as they have a limited life span and usually do not proliferate. Nevertheless, the presence of these DTCs accurately predicts the development of distant metastases. Some of the disseminated tumor cells start to proliferate at the distant site and acquire genetic mutations independent of those in the primary tumor. Some factors contributing to metastasis formation are listed in Table 6 (sectio 8, page 39).

7. TREATMENT OF BREAST CANCER

TREATMENT OF EARLY BREAST CANCER

Early breast cancer means a state in breast cancer development in which the disease is detected only in the breast, or in the case of axillary node-positive women, the breast and locoregional lymph nodes, and the entire detected disease can be removed surgically. However, undetected deposits of disease may remain either locally or at distant sites and if untreated, could over the next 5, 10, 15, or more years develop into a life-threatening clinical recurrence. The main aim of systemic adjuvant treatment is to control any remaining deposits of disease, to reduce the recurrence rate.

The 11th St Gallen (Switzerland) Conference on the Primary Therapy of Early Breast Cancer in 2009 proposed a new treatment selection algorithm for the management of early breast cancer (Goldhirsh *et al.* 2009). The Consensus Meeting considered the aspects of local and regional treatments, including surgical margins, indication for sentinel node biopsy, and the role of prophylactic mastectomy. Re-excision was considered mandatory if invasive cancer or DCIS is present at the inked surgical margin.

The Consensus listed the different criteria to clarify the therapeutic decision-making algorithm, which addresses the three distinct questions: What justifies the use of endocrine therapy?; What justifies the use of anti-HER2 therapy? and; What justifies the use of chemotherapy? (Goldhirsch *et al.* 2009, Table 3).

Any positive level of ER expression is considered sufficient to justify the use of endocrine adjuvant therapy in almost all patients. Over-expression or amplification of HER2 by standard criteria is an indication for anti-HER2 therapy for all but the very lowest risk invasive tumors.

The threshold for using cytotoxic chemotherapy is the most difficult one to define. Patients receiving anti-HER2 therapy conventionally also receive chemotherapy either preceding or concurrent with anti-HER2 treatment. Chemotherapy is the mainstay of the adjuvant therapy of patients with triple-negative disease who are at sufficient risk of relapse so as to justify its utilization.

The threshold for recommending chemotherapy for patients with ER-positive, HER2-negative disease is particularly difficult to define. These patients include a spectrum ranging from those at low risk for whom there is little evidence supporting the addition of chemotherapy to endocrine therapy, to those with a high-risk disease and limited ER expression, for whom chemotherapy appears clearly justified. Chemoendocrine therapy guidelines in ER-positive HER2-negative disease are summarized in Table 4 (Goldhirsch *et al.* 2009).

Table 3. Thresholds* for treatment modalities

Treatment modility	Indication	Comments
Endocrine therapy	Any ER staining#	ER-negative and PR-positive are probably artefactual (Ibrahim et al. 2008)
Anti-HER2 therapy	ASCO/CAP HER2-positive (>30% intense and complete staining (IHC) or FISH>2.2+)#	May use clinical trial definitions
Chemotherapy in HER2-positive disease (with anti-HER2 therapy)	Trial evidence for trastuzumab is limited to use with chemotherapy or after it	Combined endocrine therapy + anti-HER2 therapy without chemotherapy in strongly ER-positive, HER2-positive is logical but unproven
Chemotherapy in triple-negative disease	Most patients#0	No proven alternative; most at elevated risk
Chemotherapy in ER-positive, HER2-negative disease (with endocrine therapy)	Variable according to risk#	See Table 4

* Most factors are continuous, but a binary decision needs to be made at some level.

\# Patients with tumors >1cm in size without axillary nodal involvement and without other features indicating increased metastatic potential (e.g. vascular invasion) might not need adjuvant systemic therapy. If the tumor is, however endocrine-responsive, endocrine therapy should be considered.

0 Medullary carcinoma, apocrine carcinoma, and adenoid cystic carcinoma do not require chemotherapy due to their low risk despite being triple negative (provided that, as is usually the case, they have no axillary node involvement and no other signs of increased metastatic risk).

ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists

Goldhirsch et al 2009

Table 4. Chemoendocrine therapy in patients with ER-positive, HER2-negative disease

	Relative indications for chemoendocrine therapy	Factors not useful for decision	Relative indications for endocrine therapy alone
Clinicopathological features ER and PR	Lower ER and PR level		Higher ER and PR level
Histopathological grade	Grade 3	Grade 2	Grade 1
Proliferation	High*	Intermediate*	Low*
Nodes	Node positive (4 or more involved nodes)	Node positive (1–3 three involved nodes)	Node negative
PVI	Presence of extensive PVI		Absence of extensive PVI
pT size	>5 cm	2.1–5 cm	<2 cm
Patient's preference	Use all available treatments		Avoid chemotherapy-related side-effects
Multigen assays Gene signature0	High score	Intermediate score	Low score

* Conventional measures of proliferation include assessment of Ki67-labelling index (e.g. low, <15%; intermediate, 16–30%; high, >30%) and pathological description of the frequency of mitoses.

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Table 5. Systemic treatment recommendations for subtypes

Subtype	Type of Therapy	Note of therapy
Luminal A	Endocrine therapy only	Few require cytotoxins (e.g. high nodal status or other indicator of risk: see text).
Luminal B (HER2 negative)	Endocrine ± cytotoxic therapy	Inclusion and type of cytotoxins may depend on level of endocrine receptor expression, perceived risk and patient's preference.
Luminal B (HER2 positive)	Cytotoxic + anti-HER2 + endocrine therapy	No data are available to support the omission of cytotoxins in this group.
HER2 positive (nonluminal)	Cytotoxins + anti-HER2	Patients at very low risk (e.g. pT1a and node-negative) may be observed without systemic adjuvant treatment.
Triple negative (ductal)	Cytotoxins	
*Special histological types		
A. Endocrine responsive	Endocrine therapy	
B. Endocrine nonresponsive	Cytotoxins	Medullary and adenoid cystic carcinomas may not require any adjuvant (if node-negative).

* Special histologic types: Endocrine responsive (cribriform, tubular, and mucinous); Endocrine nonresponsive (apocrine, medullary, adenoid cystic and metaplastic).

Goldhirsch et al 2011

The 12th St Gallen International Breast Cancer Conference in 2011 adopted a new approach to the classification of patients for therapeutic purposes based on the recognition of intrinsic biological subtypes within the breast cancer spectrum. For practical purposes, the subtypes may be approximated using clinicopathological rather than gene expression array criteria. In general, systemic therapy recommendations follow the subtype classification, and the systemic treatment recommendations for subtypes are shown in Table 5 (Goldhirsch *et al.* 2011).

The primary treatment of early breast cancer is based on surgery of the primary tumor and the surgical manipulation of the axilla. In the last decade the method of sentinel node biopsy has partly replaced the conventional evacuation of the axillary lymph nodes.

The Panel in the 2011 Consensus meeting expressed the view that the routine use of IHC to search for low-volume metastatic disease in sentinel nodes was not indicated, since metastases revealed only by IHC would not alter the management protocol. Furthermore, isolated tumor cells, and even metastases up to 2 mm (micrometastases) in a single sentinel node, were not considered to constitute an indication for axillary dissection, regardless of the type of breast surgery carried out. The Panel accepted the option of omitting axillary dissection of macrometastases

in the context of lumpectomy and radiation therapy for patients with a clinically node-negative disease and 1–2 positive sentinel lymph nodes, as reported from ACOSOG trial Z0011 with a median follow-up of 6.3 years. This practice should not, however, be extended more generally, for instance to patients undergoing mastectomy, those who will not receive whole-breast tangential field radiation therapy, or those with involvement of more than two sentinel nodes, as well as patients receiving neoadjuvant therapy (Goldhirsch *et al.* 2011).

TREATMENT OF ADVANCED BREAST CANCER

Overall, survival of patients with metastatic breast cancer (MBC) is slowly but steadily improving. Every year, the risk of death has decreased by 1–2% (Giordano *et al.* 2004). The greatest improvement is most probably related to the development and widespread availability of modern systemic therapies. A distinct subset of MBC patients who are most likely to gain substantial benefit from an intensified multidisciplinary therapeutic approach is represented by 'oligometastatic' disease, which is characterized by solitary or only few detectable metastatic lesions that are usually limited to a single organ (Pagani et al. 2010). This population of "potentially curable" Stage IV disease is estimated to be 1–10% of newly diagnosed MBC patients (Hanrahan *et al.* 2005).

In contrast to early stage disease, for which Level 1 evidence exists for the majority of treatment options, there are few recognized therapeutic standards for advanced breast cancer (ABC), particularly after 1st line treatment. The advances have been slow and the median overall survival for patients with MBC is still only 2–3 years, although the range is wide. For HER2-positive ABC the development of anti-HER2 agents has effectively led to a substantial improvement in the survival of these patients. However, for triple-negative ABC no significant improvement in survival has yet been achieved, and ER-positive ABC, the most common subtype, overall survival has remained stable since the early nineties (Foukakis *et al.* 2011).

There are several widely used international and national guidelines for early stage breast cancer. The situation in markedly different for ABC and particularly MBC, for which only national efforts have been made. Acknowledging the urgent need for international concord in this field, the European School of Oncology (ESO) created an ABC Task Force in 2005, aiming to develop international consensus guidelines for managing ABC which could be applied worldwide, and also to identify areas where research/clinical trials are urgently needed. This Task Force has held public and interactive sessions during three consecutive European Breast Cancer Conferences, followed by the publication of manuscripts reviewing the available data and issuing the Task Force's recommendations on several issues. This work also led

to the establishment of the 1st International Consensus Guidelines Conference on ABC (ABC 1) held in November 2011. Cardoso *et al.* (2012) published the summary of the guidelines developed on ABC 1.

8. THE PHENOMENON OF TUMOR DORMANCY

BACKGROUND OF TUMOR DORMANCY

Cancer dormancy is considered to be a protracted stage in progression, during which tumors remain occult and asymptomatic for a prolonged period of time. Tumor dormancy can present one of the earliest stages in tumor development, as well as a stage in micrometastasis or minimal residual disease left after surgical removal and treatment of primary tumors. Tumor dormancy, therefore, can occur in primary as well as in secondary tumors (Udagawa 2008). The former type of dormancy can be called primary dormancy and the other metastatic dormancy (Klein 2011). Delayed recurrences are typical in breast cancer.

The fact that dormant tumors are highly prevalent in the general population is of clinical importance (Harach *et al.*1985, Black and Welch 1993, Nielsen *et al.* 1987). In addition, the dormant tumor cells remaining after primary tumor removal or treatment are commonly refractory to chemotherapy (Aguirre Chiso 2007, Wikman *et al.* 2008).

The progression of a malignant solid tumor capable of invading the surrounding stromal tissue and of disseminating, *i.e.* sending metastatic tumor cells from the primary tumor to distant organs, was earlier thought to have a continuous pattern. Several mathematical models for tumor growth have been developed, such as the exponential pattern of tumor growth, the continuous deceleration theory by Mayneord 1932, and the model of tumor cell proliferation by the Compertz equation. Compertzian growth kinetics, *i.e.* near-regular exponential growth at small cell numbers with decelerated growth at larger numbers, has been widely utilized for planning treatment regimens (Norton 1988). A continuous growth model, however, is not consistent with the results of several clinical studies.

Continuous growth was unable to explain the time distribution of first-treatment failure in 1173 breast cancer patients undergoing mastectomy alone (Demiceli *et al.* 1996). Indeed, the cause-specific hazard function for local-regional recurrences and distant metastases displayed an early peak at approximately 18 months, a second peak at about 60 months, and then a tapered plateau-like tail extending up to 15 years.

As an alternative to the continuous growth model, the tumor dormancy hypothesis was considered to provide a more reasonable description of tumor recurrence (Demiceli *et al.* 1997). The term "dormancy" dates back to Hadfield (1954) in the first half of the 20th century, and was introduced by the pathologist Rubert A. Willis in his book "The spread of tumors in the human body". The current

definition of tumor dormancy is mainly based on observations and hypotheses. Based on observations, dormancy has been defined as a prolonged latent phase (at least 5 years) (Hadfield 1954) that occurs between primary treatment and further disease progression. It was assumed that the dormant tumor cells are in a state of temporary mitotic arrest during the long latent, dormant period of cancer (Hadfield 1954). It has been hypothesized that primary tumors shed tumor cells already at an early stage into the blood circulation (Butler and Gullino 1975, Fidler 1970). Today, tumor dormancy is simply understood as a stage in cancer progression in which residual disease is present, but not clinically apparent (Páez *et al.* 2011). The prevalence of clinical dormancy is unknown. When 20-year follow-up data from different periods in medical history are compared, the percentage of dormancy cases seems to have doubled in 40 years (Klein 2011, Joensuu and Toikkanen 1991). A clinical dormancy definition would thus need to be re-adjusted to actual diagnostic and therapeutic procedures after decades of observation, and therefore would be always out-dated.

CLINICAL AND MOLECULAR MECHANISMS UNDERLYING TUMOR DORMANCY

The detection of dormant tumor cells has been extremely difficult in clinical settings. Evidence of clinical tumor dormancy has been collected from autopsies of trauma victims (Harach *et al.* 1985, Nielsen *et al.* 1987), from clinical data on late recurrence or relapse, and from findings of disseminated tumor cells (DTCs) and circulating tumor cells (CTC) in cancer patients, even years after the treatment of primary tumors.

In recent years, extensive molecular and genetic characterization of DTCs and CTCs has contributed significantly to our understanding of the frequency and prevalence of tumor dormancy (Riethdorf *et al.* 2008, Pantel *et al.* 2009). Isolated tumor cells and micrometastases have been detected in the bone marrow of primary breast cancer patients (Wiedswang *et al.* 2003, Diel *et al.* 1996). Although patients with disseminated tumor cells have a higher risk of relapse, not all patients will develop recurrent disease (Janni *et al.* 2006).

Experimental studies have shown that early steps in hematogenous metastasis (intravasation, survival, arrest, and extravasation) can be remarkably efficient, with greater than 80% of the cells successively completing the metastatic process to this point.

However, only a small subset of these cells (~ 2%, depending on the experimental model) can initiate growth as micrometastases, and an even smaller subset (~ 0.02%, depending on the experimental model) are able to persist and grow into macroscopic tumors (Allan *et al.* 2007).

The poorly understood mechanisms leading to growth activation and establishment of metastases have been intensively studied. Evidence shows that several different factors may contribute to growth activation, including the genetic predisposition of the dormant cells, as well as the immunological and angiogenetic impact of the surrounding environment (Fehm *et al.* 2008)

In order for the cancer cell to succeed in developing a late, detectable metastatic tumor, it must first be able to escape from the cohesion of its primary tumor, and to invade the surrounding stromal tissue. Thereafter, the disseminated tumor cell (DTC) must reach the lymph or blood as a circulating tumor cell (CTC), and then extravasate to the target organ parenchyma. At this stage, tumor cells have four possible fates: 1) they die (the vast majority of cells undergo apoptosis or are killed by immune cells); 2) they can enter a state of quiescence or dormancy – either as a single solitary cell, or; 3) as a micrometastatic lesion without the capacity of proliferative expansion or they can recruit a vascular bed, or; 4) they can resume proliferation and form growing micrometastases.

Based on cell culture and animal models, dormancy can occur at two different levels, as single dormant cells and as micrometastases. During the dormancy stage, sub-clinical disease may be caused by dormant cells that have entered a G0-G1 arrest (cellular dormancy), and these cells may develop mechanisms to evade immune system recognition and eradication. In the dormancy of micrometastases, there is a balance between the proliferation rate and apoptosis, with no net increase in the cell number.

GENETIC AND EPIGENETIC MECHANISMS OF TUMOR DORMANCY

Genetic mechanisms

Earlier, it was thought that genetically fit tumor cells that emerge from the primary tumor (which is proposed to be a late event in cancer progression) are able to metastasize (Hanahan *et.* Weinberg 2000, Talmadge *et al.* 1982). This was thought to be due to the time needed for tumor cells to mutate and acquire traits that allow them to pass through the different steps of metastatic development. However, recent studies suggest that tumor cell dissemination might occur early (i.e. is, by less genetically altered cells) and the disseminated tumor cells then progress towards more aggressive phenotypes that lead to metastatic growth in parallel with the primary tumor (Schardt *et al.* 2005). If disseminated tumor cells were to remain after treatment in a continuously proliferative phase, the relapse would be expected to occur much earlier than is actually observed (Demiceli 2001). Therefore, a decline in progressive properties (i.e., dormancy) is considered to be the most likely

explanation for the discrepancy between the estimated and observed disease-free periods (Demiceli 2001). Tumor dormancy is observed in local recurrences or distant metastases. In primary tumors, the term commonly used is latency: the time that separates the carcinogenic insult from the clinical detection of the primary tumor.

With the development of techniques to detect rare cells, including phenotyping and genotyping of minimal residual disease, new insights and theories are beginning to emerge (Meng *et al.* 2004, Fehm *et al.* 2005). Klein *et al.* (1999) developed a PCR strategy which enables a comprehensive analysis of the entire genome on a single-cell level. The ectopic localization of epithelial cells in bone marrow by itself does not prove their malignant nature. However, comparative genomic hybridization (CGH) and sequence findings from the isolated cytokeratin-positive cells indicate that these cells are indeed tumor cells (Klein et al. 1999).

When Klein and Hölzel analyzed the genom of DTCs, they found considerable differences in chromosomal abnormalities between primary tumors and the tumor cells isolated from bone marrow of breast cancer patients without manifested metastases (Klein et Hölzel 2006). Indeed, the tumor cells taken from bone marrow harbored significantly less genomic aberrations than did the primary tumors. For example, only 14% of M0 stage breast cancer patients with single chromosomally abnormal DTCs in their bone marrow showed shared aberration between primary tumors and the analyzed cytokeratin-positive cells. When the authors analyzed the normal-appearing cells at higher resolution for small DNA deletions or amplifications, they observed that most of these normal-appearing cells were indeed tumor cells derived from breast cancer. Therefore, in at least 95% of cases, no similarity for chromosomal aberrations could be established between the primary tumors and the cytokeratin-positive tumor cells in the bone marrow of breast cancer patients (Klein *et* Hölzel 2006).

In summary, based on the above observations (Klein *et* Hölzel 2006), the model of linear progression of cancer is challenged by the following facts: first, since CGH detects genetic changes that are present in at least 60% of primary tumor cells, this predominant cell population disseminates only exceptionally. Second, since DTCs display lower numbers of genetic changes per cell than the abnormalities found in the primary tumors, this may mean that they are not derived from the primary tumors in the phase present at diagnosis. Rather, they may originate from earlier stages of the cancerous lesion. Third, both the reduced relative number of migrating cancer cells in large tumors and the absence of typical genetic changes in the DTC population suggest that the cells in the primary tumor are highly selected for stationary growth and are probably unable to disseminate. Thus there is no evidence for genetically more advanced, fully malignant cells leading to human breast cancer metastasis.

At the time of surgery, more than 95% of disseminated breast cancer cells in the bone marrow display either none, fewer or different chromosomal abnormalities, compared to the primary tumor (Klein et Hölzel 2006). The findings from DTCs

suggest that a long time is needed to accumulate genetic changes required for metastasis. The cytokeratin-positive single DTCs from patients without manifested metastases displayed very different chromosomal changes – as long as the patients remained in the stage of minimal residual disease. However, in more than 95% of patients who presented metastases, the individually isolated and analyzed cytokeratin-positive cells from the bone marrow were highly similar. This suggests that tumor cells that have disseminated early are genetically unstable; they diverge considerably until one clone acquires genetic abnormalities enabling colonization and growth at a distant site (Klein et Hölzel).

The interesting question is: how can a dormant single cancer cell or micrometastasis be activated to escape the dormant state and to manifest metastasis?

The different factors contributing to tumor dormancy can be divided roughly into those awakening cells from dormancy, *i.e.* activating metastatic development, and into those suppressing metastatic development and tumor dormancy (Pantel *et al.* 2009) (Table 6).

Experimental studies on tumor dormancy have disclosed genes that encode the MKK4 and Kiss1 metastasis suppressors, as well as Bcl-xL and alpha5beta1 integrin-fibronectin as apoptotic inhibitors, and stanniocalcins as survival factors (Pantel *et al.* 2009). Analyses of bone marrow and blood from patients with breast cancer have revealed that upregulation or re-expression of HER2 and the urokinase-type plasminogen activator receptor (uPAR) in DTCs could serve as a switch to interrupt dormancy (Wülfing *et al.* 2006, Solomayer *et al.* 2006).

Epigenetic mechanisms

Although the effects of epigenetic changes on cancer development have been extensively investigated, their influence on tumor dormancy has not been resolved. Epigenetic changes, such as pathological methylation patterns, have been postulated to occur in an early stage of tumor progression, before chromosomal aberrations (Jones *et* Baylin 2007). Methylation of homeobox genes is a frequent and early epigenetic event in breast cancer (Tommasi *et al.* 2009). Theoretically, it is possible that such mechanisms contribute to latency (dormancy) in the development of the primary tumor, *i.e.* the time between the carcinogenic insult and the clinical detection of the tumor.

Research in the past two decades has found a group of genes specifically targeted at metastatic suppression. The genes influencing the formation of distant metastases are distinct from the genes involved in the growth of the primary tumor (Metge *et al.* 2008, Shevde and Welch 2003). Metge *et al.* showed that BRMS1 (breast cancer metastasis suppressor-1) prevents metastasizing of breast cancer and melanoma in athymic mice. They showed that the preventive function is down-regulated in a

Table 6. Factors regulating the balance between sustaining the dormant state of a tumor (+) or escape from dormancy (- onset of metastasis)

Dormancy	Protein	Effect
+	p16ink4A	tumor-suppressing factor
+	MKK4, MAP2K4- mitogen-activated protein kinase 4, metastasis suppressor	induces apoptosis, regulates apoptosis induced by TNF (tumor necrosis factor)
+	KISS1, Metastin, metastasis suppressor	reduces the activity of the matrix metalloproteinase (MMP-9) and invasion
+	BRMS1, Breast cancer metastasis suppressor	suppresses anti-apoptotic genes controlled by NF (necrosis factor) -kappa-B
+	Stanniocalcin, STC1 ja STC 2, glycoproteinlike hormone	survival and differentation factors
+	TSP thrombospondin	anti-angiogenic factor
+	Type I interferons, IL-7 and IL-15	T-cell survival cytokine, anti-tumor T-cell memory
+	p38alpha and/or beta, mitogen activator apoptotic/growth suppressive stress-activated protein kinase 2 (p38)	induce apoptosis, arrest growth, promote senescence
+	p53	tumor suppressor, activates p38
+	Maspin	tumor suppressor, apoptotic inducer, anti-angiogenic factor, metastatic suppressor
-	Estrogen	cell proliferation, tumor growth
-	Progesterone	activates breast cancer stem cells
-	Bcl-2, Bcl-xL	inhibit apoptosis
-	alpha5beta1 integrin-fibronectin	inhibits apoptosis
-	VEGF, vascular endothelial growth factor	stimulation of 'angiogenic switch'
-	HER2/neu, human epidermal growth factor 2, proto-oncogene	encodes the cell membrane receptor tyrosine kinase, involved in the signal transduction pathways leading to cell growth and differentiation
-	uPAR urokinase receptor	regulation of ERK/p38, activation of alpha5beta1 integrin
-	e-cadherin	stimulates the EMT
-	CD44+/CD24-/CK+	stem cell properties

metastatic disease and that this down-regulation is not caused by deletion of the gene, but via epigenetic silencing. This kind of altered methylation of the *BMRS1* gene promoter is an example of epigenetic silencing of gene expression (Metge *et al.* 2008).

Micro-RNAs

It is becoming accepted that micro-RNAs play a critical role in cancer. Micro-RNAs are small (approximately 22 nucleotides long), non-coding RNA molecules, which regulate gene expression after transcription. They are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression and gene silencing (Bartel 2009).

Micro-RNAs can act as either oncogenes or tumor suppressors in tumor development (Nicoloso *et al.* 2009). Moreover, one micro-RNA can presumably affect the transcription of hundreds of target genes. Identification of micro-RNAs associated with tumor dormancy and manipulation of their expression levels could lead to prolonging or preventing the dormancy periods.

The micro-RNA molecules are tissue-specific. For example, in epithelial cancers and lymphomas, there are high levels of mi-155 expression, and the influence is oncogenic, whereas in endocrine tumors this micro-RNA is highly down-regulated and possibly has suppressive functions (Calin *et* Croce 2006).

The expression of miR-126 and miR-335 is lost in the majority of primary breast tumors from patients who undergo relapse, and the loss of expression of either micro-RNA is associated with poor distant metastasis-free survival. In human breast cancer, MiR-335 and miR-126 are thus considered to be micro-RNAs that suppress metastasis and support tumor dormancy (Tavazoie *et al.* 2008). An increasing amount of data suggests that micro-RNAs may affect and therefore connect stemness and metastasis through the regulation of epithelial-mesenchymal transition (EMT), which is a genetic developmental program shared by both phenomena (Nicoloso *et al.* 2009).

Dormancy and cancer stem cells

The idea that cancer initiates from stem cells goes back to the 19th century concept of "embryonic rest". Already Rudolf Virchow noticed similarities between embryonic and cancer tissues (Virchow 1858, Hendrix *et al.* 2007). They both have the capability of renewing and differentiating. Over one hundred years later, the resemblance between this old assumption and the theory of cancer stem cells was confirmed.

By examining embryonic and hematopoietic stem cells, it has been possible to get information on the cell surface marker expression of the stem cells. The cancer stem cell theory was first proposed by Hamburger and Salmon, who demonstrated that only a small percentage of tumor cells were able to form colonies in soft agar (Hamburger and Salmon 1977).

In 1994, John Dick's laboratory first succeeded in transferring a cancer stem cell (CSC) in an animal model by transplanting an AML-initiating cell into severe combined immune-deficient (SCID) mice (Lapidot *et al.* 1994). In 2003, Michel Clarke *et al.* first found cancer stem cells in a solid tumor (Al-Hajj *et al.* 2003). They were able to distinguish the tumorigenic (tumor initiating) from the non-tumorigenic cells as CD44+CD24-/low-Lineage in eight of nine breast cancer patients. As few as 100 cells with this phenotype were able to form tumors in mice, whereas tens of thousands of cells with alternative phenotypes failed to form tumors (Al-Hajj *et al.* 2003).

If cancer cells are able to remain dormant for prolonged periods, and if they can be reactivated to renewed proliferation, the stem cell properties of dormant cancer cells could explain these events. Stem cells can stay dormant for a long time, and reversible quiescence might be observed in stem cells or in cells that withdraw into a G0 arrest because of lack of growth-promoting signals (e.g., serum withdrawal and contact inhibition) (Pelayo *et al.* 2006, Zhang *et al.* 2006).

Stem cells also have the capacity for self-renewal. This is a unique type of cell division in which the capacity of one or both daughter cells to proliferate and differentiate is similar to those of the parental cell (Al-Hajj *et* Clarke 2004). In some patients with early breast cancer, disseminated cytokeratin-positive tumor cells can be detected in the bone marrow of patients who never suffer relapse. It has been suggested that in these patients, the cancer cells lie dormant until some unknown event triggers renewed proliferation. Alternatively, it is possible that the DTSs in this group arise from non-tumorigenic cells, and only when CSCs disseminate and subsequently self-renew, will the patients relapse with macroscopic metastases (Reya *et al.* 2001).

In addition to the capability of self-renewal, the stem cells are able to protect themselves from cell injury and achieve a long life span. It has been shown that both normal stem cells and cancer cells can resist apoptotic proteins by a number of parallel mechanisms, including activation of the Hedgehog (HH) pathway, dysregulated transforming growth factor beta (TGF-beta) signalling, and provoke other anti-apoptotic proteins, such as members of the Bcl-2 family (Liu *et al.* 2006, Wang *et al.* 2003).

Since chemotherapeutic agents are targeted at rapidly dividing tumor cells, the non-dividing quiescent stem cells and the dormant tumor cells supposed to have stem cell properties are more resistant to chemotherapy. Even therapies that

cause complete regression of tumors might spare enough cancer stem cells to allow regrowth of the tumor later (Reya *et al.* 2001).

Both stem cells and supposed dormant cancer cells need a specialised safe place, a niche or a microenvironment that contains the signalling molecules required to maintain stem cell identity. Such microanatomical niches maintaining stem cells have been found in different tissue types (Li and Neaves 2006). The important role of these microenvironments is to maintain undisturbed signalling pathways and to protect the cell from uncontrolled division and cancer initiation.

Microenvironment and tumor dormancy

Tumor development and progression is dependent on the balance of biochemical and biophysiological influence of the microenvironment (Kenny and Bissell 2003). Already in 1863, Virchow suggested that there is a connection between epithelial and stromal cells. In later investigations, the role of stromal cells in the differentiation and growth of epithelial cells, and in the prerequisite of their growth, has been clarified. Bone marrow offers a natural milieu for examining the phenomenon of tumor dormancy, since the dormant cells appear to accumulate there. In bone marrow, there is a wide spectrum of chemokines and cytokines which contribute to wound healing. Bone marrow also harbors different stem cell lines, such as a wide spectrum of mesenchymal stem cells, with the potential to differentiate into myofibroblasts, endothelial cells, leukocytes, and cells producing the intercellular matrix. The interaction between stromal cells, endothelial cells and immunological mechanisms may contribute to changes for example in fibroblasts, which can promote tumor growth and metastasis.

Epithelial-mesenchymal transition (EMT) is an important event in the crosstalk between cancer and the microenvironment. Changes that influence the cell structure and adhesional properties, and which facilitate invasion, have been called EMT. In breast cancer, e-cadherin is known as a trigger for EMT (Gould and Gould 1999). The fibroblasts of the tumor induce EMT by secreting transforming growth factor (TGF-beta). Thereafter, cancer cells are released due to loosened cell-to-cell contacts and proteolytic remodelling of the intercellular matrix.

Microenvironmental factors contributing to single dormant tumor cells

It has been shown that a large proportion of DTCs remain in distant organs as single dormant cells (Goodison *et al.* 2003, Naumow *et al.* 2001). This phenomenon may be partially mediated by interactions between the tumor cells and the extracellular matrix. Furthermore, studies on experimental models have shown involvement of

the urokinase-type plasminogen activator receptor (uPAR), the epidermal growth factor receptor (EGFR), extracellular signal-regulated kinases (ERK), and p38 in the regulation of quiescence-based dormancy of tumors (Aguirre-Ghiso *et al.* 2001 , Ranganathan *et al.* 2006, Allgayer *et* Aguirre Ghiso 2008). A balance, regulated by uPAR, that favors p38 activation over ERK, can induce persistent dormancy *in vivo* (Raganathan *et al.* 2006). uPAR seems to be a central regulator of the balance between tumor cell proliferation and quiescence in induced tumor dormancy. Although uPAR expression correlates with *in vivo* cell proliferation, uPAR is also expressed in DTCs (Allgayer and Aguirre-Ghiso 2008). uPAR expression can potentially be used as a predictive marker for clinical prognosis. It was found to be associated with higher tumor cell counts in bone marrow and with an unfavorable prognosis, also in breast cancer. In addition, the transcription factor ATF6alpha, which is regulated in part by p38, was identified as a pivotal survival factor for quiescent dormant tumor cells *in vivo* (Schewe and Aguirre-Ghiso 2008).

Systemic signals that influence both stromal and tumor cells may play a role in the regulation of dormancy. The hormonal influence on dormancy has been studied by transplanting pregnancy-dependent tumors into virgin female mice. Stimulation by hormones that are produced during pregnancy, or hormonal treatment, caused these tumors to emerge from dormancy (Gatteli *et al.* 2004).

It is suggested that reduced crosstalk of DTCs with its microenvironment induce quiescence or 'differentiation' and thus dormancy of tumor cells. Although the DTCs in mouse models of experimental breast cancer metastases die, a fraction of them remains viable and does not proliferate in other tissue, as lung and liver, but forms tumors in orthotopic mammary fat pads (Chambers *et al.* 2002, Naumow *et al.* 2002). It is important to emphasize that at an early stage, solitary dormant tumor cells are surrounded by normally functioning tissue vasculature. Therefore, rather than invoking a mechanism that depends on neovascularization, it is more likely that deficient crosstalk with the new microenvironment and/or stress signalling explains the impaired proliferation of these tumor cells.

Angiogenesis-related tumor dormancy

The vast majority of tumors depend on recruitment of functional blood vessels to support the growth of the tumor mass. Tumors that are unable to induce successful angiogenesis remain avascular and microscopic in size (Holmgren 1996, Hart 1999, Watnick *et al.* 2003). Tumor cells in avascular dormant tumors typically exhibit a high proliferation rate that is balanced by elevated apoptosis (Holmgren et al 1995). The transition from a prevascular lesion to a highly vascularized and progressively outgrowing tumor is referred to as the "angiogenic switch" (Hanahan *et* Weinberg 2000, Baeriswyl *et* Christofori 2009).

Tumor cells that form dormant tumors and those forming fast-growing tumors have been compared regarding their molecular and cellular characteristics (Almong *et al.* 2006, Naumov *et al.* 2006). Although both dormant and fast-growing tumors contain cells that have similar proliferation rates *in vitro*, they may have strikingly different growth kinetics *in vivo*. In contrast to the fast-growing tumors, which were detectable a few weeks after tumor cell injections, the dormant tumors remained microscopic and did not expand in size for prolonged periods of time. A significant difference was observed in the structure of the tumor vasculature between dormant and fast-growing tumors (Almong *et al.* 2006, Naumov *et al.* 2006). In non-angiogenic dormant tumors, small clusters of endothelial cells without lumina were commonly observed, indicating that the process of tumor angiogenesis was incomplete. Cells from non-angiogenic dormant tumors secreted relatively high levels of the potent angiogenesis inhibitor, thrombospondin. These experiments indicate that angiogenesis is a necessary event for the shift from dormancy and tumor progression.

The immune system and tumor dormancy

It has been known for decades that the immune system has a role in controlling tumor growth (Aquirre-Ghiso 2007, Finn 2006, Weinhold *et al.* 1979, Zou 2005). Studies using syngeneic animals that are immunized with a subcutaneous implantation of tumor cells and then challenged by intraperitoneal injection of cells have shown that the immune system can target and kill most of the tumor cells in the challenge injection (Finn 2006, Weinhold *et al.* 1979). The immune responses are mostly mediated by cytotoxic CD8+ T lymphocytes, which mediate cytolysis of the tumor cells (Finn *et al.* 2006). However, it seems that some residual cells still persist, and this population may be kept clinically dormant by the immune system (Weinhold *et al.* 1979, Matsuzava *et al.* 1991). Thus, the immune system prevents the expansion of proliferating tumor cells.

Additional studies have shown that proliferating mouse lymphoma cells are kept at a low number in bone marrow due to persisting antigen and memory T cells that are able to coordinate the CD4+ and CD8+ T-cell-mediated response (Mahnke *et al.* 2005, Feuerer *et al.* 2001, Willimsky *et al.* 2005). These results are in line with clinical studies showing that the bone marrow of breast cancer patients contains cytokeratin-positive cells (breast cancer cells), and a higher proportion of memory T cells among the CD4+ and CD8+ cells correlated with larger tumors (Feuerer *et al.* 2001). These results suggest that in some situations, the immune system might still be operating to suppress residual tumor cell expansion, whereas other mechanisms of dormancy favor immune system evasion.

When immunologists in the 1950s gained a deeper understanding of transplantation, tumor immunobiology and immunogenetics, Burnet and

Thomas (Burnet 1957) developed the immunosurveillance theory. They proposed that lymphocyte populations continuously recognize and eliminate cancerous/precancerous cells in the host before they develop into invasive tumors with metastatic potential.

New studies in the 1990s, encouraged by technological advances in mouse genetics and monoclonal antibody (mAb) production, reinvigorated and ultimately validated the concept of cancer immunosurveillance (Smyth *et al.* 2001 b, Dunn *et al.* 2004, Dunn *et al.* 2002) and expanded it to incorporate the contributions of both innate and adaptive immunity.

However, immunosurveillance represents only one dimension of the complex relationship between the immune system and cancer (Dunn *et al*, 2002, 2004, Screiber *et al.* 2004). The immune system is capable of sculpting the cancer phenotype by interacting with tumors. Tumor cells can be modified to less immunogenic variants which can escape immune recognition and destruction (Teng *et al.* 2008).

These findings prompted the development of the cancer immunoediting hypothesis to more broadly encompass the potential host-protective and tumor-sculpting functions of the immune system throughout tumor development (Dunn *et al.* 2002, 2004).

Cancer immunoediting is a dynamic process composed of three phases: elimination, equilibrium, and escape. Elimination represents the classical concept of cancer surveillance, where cells and molecules of the innate and adaptive immune systems may eradicate the developing tumor and protect the host against tumor formation. However, if this process is not successful, the tumor cells may enter an equilibrium phase where they may either be maintained chronically or immunologically sculpted by immuno-'editors' to produce new populations of tumor variants. The equilibrium is the period of immune-mediated latency (dormancy) after incomplete tumor destruction in the elimination phase. Escape refers to the final outgrowth (awakening from dormancy) of tumors that have outstripped the immunological restrains of the equilibrium phase.

MARKERS ASSOCIATED WITH TUMOR DORMANCY

Stanniocalcins (STC-1 and STC-2)

Stanniocalcin-1 (STC-1) is a glycoprotein that is highly conserved in vertebrates – from fish to mammals (Chang *et al.* 2003). In fish, it functions as a classical hormone, regulating calcium and phosphate homeostasis and protecting against toxic hypercalcemia (Wendelaar Bonga and Pang 1991, Lu *et al.* 1994).

There is, however, no evidence that the mammalian homolog STC would have any physiological role in regulating serum calcium. Instead, mammalian STCs are expressed in a variety of tissues. STC-1 is particularly expressed in cells undergoing terminal postmitotic differentiation, such as brain neurons (Zhang *et al.* 1998), adipocytes (Serlachius *et* Andersson 2004), striated muscle (Jiang *et al.* 2000) and megakaryocytes (Serlachius *et al.* 2002). STC-1 is also found in heart, bone (Serlachius *et al.* 2002), kidneys and ovaries (Worthington *et al.* 1999). Expression of STC-2 has been found in kidney, heart, pancreas and spleen (Chang and Reddel 1998, Ishibashi *et al.* 1998, DiMattia *et al.* 1998).

STC-1 and STC- 2 expression has also been found in neoplastic mammary tissue and a correlation between ER expression and the presence of STC-1 and STC-2 has been shown in breast cancer (Bouras *et al.* 2002).

The physiological functions of stanniocalcins in mammalians are incompletely understood. STC-1 is reported to localize to mitochondria, and it induces enhanced electron transport in submitochondrial particles. Furthermore, STC-1 is regulated by the tumor suppressors BRCA1 (Welcsh *et al.*2002) and p53 (Lai *et al.* 2001). STC1 and STC2 have also been found to act as survival factors and to protect the cell against hypoxic and hypercalcemic stress, particularly in terminally differentiated slowly proliferating cells (Zhang *et al.* 2000). High expression of STC-1 was detected in slowly proliferating, well-differentiated, low-grade liposarcomas, whereas high-grade liposarcomas did not express STC-1 (Serlachius *et* Andersson 2004). Exposure of mice to low oxygen levels was shown to upregulate STC-1 in the brain *via* IL-6 dependent signalling (Westberg *et al.* 2007). Stanniocalcins may thus be regarded as tumor-suppressive factors.

There is also evidence that stanniocalcins have tumor-progressive properties. The growth of solid tumors, such as breast cancers, is usually associated with hypoxia and endoplasmic reticulum stress. STC-2 promotes cell proliferation under hypoxia, and is a hypoxia-inducing factor-1 (HIF-1)-responsive gene. It has been demonstrated that the epigenetical silencing of STC-2 may interfere with HIF-1-mediated activation of STC-2 expression, since STC-2 was aberrantly hypermethylated in human ovarian epithelial cancer (SKOV3) cells (Law *et al.* 2008).

Recently, new insight has been reported on the relation of STC-1 to breast cancer, as protein kinase Calpha (PKCalpha) suppresses the expression of STC-1 mRNA in MDA-MB-231 breast cancer cells (Cornmark *et al.* 2011). High levels of PKCalpha correlate with an aggressive breast cancer phenotype and predict poorer survival (Lonne *et al.* 2010).

STC-1 has been associated with both antiapoptotic (Zhang *et al.* 2000) and cell death-inducing effects (Nguyen *et al.* 2009). Stanniocalcin may therefore have either pro- or anti-tumorigenic effects, depending on the cellular context. As a consequence, the stanniocalcins can either function as tumor dormancy-supportive or recurrence/metastasis-activating factors.

p53

The p53 tumor suppressor gene has a regulatory function in defending against various kinds of cancer, including breast cancer (Attardi et Donehower 2005, Børresen-Dale 2003, Meek 2009). p53 is regarded as a central player in tumor suppression. It senses DNA damage and responds by inducing a transient growth arrest, allowing DNA repair or, in the case of extensive damage, promoting irreversible growth arrest (senescence) or programmed cell death (apoptosis) (Lowe *et al.* 2004, Vousden 2009).

The critical role of p53 in the prevention of cancer development is demonstrated by the presence of mutated p53 in approximately 50% of human cancers (Hollstein et al 1991). The frequency of p53 mutations in breast cancer ranges from 15 to 71% (Borresen-Dale 2003). The simplest way to measure p53 function is to look for its overexpression by IHC with an antibody to p53. Wild type p53 is normally rapidly degraded, but most p53 mutations, even as they disrupt function, lead to accumulation of the protein that is strongly reactive with antibodies to p53 (Bartley and Ross 2002). Although most studies have shown a poorer prognosis for breast cancers with increased p53 expression (Thor and Yandell 1993, Allred *et al.* 1993, Kovach *et al.* 1996, Blaszyk *et al.* 2000), some studies have found no correlation between prognosis and p53 expression (Isola *et al.* 1992, Reed *et al.* 2000, Pietiläinen *et al.* 1995).

Given that p53 is inactivated in almost all types of human cancers, a number of innovative therapeutic strategies have been developed to restore p53 function in cancer (El-Deiry 2003, Nikitina *et al.* 2002). p53 may play a role in an angiogenic tumor dormancy experiment. Angiogenic dormancy results from the balance between pro- and anti-angiogenic factors (such as vascular endothelial growth factor (VEGF) and thrombospondin (TSP), respectively. Genetic alterations in the pathways that maintain angiogenic dormancy or an exogenous angiogenic 'spike' might restore tumor growth. Oncogenic Ras can induce expression of VEGF and repress TSP. In contrast, the stress-activated kinase p38 and the tumor suppressor p53 can induce TSP or repress VEGF. Loss of function of p53 and/or p38 might tip the balance towards enhanced angiogenesis and awakening from dormancy (Aguirre-Chiso 2007).

Bcl-2

Bcl-2 is a member of a family of cytoplasmic proteins (the Bcl-2 family) whose transcription is regulated by p53. Bcl-2 was first discovered in B-cell lymphomas in which, as a result of a chromosomal translocation t (14, 18), the Bcl-2 gene is juxtaposed to the immunoglobulin heavy chain gene (Chen-Levy *et al.* 1989). This alteration drives constitutive expression of Bcl-2, which localizes to the inner

mitochondrial membrane and blocks programmed cell death (Hockenbery *et al.* 1990). According to a study by Joensuu *et al.*, expression of the oncoprotein Bcl-2 (B-cell lymphoma-2) in breast cancer is associated with a favorable prognosis (Joensuu *et al.* 1994). The reason for the apparently paradoxical favorable prognostic impact of Bcl-2 on breast cancer is not clear. Knowlton *et al.* (1998) showed that expression of Bcl-2 retards the cell cycle of breast cancer cells. A decreased proliferation rate of tumor cells may thus account for the association of Bcl-2 expression with a favorable outcome in breast cancer.

As an anti-apoptotic protein, Bcl-2 belongs to the factors which should support metastatic progression and escape from tumor dormancy (Pantel *et al.* 2009).

Maspin

Maspin is a mammary serine protease inhibitor (SERPINB5) with tumor suppressor activity, first isolated from normal mammary epithelial cells (Zou *et al.* 1994). The mechanism underlying the biological activity of maspin is largely unknown. Contradictory results have been reported on the role of maspin in breast cancer and its prognostic impact. Some earlier studies on maspin demonstrate its tumor-suppressive properties (Sheng *et al.* 1996, Zhang *et al.* 1997). In breast cancer, the tumor suppressor activity of maspin was attributed to its ability to inhibit cell motility, invasion and metastasis (Maass *et al.* 2001). High levels of maspin are also associated with decreased angiogenesis (Zhang *et al.* 2001). Maspin-mediated reduction of tumor growth is at least partially attributed to the enhancement of apoptosis (Liu *et al.* 2004, Zhang *et al.* 2005).

Contradictory findings on the function of maspin in breast cancer have, however, also been published. Umekita *et al.* reported that expression of maspin in breast cancer was associated with significantly shorter relapse-free survival (Umekita *et al.* 2002), and that the expression of maspin is upregulated during the progression of ductal breast carcinoma (Umekita *et* Yoshida 2003). In breast cancer, Mohsin *et al.* (2003) showed that nuclear immunopositivity for maspin was associated with a favorable prognosis, such as ER and PR positivity, whereas cytoplasmic staining was related to ER and PR negativity. However, there is no certainty of whether the different cellular location of maspin protein, either nuclear or cytoplasmic, is of functional importance.

Maspin is not mutated or rearranged in tumor cells, but the gene is epigenetically silenced during metastatic transaction by aberrant cytosine methylation of the maspin promoter (Domann *et al.* 2000). The reversible nature of epigenetic silencing of maspin offers a unique opportunity for therapeutic interventions through specific re-activation of the endogenous gene. (Beltran *et al.*2008).

Several chromatin-remodelling drugs have been developed to release the repressed state of tumor suppressor genes. These drugs act by inhibiting DNA methyl transferases or histone deacetylases (HSAC), resulting in increased promoter accessibility and enhanced tumor suppressor gene transcription (Cameron *et al.* 1999, Oshiro *et al.* 2003, Hellebrekers *et al.* 2007).

Because maspin displays tumor suppressor properties, and inhibits breast cancer cell motility, invasion, metastasis and angiogenesis, it most probably functions as a supportive factor in tumor dormancy.

Bmi-1

Bmi-1 belongs to the mammalian polycomb group (PcG) of proteins. They form multimeric complexes that regulate gene activity at the chromatin level (Satijn *et al.* 2001). Bmi-1 contributes to cell cycle regulation by acting as a transcriptional repressor of the INK4a/ARF (=human p19ARF) locus (Jacobs *et al.* 1999).

Kim *et al.* (2004) demonstrated that overexpression of *bmi-1* transcripts and Bmi-1 protein in breast cancer correlated with axillary lymph node metastases. They suggested that cell cycle deregulation by Bmi-1 might play a role in the progression of breast cancer and lymph node metastasis. Al-Hajj *et al.* (2003) described the existence of a cancer stem cell population in human breast cancers. These cancer stem cells displayed increased expression of Bmi-1 (Liu *et al.* 2006).

Expression of Bmi-1 contributes to the stem cell phenotype. Such cells can settle down in a non-proliferative dormant state and preserve the properties of self-renewal and the possibility to metastasize even long after treatment of the primary tumor (Pantel *et al.* 2009).

c-myc

Bishop and co-workers discovered c-Myc in the late 1970s (Bishop 1982, Vennstrom *et al.* 1982). c-Myc is a DNA-binding, nuclear transcription factor involved in the regulation of the cell cycle (Rabbits *et al.* 1985), programmed cell death, and tumorigenesis (Harrington *et al.* 1994, Amundadottir *et al.* 1995).

c-Myc was first detected in Burkitt's lymphoma, but has later been connected to many other cancers, including breast and colon cancer, neuroblastoma, osteosarcoma, and melanoma (Pelengaris *et al.* 2002).

The clinical relevance of the overexpression of c-myc protein in breast cancer is not well known (Rodriguez-Pinilla *et al.* 2007, Liao *et* Dicson 2000). On the other hand, *MYC* gene amplification has been associated with a high histological tumor grade, the presence of lymph node metastasis, a lack of PR, and poor survival in

breast cancer (Deming *et al.* 2000, van Lohuizen *et al.* 1991). Bmi-1 has an impact on c-Myc activity and *vice versa*; both of these oncogenes are able to immortalize certain cells (van Lohuizen *et al.* 1991, Haupt *et al.* 1993, and Levy *et. al.* 1993).

The *Myc* gene regulates apoptosis and downregulates Bcl-2, which mediates apoptosis-inducing effects and suppresses the onset of metastasis. c-Myc can also repress thrombospondin-1 expression and increase angiogenesis by cooperative activity with Ras (Watnick *et al.* 2003) and thereby support the onset of metastasis and escape from tumor dormancy.

Snail

Snail is a zinc-finger transcription factor essential for EMT. It downregulates the expression of cell adhesion and basement membrane proteins, most importantly cadherins (Battle *et al.* 2000). Snail increases migration and invasion in various physiological and pathological conditions (Battle *et al.* 2000, Cano *et al.* 2000, and Peinano et al 2007). The involvement of Snail in tumor progression is supported by findings of Snail expression in carcinoma cell lines that have invasive and metastatic properties (Cano *et al.* 2000, Cheng *et al.* 2001, Poser *et al.* 2001) and in de-differentiated breast cancer, and hepatocellular carcinoma (Blanco *et al.* 2002, Sugimachi *et al.* 2003). Snail is required for tumor growth and lymph node metastasis of heterotransplanted human breast carcinoma MDA-MB-231 cells (Olmeda *et al.* 2007).

Yook *et al.* have reported WNT-dependent regulation of Snail functionality in breast cancer cells. WNT signalling can induce increased expression of Axin2, leading to the redirection of GSK-3B to the cytosol, leaving Snail in its non-phosphorylated, transcriptionally active form (Yook et al 2006). Angiopoietin 2 (Ang2), a growth factor, promotes tumor angiogenesis and has been previously shown to increase nuclear Snail1 expression, downregulates E-cadherin, and increases the metastatic potential of primary breast cancers (Imanishi et al 2007).

CK5

Keratin filaments constitute a group of 8-nm fibres that form an integral part of the cytoskeleton of eukaryotic epithelial cells (Lersch *et* Fuchs 1988). There are more than 20 different human keratins encoded by a large multigene family, which can be divided into two distinct sequence classes, Type I and Type II. Type I keratins are small (40 to 56.5 kDa), while Type II keratins are larger (53 to 67 kDa). Cytokeratin 5 and its pair, cytokeratin 14, are expressed abundantly in the basal layer of the epidermis (Lersch *et* Fuchs 1988).

In normal breast, both luminal epithelial and the myoepithelial cells exhibit different and distinctive keratins. The luminal cells express CK7, 8, 18 and 19, while smooth muscle actin (SMA) and CKs 5, 14 and 17 are found in the myoepithelial/basal cells (Taylor-Papadimitriou *et al.* 1989).

The IHC expression of CK5 can be used to identify basal-like carcinomas in tissue sections (Banerjee *et al.* 2006, van de Rijn *et al.* 2002).

Abd El-Rehim *et al.* (2004) examined a tissue microarray from a large cohort of breast cancers. They observed that the tumors expressing the luminal markers, CKs 7, 8, 18 and 19, associated with a good prognosis, in contrast to the tumors expressing basal markers, particularly CK5, which were associated with a poor outcome (Abd El-Rehim *et al.* 2004).

Possibilities for application of dormancy mechanisms in cancer treatment

Identification of the different mechanisms underlying tumor dormancy has given researchers tools for therapeutical interventions to prevent cancer recurrence. Because DTCs are already present in the target organs after primary tumor removal, and cancer cells can coexist in different states in the metastatic organ, they represent very different therapeutic targets. Metastatic cells can be present as dormant, quiescent solitary cells, dormant pre-angiogenic micrometastases, and actively growing vascularized metastases (Goss *et* Chambers 2010).

Preclinical models have shown that cytotoxic chemotherapy effectively inhibits the growth of metastases, but has no effect on the dormant cells residing in the same organ in a liver metastasis model of breast cancer (Naumov *et al.* 2003).

In an experiment using mouse mammary carcinoma cell lines, treatment with doxorubicin reduced the size of large metastases, but did not reduce the number of solitary dormant cells (Naumov *et al.* 2003). Although quiescent, dormant cells are difficult to kill, it could be possible to tackle them after they have reinitiated growth.

Ongoing clinical trials in hormone-dependent breast cancer suggest that anti-strike therapies introduced late in follow-up can reduce delayed clinical recurrences. The Letrizole after Tamoxifen in Treating Women with Breast Cancer (NCIC CTC MA17) trial demonstrated a significant improvement in disease-free survival in early breast cancer by extending adjuvant (postoperative) endocrine therapy with an aromatase inhibitor, letrozole, for an additional 5 years after the initial 5 years of tamoxifen treatment (Goss *et al.* 2003).

The antibody to HER2, trastuzumab (Herceptin), is an example of a drug that targets signal transduction, and it can be exploited in adjuvant therapy in early HER2-positive breast cancer. When trastuzumab was given concomitantly with paclitaxel or after chemotherapy for 12 months as adjuvant treatment, it reduced significantly the risk of recurrence (Joensuu *et al.* 2009). The Tykerb Evaluation

After Chemotherapy (TEACH) trial is investigating the natural history of dormancy in HER2-positive breast cancer (Goss *et* Chambers 2010).

One candidate for a tumor dormancy markers is the mitogen activated protein kinase p38. Aguirre-Ghiso *et al.* (2001) studied the role of p38 in immunodeficient chick embryos using human epidermoid carcinoma Hep3 cells. p38 is highly expressed in dormant human carcinoma (Hep3) cells and it plays a critical role in the induction of tumor dormancy (Ranganathan *et al.* 2006, Adam *et al.* 2009). When p38 activity was inhibited by pharmacological (SB203580) or genetic (dominant negative-p38) approaches, dormancy was interrupted.

Interestingly, tamoxifen treatment can activate p38 signalling and quiescence (Buck *et al.* 2004), suggesting that tamoxifen 'dormancy' treatment could be considered as a maintenance therapy to prevent DTCs from exiting their state of growth arrest (*i.e.* dormancy maintenance) or by inducing growth arrest (*i.e.* dormancy induction). However, such a strategy might be selective for ER-negative tumor cells (Goss *et* Chambers 2010). p38 inhibitors (such as SCIO-469, RO4402257, PH-797804, SB681323 and BMS-5) are currently used in clinical trials to treat several neoplastic and non-neoplastic diseases (http://www.clinicaltrials.gov.)

While DTCs are already in the target organs after primary surgery, adjuvant therapy should target not only individual tumor cells, but also the surrounding microenvironment. One such treatment option is offered by Bisphonates, which are potent inhibitors of osteoclast-mediated bone resoprtion. Bisphosphonates inhibit osteoclast precursor cells, induce apoptosis of osteoclasts, and alter the growth factor and cytokine secretion of the microenvironment. They also reduce tumor cell adhesion, induce apoptosis in tumor cells, have an anti-angiogenic effect and increase anti-tumor activity of cytotoxic agents (Fehm *et al.* 2008, Santini *et al.* 2003).

Micrometastases found in bone marrow are known to worsen the prognosis – their presence at the time of the diagnosis predicts later recurrence of cancer, but not necessarily in bone marrow!

An interesting strategy in preventing cancer recurrence may be the use of cancer vaccines (Curigliano *et al.* 2007). Individuals are believed to have cytotoxic T-cell precursors specific for tumor-associated antigens (TAA) and it is assumed that the patient's immune system can be sensitized to TAAs of the patient's own tumor. TAAs (*e.g.* CEA, HER2, MUC-1) recognized by the immune effector cells have already been identified and used for preparing TAA-dependent vaccines (Ko *et al.* 2003).

It would be highly desirable to find a simple indicator that allows risk assessment of late breast cancer recurrence and metastasis development. In contrast to predictors based on tumor characteristics at the time of surgery, serum is a particularly valuable source, because it is useful not only for the initial screening for the disease but also for continuous monitoring of the therapeutic effect.

The newly developed antibody microarray-based technology, which enables the simultaneous detection of multiple proteins in serum, has been recently exploited to predict the development of distant metastases in breast cancer patients. Carlsson *et al.* (2011) identified a 21-protein signature from 240 sera of 64 patients with primary breast cancer. They were able to assess the risk of developing distant recurrence after the primary operation for each patient, using her molecular portrait. This risk assessment was not dependent on the type of adjuvant therapy given to the patients.

AIMS OF THE STUDY

This thesis was undertaken to clarify the phenomenon of tumor dormancy in breast cancer. In order to achieve this purpose, a retrospective set of primary breast cancers and their corresponding early and late relapses were collected.

Factors known to have a role in tumor dormancy, either with activating (escape from dormancy) or suppressing (supporting dormancy) properties to develop cancer metastases, have been included in this study.

The specific aims in these tumor series were:

- to investigate the relationship between tumor dormancy and the established biomarkers, ER, PR, HER2, Ki67, used for the clinicopathological treatment decision (Study IV)
- to investigate whether the expression patterns of stanniocalcins (STC-1 and STC-2) are in agreement with their presumed roles in cell survival and in supporting tumor dormancy (Study I)
- to investigate the expression of the tumor suppressive factors maspin and p53, and the antiapoptotic/dormancy-inhibiting factor Bcl-2, and their association with early or late tumor recurrence (Study II)
- to investigate whether proteins with stem cell properties – Bmi-1, c-myc and Snail – have the potential to activate tumor growth after prolonged tumor dormancy (Study III)

MATERIALS AND METHODS

PATIENTS AND TUMORS

We collected cases of female patients who had had relapsed breast cancer, and for whom representative paraffin-embedded tumor samples from both their primary tumor and the corresponding recurrent/metastatic lesion were available. The cases and the clinicopathological information on the patients were derived from the database of the Department of Pathology, Helsinki University Hospital. Recurrence/metastasis was defined as any local or regional recurrence or any distant metastatic disease. We selected the cases according to the time of relapse, and divided them into three groups: in Group 1, recurrences/metastases were detected within 2 years, in Group 2, at 5–10 years, and in Group 3 after 10 years (range, >10 up to 23 years). Only tumors of epithelial origin, i.e., the major type of malignant breast tumors, were included in the study. The patients had undergone breast cancer surgery during 1974–2006.

Paraffin-embedded tissue blocks were collected from altogether 73 primary breast cancers and their respective recurrent/metastatic lesions from the archives of the Department of Pathology, Helsinki University Hospital. The histological tumor type and grade were assigned according to the criteria of Elston and Ellis (1991).

The information of clinicopathological characteristics of the patients and their cancers are summarized in Table 7. The Ethics Committee of the Helsinki University Central Hospital approved the study protocol.

IMMUNOHISTOCHEMISTRY

Four µm thick sections of paraffin-embedded breast cancer tissue were deparaffinized in xylene and rehydrated. Antigen retrieval was done by microwaving in 10 mM citric acid monohydrate for 5 min at 900 W and for 3 x 5 min at 600W. Endogenous peroxidase activity was blocked by treatment with 0.5% H2O2. The slides were incubated overnight in a refrigerator at + 4° C with appropriate dilutions of the primary antibodies. The same procedure was used for negative controls, except that the overnight incubation took place in PBS diluent without antibody. The reaction was visualized by the Elite ABC Kit (Vectastain, Vector Laboratories, Burlingame, CA, USA) for PR and ER, Stanniocalcin 1 and 2 and maspin. HER2 and p53 staining was performed using the Envision kit (Dako, Copenhagen, Denmark).

Table 7. Clinicopathological parameters of the 73 breast cancer patients and the tissue site of the metastatic recurrence of their tumors. The patients are divided into Groups 1, 2 and 3 according to the time of relapse after primary diagnosis

	Group 1 n=19	Group 2 n=33	Group 3 n=21
Age at surgery of primary tumor			
< 50 years	10	14	13
≥ 50 years	9	19	8
Surgery			
Mastectomy	18	20	13
Partial mammary resection	1	11	8
Axillary lymph node evacuation	17	31	20
No axillary evacuation	1		
Tumor size			
< 20 mm	3	16	12
> 20 mm	16	17	9
Lymph node			
negative	3	20	11
positive	16	13	10
Grade			
1	0	5	3
2	8	19	14
3	11	9	4
Histological type			
ductal	16	27	12
lobular	3	5	9
mucinous	0	1	0
Tissue site of metastasis			
skin	5	8	11
soft tissue	6	11	6
subcutaneous tissue	0	5	2
lung	0	4	0
lymph node	1	0	0
liver	5	1	0
brain	2	2	0
bone	0	1	1
ovary	0	1	0
stomach	0	0	1

Note. In Group 1, metastatic recurrences were detected within 2 years; in Group 2, from 5–10 years; and in Group 3, after 10 years.

Breast surgery data are not available in two cases of Group 2. Axillary evacuation data were not available in one case of Group 1, in one case of Group 2 and in one case of Group 3.

Table 8. List of the antibodies, the laboratories manufacturing them, the dilutions used in IHC staining, and the cut-off (≥%) points for Pearson's Chi-square tests

Antigen	Antibody	Laboratory	Dilution	cut-off ≥%
ER alpha	Mouse monoclonal clone 6F11	Novo Castra, Newcastle, UK	1:50	1% (Study IV), 10% (Studies I,II,III)
STC-1	Polyclonal	ref. Olsen et al. 1996	1:1500	10%
STC-2	Polyclonal	ref. Olsen et al. 1996	1:1600	10%
Maspin	Mouse monoclonal clone G167-70	B.D. PharMingen, San Diego, CA, USA	1:1000	10%
Bcl-2	Mouse monoclonal clone 124	Dako Cytomation, Glostrup, Denmark	1:25	10%
p53	Mouse monoclonal clone DO-7	Dako Cytomation, Glostrup, Denmark	1:100	10%
Bmi-1	Mouse monoclonal clone ab14389	Abcam, Cambridge, UK	1:400	10%
c-myc	Mouse monoclonal clone 9E10	Santa Cruz, UK	1:400	10%
Snail	Polyclonal rabbit clone ab17732	Abcam, UK	1:2000	80%
PR alpha	Mouse monoclonal clone PgR636	Daco Cytomation Denmark	1:100	1% (Study IV), 10% (Studies I,II,III
HER2	Mouse monoclonal clone CB11	Novo Castra, UK	1:700	Wolff et al. 2007
Ki67	Mouse monoclonal clone MIB-1	Daco Cytomation Denmark	1:75	14%
CK5	Mouse monoclonal XM26	Leica, UK	1:100	1%

The slides for Bmi-1, c-myc, and Snail immunolabeling were pretreated in a PT-module (LabVision UK Ltd, UK) in Tris-HCl buffer (pH 8.5) for 20 min at 98°C and with 0.3% Dako REAL Peroxidase-blocking solution for 5 min to block endogenous peroxidase. Immunostaining was done by incubating each antibody for 1 h, followed by 30-min incubation with Dako REAL EnVision/HRP secondary antibodies. The bound antibodies were finally visualized by Dako REAL DAB+ Chromogen reaction for 10 min. The slides were washed between each step with PBS-0.04% Tween20. The antibodies used, their dilutions and the manufacturers are presented in Table 8.

The result was quantified as the proportion of positively stained tumor cells (range, 0–100%). For the analyses, the tissue samples were classified as positive for ER and PR, when ≥ 10% of the tumor cells showed positive nuclear staining in Studies I,II and III. In Study IV, the tissue samples were classified positive when ≥ 1% (Hammond *et al.* 2010) of the tumor cells showed positive nuclear staining. The 1% cut-off was also used for CK5 (Haughian *et al.* 2011).

The IHC scoring method used for HER2 was based on the intensity and percentage of positive cells, giving a score between 0 and 3+. Cases were reported as 0 (negative) if no staining or membrane staining in less than 10% of invasive tumor cells was seen. A 1+ (negative) result was reported if faint/barely perceived membrane staining was detected in more than 10% of invasive tumor cells. The scoring report was 2+ (positive), if weak to moderate complete membrane staining was seen in more than 10% of tumor cells, or < 30% with strong complete membrane staining. A 3+ (positive) result was reported, if strong complete membrane staining in more than 30% of invasive tumor cells was seen. (Wolff *et al.* 2007). The tumor was considered positive for Ki67, if ≥14% of the tumor cells showed positively stained nuclei (Cheang *et al.* 2009).

A cut-off point of 10% of positivity was used for stanniocalcins (Bouras *et al.* 2002), maspin, Bcl-2, p53 (Elledge *et al.* 1998), Bmi-1(Kim *et al.* 2004) and c-myc (Pavelic *et al.* 1992). For Snail, the tumors were divided into high- and low-expressing groups, using a proportion of 80% of positively stained cells as the cut-off point (Häyry *et al.* 2008).

We evaluated the entire tumor area from one representative section of the primary tumor and metastasis. The results were scored independently by two pathologists (KJ, PH). The antibody clones and the laboratories manufacturing them, as well as the dilutions that were used for each antibody, and the cut-off (≥%) points for Pearson's Chi-square tests are shown in Table 8.

IHC SUBTYPES

For analysing the subtype approximations to the subtypes defined by genetic array testing, the tumors were divided to seven subtypes by using IHC: luminal A (ERorPR+HER2-, luminal B (ERorPR+HER2+), HER2 overexpressing (ER-PR-HER2+). triple-negative (ER-PR-HER2-), basal-like (ER-PR-HER2-CK5+), unclassified (ER-PR-HER2-K5-), luminobasal (ERorPR+CK5+).

CHROMOGENIC *IN SITU* HYBRIDIZATION (CISH)

CISH was performed on all tumors with protein overexpression of HER2 (2+ and 3+) by IHC.

The slides with 4 μm paraffin sections were preheated at +55-58°C for about 2–6 h and then dried overnight at 37°C, to avoid detachment. Thereafter they were deparaffinized in xylene, rehydrated, and incubated in Tris-EDTA buffer (pH 9.0) for 25 min at 98°C. After digestion in 0.1% trypsin for 40 sec, the slides were post-

fixed in 10% formalin for 10 min and dehydrated in an increasing alcohol series. Then 0.4 ml digoxin-labeled ZytoDotSPECHER2 probe was applied, sealed and denatured on a heat plate at 95° for 4 min. Finally, the slides were incubated at 37°C overnight for hybridization. On the second day, the slides were opened and washed in 78°C standard saline citrate for 2 x 2 min to remove the unspecifically bound probe. The digoxigen-labeled *HER2* probe was recognized by the primary mouse monoclonal antibody (clone 1.71.256, cat no. 11333062910, Roche), and the amplified signals were visualized with DAB. Immunodetection was performed in a LabVision autostainer using the PowerVision Poly-HRP IHC Detection kit (DPVB+110DAB/ ImmunoVision Technologies Co). The slides were then counterstained with Mayers Hematoxylin and covered.

The stainings were examined under a light microscope (40 x); small dots (signals) in the nuclei represented the gene copies. We considered 5 or less signals as no amplification, 6–10 signals as low amplification, and more than 10 verified signals as high amplification. Stromal cells with the normal two gene copies/nucleus served as a negative control. The amplified cells should represent at least 10% of the entire tumor. In the case of low amplification, the chromosome 17 centromere probe was used to determine whether the extra copies are caused by chromosomal aneuploidy. In these cases, the *HER2* status was set as the ratio of the average number of *HER2* gene copies to the average number of copies of chromosome 17. If the average *HER2*/ Chr17 was ≥ 2, the result was interpreted as positive for *HER2* gene amplification. Stromal cells with the normal two gene copies/nucleus served as a negative control. The amplified cells should represent at least 10% of the entire tumor.

STATISTICAL METHODS

All statistical analyses were performed using SPSS 13.0 for Windows (SPSS Incorporation, Chicago, ILL, USA). The differences between the primary tumors and their corresponding metastases were analyzed by the paired samples *t*-test in Studies I, II, III and IV. For comparing differences between the groups, the independent samples *t*-test was used in Study I. In Studies II and III, Kruskal-Wallis and Mann-Whitney *U*-tests were used for comparing the groups. In Study IV, the differences between the groups were analyzed by using the categorical two-tailed Pearson's Chi-square test in univariate analyses, and in Study IV the regression ordinal test was used for multivariate analysis. For comparing the expression of the different proteins, the categorical two-tailed Pearson's Chi-square test was used. Probability values of $p > 0.05$ were considered significant in all analyses except in Mann-Whitney *U*-tests, were a p value < .0167 (=<.05/3) was considered significant.

RESULTS

This study investigated the expression of different biomarkers known to contribute to the progression of breast cancer. These biomarkers were evaluated in early and late-relapsing tumors in a series of 73 primary breast cancers and their corresponding metastases. The expression levels were compared between primary tumors and metastases, between the different groups according to the time of relapse after initial treatment, and with clinicopathological parameters, in order to analyze their putative roles in breast cancer progression.

The primary tumors belonged to the most common histological types of breast cancers: 55 ductal and 17 lobular adenocarcinomas, and additionally, one mucinous carcinoma. There was no difference in the distribution of the tumor types between the early and late relapsing tumors. All corresponding metastases were morphologically similar to their primary counterparts. The clinicopathological parameters are presented in Materials and methods, Table 7.

STUDY I

STANNIOCALCINS AND ER

Both STC-1 (Fig. 5) and STC-2 expression were elevated in late-relapsing tumors, especially in the recurrences. The mean frequency of positively stained tumor cells for STC-1 showed a non-significant tendency to higher initial expression in the late recurring tumors compared to the early relapsing ones. The mean frequency of STC-1 stained tumor cells was 28% in primary cancers with contemporary or early recurrence (Group 1, Table 9). In the primary tumors of Group 2 (recurrence at 5–10 years after primary surgery) the frequency was 31%, and in the latest relapsing tumors (Group 3) the frequency was 39% (Table 9). A more strongly increasing tendency of STC-1 expression was seen in the recurrent/metastatic tumors. The STC-1 positivity was 25% in Group 1, 53% in Group 2 and 71% in Group 3 (Table 9).

STC-2 displayed a similar tendency of higher expression in late relapsing tumors than in the early relapsing ones. The mean frequency of STC-2 positively stained tumor cells was 3% in the primary tumors of Group 1, 13% in Group 2 and 17% in Group 3 (Table 9). In the recurrent/metastatic tumors the STC-2 frequency of positive tumor cells was 3% in Group 1, 26% in Group 2 and 58% in Group 3, the latest relapsing tumors (Table 9).

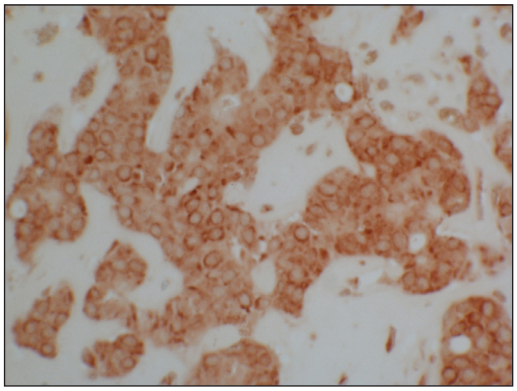


Fig. 5 STC-1 staining in a metastatic breast cancer of the latest relapsing tumors (Group 3)

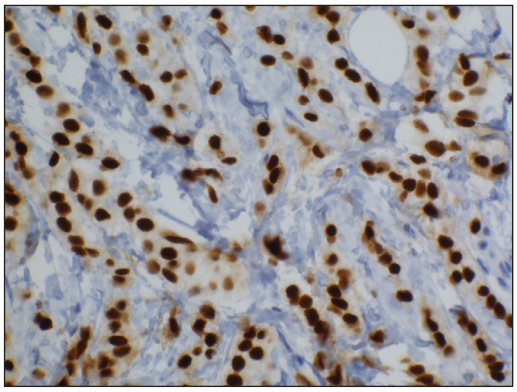


Fig. 6 ER staining in a metastatic breast cancer of the latest relapsing tumors (Group 3)

In the late-relapsing tumors (Groups 2 and 3), the frequency of positively stained cells for STC-1 and STC-2 was significantly higher in the recurrent tumors than in their primary counterparts (p=.0012, p=.0017 for STC1 and p=.004 and p=.0001 for STC-2, respectively) (Table 9).

Stanniocalcins were not associated with ER expression in the primary cancers, but STC-1 was associated with ER in the recurrent/metastatic tumors (p=.019) (Table 12).

ER

The frequency of ER-positive (Fig. 6) tumor cells did not significantly vary between the three groups, regarding the times of recurrence of the primary tumors. In the primary tumors, the ER mean expression was 30% in Group 1, 29% in Group 2 and 24% in Group 3. In the recurrent/metastatic tumors the tendency was to an increased frequency of ER-positive stained tumor cells in the later relapsed tumors (30% in Group 1, 34% in Group 2 and 46% in Group 3) (Table 9). In the recurrences of the latest relapsing tumors (Group 3) the frequency of ER- positive tumor cells was significantly higher compared to the corresponding primary tumors (p=.019) (Table 9).

STUDY II

MASPIN, BCL-2, P53

Maspin

In this study the cytoplasmic and nuclear maspin were separately evaluated. The cytoplasmic maspin expression (Fig. 7) was higher in the early relapsing tumors than in the late relapsing ones (Table 9) The mean proportion of positively stained tumor cells for cytoplasmic maspin was 19% in the primary cancers of the early relapsing tumors of Group 1, 9% in Group 2 and 4% in the latest relapsing tumors Group 3. The corresponding values in the recurrent/metastatic tumors were 23% in Group 1, 9% in Group 2 and only 1% in the latest tumor Group 3 (Table 9). Cytoplasmic maspin expression varied significantly between the groups, both in the primary cancers and in the recurrent/metastatic tumors when tested by Kruscall-Wallis test (p=.019 in primary tumors and p=.023 in the recurrences) (Table 10). When tested by Mann-Whitney *U*-test, there was a significantly higher expression of cytoplasmic maspin in the early relapsing primary tumors of Group 1 than in the later relapsing tumors of Group 2 (p=.009) (Table 11). In the recurrent/metastatic tumors, the expression of cytoplasmic maspin tended to be higher in the early relapsing tumors in Group 1 compared to the latest relapsing tumors in Group 3 (p=.010, Mann-Whitney *U*-test) (Table 11). The mean frequency of cytoplasmic maspin expression did not differ significantly between the primary tumors and their corresponding metastases (Table 9).

Nuclear maspin expression was significantly higher in the primary cancers of the early relapsing tumors of Group 3, than in the corresponding recurrent/metastatic tumors (p=.033) (Table 9). Cytoplasmic maspin positivity correlated with the p53 positivity both in the primary tumors and in the recurrences (p=004 in primary and p=.042 in the recurrent/metastatic tumors) (Table 12).

Bcl-2

In contrast to the expression levels of cytoplasmic maspin, the levels of Bcl-2 staining (Fig. 8) were significantly higher in late-relapsing cancers, both primary cancers and the recurrent/metastatic tumors, than in the early relapsing tumors (Table 9). The mean frequency of positively staining tumor cells for Bcl-2 was 25% in primary tumors of the early relapsing cancers of Group1, 37% in Group 2 and 55% in the

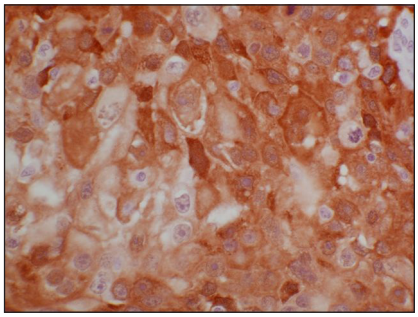


Fig. 7 Maspin staining in an early-relapsing primary tumor (Group 1)

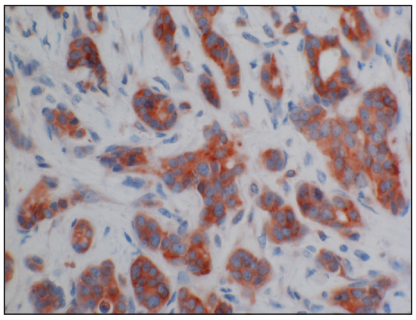


Fig. 8 Bcl-2 staining in a primary tumor of the latest relapsing tumors (Group 3)

latest relapsing tumors of Group 3 (Table 9). In the recurrent/metastatic tumors, the mean expression of Bcl-2 was 11% in Group 1, 31% in Group 2 and 40% in the latest relapsing tumors of Group 3 (Table 9). There was significantly more Bcl-2 positivity in the recurrent cancers of the latest relapsing tumors of Group 3 compared to the recurrent tumors of early relapsing tumors of Group 1, when tested by the Kruscall-Wallis test (p=.005) (Table 10) and the Mann-Whitney U-test (p=.001) (Table 11).

Bcl-2 positivity correlated significantly with ER positivity, in both primary tumors (p=.007) and the recurrences (p=.0001) and with axillary node negativity in primary tumors (p=.004) (Table 12).

p53

The mean frequency of p53 positive (Fig. 9) stained tumor cells was higher in the early relapsing tumors than in the late relapsing ones, both in the primary cancers and in the recurrences (Table 9). The mean positivity of p53 was 25% in the primary

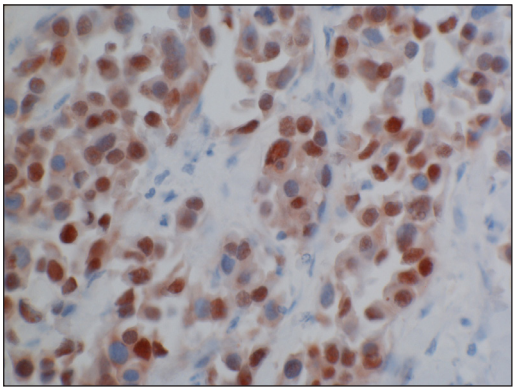


Fig. 9 p53 staining in an early relapsing primary tumor (Group 1)

tumors of the early relapsing cancers of Group 1, 19% in Group 2 and 5% in the latest relapsing tumors of Group 3 (Table 9). The corresponding values in the recurrent/metastatic tumors were 28% in Group 1, 12% in Group 2 and only 4% in the latest relapsing tumors of Group 3 (Table 9). p53 expression did not differ significantly between the primary tumors and the corresponding recurrences/metastases (Table 9). The mean P53 positivity was significantly higher in the recurrences of the early relapsing tumor Group 1 than in the latest relapsing tumors of Group 3 when tested with the Kruskall-Wallis test (p=.006) (Table 10) and the Mann-Whitney U-test (p=.002) (Table 11).

p53 negativity was associated with cytoplasmic maspin negativity both in primary tumors (p=.004) and in the recurrent/metastatic tumors (p=.042) (Table 12). p53 positivity also associated with ER negativity in recurrent/metastatic tumors (p=.008) (Table 12).

STUDY III

BMI-1, C-MYC AND SNAI1

Bmi-1

The mean frequency of Bmi-1 positive (Fig. 10) stained tumor cells was 30% in the primary tumors of the early relapsing cancers (Group 1), 32% in Group 2 and 42% in the latest relapsing tumors of Group 3 (Table 9). The corresponding values in

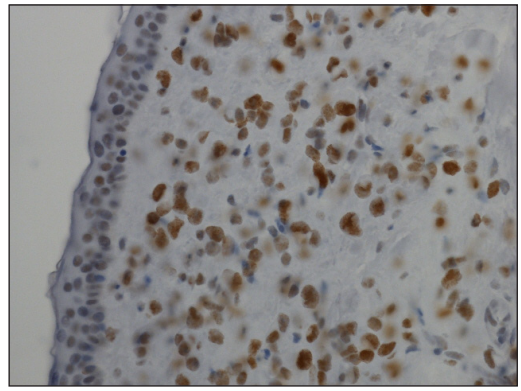


Fig. 10 Bmi-1 staining in a breast cancer metastasis of the latest metastasizing tumor group (Group 3).

recurrent/metastatic tumors were 53% in Group 1, 37% in Group 2 and 65% in Group 3. The mean Bmi-1 positivity was significantly higher in the tumor recurrences/metastases than in the primary cancers, both in the early recurring tumors of Group 1, and in the latest recurring tumors of Group 3 (p=.004 and p=.019, respectively) (Table 9). The mean expression of Bmi-1 in the primary tumors did not differ between the three groups (Table 10). In the recurrent/metastatic tumors there was significantly more Bmi-1 expression in the latest relapsing tumor Group 3 than in Group 2 (p=.007; Kruskal-Wallis test, Table 10, and p=.003; Mann-Whitney U test, Table 11).

c-myc

The mean nuclear positivity of c-myc in primary tumors was 12% in Group 1, 2% in Group 2 and 16% in Group 3 (Table 9). The corresponding ratios in recurrence were 5% in Group 1, 9% in Group 2 and 19% in Group 3. The mean cytoplasmic c-myc positivity in primary tumors was 18% in Group 1, 11% in Group 2 and 7% in Group 3 and the corresponding ratios in recurrences were 12% in Group 1, 25% in Group 2 and 8% in Group 3 (Table 9). The nuclear or cytoplasmic expression of c-myc did not differ significantly between the groups, neither in the primary tumors nor in the recurrences.

There was significantly more both nuclear and cytoplasmic c-myc positivity in the metastases of Group 2 than in the corresponding primary tumors (p=.013 for nuclear c-myc and p=.033 for cytoplasmic c-myc) (Table 9). Nuclear c-myc in the primary tumors associated significantly with axillary node positivity (p=021) (Table 12). Cytoplasmic c-myc positivity in the primary tumors associated with high Snail expression (p=.002 for invasive front Snail and p=.011 for central Snail) (Table 12).

Snail

The mean Snail positivity was evaluated separately in the invasive front and central area of the tumors. Sometimes there was greater expression of Snail in invasive front than in central areas of the tumors, but generally the mean Snail positivity was equally high in the tumors – both in the primary cancers (range 85.5 – 88.7) and in the recurrences/metastases (range 78.5 – 91.3) (Table 9). Snail expression did not differ significantly between the primary tumors and their metastases in any of the three groups (Table 9), nor did the three groups differ statistically in Snail expression. High Snail expression was associated with cytoplasmic c-myc positivity in the primary tumors, as mentioned in the preceding section on c-myc. High invasive front and central Snail positivity was associated with Bmi- positivity in the recurrent/metastatic tumors (p=.0001 for invasive front Snail and p=.009 for central Snail (Table 12).

Table 9. Differences in the mean proportion of positive stainined tumor cells for STC-1, STC-2 and ER (%; range, 0–100%) between the primary tumors and their corresponding recurrent/ metastatic tumors. The patients are divided in to Groups 1, 2 and 3 according to the time of relapse after primary diagnosis. Paired samples t- test was used. Values are shown as % mean (SD deviation).

Group	n	Primary	Recurrent/metastatic	p
		STC-1		
1	19	27.7 (32.3)	25.2 (31.3)	.66
2	35	30.8 (27.2)	53.3 (33.7)	.0012*
3	18	39.2 (32.7)	70.5 (32.7)	.0017*
		STC-2		
1	19	2.5 (3.3)	3.3 (5.4)	.54
2	35	13.2 (19.6)	26.1 (26.2)	.004*
3	18	17.4 (16.3)	57.5 (36.9)	.0001*
		ER		
1	19	30.3 (35.4)	30 (40.6)	.95
2	35	29.2 (37.5)	34.4 (38.7)	.38
3	18	24.1 (35.5)	45.6 (40.2)	.019*
		Maspin cytoplasmic		
1	19	18.8 (23.6)	23.2 (30.2)	.409
2	34	8.5 (23.1)	9 (22.1)	.860
3	20	4.2 (9.3)	1 (3.3)	.149
		Maspin nuclear		
1	19	4.8 (8.6)	8.1 (17.4)	.305
2	34	2.8 (10.8)	3 (6.9)	.908
3	20	9.7 (17.3)	1.6 (4)	.033*
		Bcl-2		
1	19	25.4 (33.1)	10.5 (20)	.037*
2	34	37 (35.8)	30.8 (36.5)	.098
3	20	54.8 (40.2)	40 (39.9)	.408
		p53		
1	19	24.5 (35.2)	27.5 (34.8)	.621
2	34	18.8 (32.5)	11.9 (25.3)	.098
3	20	5.3 (20.6)	4.2 (16.1)	.408
		Bmi-1		
1	19	30.1 (27.3)	53 (28.4)	.004*
2	33	32 (35.7)	37 (33.4)	.466
3	21	41.9 (36.9)	64.9 (25.1)	.019*
		c-myc nuclear		
1	19	11.5 (17.9)	4.7 (9.9)	.168
2	33	2.3 (4.1)	8.6 (13.9)	.013*
3	21	16.0 (28.0)	18.9 (31.6)	.783
		c-myc cytoplasmic		
1	19	17.9 (25.8)	12.4 (22.1)	.306
2	33	10.6 (20.9)	25.0 (31.6)	.033*
3	21	6.8 (15.9)	18.9 (31.6)	.897
		Snail frontal		
1	19	88.7 (17.5)	93.3 (8.2)	.280
2	33	85.5 (23.7)	88.5 (21.5)	.497
3	21	88.1 (21.7)	88.5 (21.5)	.479
		Snail central		
1	19	85.7 (14.0)	90.3 (10.7)	.246
2	33	77.0 (30.2)	78.5 (26.3)	.801
3	21	79.0 (30.5)	91.3 (10.8)	.120

Note. Group 1 (n= 19) includes patients with recurrence or metastasis detected within 2 years of diagnosis. Group 2 (n=35) and 3 (n=18) include patients with recurrence or metastasis detected between 5-10 and after 10 years of follow-up, respectively.

* statistically significant

Table 10. Differences in mean proportion of positively stained tumor cells between the 3 groups of 73 breast cancers,in primary tumors and in their recurrent/metastatic tumors. The patients are divided into 3 groups, according to the time of relapse after primary diagnosis. The Kruskal-Wallis test was used.

Staining	Group	n	Mean rank	p
Maspin cytoplasmic				
Primary	1	19	47.2	.019*
	2	34	32.2	
	3	20	35.4	
Recurrent/metastatic	1	19	46.1	.023*
	2	34	35.3	
	3	20	31.3	
Bcl-2				
Primary	1	19	29.9	.061
	2	34	35.9	
	3	20	45.5	
Recurrent/metastatic	1	19	25.6	.005*
	2	34	37.6	
	3	20	46.8	
p53				
Primary	1	19	40.6	.089
	2	34	39.4	
	3	20	29.5	
Recurrence/metastatic	1	19	46	.006*
	2	34	36.1	
	3	20	27.7	
Bmi-1				
Primary	1	19	37	.397
	2	33	38.8	
	3	21	41.8	
Recurrent/metastatic	1	19	39.7	
	2	33	28.9	
	3	21	47.2	.007*

Note: Group 1 includes patients with a recurrence or metastasis detected within 2 years of diagnosis. Groups 2 and 3 include patients with a recurrence or metastasis detected at 5–10 years, or after 10 years of follow-up, respectively.

* statistically significant

Table 11. Staining differences of cytoplasmic maspin, Bcl-2, p53 and Bm1-1 between 2 groups. The patients are divided into 3 groups, according to the time of relapse after primary diagnosis. Mann-Whitney U test was applied, if Kruskal-Wallis test showed a significant difference between the 3 groups. P value was considered statistically significant*, if it was < 0.0167 (<0.05/3).

		U	Z	p
Maspin cytoplasmic				
Primary	Group 1 vs group 2	198	-2.626	.009*
	Group 1 vs group 3	121.5	-2.048	.041
	Group 2 vs group 3	303	-.805	.421
Recurrent/metastatic	Group 1 vs group 2	229.5	-2.022	.043
	Group 1 vs group 3	111.5	-2.571	.010
	Group 2 vs group 3	304.5	-.848	.396
Bcl-2				
Primary	Group 1 vs group 2	215.5	-2.141	.032
	Group 1 vs group 3	81	-3.200	.001*
	Group 2 vs group 3	252.5	-1.600	.110
p53				
Recurrent/metastatic	Group 1 vs group 2	231	-1.876	.061
	Group 1 vs group 3	92	-3.055	.002*
	Group 2 vs group 3	244	-1.889	.059
Bmi-1				
Recurrent/metastatic	Group 1 vs group 2	215.0	-1.880	.060
	Group 2 vs group 3	178.0	-2.996	.003*
	Group 1 vs group 3	153.0	-1.266	.206

Note. Group 1 (n=19) includes patients with a recurrence or metastasis detected within 2 years of diagnosis. Groups 2 (n=34, for Bmi-1 n=33) and 3 (n=20, for Bmi-1 n=21) include patients with a recurrence or metastasis detected at 5–10 years, or after 10 years of follow-up, respectively.

Table 12. Relationship between cytoplasmic maspin, Bcl-2, p53, ER, c-myc, Snail and clinicopathological parameters in 73 primary breast cancers and recurrent/metastatic tumors. Categorical Pearson's Chi-square test was used. Only statistically significant results are shown.

Primary

	Maspin cytoplasmic negative n (%)	Maspin cytoplasmic positive n (%)	p
p53 neg	44 (81)	9 (47)	
p53 pos	10 (19)	10 (53)	.004
	Bcl-2 negative n (%)	Bcl-2 positive n (%)	
ER neg	21 (78)	21 (46)	
ER pos	6 (2)	25 (54)	.007
Node neg	7 (26)	28 (61)	
Node pos	20 (74)	18 (39)	.004
	Bmi-1 negative n (%)	Bmi-1 positive n (%)	
ER negative	19 (46)	22 (54)	
ER positive	6 (19)	26 (81)	.014
	Nuclear c-myc negative n (%)	Nuclear c-myc positive n (%)	
Node negative	31 (91)	3 (9)	
Node positive	27 (69)	12 (31)	.021
	Cytoplasmic c-myc negative n (%)	Cytoplasmic c-myc positive n (%)	
Snail invasive front low	13 (100)	0	
Snail invasive front high	33 (55)	27 (45)	.002
Snail invasive front low	18 (86)	3 (14)	
Snail invasive front high	28 (54)	24 (46)	.011

Recurrent/metastatic

	STC-1 negative n (%)	STC-1 positive n (%)	
ER negative	11 (38)	18 (62)	
ER positive	6 (14)	37 (86)	.019
	Maspin cytoplasmic negative n (%)	Maspin cytoplasmic positive n (%)	
p53 negative	43 (78)	9 (53)	
p53 positive	12 (8)	8 (47)	.042
	Bcl-2 negative n (%)	Bcl-2 positive n (%)	
ER negative	23 (59)	6 (18)	
ER positive	16 (41)	28 (82)	.0001
	p53 negative n (%)	p53 positive n (%)	
ER negative	16 (31)	13 (65)	
ER positive	36 (69)	7 (35)	.008
	Bmi-1 negative n (%)	Bmi-1 positive n (%)	
ER negative	10 (26)	28 (74)	
ER positive	1 (3)	34 (97)	.005
Snail invasive front low	4 (67)	2 (33)	
Snail invasive front high	7 (10)	60 (90)	.0001
Snail invasive front low	5 (39)	8 (62)	
Snail invasive front high	11 (18)	49 (82)	.009

STUDY IV

ER, PR, HER2, KI67, CK5, THE CLINICOPATHOLOGICAL PARAMETERS AND THE IHC DEFINED APPROXIMATIONS TO THE INTRINSIC GENETICALLY DEFINED BREAST CANCER SUBTYPES

Clinicopathological parameters

The results of the univariate analysis of clinicopathological parameters are shown in Table 13. Axillary node positivity was associated with early tumor relapse (p=.006). High tumor grade and tumor size greater than 20 mm was also associated with early tumor recurrence (p=.008 for tumor grade and p=.021 for tumor size). No significant differences were seen between the groups in regard to age or histological type of tumor. In multivariate analysis, increasing tumor size significantly enhanced the risk of early tumor relapse (OR 1.07, CI 1.01 – 1.12) (Table 16). An increase of 1 mm in tumor size thus increased the risk of early breast cancer relapse by 7%.

ER

ER positivity (Fig. 6) in the primary tumors in this cohort of 72 tumors was 68%. The positivity between primary and recurring tumors changed most in the early relapsing tumors of Group 1, where 3 ER-positive tumors became ER-negative in the recurrences (15% decrease) (Table 14). ER positivity was associated with late tumor relapse (p=0.47) (Table 13). In multivariate analysis, ER was not a significant risk factor for late relapse. ER positivity was associated with axillary node negativity (p=.014), low grade (p=.024), and HER2 negativity (p=.046) in the primary tumors, data not shown.

PR

There were significantly more PR-positive (Fig, 13) primary tumors (38%) compared to the recurrent cancers (24%) in the entire tumor set (n=72, p=.005) (Table 14). A decrease in PR positivity was also seen in the recurrent/metastatic tumors of all three groups. The decrease in the number of PR-positive tumors was most significant (31%) in the latest relapsing tumors of Group 3 (p=.030) (Table 14). There were

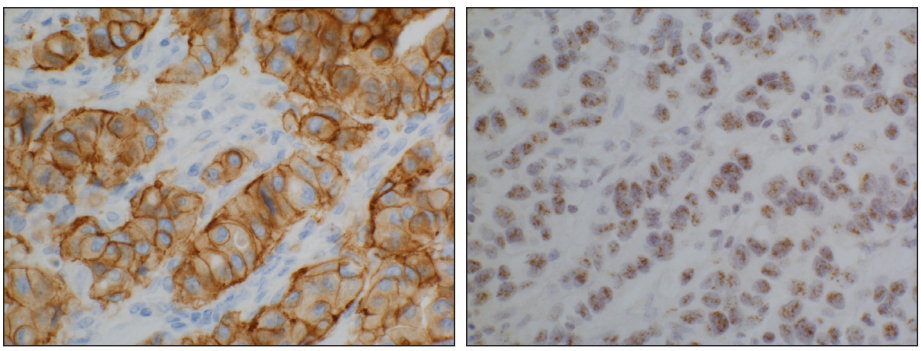
no significant differences in PR expression between the groups. PR positivity in the primary tumors was associated with node negativity (p=.017), data not shown.

HER2

There was a high concordance (97%) of HER2 overexpression (Fig. 11) measured by both IHC and CISH between the primary tumors and the corresponding metastases in all three groups, according to the time after primary detection of the cancers (Table 14). There were 15 (21%) HER2-positive primary and 15 (21%) positive recurrent/metastatic tumors in the whole tumor set (Table 14). Only three pairs with discordant HER2 status were found in the entire tumor material of paired primary and metastatic tumors. One primary tumor, belonging to the early relapsing Group 1, was HER2-positive by IHC and CISH, but its metastasis was CISH-negative. Another case of discordance was noted in Group 1: the primary tumor was HER2-positive by both IHC and CISH, and the recurrent tumor was negative (1+) by IHC, but CISH-positive. In the third case of discordance, a primary tumor from Group 3 was negative for HER2 by both IHC and CISH, but the metastasis was positive by both IHC and CISH. One tumor of all CISH-positive cases displayed low amplification. When tested with the chromosome 17 centromere probe, this case showed an average HER2/Chr17 ratio over 2, and turned out to be HER2 gene amplified.

HER2 over-expression signified early tumor relapse (p=.003) (Table 13). In multivariate analysis, HER2 negativity significantly lowers the risk of early tumor relapse (OR 0.19 95% CI 0.04–0.83) (Table 16) or inversely, HER2 positivity increases the risk of early tumor relapse by 1/0.19 = 5.3. HER2 negativity was associated with node negativity (p=.018), low tumor grade (p=.021), and Ki67 negativity (p=.008) in the primary tumors, data not shown.

Fig. 11 HER2 over-expression in an early relapsing breast cancer of Group 1, a (IHC), b (CISH)



a b

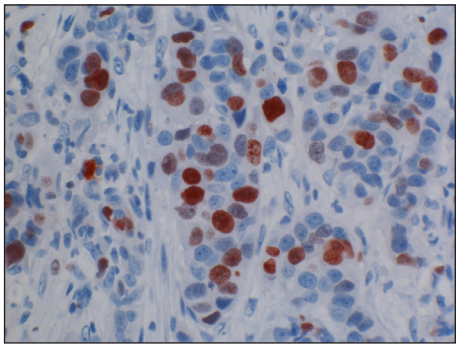


Fig. 12 Ki67 positive staining in a metastatic tumor of the early relapsing breast cancers (Group 1)

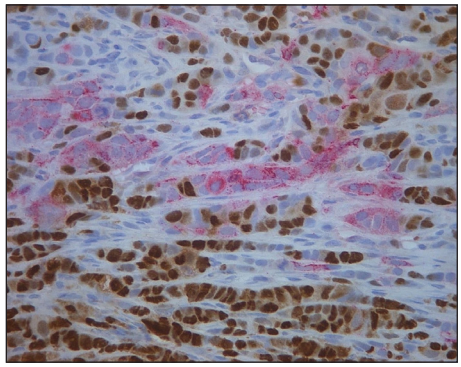


Fig. 13 CK 5 (red)/PR (brown) dual staining in early relapsing breast cancer (Group 1)

Ki67

The expression of Ki67 (Fig. 12) was associated with early tumor relapse (p=.012) (Table 13) in univariate analysis, but not in the multivariate test. The positivity of Ki67 (>14% positively stained tumor cells) declined gradually from the early relapsing tumors (Group 1), toward the latest relapsing tumors of Group 3 (Table 14) There were 68% primary Ki67 positive breast cancers in the early relapsing tumor Group 1, 42% in Group 2 and 21% in the latest relapsing tumors of Group 3. The corresponding ratios in recurrent/metastatic tumors were 74% in Group 1, 62% in Group 2 and 21% in Group 3. There were significantly more Ki67-positive recurrent/metastatic cancers in the tumors of Group 2 (recurrences at 5–10 years) than in the corresponding primary tumors (p=.017) (Table 14).

Ki67 positivity was associated with axillary node positivity (p=.027), high grade (p=.0001), ductal histological type (p=.026), and HER2 positivity (p=.008) in the primary tumors, data not shown.

CK5

In the whole tumor set of 72 breast cancers, there were significantly more CK5-positive (Fig. 13) primary cancers compared to the recurrent/metastatic tumors (31% and 11%, respectively, p=.0001) (Table 14). The expression of CK5 declined gradually from the early relapsing tumors toward the late relapsing ones. There were 58% CK5 positive tumors in Group 1, 24% in Group 2 and 16% in Group 3. The corresponding ratios in recurrences were 26% in Group 1, 9% in Group 2 and none in Group 3 (Table 14). As in the whole tumor set, there was also a significant

loss of CK5 positivity in the recurrent tumors of the early relapsing cancers of Group 1 (p=.010) (Table 14).

In univariate analysis, CK5 expression associated significantly with early tumor relapse (p=.009) (Table 13), but not in multivariate analysis. CK5 positivity correlated significantly with metastases to the axillary lymph nodes (p=.025), high tumor grade (p=.0001), ductal histological type (p=.003), ER negativity (p=.029), and Ki67 positivity (p=.0001), data not shown.

Table 13. Relationship of clinicopathological parameters, ER, PR, HER2, Ki67 and CK5 protein expression in primary tumors of 72 breast cancer patients. The patients are divided into 3 groups, according to the time of relapse after primary diagnosis. Categorical Pearson's Chi-square test was used.

Group	Node negative n (%)	Node positive n (%)		p
1	3 (16)	18 (84)		
2	20 (59)	14 (41)		
3	11 (58)	8 (42)		.006*
	Grade 1	Grade 2	Grade 3	
1	0	8 (42)	11 (58)	
2	5 (15)	19 (56)	10 (29)	
3	3 (16)	15 (79)	1 (5)	.008*
	size < 20 mm	size > 20 mm		
1	3 (16)	16 (84)		
2	16 (47)	18 (53)		
3	11 (58)	8 (42)		.021*
	ER negative	ER positive		
1	10 (53)	9 (47)		
2	10 (29)	24 (71)		
3	3 (16)	16 (84)		.047*
	PR negative	PR positive		
1	13 (68)	6 (32)		
2	15 (44)	19 (56)		
3	6 (32)	13 (68)		.066
	HER2 negative	HER2 positive		
1	10 (53)	9 (47)		
2	29 (85)	5 (15)		
3	18 (95)	1 (5)		.003*
	Ki67 negative	Ki67 positive		
1	6 (32)	13 (68)		
2	20 (59)	14 (41)		
3	15 (79)	4 (21)		.012*
	CK5 negative	CK5 positive		
1	8 (42)	11 (58)		
2	26 (77)	8 (24)		
3	16 (84)	3 (16)		.009*

Note: Group 1 (n=19) includes patients with a recurrence or metastasis detected within 2 years of diagnosis. Groups 2 (n=34) and 3 (n=19) include patients with a recurrence or metastasis detected at 5–10 years, or after 10 years of follow-up, respectively.

* statistically significant

Table 14. Expression of CK5, ER, PR, HER2 and Ki67 as compared in primary and metastatic tumors of 72 breast cancer patients. The patients are divided into 3 groups, according to the time of relapse after primary diagnosis. Paired samples t-test was used.

	Primary positive n (%)		**Recurrent/metastatic positive n (%)**	***p***
	ER			
All cases n=72	49 (68)		47 (65)	.596
Group				
1	9 (47)		6 (32)	.083
2	24 (71)		25 (74)	.711
3	16 (84)		16 (84)	1.000
	PR			
All cases n=72	38 (53)	.	24 (33)	.005*
Group				
1	6 (32)		4 (21)	.331
2	19 (56)		13 (38)	.110
3	13 (68)		7 (37)	.030*
	HER2			
All cases n=72	15 (21)		15 (21)	1.000
Group				
1	9 (47)		8 (42)	.331
2	5 (15)		5 (15)	1.000
3	1 (5)		2 (11)	.331
	Ki67			
All cases n=72	31 (43)		39 (54)	.059
Group				
1	13 (68)		14 (74)	.667
2	14 (42)		21 (62)	.017*
3	4 (21)		4 (21)	1.000
	CK5			
All cases n=72	22(31)		8 (11)	.0001*
Group				
1	11(58)		5 (26)	.010*
2	8(24)	.43	3 (9)	.058
3	3(16)	.37	0	.083

Note: Group 1 (n=19) includes patients with a recurrence or metastasis detected within 2 years of diagnosis. Groups 2 (n=34) and 3 (n=19) include patients with a recurrence or metastasis detected at 5–10 years, or after 10 years of follow-up, respectively.

* statistically significant

Relationship of the IHC surrogates to the intrinsic genetical defined subtypes, luminal A (ER or PR+HER2-), luminal B (ER or PR+HER2+), HER2 over-expressing (ER-PR-HER2+), triple-negative (ER-PR-HER2-), basal-like (ER-PR-HER2-CK5+), unclassified (ER-PR-HER2-CK-) and luminobasal (ER or PR+CK+) to early and late recurrence of tumors.

There were altogether 35 (49 %) primary tumors of luminal A (ER or PR+HER2-) type and this subtype signified late tumor recurrence (p=.0001, Table 15) in univariate analysis. In multivariate analysis, the non-luminal A phenotype of tumors increased significantly the risk of early tumor relapse (OR 3.26 95% CI 1.01–10.59) (Table 16), i.e. the subtype luminal A of tumors with this changing coefficient of 0.3067 (1/3.26=0.3067) lowers the risk 100*(1–0.3067) = 69.33%.

There were only 2 (11%) luminal A type tumors in the early relapsing tumors of Group 1, 20 (59%) in Group 2 and 13 (68%) in the latest relapsing tumors of Group 3 (Table 15). The other subtypes were rare, and they mostly represented early relapsing tumors, but there were no significant differences. There were only 4 (11 %) luminal B type primary tumors in the whole tumor set, 2 (11%) in the early relapsing tumor Group1 , 2 (6%) in Group 2 and none in the latest recurring tumor group (Group 3) (Table 15). There were 8 HER2 over-expressing (ER-PR-HER2+) type primary tumors, 5 (26%) in the early relapsing tumors of Group 1, 2 (6%) in Group 2 and 1 (5%) in the latest relapsing tumors of Group 3. There were 14 luminobasal type of cancers, 6 (32%) in Group 1, 5 (15%) in Group 2 and 3 (16%) in Group 3. From the all 7 basal-like tumors 4 (21%) were in Group 1, 3 (9%) in Group 2 and none in Group 3. There were 4 non-classified types of tumors, none in Group 1, 2 (6%) in Group 2 and 2 (11%) in Group 3. There were 11 primary triple-negative type of tumors, 4 (21%) in Group 1, 5 (15%) in Group 2 and 2 (11%) in Group 3.

The triple-negative CK5 positive, basal-like tumors tended to associate with early tumor relapse (p=.055, Table 15). A total of 11 triple-negative pimary tumors occurred in the whole tumor set, 4 (21%) in the early relapsing tumors of Group 1, 5 (15%) in tumor Group 2 (recurrence after 5 years) and 2 (11%) in the latest relapsing tumor Group 3. The 4 triple-negative tumors in the early relapsing tumors (Group 1) were all CK5-positive, whereas the two triple-negative tumors in the latest recurring tumors (Group 3) were all CK5-negative (Table 15).

Ki67 positivity associated with early tumor relapse in luminal B phenotype of tumors but not in luminal A type of cancers (p=.0001 in luminal A and p=.364 in luminal B type, data not shown).

Table 15. Distribution of seven subtypes of tumors defined by IHC, in primary tumors of 72 breast cancer patients. The patients are divided into 3 groups, according to the time of relapse after primary diagnosis. Categorical Pearson's Chi-square test was used.

Group	Luminal A n (%)	all others n (%)	p
1	2 (11)	17 (90)	
2	20 (59)	14 (41)	
3	13 (68)	6 (32)	.0001*
	Luminal B	**all others**	
1	2 (11)	17 (90)	
2	2 (6)	32 (94)	
3	0	19 (100)	.364
	HER2 overexpressing	**all others**	
1	5 (26)	14 (74)	
2	2 (6)	32 (94)	
3	1 (5)	18 (95)	.049*
	Luminobasal	**all others**	
1	6 (32)	13 (68)	
2	5 (15)	29 (85)	
3	3 (16)	16 (84)	.296
	Basal-like	**all others**	
1	4 (21)	15 (79)	
2	3 (9)	31 (91)	
3	0	19 (100)	.088
	Non-classified	**all others**	
1	0	19 (100)	
2	2 (6)	32 (94)	
3	2 (11)	17 (90)	.364
	Triple-negative	**all others**	
1	4 (21)	15 (79)	
2	5 (15)	29 (85)	
3	2 (11)	17 (90)	.660
	Triple-negative CK5+	**Triple-negative CK5-**	
1	4 (100)	0	
2	3 (60)	2 (40)	
3	0	2 (100)	.055

IHC subtypes: Luminal A (ER or PR+HER2-), Luminal B (ERorPR+HER2+), HER2 overexpressing (ER-PR-HER2+), Basal-like (ER-PR-HER2-CK5+), Luminobasal (ERorPR+CK5+), Non-classified (ER-PR-HER2-CK5-), Triple-negative CK5 positive (ER-PR-HER2-CK5+).

Basal-like (ER-PR-HER2-CK5+) = Triple negative CK5+

Non-classified (ER-PR-HER2-CK5-) = Triple negative CK5-

Note. Group 1 (n=19) includes patients with a recurrence or metastasis detected within 2 years of diagnosis. Groups 2 (n=34) and 3 (n=19) include patients with a recurrence or metastasis detected at 5–10 years, or after 10 years of follow-up, respectively.

* statistically significant

Table 16. Dependence of the time of tumor recurrence on different factors. Model 1 includes clinicopathological parameters and HER2, ER, CK5, Ki67. Model 2 includes the clinicopathological parameters and the subgroups luminal A and HER2 overexpressing, defined by IHC. The regression ordinal test was used.

Model 1	Variable	Wald Sig.	OR	95% Confidence interval
	size (2mm–90mm)	0.011	1.07	1.02 – 1.13
	HER2-negative	0.027	0.19	0.04 – 0.83
	ER-negaative	0.228	2.03	0.64 – 6.41
	CK5-negative	0.146	0.35	0.09 – 0.69
	Ki67-negative	0.344	0.53	0.15 – 1.96
	Node-negative	0.431	1.64	0.48 – 5.60
	Grade 1	0.647	0.62	0.08 – 4.77
	Grade 2	0.799	0.83	0.19 – 3.58

Model 2	Variable	Wald Sig.	OR	95% Confidence interval
	size (2mm–90mm)	0.013	1.07	1.01 – 1.12
	Non-luminal A	0.049	3.26	1.01 – 10.59
	Non-HER2-overexpressing	0.211	0.34	0.06 – 1.85
	Node-negative	0.593	1.39	0.41 – 4.67
	Grade 1	0.153	0.26	0.04 – 1.65
	Grade 2	0.277	0.47	0.12 – 1.84

Note: IHC subtypes: luminal A(ERorPR+HER2-), HER2 overexpressing(ER-PR-HER+).

DISCUSSION

This summary is based on four studies, in which the expression of different proteins is described in a series of 73 primary breast cancers and their corresponding recurrences. 19 of the cancers were early relapsing tumors and 53 late recurring ones (after 5 or 10 years). Immunoexpressing levels of proteins were compared between the primary tumors and their metastases, as well as between early and late recurring cancers, and also with clinicopathological parameters, in order to analyze their putative role in breast cancer progression.

STUDY IV

In Study IV, the established markers guiding breast cancer treatment after surgery of the primary tumor, i.e., ER, PR, HER2, Ki67, together with the basal-like cytokeratin CK5, were evaluated. The results were analysed with the clinicopathological parameters and the IHC surrogates of the intrinsic genetically defined subgroups of breast cancers, luminal A (ERorPR+HER2-), luminal B (ErorPR+HER+), HER2 over-expressing (ER-PR-HER2+), triple-negative (ER-PR-HER2-), basal-like, (ER-PR-HER-CK5+), non-classified (ER-PR-HER2-CK5-) and luminobasal (ERorPR+CK5+).

CLINICOPATHOLOGICAL PARAMETERS

The well known parameters for poor outcome in breast cancer, i.e., axillary node positivity, high tumor grade, and tumor size (Elston *et al.*1982) were associated with early tumor recurrence in univariate analysis. In multivariate analysis, tumor size was the most significant risk factor for early recurrence. Galea *et al.* (1982) have also shown tumor size to be a significant risk factor for poor prognosis in breast cancer patients in multivariate analysis. Excellent estimates for breast cancer growth rates, based on cancer incidence and tumor measurements from almost 400,000 women who were 50–69 years of age, have been calculated from Norwegian mammography screening studies (Weedon-Fekjaer *et al.* 2008). The 5% fastest growing tumors took 1.2 months to grow from 10 to 20 mm in diameter, whereas the 5% slowest growing tumors needed more than 6.3 years. Assuming constant exponential or logistic growth for small tumors (i.e., without a dormancy period or retarded growth), the fast growing tumors reached a size of 10 mm within one year, and the slow growing tumors in more than 50 years. For the 5% fast growing

cancers, a period of dormancy is very likely when no metastasis is found within two years after diagnosis. In the second group, dormancy is highly unlikely, even when it is assumed that the dissemination of tumor cells and initiation of metastasis occurred shortly after malignant transformation. Therefore, metastasis dormancy may occur in cases of early relapse, although absent in very late recurrences. This individual variation may be one reason why it is not possible to deduce the state of dormancy from clinical studies or epidemiological data, and has led to conflicting results (Klein 2011, Demiceli 2001, Demiceli *et al.* 1996).

ER AND PR

In this study, ER positivity was associated with very late tumor relapse, which is in accordance with previous reports (Hess *et al.* 2003, Brewster *et al.* 2008). The heterogeneity of ER expression within an individual tumor, between different tumors, and also between primary tumors and their metastases, is a well known phenomenon.

The discordance of the receptor status between primary and metastatic breast cancers has been known for over 30 years (Brennan *et al.* 1979). Such discordance in estrogen and progesterone receptors can occur in as many as 40% of breast cancers (Li *et al.* 1994, Kuukasjärvi *et al.* 1996).

In my tumor material, there were 15% more ER-positive primary tumors compared to their metastases in the early relapsing cancers. In a study of 75 patients, Sari et al. demonstrated that 21% of ER-positive tumors did not express ER in their metastases and, in contrast, 15% of ER-negative primary tumors were positive in their metastases (Regitnig *et al.* 2004). In our study, only one (3%) primarily ER-negative tumor had an ER-positive metastasis. Hoefnagel *et al.* (2010) showed inversion of primary ER-positive tumors into negative metastases in 10.7 % and from negative into positive in 3.4% of their patients. The corresponding numbers in the study of Thomson *et al.* (2010) were 8% from positive into negative, and vice versa in 2.2% of their cases.

Several studies have shown that in breast cancer late metastases mostly display higher ER and PR expression than do the primary cancer and early metastases (Spataro *et al.* 1992).

The mechanism of ER in tumor dormancy is likely to be related to its role as a receptor of estrogen, which is the most potent breast cancer growth factor. By expressing functional ER, the cancer cells retain their receptor-mediated capability to connect with their ligand, the transcription and mitogen factor estrogen, and to maintain tumor proliferation and growth. ER may also act as a breast cancer survival factor. After a long period of silence in cancer manifestation after the primary

endocrine treatments, residual tumor cells are left with functional estrogen receptors. It may thus be possible that the cells can be reactivated by new hormonal influence.

This study supports the conclusion of Brewster *et al.* (2008), patients with early-stage breast cancer who are disease-free at 5 years after adjuvant systemic therapy (AST) have a substantially increased residual risk of recurrence. The findings in this study confirm that ER acts as a DTC and micrometastasis growth factor, and is a risk factor for a tumor to escape from dormancy.

A positive estrogen receptor status in breast cancer is associated with a good response to hormonal therapy, a good prognosis, and a long disease-free and overall survival (Vollenweider *et al.* 1986). The additional prognostic and predictive value of the progesterone receptor has remained controversial. Liu *et al.* (2010) examined the value of PR for prognosis and response to tamoxifen in a population-based series of 4046 invasive early stage breast cancer patients. Survival analyses for both the whole cohort and the ER-positive cases that were given tamoxifen therapy showed that patients with PR-positive tumors had better breast cancer specific survival. Cui *et al.* (2003) demonstrated that IGF-I (insulin-like growth factor-1) inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/akt/mammalian target of the rapamycin pathway. The authors suggested that low PR expression may indicate activated growth factor signalling in breast cancer cells, and therefore point to an aggressive tumor phenotype and resistance to hormonal therapy.

PR expression may define a subpopulation of breast cancer patients who have a stronger dependence on hormone receptor-associated growth, and consequently a superior response to hormone therapy (Cui *et al.* 2005).

In this study, PR positivity between metastases and primary tumors changed mostly in the latest relapsing tumors, where 6 out of 13 PR-positive tumors turned out to be negative in the metastases. The role of progesterone in early or late tumor recurrence in the present study remains unclear. It is possible that PR is more an ER-dependent factor than an individual factor in breast cancer progression.

The change in hormone receptor status during tumor progression has also been demonstrated by Aktas *et al.* (2011), who compared hormone receptor expression of CTCs with the primary tumors of patients who had developed metastases. They showed that most of the CTCs were ER/PR-negative, despite the presence of an ER/PR-positive primary tumor. Another recent study compared the hormone receptor status between primary tumors and the CTCs, and showed that the most common CTC phenotype was triple-negative, followed by HER2+/ER-PR subtype and ERorPR-positive, while 95% of the corresponding primary tumors were ER- and PR-positive. Furthermore, before as well as after surgery, CTC phenotype generally remained identical, but could differ from that of the primary tumor (Banys *et al.* 2011).

HER2

As anticipated, HER2 over-expression was associated with early tumor recurrence, and it was highest in the early-relapsing tumors. HER2 over-expression was also associated with other poor prognostic factors, such as node positivity, high tumor grade, ER negativity, and high Ki67 expression. This is in agreement with earlier reports (Ross and Fletcher 1998). There was a high concordance of HER2 over-expression between the primary tumors and the metastases. In the late-relapsing tumor group (metastasis after 10 years), one primarily HER2-negative tumor was found to over-express HER2 in metastasis. In contrast, Regitnig *et al.* (2004) detected HER2 over-expression *de novo* in 9.7% of distant breast cancer metastases at a late disease stage. In the present study, HER2 positivity was a significant risk factor in multivariate analysis for early tumor recurrence. Because HER2 is a strong breast cancer growth factor, it promotes metastatic progression and escape from tumor dormancy (Pantel 2009).

The impact of HER2 in predicting metastatic growth and escape from tumor dormancy was also shown by Hayashi *et al.* (2012), who demonstrated that patients with HER2-positive CTCs had a significantly shorter progression-free and overall survival than did patients without HER2-positive CTCs. They also noted that 24.2% of patients with HER2-negative primary tumors had HER2-positive CTCs during the study period (Hayashi *et al.* 2012).

KI67

In this tumor material, Ki67 expression with a cut-off point of 14% clearly differentiated tumors into slowly progressing tumors and those progressing more aggressively. Ki67 expression denoted early-relapsing tumors and correlated linearly with tumor progression, since Ki67 positivity declined gradually from early-relapsing toward late-recurring tumors. Luminal A-type tumors were associated with low Ki67 expression, and Ki67 positivity was associated with late tumor recurrence in Luminal A type tumors, but not in luminal B type cancers. This is in agreement with Cheang *et al.* (2009), who described an immunopanel of ER, PR, HER2, and Ki67 that can separate the Luminal A and B subtypes. As a proliferating marker, Ki67 supports tumor growth and metastatic progression. It is a marker that supports escape from tumor dormancy, and Ki67 positivity in CTCs indicates that these tumor cells are non-dormant (Aguirre-Chiso 2007).

CK5

The basal type cytokeratin CK5 expression correlated with poor prognostic features, such as early recurrence, axillary lymph node positivity, high tumor grade, Ki67 positivity, and ER negativity in this tumor material. The results are in line with those of Banerjee *et al.* (2006) and Choccalingam *et al.* (2012), who also demonstrated that basal-like breast cancer, defined by basal cytokeratin expression, correlated with a negative hormonal status and shorter disease-free intervals.

In this study, there was a significant loss of CK5 expression in the metastases. In contrast, Tot (2000) demonstrated a high concordance of CK5 positivity between primary breast cancers and their metastases, which seems to be explained with the medullary histological breast cancer type in their study differing to the ductal and lobular cancers in this tumor material. Su *et al.* (1996) described alterations in cytokeratin expression and partial loss of the normal regulation of cytokeratin expression during carcinogenesis and tumor progression. In normal mammary gland, a small number of cells that are positive for CK5 are located in the luminal compartment; they display morphological features of stem cells and have the capacity to differentiate towards either glandular or basal phenotype (Boecker and Buerger 2003). It is postulated that tumors containing a sufficient population of cancer stem cells are capable of self-renewal and of forming new tumors. CK5 can be regarded as a factor that supports tumor metastatic progression and escape from dormancy.

RELATIONSHIP BETWEEN THE IHC SURROGATES TO THE INTRINCIC GENETICAL DEFINED BREAST CANCER SUBTYPES, LUMINAL A, LUMINAL B, HER2-OVER-EXPRESSING, TRIPLE-NEGATIVE , BASAL-LIKE , UNCLASSIFIED AND LUMINOBASAL WITH EARLY AND LATE RECURRENCE OF TUMORS

The most significant and well known factor which lowers the risk of early tumor relapse in this study was the luminal A tumor type. The behavior of this tumor type can easily be seen to reflect HER2 negativity, low Ki67 expression and slow proliferation of these tumors, as well as the endocrine adjuvant therapies of the patients after repression of their ER-positive tumors.

The luminal A subtype of tumors correlated with low Ki67 expression. This is in agreement with the study of Cheang et al. (2009), who described an immunopanel of ER, PR, HER2, and Ki67 that can segregate the luminal A and B subtypes. Luminal breast cancers with Ki67 levels of at least 14% had a worse prognosis for both cancer recurrence and death, compared with tumors whose Ki67 levels were below 14%.

In this study, CK5-positive 'Triple-negative' basal-like tumors associated with early tumor recurrence, which is in line with the results of Cheang *et al.* (2008). They showed that patients with CK5- positive triple-negative breast cancers had worse overall survival than the subgroup of CK5 negative triple-negative cancers. In this study there were 11 triple-negative primary tumors, of which 4 triple-negative

tumors belonged to early relapsing tumors and were all CK5-positive, whereas there was no CK5-positive triple-negative tumor among the latest relapsing tumors.

STUDY I

STANNIOCALCINS

The expression of both STC-1 and STC-2 was significantly elevated in late-recurring tumors, expecially in the metastases, indicating that the results were in agreement with the presumed roles of stanniocalcins in maintaining cell survival, and supporting tumor dormancy. Stanniocalcins have been found to act as survival factors. They confer cytoprotection against hypoxic and hypercalcemic stress, particularly in terminally differentiated normal cells with limited or absent proliferative capability (Serlachius *et* Andersson 2004). In addition to its various biological processes, STC-1 has been associated with angiogenesis (Kahn *et al.* 2000). The role of this gene in angiogenesis is not known, but a significant increase in STC-1 mRNA levels was shown in an *in vitro* experimental model of angiogenesis (Kahn *et al.* 2000). The role of stanniocalcins in angiogenesis may be related to their capability to respond to cellular hyperoxide, hypoxive and osmotic stress (Kahn *et al.* 2000, Zhang et al. 2000, Westberg *et al.* 2007). The association of elevated expression of stanniocalcins in late relapsing tumors and correlation with favorable early outcome of the patients as well as tumor dormancy in this study is supported by Nguyen *et al.* (2009). They found that STC1 acts in a negative feedback loop in the prosurvival ERK1/2 signalling pathway during oxidative stress.

Contrary to the stanniocalcins in fish, where they act as secreted hormones of the endocrine glands, the stanniocalcins in mammals are thought to have an autocrine or paracrine role (Chang *et al.* 1995), although their function as a circulating hormone has also been suggested (James *et al.* 2005). The possibility of an intracellular role of STC-1 has been suggested because STC-1 is localized in the inner mitochondrial membrane which regulates cellular metabolism (McCudden *et al.* 2002, Westberg *et al.* 2007). It would be interesting to investigate the relationship and interactions between stanniocalcins in the tumor cells and the extracellular matrix, in order to gain a better understanding of the function of stanniocalcins in tumor cell survival.

Stanniocalcins have also been shown to play a role in inflammation (Iyer *et al.* 1999). In an experiment on hypoxic preconditioning in mice, they upregulated the expression of STC-1 in the brain via IL-6-dependent signalling (Westberg *et al.* 2007); this indicates that inflammatory cytokines, particularly of the IL-6 family,

may contribute to STC-1 levels in tumors (Knüpfer and Preiss 2007, Underhill-Day and Heath 2006).

All of these findings give reason to assume that stanniocalcins participate in tumor dormancy by facilitating tumor cells to enter and remain in a quiescent state as a micrometastatic lesion. In such a lesion, the tumor cells are in balance with cell proliferation and apoptosis, thus contributing to angiogenic dormancy, or dormancy related to immunosurveillance.

ER

The relationship of ER to stanniocalcins is discussed here, while the role of ER in breast cancer progression and tumor dormancy is described more thoroughly in the previous section (Study IV). In this study, ER expression was also found to associate with late recurrence of breast cancer. The expression of ER did not correlate with STC-1 or STC-2 expression in the primary cancers in this tumor material, but a positive correlation was found between ER and STC-1 in the recurrent/metastatic tumors.

STC-2 has been found to be an estrogen-responsive gene. Yamamura *et al.* (2004) found that expression of STC-2 was associated with a more favorable prognosis in hormone receptor-positive (ER and/or PR) tumors treated with adjuvant hormone therapy, whereas STC-2 expression did not have any impact on the prognosis of patients with hormone-negative tumors. The expression of STC-1 in breast cancer is less dependent on steroid hormones.

STUDY II

In Study II, the markers maspin, Bcl-2 and p53 were investigated. The association of these markers with tumor dormancy contradicted their presumed functional properties to some extent. The maspin and p53 with tumor suppressive properties were associated with early tumor relapse in this study. On the other hand, the Bcl-2 as an antiapoptotic factor, presumed to support metastatic growth and act as a dormancy-inhibiting factor, was associated with late tumor recurrence.

MASPIN

In this study, cytoplasmic maspin correlated significantly with early tumor relapse. The mean expression of cytoplasmic maspin was significantly higher in the early-metastasizing breast cancers than in the late-metastasizing ones. The proportion

of tumors with nuclear positivity for maspin in this material was lower than the proportion of cytoplasmic maspin-positive tumors. Additionally, the nuclear maspin positivity tended to be stronger in the late-relapsing tumors. This indicates that cytoplasmic maspin or lack of nuclear maspin was associated with early progression of cancer. Maspin expression has been associated with both favorable outcome (Maass *et al.* 2001, Mohsin *et al.* 2003), as well as poor prognosis in breast cancer (Umekita *et al.* 2002, Yoshihisa *et al.* 2002). It has previously been proposed that these controversial results could be related to the subcellular location of maspin, and that nuclear localization is essential for maspin to inhibit tumor growth (Goulet *et al.* 2011). As a tumor-suppressing factor, and as a serine protease inhibitor (Bailey *et al.* 2006) maspin should prevent tumors from metastasizing. It should prevent primary tumor invasion through the basement membrane, through extracellular matrix, and through blood and lymph vessel walls. The findings of this study demonstrate that the anti-tumor dormancy role of maspin is related to its cytoplasmic localization.

BCL-2

The levels of Bcl-2 staining were significantly lower in the early-relapsing cancers, both in primary tumors and their metastases, compared to late-recurring tumors.

As an inhibitor of apoptosis, Bcl-2 expression in breast cancer should inhibit apoptosis, and therefore predict a worse outcome and function as an anti-tumor-dormancy factor (Pantel 2009). In contrast, in line with the results in this study, it is well known that the expression of Bcl-2 in breast cancer signifies a favorable prognosis (Gasparini *et al.* 1995, Lê *et al.* 1999, Neri *et al.* 2006). This apparently paradoxical favorable prognostic value of Bcl-2 expression may be explained partly by the pro-apoptotic activity of other members of the Bcl-2 family (Neri *et al.* 2006, Takayama *et al.* 1995), the inhibitory activity of Bcl-2 on cell proliferation (Knowlton *et al.* 1998), or the estrogen-inducibility of Bcl-2 expression (Krajewski *et al.* 1999).

The strong association of Bcl-2 with ER in this study is in accordance with previous results (van Slooten *et al.* 1996, Lê *et al.* 1999). Bcl-2 is present in the normal breast; it may thus have a role in normal cyclical breast development, and may be controlled by an estrogen-dependent transcriptional pathway (Gasparini 1995, Leek 1994).

P53

p53 expression in this study signified early tumor relapse. Many studies have investigated the association between *p53* gene over-expression and the clinical outcome of breast cancer. Most of these studies show a poorer overall and disease-

free survival for breast cancer patients with *p53* mutation (Elledge and Allred 1998). The p53 expression in this study did not significantly differ between primary tumors and the metastases. This has also been shown by others (Shimizu *et al.* 2000).

The expression of p53 in this study associated significantly with cytoplasmic maspin, both in primary tumors and in their recurrences. p53 regulates the expression of the maspin gene (Zou *et al.* 2000). Both positive correlation (Umekita *et al.* 2002) and negative correlation (Hojo *et al.* 2001) between maspin and p53 have been reported. p53 is encoded by the *TP53* gene known as a tumor suppressor gene.

p53 has many mechanisms of anticancer function, and it plays a role in apoptosis, genomic stability, and inhibition of angiogenesis. When the *TP53* is damaged, tumor suppression is severely reduced. The mutated p53 protein, as in this tumor material, can be measured by IHC, and the high expression of mutated p53 is associated with decreased apoptotic and antiproliferative function. The mutant p53 protein itself can inhibit normal p53 function. The mutant p53 measured in this study thus belongs to the factors that support metastatic progression and escape from tumor dormancy.

In this study, p53 positivity also associated significantly with other factors contributing to early tumor relapse, *i.e.* CK5, cytoplasmic maspin and Ki67 positivity.

In conclusion, the main result in Study II is that cytoplasmic maspin and p53 positivity, together with low Bcl-2 and ER expression, are associated with early tumor recurrence.

STUDY III

The expression of polycomb protein and oncogene Bmi-1, proto-oncogene c-myc, and transcription factor Snail were analysed in Study III**.** As postulated, stem-cell markers, all of which are members of the hedgehog signalling pathway (Liu *et al.* 2006), are believed to suppress tumor dormancy. In these tumor series, the c-myc and Snail expression were not clearly associated with early or late tumor recurrence. C-myc was associated significantly with axillary node positivity in breast cancer patients; this is also in agreement with other studies (Lê *et al.* 1999).

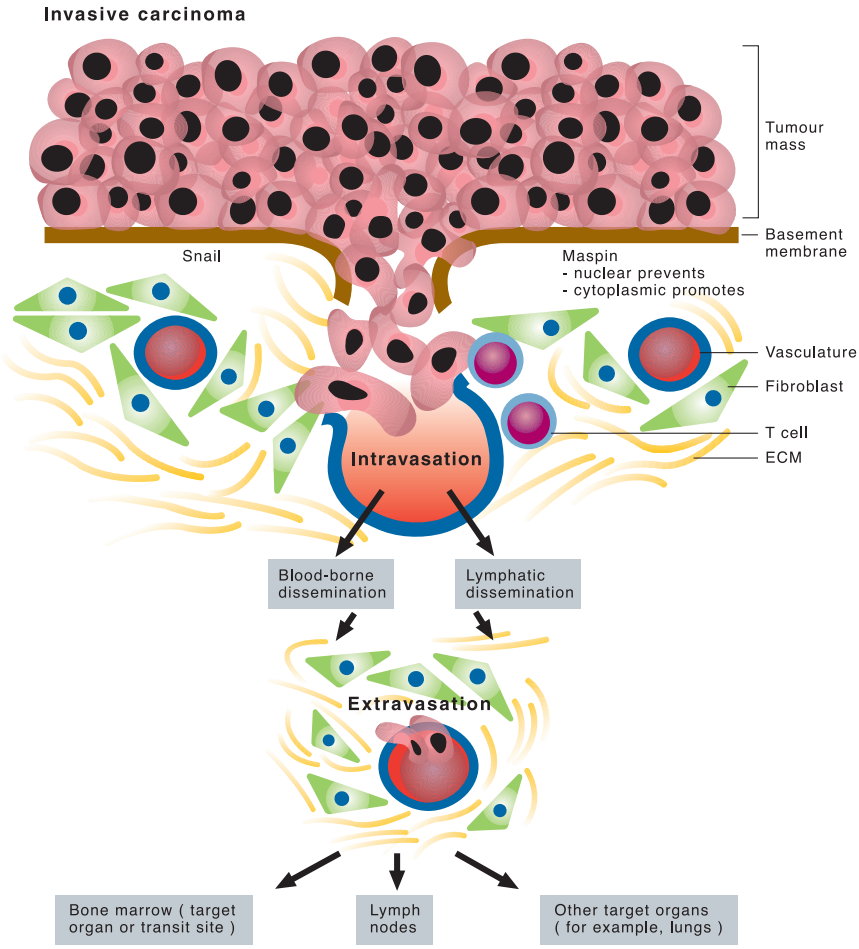
Only Bmi-1 expression in this study was associated with the time of tumor recurrence. The highest expression was seen in the metastases of the latest recurring cancers, indicating that Bmi-1 expression correlates with a slow initial progression of cancers. These results suggest that metastases, in contrast to primary tumors, arise from tumor cells that have retained their stem cell properties. Bmi-1 expression has been associated with both favorable and poor prognosis in breast cancer. In their study, Wang *et al.* (2012) showed that Bmi-1 status correlated with high tumor grade, basal-like phenotype, and decreased 5-year overall survival of the patients. Silva *et al.* (2007) demonstrated that the circulating Bmi-1 mRNA is a poor prognostic factor in breast cancer patients. Bmi-1 protein expression in breast cancer tissue has

also been associated with favorable overall survival, but only in ER-positive patients (Choi *et al.* 2009). In this tumor material, Bmi-1 positivity correlated significantly with ER positivity, in both primary tumors and metastases.

Al-Hajj *et al.* (2003) were the first to describe a cancer stem cell population in human breast cancers; these cancer stem cells displayed increased expression of Bmi-1 (Liu *et al.* 2006). The Bmi-1 expression in these tumors may therefore reflect stem cell properties, enabling the tumor cells to remain inactive for a long time and to maintain their self-renewal and tumor-initiating capability. Later, in appropriate circumstances, they may begin metastatic growth.

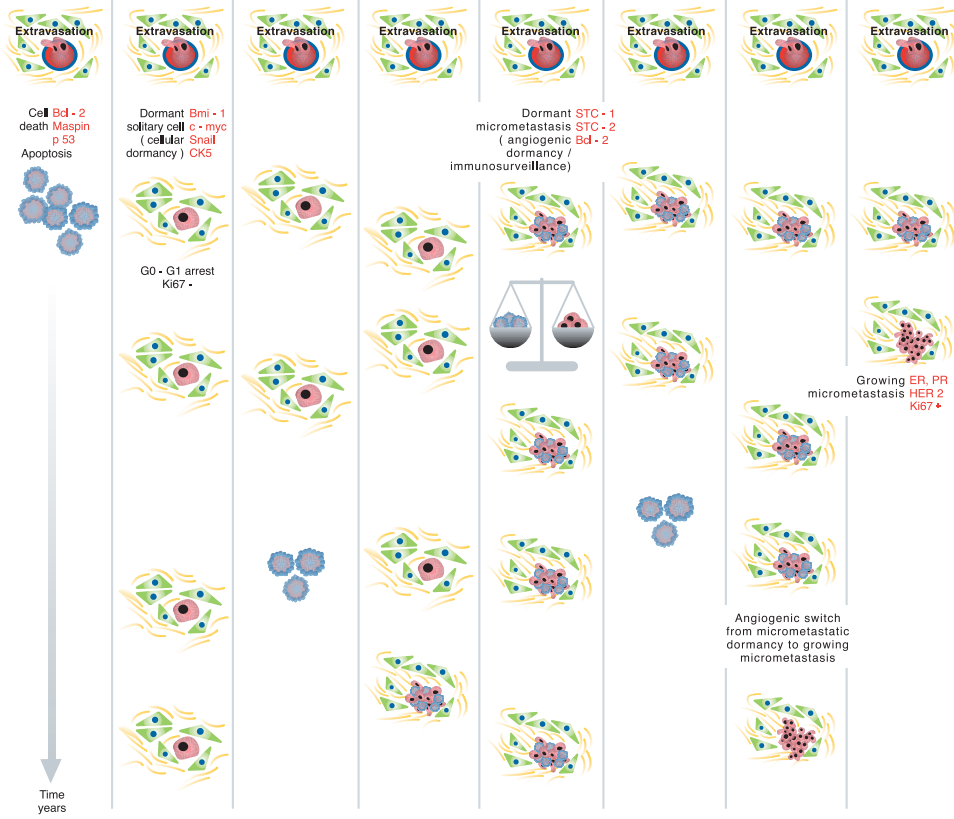
In figure 14 the imaginary pathway-schema of disseminated cancer cells and tumor dormancy as a component of breast cancer progression is illustrated.

Figure 14. Tumor dormancy as a component of cancer progression



Cancer cells that have undergone malignant genetic or epigenetic changes have acquired motile and invasive properties which can degrade the basement membrane and invade the underlying stroma. The invading tumor cells interact with fibroblasts or immune cells and the stromal matrix. Tumor cells, in cooperation with stromal cells, can degrade the extracellular matrix (ECM) and the vascular walls and intravasate (through arterial or lymphatic routes). Tumor cells that are arrested in the vasculature of the bone marrow can either proliferate or remain dormant. The bone can be a target organ, but it may also serve as a site of transit from which cells can again disseminate to their final destination (i.e., lungs, liver etc., where they metastasize). Tumor cells in the bone marrow have the potential to become secondary lesions, and they can also carry the information about the future progression of the disease. Tumor cells can arrest in lymph nodes or in the target organ vasculature where they can extravasate into organ parenchyma.

Possible fates of tumor cells after extravasation



Intra- or extra-vascularly lodged tumor cells in organ parenchyma, in the bone marrow or lymph nodes have four alternative fates: 1) they die (the vast majority of cells undergo apoptosis), 2) they can enter a state of quiescence or dormancy, either as a single solitary cell (G0-G1 arrest), or 3) as a micrometastatic lesion that undergoes proliferative expansion and cannot recruit a vascular bed, or 4) they can resume proliferation and form growing micrometastases. With time, the minimal residual cancers can have other fates. They can stay dormant indefinetly, go to apoptosis, or begin to proliferate and resume metastatic growth. The dormant cells in the micrometastatic lesions (angiogenic dormancy) can also begin to proliferate and switch to growing micrometastases (angiogenic switch). It is possible that cellular dormancy often precedes the immunosurveillance or the angiogenic dormant phase. In the figure the markers studied in this thesis have been placed on the steps in tumor progression according to their functional properties. Adapted with permission from Aguirre-Ghiso and Nat Rev Cancer (Aguirre-Ghiso 2007).

CONCLUDING REMARKS

The aim of these four studies was to investigate the relationship between the expression of different proteins and the clinicopathological parameters in the tumors of breast cancer patients in respect to tumor dormancy. The studies of this thesis focus on metastatic tumor dormancy, in other words, the latency period between detection of the primary tumor and manifestation of the recurrent/metastatic lesion.

The results were more in accordance with the results of clinical studies in which protein or gene expression has been evaluated in the tumor tissue of breast cancer patients, than with the knowledge of the functional properties of the markers obtained from in vitro and in vivo experimental models. This is understandable because the functioning of the proteins in the tumor tissue of patients is differentially controlled by signals from the surrounding stromal tissue, including inflammatory cells, immunological and hormonal influence, and the effects of the treatment, compared to laboratory conditions.

In this study, the markers that correlated most strongly with the late relapse of the tumors, i.e., to tumor dormancy, were stanniocalcins, ER, Bcl-2 and Bmi-1, and the IHC phenotype 'luminal A'. Markers that associated significantly with early tumor relapse were HER2, Ki67, maspin, p53, and CK5.

This work showed that the expression of proteins measured by IHC differs during the different developmental stages of breast cancer. It is nevertheless extremely difficult to deduce the phenomenon of tumor dormancy from IHC staining results to cancer progression.

Currently, the selection of patients who would benefit from additional systemic therapies after surgical resection is based on their statistical risk of developing tumor recurrence. The statistical risk is based on the clinicopathological parameters and the IHC evaluation of ER, PR, HER2 and Ki67 expression of the primary tumor tissue, without knowledge of whether they actually harbor DTCs or CTCs. This uncertainty may lead to over-treatment.

DTCs/CTCs are cells which can potentially initiate new growth and escape from tumor dormancy. In future it is essential to continue the efforts to elucidate the entire chain in cancer progression, i.e., from manifestation of the primary tumor to initiation of the recurrent tumor, and the clinically dormant state between these. So, in addition to the characterization of the primary tumor, it is essential to include DTCs/CTTs in the analysis. This information is also important for the design of clinical trials which apply biological therapies directed at specific targets.

In this study, the most interesting proteins related to late relapse of tumors were ones which were enriched in metastases that manifested 10 years after the primary treatment, namely stanniocalcins, ER, bcl-2 and Bmi-1. Especillay Bm-1,

which has stem cell properties, appears to be a promising marker in CTCs/DTCs to be screened from the blood or BM of breast cancer patients.

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