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The Prognostic Impact of Proliferation Markers in Breast Cancer with Emphasis on Cyclin B1 and PPH3

ANTHOULA KOLIADI

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Abstract

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The aim of this thesis was to investigate the prognostic role of the proliferation markers cyclin B1 and Phosphorylated Histone 3 (PPH3) in breast cancer (BC).

In paper I we used an experimental study design, we compared women dying early from their BC with women free from relapse more than eight years after initial diagnosis. All women had stage I, node-negative and hormone receptor positive disease. None had received adjuvant chemotherapy. We found that low-risk node negative patients with high expression of cyclin B1 had a significantly worse outcome than patients with low expression of cyclin B1.

In paper II a population-based case control study was performed to further investigate the prognostic value of cyclin B1. One hundred and ninety women who died from BC were defined as cases and 190 women alive at the time for the corresponding case's death were defined as controls. Inclusion criteria were tumor size 50 mm, no lymph node metastases, and no adjuvant chemotherapy. Two investigators evaluated the stainings independently. Cyclin B1 was found to be a prognostic factor for BC death that could identify high-risk patients with a good to very good reproducibility.

Paper III aimed to investigate the role of proliferation in male breast cancer (MBC). One hundred and ninety-seven MBC tumors were stained for cyclin A, B1, D1 and Ki67. Overexpression of cyclin A and B1 and elevated mitotic count were predictive of breast cancer death. Ki67 was re-evaluated and different cut-offs were used, but no prognostic value could be demonstrated. On the other hand high levels of cyclin D1 were associated with better outcome in MBC.

In paper IV we applied the immunohistochemichal panel suggested from international guidelines to the same patient material as in paper II, to discriminate luminal A from luminal B BC. We wanted to evaluate if different cut-off values of Ki67, cyclin A or B1 could more clearly separate luminal A from B. Cyclin A, B1 and Ki67 (cut-off 20%) could detect difference in outcome between these subtypes with cyclin A showing greater prognostic value.

The aim of paper V was to examine the prognostic role of PPH3 compared to the proliferation markers Ki67, cyclin A and cyclin B1 with focus on ER positive disease. PPH3 was found to be a prognostic factor for breast cancer death but in the multivariate analysis including all proliferation markers, only cyclin A remained a prognostic factor.

Finally, we conclude that both cyclin B1 and PPH3 are prognostic factors for breast cancer death, but are outperformed by cyclin A in ER positive patients. In male breast cancer prognostic factors need to be further studied.

Keywords: proliferation, cyclin B1, Phosphorylated Histone 3, cyclin A, breast cancer, node negative, prognostic factor, luminal

Anthoula Koliadi, Department of Radiology, Oncology and Radiation Science, Oncology, Akademiska sjukhuset, Uppsala University, SE-751 85 Uppsala, Sweden.

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To Dimitris, Maria and Chrissí

To my mother

List of Papers

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

I. **Koliadi A,** Nilsson C, Holmqvist M, Holmberg L, De La Torre M, Wärnberg F, Fjällskog M-L (2010). Cyclin B is an immunohistochemical proliferation marker which can predict breast cancer death in low-risk node negative breast cancer. *Acta Oncologica,* 2010; 49: 816-820

II. Niméus-Malmström E, **Koliadi A,** Ahlin C, Holmqvist M, Holmberg L, Amini R-M, Jirström K, Wärnberg F, Blomqvist C, Fernö M, Fjällskog M-L (2009). Cyclin B1 is a prognostic proliferation marker with high reproducibility in a population-based lymph node negative breast cancer cohort. *Int. J. Cancer:* 2010; 127: 961-967

III. Nilsson C, **Koliadi A,** Johansson I, Ahlin C, Thorstenson S, Bergkvist L, Hedenfalk I, Fjällskog M-L (2013). High proliferation is associated with inferior outcome in male breast cancer patients. *Modern Pathology* 2013; 26: 87-94

IV. **Koliadi A**, Ahlin C, Amini R-M, Holmqvist M, Holmberg L, Fjällskog M-L. Cyclin A is an excellent proliferation marker for identifying luminal breast cancer subgroups using immunohistochemistry. (manuscript)

V. **Koliadi A,** Grönberg M, Valachis A, Olofsson H, Ahlin C, Tiensuu Janson E, Fjällskog M-L. PPH3 is an independent prognostic factor in node negative breast cancer, though outperformed by cyclin A in the ER positive patients. (manuscript)

Abbreviations

AI	Aromatase inhibitor
ALND	Axillary lymph node dissection
BC	Breast cancer
BMI	Body mass index
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CMF	Cyclofosfamide, methotrexate, fluorouracil
DFS	Disease free survival
DNA	Deoxyrebonucleic acid
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ESMO	European Society of Medical Oncology
FAC	Fluorouracil, adriamycin, cyclofosfamide
FEC	Fluorouracil, epirubicin, cyclofosfamide
FISH	Fluoroscence in situ hybridization
GGI	Genomic grade index
HER2	Human epidermal growth factor receptor 2
^{3}HTdR	Tritiated thymidine index
IHC	Immunohistochemical
ITC	Isolated tumor cells
LHRH	Luteinizing hormone releasing hormone
MAI	Mitotic activity index
MBC	Male breast cancer
MPF	Maturation promoting factor
MRI	Magnetic resonance imaging
m-TOR	Mammalian target of rapamycin
NGS	Nottingham grading system
NPI	Nottingham prognostic index
OS	Overal survival
PPH3	Phosphorylated Histone 3
RT	Radiotherapy
RNA	Ribonucleic acid
SNB	Sentinel node biopsy

SPF	S-phase fraction
TMA	Tissue microarray
TNM	Tumor size, node, metastasis

Contents

Introduction

Epidemiology

Breast cancer (BC) is the most common female malignancy and the leading cause of cancer death among women worldwide, accounting for 25% (1.7 million) of the total new cancer cases and 14.7% (522.000) of the total cancer deaths in 2012 [1][2]. Although the overall number of new diagnoses was similar in developed countries compared with developing countries, incidence rates were almost two and a half times higher in developed countries (71.7/100.000 and 29.3/100.000, respectively) after adjusting for population size and age structure [3]. In Sweden, 8.382 new cases of female BC were diagnosed in 2011, which constituted 30.3% of all female cancers and 45 new cases of male BC, which comprise 0.1% of male cancer. The cumulative probability of developing BC before the age of 75 years is 10% in Swedish women [4] . BC incidence in women has increased with 1.4 per cent annually during the last 20 years. It is also common to diagnose BC more than once in the same woman [5].

Etiology and Risk Factors

About 5-10% of all BCs are due to mutations in certain genes; 2.5-5% represent mutations in the BRCA1 and BRCA2 genes and women bearing these genes have a higher risk of developing BC, ovarian cancer and several other types of cancer. Li Fraumeni syndrome, ataxia telangiectasia and Peutz-Jeghers syndrome are other hereditary diseases associated with a high risk for developing BC. Traditionally, early age at menarche, high age at first full-term birth, nulliparity, history of benign breast disease, current or recent oral contraceptive use, and current or long-term combinational replacement therapy represent risk factors for BC. However, many of those factors are actually associated with luminal A and B tumors[6-8]. Furthermore, high body mass index (BMI) or obesity increases the risk for luminal B tumors in premenopausal women whereas

the risk for luminal A tumors is decreased [6, 7]. Short duration of breast-feeding, obesity and family history of BC are thought to be risk factors for triple negative BC among premenopausal women [7]. The duration of lactation is also inversely associated with the relative risk of both triple negative and luminal subtypes. Alcohol consumption, low physical activity, ionization radiation are some other risk factors. The findings that risk factors differ between molecular subtypes suggest etiological heterogeneity in breast carcinogenesis.

Diagnosis

The procedures used to diagnose BC include clinical examination, mammography, often suppplemented by ultrasonography and fine-needle aspiration or core biopsy for pathological examination. Magnetic resonance imaging (MRI) of the breast is another radiologic method able to detect BC but not established as a routine method in Sweden, with the exception of young BRCA-mutation carriers who have dense breasts where the sensitivity of screening mammography is reported to be lower [9].

Screening mammography detects half of BCs in Sweden and the others are clinically detected. Regular mammography screening for women aged 40-69 years was introduced gradually in Sweden from 1974, achieving nationwide coverage across all 21 counties in 1997. Mammography has an estimated sensitivity of 92% and a specificity of 90%. Some studies have shown that the method of diagnosis is an independent prognostic factor and patients with mammography-detected tumors have better outcomes than patients with tumors detected by other methods, even 15 years after diagnosis [10, 11].

Pathology

Information from the clinical examination and radiological imaging is used before surgery to describe the BC according to TNM staging system. The tumor size, lymph node status and distant metastases (TNM) are important factors determining systemic therapy. After surgery, the pathological TNM, denoted by the prefix p (pTNM) based on histopathological examination, adds accurate information about the size of tumor and especially the lymph node status, usually the sentinel node status. BC is classified in different stages based on the TNM system (Table 1).

Table 1. *TNM classification of breast cancer (7th edition).*

Tx — Primary tumor cannot be assessed	
T0 — No evidence of primary tumor	
Tis — Carcinoma in situ.	
- Tis (DCIS) — Intraductal carcinoma in situ.	
- Tis (LCIS) — Lobular carcinoma in situ.	
T1 — ≤2 mm	
T2 — >20 mm but ≤50 mm	
T3 — >50 mm	
T4 — Tumor of any size with direct extension to the chest wall and/or the skin, inflammatory	
NX — Regional lymph nodes cannot be assessed (eg, previously removed).	
N0 — No regional lymph node metastases.	
N1 — Metastasis to movable ipsilateral level I, II axillary lymph nodes	pN1 — 1-3 axillary nodes
N2 — Metastasis to ipsilateral level I, II axillary lymph nodes that are clinically fixed	pN2 — 4-9 axillary nodes
N3 — Metastases in ipsilateral infraclavicular (level III axillary), supraclavicular, internal mammary lymph nodes	pN3 — ≥ 10 axillary nodes or infraclavicular nodes
M0 — No clinical or radiographic evidence of distant metastases	
M1 — Distant detectable metastases as determined by classic clinical and radiographic examination	

Prognosis

The relative 10-year survival was almost 80% in the year 2009 in a Swedish population, which was a remarkable improvement compared with approximately 50% in the 1960s in the same population [12][13]. The survival gain is likely to have several different causes, including improved detection through screening, increasing specialization of care and better access to more effective treatments, such as improved surgical techniques, targeted use of radiotherapy and use of adjuvant therapies including the widespread adoption of tamoxifen and aromatase inhibitors (AIs). A BC mortality reduction of 26% after 6–11 years of follow-up seems to be the likely impact of the European service mammography screening program offered to women aged 50–69 [14].

Treatment

Surgery

Patients with primary BC are operated using either breast conservative surgery (lumpectomy) or modified radical mastectomy. The latter is preferred for large (> 50 mm), multifocal, inflammatory tumors, lesions that infiltrate the chest wall and skin. Mastectomy can also be the patient's preference or recommended when an acceptable cosmetic result is not possible with breast conserving surgery, often due to the size of tumor in relation to the breast volume. For patients with locally advanced disease or for downstaging, neoadjuvant treatment is indicated before any surgery is feasible. No surgical treatment is recommended routinely in patients with metastatic BC, but exceptions exist.

In common routine breast conservative surgery is followed by sentinel node biopsy (SNB). If the SNB is negative no further surgery is required but if the SNB is positive then axillary lymph node dissection (ALND) has been recommended. However, recent studies show that knowledge of further nodal status beyond SNB does not significantly influence the decision for adjuvant systemic treatment, which is mostly based on tumor characteristics and SNB status alone [15]. In patients with limited sentinel node metastasis treated with breast-conserving surgery, whole-breast irradiation, and adjuvant systemic therapy, the use of ALND compared with SNB alone do not result in superior survival [16]. Therefore, ALND is no longer justified in patients with clinically negative axillary nodes and seems to be unnecessary in patients with pathological positive SNB [17, 18]. Consequently, the limited surgical procedure of SNB minimizes the risk of developing lymph edema in the arm, the most common morbidity after ALND.

Radiotherapy

Postoperative radiotherapy (RT) is delivered to women treated with breast conservative surgery and in patients with positive lymph nodes, regardless of the surgical method. For patients treated with mastectomy postoperative irradiation is recommended in cases of multiple tumors, tumor > 5cm and uncertain surgical margins and skin involvement. Radiotherapy is delivered within 4-8 weeks after breast conservative surgery, excluding patients receiving chemotherapy where radiotherapy starts usually within 3–4 weeks after the last chemotherapy cycle. Conventional radiotherapy is represented by whole breast irradiation generally using two tangential fields and a standard regimen of 2 Gy x 25 = 50 Gy. A boost irradiation of 2 Gy x 5-8 = 10-16 Gy is recommended in young patients, in cases of uncertain surgical margins and multiple tumors in the removed sector. Hypofractionated radiotherapy has been evaluated in several trials [19] and has demonstrated comparable outcomes and toxicity as conventional fractionation, suggesting that radiotherapy can be given under a shorter time period using larger doses per fraction [20, 21].

In 2011, the EBCTCG meta-analysis of women receiving radiotherapy after breast conserving surgery and reduced the 10-year risk of any (ie, locoregional or distant) first recurrence from 35.0% to 19.3%(absolute reduction 15.7%) and reduced the 15-year risk of BC death from 25.2% to 21.4% (absolute reduction 3.8%). The new fact here is that radiotherapy after breast conserving surgery reduces BC death, thus also reduces distant recurrence [18]. Because the risk of loco-regional recurrence increases with the number of positive axillary lymph nodes, a widely adopted approach has been to accept RT only for patients with four or more positive axillary nodes. The indication of loco-regional RT in the presence of one to three positive lymph nodes has been the subject of controversy. New data support that patients with 1-3 positive lymph nodes have a similar benefit from RT as those having > 4 positive lymph nodes [22, 23]. RT, does have a strong systemic effect, significantly reducing the rate of systemic recurrences and thus improving overall survival, both in the setting of post mastectomy and after conservative surgery.

Endocrine therapy

Tamoxifen was the first medicine used for the endocrine therapy of metastatic BC in the late 1970s and introduced as adjuvant treatment for pre- and postmenopausal women with estrogen receptor (ER) positive BC between 1985-1990. In the late 1990s the first generation of aromatase inhibitors (AI) was introduced initially as second line and later as first line endocrine therapy for postmenopausal women with metastatic and early BC [24].

The recommended endocrine treatment duration has so far been five years [25]. Tamoxifen is the recommended endocrine therapy for premenopausal women. For those women even ovarian suppression with luteinizing hormone releasing hormone (LHRH) analogues or ablation can be used to reduce estrogen levels to postmenopausal status. Combination of letrozole usually for 2 years and switch to tamoxifen for the rest of the 5-year period is recommended for postmenopausal women. AI alone for five years is the treatment choice for women with > 4 positive lymph nodes. The use of AI in premenopausal women is not recommended because the ovarian estrogen secretion is not affected of these medicines.

Recently presented studies reveal new data and bring into discussion the duration of endocrine therapy. The Adjuvant Tamoxifen: Longer Against Shorter (ATLAS) trial has shown that for women with ER-positive disease, continuing tamoxifen to 10 years rather than stopping at 5 years produces a further reduction in recurrence and mortality, particularly after year 10. These results suggest that 10 years of tamoxifen treatment can approximately halve BC mortality during the second decade after diagnosis [26]. Furthermore the MA 17 trial suggests that extended adjuvant therapy achieved a significant improvement in overall survival in postmenopausal women with node-positive disease [27]. The 12-year update of the BIG 1-98 adjuvant trial, comparing letrozole with tamoxifen monotherapy, was presented at a San Antonio Breast Cancer Symposium in December 2013 and revealed the benefit of letrozole over tamoxifen with a reduction in the risk of a DFS event in Luminal B BC for both ductal and lobular histology. There was also a trend for OS favoring letrozole in women with lobular cancer [28].

Chemotherapy

Adjuvant chemotherapy in BC was initially administered to lymph node-positive patients and the first established regimen CMF (cyclophosphamide, methotrexate and 5-fluorouracil) reduced the relative risk of relapse by 34% and the relative risk of death by 26% [29]. Anthracyclines were introduced in the 1990s and a EBCTCG meta-analysis showed that anthracycline-containing polychemotherapy regimens produced greater positive effects at regards recurrence, and on mortality [30]. Adriamycin or epirubicin are the commonest anthracyclines used in FAC (Fluorouracil, adriamycin, cyclofosfamide) or FEC (Fluorouracil, epirubicin, cyclofosfamide) polychemotherapy regimens. Anthracycline-based chemotherapy has decreased the probabilities of recurrence, BC mortality and overall mortality by 3% at 5 years and 4% at 10 years [31], compared with CMF, leading to a moderate but highly significant advantage for anthracyclines.

The next step in adjuvant chemotherapy of BC was the introduction of taxanes (docetaxel and paclitaxel) in the 2000s. Several studies and a meta-analysis of 19 adjuvant taxane trials demonstrated a 19% reduction in the hazard ratio for DFS and OS for the taxane-containing regimens [32].

Antibody-based treatment

Trastuzumab is a monoclonal antibody that binds to HER2 and leads to cell proliferation arrest. Adjuvant trastuzumab is offered to patients with overexpression of the HER2. Treatment with a trastuzumab containing regimen has improved the DFS and OS irrespective of tumor size, nodal and hormonal status and age [33]. The DFS event rate was reduced by 48% and the mortality by 39%, as presented in previous studies [34, 35]. The optimum duration of trastuzumab treatment has been a matter of debate. The HERA trial demonstrated no benefit for 2 years trastuzumab treatment versus 1-year treatment while the PHARE trial did not show that 6 months of treatment with trastuzumab in combination with chemotherapy was non-inferior to 12 months of such treatment [36, 37]. Thus, 1 year of treatment with trastuzumab in combination with chemotherapy remains the standard.

Pertuzumab is another humanized monoclonal antibody that inhibits heterodimerization and downstream signaling and is approved for use in combination with trastuzumab. The NeoSphere trial showed that patients given pertuzumab and trastuzumab plus docetaxel had a significantly improved pathological complete response rate compared with those who only received trastuzumab and docetaxel [38, 39].

New drugs

Apart from adjuvant use endocrine therapy is important in metastatic disease. The development of resistance in hormonal therapy in up to 50% of BC patients remains a topic under investigation. The BOLERO-2 trial compares a combination arm of an m-TOR inhibitor (everolimus) plus a steroidal aromatase inhibitor (exemestane) with a control arm of placebo and exemestane in postmenopausal and hormone-resistant metastatic disease. Patients receiving the combination of everolimus with exemestane had a 4-6 month prolonged progression-free survival. The S1207 ongoing trial attempts to answer the question whether the addition of evorolimus to standard adjuvant endocrine therapy improves the invasive disease-free survival in patients with high risk ER-positive BC [40][41].

Male breast cancer

Male breast cancer (MBC) is an uncommon disease representing 0.5-1% of all breast cancers in North America and Europe. Higher incidence in certain African countries explained by infectious diseases that leads to higher estrogen levels [42]. In the Nordic countries MBC accounts for 0.2% of all invasive cancer in men, resulting to approximately 100 new diagnoses every year [43].

Risk factors associated with an increased risk for MBC are obesity, physical inactivity, gynecomastia, orchitis/epididymitis, liver cirrhosis and radiation exposure [44]. Many of these factors alter the ratio of estrogen to androgen resulting in estrogen excess. Four percent to 40% of MBC is thought to result from autosomal dominant inheritance in BRCA1 and BRCA2 mutations, whereas BRCA2 mutations are much more common. In men with Kleinefelter's syndrome (usually 47XXY) the risk for BC is 20 to 50 times higher than in 46XY men.

In men BC presents usually as a painless lump in the breast and especially in the retroareolar area. The diagnostic procedure involves clinical examination, mammography and/or ultrasonography and fine-needle aspiration or core biopsy for histopathological verification. In men the most common histology is ductal carcinoma (>90%) while lobular carcinoma is very rare. The prevalence of MBC increase with age and the peak incidence occurs at an average age of 60 years.

MBC has been reported to be in more advanced stage at diagnosis compared to female tumors. Stage T4 because of skin and nipple involvement, lymphovascular invasion and positive lymph nodes in 40-50% are reported. Anatomic differences like the smaller distance between breast tissue and skin could possibly explain the higher rate of axillary nodal involvement[45]. It is reported that 5% of all MBC in situ cancers are diagnosed while still in situ [46].

Comparisons of outcome between male and female BC are contradictory. Several studies indicate a poorer survival for men when compared with women with BC while others found no difference in disease specific survival [47-49].

The management of MBC involves surgery, radiotherapy, endocrine therapy, chemotherapy and antibody therapy. MBC patients receive treatment according to guidelines in female BC. Today most patients undergo radical modified mastectomy, preferable because of the central location of tumors. In one of the largest series of MBC, where 85% of patients received radiotherapy the rates of chest and nodal recurrence were low, suggesting benefit from radiotherapy [46]. For patients treated with mastectomy same principles as in females are followed regarding the radiotherapy.

The majority of studies in MBC show that ER and PgR expression is greater compared to female BC (80-90% versus 75 and 73-81% versus 65%, respectively). Endocrine treatment is therefore considered an important part of the MBC management. Tamoxifen is the first choice in the adjuvant and in metastatic disease. Adjuvant treatment with tamoxifen in MBC is associated with significantly improved outcome according to retrospective studies [46]. Today, 5 years tamoxifen adjuvant treatment is recommended in men with BC.

The role of chemotherapy in MBC is not well examined. Randomized studies with high power will probably never be feasible because of the rarity of MBC. Men with MBC should recommended chemotherapy according to the guidelines regarding women.

Due to the rarity of MBC, limited information is available about the role of proliferation. Overexpression of Ki67 is associated to poorer outcome in some studies whereas other studies do not detect any prognostic value [50, 51]. To date and to our knowledge cyclin A and B1 have not been investigated in MBC, while the prognostic value of cyclin D1 have been examined in a few small series of MBC with contradictory results [50, 51]. The recommended adjuvant treatment in MBC is tamoxifen and previous studies in female BC have associated cyclin D1 with tamoxifen resistance [52].

Prognostic factors

Prognostic factors aim to foresee the outcome of patients, risk of relapse and death, irrespective of treatment. Such factors are usually indicators of growth, invasion and metastatic potential. Predictive factors intend to assess the outcome of patients receiving a certain systemic therapy and thus are intimately associated with sensitivity or resistance to therapy.

Histological grade, lymph node status and size are well-studied and established prognostic markers in early BC. The prognostic value of the proliferation marker Ki67 concerns mainly the ER-positive BC. Age and peritumoral vascular invasion are also known prognostic markers that are taken into account before therapy decisions are made.

Estrogen receptors (ER), progesterone receptors (PgR) and HER2 are both prognostic and predictive factors.

Histological grade

The first approach to a BC histological grading system was established by Bloom and Richardson [53]. The system used today, called the Nottingham Grading System (NGS) is a modification of the above, introduced by Elston and Ellis in 1991 [54]. It is based on pathological evaluation of tubular formation, nuclear atypia and number of mitoses. The scores for the three components are summated and categorized as follow: grade 1 (3-5) is well-differentiated, grade 2 (6-7) is moderately differentiated and grade 3(8-9) is poorly differentiated. Grade 1 tumors have the best prognosis, grade 3 characterizes more aggressive lesions with a poor prognosis tumors and grade 2 is in-between, not informative for clinical decision-making and represents about 30-60% of all tumors. The histological grade is an independent prognostic factor in breast cancer DFS and OS, but controversial opinions on the reproducibility of the system have been demonstrated. NGS will always have an underlying subjective element, which entails moderate reproducibility [55, 56]. Among the

NGS three variables, mitotic count has an independent role in predicting overall survival in multivariate analysis [57-60] and, in some cases, provides even additional prognostic information. The moderate reproducibility of the NGS and the fact that the mitotic index has the strongest prognostic value is a subject of debate and a proposal of modifications and simplifications of the system have been suggested [59, 60]. The gene expression grade index (GGI), developed from genes associated with cell cycle and proliferation differentially expressed between grade 1 and 3 tumors could more accurately grade tumors into two categories. Histologic grade 2 tumors were reclassified into grade 1 or 3, simplifying the grading system and suggesting the use of GGI as a substitute for or complementary with histologic grade in the Nottingham Prognostic Index (see above).

Lymph node status

Axillary lymph node status remains one of the most important factors in staging, adjuvant treatment choice and prognosis of survival in BC. The number of positive lymph nodes is utilized to divide BC patients into four groups: negative nodes, 1-3 positive nodes, 4-9 positive nodes and > =10 positive nodes. The 5-year survival for patients with node-negative disease is 82.8% compared with 73% for 1- 3 positive nodes, 45.7% for 4-12 positive nodes, and 28.4% for ≥13 positive nodes. There is also a direct relationship between the number of positive axillary lymph nodes and the risk of distant recurrence [61].

Since the sentinel node era, new terms have been introduced to precisely define the metastases in the axillary lymph nodes as macrometastases when >2mm tumor deposits in diameter are found, micrometastases are defined as measuring between 0.2mm and less than 2mm. The existence of isolated tumor cells in an area of less than 0.2 mm in largest diameter is denoted as lymph node negative. Some studies suggest that independently of the size (micro-, or macro-), lymph node metastasis is negatively associated with prognosis, indicating adjuvant systemic chemotherapy and local irradiation [58, 62, 63]. However, other tumor characteristics are taken into consideration especially when ITC or micrometastases are found in ER-positive lesions, with favorable outcome tumors (luminal A) that can be treated with endocrine therapy alone.

Size

Tumor size at diagnosis correlates with the histological grade and the number of positive lymph nodes and is also an independent factor with strong prognostic value even after 20 years of follow-up [58, 64, 65]. In a study of 13,464 patients with node-negative BC, those with tumors <1 cm had a 5-year OS of close to 99% compared with 89% for tumors between 1 cm and 3 cm and 86% for tumors between 3 cm and 5 cm. Similar results were also seen in longer follow-up. The 20-year recurrence-free survival is 88% for tumors ≤1 cm, 72% for tumors between 1.1 cm to 3 cm, and 59% for tumors between 3.1 cm and 5 cm in a study of node-negative BC patients [66].

Histological grade, lymph node status and tumor size often remain significant in multivariate analysis and are the three equally contributing elements composing the Nottingham Prognostic Index (NPI) [67]. The NPI has been further validated in many studies and can predict survival patterns in stratified groups [68, 69]. Studies and meta-analysis of gene expression signatures show that in multivariate models lymph node status and size remain independent factors for BC death [70, 71].

Age

Young age at diagnosis of BC is often associated with clinically detected aggressive tumors, with lymph node metastases, triple negative profile and larger size. However, adjuvant treatment diminishes the poor prognostic value of young age.

ER and PgR

ER and PgR are both prognostic and predictive factors used to decide whether the patient will benefit from endocrine treatment or not[72, 73]. Approximately 80% of all BCs have ER-positive tumor cells [74]. Two types of ER are recognized, ERα and ERβ with the former much more frequently expressed. IHC methods using monoclonal antibodies visualize the ER located in the nucleus. International guidelines (St Gallens, ESMO, ASCO) have recommended a cut-off >1% ER-positive tumor cells for the likelihood of response to endocrine therapy [75-77]. On the

other hand European and National guidelines have recommended ER ≥ 10% as the optimal cut-off [77]. This discrepancy influences the decision whether patients with ER expression between 1-9% should receive endocrine therapy or not. A recent study showed that approximately 3% of all BC express ER in the range of 1-9% and have clinical and pathological characteristics different from ER-positive tumors [78].

PgR is also a nuclear receptor and its expression is induced by ER activation [79]. PgR-negativity is associated with poorer outcome in BC and is en independent prognostic factor for BC death[80, 81]. However, a recent meta-analysis of randomized trials evaluated adjuvant Tamoxifen vs no adjuvant Tamoxifen suggested that the effect of Tamoxifen in ER-positive patients is independent of PgR level [77]. A recent study showed that high expression of PgR is more frequently observed in luminal A than luminal B tumors, although approximately 50-75% of luminal B tumors express PgR but may be lower than luminal A [80].

HER2

HER2 is a protein encoded by ERBB2 proto-oncogene located on chromosome 17 and belongs to the epidermal growth factor receptor (EGFR) family. Amplification of this gene is reported in 11-30% of early BC patients and associated with a poor outcome [82]. The high expression of HER2 assessed by immunohistochemistry or the amplification of the HER2 gene is associated with aggressive tumor growth and poor prognosis[82]. On the other hand, HER2 is a predictive factor for the treatment with the antibody trastuzumab in the adjuvant and metastatic setting [33].

Proliferation markers

The normal process of cell development and division is called cell growth or proliferation. Uncontrolled proliferation is the cancer cell's main characteristic. Immunohistochemistry is one of the methods used to visualize proliferated cells, by staining for several proliferation biomarkers.

Mitotic activity index (MAI) is the most widely used proliferation marker and defined as the number of mitoses per 10 high power fields at the periphery of the tumor and carries the highest prognostic value among the components of the Nottingham Grading System (NGS). The independent prognostic value of MAI regarding the development of metastases or death has been confirmed in meta-analyses in both univariate and multivariate models [83]. Studies have also demonstrated that MAI is independent of tumor size in node-negative patients [59, 84],

Ki67 is a nuclear protein encoded by the MKI67 gene and is strictly associated with cell proliferation. It is present in all phases of the cell cycle and reaches its level peak during mitosis [85]. Ki67 is not expressed in the quiescent phase (G0) [86]. The Ki67 and MIB-1 monoclonal antibodies bind different epitopes of the Ki67 antigen but the main difference between them is that MIB-1 can be used in formalin embedded tissues, whereas Ki67 only works on fresh or frozen tissue, which reduces its clinical usefulness. Several antibodies have been developed for use in paraffin sections but MIB-1 has been validated more frequently [87].

Even though the exact function of the Ki67 protein remains unknown, several investigations in the majority of retrospective studies have shown that its overexpression in BC tissue is a prognostic factor for poor outcome both in node-positive and node-negative patients [85, 86, 88-91]. There is high positive correlation between Ki67 and histological grade, MAI, S-phase, tp53 and HER2, while ER status is more often reported inversely correlated with Ki67 [92, 93].

Studies have investigated the role of Ki67 as a predictive factor for adjuvant treatment in ER-positive tumors but the results are contradictor [94-96]. On the other hand, reduction in Ki67 is a strong predictor of long-term outcome in neoadjuvant chemotherapy and, moreover, in endocrine therapy [93, 97, 98].

However, unstandardized staining and scoring methodology, lack of reproducibility and differently used cut-off values limit its clinical validity [99]. The International Ki67 in the Breast Cancer Working group presented recommendations for Ki67 assessment in 2011; however, most laboratories have adjusted their methodology over a period of time to achieve results with reasonable clinical utility and the incorporation of new routines requires such efforts [91].

The S-phase fraction (SPF) is a proliferation marker with prognostic value in BC. It represents the percentage of cells in a tumor that synthesize DNA in the S phase of the cell cycle. The tritiated thymidine index (^{3}HTdR) was the first method used to measure SPF and required fresh tissue and autoradiography [100]. This method allowed the recognition of proliferative cells, even if the proliferation rate was slow. Flow cytometry measurement of SPF is the most validated method since fresh, frozen or paraffin-embedded tissue can be used [101]. An immunohistochemical (IHC) method using specific antibodies for 5-bromodeoxyouridine (BrdU) allows the measurement of SPF with a better resolution than ^{3}HTdR [102]. A high SPF is correlated to a poor prognosis; both relapse-free survival and overall survival were significantly higher in patients with low SPF tumors [103-105]. The technique can be performed on both fresh-frozen and paraffin embedded tissue but the latter is often associated with more debris and, therefore, a higher proportion of tumors which SPF cannot be assessed.

The study of other proliferation markers such as topoisomerase IIa and cyclin-dependent kinase inhibitors (CDKIs) has not yet resulted in useful clinical markers but improves the understanding of proliferation complexity.

Gene expression profiling assays

The simultaneous quantitative assessment of thousands of expressed genes in BC series from individual patients led to the molecular classification of BC. The major molecular BC subtypes with prognostic and or predictive value, identified by gene expression profiling assays, are called luminal A, luminal B, HER2-enriched, basal like [106-110]. The four intrinsic subtypes are shown in Table 2.

Table 2. *Molecular classification of breast cancer.*

Luminal A	High expression of ER, ER-related genes
Luminal B	High expression of ER, ER-related genes, proliferation genes and in some cases HER2
HER2-enriched	Expression of HER2 and related genes
Basal-like	Low expression of ER, PR and HER2, expression of cytokeratins (5,6)

The luminal BCs are characterized by estrogen positivity, the expression of estrogen-associated genes and are sub-classified into two distinct subgroups, luminal A and B. The main biological distinction between luminal A and B subtypes is based on proliferation signatures. Luminal B tumors express proliferation genes, such as MKI67 and CCNB1, and are significantly associated with poorer outcomes. In 2009, Cheang et al examined a simplified classification based on the IHC markers ER, PgR, HER2 and Ki67. Ki67's ability to distinguish luminal A from B was investigated. Firstly, the gene signature PAM50 was used to identify luminal A and B, then the value of Ki67 able to distinguish the two groups was defined by IHC at 14% and validated in an independent cohort [111]. This definition was adopted by the 2011 St Gallen guidelines for the systemic treatment of early BC recommending adjuvant endocrine therapy alone for patients with luminal A tumors and the addition of chemotherapy for patients with luminal B BC [112]. The 2013 St Gallen guidelines included the requirement of substantial PgR positivity (≥20%) in the definition of luminal A to achieve a greater clarity of distinction between luminal A and B tumors [75].

HER2-enriched and basal-like BC have poor prognosis; however, they are chemotherapy sensitive [113, 114]. The OS and the DFS in HER2-enriched group is prolonged by the combination of trastuzumab and chemotherapy.

Today, it is well known that gene signatures are mostly available for patients with ER-positive early BC whereas no established tests exist for the other BC subtypes. The best-known gene-arrays are Oncotype DX Recurrence Score and MammaPrint. The Oncotype DX is developed to identify patients with ER-positive, node-negative disease treated with adjuvant tamoxifen who will benefit from chemotherapy. A recurrence score is attained by scoring and plugging ER-, HER2-, proliferation-, invasion- related genes into an algorithm. Proliferation genes are the most heavily weighted components in the formula [70].

Cell cycle

The cell cycle is a sequence of reproducible events leading to the growth and development of all eukaryotic cells. DNA replication occurs in the S-phase and mitosis in the M phase. These events are contemporarily separated by two gaps known as G1, where the cells prepare for DNA synthesis and G2, where the cells grow before entering mitosis. During mitosis, the duplicated chromosomes condense and join into fibers that pull sister chromatids to opposite poles of the cell. Non-dividing cells remain in G0 or the quiescent phase. See Figure 1 for overview.

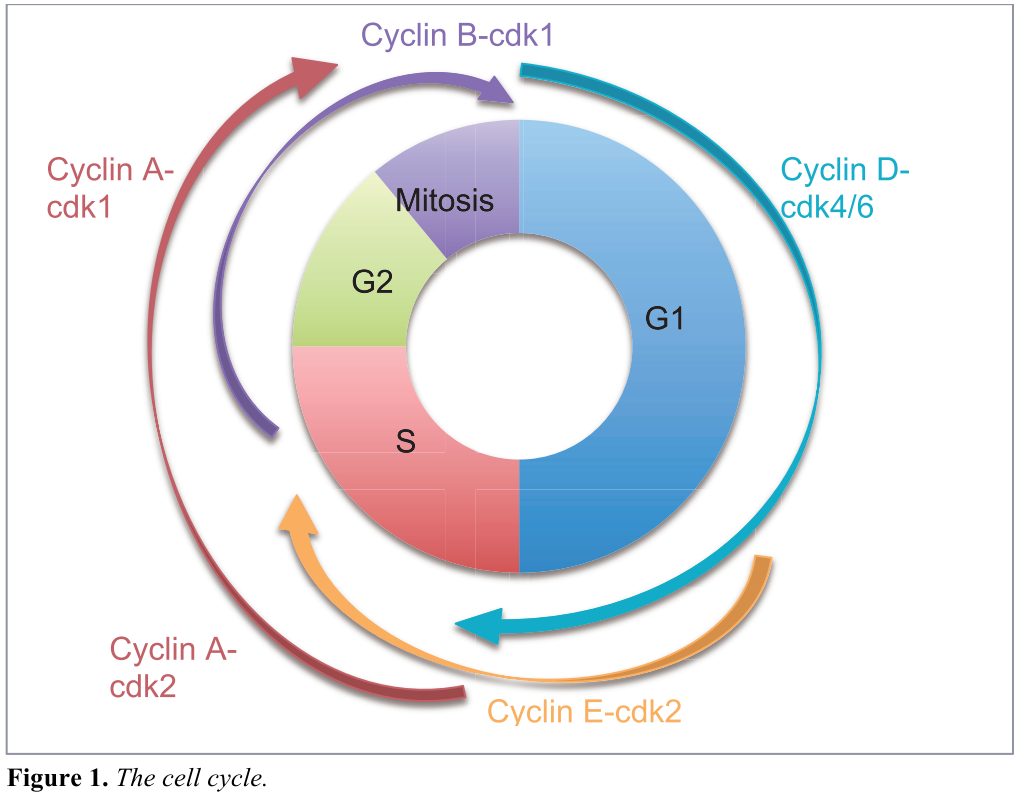


Figure 1. *The cell cycle.*

Complexes consisting of cyclins and the dependent kinases (CDK) accomplish the progress into the different steps of the cell cycle. Cyclins were discovered in the early 1980s and were so named because of the cyclical changes in their concentrations during the cell cycle. The cyclin-CDK units activate different target proteins via phosphorylation. There are many families of cyclins but the main groups are two: G1/S phase cyclins including cyclin A, D and E and G2/M cyclins including cyclin B.

The progression from one phase into the next is controlled by mechanisms called cell cycle checkpoints. Their role is to ensure that processes at each phase of the cell cycle have been accurately completed before progression to the next phase. The presence of DNA damage leads to the arrest of the cell cycle, mediated by P53, retinoblastoma protein (RB) and cyclin dependent kinase inhibitors (CDKI). There are three checkpoints: the G1 or restriction, the G2 and the mitotic spindle checkpoint.

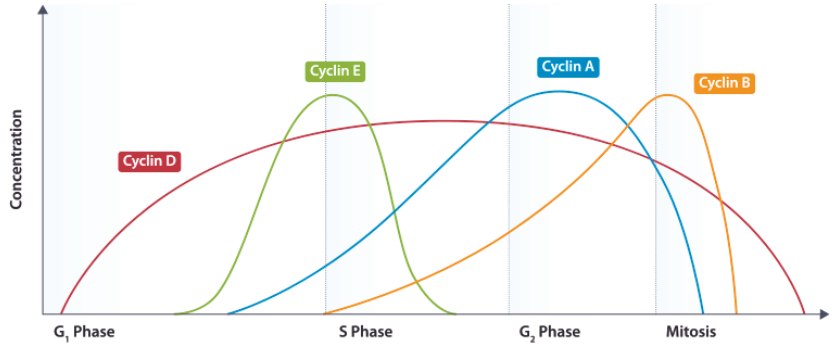


Figure 2. *Expression of cyclins through the cell cycle. (en.wikipedia.org)*

The first cyclin produced in the cell cycle and answering to growth signals is cyclin D. There are at least three different cyclin D of which cyclin D1 has been most thoroughly investigated. Cyclin D1 controls the G1 to S phase transition and promotes cell progression. Cyclin D1 activates the CDK 4/6 causing hyperphosphorylation and inactivation of the retinoblastoma protein (RB). The RB disassociates from the E2F/DP/RB complex thus activating the E2F gene resulting in transcription of genes such as cyclin A and E. The stabilization of the cyclin D1-CDK4/6 complexes depends on the interaction with the CDKIs, p21/27 [115]. There are two families of CDKIs: INK4 and CIP/KIP [115]. Cyclin D1 undergoes ubiquitination-mediated degradation during early S-phase. In BC

overexpression of cyclin D1 occurs via oncogene signaling, cyclin D1 locus amplification (10-20%) or mutations affecting degradation [116]. Furthermore, Cyclin D1 can act as a co-factor for ER-a, independently of ligand and CDK coordination [117].

The overexpression of cyclin D1 has reported contradictory results in BC. In some studies, high Cyclin D1 levels are associated with non-aggressive features such as positive ER status, small tumors and low grade and thus linked to better outcome [118]. On the other hand, other studies have demonstrated the opposite, showing that the overexpression of cyclin D1 is a predictive factor for tamoxifen resistance and poor prognostic features in ER-positive BC [52, 119, 120]. The maintenance of ERB2-induced tumors in mice is dependent on the continued expression of cyclin D1. Deletion of cyclin D1 led to a reduced animal tumor burden and decreased Ki67 expression in these tumors, indicating the involvement of cyclin D1 in proliferative activity [121].

Cyclin E binds to CDK2 and the activated complex phosphorylates target proteins involved in DNA replication. Cyclin E levels peak during G1-phase. There are two types of cyclin E but cyclin E1 has been more extensively studied. The cyclin E-CDK2 complex completes the inactivation of the RB protein through phosphorylation resulting in E2F repression and transcriptional activity in the S-phase. After this point, further cell cycle progression becomes independent of mitogenic stimulation. As the S-phase progresses cyclin E becomes degraded by mechanisms involving the ubiquitin-proteasome system.

Cyclin A is produced at the onset of DNA synthesis in the S-phase and is associated with the CDK2. Cyclin A-CDK2 is important for S phase progression and regulates the nuclear accumulation and activation of cyclin B1-CDK1. Association with CDK1 leads to the active complex of Cyclin A-CDK1 that is present in G2 and regulates the G2 to M transition. Cyclin A is involved in cellular activities that lead to a progression of replication and transcription and is a marker of tumor proliferation activity. Degradation of cyclin A occurs during mitosis. Some previous studies have reported that overexpression of cyclin A is associated with worse outcome in early BC [122-124] while other publications have shown contrary results [125].

Cyclin B1 accumulation starts in the S-phase, reaches the maximum level at mitosis and is rapidly degraded during the metaphase-anaphase transi-

tion. The cyclin B1-CDK1 complex is called the maturation-promoting factor (MPF) and is important for the chromosome condensation, nuclear envelope breakdown and spindle pole assembly; these are necessary mechanisms for cell progression into and out of mitosis. Before mitosis, cyclin B1 is located in the cytoplasm but, in late prophase, it is phosphorylated and relocates to the nucleus. At the end of mitosis, cyclin B1 becomes the substrate for the anaphase-promoting complex that degrades cyclin B1 and permits the cell to exit mitosis. The CCNB1 gene that encodes cyclin B1 is located in chromosome 5.

Overexpression of cyclin B1 in BC studies is associated with poorer outcomes [125-128] and correlated to histological grade and Ki67 but not to metastasic development [129]. Looking closer at BC subtypes, high cyclin B1 expression is found in Luminal B and basal like tumors and, furthermore, in ductal versus lobular and mixed histological types [130].

Unlike other cyclins (cyclins A and E), cyclin B1 is not overexpressed as a result of its gene CCNB1 amplification. Agwahl et al hypothesize that other oncogenic events such as MYC amplification and/or TP53 mutation, drive the overexpression of cyclin B1 in basal-like and luminal B tumors. In that study, high cyclin B1 was also correlated to an overexpression of Aurora kinase B and other genes indicating that inhibitors of these pathways may be beneficial in the treatment of basal-like and luminal B tumors where cyclin B1 is overexpressed [130].

The importance of proliferation in tumorigenesis is robust and antiproliferative agents have been suggested as potential treatment strategies. Hence, several inhibitors of the CDK-1 subunit of the MPF (cyclin B1-CDK1) have been previously investigated but most of them lack sensitivity [131]. The theory that cyclin B1 could be the target for antiproliferative drugs is investigated in another study. Yuan et al used small interfering RNAs (siRNAs) to target cyclin B1 in several human tumor cell lines and found that the protein level of cyclin B1 was reduced and the cells were arrested in G2/M phase in all cell lines tested, among them MCF-7 BC cells. The proliferation of tumor cells was suppressed and apoptosis increased [132]. In contrast to tumor cells, proliferation and apoptosis were not influenced in normal vein epithelial cells treated with siRNAs, making the antiproliferative drug strategy with siRNAs more attractive.

During the cell cycle, DNA is packed into structural units called nucleosomes. The protein component of nucleosomes includes histones and

non-histone proteins. There are 5 major histone families H1/H5, H2A, H2B, H3 and H4. Each individual nucleosome consists of an octameric histone core (pairs of H2A, H2B, H3 and H4) and double-stranded DNA. Histone modifications with methylation, acetylation and phosphorylation of several bases play a fundamental role in DNA function by controlling chromatin structure. The phosphorylation of histone H3 (PPH3) in serine 10 and 28 takes place during chromosome condensation and remains until the end of mitosis [133, 134]. Aurora B kinase has been identified to be the enzyme that is responsible for histone H3 phosphorylation at Ser10. Some studies indicate that PPH3 and Aurora B kinase are associated during the end of cytokinesis and have an important role in the formation of daughter cells [134, 135]. In vitro, small molecules inhibiting Aurora kinases have been reported to enhance the anti-proliferative effect of taxanes in metastatic triple-negative BC [136].

Antibodies against the phosphorylated Histone H3 are used as a tool to recognize cells in mitosis. The stable and contrast-rich staining has contributed to a high reproducibility between observers and remains consistent with digital image analyses [137].

The mitotic activity index (MAI) is one of three parameters of the Nottingham Grading System for BC and influences therapy decision making. However, the lack of reproducibility in MAI evaluation in hematoxylin-eosin stained sections has been discussed and considered to be a limitation. PPH3 has been investigated in node-negative BC patients and showed to correlate strongly with MAI and in certain studies even had the strongest prognostic value [137-139].

Aims of the thesis

The aim of this thesis was to investigate the prognostic value of proliferation markers, especially cyclin B1 and PPH3, in BC.

The specific aims were:

Paper 1: To investigate whether the proliferation marker cyclin B1 could differentiate poor from good prognosis patients in a subgroup of low-risk BC patients.

Paper 2: To further examine the prognostic value of the proliferation marker cyclin B1 in a lymph-node-negative and chemotherapy-untreated patient cohort using immunohistochemistry and to evaluate its reproducibility.

Paper 3: To evaluate the prevalence and prognostic value of the proliferation markers cyclins A and B1 and Ki67 in male breast cancer.

Paper 4: To apply the IHC panel suggested in the 2011 St Gallen guidelines to our material and to investigate whether an alternative Ki67 cut-off, cyclin A or B1 can distinguish luminal A from luminal B in a reliable fashion.

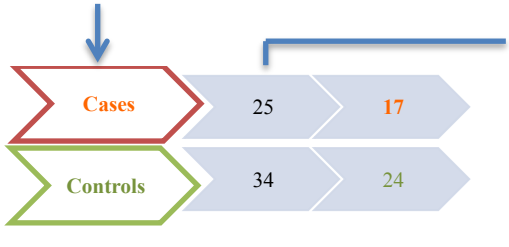
Paper 5: To investigate the prognostic role of PPH3 compared with the proliferation markers Ki67, cyclin A and B1 in lymph-node-negative BC with focus on ER-positive patients.

Materials

Paper I

Patients from a defined cohort of women diagnosed with BC in the Uppsala-Örebro region between 1993 and 2001 were analyzed. The patient cohort was identified from the regional BC registry, that has a population coverage of 99% and a high level of agreement regarding data on primary treatment retrieved from patients files. They all had tumors < 2 cm, no lymph node metastases, a histologic grade of I/II or low proliferation (SPF), ER and/or PgR receptor positive tumors and none had received adjuvant chemotherapy. Women who died of their BC denoted as cases. In this study we chose as cases the 25 with the shortest survival after their BC diagnosis and as controls 25 women that were alive and without relapse eight years after the BC diagnosis. Since only 34 women fulfilled the control inclusion criteria, all were included. After data review 17 cases and 24 controls remained (figure 3).

Inclusion Criteria
Tumor size <= 20mm
No lymph node metastases
Histological grade I-II
Low proliferation (S-phase)
ER- or/and PgR- positive receptors
No adjuvant chemotherapy



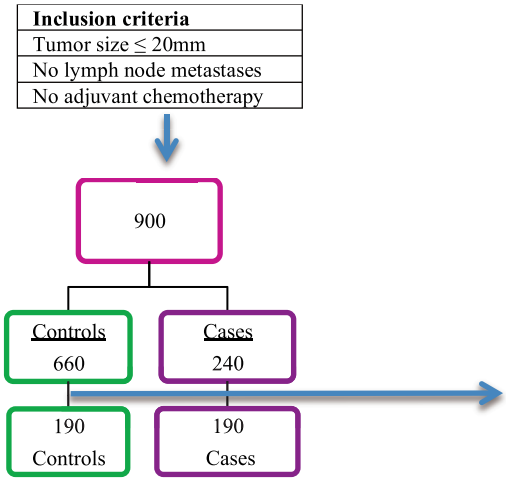
Exclusion criteria	
No paraffin blocks	5
Controlateral breast cancer	3
Tumor size > 2cm	1
Distant metastasis or lymph node at diagnosis	2
Non breast cancer death	1
Relapse or death of breast cancer	3
Diagnosis or death of concurrent cancer	3
	18

Figure 3. *Inclusion criteria and selection of 17 cases and 24 controls, patients dying early and being alive 8 years after BC diagnosis.*

Papers II, IV,V

The defined cohort of women with BC in the Uppsala-Örebro region 1993-2004 was studied and women with a tumor size ≤ 50mm, no lymph node metastases and no adjuvant chemotherapy were identified. Originally 900 women fulfilled the above described inclusion criteria. In this cohort, 240 women died of BC and were denoted as cases. For each case one control was used and controls were women alive at the time of the corresponding case's death. Fifty cases and corresponding controls were excluded because they did not fulfill the inclusion criteria or no paraffin blocks were found when the medical records were reviewed (figure 4).

In paper II controls were selected by incidence density sampling, which involves matching each case to a sample of those who were at risk at the time of the case occurrence. All eligible women with an event were studied as cases and about 20% of the entire cohort was chosen as controls. With this study design the loss in power in comparison with a complete analysis of all cohort members was small. Having 190 cases yields a power of 90% to detect an odds ratio of 2,5 on a statistical significance level of 5%, assuming a prevalence of 30% cyclin B1 overexpression in the cohort.



Exclusion reason	n
New/ contralateral or locally advanced BC	26
No paraffin blocks	12
No breast cancer death	6
Distant metastases at diagnosis	4
Adjuvant chemotherapy	1
No breast surgery	1
	50

Figure 4. *Inclusion, exclusion criteria and selection of 190 cases who died of breast cancer and 190 controls alive at the time of the corresponding case's death.*

Table 3. *Patient and tumor characteristics of 190 cases and corresponding controls with no lymph node metastases, tumor size ≤50 mm, and chemotherapy naïve.*

	Case n	(%)	Control n	(%)
Tumor histology				
Ductal	163	(86)	145	(76)
Lobular	20	(10)	23	(12)
Others	7	(4)	22	(12)
Histologic grade				
1	19	(10)	48	(25)
2	94	(50)	105	(55)
3	76	(40)	34	(18)
Not known	1	(0)	3	(2)
ER				
Positive	103	(54)	147	(77)
Negative	79	(42)	41	(22)
Not known	8	(4)	2	(1)
PgR				
Positive	73	(38)	127	(66)
Negative	108	(57)	60	(32)
Not known	9	(5)	3	(1)
HER2				
Overexpression (IHC 3+ or FISH pos)	18	(10)	13	(7)
Normal	158	(83)	161	(85)
Not known	14	(7)	16	(8)
Adjuvant radiotherapy				
Yes	101	(53)	116	(61)
No	89	(47)	74	(39)
Adjuvant endocrine therapy				
Yes	53	(28)	47	(25)
No	137	(72)	143	(75)
Total	190		190	

Paper III

The National Breast Cancer Register in two regions of Sweden was used to identify male patients with invasive BC diagnosed between 1990-2007. The population covered was 1,96 million people in Uppsala-Örebro region and 1,70 in the Lund region. Initially 131 patients in Uppsala-Örebro and 118 patients in Lund were identified. Paraffin tumor blocks were missing in 18 cases from Uppsala-Örebro and 26 cases from Lund region. Patients with in situ cancer (Uppsala-Örebro n=4, Lund n=2) and cases with missing patients' records (Uppsala-Örebro n=0, Lund n=2) were excluded. 109 patients from Uppsala-Örebro and 88 patients from Lund with available paraffin-embedded tumor blocks, clinic-pathological data and outcome data were included in the study. The National Population Register was used for updated information on patients' vital status and cause of death.

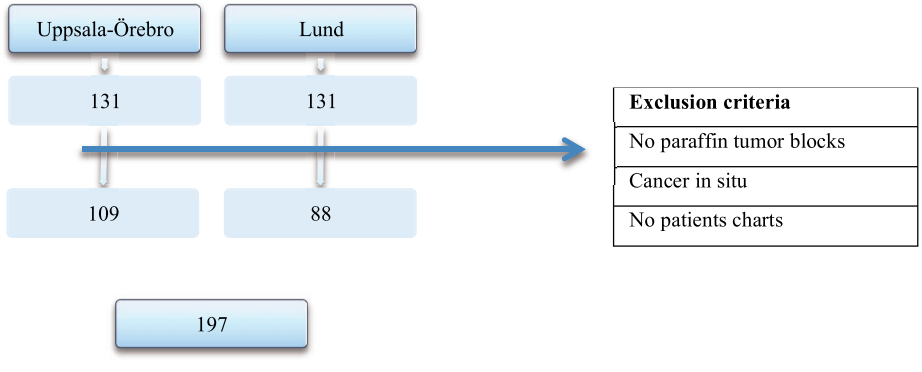


Figure 5. *Male breast cancer patients from Uppsala-Örebro and Lund region between 1990-200*

Table 4. *Clinico-pathological characteristics.*

Variable	n	%	*Variable*	n	%
Age (years)	Mean 70 (range 23–98)				
Tumor size (mm)	Median 20 (range 4–100)				
Tumor side			***ER***		
Right	85	43	Positive (>10%)	183	93
Left	108	55	Negative (≤10%)	9	5
Data missing	3	2	NA	5	3
Tumor size			***PR***		
pT1	91	46	Positive (>10%)	152	77
pT2	60	31	Negative (≤10%)	38	19
pT3	6	3	NA	7	4
T4	20	10			
NA	20	5			
Node status			***Ki67***		
0	88	45	High (>14%)	92	47
Positive	78	40	Low (≤14%)	99	50
NA	31	15	NA	6	3
Metastases			***HER2***		
No	178	90	Positive by IHC or SISH	21	11
Yes	9	5			
Data missing	10	5			
Grade					
I (NHG 3–5)	15	8			
II (NHG 6–7)	96	49			
III (NHG 8–9)	81	41			
NA	5	3			

Abbreviations. NA: not analyzed SISH: silver in situ hybridization

Methods

Paper I

Immunohistochemical staining was performed on paraffin-embedded tissue sections from BC tumors. Four to 5 μm thick sections were cut from the paraffin blocks and mounted on super-frost slides. Conventional slides were deparaffinized in xylene and rehydrated through a ladder of graded ethanol (absolute ethanol, 95%, 80% and distilled water). Antigen retrieval was done in tris buffer (pH=8) using a pressure cooker before processing the sections in an automatic immunohistochemistry-staining machine according to standard procedures (Autostainer; Dako, Sweden). The primary antibody for the detection of cyclin B1 (rabbit monoclonal; Y106; Epitomics Inc, Burlingame, CA, USA) was applied for 30 minutes at room temperature. Immunostainings were detected via DAKO Cytomation envision/HRP kit K5007. Tonsil samples were used as positive controls. The primary antibody was omitted from negative controls. In most tumors 1 000 cells were counted, with a minimum level set at 200 cells.

Nuclear as well as cytoplasmic stainings were assessed in a comparative study. A cut-off value separating high and low cyclin B total (total =nuclear and/or cytoplasmic staining) was set at ≥ 9% corresponding to the 7th decile of all patients ' values. Both staining and scoring were performed blinded to case and control status.

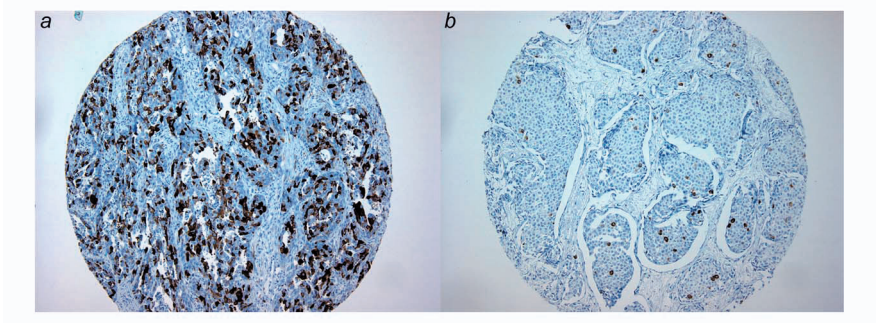
Papers II, IV, V

Immunohistochemistry was used and performed on formalin-fixed paraffin-embedded tumors. Haematoxylin and eosin sections were reviewed and areas with invasive tumor were selected. Histological grade was evaluated according to the Elston and Ellis grading system[54]. Representative areas from each tumor were punched and brought into recipient

paraffin blocks to construct TMAs (tissue microarray) consisting of two cores (diameter 1 mm) from each tumor. The TMA slides were deparaffinized and rehydrated as described above in paper I. Antigen retrieval was performed in TE (Tris-EDTA pH 9 buffer) in a microwave oven for 10 minutes (750W) 15 min (350W) before being processed in an automatic immunohistochemistry staining machine according to standard procedures (Autostainer; Dako, Sweden). All antibodies were applied for 30 min at room temperature. The following monoclonal antibodies were used: cyclin A (NCL-Cyclin A 1:100 NovoCastra Laboratories, UK), cyclin B1 (Y106 1:200 Epitomics Inc, Burlingame, CA, USA), Ki67 (1:200 M7240, Dako, Sweden), ER (NCL-ER-6F11 1:150 NovoCastra Laboratories, UK), and PgR (NCL-PGR 1:100 NovoCastra Laboratories, UK). For PPH3 the rabbit polyclonal anti-phosphohistone H3 (ser10) was used (Upstate #06.570 1:1500 Lake-Placid, NY, USA). Immunostainings were detected via DAKO Cytomation envision/HRP kit K5007. For cyclin A, B1, Ki67 and PPH3 stainings, tonsil samples were used as positive controls; and for ER and PgR stainings, BC tissue was used. The primary antibody was omitted from negative controls. HER2 status was determined using IHC staining with HercepTest (DAKO) and cases were classified as0, 1+, 2+ or 3+ according to standard procedures. All cases considered as 2+ were further analyzed by fluorescent in situ hybridization, as well as cases with a cytoplasmic and/or doubtful IHC staining pattern. All patients with HER2 amplified tumors or with IHC score of 3+ were considered HER2-positive.

Evaluation of immunoreactivity scores

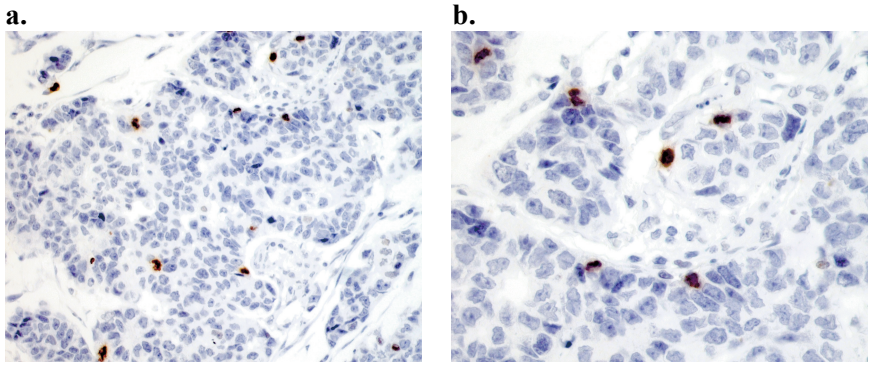
In paper II two independent investigators evaluated cyclin B1 by counting several numbers of cells in hotspots (100 cells, 200 cells, 500 cells and 1000 cells). One investigator counted cells on a computer screen using digitally acquired images that a light microscope had provided. The other investigator counted cells in high-power fields using a light microscope (an ocular graticule consisting of 10 x 10= 100 grids was used). 1000 tumor cells were counted in most TMAs (minimum 200cells). The percentage of positive cells in approximately 100, 200, 500 cells was calculated and to get the average percentage of positive cells we divided all positive cells from the two cores of biopsies with the entire or largest number of cells (approximately 1000) from the same biopies. The 7^{th} decile was used to define the cut-off values. High and low staining of cyclin B1 on TMA slides from BC are shown in picture 1.



Picture 1. *High (a) and low (b) staining of cyclin B1 (Epitomics Inc.) on tissue microarray slides from breast cancer cells with 100x magnification.*

In paper V, PPH3 evaluation was assessed blindly by an investigator (AK) and supervised by a board-certified breast pathologist (HO). PPH3 showed both nuclear staining and staining of mitotic figures. Nuclei with fine granular staining, intact membrane and presence of nucleolus were excluded from the quantification since they belong to cells in interphase [137]. Mitotic figures characterized by chromatin clumps, had strong and dense staining and were accepted as positive.

We used two methods to evaluate the PPH3 staining. The percentage of positive stained cells counting 200 and 500 tumor cells were calculated and the number of positive stained cells in 10 fields of view. Tumor cells were counted in high-power fields (40X objective) using a light microscope with an ocular graticule consisting of 10 X 10 = 100 grids. As the TMA area core is approximately 7 FOVs, the results were extrapolated to 10 FOVs (PPH3 index). The most mitotic active TMA core was counted, multiplied by 1.59 and divided by the whole area of the TMA, 1.13 mm^2. The cut-off value was defined by the 7^{th} decile as has been shown in previous studies evaluating proliferation markers in TMAs. The distinct staining of PPH3 in BC cells with 200 and 400x magnification is shown in picture 2.



Picture 2. *PPH3 staining (Lake-Placid) in breast cancer cells with 200x (a) and 400x (b) magnification.*

Paper III

The tissue microarray slides were deparaffinized in xylene and rehydrated through a ladder of graded ethanol (absolute ethanol, 95%, 80% and distilled water). For cyclin A (NCL-Cyclin A; Novo Castra Laboratories) and cyclin D1 (RM-9104-S; Neo Markers) antigen retrieval was performed in a micro- wave oven for 10 min (750 W) + 15 min (350 W) using a TE-buffer. For Cyclin B (1495-1; Epitomics) antigen retrieval was performed in a pressure cooker for four minutes with the use of TRS buffer. After antigen retrieval, all tissue microarray slides were processed in an automatic immunohistochemistry staining machine according to the standard procedures (Autostainer, Dako, Sweden).

Evaluation of immunoreactivity scores. Cyclins A and D1 were analyzed by one investigator (CN) and cyclin B1 by a second investigator (AK). The percentage of positively cells was assessed by choosing the high-power field with the largest number of positive stained cells out of the two biopsies and dividing by the entire number of cells from the same high-power field. A minimum of 200 cells per tumor was counted. For cyclins A and D1, cells with unequivocal nuclear staining and for cyclin B, nuclear and cytoplasmic staining, were considered as positive. Cells were manually counted in high-power fields using a light microscope.

Statistics

Paper I

The study was designed to compare two groups of women with low-risk BC experiencing different outcomes, those dying early of their BC and denoted as cases and those alive and without relapse eight years after the BC diagnosis. Sample size was calculated using Lehr's formula: to detect a minimum difference in the distribution of a continuous variable of one standard deviation 16 patients (α=0.05; β=0.20) or 21 patients (α=0.05; β=0.10) were needed in each group. Since the patients were selected, Fisher's exact test was used comparing cases and controls. For comparison of means, Mann-Whitney was used. Differences were considered statistically significant if $p < 0.05$.

Paper II

Prognostic factors such as age, tumor size, hormone receptors, histological grade, mitotic count, tubuli, nuclear atypia, HER2, Ki67, cyclin A and cyclin B1 were analyzed univariately, using conditional logistic regression analysis to estimate odds ratios. Multivariate conditional logistic regression analysis including these factors considered not to be appropriate because histological grade, mitotic count, nuclear atypia, Ki67, cyclin A and Cyclin B1 were highly correlated. In addition, models adjusted for age and tumor size were performed. For cyclin B1 endocrine therapy was also included in the adjusted model. Correlations between cyclin B1 and other clinicopathological parameters were assessed with Spearman's correlation test.

The data were dichotomized in order to examine the reproducibility of the IHC analysis of cyclin B1. The degree of concordance between the two investigators was quantified as the chance corrected measure of agreement, known as kappa[140].

One commonly applied definition for the interpretation of different kappa values is as follows: <0.20 poor; 0.21–0.40 fair; 0.41–0.60 moderate; 0.61–0.80 good; 0.81–1.00 very good.

Paper III

Correlations between variables were analyzed using Spearman's correlation test. The association between disease stage and treatment was tested using χ^2 test. Cox proportional regression was used for uni- and multivariate analyses. Survival estimates were calculated with Kaplan–Meier and log-rank statistics. Breast cancer death was chosen as the end-point, other cases were censored. All p values are two-sided with statistical significance set at 0.05. For evaluation of cyclins in comparison with other clinico-pathological variables, we used the results from a previous study on the same patient material.

Paper IV

In paper IV conditional logistic regression analysis for matched case-control study was performed to estimate odds ratios (ORs) and confidence intervals (CIs) using the PHREG procedure in SAS (SAS Institute Inc).

Paper V

The PPH3 was first analyzed univariately in the entire material (190 cases/190 controls) using conditional logistic regression analysis to estimate odds ratios using SPSS statistics.

The primary study was designed as a matched case-control and the sample size was calculated to reveal a difference for prognostic factor levels in cases and controls with an odds ratio of 2.5. One aim of this study was to examine only ER-positive patients. In total, 77 out of 190 matched pairs were available for the analysis. Because of a great loss of data with restriction to matched pairs, we conducted an unmatched analysis of the whole cohort of ER-positive women by using unconditional logistic regression. Univariate analysis of well-documented clinico-pathological

prognostic factors (age, tumor size, histologic grade, PgR, HER2 status, adjuvant endocrine therapy) and proliferation markers (cycklin A, cyclin B, PPH, Ki67) was performed.

In the ER-positive group the variance inflation factor (VIF) was calculated for each proliferation marker to assess multicollinearity in multivariate models. VIF values were < 2.5 for all proliferation markers, indicating that multicollinearity was unlikely to be present in this study. Multivariate analyses of proliferation markers (adjusted for age, tumor size, histologic grade, PgR, adjuvant endocrine therapy) were therefore performed. As a further sensitivity analysis, we used conditional logistic regression to include only the 77 matched pairs.

Spearman's analyses between $PPH3_{200}$, $PPH3_{500}$, PPH3 index and age, tumor size, histologic grade, mitotic count, tubuli, nuclear atypia, ER, PgR, HER2, Ki67, cyclin A and B1 were performed for all patients and ER-positive patients to estimate correlations between those factors. The analyses were performed using the StatsDirekt statistical software.

Results

Paper I

Women that died early of their BC (cases) and women alive and without relapse eight years after BC diagnosis where accurately separated by the proliferation marker cyclin B1 using immunohistochemistry.

The mean value cyclin $B1_{total}$ was 12% (95%CI 8-16%) for cases and 5% (95%CI 4-6%)for controls and cyclin $B1_{nuclear}$ was 2% (95%CI 0-5%) for cases and 0.5% (95%CI 0-1%) for controls. Cut-off value separating high and low cyclin $B1_{total}$ was set at ≥ 9% and high cyclin $B1_{total}$ could accurately identify 11 of 17 patients with early death and low cyclin $B1_{total}$ could accurately identify 22 of 24 patients that were free from relapse eight years after the initial diagnosis. The specificity was 92% and the sensitivity 65%. The overall accuracy for identifying women died from BC and long time survivors using cyclin $B1_{total}$ was 80% (33/41) and using cyclin $B1_{nuclear}$ 65% (27/41).

Paper II

For cases, the median cyclin $B1_{100}$ was 9% (range 0-48%), for cyclin $B1_{200}$ 8% (range 0-48%), for cyclin $B1_{500}$ 8% (range 0-46%), and for cyclin $B1_{tot}$ 7% (range 0-46%). The corresponding values for controls were cyclin $B1_{100}$ 5% (range 0-46%), for cyclin $B1_{200}$ 5% (range 0-46%), for cyclin $B1_{500}$ 4% (range 0-37), and for cyclin $B1_{tot}$ 4% (range 0-35%).

Cyclin B1 was highly correlated to histological grade, mitotic count, other proliferation markers as Ki67 and cyclin A(r=0.63-0,80) and inversely correlated to ER and PgR (r=-0.25-0.33). Weaker correlation was seen between cyclin B1 and HER2 and tumor size (r=0.25-0.27).

A statistically significant association was observed between BC death and expression of cyclin $B1_{100}$, cyclin $B1_{200}$, cyclin $B1_{500}$, cyclin $B1_{tot}$, Ki67, cyclin A, histological grade, mitotic count, tubuli, nuclear formation, tumor size, ER, and PgR using conditional logistic regression in a univariate model, see table 5. All these differences remained after adjustment for tumor size and age at diagnosis. Furthermore, the independent prognostic value of cyclin B1 persisted when endocrine therapy was included in the multivariate analysis (cyclin $B1_{tot}$: OR 3.3, 95% CI 1.9–5.9). Overexpression of HER2 was not statistically associated with BC death.

Within the case group studied on its own, there was a difference in time from diagnosis to distant metastasis comparing patients with low cyclin $B1_{200}$ to high cyclin $B1_{200}$ (43 and 33 months, respectively). There was also a difference in survival from diagnosis between women with low cyclin $B1_{200}$ and high cyclin $B1_{200}$ (56 and 47 months, respectively). However, there was no difference in time to BC death from distant metastasis comparing patients with low cyclin B1 with those with high cyclin B1 (15 and 14 months, respectively).

The two investigators examined the cyclin B1 stainings blindly and separately for approximately 100, 200, 500, and 1000 cells and the kappa value was between 0.74-0.82. For both investigators, cyclin B1 was a significant prognostic factor (ORs > 2) in both uni- and multivariate analyses (adjusted for tumor size, age, and endocrine therapy), independent of the way of counting.

Table 5. *Univariate and multivariate analysis of prognostic factors.*

		Univariate analysis		Models adjusted for age and tumor size	
		N=380 (190 cases/190 controls)		N=380 (190 cases/190 controls)	
	Reference	**OR**	**95% CI**	**OR**	**95% CI**
Tumor size	≤20	2.3	1,4-3.5		
Age at diagnosis					
<50	50-69	0.7	0.5-1.9		
≥70	50-69	2.2	1.3-3.5		
ER	>10	2.6	1.6-4.2	2.5	1.5-4.3
PgR	>10	2.5	1.6-3.8	2.3	1.5-3.6
Grade	I-II	3.1	1.8-5.1	3.9	2.2-7.0
Mitotic count	I-II	2.5	1.5-4.2	3.1	1.7-5.6
Tubuli	I-II	3.2	1.9-5.6	3.3	1.9-5.9
Nuclear atypia	I-II	2.5	1.5-4.0	2.8	1.6-4.8
Ki67	≤22	1.7	1.1-2.7	1.6	1.0-2.5
Cyclin A	≤11	3.4	2.1-5.5	3.4	2.1-5.7
HER2	Negative	1.0	0.4-2.3	0.9	0.4-2.2
Endocrine treatment	No endocrine treatment	0.8	0.5-1.3	0.5	0.3-0.9
Cyclin $B1_{100}$	≤8	3.0	1.9-4.8	2.8	1.7-4.8
Cyclin $B1_{200}$	≤7	2.8	1.8-4.5	2.8	1.7-4.6
Cyclin $B1_{500}$	≤7	2.5	1.5-4.0	2.4	1.4-4.0
Cyclin $B1_{tot}$	≤6	2.9	1.8-4.6	2.9	1.7-4.9

Paper III

Cyclins A and B, mitotic count and Ki67 demonstrated strong positive correlation with each other. Cyclin A showed an inverse correlation to PR. None of cyclins A and B1 demonstrated any correlation with ER. Cyclin D1 was correlated to ER, but did not correlate to the other cyclins, Ki67 or the mitotic count.

The mean values of expression for cyclin A, cyclin B1 and Ki67 were the following: cyclin A, 10% (range 1– 42%); cyclin B1, 9% (range 0–29%); and Ki67, 16% (range 0–83%). The cut-off values for the 5th (median) and 7th decile were 9 and 12% for cyclin A, 8 and 11% for cyclin B1, and 14 and 21% for Ki67, respectively. The mitotic count ranged from 0 to 51, with a median value of 11. The 7th decile cut-off for cyclin A (12%) corresponded well to the previously defined optimal cut-off in female BC (11%). When evaluating these cut-offs, overexpression of cyclin A showed a trend toward poorer BC survival (p-value 0.11 and

0.07, respectively). The prognostic impact was strengthened by using the cut-offs corresponding to the 8th (14%) and 9th decile (17%) with HR 2.1, p-value = 0.05 and HR 3.7, p-value= 0.001, respectively. When the cut-off 15%, corresponding to the 9th decile was used, then, the overexpression of cyclin B1 was associated with an increased risk of BC death with a HR of 2.7, p-value= 0.02. Mitotic count >10 per field of view was predictive of poorer survival (HR 2.5,p-value 0.01), whereas no difference between tubuli 1-2 vs 3 or nuclear atypia 1-2 vs 3 could be demonstrated (data not shown). The highest prognostic impact for cyclin D1 was found when the cut-off (62%) corresponding to 3rd decile was used. However, in contrast to the other cyclins, overexpression of cyclin D1 was predictive of better survival (HR 0.3 p=0.001). Cyclin D1 and nodal status remained independent prognostic factors in a multivariate model including nodal status, tumor size (HR for cyclin D1 0.48, p-value= 0.05)

Sixty percent (n =119) of patients underwent adjuvant endocrine treatment, and all but one (118 of 119) received tamoxifen. Endocrine therapy was administered more often in higher-stage disease: 79.5% in stage III, whereas only 46.9% in stage I. Cyclin D1 overexpression did not influence the response to endocrine treatment. An analysis of adjuvant endocrine treatment in patients with ER positive tumors adjusted for cyclin D1 did not result in any notable change in the HR for endocrine treatment; 2.34 (95% CI, 1.02–5.37) vs 2.14 (95% CI, 0.88–5.19) in the unadjusted and adjusted analyses, respectively.

Paper IV

Out of 380 tumors, results from 30 (8%) stainings were missing and therefore not included to the classification. According to Cheang's classification, 122 patients (32%) were classified as luminal A, 119 (31%) luminal B, 13 (3%) luminal HER2, 18 (5%) HER2 enriched and 78 (21%) triple negative.

A statistically significant association was observed between BC death and BC subtypes defined by proliferation markers (Ki67, cyclin A and cyclin B1) using conditional logistic regression in a univariate model, see Table 6. Using the $Ki67_{14\%}$ we found that luminal B was not significantly associated with poorer survival compared with luminal A (OR 1.4 95%CI 0.8-2.6 p-value 0.24). However, using the $Ki67_{20\%}$, we found that patients with luminal B tumors showed poorer survival compared with those with luminal A tumors with OR 2.0 (95%CI 1.0-3.9 p-value 0.04). Cyclin B1

could also separate luminal B from A with an OR of 2.2 (95%CI 1.1-4.5 p-value 0.03). Cyclin A could separate the luminal B from luminal A with OR for BC death of 3.6 (95%CI 1.8-7.0 p-value <0.001). See Table 6 for overview.

Table 6. *Comparisons between luminal subtypes, using different proliferation markers.*

Luminal B vs luminal A			
Reference	**OR**	**95 %CI**	**P value**
Ki67 ≥14%	1.4	0.8-2.6	0.24
Ki67 ≥ 20%	2.0	1.0-3.9	0.04*
Cyclin A > 11%	3.6	1.8-7.0	<0.001*
Cyclin B1 >7%	2.2	1.1-4.5	0.03*

Significance level * ≤ 0,05

Paper V

PPH3 was strongly correlated to mitotic count, cyclin A, Ki67 and histological grade when all patients were examined while the PPH3 index was strongly correlated only to mitotic count in the ER-positive patients. PPH3 evaluated both as percentage and as positive cells in 10 FOVs had a prognostic value for BC death with OR around 2 and 3. PPH3 index is a more accurate and time-saving method, see Table 7. When all patients were examined a statistically significant association was observed between the expression of PPH3 index and BC death, using conditional logistic regression in a univariate model (OR 2.6 95%CI 1.6-4.5 p-value <0.001) and, in a model adjusted for age and size (OR 2.2 95%CI 1.2-3.9 p-value 0.01). We stratified the analysis for patients with ER-positive disease and found that a high PPH3 was associated with BC death in a univariate model using unconditional logistic analysis (OR 2.9 95%CI 1.6-5.2 p-value <0.001), see table 8. Conditional regression analysis did not change the result (OR 4.2 95% CI 1.7-10.2 p-value <0.002). In the multivariate analyses with unconditional and conditional regression cyclin A was the only independent prognostic factor for BC death in ER-positive patients with an OR 3.6 (95% CI 1.6-8.1 p-value 0.002) for the unconditional and 4.2 (95% CI 1.1-16.2 p-value 0.004) for the conditional analyses.

Table 7. *Conditional logistic regression analysis. Univariate and multivariate analysis of PPH3 in 190 cases/190 controls.*

	Univariate analysis			Models adjusted for age and tumor size			
	Cut-off	**OR**	**95%CI**	**p value**	**OR**	**95%CI**	**p value**
$PPH3_{200}$	≤1%	1.9	1.2-3.1	0.009*	2.0	1.2-3.3	0.01*
$PPH3_{500}$	≤1%	2.4	1.4-4.1	<0.001*	2.1	1.2-3.9	0.02*
PPH3 index	≤8	2.6	1.6-4.5	<0.001*	2.2	1.2-3.9	0.01*

Significance level * ≤ 0,05

Table 8. *Unconditional logistic regression analysis for the ER-positive patients. Univariate and multivariate model.*

	Univariate analysis				Multivariate analysis			
	OR	**95% CI**		**p-value**	**OR**	**95% CI**		**p-value**
		Low	High			Low	High	
Age								
<70	1				1			
≥ 70	2.3	1.3	3.9	0.002*	3.0	1.5	5.9	0.001*
Tumor size								
≤ 20 mm	1							
>20 mm	1.9	1.1	3.5	0.031*	1.3	0.6	1.9	0.448
Histologic grade								
I-II	1				1			
III	3.4	1.7	6.8	0.001*	1.5	0.5	4.1	0.438
PgR								
Positive (> 10%)	1				1			
Negative (≤ 10%)	2.0	1.3	3.3	0.008*	2.8	1.4	5.8	0.005*
HER2								
Positive	1							
Negative	1.7	0.4	6.6	0.465				
Adjuvant endocrine therapy								
Yes	1				1			
No	1.0	0.6	1.6	0.898	0.8	0.4	1.6	0.562
Ki67								
≤ 22	1				1			
>22	1.6	0.9	2.9	0.093	1.9	0.8	4.5	0.128
Cyclin A								
<11	1				1			
≥11	3.8	2.2	6.6	<0.001*	3.6	1.6	8.1	0.002*
Cyclin B								
<7	1				1			
≥ 7	2.9	1.7	4.9	<0.001*	1.8	0.8	4.0	0.139
PPH3 index								
<8	1				1			
≥8	2.9	1.6	5.2	<0.001*	1.5	0.7	3.2	0.359

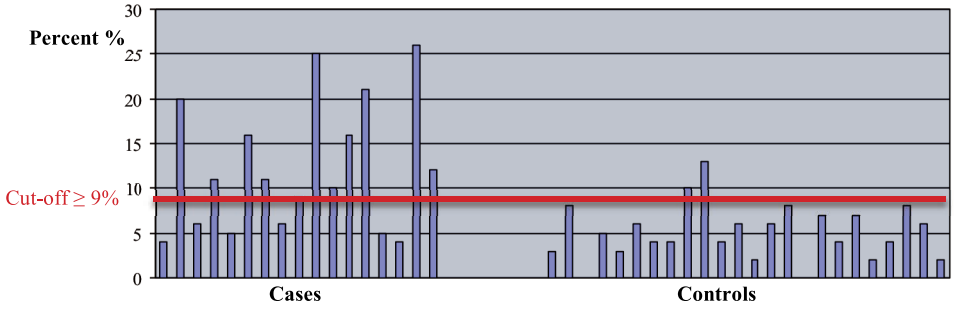
Significance level * ≤ 0,05

Discussion and conclusion

In this thesis we present five papers aimed at increasing our understanding of the role played by proliferation in the prognosis of BC by examining cyclin B1 and PPH3. The prognostic significance of cell proliferation in BC has been extensively studied by various methods including determination of the MAI, SPF and immunohistochemistry for Ki67 [86]. Cyclins, on the other hand, have been examined in BC to a lesser extent. In paper I the aim was to examine whether cyclin B1 could predict for BC death in patients with early BC. A subgroup of node-negative, ER-positive patients with stage I disease has en excellent prognosis with a BC mortality of only 5% at ten years. This means that we should identify these 5% of patients who have a poor prognosis and might have benefited from adjuvant systemic treatment.

In paper I, we deliberately chose to investigate two extreme groups with same favorable clinicopathological characteristics but with different outcomes. In this study we have shown that assessing cyclin B1 expression in tumors can help separate patients with a poor outcome from those with a good outcome in a subgroup of patients with low-risk node-negative BC, see figure 6.

Figure 6. *Expression of cyclin B1 in low-risk node-negative breast cancer.*



Low levels of cyclin $B1_{total}$ were seen in 22 of 24 women in the control group and high levels in 11 of 17 women in the case group resulting to an overall accuracy of 80% (33/41). In a previous study, the accuracy of correctly predicting metastases using gene signatures varied from 48-75%. The authors also tested their 32-gene profile (HUMAC32), which was previously developed from this data set and found that their accuracy was 78% [141]. Interestingly the simultaneously evaluation of many genes resulted to a slightly lower accuracy compared with the accuracy achieved using cyclin B1, an IHC assessed single marker.

In previous papers the prognostic capacity of cyclin B1 was based on the cyclin $B1_{total}$, while Suzuki et al claimed that only cyclin $B1_{nuclear}$ is of prognostic significance [125-127]. We evaluated cyclin B1 and found that both nuclear and total (nuclear and/or cytoplasmic) staining of Cyclin B1 could separate patients with a poor outcome from those with a good outcome. However, the difference between the two groups was greater using cyclin $B1_{total}$ than cyclin $B1_{nuclear}$ (12-5% and 2-0.5%, respectively), making cyclin $B1_{nuclear}$ impractical in the clinical setting. It is also of importance to mention that the counting of nuclear reactivity was difficult in the presence of cytoplasmic staining as the cytoplasm often overlapped the nucleus making the evaluation uncertain. Moreover, the accuracy of correctly identifying patients dying of BC and survivors was higher using cyclin $B1_{total}$ than cyclin $B1_{nuclear}$, 80% and 66%, respectively.
The promising results from paper I on the prognostic value of the proliferation marker cyclin B1 and the need to evaluate them in a larger sample size motivated further studies and led to Paper II. In concordance with our results from Paper I, cyclin $B1_{total}$ was evaluated. Prior to the patients' enrolment in the study a power calculation was also performed. To achieve statistical power a case-control design was chosen. Assuming a power of 80%, alpha of 0.05, a prevalence 0.3-0.5 and OR=2,5 enrolment required 125-150 patients in each group. In our study a total of 190 cases and 190 controls were identified, none of which had received adjuvant chemotherapy, resulting in an increased power (90%). To measure the prognostic value of cyclin B1, we selected patients without lymph nodes metastases and not treated with chemotherapy, which is known to diminish the prognostic impact of proliferation markers [142, 143]. A complete analysis of all cohorts' members is often impossible but by using incidence density sampling the loss in power was limited. Established prognostic factors such as tumor size, age, hormone receptors and histological

grade proved to be prognostic factors in our material with an OR of 2-3 in univariate analyses [84, 144]. These results are in line with several studies in this field. However, in our study HER2 did not turn out to be a prognostic factor, probably due to few patients with an overexpression of HER2 (10% in cases and 7% in controls) and consequently low statistical power.

Regarding the evaluation of immunoreactivity scores, we found that ORs remained constant at 2-3 independently of the number of cells counted and suggest that counting 200 tumor cells is sufficient to obtain a stable OR and, at the same time, is a less time-consuming method.

The prognostic importance of cyclin B1 was strong and other investigators have previously obtained similar results [125, 127] although we could not perform a direct comparison since the cohort material and the staining methods differed. Suzuki et al demonstrated that only nuclear and not cytoplasmic cyclin B1 was significantly associated with a poorer outcome. Their material included both women with lymph-node-negative and positive disease and the majority was treated with adjuvant chemotherapy. The cut-off value was set at 10%. Rudolph et al and Kuhling et al included in their material women without lymph node metastases, not treated with adjuvant chemotherapy and diagnosed with BC before the introduction of mammographic screening. They used the median value as cut-off [125, 126]. In another study, Aaltonen et al found that cyclin B1 is a prognostic factor for BC death but their material did not comprise patients with sporadic cancer [128].

In paper II, we found that, within the case group there was a difference in time from the primary diagnosis to the occurrence of distant recurrence or death in BC, when comparing patients with low and high cyclin B1 content. These findings are in line with other studies showing that the recurrence of BC is faster in highly rather than slowly proliferating tumors [145-147]. Today, an association between high proliferation and early recurrence is reported in all BC subtypes [146]. Aleskandarany et al have reported that the proliferation assessment using Ki67 and MAI could predict BC death in the luminal subtype but failed to detect differences in survival in triple negative and HER2-enriched [148]. These subtypes have high proliferation, which limits the ability of proliferation factors to detect clinically different subgroups. The time between the occurrence of distant recurrence and the time of death in BC did not differ when comparing low and high cyclin B1. The palliative chemothera-

py given in recurrent BC is more effective in highly proliferating tumors and that could be a possible explanation for this finding [149]. This is also supported from studies in the neoadjuvant setting.

In paper III we found that overexpressions of cyclin A and cyclin B1 could predict poorer BC survival in male BC. The poorer outcome for cyclin A and B1 overexpression was found when the cut-offs corresponding to the subset of tumors with the highest overexpression were used. Furthermore, we could show that a high mitotic count was associated with an increased risk of BC death. Some previous studies have also demonstrated similar results, while others have reported contradictory results [46, 50, 150]. Ki67 was re-evaluated and different cut-offs were used, but we could not detect any prognostic value in this cohort. In accordance with our results in female BC, shown in paper II, IV and V, Ki67 seems to be a weaker prognostic factor than cyclin A and B1. At this point we should mention that a number of patients had been treated with adjuvant and/or palliative chemotherapy treatment which can diminish the prognostic impact of proliferation markers [143]. Contrary to other cyclins, the overexpression of cyclin D1 was an independent predictor of a better BC survival. Cyclin D1 did not correlate to the other cyclins, Ki67 or mitotic count in our material, suggesting that cyclin D1 is involved in tumor biological processes other than proliferation. This is in accordance with the reports in female BC showing that an overexpression of cyclin D1 at the protein level does not correlate to the other cyclins and is more often seen in ER-positive and well-differentiated tumors [129]. In our study, we could demonstrate that cyclin D1 is predictive of better BC survival in male BC, which it has also been shown in previous studies in MBC [50, 151]. Although cyclin D1 participates through out the cell cycle in proliferation, its overexpression is associated with a better outcome and this is something that is not fully understood. In female BC, high cyclin D1 has been associated with tamoxifen resistance[52]. One explanation could be that cyclin D1 has the ability to stimulate ERa in a ligand-independent manner acting as co-activator and cooperating with other ER co-activators. Increased levels of co-activators can enhance tamoxifen's agonist effect and thereby cause tamoxifen resistance [52, 120, 152]. This could be of great importance in MBC since tamoxifen is the standard treatment for ER positive disease. However, adjusting for endocrine treatment did not change the prognostic value of cyclin D1, indicating that cyclin D1 does not influence the response to endocrine treatment in MBC. This could be explained by different ER signaling or reduced functionality of ER receptors supported by IHC and gene expres-

sion profiling analyses [153, 154]. Cyclin D1 gene amplification has been reported in about 15-30% of the female BCs and is also correlated to tamoxifen resistance, even in the absence of protein overexpression [117]. Studies have also shown that mice lacking cyclin D1 did not develop BC despite induced HER2 expression, indicating that presence of cyclin D1 was necessary for tumor growth in lesions expressing HER2 [121]. Not surprisingly the optimal cut-offs for cyclin A and B1 in MBC were not the same as those for female BC since MBC is a unique BC entity different from female BC [49]. We conclude that proliferation is of great importance in MBC but molecular pathways and their cross- talking should be further investigated to help us understand the role of proliferation in MBC.

It has been shown that genes linked to proliferation drive the performance of gene arrays that are mostly applicable in the case of ER-positive patients [70, 71, 155]. The limited availability of gene expression analysis has contributed to the development of surrogate markers to identify BC subgroups using IHC. The Ki67 is the proliferation factor recommended from international guidelines to identify luminal A and B subtypes. In Paper IV we used the IHC panel, suggested from the St Gallen guidelines, consisting of ER, PgR, HER2 to examine whether the $Ki67_{14\%}$, $Ki67_{20\%}$ or the proliferation markers cyclin A and B1 could more clearly separate luminal A from B [75, 111, 112]. Although Ki67 is extensively studied, lack of standardization in staining and scoring remain a debatable issue while the poor to fair reproducibility limits its clinical utility [90, 91]. Cyclin A and B1, have been evaluated and proven to be prognostic factors of BC death, and, in several studies with greater value than Ki67 with predominantly prognostic value ER-positive disease [122, 130]. Using $Ki67_{14\%}$ we found that luminal B was not significantly associated with poorer survival compared with luminal A, which is in line with previous studies [156, 157]. On the other hand, when evaluating the $Ki67_{20\%}$ cyclin A and B1, we found that patients with luminal B tumors showed a poorer survival compared with those with luminal A tumors. Some previous studies have also reported that $Ki67_{20\%}$ could define a high risk ER-positive population likely to benefit from adjuvant chemotherapy [143, 158-160]. However, cyclin A, showed an numerically higher odds ratio, indicating a stronger prognostic value than Ki67 and cyclin B1. This result is in line with other publications and paper V where cyclin A outperformed Ki67, cyclin B1 and PPH3 in multivariate analysis including all proliferation markers [122, 158].

In paper V we examined the prognostic role of PPH3 in the same material as in paper II but with a special focus on ER-positive patients. We found that PPH3 examined in all patients and evaluated both as percentage and as index had a prognostic value for BC death. However, the numerical values of $PPH3_{200}$ and $PPH3_{500}$ were low and thereby even a small number of incorrectly evaluated mitotic figures could negatively influence the grading. Thus in concordance with other studies we recommend the counting of PPH3 index as a more accurate and time saving evaluation method [138, 158].

The primary study was designed as a matched case-control study. In the present investigation, we included all ER-positive patients in an unmatched analysis to gain more power to reveal differences. In line with another publication, we found that in ER-positive patients, the elevated PPH3 index was associated with BC death [158]. In paper V, we furthermore compared the PPH3 index with the proliferation markers Ki67, cyclin A and B1 and found that the prognostic value of cyclin A was stronger, while cyclin B1 performed similar to the PPH3 index. As a further sensitivity analysis we used conditional logistic regression for 77 out of 190 available ER-positive matched pairs and found that the PPH3 index was a strong prognostic factor for BC death in the univariate analyses but not in a multivariate model. Interestingly, cyclin A was the only proliferation factor that retained prognostic significance in the multivariate model, thus outperforming the prognostic value of Ki67, cyclin B1, PPH3 and histological grade.

Some previous studies have demonstrated that PPH3-positive mitotic figures and Oncotype DX were significantly correlated. The authors suggested that PPH3 could provide similar information to the gene test [161]. However, it is reported that inflammatory cells or stromal cells especially when mitotically active can influence the test's result while a pathologist does not take these cells into account when PPH3 and cyclins are evaluated. In paper V, histological grade could predict BC death with an odds ratio higher than that reported from other studies [144]. The good result may depend on the re-evaluation of grade by an experienced pathologist, a conclusion which is in line with reports from other studies when evaluation of histological grade is centrally assessed [162]. The contrast-rich PPH3 staining makes the evaluation less subjective and can be applied even in cases where tumor morphology is not perfectly preserved. Moreover, it facilitates the use of automated image analysis sys-

tems; systems that are still under development but will be less time consuming and highly reproducible for the evaluation of IHC markers [137].

Taking all this into account a number of limitations concerning this thesis need to be addressed. In all papers, the IHC analysis of cyclins and proliferation markers is evaluated on TMA. Hot-spots were chosen and a minimum of 200 cells per tumor and TMA were counted. The heterogeneity of tumors could in that case affect the results. Only a small tissue amount is analyzed and leads to the question of how representative the core is. On the other hand, the TMA technique enables the evaluation of a large number of tumors on a microscopic slide and a previous study has shown that TMA is as good as large sections in the scoring of cyclin A in BC [163]. In paper II, the reproducibility study should also include preanalytical and analytical methods and not only the IHC staining. In paper V the stratification for ER-positive patients was not preplanned and further investigation and validation should be carried out for this subpopulation. Using unconditional regression analysis we break the matching coding but we gain more power. The evaluation of PPH3 by two observers would have contributed to the validation of the reproducibility reported in other studies and thus improve the reliability of the data [164]. It should also be mentioned that women treated with endocrine therapy were included in the study. However, endocrine therapy was not a statistically significant prognostic factor for BC death in the univariate and multivariate analysis.

Our interest lies in finding prognostic factors that many oncologists will have the ability to use in clinical practice. In paper I, we found that cyclin B1 as an easily assessable IHC proliferation marker that can be clinically useful to predict for BC death in a subgroup of low-malignant BC patients with high specificity. These results have then validated in a case-control study with node-negative tumors, not treated with adjuvant chemotherapy as demonstrated in paper II. Cyclin B1 is a strong prognostic factor for BC death both in female and MBC and has good reproducibility. PPH3 is a prognostic factor for BC related death in node-negative BC patients. The prognostic value of PPH3, cyclin A and B1 remains in ER-positive patients, while Ki67 loses its value. However, cyclin A operates as the dominant proliferation factor to predict BC death, outperforms PPH3 and cyclin B1 in the ER-positive patients and may distinguish luminal A from B BC.

Future perspectives

The present thesis highlights the need of further investigation into distinct BC subtypes. Since the innovative publication of Perou and co-workers, it is accepted that BC can be divided into at least four subtypes with different phenotype and molecular profile [106, 110]. It is known that in ER-positive patients the proliferation genes and histologic grade can predict for BC death, while in HER2-positive patients the tumor invasion and immune response are associated with poorer outcomes. In the triple-negative population, a high expression level of the immune response genes is associated with better outcome [165]. The triple negative BC is furthermore associated with BRCA1 mutation [166]. However, prognostic and predictive markers based on proliferation genes have been developed for the ER-positive patients but not for ER-negative and HER2 enriched patients.

A promising field of research would be the investigation of the the hypothesis that different factors and biologic pathways may be involved in determining prognosis and moreover, response, and resistance to therapy in different subgroups of BC. This should enable subdivision of previously large patients groups into smaller in order to better understand BC biology and personalize the treatment.

In this thesis cyclins have been investigated as prognostic factors in BC, while recent studies focus on the inhibition of CDKs as potential therapeutic strategies. Studies in mice have shown that a small-molecule inhibitor of CDK4/6 inhibited the growth of HER2-positive tumors and is currently investigated in clinical trials [121]. Palbociclib is a highly selective inhibitor of CDK4/6 and combination with letrozole has shown promising clinical results in the ER-positive and metastatic BC[167][168].

Taken together, we expect future clinical trials to address the issue of BC heterogeneity by investigating relevant prognostic/predictive factors in different BC subtypes as well as developing new targeted drugs specific for the different BC groups.

Περίληψη

Ο καρκίνος του μαστού είναι η πιο συχνή κακοήθεια στις γυναίκες και παγκοσμίως η πρώτη αίτια θανάτου από καρκίνο στις γυναίκες. Στην Ελλάδα περίπου 4.500 γυναίκες διαγιγνώσκονται με καρκίνο του μαστού ετησίως, ενω 1 στις 12 Ελληνίδες αναμένεται να νοσήσει από καρκίνο του μαστού κάποια στιγμή στη ζωή της ενώ παρατηρείται ανοδική τάση αυτού του ποσοστού. Η θεραπεία του καρκίνου του μαστού μπορεί να αποτελείται απο ένα ή συνδυασμούς των παρακάτω βημάτων ανάλογα με την παρουσία ή απουσία διαφόρων κλινικοπαθολογικών παραγόντων: χειρουργική αφαίρεση τμήματος ή ολόκληρου του μαστού, ακτινοθεραπεία, ενδοκρινική θεραπεία, χημειοθεραπεία και αγωγή με αντισώματα. Στο σημείο αυτό αξίζει να σημειωθεί ότι στην πλειοψηφία τους οι γυναίκες με λεμφαδενικές μεταστάσεις θα πάρουν χημειοθεραπεία. Οι γυναίκες χωρίς λεμφαδενικές μεταστάσεις αναμένεται σε ποσοστό 80-90% να είναι ζωντανές και χωρίς υποτροπή 10 χρόνια μετά την χειρουργική αφαίρεση του όγκου. Ωστόσο οι υπόλοιπες διατρέχουν τον κίνδυνο υποτροπής και θανάτου κι είναι αυτές που πρέπει να αναγνωρίσουμε για να τους χορηγήσουμε συμπληρωματική χημειοθεραπεία μετά το χειρουργείο.

Προγνωστικοί παράγοντες μπορούν ανεξάρτητα από τη θεραπεία να προβλέψουν το κλινικό αποτέλεσμα, τον κίνδυνο υποτροπής και θανάτου από καρκίνο του μαστού. Εδραιωμένοι προγνωστικοί παράγοντες είναι το μέγεθος του όγκου, ο βαθμός κακοήθειας, ο αριθμός διηθημένων λεμαφαδένων, η κατάσταση των ορμονικών υποδοχέων για τα οιστρογόνα (ER) και την προγεστερόνη (PgR) καθώς και η έκφραση του ογκογονιδίου HER2. Ο κυτταρικός πολλαπλασιασμός είναι επίσης παράγοντας πρόγνωσης αλλά έχει κυρίως αξία στην υποομάδα των όγκων που είναι θετικοί για ορμονικούς υποδοχείς.

Ο κυτταρικός πολλαπλασιασμός είναι μια φυσιολογική διαδικασία της κυτταρικής ανάπτυξης. Ο ανεξέλεγκτος κυτταρικός πολλαπλασιασμός

είναι χαρακτηριστικό γνώρισμα του καρκίνου και η αξία του στην πρόγνωση του καρκίνου του μαστού έχει αναφερθεί σε πολλές μελέτες. Ο δείκτης κυτταρικού πολλαπλασιασμού Ki67 έχει μελετηθεί περισσότερο από κάθε άλλον και η υπερέκφραση του σχετίζεται με κακή πρόγνωση.

Σύμπλοκα αποτελούμενα από κυκλίνες και κυκλινο-εξαρτώμενες κινάσες έχουν κυρίαρχο ρολο στη ρύθμιση του κυτταρικού κύκλου. Η κυκλίνη Α είναι σημαντική στη φάση S όπου γίνεται η σύνθεση του DNA και απαραίτητη για τη μετάβαση στην φάση της μίτωσης, όπου το κύτταρο διαιρείται σε δυο ομοια κύτταρα. Η κυκλίνη Β1 φτάνει στο υψηλότερο επίπεδο της στη φάση της μίτωσης όπου και διαδραματίζει σημαντικό ρόλο. Η κυκλίνη D1 είναι παρούσα στη φάση όπου το κύτταρο προετοιμάζεται για τη σύνθεση του DNA και σημαντική για τη μετάβαση στη φάση της μίτωσης. Προηγούμενες μελέτες στον πρώιμο καρκίνο του μαστού έχουν δείξει ότι η υπερέκφραση της κυκλίνης Α και Β1 σχετίζεται με κακή πρόγνωση, ενώ η υπερέκφρασης της κυκλίνης D σχετίζεται με καλή πρόγνωση. Επιπλέον φωσφορυλίωση της πυρηνικής πρωτεΐνης Ιστόνη Η3 (PPH3) είναι απαραίτητη για τη συσπείρωση των χρωμοσωμάτων στη διάρκεια της μίτωσης και προηγούμενες μελέτες έχουν δήξει ότι η PPH3 έχει ισχυρή προγνωστική αξία στο μη μεταστατικό καρκίνο του μαστού.

Την τελευταία δεκαετία οι γονιδιακές αναλύσεις έχουν δείξει την παρουσία τουλάχιστον τέσσερις κατηγορίες καρκίνου του μαστού με διαφορετική πρόγνωση η κάθε μία. Οι κατηγορίες είναι η luminal A, luminal B, HER2 enriched και Basal like. Οι κατηγορίες luminal A και Β χαρακτηρίζονται από έκφραση των γονιδίων σχετικούς με τους ορμονικούς υποδοχείς. Το κύριο διαχωριστικό σημείο μεταξύ luminal A και luminal B είναι γονίδια που καθορίζουν τον κυτταρικό πολλαπλασιασμό. Χαμηλός ρυθμός κυτταρικού πολλαπλασιασμού και καλή πρόγνωση χαρακτηρίζουν την luminal A καθιστώντας δυνατή τη μετεγχειρητική θεραπεία μόνο με ενδοκρινική αγωγή, ενώ η luminal B έχει υψηλό ρυθμό κυτταρικού πολλαπλασιασμού και κακή πρόγνωση, καθιστώντας απαραίτητο το συνδυασμό ενδοκρινικής αγωγής με χημειοθεραπεία. Ωστόσο εξαιτίας οικονομικών κυρίως παραγόντων οι γονιδιακές αναλύσεις δεν είναι διαθέσιμες σε πολλές χώρες και αναπληρωματικές πλατφόρμες με ανοσοιστοχημικούς παράγοντες επιχειρούν το διαχωρισμό του καρκίνου του μαστού με παρόμοιο των γονιδιακών αναλύσεων τρόπο. Οι κατευθυντήριες γραμμές για τη θεραπεία του καρκίνου του μαστού όπως αυτές καθορίζονται από το

διεθνές συνεδριο στο St Gallen και το ESMO συμπεριέλαβαν το 2011 το δείκτη κυτταρικού πολλαπλασιασμού Ki67 για το διαχωρισμό των κατηγοριών luminal A και luminal B.

Στην πρώτη εργασία ο στόχος μας ήταν να ερευνήσουμε αν η κυκλίνη B1 έχει προγνωστική άξια στον μη μεταστατικό και χαμηλού κινδύνου καρκίνο του μαστού. Επιλέξαμε συνειδητά να μελετήσουμε γυναίκες που ανήκαν σε δυο ακραίες ομάδες. Συγκρίναμε γυναίκες που πέθαναν νωρίς εξαιτίας του καρκίνου του μαστού (17) και γυναίκες χωρίς υποτροπή οκτώ χρόνια μετά την αρχική διάγνωση (24). Όλες οι γυναίκες προέρχονταν απο την περιοχή Ουψάλα/Ορεμπρού της Σουηδίας και είχαν διαγνωσθεί με καρκίνο του μαστού απο το 1993/2001. Όλοι οι ασθενείς είχαν όγκους μεγέθους ≤ 2εκ, χωρίς λεμφαδενικές μεταστάσεις, ιστολογικό βαθμό I/II, χαμηλό κυτταρικό πολλαπλασιασμό (SPF) και ήταν θετικοί για υποδοχείς οιστρογόνων και προγεστερόνης. Σε καμιά ασθενή δεν ειχε χορηγηθεί χημειοθεραπεία, αφού τα χαρακτηριστικα των όγκων τους ήταν ευνοϊκά και η πρόγνωση θεωρούνταν πολύ καλή. Χρησιμοποιήσαμε την ανοσοιστοχημική μέθοδο και με μικροσκόπιο εξετάσαμε τα δείγματα ιστού. Το συμπέρασμα μας ήταν οτι τα υψηλά επίπεδα της κυκλινης B1 σε όγκους ασθενών με μη μεταστατικό καρκίνο του μαστού συσχετίζονταν με χειρότερη έκβαση συγκριτικά με ασθενείς που είχαν χαμηλά επίπεδα κυκλίνης B1.

Τα ενθαρρυντικά αποτελέσματα της πρώτης εργασίας μας οδήγησαν στη δεύτερη όπου χρησιμοποιήσαμε μια μελέτη ασθενών-μαρτύρων (case-control) αποτελούμενη από 190 γυναίκες που πέθαναν από καρκίνο του μαστού και ορίστηκαν ως ασθενεις και 190 γυναίκες που ζούσαν κατά το χρόνο θανάτου του αντίστοιχου ασθενή και ορίστηκαν ως μάρτυρες. Τα κριτήρια ένταξης ήταν, μέγεθος του όγκου ≤ 50 mm χωρίς λεμφαδενικές μεταστάσεις, και οχι επικουρική χημειοθεραπεία. Δύο ανεξαρτητοι ερευνητές αξιολόγησαν τη χρώση της κυκλίνης B1 καταμετρώντας 100 , 200 , 500 και 1000 κύτταρα από κάθε όγκο. Τα αποτελέσματα έδειξαν οτι η υπερέκφραση της κυκλίνης B1 σχετίζεται με αυξημενο κίνδυνο για θάνατο από καρκίνο του μαστού. Η συμφωνία μεταξύ των δύο ερευνητων ήταν καλή έως πολύ καλή , ανεξάρτητα από τον αριθμό των κυττάρων που μετρήθηκαν. Από αυτή τη μελέτη καταλήξαμε στο συμπέρασμα ότι η κυκλίνη B1 είναι ένας αρνητικός παράγοντας πρόγνωσης στον καρκίνο του μαστού. Γυναίκες με υψηλά επίπεδα κυκλίνης B1 στους όγκους τους διατρέχουν περίπου 2-3 φορές μεγαλύτερο κίνδυνο για θάνατο από καρκίνο του μαστού σε σχέση με ασθενείς που είχαν χαμηλά επίπεδα.

Ο σκοπός της τρίτης εργασίας ήταν η μελέτη του κυτταρικου πολλαπλασιασμού σε άνδρες με καρκίνο του μαστού. Ο καρκίνος του μαστού στους άνδρες είναι πολύ σπάνιος αλλά μελέτες έχουν δείξει ότι διαφέρει από τον καρκίνο του μαστού στις γυναίκες. Οι κυκλίνες Α, Β1, D1 και ο δείκτης μίτωσης μελετήθηκαν. Συνολικά 197 άνδρες με καρκίνου του μαστού από δυο επαρχίες της Σουηδίας, με προσβάσιμο υλικό και κλινικά δεδομένα ερευνήθηκαν. Οι ασθενείς με υψηλή έκφραση της κυκλίνης Α και Β1 είχαν φτωχότερη επιβίωση . Επιπλέον , η υψηλός δείκτης μίτωσης συσχετίστηκε με αυξημένο κίνδυνο θανάτου από καρκίνο του μαστού. Αντίθετα η υπερέκφραση της κυκλίνης D1 ήταν θετικός παράγοντας πρόγνωσης και συσχετιζόταν με καλύτερη επιβίωση. Συμπερασματικά , τα υψηλά επίπεδα της κυκλίνης Α και Β1 και ο δείκτης μίτωσης συσχετισθηκαν με 2-3 φορές υψηλότερο κίνδυνο για θάνατο από καρκίνο του μαστού. Αντίθετα η υπερέκφραση της κυκλίνης D1 μείωνε τον κίνδυνο.

Ο κυτταρικός πολλαπλασιασμός έχει προγνωστική αξία και κατευθύνει τις θεραπευτικές επιλογές ειδικά στους ασθενείς με θετικούς υποδοχείς οιστρογόνου, δηλαδή τις κατηγορίες luminal Α και Β. Το 2011 οι οδηγίες που στοιχειοθετήθηκαν απο διεθνή συνέδρια, ESMO και St Gallen, πρότειναν τη χρήση ανοσοιστοχημικών δεικτων ER, PgR, HER2 και Ki67 cut-off 14% ($Ki67_{14\%}$) για τον διαχωρισμό της κατηγορίας luminal Α από τη Β. Η τιμή cut-off αντιπροσωπεύει το ποσοστό των κυττάρων που είναι θετικά για την πρωτεΐνη Ki67 και που μπορεί να διαχωρίσει τους όγκους με υψηλό ή χαμηλό κυτταρικό πολλαπλασιασμό. Σκοπός της τέταρτης εργασίας ήταν να αξιολογήσουμε αν κάποια απο τις προτεινόμενες τιμές cut-off για την πρωτεΐνη Ki67, η κυκλίνη Α ή η Β1 θα μπορούσε πιο αξιόπιστα να διαχωρίσει την κατηγορια luminal Α απο τη Β. Στη μελέτη μας, η $Ki67_{14\%}$ απέτυχε να ανιχνεύσει οποιαδήποτε διαφορά στην έκβαση μεταξύ των κατηγοριών luminal Α και Β. Αντίθετα, με τη χρήση της κυκλίνης Α η κατηγορια luminal Β βρέθηκε να έχει σχεδόν 3,5 φορές υψηλότερο κίνδυνο θανάτου από καρκίνο του μαστού. Η κυκλίνη Β1 και η $Ki67_{20\%,}$ μπορούσαν, επίσης, να διαχωρίσουν αυτές τις κατηγορίες, αλλά η κυκλίνη Α ήταν πιο αποτελεσματική κι ακριβής. Έτσι καταλήξαμε στο συμπέρασμα ότι η κυκλίνη Α ευδιάκριτα διαχωρίζει τη κατηγορία luminal Α που έχει καλη πρόγνωση από τη Β που έχει κακή πρόγνωση.

Πρόσφατες μελέτες στον καρκίνο του μαστού υποδεικνύουν ότι η φωσφορυλιωμένη ιστόνη 3 (PPH3) μπορεί να ειναι επίσης παράγοντας πρόγνωσης για το θανάτο από καρκίνο του μαστού. Στόχος μας ήταν να

εξετάσουμε την προγνωστική αξία της PPH3 συγκριτικά και με αλλους γνωστους δείκτες πολλαπλασιασμού οπως Ki67, κυκλίνη Α και Β1 στο ίδιο υλικο ασθενων που χρησιμοποιήσαμε στη δεύτερη και τέταρτη εργασία αλλά με ιδιαίτερη έμφαση στους ασθενείς με θετικους ορμονικούς υποδοχείς (ER), δηλαδή τις κατηγορίες luminal Α και Β. Η PPH3 έδειξε να είναι αρνητικός προγνωστικός παράγοντας για το θάνατο από καρκίνο του μαστού. Στους ER θετικούς ασθενείς η PPH3 καθώς και η κυκλίνη Α και Β 1, αλλά οχι η Κί67 μπορούσε να προβλέψει το θανάτο από καρκίνο του μαστού. Ωστόσο, στην πολυπαραγοντική ανάλυση των δεικτών πολλαπλασιασμού, μόνο η κυκλίνη Α παράμεινε ανεξαρτητος και ισχυρός παράγοντας πρόγνωσης.

Το συμπέρασμά μας είναι ότι ο κυτταρικός πολλαπλασιασμός έχει μεγάλη σημασία στον καρκίνο του μαστού τόσο στις γυναίκες όσο και στους άντρες. Η ιατρική κοινότητα θα πρέπει να καταβάλει προσπάθειες για την ακριβή συμφωνία και υψηλή επαναληψιμότητα στην αξιολόγηση της τρέχουσων και νέων IHC διαγνωστικών δείκτων ώστε τα αποτελεσμάτα να ειναι συγκρίσιμα και να επιτυγχάνεται συμφωνία στην επιλογή της θεραπείας. Τα αποτελέσματα των μελετών μας δείχνουν οτι η κυκλίνη Β1 και η μπορούν να συνεισφέρουν στο διαχωρισμού ασθενών με υψηλού κινδύνου καρκίνο του μαστού, ωστόσο η κυκλίνη Α αποδείχθηκε ακόμα πιο αποτελεσματική και ειδικά στους ασθενείς με θετικούς ορμονικούς υποδοχείς. Η χορήγηση συμπληρωματικής χημειοθεραπείας σε αυτήν την καλής πρόγνωσης κατηγορία ασθενών είναι μια δύσκολη απόφαση εξαιτίας της αδυναμίας μας να αναγνωρίσουμε τους ασθενείς υψηλού κινδύνου. Καταλήγουμε λοιπόν στο συμπεέρασμα ότι η χρήση των δεικτών κυτταρικού πολλαπλασιασμού κυκλίνη Β1 και PPH3 είναι μεγάλης σημασίας στην αναγνώριση ασθενών με πρώιμο καρκίνο του μαστού που διατρέχουν αυξημένο κίνδυνο υποτροπής ή θανάτου. Ακόμη πιο ισχυρή είναι η προγνωστική δύναμη της κυκλίνης Α και ειδικά στους όγκους με θετικούς ορμονικούς υποδοχείς. Η εφαρμογή της στην καθημερινή διαγνωστική προσέγγιση του καρκίνου του μαστού μπορεί συνεισφέρει στην αναγνώριση ασθενών υψηλού κινδύνου στους οποίους η χορήγηση συμπληρωματικής μετεγχειρητικής χημειοθεραπείας θα έχει όφελος.

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References

1. Latest world cancer statistics. Global cancer burden rises to 14.1 million new cases in 2012: Marked increase in breast cancers must be addressed
2. DeSantis, C., et al., *Breast cancer statistics, 2013.* CA Cancer J Clin, 2014. **64**(1): p. 52-62.
3. Youlden, D.R., et al., *The descriptive epidemiology of female breast cancer: an international comparison of screening, incidence, survival and mortality.* Cancer Epidemiol, 2012. **36**(3): p. 237-48.
4. Cancer incidence in Sweden 2011, The Swedish National Board of Health and Welfare, 2012, Available from http://www.socialstyrelsen.se
5. Cancer i siffror 2013. http://www.socialstyrelsen.se
6. Tamimi, R.M., et al., *Traditional breast cancer risk factors in relation to molecular subtypes of breast cancer.* Breast Cancer Res Treat, 2012. **131**(1): p. 159-67.
7. Gaudet, M.M., et al., *Risk factors by molecular subtypes of breast cancer across a population-based study of women 56 years or younger.* Breast Cancer Res Treat, 2011. **130**(2): p. 587-97.
8. Turkoz, F.P., et al., *Association between common risk factors and molecular subtypes in breast cancer patients.* Breast, 2013. **22**(3): p. 344-50.
9. Kuhl, C.K., et al., *Mammography, breast ultrasound, and magnetic resonance imaging for surveillance of women at high familial risk for breast cancer.* J Clin Oncol, 2005. **23**(33): p. 8469-76.
10. Lehtimaki, T., et al., *Long-term prognosis of breast cancer detected by mammography screening or other methods.* Breast Cancer Res, 2011. **13**(6): p. R134.
11. Mook, S., et al., *Independent prognostic value of screen detection in invasive breast cancer.* J Natl Cancer Inst, 2011. **103**(7): p. 585-97.
12. Cancer i siffror. The Swedish national board of Health and Welfare. Available from: http://www.socialstyrelsen.se
13. Tryggvadottir, L., et al., *Trends in the survival of patients diagnosed with breast cancer in the Nordic countries 1964-2003 followed up to the end of 2006.* Acta Oncol, 2010. **49**(5): p. 624-31.

14. Njor, S., et al., *Breast cancer mortality in mammographic screening in Europe: a review of incidence-based mortality studies.* J Med Screen, 2012. **19 Suppl 1**: p. 33-41.
15. Straver, M.E., et al., *Role of axillary clearance after a tumor-positive sentinel node in the administration of adjuvant therapy in early breast cancer.* J Clin Oncol, 2010. **28**(5): p. 731-7.
16. Giuliano, A.E., et al., *Axillary dissection vs no axillary dissection in women with invasive breast cancer and sentinel node metastasis: a randomized clinical trial.* JAMA, 2011. **305**(6): p. 569-75.
17. Galimberti, V., et al., *Positive axillary sentinel lymph node: is axillary dissection always necessary?* Breast, 2011. **20 Suppl 3**: p. S96-8.
18. Darby, S., et al., *Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials.* Lancet, 2011. **378**(9804): p. 1707-16.
19. Schoenfeld, J.D. and J.R. Harris, *Abbreviated course of radiotherapy (RT) for breast cancer.* Breast, 2011. **20 Suppl 3**: p. S116-27.
20. Ishihara, T., et al., *Long-term outcome of hypofractionated radiotherapy to the whole breast of Japanese women after breast-conserving surgery.* Breast Cancer, 2014. **21**(1): p. 40-6.
21. Whelan, T.J., et al., *Long-term results of hypofractionated radiation therapy for breast cancer.* N Engl J Med, 2010. **362**(6): p. 513-20.
22. Ragaz, J., et al., *Locoregional radiation therapy in patients with high-risk breast cancer receiving adjuvant chemotherapy: 20-year results of the British Columbia randomized trial.* J Natl Cancer Inst, 2005. **97**(2): p. 116-26.
23. Overgaard, M., et al., *Postoperative radiotherapy in high-risk premenopausal women with breast cancer who receive adjuvant chemotherapy. Danish Breast Cancer Cooperative Group 82b Trial.* N Engl J Med, 1997. **337**(14): p. 949-55.
24. Davies, C., et al., *Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials.* Lancet, 2011. **378**(9793): p. 771-84.
25. Sainsbury, R., *The development of endocrine therapy for women with breast cancer.* Cancer Treat Rev, 2013. **39**(5): p. 507-17.
26. Davies, C., et al., *Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial.* Lancet, 2013. **381**(9869): p. 805-16.
27. Goss, P.E., et al., *Impact of premenopausal status at breast cancer diagnosis in women entered on the placebo-controlled NCIC CTG MA17 trial of extended adjuvant letrozole.* Ann Oncol, 2013. **24**(2): p. 355-61.
28. O Metzger, A Giobbie-Hurder, E Mallon, G Viale, E Winer, B Thürlimann, RD Gelber, M Colleoni, B Ejlertsen, H Bonnefoi, AS Coates, A Goldhirsch, B Gusterson, BIG 1-98 Collaborative Group, and International Breast Cancer Study Group. Relative effectiveness of

letrozole compared with tamoxifen for patients with lobular carcinoma in the BIG 1-98 trial. Cancer Research: December 15, 2012; Volume 72, Issue 24, Supplement 3 doi: 10.1158/0008-5472.SABCS12-S1-1

29. Bonadonna, G., et al., *Adjuvant cyclophosphamide, methotrexate, and fluorouracil in node-positive breast cancer: the results of 20 years of follow-up.* N Engl J Med, 1995. **332**(14): p. 901-6.
30. *Polychemotherapy for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group.* Lancet, 1998. **352**(9132): p. 930-42.
31. *Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials.* Lancet, 2005. **365**(9472): p. 1687-717.
32. Qin, Y.Y., et al., *Adjuvant chemotherapy, with or without taxanes, in early or operable breast cancer: a meta-analysis of 19 randomized trials with 30698 patients.* PLoS One, 2011. **6**(11): p. e26946.
33. Romond, E.H., et al., *Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer.* N Engl J Med, 2005. **353**(16): p. 1673-84.
34. Perez, E.A., et al., *Sequential versus concurrent trastuzumab in adjuvant chemotherapy for breast cancer.* J Clin Oncol, 2011. **29**(34): p. 4491-7.
35. Perez, E.A., et al., *Four-year follow-up of trastuzumab plus adjuvant chemotherapy for operable human epidermal growth factor receptor 2-positive breast cancer: joint analysis of data from NCCTG N9831 and NSABP B-31.* J Clin Oncol, 2011. **29**(25): p. 3366-73.
36. Goldhirsch, A., et al., *2 years versus 1 year of adjuvant trastuzumab for HER2-positive breast cancer (HERA): an open-label, randomised controlled trial.* Lancet, 2013. **382**(9897): p. 1021-8.
37. Pivot, X., et al., *6 months versus 12 months of adjuvant trastuzumab for patients with HER2-positive early breast cancer (PHARE): a randomised phase 3 trial.* Lancet Oncol, 2013. **14**(8): p. 741-8.
38. Nielsen, D.L., et al., *Efficacy of HER2-targeted therapy in metastatic breast cancer. Monoclonal antibodies and tyrosine kinase inhibitors.* Breast, 2013. **22**(1): p. 1-12.
39. Gianni, L., et al., *Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial.* Lancet Oncol, 2012. **13**(1): p. 25-32.
40. Beaver, J.A. and B.H. Park, *The BOLERO-2 trial: the addition of everolimus to exemestane in the treatment of postmenopausal hormone receptor-positive advanced breast cancer.* Future Oncol, 2012. **8**(6): p. 651-7.
41. Cancer Research: December 15, 2012; Volume 72, Issue 24, Supplement 3 doi: 10.1158/0008-5472.SABCS12-OT2-2-04 Abstracts: Thirty-Fifth Annual CTRC-AACR San Antonio Breast Cancer

Symposium-- Dec 4-8, 2012; San Antonio, TX © 2012 American Association for Cancer Research
42. Fentiman, I.S., A. Fourquet, and G.N. Hortobagyi, *Male breast cancer.* Lancet, 2006. **367**(9510): p. 595-604.
43. NORDCAN.Cancer Incidence, Mortality, Prevalence and Prediction in the Nordic Countries http://www.ancr.nu 2009
44. Brinton, L.A., et al., *Etiologic factors for male breast cancer in the U.S. Veterans Affairs medical care system database.* Breast Cancer Res Treat, 2010. **119**(1): p. 185-92.
45. Joshi, M.G., et al., *Male breast carcinoma: an evaluation of prognostic factors contributing to a poorer outcome.* Cancer, 1996. **77**(3): p. 490-8.
46. Cutuli, B., et al., *Male breast cancer. Evolution of treatment and prognostic factors. Analysis of 489 cases.* Crit Rev Oncol Hematol, 2010. **73**(3): p. 246-54.
47. Czene, K., et al., *How to treat male breast cancer.* Breast, 2007. **16 Suppl 2**: p. S147-54.
48. Marchal, F., et al., *Men with breast cancer have same disease-specific and event-free survival as women.* Ann Surg Oncol, 2009. **16**(4): p. 972-8.
49. Nilsson, C., et al., *Similarities and differences in the characteristics and primary treatment of breast cancer in men and women - a population based study (Sweden).* Acta Oncol, 2011. **50**(7): p. 1083-8.
50. Rayson, D., et al., *Molecular markers in male breast carcinoma.* Cancer, 1998. **83**(9): p. 1947-55.
51. Wang-Rodriguez, J., et al., *Male breast carcinoma: correlation of ER, PR, Ki-67, Her2-Neu, and p53 with treatment and survival, a study of 65 cases.* Mod Pathol, 2002. **15**(8): p. 853-61.
52. Stendahl, M., et al., *Cyclin D1 overexpression is a negative predictive factor for tamoxifen response in postmenopausal breast cancer patients.* Br J Cancer, 2004. **90**(10): p. 1942-8.
53. Bloom, H.J. and W.W. Richardson, *Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years.* Br J Cancer, 1957. **11**(3): p. 359-77.
54. Elston, C.W. and I.O. Ellis, *Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up.* Histopathology, 1991. **19**(5): p. 403-10.
55. Boiesen, P., et al., *Histologic grading in breast cancer--reproducibility between seven pathologic departments. South Sweden Breast Cancer Group.* Acta Oncol, 2000. **39**(1): p. 41-5.
56. Dalton, L.W., D.L. Page, and W.D. Dupont, *Histologic grading of breast carcinoma. A reproducibility study.* Cancer, 1994. **73**(11): p. 2765-70.
57. Frkovic-Grazio, S. and M. Bracko, *Long term prognostic value of Nottingham histological grade and its components in early (pT1N0M0) breast carcinoma.* J Clin Pathol, 2002. **55**(2): p. 88-92.

58. Joensuu, H., L. Pylkkanen, and S. Toikkanen, *Late mortality from pT1N0M0 breast carcinoma.* Cancer, 1999. **85**(10): p. 2183-9.
59. Volpi, A., et al., *Prognostic relevance of histological grade and its components in node-negative breast cancer patients.* Mod Pathol, 2004. **17**(9): p. 1038-44.
60. Meyer, J.S., et al., *Breast carcinoma malignancy grading by Bloom-Richardson system vs proliferation index: reproducibility of grade and advantages of proliferation index.* Mod Pathol, 2005. **18**(8): p. 1067-78.
61. Fisher, B., et al., *Breast cancer estrogen and progesterone receptor values: their distribution, degree of concordance, and relation to number of positive axillary nodes.* J Clin Oncol, 1983. **1**(6): p. 349-58.
62. de Boer, M., et al., *Breast cancer prognosis and occult lymph node metastases, isolated tumor cells, and micrometastases.* J Natl Cancer Inst, 2010. **102**(6): p. 410-25.
63. Grabau, D., *Breast cancer patients with micrometastases only: is a basis provided for tailored treatment?* Surg Oncol, 2008. **17**(3): p. 211-7.
64. Warwick, J., et al., *Time-dependent effects on survival in breast carcinoma: results of 20 years of follow-up from the Swedish Two-County Study.* Cancer, 2004. **100**(7): p. 1331-6.
65. Arriagada, R., et al., *Twenty-five years of follow-up in patients with operable breast carcinoma: correlation between clinicopathologic factors and the risk of death in each 5-year period.* Cancer, 2006. **106**(4): p. 743-50.
66. Cianfrocca, M. and L.J. Goldstein, *Prognostic and predictive factors in early-stage breast cancer.* Oncologist, 2004. **9**(6): p. 606-16.
67. Haybittle, J.L., et al., *A prognostic index in primary breast cancer.* Br J Cancer, 1982. **45**(3): p. 361-6.
68. Sundquist, M., et al., *Applying the Nottingham Prognostic Index to a Swedish breast cancer population. South East Swedish Breast Cancer Study Group.* Breast Cancer Res Treat, 1999. **53**(1): p. 1-8.
69. Balslev, I., et al., *The Nottingham Prognostic Index applied to 9,149 patients from the studies of the Danish Breast Cancer Cooperative Group (DBCG).* Breast Cancer Res Treat, 1994. **32**(3): p. 281-90.
70. Sotiriou, C., et al., *Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis.* J Natl Cancer Inst, 2006. **98**(4): p. 262-72.
71. Wirapati, P., et al., *Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures.* Breast Cancer Res, 2008. **10**(4): p. R65.
72. Ferno, M., et al., *Results of two or five years of adjuvant tamoxifen correlated to steroid receptor and S-phase levels. South Sweden Breast Cancer Group, and South-East Sweden Breast Cancer Group.* Breast Cancer Res Treat, 2000. **59**(1): p. 69-76.
73. Dowsett, M., et al., *Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen.* J Clin Oncol, 2010. **28**(3): p. 509-18.

74. Anderson, W.F., et al., *Estrogen receptor breast cancer phenotypes in the Surveillance, Epidemiology, and End Results database.* Breast Cancer Res Treat, 2002. **76**(1): p. 27-36.
75. Goldhirsch, A., et al., *Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013.* Ann Oncol, 2013. **24**(9): p. 2206-23.
76. Aebi, S., et al., *Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol, 2011. **22 Suppl 6**: p. vi12-24.
77. Early Breast Cancer Trialists' Collaborative, G., et al., *Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials.* Lancet, 2011. **378**(9793): p. 771-84.
78. Yi, M., et al., *Which threshold for ER positivity? A retrospective study based on 9639 patients.* Ann Oncol, 2014.
79. Conneely, O.M., B.M. Jericevic, and J.P. Lydon, *Progesterone receptors in mammary gland development and tumorigenesis.* J Mammary Gland Biol Neoplasia, 2003. **8**(2): p. 205-14.
80. Prat, A., et al., *Prognostic significance of progesterone receptor-positive tumor cells within immunohistochemically defined luminal A breast cancer.* J Clin Oncol, 2013. **31**(2): p. 203-9.
81. Klintman, M., et al., *A prospective, multicenter validation study of a prognostic index composed of S-phase fraction, progesterone receptor status, and tumour size predicts survival in node-negative breast cancer patients: NNBC, the node-negative breast cancer trial.* Ann Oncol, 2013. **24**(9): p. 2284-91.
82. Owens, M.A., B.C. Horten, and M.M. Da Silva, *HER2 amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues.* Clin Breast Cancer, 2004. **5**(1): p. 63-9.
83. Stuart-Harris, R., et al., *Proliferation markers and survival in early breast cancer: a systematic review and meta-analysis of 85 studies in 32,825 patients.* Breast, 2008. **17**(4): p. 323-34.
84. Baak, J.P., et al., *Proliferation accurately identifies the high-risk patients among small, low-grade, lymph node-negative invasive breast cancers.* Ann Oncol, 2008. **19**(4): p. 649-54.
85. Kontzoglou, K., et al., *Correlation between Ki67 and breast cancer prognosis.* Oncology, 2013. **84**(4): p. 219-25.
86. Colozza, M., et al., *Proliferative markers as prognostic and predictive tools in early breast cancer: where are we now?* Ann Oncol, 2005. **16**(11): p. 1723-39.
87. Yerushalmi, R., et al., *Ki67 in breast cancer: prognostic and predictive potential.* Lancet Oncol, 2010. **11**(2): p. 174-83.
88. Brown, D.C. and K.C. Gatter, *Ki67 protein: the immaculate deception?* Histopathology, 2002. **40**(1): p. 2-11.

89. de Azambuja, E., et al., *Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12,155 patients.* Br J Cancer, 2007. **96**(10): p. 1504-13.
90. Luporsi, E., et al., *Ki-67: level of evidence and methodological considerations for its role in the clinical management of breast cancer: analytical and critical review.* Breast Cancer Res Treat, 2012. **132**(3): p. 895-915.
91. Dowsett, M., et al., *Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group.* J Natl Cancer Inst, 2011. **103**(22): p. 1656-64.
92. Spyratos, F., et al., *Correlation between MIB-1 and other proliferation markers: clinical implications of the MIB-1 cutoff value.* Cancer, 2002. **94**(8): p. 2151-9.
93. Urruticoechea, A., I.E. Smith, and M. Dowsett, *Proliferation marker Ki-67 in early breast cancer.* J Clin Oncol, 2005. **23**(28): p. 7212-20.
94. Penault-Llorca, F., et al., *Ki67 expression and docetaxel efficacy in patients with estrogen receptor-positive breast cancer.* J Clin Oncol, 2009. **27**(17): p. 2809-15.
95. Viale, G., et al., *Predictive value of tumor Ki-67 expression in two randomized trials of adjuvant chemoendocrine therapy for node-negative breast cancer.* J Natl Cancer Inst, 2008. **100**(3): p. 207-12.
96. Criscitiello, C., et al., *High Ki-67 score is indicative of a greater benefit from adjuvant chemotherapy when added to endocrine therapy in Luminal B HER2 negative and node-positive breast cancer.* Breast, 2014. **23**(1): p. 69-75.
97. Assersohn, L., et al., *Studies of the potential utility of Ki67 as a predictive molecular marker of clinical response in primary breast cancer.* Breast Cancer Res Treat, 2003. **82**(2): p. 113-23.
98. Ellis, M.J., et al., *Outcome prediction for estrogen receptor-positive breast cancer based on postneoadjuvant endocrine therapy tumor characteristics.* J Natl Cancer Inst, 2008. **100**(19): p. 1380-8.
99. Mengel, M., et al., *Inter-laboratory and inter-observer reproducibility of immunohistochemical assessment of the Ki-67 labelling index in a large multi-centre trial.* J Pathol, 2002. **198**(3): p. 292-9.
100. Meyer, J.S. and R.W. McDivitt, *Reliability and stability of the thymidine labeling index of breast carcinoma.* Lab Invest, 1986. **54**(2): p. 160-4.
101. Duigou, F., et al., *Variation of flow cytometric DNA measurement in 1,485 primary breast carcinomas according to guidelines for DNA histogram interpretation.* Cytometry, 2000. **42**(1): p. 35-42.
102. Moriki, T., et al., *Proliferation marker MIB-1 correlates well with proliferative activity evaluated by BrdU in breast cancer: an immunohistochemical study including correlation with PCNA, p53, c-erbB-2 and estrogen receptor status.* Pathol Int, 1996. **46**(12): p. 953-61.
103. Malmstrom, P., et al., *S-phase fraction and urokinase plasminogen activator are better markers for distant recurrences than Nottingham*

Prognostic Index and histologic grade in a prospective study of premenopausal lymph node-negative breast cancer. J Clin Oncol, 2001. **19**(7): p. 2010-9.
104. Beresford, M.J., G.D. Wilson, and A. Makris, *Measuring proliferation in breast cancer: practicalities and applications.* Breast Cancer Res, 2006. **8**(6): p. 216.
105. Stal, O., et al., *S-phase fraction is a prognostic factor in stage I breast carcinoma.* J Clin Oncol, 1993. **11**(9): p. 1717-22.
106. Sorlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10869-74.
107. Sorlie, T., et al., *Repeated observation of breast tumor subtypes in independent gene expression data sets.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8418-23.
108. Sotiriou, C., et al., *Breast cancer classification and prognosis based on gene expression profiles from a population-based study.* Proc Natl Acad Sci U S A, 2003. **100**(18): p. 10393-8.
109. van 't Veer, L.J., et al., *Gene expression profiling predicts clinical outcome of breast cancer.* Nature, 2002. **415**(6871): p. 530-6.
110. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
111. Cheang, M.C., et al., *Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer.* J Natl Cancer Inst, 2009. **101**(10): p. 736-50.
112. Goldhirsch, A., et al., *Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011.* Ann Oncol, 2011. **22**(8): p. 1736-47.
113. Rouzier, R., et al., *Breast cancer molecular subtypes respond differently to preoperative chemotherapy.* Clin Cancer Res, 2005. **11**(16): p. 5678-85.
114. Gluck, S., et al., *Molecular subtyping of early-stage breast cancer identifies a group of patients who do not benefit from neoadjuvant chemotherapy.* Breast Cancer Res Treat, 2013. **139**(3): p. 759-67.
115. Sherr, C.J. and J.M. Roberts, *Inhibitors of mammalian G1 cyclin-dependent kinases.* Genes Dev, 1995. **9**(10): p. 1149-63.
116. Casimiro, M.C., et al., *Overview of cyclins D1 function in cancer and the CDK inhibitor landscape: past and present.* Expert Opin Investig Drugs, 2014. **23**(3): p. 295-304.
117. Jirstrom, K., et al., *Adverse effect of adjuvant tamoxifen in premenopausal breast cancer with cyclin D1 gene amplification.* Cancer Res, 2005. **65**(17): p. 8009-16.
118. Hwang, T.S., et al., *Prognostic value of combined analysis of cyclin D1 and estrogen receptor status in breast cancer patients.* Pathol Int, 2003. **53**(2): p. 74-80.

119. Aaltonen, K., et al., *Cyclin D1 expression is associated with poor prognostic features in estrogen receptor positive breast cancer.* Breast Cancer Res Treat, 2009. **113**(1): p. 75-82.
120. Ahnstrom, M., et al., *Role of cyclin D1 in ErbB2-positive breast cancer and tamoxifen resistance.* Breast Cancer Res Treat, 2005. **91**(2): p. 145-51.
121. Choi, Y.J., et al., *The requirement for cyclin D function in tumor maintenance.* Cancer Cell, 2012. **22**(4): p. 438-51.
122. Ahlin, C., et al., *Cyclin A is a proliferative marker with good prognostic value in node-negative breast cancer.* Cancer Epidemiol Biomarkers Prev, 2009. **18**(9): p. 2501-6.
123. Michalides, R., et al., *Cyclin A is a prognostic indicator in early stage breast cancer with and without tamoxifen treatment.* Br J Cancer, 2002. **86**(3): p. 402-8.
124. Bukholm, I.R., G. Bukholm, and J.M. Nesland, *Over-expression of cyclin A is highly associated with early relapse and reduced survival in patients with primary breast carcinomas.* Int J Cancer, 2001. **93**(2): p. 283-7.
125. Kuhling, H., et al., *Expression of cyclins E, A, and B, and prognosis in lymph node-negative breast cancer.* J Pathol, 2003. **199**(4): p. 424-31.
126. Rudolph, P., et al., *Differential prognostic impact of the cyclins E and B in premenopausal and postmenopausal women with lymph node-negative breast cancer.* Int J Cancer, 2003. **105**(5): p. 674-80.
127. Suzuki, T., et al., *Nuclear cyclin B1 in human breast carcinoma as a potent prognostic factor.* Cancer Sci, 2007. **98**(5): p. 644-51.
128. Aaltonen, K., et al., *High cyclin B1 expression is associated with poor survival in breast cancer.* Br J Cancer, 2009. **100**(7): p. 1055-60.
129. Bostrom, P., et al., *Analysis of cyclins A, B1, D1 and E in breast cancer in relation to tumour grade and other prognostic factors.* BMC Res Notes, 2009. **2**: p. 140.
130. Agarwal, R., et al., *Integrative analysis of cyclin protein levels identifies cyclin b1 as a classifier and predictor of outcomes in breast cancer.* Clin Cancer Res, 2009. **15**(11): p. 3654-62.
131. Davies, T.G., et al., *Structure-based design of a potent purine-based cyclin-dependent kinase inhibitor.* Nat Struct Biol, 2002. **9**(10): p. 745-9.
132. Yuan, J., et al., *Cyclin B1 depletion inhibits proliferation and induces apoptosis in human tumor cells.* Oncogene, 2004. **23**(34): p. 5843-52.
133. Goto, H., et al., *Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation.* J Biol Chem, 1999. **274**(36): p. 25543-9.
134. Li, D.W., et al., *Dynamic distribution of Ser-10 phosphorylated histone H3 in cytoplasm of MCF-7 and CHO cells during mitosis.* Cell Res, 2005. **15**(2): p. 120-6.
135. Song, L., et al., *Ser-10 phosphorylated histone H3 is involved in cytokinesis as a chromosomal passenger.* Cell Biol Int, 2007. **31**(10): p. 1184-90.

136. Bush, T.L., et al., *AMG 900, a small-molecule inhibitor of aurora kinases, potentiates the activity of microtubule-targeting agents in human metastatic breast cancer models.* Mol Cancer Ther, 2013. **12**(11): p. 2356-66.
137. Bossard, C., et al., *Phosphohistone H3 labelling for histoprognostic grading of breast adenocarcinomas and computer-assisted determination of mitotic index.* J Clin Pathol, 2006. **59**(7): p. 706-10.
138. Skaland, I., et al., *Phosphohistone H3 expression has much stronger prognostic value than classical prognosticators in invasive lymph node-negative breast cancer patients less than 55 years of age.* Mod Pathol, 2007. **20**(12): p. 1307-15.
139. Baak, J.P., et al., *Proliferation is the strongest prognosticator in node-negative breast cancer: significance, error sources, alternatives and comparison with molecular prognostic markers.* Breast Cancer Res Treat, 2009. **115**(2): p. 241-54.
140. Altman D. Practical Statistics for Medical Research. London, UK: Chapman & Hall; 1991
141. Thomassen, M., et al., *Prediction of metastasis from low-malignant breast cancer by gene expression profiling.* Int J Cancer, 2007. **120**(5): p. 1070-5.
142. Sjostrom, J., *Predictive factors for response to chemotherapy in advanced breast cancer.* Acta Oncol, 2002. **41**(4): p. 334-45.
143. Ahlin, C., et al., *Ki67 and cyclin A as prognostic factors in early breast cancer. What are the optimal cut-off values?* Histopathology, 2007. **51**(4): p. 491-8.
144. Rakha, E.A., et al., *Prognostic significance of Nottingham histologic grade in invasive breast carcinoma.* J Clin Oncol, 2008. **26**(19): p. 3153-8.
145. Hilsenbeck, S.G., et al., *Time-dependence of hazard ratios for prognostic factors in primary breast cancer.* Breast Cancer Res Treat, 1998. **52**(1-3): p. 227-37.
146. Ribelles, N., et al., *Pattern of recurrence of early breast cancer is different according to intrinsic subtype and proliferation index.* Breast Cancer Res, 2013. **15**(5): p. R98.
147. Bianchini, G., et al., *Proliferation and estrogen signaling can distinguish patients at risk for early versus late relapse among estrogen receptor positive breast cancers.* Breast Cancer Res, 2013. **15**(5): p. R86.
148. Aleskandarany, M.A., et al., *Prognostic value of proliferation assay in the luminal, HER2-positive, and triple-negative biologic classes of breast cancer.* Breast Cancer Res, 2012. **14**(1): p. R3.
149. Poikonen, P., et al., *Cyclin A as a marker for prognosis and chemotherapy response in advanced breast cancer.* Br J Cancer, 2005. **93**(5): p. 515-9.

150. Pich, A., E. Margaria, and L. Chiusa, *Proliferative activity is a significant prognostic factor in male breast carcinoma.* Am J Pathol, 1994. **145**(2): p. 481-9.
151. Kanthan, R., et al., *Expression of cell cycle proteins in male breast carcinoma.* World J Surg Oncol, 2010. **8**: p. 10.
152. Lamb, J., et al., *Regulation of the functional interaction between cyclin D1 and the estrogen receptor.* Mol Cell Biol, 2000. **20**(23): p. 8667-75.
153. Weber-Chappuis, K., S. Bieri-Burger, and J. Hurlimann, *Comparison of prognostic markers detected by immunohistochemistry in male and female breast carcinomas.* Eur J Cancer, 1996. **32A**(10): p. 1686-92.
154. Johansson, I., et al., *Gene expression profiling of primary male breast cancers reveals two unique subgroups and identifies N-acetyltransferase-1 (NAT1) as a novel prognostic biomarker.* Breast Cancer Res, 2012. **14**(1): p. R31.
155. Paik, S., et al., *A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer.* N Engl J Med, 2004. **351**(27): p. 2817-26.
156. Braun, L., et al., *Intrinsic breast cancer subtypes defined by estrogen receptor signalling-prognostic relevance of progesterone receptor loss.* Mod Pathol, 2013. **26**(9): p. 1161-71.
157. Ferguson, N.L., et al., *Prognostic value of breast cancer subtypes, Ki-67 proliferation index, age, and pathologic tumor characteristics on breast cancer survival in Caucasian women.* Breast J, 2013. **19**(1): p. 22-30.
158. Klintman, M., et al., *The Prognostic Value of Mitotic Activity Index (MAI), Phosphohistone H3 (PPH3), Cyclin B1, Cyclin A, and Ki67, Alone and in Combinations, in Node-Negative Premenopausal Breast Cancer.* PLoS One, 2013. **8**(12): p. e81902.
159. Klintman, M., et al., *The prognostic value of Ki67 is dependent on estrogen receptor status and histological grade in premenopausal patients with node-negative breast cancer.* Mod Pathol, 2010. **23**(2): p. 251-9.
160. Nishimura, R., et al., *Ki-67 as a prognostic marker according to breast cancer subtype and a predictor of recurrence time in primary breast cancer.* Exp Ther Med, 2010. **1**(5): p. 747-754.
161. Williams, D.J., et al., *Proliferation (Ki-67 and phosphohistone H3) and oncotype DX recurrence score in estrogen receptor-positive breast cancer.* Appl Immunohistochem Mol Morphol, 2011. **19**(5): p. 431-6.
162. Postma, E.L., et al., *Discrepancy between routine and expert pathologists' assessment of non-palpable breast cancer and its impact on locoregional and systemic treatment.* Eur J Pharmacol, 2013. **717**(1-3): p. 31-5.
163. Aaltonen, K., et al., *Reliability of cyclin A assessment on tissue microarrays in breast cancer compared to conventional histological slides.* Br J Cancer, 2006. **94**(11): p. 1697-702.
164. Skaland, I., et al., *Validating the prognostic value of proliferation measured by Phosphohistone H3 (PPH3) in invasive lymph node-*

negative breast cancer patients less than 71 years of age. Breast Cancer Res Treat, 2009. **114**(1): p. 39-45.
165. Desmedt, C., et al., *Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes.* Clin Cancer Res, 2008. **14**(16): p. 5158-65.
166. Foulkes, W.D., et al., *Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer.* J Natl Cancer Inst, 2003. **95**(19): p. 1482-5.
167. Finn RS, Crown JP, Boer K, et al. Results of a randomized phase 2 study of PD 0332991, a cyclin-dependent kinase (CDK) 4/6 inhibitor, in combination with letrozole vs letrozole alone for first- line treatment of ER+/HER2- advanced breast cancer (BC) [abstract 100O]. Ann Oncol 2012;23:ii43-5
168. Finn RS, Dieras V, Gelmon KA, et al. A randomized, multicenter, double-blind phase III study of palbociclib (PD- 0332991), an oral CDK 4/6 inhibitor, plus letrozole versus placebo plus letrozole for the treatment of postmenopausal women with ER(+), HER2(-) breast cancer who have not received any prior systemic anticancer treatment for advanced disease. J Clin Oncol 2013;31s:abstract TPS652

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