Cyclin A and cyclin E as prognostic factors in early breast cancer

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Abstract

Breast cancer is one of the most common malignancies in women. Due to early detection and the use of screening programs approximately 60% of all new cases lack lymph node involvement. Today, a substantial proportion of these women will be offered adjuvant systemic chemotherapy. However, better proliferation markers are needed to predict patient outcome and to avoid overtreatment.

Cyclin A, cyclin E and Ki-67 are all markers for proliferation and involved in the regulation of the cell cycle. Overexpression has been associated with disease recurrence in several studies, but the results have not been consistent. However, none of these studies has investigated aberrant expression of cyclin E (the expression of cyclin E during phases of the cell cycle other than late G1 and early S-phase). Studies have shown that aberrant cyclin E might provide additional prognostic information compared to cyclin E alone.

The aims of this thesis were 1.to investigate the prognostic value of cyclin A, cyclin E and aberrant cyclin E in early breast cancer. 2.to validate the tissue microarray (TMA) technique for cyclin A and 3.to define the most optimal cut-off values for cyclin A and Ki-67.

We found that the agreement of TMA and large section results was good with kappa values 0.62-0.75 and that the reproducibility of the two readers’ results was good or even very good, with kappa values 0.71 – 0.87.

The optimal cut-off value for cyclin A average was 8% and for cyclin A maximum value 11%. The corresponding values for Ki-67 were 15 and 22%.

Neither cyclin E nor aberrant cyclin E was a prognostic factor in low-risk node negative breast cancer patients.

Finally, we conclude that cyclin A is a prognostic factor in node negative breast cancer (univariate analysis average value OR=2.9 95% CI 1.8-4.6; maximum value OR=3.7 95% CI 2.3-5.9).

Keywords: breast cancer, chemotherapy, cyclin A, cyclin E, aberrant cyclin E, Ki-67, TMA

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LIST OF PAPERS

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


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEC</td>
<td>Amino-ethyl-carbazole</td>
</tr>
<tr>
<td>BCSS</td>
<td>Breast cancer specific survival</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin dependant kinase</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CIP</td>
<td>Cdk interacting protein</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin dependent kinase inhibitor</td>
</tr>
<tr>
<td>CMF</td>
<td>Cyclophosphamide, methotrexate, 5-fluorouracil</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>2-4-amidinophenyl-6-indolecarbamidine dihydrochloride</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease-free survival</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E2F</td>
<td>E2 promotor binding factor</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>FAC</td>
<td>5-fluorouracil, adriamycin, cyclophosphamide</td>
</tr>
<tr>
<td>FEC</td>
<td>5-fluorouracil, epirubicin, cyclophosphamide</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>Her2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>INK4</td>
<td>Inhibitor of cdk4</td>
</tr>
<tr>
<td>KIP</td>
<td>Cdk inhibitory protein</td>
</tr>
<tr>
<td>LMW</td>
<td>Low-molecular-weight</td>
</tr>
<tr>
<td>MFS</td>
<td>Metastasis free survival</td>
</tr>
<tr>
<td>NHG</td>
<td>Nottingham histological grade</td>
</tr>
<tr>
<td>NNT</td>
<td>Number needed to treat</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PgR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>pRb</td>
<td>The retinoblastoma gene product</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROC</td>
<td>Regionalt Onkologiskt Centrum</td>
</tr>
<tr>
<td>RR</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>SPF</td>
<td>S-phase fraction</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour size, node, metastasis</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
</tbody>
</table>
INTRODUCTION

Breast cancer is the most common cancer among women in the Western world today. Extensive research during recent decades has focused on tumour growth, invasion and metastasis as well as the development of effective and safe treatments against breast cancer. Despite this, approximately 1500 patients die from breast cancer every year in Sweden.

One major question that challenges oncologists around the world on daily basis is how to select patients that are at high risk for recurrence after breast cancer surgery and that will benefit from adjuvant chemotherapy. One way to tackle this issue is to find better prognostic markers in the attempt to avoid relapse and overtreatment.

This thesis is focused on studies of cyclin A, cyclin E and aberrant cyclin E as prognostic factors in early breast cancer as well as validation of the tissue microarray (TMA) technique and the definition of the most optimal cut-off point for cyclin A and Ki-67.

Epidemiology

Worldwide, breast cancer is the most common cancer among women and the second leading cause of cancer deaths. In 2002, 1.15 million new breast cancers were diagnosed (1). The incidence varies between countries being the highest in industrialized countries such as the United States, western and northern Europe, Australia and New Zealand. One reasonable explanation for these rates is probably the introduction of screening programs that detect small cancers, some of which would otherwise have been diagnosed later or not at all. Age-standardized incidence and mortality rates for breast cancer are shown in Figure 1.
In 2006, there were 429 900 cases (29% of total) of breast cancer diagnosed and 131 900 (8%) deaths in Europe (2). Several studies in the 1990s reported a fall in breast cancer mortality due to earlier detection and better adjuvant treatment (3-4). These improvements were mainly seen in younger women. As a result of an ageing population, the breast cancer rate is beginning to rise anew (130 000 in 2004, 132 000 in 2006) (2).

In Sweden the incidence is more than 7000 per year, which is 29% of all cancers among females. The annual increase over the last 20 years has been 1.3 %. The disease is unusual before the age of 40, but increases rapidly thereafter (5).
Risk factors

About 5-10% of breast cancer is thought to be linked to changes (mutations) in certain genes. The most common are those of the BRCA 1 and BRCA 2 genes. Women with mutations in BRCA 1 or BRCA 2 have a high risk of developing breast cancer, ovarian cancer, and several other types of cancer during their lifetimes (6). However, most cases of breast cancer occur “by chance”. The causes are still unknown, but there is probably a combination of factors including lifestyle factors, environmental factors and hormone factors. A list of established and probable risk factors for breast cancer are shown in table 1.

Table 1. Established and probable risk factors for breast cancer. Table from McPherson et al. BMJ 2000 (6).

<table>
<thead>
<tr>
<th>Factor</th>
<th>RR</th>
<th>High risk group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>&gt;10</td>
<td>Elderly</td>
</tr>
<tr>
<td>Geographical location</td>
<td>5</td>
<td>Developed country</td>
</tr>
<tr>
<td>Age at menarche</td>
<td>3</td>
<td>Menarche before age 11</td>
</tr>
<tr>
<td>Age at menopause</td>
<td>2</td>
<td>Menopause after age 54</td>
</tr>
<tr>
<td>Age at first full pregnancy</td>
<td>3</td>
<td>First child in early 40s</td>
</tr>
<tr>
<td>Family history</td>
<td>≥2</td>
<td>Breast cancer in first degree relative when young</td>
</tr>
<tr>
<td>Previous benign disease</td>
<td>4-5</td>
<td>Atypical hyperplasia</td>
</tr>
<tr>
<td>Cancer in other breast</td>
<td>&gt;4</td>
<td></td>
</tr>
<tr>
<td>Socioeconomic group</td>
<td>2</td>
<td>Groups I and II</td>
</tr>
<tr>
<td>Diet</td>
<td>1.5</td>
<td>High intake of saturated fat</td>
</tr>
</tbody>
</table>

**Body weight:**

<table>
<thead>
<tr>
<th>Factor</th>
<th>RR</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Premenopausal</td>
<td>0.7</td>
<td>Body mass index &gt;35</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>2</td>
<td>Body mass index &gt;35</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>1.3</td>
<td>Excessive intake</td>
</tr>
<tr>
<td>Exposure to ionising radiation</td>
<td>3</td>
<td>Abnormal exposure in young females after age 10</td>
</tr>
</tbody>
</table>

**Taking exogenous hormones:**

<table>
<thead>
<tr>
<th>Factor</th>
<th>RR</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral contraceptives</td>
<td>1.24</td>
<td>Current use</td>
</tr>
<tr>
<td>Hormone replacement therapy</td>
<td>1.35</td>
<td>Use for ≥10 years</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>2</td>
<td>Use during pregnancy</td>
</tr>
</tbody>
</table>

RR= relative risk.
Diagnosis

Early breast cancer does not usually cause pain. When the cancer grows, it causes changes in the size or shape of the breast: a lump or thickening may be noticeable. In advanced cases the tumour can show signs of ulceration of the skin and fixation to the chest wall, and in the worst cases large lymph nodes may be present.

If any of these symptoms appears a proper investigation should be initiated. The “triple diagnosis” includes clinical examination, mammography and/or ultrasonography and fine-needle aspiration for cytology or core-needle biopsy for histopathological examination.

Mammographic screening was first introduced in Sweden in the late 1980s. The Swedish Board of Health and Welfare recommends that all women between 40 to 74 years of age participate (7). Several studies have reported that mammographic screening reduces breast cancer mortality by 23% (8).

Staging

The TNM staging system was designed to be a useful instrument in determining the prognosis of cancer patients and in planning their treatment. The system is derived from tumour size (T), lymph node status (N) and distant metastasis (M) (9). Clinical stage is based on all information, including physical examination and imaging before surgery. Pathological staging (pTNM) adds additional information gained by examination of the tumour microscopically by a pathologist.

Definition of pTNM (9):

*Primary tumour (T)*: Tx, primary tumour cannot be assessed; T0, no evidence of primary tumour; Tis, carcinoma in situ or Paget's disease of the nipple; T1, tumour 20 mm or less; T2, tumour more than 20 mm but nor more than 50 mm; T3, tumour more than 50 mm; T4, tumour of any size with direct extension to chest wall or skin, or inflammatory breast cancer.  

*Regional lymph nodes (N)*: N0, no node metastasis (includes cases with only isolated tumour cells, or small clusters of cells, not more than 0.2 mm); N1mi, micrometastasis (larger than 0.2 mm, but none larger than 2 mm); N1, metastasis in 1-3 ipsilateral axillary node(s), and/or in ipsilateral internal mammary nodes with microscopic metastasis detected by sentinel lymph node dissection but not clinically apparent; N2 metastasis in 4-9 ipsilateral axillary lymph nodes or in clinically apparent internal mammary lymph node(s); N3, metastasis in 10 or more ipsilateral axillary lymph nodes, or in infra- or supraclavicular lymph nodes, or in both ipsilateral axillary lymph nodes and clinically apparent ipsilateral internal mammary lymph nodes.
**Distant metastasis (M):** M0, no distant metastasis; M1, presence of distant metastasis.

Table 2. Stage grouping based on the three parameters T, N and M.

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T0</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>T0</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1, N2</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>Any</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any</td>
<td>Any</td>
<td>M1</td>
</tr>
</tbody>
</table>
TREATMENT

Surgery

Several randomised trials have shown that breast conserving surgery plus radiotherapy is as effective as mastectomy for most women with stage I and II breast cancer (10-12).

Meta-analysis, done by the Early Breast Cancer Trialists’ Collaborative Group (EBCTCG), confirmed the equivalence in survival rates for women treated with these two approaches (13). However, breast conserving surgery is not an option for all women. If the tumour is ≥4cm, multifocal/multicentric, or if radiotherapy has to be avoided, mastectomy is the method of choice.

Regardless of the method used, an axillary lymph node dissection is always mandatory.

The reason for this is that we know from several studies that the axillary lymph node status is the most important prognostic factor for recurrence and survival (14-15). Two different operations of the axilla can be performed. Traditional axillary lymph node dissection or sentinel lymph node biopsy. The former has been the standard procedure for a long time with additional side effects such as sensory disturbances, lymphedema, pain, seroma formation, poorer cosmetics and infections (16-18). The sentinel node biopsy is by definition the first lymph node to receive lymphatic drainage from a tumour. Today, the technique is considered to be standard procedure (19).

Postoperative radiotherapy

Postoperative radiotherapy is known to substantially reduce the risk of locoregional recurrence and improve breast cancer mortality, both when given after mastectomy and after breast-conserving surgery (20). The meta-analysis by the EBCTCG included a total of 7300 patients who underwent breast-conserving surgery +/- postoperative radiotherapy towards the remaining breast. The locoregional recurrence rate after 5 years was 7% versus 26% (reduction 19%) and 15 years breast cancer mortality risks 30.5% versus 35.9% (reduction 5.4%) (20).

The most common fractionation schedule used is 50Gy in 25 fractions to the whole breast. For younger women an additional booster dose of 16Gy to
the tumour bed is recommended, as this reduces the local recurrence rate by almost 50% (21).

According to the American Society of Clinical Oncology (ASCO) guidelines postoperative radiotherapy after mastectomy, is recommended to patients with tumours >5cm regardless of lymph node status and to patients with four or more positive lymph nodes (22).

This recommendation is somewhat controversial, as two Danish randomised studies have shown a survival benefit from radiotherapy in patients with tumours <5cm and one-three positive lymph nodes (23-24).

Adjuvant chemotherapy

The use of adjuvant chemotherapy was first introduced in the mid-1970s. Two prospective randomised trials in patients with node positive patients showed promising results in terms of delayed tumour recurrence (25-26). Despite the fact that both studies had very short follow-up times (18 and 27 months, respectively) adjuvant chemotherapy was considered the treatment of choice for many women in most developed countries.

The original regimen used was cyclophosphamide, methotrexate, 5-fluorouracil (CMF). Thereafter, many other regimes have been used.

According to the meta-analysis by EBCTCG, adjuvant poly chemotherapy, consisting of either CMF, 5-fluorouracil, adriamycin, cyclophosphamide (FAC) or 5-fluorouracil, epirubicin, cyclophosphamide (FEC), reduces both recurrence and mortality from breast cancer (27). The absolute reduction in breast cancer mortality for women <50 years was 10% and for women aged 50-69 3%. When CMF-based poly chemotherapy was compared with anthracycline-based there was a moderate but significant advantage for anthracyclines, especially in women <50 years (27).

Adjuvant chemotherapy reduces the annual breast cancer death in both node negative and node positive patients. The absolute improvement in 15-years survival is 5% (intention to treat 20) for node negative and 15% for node positive patients (intention to treat 6) (27). Hence, overtreatment with systemic chemotherapy is a common problem in node negative patients, especially as the side effects generally increase as the drugs get more effective. For example, the FEC regimen induces cardiotoxicity and the incidence of secondary leukaemia (28-29).

The role of taxanes (docetaxel and paclitaxel) has been investigated in several studies (30-32). A recent meta-analysis, including 15,500 patients treated with either docetaxel or paclitaxel, shows an absolute survival gain for node positive patients of 3% compared to anthracyclines (33). Due to increased toxicity, taxanes are only recommended for patients at moder-
Adjuvant Trastuzumab

Trastuzumab is a humanized mouse monoclonal antibody that binds to the extracellular domain of the Her2 receptor. Approximately 25-30% of breast cancer tumours have an amplification of the Her2 gene or overexpression of its protein product (34). Overexpression of the receptor is associated with increased disease recurrence and worse prognosis. Adjuvant trastuzumab in combination with or followed chemotherapy has been investigated in five randomised studies (35-38). Pooled results from the four major trials and the smaller FinHer study have recently been analysed in a meta-analysis by Viani et al (39). It concluded that trastuzumab showed a significant reduction of mortality (p<0.00001), recurrence (p<0.00001) and metastases rates (p<0.00001) compared to patients never treated with adjuvant trastuzumab (39).

Due to these results, all Her2 positive patients treated with adjuvant chemotherapy should be considered for one year treatment with trastuzumab.

Side effects such as cardiac toxicity grade III or IV was reported in all five studies especially after treatment with antracyclines. The risk for cardiac toxicity was 2.45 fold higher (95% CI 1.89-3.16) in the group of patients treated with trastuzumab (39). However, patients that developed cardiac heart failure generally improved on removal of the agent. Despite this, regularly monitoring of the heart is recommended throughout treatment. In Sweden, an echocardiogram is preformed prior to starting the treatment and thereafter every third month.

Endocrine therapy

Tamoxifen is a non-steroidal anti-oestrogen that was developed over thirty years ago. It works by competing with oestrogen to bind to the oestrogen receptor (ER) in breast cancer cells.

Several overviews of randomised trials have shown reduced mortality in the adjuvant setting. The latest Oxford overview (15 years follow-up) confirmed a 31% reduction in mortality in women with ER-positive disease who received tamoxifen for five years, regardless of age, menopausal status or nodal status, and a 39% reduction in the incidence of contralateral breast cancer (27).

Aromatase is an enzyme that naturally converts oestrogen from androgen. In premenopausal women, most of the oestrogen is produced in the ovaries,
but in postmenopausal women, most oestrogen is synthesised in peripheral tissue from conversion of androgens (40).

Several trials have investigated the effectiveness of aromatase inhibitors in postmenopausal women with ER-positive, early breast cancer. Regardless of whether it is given “up front” or sequentially after tamoxifen an improvement in treatment outcomes have been noted (41-44).

Furthermore, the MA17 trial showed that the aromatase inhibitor letrozole further decreased the risk of recurrence and improved overall survival (OS) for node positive patients when given as extended treatment after five years of tamoxifen (45).
A prognostic factor predicts disease recurrence or death from breast cancer irrespective of the systemic or local therapy given. The prognostic factors recommended by the St Gallen guidelines are: lymph node status, tumour size, Nottingham histological grade (NHG), peritumoural vascular invasion, age, ER status, progesterone receptor (PgR) status and Her2 (46-51). The recommendations from the Swedish Breast Cancer Group are similar except for S-phase fraction (SPF), ER, PgR status and peritumoural vascular invasion (52).

Adjuvant systemic treatment, especially chemotherapy, is often associated with severe side effects. Therefore it would be of great interest to have better tools to select patients who are likely to benefit.

Lymph node status

Axillary lymph node status in early breast cancer is the most important factor for determining stage, prognosis and adjuvant systemic therapy (53-55). Approximately 80-90% of patients with node negative disease are expected to be alive and free from disease recurrence at ten years after surgery (56-58). The absolute number of involved nodes is also of prognostic importance. Patients with four or more involved lymph nodes have a worse prognosis compared to patients with less than four involved nodes (59).

Tumour size

Numerous studies have shown that tumour size correlates with the number of positive lymph nodes involved and distant recurrence rates increase with larger tumour size (60). In a study that included a total of 13,464 patients with node negative disease, the five-year OS was close to 99% in the group of patients with tumours <1cm. For patients with tumours between 1-3cm and 3-5cm the OS for the same period of time was 89% and 86%, respectively (61).
NHG

The histologic grading system used today was first introduced by Elston and Ellis (48). The system was a modification of the Bloom and Richardson grading system from the 1950’s (62). The reproducibility of the system is moderate on condition that the protocol drawn up by the Nottingham group is applied (48, 63-66). The grading system was first adopted in the Nottingham/Tenovus study which included a total of 2000 patients with clinically (not mammographically) detected breast cancer (48). Several other studies have confirmed its importance as prognostic factor in early breast cancer (50, 65, 67-68).

In a Swedish study by Sundqvist et al, NHG was compared to SPF in a study population consisting of 654 patients. The best predictor for breast cancer death in the multiple regression analysis was grade, regardless of tumour size and lymph node status (51).

Using NHG three features are analysed: the degree of tubule formation, variation in the size and shape of nuclei and mitotic rate. Each feature is given a score of 1-3. The total sum of scores from the three features according to NHG, are as follows: Grade I, well differentiated, 3-5 points; grade II moderately differentiated, 6-7 points; grade III poorly differentiated 8-9 points.

The Nottingham prognostic index was created in 1982. It was based on a retrospective analysis of 9 factors in 387 breast cancer patients. Only 3 of the factors (lymph node status, tumour size and tumour grade) remained significant when a multivariate analysis was preformed (68). The index has also been validated in a prospective study consisting of 320 patients (69).

SPF

Flow cytometric measurement of the percentage of cells in a tumour that are in the DNA synthesis phase of the cell cycle is represented by the SPF. The SPF is one of the most thoroughly investigated proliferation markers in breast cancer. Several studies have shown a correlation to poor prognosis (49-52, 70). However, the technique has several disadvantages. For example, larger tumour volumes are needed than for immunohistochemistry (IHC) methods. The technique can be performed on both freshly-frozen and paraffin-embedded tissue, though more debris is often associated with the latter. Two reproducibility studies have been preformed in Sweden. Both resulted in improved interlaboratory agreements for cytometric DNA ploidy and SPF analysis (71-72).
Ki-67

The Ki-67 protein is a cellular marker for proliferation. It is present in the mid-G1, S, G2 phases and the entire M phase of the cell cycle (73). Ki-67, as a prognostic factor was recently reviewed in a meta-analysis involving 12155 patients (74). Regarding OS results subgroups analysis was eligible in nine studies with node negative patients and in two studies that only included untreated patients (75-84). The main characteristics and OS for these studies are given in table 3.

In conclusion, an association between overexpression of Ki-67 and worse outcome in early breast cancer was seen for the entire study populations and also in subgroups analysis for node negative, node positive and untreated patients (74). Despite this, it is not an established prognostic factor recommended in clinical use (46). The reasons are lack of consensus concerning methodology, especially the definition of cut-off value for high/low proliferation rates in tumour cells. Some authors have used mean, median or the tertile distribution as the cut-off value (83-86). Furthermore, different antibodies and scoring systems have been used without any description of how many tumour cells that were actually counted.
Table 3. Main characteristics and OS for studies including only node negative and untreated patients. Table from de Azambuja et al. Br J Cancer 2007 (74).

<table>
<thead>
<tr>
<th>Ref</th>
<th>N</th>
<th>Systemic treatment</th>
<th>Antibody</th>
<th>Threshold (chosen by)</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bevilacqua 1996</td>
<td>107</td>
<td>Untreated</td>
<td>Anti-Ki-67</td>
<td>10% (arbitrary)</td>
<td>2.8</td>
<td>1.02-7.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Anti-MIB-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brown 1996</td>
<td>674</td>
<td>156 CT and/or HT</td>
<td>Anti-Ki-67</td>
<td>5% (optimal cut-off)</td>
<td>1.2</td>
<td>0.8-1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Domagala1996</td>
<td>111</td>
<td>47 CT or HT</td>
<td>Anti-MIB-1</td>
<td>10% (median value)</td>
<td>3.0</td>
<td>1.03-9.0</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erdem 2005</td>
<td>47</td>
<td>All adjuvant CT (?)</td>
<td>Anti-Ki-67</td>
<td>10% (median value)</td>
<td>17.2</td>
<td>2.4-122.4</td>
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<tr>
<td>Fresno 1997</td>
<td>146</td>
<td>13 CMF 80 TAM</td>
<td>Anti-MIB-1</td>
<td>10% (arbitrary)</td>
<td>1.8</td>
<td>0.7-4.6</td>
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<tr>
<td>Jensen 1995</td>
<td>118</td>
<td>3 CT or HT</td>
<td>Anti-MIB-1</td>
<td>17% (median value)</td>
<td>3.4</td>
<td>1.4-8.1</td>
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<tr>
<td>Pinder 1995</td>
<td>177</td>
<td>Untreated</td>
<td>Anti-MIB-1</td>
<td>34% (tertile distribution)</td>
<td>1.7</td>
<td>1.1-2.5</td>
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<td>Rudolph 1999</td>
<td>863</td>
<td>531 CT or HT</td>
<td>Anti-Ki-S11</td>
<td>25% (median values)</td>
<td>1.9</td>
<td>1.5-2.4</td>
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<tr>
<td>Trihia 2003</td>
<td>188</td>
<td>125 CMF</td>
<td>Anti-MIB-1</td>
<td>16% (proportion of scored cells)</td>
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<td>1.2-3.1</td>
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</tr>
<tr>
<td>Weikel 1995</td>
<td>234</td>
<td>Mostly TAM</td>
<td>Anti-Ki-67</td>
<td>20% (groups)</td>
<td>1.7</td>
<td>0.8-3.5</td>
</tr>
</tbody>
</table>

Cl= confidence interval; CMF= cyclophosphamide, methotrexate, 5-fluorouracil; CT= chemotherapy; HR= hazard ratio; HT= hormonotherapy; N= number of patients included; TAM= tamoxifen; Ref= reference.

Her2

Her2 is a member of the erbB (Her) or epidermal growth factor (EGF) receptor family, which participates in cell growth and proliferation (87). An overexpression or amplification of the Her2 gene is seen in approximately 25-30% of all breast cancer cases and is associated with aggressive growth and poor prognosis (34).

The determination of Her2 overexpression/amplification in the diagnosis of early breast cancer is today a routine procedure in Sweden (88). There are two tests that are available to determine if a tumour is Her2 positive. One, IHC assesses the overexpression of the Her2 protein and the other test, fluo-
rescence in situ hybridization (FISH) assesses whether there is an amplification of the gene or not. The current practice in Sweden today is to screen tumour samples with the IHC test. Only tumours with 3+ detected by IHC are considered as Her2 positive. If the tumour tests 2+, the tissue is retested with FISH. A FISH score greater than 2 means that the tumour is Her2 positive (88). Her2 is the target for the humanized monoclonal antibody trastuzumab (Herceptin). Trastuzumab, with or without chemotherapy such as antracyclin, taxanes and platinum salt, has been associated with increased MFS in the adjuvant setting (35-38). Due to these results trastuzumab is recommended to all women with Her2 positive decease (IHC 3+ or FISH+) (89).
THE CELL CYCLE

General aspects

The cell cycle is a series of events that is fundamental to the growth and development of all eukaryotic cells. It consists of four distinct phases: G1-phase, S-phase, G2-phase and M-phase (Figure 2). Deregulation of these steps is important in the development of tumours.

Figure 2. Illustration of the cell cycle. Figure made from P. Slichter, Gresham High School.

The last phase in the cell cycle is the M-phase, when nuclear and cytoplasmic division takes place leading to two new daughter cells that enter G1-phase. The cells will only enter G1-phase if the surrounding environment is favourable for proliferation. If not, the cells will enter a quiescent state, the G0-phase (90-91).

If nutrients and growth factors are available, the cell will continue through G1-phase until it reaches the so-called restriction point. After this point,
extracellular growth factors are not needed for cell cycle progression (90, 92).

The cyclin/CDK/p16^{INK4A} retinoblastoma protein (pRb) pathway is another important regulatory pathway of the G1-phase (93). When pRb protein is hypophosphorylated it binds to E2F transcription factor and acts as a transcriptional repressor. Mitogenic stimulation leads to a series of phosphorylations, performed by the D-type cyclins (D1, D2 and D3) and cyclin E (E1 and E2). The cyclins must be associated with cyclin-dependent kinases (cdks) to be active. The D family is associated with cdk4 and 6 while the partner of cyclin E is cdk 2. This mitogenic stimulation results in hyperphosphorylation of pRb, release of E2F and passage through the restriction point. The hyperphosphorylated state of pRb is maintained and increased by cyclins E/cdk2, A/cdk2 and B/cdk1 as the cell continues through the cell cycle (94).

G1 phase progression is negatively regulated by the cdk inhibitors (CKIs) (95). There are two families of CKIs: INK 4 and CIP/KIP. The INK 4 inhibitors consist of four proteins, p16, p15, p18 and p19. Their main function is to inhibit the function of cdk 4 and cdk6. The CIP/KIP inhibitors include p21, p27 and p57. These inhibitors interact with many cdks, including the cyclin D and cyclinE-cdk complexes (96) (Figure 3).

![Figure 3. Schematic overview of the cyclin/CDK,p16^{INK4A} retinoblastoma protein pathway. Figure from Yasmeen et al. Expert Rev. Mol. Diagn 2003 (117).](image)

**Cyclin E**

In normal dividing cells cyclin E appears in G1, peaks in late G1 and is degraded in early S-phase (97-98). Cyclin E is expressed precisely when needed in every phase and is then rapidly degraded. Cyclin E is responsible for the phosphorylation of the retinoblastoma protein and the release of E2F. Once E2F is released, DNA synthesis is initiated, thus cyclin E plays an important role in G1- to S transition. For example, when anti-cyclin E anti-
bodies were injected into fibroblasts during G1 phase, cell cycle arrest were induced (98). Conversely, overexpression of cyclin E leads to shortening of the G1 phase and diminished requirements for growth factors (99).

Different mechanisms responsible for deregulated expression of cyclin E have been reported. Gene amplification and defective degradation caused by mutated hCDC4 are two examples (100-101). Another example is the presence of hyper active low-molecular-weight (LMW) isoforms of cyclin E (102).

In addition to full length cyclin E (50-kDa) seen in both normal and tumour cells, up to five different LMW isoforms of cyclin E have been expressed in some breast cancer lines (103-106). These isoforms, which lack the amino terminus, are formed by the cleavage of full length cyclin E by elastase and by calpain 2 (107-108). In comparison to full length cyclin E, they provide a growth advantage to tumour cells due to greater affinity to cdk2, induced genomic instability and resistance to p21, p27 and anti oestrogens (109-110).

Many research groups have investigated the prognostic value of cyclin E in breast cancer, although with conflicting results (111-112). The reason for this might be due to methodological differences (IHC versus Western blotting), patient settings (stage I-III), small study size and the fact that many patients have received adjuvant chemotherapy (112).

In a recent meta-analysis of cyclin E overexpression of 2,534 patients in 12 different studies, overexpression of cyclin E was associated with an 2.32 fold (95% CI 1.25-4.30 fold) increased risk for relapse in univariate analysis and a 1.72 fold (95% CI 0.95-3.10) increased risk in multivariate analysis. The combined hazard ratio (HR) for OS and breast cancer specific survival (BCSS) was 2.98 (95% CI 1.85-4.78) and 2.86 (95% CI 1.85-4.41) in univariate analysis and multivariate analysis, respectively (112). Despite this, cyclin E is not recommended as a prognostic factor in clinical use, mainly due to reasons mention above (113).

However, none of these studies have investigated aberrant expression of cyclin E (the expression of cyclin E during other phases of the cell cycle than late G1 and early S-phase). Elevated levels of cyclin E could simply be due to increased proliferation in the tumour samples. Aberrant cyclin E has recently been investigated in patients. Both studies concluded that parallel cyclin A and E expression is an indicator of poor outcome in cervical carcinomas (114-115).

Cyclin A

The level of cyclin A rises in early S-phase and falls in mid M-phase (116). Two types of cyclin A have been described, cyclin A1 and A2. Cyclin A2 is associated with cellular proliferation and can therefore be used for molecular
diagnostics. Cyclin A1 has been observed in tissue samples from patients with acute myeloid leukaemia and testicular cancer and does not seem to be a marker for proliferation of breast cancer cells (117). At the beginning of S-phase, cyclin A binds to cdk2. As the cell moves further and enters the G-2 phase, it switches partners and binds to cdk1. Some previous studies have shown an association between high cyclin A2 expression and worse prognosis in early breast cancer, while others have failed to confirm this (118-121). The discordance between results may be due to the lack of consensus concerning methodology, especially the definition of cut-off value for high/low proliferation rates in tumour cells. Some papers have used the median cyclin A value determined for the entire cohort (119, 122-123), while others have used the average value that best corresponds to NHG III as the cut-off value (124).
VALIDATION OF TMA

The TMA technique was first developed by Kononen et al in the late 1990s (125). It allows a large number of tumours to be analysed simultaneously on a single microscopic slide. Tumour markers can be analysed on the DNA, RNA and protein levels. The TMA technique is cost-effective and tissue-saving. It requires much less manufacturing time than traditional histopathology slides and it minimises tissue damage to the donor block.

To what extent might tumour heterogeneity influence the results of an individual histopathological factor when only a small amount of tumour is studied?

This has been an issue for many studies in the past. Most of these studies have shown that there is no difference in the correlation to histopathological factors and prognostic implications between TMA and traditional histopathology slides (126-130).

Figure 4A. Breast cancer TMA.
Figure 4B. TMA biopsy stained for cyclin A.
AIMS OF THE STUDY

The purpose of this study was to investigate the prognostic value of cyclin A, cyclin E and aberrant cyclin E in early breast cancer.

The specific aims were:
I     To evaluate the representativeness of TMA in comparison to large sections in breast cancer tumours for assessing cyclin A.
II    To find the optimal cut-off values for cyclin A and Ki-67 in early breast cancer tumours.
III   To investigate cyclin E and aberrant cyclin E expression in low-risk node negative breast cancer.
IV    To investigate the prognostic value of cyclin A in node negative breast cancer.
PATIENTS AND METHODS

Patients paper I
Tumours from 200 T1-4N0-N1M0 breast cancer patients treated at Helsinki University Central Hospital between 1997 and 1998 were analysed on TMA and traditional large sections. The patients were part of a larger cohort mainly focusing on hereditary breast cancer, genetics, epidemiological and clinicopathological factors associated with breast cancer risk and prognosis (59). Pathology reports of the primary tumours were studied. Pathological data including information on tumour histology, grade, ER and PgR status, tumour diameter, nodal status and distant metastases were collected. Grading was performed according to Scarff-Bloom-Richardson modified by Elston and Ellis (48). Patients’ records were studied and information was collected on adjuvant treatment, local relapse and distant metastases as well as time of death or follow-up.

Patients paper II
Tumours from 570 T1-4N0-1M0 consecutive patients with breast cancer, treated at the Department of Oncology at Helsinki University Central Hospital between 1997-1998 and 2000 were analysed by the TMA technique. All patients underwent surgery and were treated according to guidelines that were standard at that time regarding adjuvant chemotherapy, radiotherapy and endocrine treatment. A decision on adjuvant chemotherapy was based on the patient age, PgR status, SPF, tumour size and nodal status.

A total of 231 (40.5%) of the patients received adjuvant chemotherapy. Patient follow-up information concerning distant metastases and time of death was obtained from the patient records. A total of 79 patients relapsed with distant metastases and 53 of these died from breast cancer.

Study design paper III
To test our hypothesis we compared women that died early from their breast cancer with women free from relapse > 8 years after initial diagnosis. All patients belonged to a defined cohort of women diagnosed with breast cancer in the Uppsala-Örebro region 1993-2001. They all had tumours ≤2cm, no lymph node metastases, NHG I/II or low proliferation (SPF), ER and/or PgR
positive tumours and none had received adjuvant chemotherapy. The patients were identified from our regional breast cancer quality register. Those within the cohort dying from breast cancer are hereafter denoted as cases. Women who survived without breast cancer relapse > 8 years after initial diagnosis served as a comparison group, hereafter denoted as controls. We planned on having 25 cases and 25 controls, however, since only 34 patients fulfilled the inclusion criteria for controls in the whole region, they were all included. All patients’ files and pathology reports were reviewed to validate all data received from the breast cancer quality register. Eight out of 25 cases were excluded from the study because of contralateral breast cancer with lymph node metastases or tumour size >2cm (3 patients), no paraffin blocks found (2 patients), tumour size >2cm (1 patient), distant metastases at diagnosis (1 patient) and non breast cancer death (1 patient). Ten out of 34 controls were excluded from the study because of diagnosis or death from a concurrent cancer (3 patients), relapse or death in breast cancer (3 patients), no paraffin blocks were found (3 patients) and having lymph node metastasis (1 patient). 17 cases and 24 controls remained in the study after the data review.

Patients paper III

All patients had tumours ≤2cm. Six of the cases and three of the controls had progesterone receptor negative tumours. One of the inclusion criteria was NHG I/II, however, our board certified pathologist re-graded all tumours and found that some were grade III-tumours. Mean NHG points in cases was 6.8 and in controls 6.1 (a non-significant difference), qualifying both groups as grade II tumours on average. No tumours were excluded because of differences in re-grading. Most tumours were of ductal histology: 16 ductal and 1 lobular carcinoma in the case group and 20 ductal, 3 lobular and 1 mucinous carcinoma in the control group. None of the patients received adjuvant chemotherapy. However, 11 cases and 20 controls who were surgically treated with sector resection received adjuvant radiotherapy. Three cases received adjuvant antihormonal treatment versus none of the controls. Mean time to distant metastases and survival among cases was 23 months and 43 months, respectively. Mean follow-up among controls was 139 months.

Study design paper IV

To investigate the prognostic value of cyclin A we designed a case-control study. Inclusion criteria were tumour size ≤5cm, no lymph node metastases and no adjuvant chemotherapy. All patients were selected from a defined cohort of women diagnosed with breast cancer in the Uppsala-Örebro region 1993-2004. For each of the 240 identified cases one control were used. Fifty cases were excluded due to new/contra lateral or locally advanced breast
cancer (26 patients), no paraffin blocks found (12 patients), non breast cancer death (6 patients), distant metastases at diagnosis (4 patients), adjuvant chemotherapy was given (1 patient) and no breast surgery was performed (1 patient). Cases were defined as women who died from breast cancer, controls as women alive at the time for the corresponding case’s death. All patient files and pathology reports were reviewed to validate all data received from the breast cancer quality register. The study was approved by the local ethics committee in Uppsala, Sweden.

Patients paper IV
Mean age was 66 years for the cases and 61 years for the controls. The average tumour size was 2 cm for the cases and 1.6 cm for the controls. All patients underwent surgery consisting of either modified radical mastectomy with axillary dissection, or conservative breast surgery with axillary dissection and post-operative irradiation of the breast. Fifty three cases (28%) and forty seven (25%) controls received anti-hormonal therapy. None were treated with adjuvant chemotherapy.

TMA construction paper I and II
Paraffin blocks from the patient primary tumours were collected. Haematoxylin and eosin sections were reviewed and the most representative tumour areas were selected. These areas from each tumour were punched and incorporated into recipient paraffin-blocks to produce TMAs consisting of four cores (diameter 0.6 mm) for each tumour (131-132).

TMA construction paper IV
Representative areas from each tumour were punched and brought into recipient paraffin-blocks to produce TMAs consisting of two cores (diameter 1 mm) of each tumour.

Immunohistochemistry
Sections 3-4 um thick were cut from the array blocks and transferred to glass slides (paper I, II and IV). For paper III, 4-5 um sections were cut from paraffin blocks and transferred to traditional large section slides.

Paper I
Immunostaining for cyclin A (mouse monoclonal, Novo Castra Laboratories) was done manually. Antigen retrieval was done using a pressure cooker
for 5 minutes in 0.01M citrate buffer, pH6.0. Primary antibody was diluted 1:300 and applied for overnight incubation. Staining was done using the avidin biotin peroxidase complex and amino-ethyl-carbazole (AEC) procedures (133). The peroxidase was developed using the diaminobenzidine (DAB) technique.

Paper II
Deparaffination of the TMA samples were done using xylene. The slides were rehydrated through graded alcohols to water. Immunostaining for cyclin A (mouse monoclonal, Novo Castra Laboratories) was done manually. Antigen retrieval was done using a pressure cooker for 5 minutes in 0.01 M citrate buffer, pH6.0. Primary antibody was diluted 1:300 and applied for overnight incubation. Staining was done using the avidin biotin peroxidase complex and AEC procedures (133). The peroxidase was developed using the DAB technique. Immunostaining for Ki-67 (Mib-1, Dako cytomation, Sweden) diluted 1:100 was performed in an automated immunostainer (Ventana Medical Systems Inc., Tucson, AZ, USA) using a DAB kit (Ventana). The slides were manually counterstained in Mayers haematoxylin (Sigma, St. Louis, MO, USA). Finally, the slides were dehydrated through graded alcohols to xylene and mounted in organic mounting medium (Pertex, Histolab, Gothenburg, Sweden).

Paper III
Immunofluorescence stainings were performed on paraffin-embedded tissue sections from breast cancer tumours. The sections were deparaffinised and thereafter rehydrated through a ladder of graded ethanol (absolute ethanol, 90%, 70%, 50% and 30%). An antigen retriever (2100 Retriever, PickCell Laboratories, Amsterdam, NL) was used to recover the tissue sections’ antigenicity by heating the slides in citrate acid (pH6) to 120°C for 20 minutes and thereafter slowly cooling them in room temperature during 2 hours. Blocking buffer (1% bovine serum albumin and 0.5% Tween 20 dissolved in phosphate-buffered saline) was applied for 10 minutes. A polyclonal rabbit antibody (H432; Santa Cruz Biotechnology, Santa Cruz, USA) was used to detect cyclin A and a monoclonal mouse antibody (H12; BD Pharmingen, San Diego, CA, USA) to detect cyclin E, both applied for overnight incubation. Three washing steps, 20 minutes each, were performed in washing buffer. Unspecific binding of the secondary antibodies was blocked by incubation of the slides in 4% donkey serum diluted in blocking buffer. Secondary antibodies, goat anti-rabbit Cy5 (ab 6564-100, Abcam, Cambridge, UK) and donkey anti-mouse biotinylated (7754; Dako Cytomation, Carpenteria, CA, USA), were added to the slides and incubated in room-temperature for 2 hours. An amplification step with biotin and streptavidin was used to en-
hance the signal from cyclin E. Visualisation of cyclin E was accomplished by adding Streptavidin-Cy3 (PA 43001, Amersham Life Sciences) binding to the biotinylated secondary antibody. Cover slips were mounted for fluorescence microscopy in mounting medium (Vectashield, Vector Laboratories Inc, Burlingame, CA, USA) containing 2-4-amidinophenyl-6-indolecarbamidine dihydrochloride (DAPI). As positive control we used tonsil and as negative control omission of primary antibodies. This staining protocol resulted in cell nuclei stained with DAPI, cyclin A stained with Cy5 and cyclin E with Cy3.

Paper IV

TMA slides were deparaffinised in xylene and rehydrated through a ladder of graded ethanol (absolute ethanol, 95%, 80% and distilled water). Antigen retrieval was done in TE (Tris-EDTA pH9 buffer) in a microwave oven for 10 minutes (750W) + 15 minutes (350W) before being processed in an automatic immunohistochemistry staining machine according to standard procedures (Autostainer; Dako, Sweden). All antibodies were applied for 30 minutes at room temperature. The following monoclonal antibodies were used: Cyclin A (NCL-Cyclin A 1:100 NovoCastra Laboratories, UK), Ki-67 (1:200 M7240, Dako, Sweden), ER (NCL-ER-6F11 1:150 NovoCastra Laboratories, UK ) and PgR (NCL-PGR 1:100 NovoCastra Laboratories, UK) . Immunostainings were detected via DAKO Cytomation envision/HRP kit K5007.

For cyclin A and Ki-67 stainings tonsil samples was used as positive controls and for ER and PgR stainings breast cancer tissue. The primary antibody was omitted from negative controls.

The antibodies, dilutions, vendors and clones used are summarised in table 4.
Table 4. Summary of antibodies, dilutions, vendors and clones used in paper I-IV.

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<th>Dilution</th>
<th>Paper</th>
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<td>Mouse</td>
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<td>Dako Cytomation</td>
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<td>Mouse</td>
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Evaluation of immunoreactivity scores

Paper I
Two readers (Cecilia Ahlin, Kirsimari Aaltonen) scored all the slides. The percentage of cyclin A positive breast cancer cells was counted in one high-power field (40X objective) in each of the four tissue cores for TMA and in three randomly selected and one “hot-spot” high-power field for the large sections. At least 200 breast cancer cells were counted. All statistical evaluations were done with both average and maximum values of the four counts.

Paper II
TMA slides were analysed by one investigator (Kirsimari Aaltonen). The percentage of cyclin A and Ki-67 positive breast cancer cells was counted in one high-power field (40X objective) in each of the four tissue cores for TMA. At least 200 cells were counted in each tumour. All statistical analyses were done using both average and maximal values for each patient. To determine the maximum percentage cyclin A and Ki-67 values, we counted the biopsy core with the largest number of positively stained cells out of the
four and divided by the total number of cells from that particular biopsy. To obtain the average percentage value we divided all positive cells from the four biopsies by the total number of cells from the same biopsies.

Paper III

Tumour samples were analysed for expression of cyclin A, cyclin E and double-staining using immunoflorescence staining and digital microscopy. Tumour cell nuclei were manually counted and marked with an x in the DAPI image. At least 500 cells were counted on each tissue section. Cyclin A positive cells were counted in the Cy5 image and cyclin E positive cells in the Cy3 image. Only cells marked with an x were considered true tumour cells and hence counted. Superimposition of the DAPI, Cy3 and Cy5 images showed tumour cells co-expressing cyclin A and E. Only staining arising from cell nuclei was considered truly positive. Since cyclin E is normally degraded early in S-phase (when the nuclei are weakly positive for cyclin A) and we were focusing mainly on aberrant cyclin E expression, only nuclei staining moderately or strongly positive for cyclin A (representing cells in S-phase and G2-phase) were counted as positive. All nuclei staining positive for cyclin E were counted. In order to determine which stainings were to be considered positive, we set thresholds manually by visual inspections of the images.

Paper IV

All cyclin A and Ki-67 TMAs were analysed by one investigator (Cecilia Ahlin). Hormone receptor analyses were performed by a pathologist (Wenjing Zhou). The percentage of cyclin A, Ki-67, ER and PgR positive breast cancer cells was counted in high-power fields (40X objective) in both tissue cores on TMA. In most cases 1000 cells were counted in each tumour and for all tumours a minimum of 200 cells. All cyclin A and Ki-67 statistical analyses were done using both average and maximal value for each of the patient. When calculating for cyclin A and Ki-67 maximum value in percentage, we counted the high-power field that had the largest number of positively stained cells out of the two biopsies and divided by the entire number of cells from that particular high-power field. To get the average percentage value we divided all positive cells from the two biopsies by the entire number of cells from the same biopsies.
RESULTS AND DISCUSSIONS

Paper I

Prognostic and proliferation markers, such as ER, PgR, Her2 and Ki-67 have in previous studies shown good agreement when evaluated on TMA compared to traditional large sections (125-130). In this study, TMA and traditional large sections results from 200 T1-4N0-N1M0 breast cancer tumours were analysed. The cut-off value of 10% was chosen based on a previous study in which the cyclin A count >10% identified a similar proportion of patients as the number of grade III tumours (124).

The reproducibility of the two readers’ results was good or even very good with kappa values 0.71-0.87. The agreement of TMA and large section results was also good with kappa value 0.62-0.75. Discrepant results might be due to the method used in counting cells. The percentage of cyclin A positive breast cancer cells was counted in one high-power field (40X objective) in each of the four tissue cores for TMA and in three randomly selected and one “hot-spot” high-power field for the large sections. Another reason could be the amount of cells counted. The results that differ most from each other were results from tumours with fewer cells counted.

We conclude that TMA is as good as large sections in scoring for cyclin A on breast cancer tissue.

Paper II

Ki-67 and cyclin A have shown promising results in several breast cancer studies when evaluating their prognostic values (74, 118, 120). One major problem has been the lack of consensus on cut-off values for high/low proliferation rate. Some of the previous studies, which have investigated the prognostic value of cyclin A have, for example, used the median cyclin A average value determined for the entire cohort or the average value that best corresponded to NHG III (119, 122-124). The same lack of consensus characterizes the literature on Ki-67 (74).

The purpose of this study was to evaluate the most optimal cut-off values, not the prognostic values, of cyclin A and Ki-67. For prognostic studies the material is sub optimal (heterogenic, different treatments, too few events).
To investigate the optimal cut-off values for cyclin A, Ki-67 average and maximum values we divided the material into 10 deciles. For each cut-off we calculated the risk ratio (RR) for metastasis free survival (MFS) and OS with 95% confidence interval (CI) using the Cox proportional hazard model.

The reason for choosing MFS as our primary end point and not OS was that we found no association between prognosis and proliferation rate in the group of patients that received chemotherapy. This may be an indication that tumours with very high cyclin A values may be especially sensitive to chemotherapy. For details see table 5 and 6.

In the chemotherapy naïve subgroup the highest relative risks for metastasis was at cut-off values around the 7th decile (RR 2.4-2.9) for both cyclin A and Ki-67. The 7th decile corresponds to a cyclin A average value of 8% (RR 2.4 95% CI 1.2-4.9) a cyclin A maximum value of 11% (RR 2.6 95% CI 1.3-5.1), a Ki-67 average value of 15% (RR 2.8 95% CI 1.4-5.5) and a maximum value of 22% (RR 2.9 95% CI 1.5-5.6). For details see table 5 A and B.

Table 5A and B. Correlations between different cut-off values for cyclin A average/maximum value and metastasis-free survival using the Cox proportional hazard model.

A. Chemotherapy naïve subgroup. B. Chemotherapy subgroup.

<table>
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RR= relative risk; CI=confidence interval.
During recent years mounting evidence has suggested that cyclin E is a prognostic factor for poor outcome in breast cancer (112, 119, 134-135). In these studies, the investigators have considered the total amount of cyclin E expressed, not cyclin E expression over the cell cycle. This study aimed to investigate cyclin E and aberrant cyclin E expression in low-risk node negative breast cancer. For this purpose we designed a study that compared women that died from breast cancer (n=17) with women free from relapse >8 years after initial diagnosis (n=24). All tumours were node negative. Due to our regional and national recommendations none of these patients were offered adjuvant chemotherapy. Still, some of them relapsed and might have benefited from such adjuvant treatment anyway. Our goal with this study was to separate patients that relapse from those that do not by assessing cyclin E expression.

However, no statistically significant differences regarding cyclin A expression (2%, 95% CI -3-6%, p=0.56), cyclin E expression (0%, 95 CI -2-2%, p=0.57), co-expression (0.7%, 95 CI -0.5-1.9%, p=0.27) or fraction double-stained cells (2%, 95 CI -4-8%, p=0.36) were seen between cases and controls. These results are in contrast to other papers reporting a significant prognostic relevance for cyclin E in breast cancer. One possible explanation for the discrepancy between these results is that the material in the different studies is not comparable to each other. In our study only patients with low-

<table>
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<tr>
<th>Percentile</th>
<th>Cut-Off (%)</th>
<th>RR</th>
<th>95% CI</th>
<th>P-value</th>
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<td>0.8</td>
<td>1.5</td>
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<td>15.8</td>
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</table>

RR= relative risk; CI=confidence interval.
risk node negative breast cancer, of which none received adjuvant chemotherapy was included. In Kühling et al’s study half of the patients had tumours larger than 2cm and one third of them had receptor negative disease. In Bukholm et al half of the patients had node positive disease. It is well known that cyclin E expression increases with tumour size, grade, stage and lack of hormone receptors and, consequently, our results make sense and could have been expected (111, 133, 136-137).

Another notable observation is due to methodological differences. Some studies have suggested that the LMW forms of cyclin E, detected by Western blot, facilitate the transition from the G1 phase to the S phase more effectively than the full-length form of the protein (106, 138). Substantially higher prognostic value has been reported when both the LMW forms and the full length of the protein are expressed (134). In our immunohistochemical analysis of cyclin E, we used an antibody that cannot detect the LMW forms of cyclin E reliably. Unfortunately, there are no commercial antibodies available that target the C-terminal domain of the protein thus allowing the detection of the isoforms.

Figure 5. Diagrams showing percentage(s) of all tumour cells staining positive for cyclin E and aberrant expression of cyclin E, i.e. fraction of cells co-expressing cyclins A and E, in cases and controls with node negative breast cancer.
Figure 6. Photos illustrating a. cell nuclei stained with DAPI, b. cyclin A positive cells (Cy 5), c. cyclin E positive cells and d. cells co-expressing cyclins A and E (arrows).

Paper IV

Node negative status at diagnosis has commonly been associated with a favourable patient outcome. Approximately 80-90% of these women are expected to be alive and free from disease recurrence at ten years after surgery (56-58). However, a majority of these patients will be treated with adjuvant loco-regional and systemic therapy according to the recommendations from the EBCTCG. Adjuvant chemotherapy reduces the annual breast cancer death by approximately 30% in both node negative and node positive patients, corresponding to absolute improvements in 15-years survival of 5% (intention to treat 20) and 15% (intention to treat 6) (27). However, this improvement in survival come to a price, as a substantial proportion of women will get side effects from the chemotherapy given unnecessarily.

Cyclin A’s and Ki-67’s roles as prognostic factors in node negative patients have been investigated in several studies with conflicting results, mainly due to heterogeneous material and the use of adjuvant chemotherapy (74, 118-121).

The aim of this study was to investigate cyclin A as a prognostic factor in node negative breast cancer tumours using a case-control design. Cases were defined as women who died from breast cancer and controls as women alive
at the time for the corresponding case’s death. Inclusion criteria were tumour size \( \leq 5 \text{cm} \), no lymph node metastases and no adjuvant chemotherapy. All patients were selected from a defined cohort of women diagnosed with breast cancer in the Uppsala-Örebro region 1993-2004.

One hundred and ninety cases and 190 controls were included into the study. Established and potential prognostic factors such as endocrine responsiveness, tumour size, NHG, Ki-67, cyclin A and mitotic count were included in the univariate model (46). In the multivariate models only variables associated with proliferation were included. We deliberately included NHG in the two multivariate models since it is the most used proliferation factor in the world today, due to previous reports confirming its importance as prognostic factor (48, 50, 65, 67-68).

Using conditional logistic regression in a univariate model, a statistically significant association was observed between expression of cyclin A average/maximum value, Ki-67 average/maximum value, NHG, mitotic count, tumour size, ER, PgR and breast cancer death. Cyclin A and Ki-67 (average value) lost their significance when including NHG into the multivariate model. Only NHG was independently associated with breast cancer death (table 6 and 7).

Table 6. Univariate analysis of established prognostic factors and proliferation markers for breast cancer death.

<table>
<thead>
<tr>
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<th>Model adjusted for age at diagnosis and tumour size.</th>
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<tr>
<td></td>
<td>OR</td>
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<tr>
<td>Tumour size</td>
<td>2.2</td>
</tr>
<tr>
<td>ER</td>
<td>2.6</td>
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<tr>
<td>PR</td>
<td>2.5</td>
</tr>
<tr>
<td>NHG</td>
<td>3.0</td>
</tr>
<tr>
<td>Mitotic count</td>
<td>2.5</td>
</tr>
<tr>
<td>Ki-67 maximum</td>
<td>1.8</td>
</tr>
<tr>
<td>Ki-67 average</td>
<td>2.0</td>
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<tr>
<td>Cyclin A maximum</td>
<td>3.7</td>
</tr>
<tr>
<td>Cyclin A average</td>
<td>2.9</td>
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</table>

OR= odds ratio. CI= confidence interval. Ref= reference.

These results (univariate analysis) for cyclin A are interesting as no other studies to my knowledge, have shown a correlation between breast cancer death and cyclin A in this set of patients before. Baldini et al reported that
DFS was significantly shorter in patients with cyclin A overexpression (p=0.01). Unfortunately, only 75 patients were included and chemotherapy was administrated according to individual risk factors, thus cyclin A’s true prognostic role is hard to evaluate (121). In Kuhling et al patients similar to ours were included. However, cyclin A was not associated with prognosis in this study (119).

Table 7. Multivariate analysis of cyclin A, Ki-67 average value and NHG. Models adjusted for age at diagnosis and tumour size.

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>Ref</th>
<th>95% CI</th>
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<tbody>
<tr>
<td><strong>Model 1</strong></td>
<td></td>
<td></td>
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<tr>
<td>NHG</td>
<td>3.3</td>
<td>I-II</td>
<td>1.8-6.1</td>
</tr>
<tr>
<td>Ki-67 average</td>
<td>1.3</td>
<td>≤15%</td>
<td>0.8-2.2</td>
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<tr>
<td><strong>Model 2</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NHG</td>
<td>2.6</td>
<td>I-II</td>
<td>1.3-5.2</td>
</tr>
<tr>
<td>Cyclin A average</td>
<td>1.7</td>
<td>≤8%</td>
<td>0.9-3.0</td>
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OR= odds ratio. CI= confidence interval. Ref= reference.

Before patients were enrolled in the study, a power calculation was performed. To achieve statistical power a case-control design was used. Assuming a power of 0.80 and alpha of 0.05 (prevalence 0.3-0.5, RR=2) the study design required enrolment of 125-150 patients in each group. As mentioned above, a total of 190 cases and controls were included of which none had received adjuvant chemotherapy.

The result for Ki-67 is, on the other hand not surprising given the fact that several papers have reported a correlation between Ki-67 and prognosis in node negative breast cancer (74).

In the present study, both cyclin A and Ki-67 lost their prognostic relevance when NHG was included in the multivariate model. This could be explained by their strong correlation to each other as all three variables are associated with proliferation.
GENERAL CONCLUSIONS

Paper I
I. The TMA technique is as good as traditional histological slides for analysing histological correlations and the prognostic significance of cyclin A in early breast cancer.
II. The agreement between the TMA and large section results was good with kappa values 0.62-0.75.
III. The reproducibility between the two readers’ results was good or even very good, with kappa values 0.71 – 0.87.

Paper II
I. The average and maximum cyclin A and Ki-67 values correlated strongly with ER negativity, PgR negativity, high tumour grade and large tumour size. There were no correlations between cyclin A and Ki-67 and nodal status.
II. Our results suggest that the optimal cut-off value for cyclin A average is 8% and for cyclin A maximum value 11%, for Ki-67 correspondingly 15 and 22%.

Paper III
I. Neither cyclin E nor aberrant cyclin E is a prognostic factor in low-risk node negative breast cancer.

Paper IV
I. Cyclin A is a prognostic factor in node negative breast cancer (univariate analysis average value OR=2.9 95% CI 1.8-4.6; maximum value OR=3.7 95% CI 2.3-5.9).
I would like to sincerely thank everybody who helped and supported me during the work presented in this thesis. I would especially like to thank following peoples:

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My German Shephard Ioda, if I had your self-confidence and strength I would be undefeatable!

Kenneth, my true love, thanks for all your support and encouragement. Although both you and I occasionally wanted to throw away the computer and burn the manuscripts I am glad that we did not.

Adam, thanks for all your patience and understanding and for teaching me how to think like a winner. No one loves you as I do!
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