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# Prognostic factors in breast cancer with focus on proliferation and non-linear effects

Carina Forsare



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DOCTORAL DISSERTATION

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To be defended at the lecture hall in the Radiotherapy building, Skåne Oncology  
Clinic, Lund, Sweden, Friday the 11<sup>th</sup> of November 2016, at 9:00 am

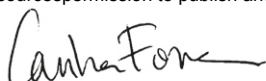
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<p><b>Abstract</b></p> <p>Breast cancer is the most common cancer today among women in the Western world. In Sweden in 2015, more than 8 000 women were diagnosed with breast cancer. For a large number of patients, the prognosis is good. The majority of the patients are cured by primary surgical treatment, and many of the recurring patients survive for long periods of time. Additional (adjuvant) treatment is administered to prevent recurrences, although many patients are already cured by the primary surgery, thereby leading to over-treatment. Despite adjuvant treatment, more than 1 400 patients die from breast cancer every year, demonstrating the need for tools to better tailor treatment. The most important and routinely used prognostic and treatment predictive factors are age at diagnosis, tumor size, spread to the lymph nodes, histological grade, hormone receptors, factors for proliferation and expression of a growth factor receptor (HER2).</p> <p>The overall aim of this thesis was to investigate prognostic and predictive markers in breast cancer and to find ways to improve the use of established factors, with a focus on proliferation and non-linear effects. Factors for proliferation were studied in different breast cancer patient cohorts and with different methods.</p> <p>In <b>Study I</b>, we investigated whether an index, CAGE, based on cyclin A, histological grade, and estrogen receptor, could provide valuable prognostic information. CAGE was evaluated on tissue microarray slides, with samples from 219 premenopausal node-negative breast cancer patients. CAGE together with HER2 identified 53% of the patients as low risk with a 5-year distant disease-free survival of 95%. In <b>Study II</b>, we investigated whether the results of <b>Study I</b> could be confirmed in independent and larger series, replacing cyclin A with the worldwide used Ki67, creating KiGE. In chemo-naïve N0/N1 patients, KiGE alone identified 57% of the patients as low risk, with a 5-year event-free survival of 92%.</p> <p>In <b>Study III</b>, we studied five factors for proliferation (mitotic activity index (MAI), phosphorylated histone H3 (PPH3), cyclin B1, cyclin A, and Ki67), separately and in combinations. Since all of the prognostic factors have pros and cons, our hypothesis was that combining factors would circumvent these issues. We demonstrated that a combination of MAI and cyclin A improved prognostication compared to use of the factors individually.</p> <p>Although it is a well-known fact that information is lost when predictors are dichotomized (divided into two groups) or categorized into three groups, for clinical decision-making, this is often performed. In <b>Study IV</b>, we investigated whether the prognostication could be improved by postponing dichotomization/categorization until the last step in the process. We concluded that dichotomization definitely leads to information loss and should be avoided. Categorization improved prognostication, whereas using non-linear transformations only moderately improved the predictions.</p> <p>In conclusion, the studies included in this thesis demonstrated that several markers for proliferation could be used and that combinations seems to improve prognostication, compared to examining factors individually. With these combinations, it was possible to identify patients with a low risk of developing recurrence so that adjuvant chemotherapy might be avoided. Dividing prognostic factors in more groups than two gives better predictive performance, but keeping them continuous was only moderately better. However, by postponing categorization until the very last step of the prognostic modeling strategy, more possibilities for individual predictions were enabled.</p>		
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# Prognostic factors in breast cancer with focus on proliferation and non-linear effects

Carina Forsare



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*Climb mountains and reach new heights*



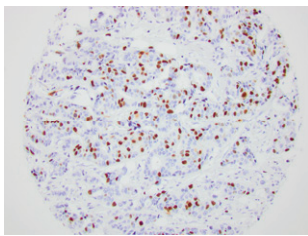
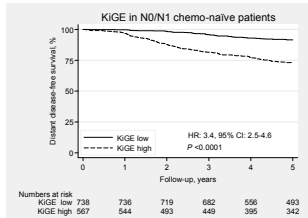
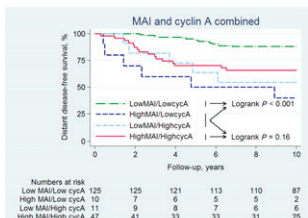
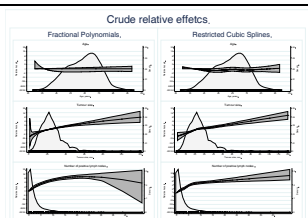
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# Thesis at a glance

Study	Question	Patients and Methods	Figure	Results
I	Could an index, CAGE, based on cyclin A, histological grade, and estrogen receptor (ER), provide valuable prognostic information?	CAGE was evaluated on tissue microarray (TMA)-slides, with samples from 219 premenopausal, node-negative breast cancer patients. Five-year distant disease-free survival (DDFS) was used as the endpoint.		CAGE together with HER2 identified 53% of the patients as low risk with a 5-year DDFS of 95% (95% CI: 89–98%).
II	In independent and larger patient series, could the results of <b>Study I</b> be confirmed by replacing cyclin A with Ki67 and creating KiGE?	KiGE was evaluated on TMA slides with samples from 1 305 chemo-naïve N0/N1 breast cancer patients. Five-year event-free survival was used as the endpoint.		The results from <b>Study I</b> were confirmed. KiGE identified 57% of the patients as low risk with a 5-year event-free survival of 92% (95% CI: 89–93%).
III	Would the use of a combination of factors for proliferation, instead of using them individually, improve prognostication?	Mitotic activity index (MAI), phosphorylated histone H3 (PPH3), cyclin B, cyclin A, and Ki67 were evaluated in premenopausal, node-negative breast cancer patients. Ten-year DDFS was used as the endpoint.		MAI together with cyclin A showed stronger prognostic value when combined than when each proliferation factor was used individually.
IV	Would prognostication be improved by keeping predictors continuous as long as possible?	Age at diagnosis, tumor size, and number of positive lymph nodes were evaluated in models based on dichotomized, categorized, and continuous predictors.		Categorization improved prognostication, whereas using fractional polynomial and restricted cubic spline transformations only moderately improved the predictions.



# Included studies

**Study I.** Combination of the proliferation marker cyclin A, histological grade, and estrogen receptor status in a new variable with high prognostic impact in breast cancer

**Carina Strand**, Cecila Ahlin, Pär-Ola Bendahl, Marie-Louise Fjällskog, Ingrid Hedenfalk, Per Malmström, Mårten Fernö, *Breast Cancer Res Treat*, 131(1):33–40 2012

**Study II.** The combination of Ki67, histological grade and estrogen receptor status identifies a low risk group among 1,853 chemo-naïve women with N0/N1 primary breast cancer

**Carina Strand**, Martin Bak, Signe Borgquist, Gunilla Chebil, Anna-Karin Falck, Marie-Louise Fjällskog, Dorthe Grabau, Ingrid Hedenfalk, Karin Jirström, Marie Klintman, Per Malmström, Hans Olsson, Lisa Rydén, Olle Stål, Pär-Ola Bendahl, Mårten Fernö, *SpringerPlus* 2(1):111 2013

**Study III.** 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.

Marie Klintman M, **Carina Strand**, Cecila Ahlin, Sanda Beglerbegovic, Marie-Louise Fjällskog, Dorthe Grabau, Einar Gudlaugsson, Emiel Janssen, Kristina Lövgren, Ivar Skaland, Pär-Ola Bendahl, Per Malmström, Jan Baak, Mårten Fernö, *PloS one* 8(12):e81902 2013

**Study IV.** Will using age, tumor size, and lymph node status as continuous predictors improve prognostication in breast cancer?

**Carina Forsare**, Martin Bak, Anna-Karin Falck, Dorthe Grabau†, Fredrika Killander, Per Malmström, Lisa Rydén, Olle Stål, Marie Sundqvist, Pär-Ola Bendahl\*, Mårten Fernö\* (\*shared senior authorship), Manuscript

† Deceased December 2015

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**Studies not included but of relevance for the thesis:**

Training artificial neural networks directly on the concordance index for censored data using genetic algorithms

Jonas Kalderstam, Patrik Eden, Pär-Ola Bendahl, **Carina Strand**, Mårten Fernö, Mattias Ohlsson, *Artificial intelligence in medicine* 58(2):125-132 2013

Histological grade provides significant prognostic information in addition to breast cancer subtypes defined according to St Gallen 2013

Anna Ehinger, Per Malmström, Pär-Ola Bendahl, Christopher Elston, Anna-Karin Falck, **Carina Forsare**, Dorthe Grabau, Lisa Rydén, Olle Stål, Mårten Fernö, *Acta Oncologica*, In press

# Abbreviations

AI	aromatase inhibitor
ANN	artificial neural network
ASCO	American Association of Clinical Oncology
BCS	breast conserving surgery
BCSS	breast cancer-specific survival
CAGE-index	cyclin A, grade and ER
CDK	cyclin dependent kinase
CI	confidence interval
CISH	chromogenic <i>in situ</i> hybridization
CTCs	circulating tumor cells
ctDNA	circulating tumor DNA
DAB	diaminobenzidine
DIA	digital image analysis
DDFS	distant disease-free survival
DMFS	distant metastasis-free survival
ER	estrogen receptor
FAC	5-fluorouracil, doxorubicin (Adriamycin), and cyclophosphamide
FEC	5-fluorouracil, epirubicin, and cyclophosphamide
FISH	fluorescence <i>in situ</i> hybridization
FOVs	fields of vision
FP	fractional polynomial
HER2	human epidermal growth factor receptor 2
HR	hazard ratio

IHC	immunohistochemistry
KiGE-index	Ki67, grade and ER
MAI	mitotic activity index
MFP	multivariable fractional polynomial
N0	lymph node negative
N1	1–3 positive lymph nodes
PAI-1	plasminogen activator inhibitor type 1
PgR	progesterone receptor
PI	prognostic index
PPH3	phosphorylated histone H3
RCS	restricted cubic spline
SERM	selective estrogen receptor modulator
SISH	silver enhanced <i>in situ</i> hybridization
SPF	S-phase fraction
TAM	tamoxifen
TMA	tissue microarray
TNM	tumor node metastasis
uPA	urokinase plasminogen activator
WHO	World Health Organization

# Abstract

Breast cancer is the most common cancer today among women in the Western world. In Sweden in 2015, more than 8 000 women were diagnosed with breast cancer. For a large number of patients, the prognosis is good. The majority of the patients are cured by primary surgical treatment, and many of the recurring patients survive for long periods of time. Additional (adjuvant) treatment is administered to prevent recurrences, although many patients are already cured by the primary surgery, thereby leading to over-treatment. Despite adjuvant treatment, more than 1 400 patients die from breast cancer every year, demonstrating the need for tools to better tailor treatment. The most important and routinely used prognostic and treatment predictive factors are age at diagnosis, tumor size, spread to the lymph nodes, histological grade, hormone receptors, factors for proliferation and expression of a growth factor receptor (HER2).

The overall aim of this thesis was to investigate prognostic and predictive markers in breast cancer and to find ways to improve the use of established factors, with a focus on proliferation and non-linear effects. Factors for proliferation were studied in different breast cancer patient cohorts and with different methods.

In **Study I**, we investigated whether an index, CAGE, based on cyclin A, histological grade, and estrogen receptor, could provide valuable prognostic information. CAGE was evaluated on tissue microarray slides, with samples from 219 premenopausal node-negative breast cancer patients. CAGE together with HER2 identified 53% of the patients as low risk with a 5-year distant disease-free survival of 95%. In **Study II**, we investigated whether the results of **Study I** could be confirmed in independent and larger series, replacing cyclin A with the worldwide used Ki67, creating KiGE. In chemo-naïve N0/N1 patients, KiGE alone identified 57% of the patients as low risk, with a 5-year event-free survival of 92%.

In **Study III**, we studied five factors for proliferation (mitotic activity index (MAI), phosphorylated histone H3 (PPH3), cyclin B1, cyclin A, and Ki67), separately and in combinations. Since all of the prognostic factors have pros and cons, our hypothesis was that combining factors would circumvent these issues. We demonstrated that a combination of MAI and cyclin A improved prognostication compared to use of the factors individually.



Although it is a well-known fact that information is lost when predictors are dichotomized (divided into two groups) or categorized into three groups, for clinical decision-making, this is often performed. In *Study IV*, we investigated whether the prognostication could be improved by postponing dichotomization/categorization until the last step in the process. We concluded that dichotomization definitely leads to information loss and should be avoided. Categorization improved prognostication, whereas using non-linear transformations only moderately improved the predictions.

In conclusion, the studies included in this thesis demonstrated that several markers for proliferation could be used and that combinations seems to improve prognostication, compared to examining factors individually. With these combinations, it was possible to identify patients with a low risk of developing recurrence so that adjuvant chemotherapy might be avoided. Dividing prognostic factors in more groups than two gives better predictive performance, but keeping them continuous was only moderately better. However, by postponing categorization until the very last step of the prognostic modeling strategy, more possibilities for individual predictions were enabled.

# Introduction

The processes by which normal cells evolve into tumor cells follow a complicated route. As described by Hanahan and Weinberg in 2000, cells must to acquire certain abilities, i.e., hallmark capabilities, to become tumorigenic and ultimately malignant [1]. These hallmark capabilities include the following: (1) sustaining proliferation, (2) evading growth suppressors, (3) resisting cell death, (4) enabling replicative immortality, (5) inducing angiogenesis, and (6) activating invasion and metastasis. In 2011, these hallmarks were revised, and two more hallmark capabilities and two enabling characteristics were added. The two added hallmarks are deregulating cellular energetics and avoiding immune destruction, and the two enabling characteristics are tumor-promoting inflammation and genomic instability and mutation [2] (Figure 1).

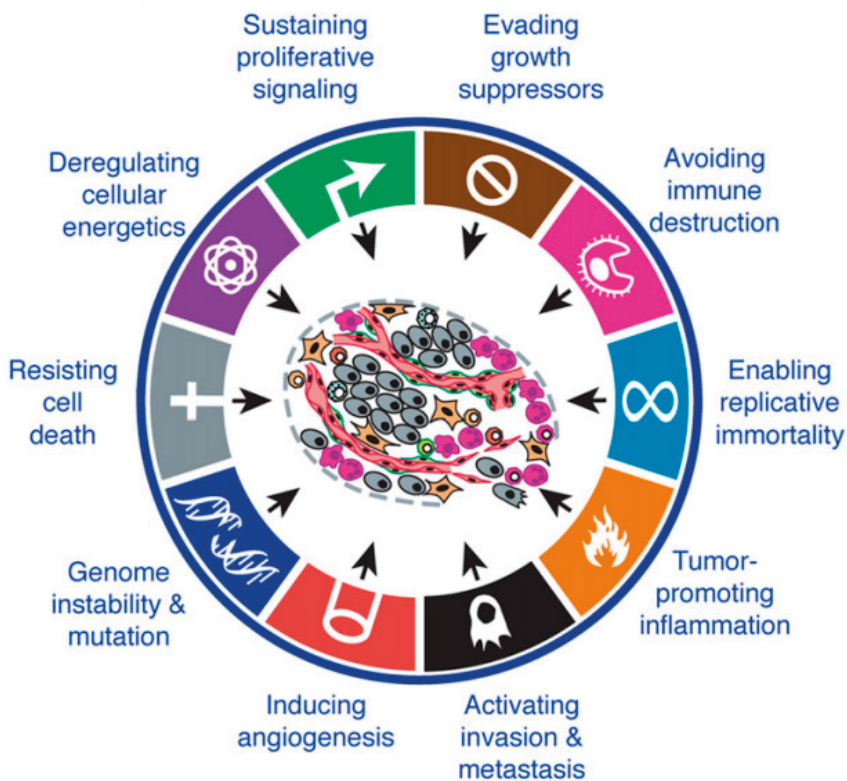
It is believed that most cancers must acquire the same hallmark capabilities and that the two enabling characteristics contribute to driving them forward. First and foremost is the development of genomic instability, which generates random mutations (including chromosomal rearrangements). The second characteristic involves tumor-promoting inflammation of (pre)-malignant lesions, which in turn promotes tumor progression [2].

As previously mentioned, one of the hallmarks of cancer is sustained proliferation, which is considered to be one of the most fundamental traits of cancer cells [2]. Cyclins regulates the cell cycle and hence the growth. Too many or too few cyclins at the wrong time point are important reasons for a cancer's uncontrolled growth [3]. The levels of cyclins vary during the cell cycle. They are synthesized and degraded as needed to drive the different stages of the cell cycle forward (Figure 2). They bind to and regulate the activity of the cyclin-dependent protein kinases (CDKs) [4]. CDKs never act on their own: they depend on the cyclins for proper functioning [5, 6]. The activities of the various cyclin/CDK-complexes must be modulated to impose control on specific steps in the cell cycle (Figure 3.). This modulation is achieved by changing the levels and availability of cyclins during various phases of the cell cycle. Tumorigenic cells have inactivated or lost the late G<sub>1</sub>-check point (R-point) [5].

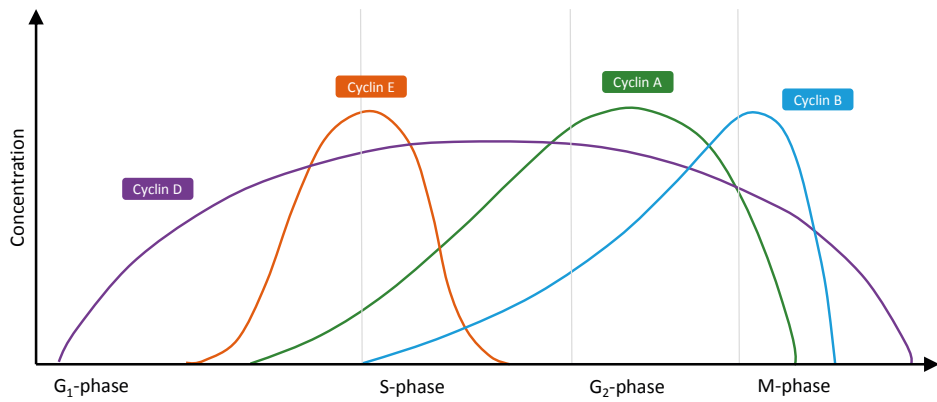
The cyclin/CDK-complexes have two main functions, as follows: (1) to activate the enzymes that drive the cell cycle forward and into respective cell phases; and (2) in a negative feedback loop, to induce degradation of the cyclins from previous

phases, thereby driving the cell into the next phase. Since the cyclins of respective phases are degraded continuously, the phase transitions are irreversible.

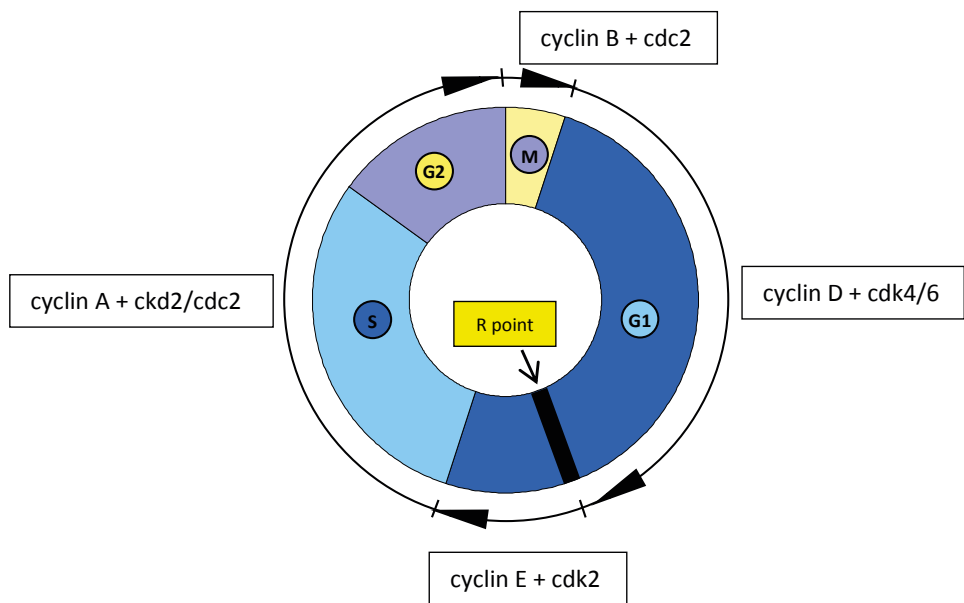
The main phases of the cell cycle are as follows.  $G_0$  (G stands for gap phase) is a non-growing state during which the cells wait for external signals before deciding to enter the active cell cycle.  $G_1$ , also known as the growth phase, is the phase during which the cells' supply of proteins is increased, the number of organelles, such as mitochondria and ribosomes are increased, and the cell grows in size.  $G_1$  is also the critical phase during which cells wait to enter into next phase. Damaged cells should be repaired before entering the S-phase. The  $G_1$  checkpoint ensures that everything is ready for DNA synthesis. The S-phase (DNA synthesis) is the phase during which DNA is replicated. In the  $G_2$ -phase, the cells prepare themselves for entering into M-phase. The  $G_2$  checkpoint ensures that everything is ready for mitosis, and finally the M-phase (mitosis) is the phase during which cells are divided [3, 5, 6] (Figure 3).



**Figure 1. Hallmarks of cancer.** Reprinted with permission from Elsevier [2]



**Figure 2. Levels of cyclins during the cell cycle**



**Figure 3. The cell cycle**

Cancer is a disease of uncontrolled cell proliferation. One might presume that the control system within cancer cells is organized in a different manner than that of normal cells. In truth, they use almost identical control systems. Cancer cells make only minor modifications to the control steps. Many types of cancer cells have inactivated one or more of the checkpoint controls, which helps them to accumulate mutant genes and altered karyotypes, driving their neoplastic growth [5-7].

# Breast cancer

## General background

Breast cancer is the most common cancer today among women in the Western world. In Sweden in 2015, 8 022 women and 41 men were diagnosed with breast cancer [8]. For a large number of patients, the prognosis is good. The majority of the patients are cured by primary surgical treatment, and many of the recurring patients survive for long periods of time.

In Sweden, the breast cancer incidence has increased by 1.6% annually over the last 20 years and by 2.6% over the last 10 years [8], but at the same time, the mortality has decreased. Due to mammographic screening (introduced in Sweden in the 1980s), breast cancers are detected at earlier stages [9]. Early detection, together with improved local and systemic adjuvant treatment, is considered to be important reasons for the improved cure rate of breast cancer patients [10-12]. However, unlike other cancers, breast cancer has no ‘cure point’, indicating that, regardless of how long after the breast cancer diagnosis, the risk of death among breast cancer patients will always be higher than for women of the same age without breast cancer [13, 14]. The survival is dependent on adequate surgery and the administration of adjuvant therapy to patients at risk of developing recurrence. Sweden has one of the best 5-year survival rates for breast cancer worldwide [15]. The 5-year survival rate is currently almost 90%, and the 10-year survival rate is greater than 80%. In 2014, 1 405 women died of breast cancer in Sweden [8]. The leading cause of breast cancer related death is distant metastasis. Metastasis is the process by which malignant cells spread from the primary tumor site to other distant vital organs. Breast cancer predominantly spreads to the lymph nodes, skeleton, lungs, liver, and/or brain.

The majority of breast cancers are sporadic, but 5-10% of patients have a family history of the disease that suggests an inherited predisposition [16]. The most obvious susceptibility genes with high penetrance mutations are BRCA1 or BRCA2 [17, 18]. However, a large proportion of patients with a family history of breast cancer do not carry germline BRCA gene mutations, and their increased risk might be caused by defects in moderate or low penetrance genes, by combinations of genes or by still unknown factors [19].

In addition to genetic inheritance and being a woman, known risk factors for developing breast cancer are increasing age, early menarche and late menopause, nulliparity or older age at first full-term pregnancy [20-22], and hormone replacement therapy after menopause (use for >10 years) [23, 24]. Current use of oral contraceptives or use during the previous ten years moderately increases the risk of developing breast cancer [25, 26]. Lifestyle factors such as high intake of saturated fat, being overweight or obese after menopause, or excessive alcohol consumption, are also risk factors for breast cancer [17, 27].

## Diagnostics

In Sweden, the majority of breast cancers are detected with mammography and the remaining cancers by the patient herself or her physician. Once a cancer is suspected, a diagnosis is reached by the triple diagnostic approach, which includes clinical examination (palpation), mammography, and investigation by fine or core needle aspiration [28, 29]. Further investigation by surgery is undertaken if at least one of these diagnostic tools provides a reason to suspect malignancy. When diagnosed, it is recommended that the patient be discussed at a multidisciplinary conference before surgery [30, 31]. Breast density, which has begun to be recognized as a risk factor for breast cancer, can decrease the diagnostic sensitivity and hence mask malignancies: therefore, ultrasound in combination with mammography might be recommended for young women (<30 years old) [32, 33].

## Prognostic and treatment predictive factors in breast cancer

Prognostic and treatment predictive factors in breast cancer can help to predict the risk of breast cancer recurrence and to facilitate the choice of optimal treatment. Although these factors have improved treatment decisions, not every patient receives optimal therapy: hence, some patients are over-treated and others are under-treated. To assess the risk of recurrence and to select patients who will benefit from adjuvant therapy, prognostic factors are used. A prognostic factor is correlated with recurrence in untreated patients. A predictive factor, in contrast, is correlated with the likelihood of a response to a certain treatment [34, 35]. The generally accepted prognostic factors are lymph nodes status, tumor size, age at diagnosis, histologic grade, hormone receptors (estrogen (ER) and progesterone (PgR)), human epidermal growth factor receptor 2 (HER2), and proliferation. ER

and HER2 are also used as predictive factors [36, 37]. Ki67, frequently used to determine the rate of proliferation, is only recommended by the St Gallen guidelines [36]. The American Society of Clinical Oncology (ASCO) guidelines, however, do not recommend the use of Ki67 for guidance in the choice of adjuvant chemotherapy [37]. The molecular profiles, *Oncotype DX*<sup>®</sup> and *MammaPrint*<sup>®</sup>, have been approved by the St Gallen and ASCO guidelines [36, 37]. Recently finished or ongoing trials evaluate the prognostic and predictive value of these profiles [38-40]. Web-based tools such as Adjuvant! Online or PREDICT can be used to estimate the risk of recurrence (within a certain time frame, e.g., 5 years) for single patients [41-43]. However, such predictions should be interpreted at the group level and not as predictions for individual patients [44].

## Proliferation

As previously mentioned, one of the hallmarks of cancer is sustained proliferation, which is considered to be one of the most fundamental traits of cancer cells [2]. Therefore, several methods have been developed to estimate the proliferation rate of tumor cells, and numerous studies have shown that high proliferation rates, irrespective of evaluation method, are associated with worse prognosis in breast cancer [4, 45-56]. Proliferation rates are, in general, higher in triple-negative (ER/PgR/HER2-negative), HER2-positive and receptor-negative tumors, compared to ER-positive tumors [57-60]. Furthermore, it seems as if the added prognostic value of proliferation is most pronounced in ER-positive/HER2-negative tumors [61, 62]. Measurement of proliferation rates can be performed either by the assessment of single factors, e.g., using immunohistochemistry (IHC), or as the main common denominator of different genetic profiles [63, 64].

A high proliferation rate has also been shown to be associated with a more favorable response to chemotherapy [65, 66]. Hence, a validated proliferation marker might be useful not only for prognostic purposes but also, more importantly, for treatment predictive purposes.

### Markers of proliferation

#### *Mitotic activity*

Mitosis count is one of the three components of histological grade (together with tubular formation and nuclear atypia) [67]. According to the Elston and Ellis grading system, mitoses should be assessed in ten fields of vision (FOVs), starting at the periphery of the tumor [67]. According to the World Health Organization



(WHO), the number of mitoses is categorized into three groups as a part of histological grading: group 1 (0–5 mitoses), group 2 (6–10 mitoses), and group 3 (>10 mitoses) [68]. The WHO has published recommendations for a more strict mitosis counting procedure: mitotic activity index (MAI) [69]. Instead of ten random FOVs, ten FOVs in ten neighboring consecutive fields should be assessed. Furthermore, the manner in which field diameter is defined is stricter. When the WHO protocol has been strictly followed, MAI has been highly reproducible [47].

Some studies have shown that the prognostic value of histological grade is mainly due to mitosis [55, 70-72]. Other studies have shown that nuclear atypia is the most important part of histological grading [73]. However, problems with reproducibility have been reported [74-77].

#### *Thymidine labeling index*

Another of the first markers for the measurement of proliferation rate was thymidine-labeling index (TLI), which measures the proportion of cells synthesizing DNA at a given time. Fresh tumor material is required. The tissue is incubated in medium containing thymidine labeled with the nucleotide tritium ( $^3\text{H}$ ). The level of incorporated  $^3\text{H}$ -thymidine into the tissue is measured, and a ratio of labeled to non-labeled DNA is calculated [78, 79]. Several studies have shown the prognostic and predictive importance of TLI [66, 78, 80-82].

#### *S-phase fraction*

Using a flow cytometer, the S-phase fraction (SPF) can be analyzed either in paraffin-embedded or fresh frozen material. However, the majority of publications showing correlations with prognosis in breast cancer were performed on fresh frozen material. The first publication showing a correlation between SPF and prognosis in breast cancer was published in 1984 [83]. Before the cells are processed through the flow cytometer, they are mechanically disrupted and stained with propidium iodide. DNA histograms are obtained, which show the distribution of cells corresponding to the phases of the cell cycle, in which the major peak usually represents the  $G_0/G_1$ -phase. Some of the disadvantages are that, for best performance, fresh frozen tissue is required, a larger tumor volume than, for instance, for IHC is needed, and a mixture of normal cells and cancer cells is analyzed. Several studies have shown a correlation between a high SPF and poor prognosis in breast cancer [84-86]. The measurement of SPF by flow cytometry, combined with PgR and tumor size, was used as a prognostic index in parts of Sweden and Finland in the 1990s [86, 87].

#### *Ki67*

Proliferation can also be evaluated by measuring Ki67, which is a cell cycle antigen. It was originally described in Kiel (1983): hence, Ki stands for Kiel, and

67 is the number of the original clone in the 96-well plate from which it was derived [88]. Ki67 is expressed by all proliferating cells in the body, and it is present in all active phases of the cell cycle: G<sub>1</sub>, S, G<sub>2</sub> and M, peaking during the mitotic phase, (hence not in G<sub>0</sub>) [89, 90]. The exact function of Ki67 is unknown, but a role has been suggested in ribosomal RNA-synthesis [91, 92].

An association between the overexpression of Ki67 and worse outcomes in early breast cancer has been observed in several studies [47, 53, 93, 94]. Ki67 might also be an indicator of better response to chemotherapy [95]. The independent prognostic utility of Ki67 has been shown, especially among patients with ER-positive tumors [61]. Expression of Ki67 can separate ER-positive tumors into two subclasses, a low proliferative group ('luminal A-like'), associated with a favorable prognosis: and a high proliferative group ('luminal B-like'), associated with a less favorable prognosis [60].

Because Ki67 carries both prognostic and predictive information, and it is affordable and easily measured on paraffin embedded tumor material, it is widely used. However, there is not yet an international consensus on the assessment of Ki67, although recommendations have been proposed [96, 97]. The ASCO guidelines from 2016 still do not recommend the use of Ki67 to guide the choice of adjuvant chemotherapy [37], because of a lack of consensus concerning methodology, especially cut-off values [98]. Different antibodies and scoring systems have been used [99]. However, in the 2011 St Gallen guidelines, Ki67 was recommended to be used to distinguish between slowly proliferating 'luminal A-like' and rapidly proliferating 'luminal B-like' tumors [100]. Ki67 can furthermore be used to subdivide histological grade 2 into groups with different prognosis [61, 62]. Studies have shown poor reproducibility, and efforts have been undertaken to standardize assessment, choice of cut-off, antibody, etc., but issues with poor reproducibility persist [96, 98, 101-103].

### *Cyclins*

Cyclins can be studied on either the DNA or the protein level, using, e.g., fluorescence *in situ* hybridization (FISH) or IHC. In this thesis the focus is on the protein level as measured by IHC.

#### Cyclin D

Cyclins D1, D2 and D3 exist: however, in breast cancer, the most studied cyclin is cyclin D1, which is associated with CDK4/6 to ensure the advancement of the G<sub>1</sub>-phase. Overexpression of cyclin D1, often due to amplification of the coding gene, is found in a large proportion of breast cancers but only rarely in benign and premalignant lesions. Although it is involved in the activation of proliferation, studies have shown links to a less malignant phenotype, with high levels of cyclin D1 correlated with better prognosis [104-108]. In contrast, an association with

favorable prognosis has been shown on the protein level, while amplification of the gene has shown an association with poor prognosis [109, 110].

### Cyclin E

Cyclins E1 and E2 localize to different sites in breast cancer cells, but they also co-localize within the cell [111]. Although genes on different chromosomes encode cyclin E1 and cyclin E2, they both activate CDK2 [111]. Together with CDK2, cyclin E2 forms a catalytically active complex, and it has been suggested that cyclin E1 and cyclin E2 phosphorylates different sets of substrates and thereby regulate the progression of cells through the G<sub>1</sub>-phase and into the S-phase [112]. The critical determinant for entering into S-phase is cyclin E in cooperation with CDK2. Several studies have identified cyclin E as important in breast cancer prognosis [113-118]. Recently, a study by Karakas et al., showed that cytoplasmic cyclin E, together with phosphorylated CDK2, could be used as a biomarker for aggressive breast cancer because it predicted shorter recurrence-free and overall survival [119].

### Cyclin A

Cyclin A1 is expressed during meiosis and embryogenesis, while cyclin A2 is expressed in dividing somatic cells [120]. Cyclin A2 is responsible for the initiation of DNA replication and it is also involved in G<sub>2</sub> to M-phase transition. It can activate two different CDKs: CDC2 (also called CDK1) and CDK2. The level of cyclin A2 rises in the early S-phase and falls in the mid M-phase. As cells enter the S-phase, cyclin A2 replaces cyclin E as the partner of CDK2, thereby enabling the S-phase to progress. As the cell moves further and enters the G<sub>2</sub>-phase, cyclin A2 switches partner and binds to CDC2. Like cyclin E, cyclin A2 has been associated with worse outcomes in breast cancer patients in some studies, but other studies have been unable to confirm this finding (perhaps due to a lack of consensus concerning cut-off values, analogous to Ki67) [4, 45, 49, 121-126]. In this thesis, cyclin A2 is hereafter referred to as cyclin A.

### Cyclin B

It has been suggested that cyclin B1 is involved in chromatin condensation and that cyclin B2 is involved in disassembling the Golgi apparatus [127]. The final step in the cell cycle is controlled by the cyclin B1–CDK1/CDC2 complex, also known as the mitosis-promoting factor. To date, only a few publications have found that high expression of cyclin B1 was correlated with poor survival [128-130].

### Cyclin H

Recently, another cyclin, cyclin H, has been shown to be correlated with outcomes in breast cancer, and once again, it was most pronounced for ER-positive breast

cancer. Patel et al., found that the majority of tumors with high expression of cyclin H were ER-positive with a better prognosis [131].

### *Phosphorylated histone H3, PPH3*

Phosphorylation of histone H3, called PPH3 or PHH3, occurs late in the G<sub>2</sub> and M-phases, and it is involved in initiating chromatin condensation. It is needed for the cell to progress to mitosis. PPH3 is assessed with IHC and is evaluated similarly to MAI. Positive cells are counted in ten consecutive FOVs, starting at the periphery in the area with the highest proliferation. Difficulties in counting mitotic figures can be associated with condensed mitoses due to hypoxia or suboptimal fixation: using PPH3 circumvents this issue. Studies have shown a correlation between high expression of PPH3 and poor prognosis [55, 132-136].

## Other prognostic and treatment predictive factors in breast cancer

### **TNM classification**

The most important prognostic factor is the TNM-classification, in which T stands for tumor size, N for spread to the regional lymph nodes, and M for the presence of distant metastasis [137]. TNM classifies the tumor and provides a strong indication of prognosis. Patients are classified into five prognostic stages (0–IV), and higher stages have worse prognosis.

Patients with larger tumors have worse prognosis than patients with smaller tumors [138]. Accordingly, tumor size is divided into four groups with increasing risk for recurrence: T1: 1–20 mm; T2: 21–50 mm; T3: >50 mm; and T4: a tumor of any size, with skin or chest wall involvement.

The more lymph nodes that are involved, the higher the risk is for developing distant metastases [35]. Lymph node involvement is divided into: N0: no involvement; N1: 1–3 positive lymph nodes involved; N2: 4–9 lymph nodes involved; and N3: ≥10 lymph nodes involved.

If the patient has a tumor that is less or equal to 2 cm in size, with no spread or dissemination to the lymph nodes in the axilla or to distant sites, the tumor is classified as stage I, with better prognosis than for higher stages [35]. Stage IV tumors are considered incurable [139].

Since the introduction of mammographic screening, the fraction of small tumors with no spread to the lymph nodes has increased: hence tumors are diagnosed and surgically removed at lower stages today [9].

### **Age at diagnosis**

Approximately 5–10% of women diagnosed with breast cancer are younger than 40 years old [8]. Studies have showed that breast cancer in young women is associated with worse prognosis [140, 141]. Fredholm et al., showed that women <35 years old had worse breast cancer-specific survival (BCSS) (69% 10-year breast cancer-specific survival) compared to 76% for 35–39 years old, and 84% for 40–49 years old [142]. Their study reported that the risk for relapse in young women was most pronounced in stage I–II and luminal B tumors. Kroman et al., found a similar age trend, with younger women having a higher risk of dying: however this trend vanished in patients who received chemotherapy. They suggested that young age alone should be considered a high risk factor [143]. Elderly patients in general have better outcomes than patients younger than 60 years old [144]. The majority of newly diagnosed breast cancer patients are 50 years old or older [8]. In Sweden in 2015, the median age at diagnosis was 64 years old [8].

### **Histological grade**

Histological grading, analyzed on hematoxylin/eosin-stained tissue slides using a light microscope, is the combined evaluation of the presence of tubules, how pleomorphic the nuclei are, and mitotic activity. Each category is scored 1–3, and these scores are added into a histological grade score (3–9 points). Grade 1 is defined as 3–5 points, grade 2 as 6–7 points, and grade 3 as 8–9 points. Grading was first described by Bloom and Richardson [145] and was later revised by Elston and Ellis in 1991 [67]. Several studies have shown correlations with prognosis [75, 146, 147], other studies have shown problems with reproducibility [67, 75, 147, 148].

### **Estrogen and progesterone receptors: ER and PgR**

Estrogens play an important role in the development of breast cancer. The majority of all breast cancers are ER-positive (85% in Sweden in 2015) [149]. The incidence of ER-positive cases increases with age. There are two types of ER, ER $\alpha$  and ER $\beta$ . ER $\alpha$  has proved to be correlated with sensitivity to endocrine treatment.

ER $\beta$  has not been as well studied. The term ER in this thesis refers to ER $\alpha$ . By also evaluating PgR, it is possible to improve the prediction of outcome, and in the St Gallen guidelines 2013, PgR was included to guide the distinction between ‘luminal A-like’ and ‘luminal B-like’ breast cancer [36]. However, according to the latest meta-analysis from EBCTCG, PgR was not predictive of response to endocrine treatment [150].

Both ER and PgR are present in the cell nucleus and can be routinely detected by IHC in the clinic.

## **Human epidermal growth factor receptor 2**

The cell surface tyrosine kinase receptor human epidermal growth factor receptor 2 (HER2) is a member of the epidermal growth factor receptor family. HER2 is both prognostic and predictive [151, 152]. Anti-HER2-targeted therapy has been administered since the late nineties for metastatic breast cancer and since 2005 as an adjuvant treatment. In Sweden, 12-15% of breast cancers overexpressed HER2 and/or were amplified [149, 153].

The HER2 protein is assessed by IHC and gene amplification by FISH, chromogenic *in situ* hybridization (CISH), or silver enhanced *in situ* hybridization (SISH). Since 2011, using the Inform HER2 Dual ISH, chromosome 17 and the HER2 gene can be chromogenically stained at the same time.

## **Molecular profiling**

Historically, prognostic factors have been identified and used to stratify patients into subgroups with distinct biology, prognosis and response to treatment. For the majority of these factors, evaluation was/is performed on a single protein level. Since the introduction of gene expression profiling techniques, it is possible to perform this analysis on a multi-gene level [154]. With distinct combinations of genes, Perou and Sorlie pioneered the use of these new techniques to identify molecular classes [57, 63, 155]. Others soon followed [58, 156-158]. Important results from these studies are that ER-positive breast cancer is a distinctively different form of breast cancer than ER-negative [159]. Furthermore, molecular subtypes identified two luminal subtypes (luminal A and B), which are ER-positive with different expression of PgR and Ki67, HER2-enriched and basal-like (ER/HER2/PgR-negative) tumors. Luminal A tumors, which are low proliferating and HER2-negative, generally have better outcomes than luminal B tumors [160]. Since the introduction of these techniques, a plethora of prognostic breast cancer profiles have been reported [156, 158, 161-164]. Many studies have shown the

importance of proliferation [60, 158, 165, 166]. To date, only a few profiles are in clinical use and are recommended in international guidelines [36]. Recommended by the ASCO guidelines are *Oncotype DX*<sup>®</sup> recurrence score and PAM50-Risk of Recurrence Score (ROR) [37, 156, 162]. Furthermore, the clinical utility and validity are being tested in recently closed or ongoing trials, such as MINDACT (testing the utility of MammaPrint<sup>®</sup>), TAILORx and RxPONDER (testing the usefulness of *Oncotype DX*<sup>®</sup>) [38-40, 167-169].

Transcriptomic-based assays are being increasingly used in clinical practice, particularly in the US. However, due to complicated technologies, high required expertise and expense, it is not available for the majority of patients worldwide. As an alternative, the St Gallen International Expert consensus 2013 panel has approved surrogate criteria for determining the molecular subtypes [36]. This surrogate classification considers different combinations of the four biomarkers, ER, PgR, HER2, and Ki67, and can be used when it is not possible to perform genomic tests, due to cost or other reasons. The markers are combined as follows:

‘Luminal A-like’ (ER-positive, PgR high, HER2-negative, Ki67 low);

‘Luminal B-like (HER2-negative)’ (ER-positive, HER2-negative, and at least one of Ki67 high or PgR low);

‘Luminal B-like (HER2-positive)’ (ER-positive, HER2-positive, any Ki67 and any PgR);

‘HER2 positive’ (ER-negative, PgR low, HER2-positive); and

‘Triple negative’ (ER-negative, PgR low, HER2-negative).

## **Other promising prognostic factors**

Examples of other promising prognostic factors are circulating tumor cells (CTCs), tumor-infiltrating lymphocytes, and circulating tumor DNA: however these factors do not have the evidence required to be included in the current guidelines [37, 97]. According to the ASCO guidelines, urokinase plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1) can be used for ER/PgR-positive, HER2-negative breast cancer: however the level of recommendation is weak [37].

## Treatment

After surgery, a combination of different treatments is administered to a large proportion of patients to prevent disseminated cancer cells from creating metastases at distant sites. Treatments can be administered as local therapy (radiotherapy) or systemic therapy (endocrine, chemotherapy, and/or anti-HER2 targeted treatment). Systemic therapy can also be administered before surgery (neoadjuvant). For patients with metastatic breast cancer, systemic palliative treatment is given. If the primary tumor is inoperable due to the size of the tumor, neoadjuvant therapy can be administered to shrink the tumor and render it surgically removable. In Sweden, postmenopausal, node-positive patients can be offered bisphosphonate treatment as an additional adjuvant treatment [31].

If not elsewhere stated, the recommendations are according to the Swedish guidelines and the presented figures represents Swedish breast cancer patients [31].

### Surgery

The primary therapy is removal of the tumor, either by performing breast-conserving surgery (BCS) or by removing the whole breast, i.e., mastectomy [170, 171]. Cancers that are detected by mammography are statistically more likely to be treated with BCS [172]. In Sweden, three quarters of breast cancer patients are operated on with BCS (if the tumors are <30mm) [149]. The sentinel lymph node procedure (introduced in 1990) is used to decide whether more lymph nodes must be removed or not [173, 174]. Injection of radioactively labeled albumin and a blue dye identifies the sentinel nodes [175]. The sentinel nodes are surgically removed and are sent to the pathology department, where they are examined perioperatively on frozen sections. If the lymph nodes contain metastases  $\geq 0.2$  mm in diameter, further axillary dissection is performed [31]. Ongoing trials are studying whether axillary dissection can also be omitted when macrometastases (>2mm) are detected [176, 177].

### Radiotherapy

Postoperative radiotherapy is administered as a precaution in order to reduce the risk of local and regional recurrences. Patients who have undergone BCS are offered radiotherapy. Furthermore, radiotherapy is offered to patients who have undergone mastectomy if the tumor is >50 mm, or at least one positive lymph node is detected in the axilla [31]. Several studies comparing mastectomy with



BCS followed by radiotherapy have shown no difference in survival between the two methods [178-181].

## **Endocrine therapy**

If a tumor is considered ER-positive, which in Sweden indicates that the tumor expresses >10% ER (the St Gallen [97] and ASCO guidelines [37] recommend a cut-off of >1% for positivity), endocrine treatment is administered to the great majority of patients [11, 31].

Endocrine treatment alternatives consist of either tamoxifen (TAM), which is an anti-estrogen and binds to ER in an antagonistic manner, aromatase inhibitors (AIs), which target the enzyme aromatase (aromatase is involved in the process of converting androgens into estrogens), or drugs suppressing the ovarian production of estrogens. Selective ER modulators (SERMs), such as TAM, antagonize ER function. TAM belongs to the SERM group of drugs that mimic the effects of estrogen in some tissues, while opposing them in others [182]. For instance, TAM inhibits ER activation in the breast while stimulating it in the endometrium [183].

Five years of adjuvant TAM (vs. no treatment) reduces the relative risk of recurrence by approximately 50%, and mortality by 30% [184]. Five years of TAM is recommended as adjuvant therapy, but a benefit of prolonged treatment has been reported [185, 186]. TAM has effects in both pre- and menopausal patients, whereas AIs are effective only in postmenopausal women because AIs do not suppress estrogen production in the ovaries [187, 188]. Compared to five years of TAM given to postmenopausal breast cancer patients, AIs reduce 10-year relative breast cancer mortality by 15% [184, 189]. Selected patient groups, mainly node-positive, are offered prolonged endocrine treatment, but it is today not recommended to administer AIs for more than five years. However, studies of more than five years of AI are ongoing [190].

## **Chemotherapy**

Adjuvant chemotherapy is recommended for patients at high-risk for recurrence, i.e., either young age, high proliferation rate, high histological grade, node-positivity, large tumor size, and HER2-positive, ER-negative and/or PgR-negative tumors. The most common chemotherapy regimen consists of an anthracycline-based combination, i.e., FEC (5-fluorouracil, epirubicin, and cyclophosphamide) or FAC (5-fluorouracil, doxorubicin (Adriamycin), and cyclophosphamide), followed by a taxane, either paclitaxel or docetaxel [191].

## **Anti-HER2 therapy**

If a patient has an HER2-positive tumor, indicating an IHC-score of 3+ and/or with the HER2 gene amplified, the monoclonal antibody trastuzumab, targeted at HER2, is recommended for use in the adjuvant setting. In 2015, HER2-positive cancers represented 14% of cases in Sweden [149]. The monoclonal antibody pertuzumab and TMD-1 are other anti-HER2 directed drugs that are being studied in the adjuvant setting [192].

## **Positive and negative effects of adjuvant treatment**

Large meta-analyses have shown that breast cancer mortality is reduced, and overall survival is improved by adjuvant treatment [11, 193]. However, all adjuvant treatment regimens also induce side effects. After radiotherapy, the typical local acute side effects are erythema and pneumonitis [194]. Late side effects of the cardiovascular system with increased cardiac mortality have been documented in several studies with long-term follow-up, among others, in the SB II:1 trial from the South Swedish Breast Cancer Group [195].

For those patients treated with adjuvant chemotherapy, the majority experience mild or moderate nausea and vomiting [196]. The most dangerous side effect of chemotherapy is bone marrow suppression and risk of neutropenic fever.

The most common side effects with TAM are menopausal symptoms and increased risk of thrombosis and pulmonary embolism [197]. A common side effect of AIs is arthralgia and AIs can also cause osteoporosis [184, 198]. TAM is generally better tolerated, at least in postmenopausal patients, and it has been reported to delay myocardial arteriosclerosis [199].

In addition to the side effects, many treatments are expensive. Nevertheless, the benefit from adjuvant therapy outperforms the side effects, and strategies for personalizing treatment are being investigated. Although a large proportion of breast cancer patients are cured by surgery alone, the majority receives systemic treatment despite the risk of recurrence being low. Number one of the top ten research questions in the web-based consultation performed by Dowsett et al., was to identify tools to better guide the selection of patients for whom adjuvant chemotherapy could be avoided [200]. This thesis focuses on improved prognostication and is thus one of many approaches for attaining this goal.

## Background to the thesis

When this thesis was initiated, other groups had shown that, using gene expression profiles, patients with ER-positive breast cancer and also breast cancer classified as histological grade 2, could be subdivided into two groups with distinctively different prognosis [59, 63, 201]. In the ER-positive subgroup, genes associated with proliferation seemed to be the most important, whereas genes associated with immune response were more important among ER-negative breast cancers [59]. Since the most important genes in the ER-positive subgroup were associated with proliferation, we wanted to investigate whether the use of a single factor (Ki67), could provide similar results. The answer was yes [61]. At the same time, Ahlin et al., showed that cyclin A could be used as a marker of proliferation [126]. Because cyclin A seemed easier to evaluate (contrast-rich and crisp staining), we wanted to investigate this further.

Although it is a well-known fact that information is lost when predictors are dichotomized or categorized, for clinical decision-making, this is often performed. One possible strategy could be to postpone dichotomization/categorization until the last step in the modeling process, thereby exploiting the information included among continuous predictors. Surprisingly few publications have addressed this issue, and we addressed it in this thesis.

# Aims of the thesis

## Overall aim

The overall aim was to investigate prognostic and predictive markers in breast cancer and to find methods of improving the use of established factors, with a focus on proliferation and non-linear effects. These results could be used in clinical decision-making to determine which patients need or do not need adjuvant systemic treatment, especially chemotherapy, after primary surgery.

## Study I

Our aim was to evaluate the performance of the new variable CAGE: a combination of cyclin A, histological grade, and ER, in premenopausal node-negative breast cancer patients.

Our hypothesis was that an index based on cyclin A, ER, and histological grade would provide valuable prognostic information. Furthermore, we wanted to investigate the prognostic importance of cyclin A alone and in subgroups with different histological grade, and in relation to genetic grade.

## Study II

The aim was, in a larger dataset, to confirm the previously defined prognostic index (CAGE) and, instead of cyclin A, to combine the worldwide used proliferation marker Ki67 with histological grade and ER (KiGE).

Our hypothesis was that KiGE was equally valid to CAGE and that it could confirm the prognostic value of the combination of a proliferation factor with histological grade and ER.

### **Study III**

The aim was to evaluate the prognostic value of MAI, PPH3, cyclin B1, cyclin A, and Ki67, alone and in combinations, in node-negative breast cancer.

Our hypothesis was that a combination of proliferation factors would improve prognostication.

### **Study IV**

The primary aim was proof of principle, i.e., to evaluate whether accounting for non-linear effects of the three factors age at diagnosis, tumor size, and number of positive lymph nodes improved prognostication.

Our hypothesis was that, by keeping the predictors continuous for as long as possible in the modeling process, prognostication would be improved.

# Patients

## Studies in thesis

## Original cohorts/studies

Study I and III, (N0)

SB 91:B

Study II, (chemo naïve N0/N1)

SB II:2 pre and post  
Malmö cohort  
Bone marrow metastases cohort  
Odense cohort  
Uppsala study

Study IV

SB II:1 pre and post  
SB II:2 pre and post  
Bone marrow metastases cohort  
Odense cohort  
Kalmar cohort

## Study I and III

The patients came from the SB 91:B study population ( $N = 237$ , 59 with distant metastases) [202], which between 1991 and 1994 included premenopausal lymph node-negative women. Adjuvant treatment was administered in 29 (13%) of the patients. It was a trial with the purpose of studying the prognostic importance of prospectively analyzed SPF. This trial was also a part of the node-negative breast cancer (NNBC)-trial [86]. Fourteen patients were excluded due to lack of paraffin blocks. Cases were also excluded if fewer than 200 cells were counted for cyclin A, leaving 219 patients included in *Study I*.

In **Study III**, samples from  $N = 221$  patients were evaluated for PPH3,  $N = 195$  for MAI,  $N = 199$  for Ki67, and  $N = 217$  for cyclin B. At the time of **Study III**, one additional case of cyclin A was excluded due to an error in the database, leaving  $N = 218$  evaluated for cyclin A in **Study III**.

## Study II

In total, 1 854 women with primary breast cancer originating from two randomized clinical studies, three cohorts and one case-control study were included. Only patients who were node-negative or with 1–3 positive lymph nodes were included. Patients were excluded if they had received chemotherapy or if information on adjuvant therapy, Ki67, histological grade or ER was missing. The patients came from the following patient materials:

SB II:2-pre ( $N = 221$ , 68 with distant recurrences): Between 1986 and 1991, women were included in this randomized trial of premenopausal stage II breast cancer, with the aim of comparing the effects of two years of TAM vs. no adjuvant systemic treatment [203, 204].

SB II:2-post ( $N = 166$ , 22 with distant recurrences): Between 1983 and 1991, women were included in this randomized trial studying two vs. five years of TAM in postmenopausal women [205].

The Malmö cohort ( $N = 217$ , 32 with recurrences): This consecutive series in Malmö enrolled breast cancer patients between 1988 and 1992 with the purpose of evaluating biomarkers on tissue microarray (TMA) slides [206].

The Bone marrow metastases cohort ( $N = 379$ , 27 with distant recurrences): This consecutive series in the South Swedish Health Care Region enrolled breast cancer patients between 1999 and 2003 with the purpose of studying cytokeratin positive cells in bone marrow aspirates from the sternum [207].

The Odense cohort ( $N = 539$ , 86 with distant recurrences): This consecutive series in Odense enrolled patients between 1980 and 1990 with the purpose of evaluating the prognostic value of Chalkley counting in a population-based cohort [208].

The Uppsala study ( $N = 166$  cases and 166 controls) was used as an additional dataset. Patients were enrolled in the study between 1993 and 2004, and within this cohort, cases were defined as women who died from breast cancer. The purpose of the study was to evaluate the prognostic value of cyclin A in node-negative patients, and furthermore to validate previously suggested cut-off values [126].

## Study IV

A derivation set was compiled of 4 477 women with primary breast cancer originating from four multicenter randomized clinical studies and three cohorts. The patients came from the following patient materials:

SB II:1-pre ( $N = 362$ , 125 with distant metastases): Between 1978 and 1983, women were included in this randomized trial with the purpose of evaluating the effects of chemotherapy and radiotherapy, alone and in combination [195].

SB II:1-post ( $N = 624$ , 237 with distant metastases): Between 1978 and 1985, women were included in this randomized trial studying the effects of TAM for one year and radiotherapy, alone and in combination, in postmenopausal women [195, 209].

SB II:2-pre ( $N = 561$ , 233 with distant metastases) [203], SBII:2-post ( $N = 1,553$ , 382 with distant metastases) [205], the Odense cohort ( $N = 841$ , 251 with distant metastases) [208], and the Bone marrow metastases cohort ( $N = 536$ , 87 with distant metastases) [207]. For more information on the last four studies, see above beneath the subheading **Study II**.

A limited number of patients ( $N = 537$ ) were excluded due to missing information on follow-up, number of positive lymph nodes, and/or tumor size.

The Kalmar cohort ( $N = 1 132$ , 311 with distant metastases), a consecutive series of primary breast cancers, was used as a validation dataset.



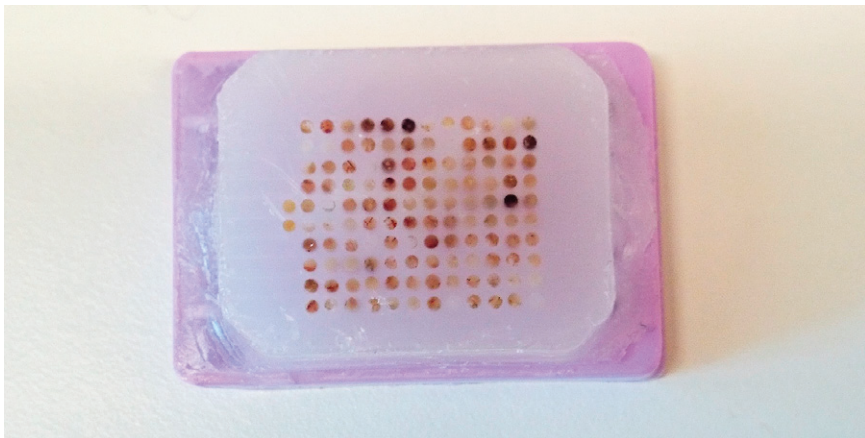


# Methods

## Tissue microarray

Tissue microarray (TMA), as a high-throughput technique for paraffin-embedded material, was introduced in 1998 [210]. One of the advantages of TMA is that it cost-effectively enables simultaneously staining of hundreds of samples on only one slide meaning that, using only a limited amount of tissue, fewer amounts of antibody are necessary, with minimal differences in staining conditions. The disadvantages include representability and heterogeneity, i.e., the risk of not capturing the region of interest in the tumor tissue due to the small tumor area in the sample.

In this thesis, TMA-blocks were prepared from paraffin-embedded blocks using a manual arrayer (Beecher Instruments Inc., Sun Prairie, WI, USA). Two 0.6-mm cores (*Studies I and II*) and cores of 1.0 mm (*Study III*) were obtained from representative areas (i.e., with tumor cells) of each primary tumor block and were transferred to a recipient block. Then, 3–4  $\mu\text{m}$  sections were cut from each block and transferred to glass slides (Menzel Superfrost<sup>®</sup> Plus), dried at room temperature and then incubated in a heat chamber for 2 hours at 60°C.



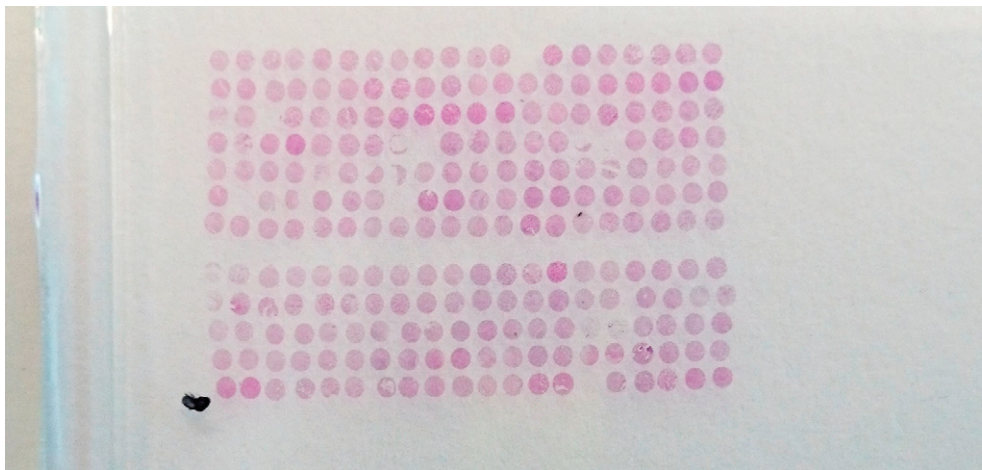
**Figure 4. Breast cancer TMA paraffin block.**

# Immunohistochemistry

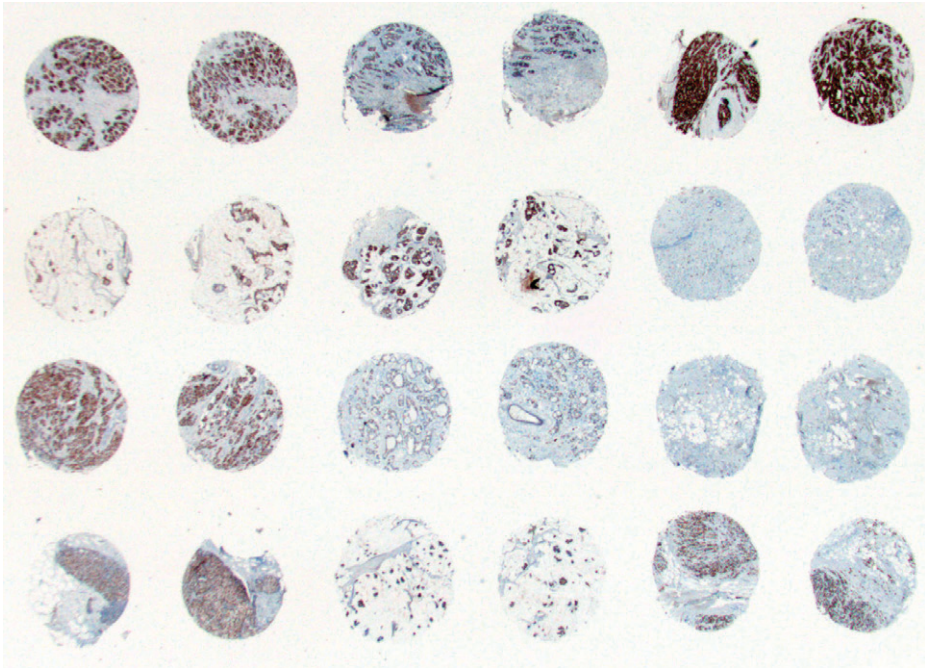
In tumor diagnosis, immunohistochemistry (IHC) is commonly used to detect antigens and to assess the distribution and localization of proteins in the tissue. Its advantages include ease of methodology, low costs, and high efficiency. Furthermore, distinct cancer cells can be evaluated, thereby avoiding evaluation of non-malignant cells in the tumor. Its disadvantages include choice of antibody, and bad staining quality depending on poor tissue fixation and poor reproducibility of evaluators. These disadvantages underpin the need for defined and validated guidelines to render the choice of cut-offs easier and the use of established antibodies, and to guide the methods of evaluation.

Immunohistochemistry was used in *Study I* (cyclin A), *Study II* (Ki67), and *Study III* (PPH3, cyclin B1, cyclin A and Ki67). To visualize the proteins of interest, primary antibodies directed against the antigen were used. A secondary antibody, usually conjugated with biotin directed to the primary antibody, was added and visualized with a chromogen, e.g., diaminobenzidine (DAB). The protein of interest was colored brown, and the surrounding tissue was colored blue with hematoxylin.

The TMA-slides were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol solutions (from absolute ethanol to distilled water). Immune staining was performed in an automated Autostainer *Plus* (Dako Denmark A/S), according to the manufacturers procedures<sup>7</sup>.



**Figure 5. Breast cancer TMA-slide.** Stained with hematoxylin/eosin. White areas in the rows represent missing TMA-cores.



**Figure 6. Breast cancer TMA-slide.** TMA-cores stained for Ki67 and counterstained with Mayer's hematoxylin (blue). Brown color represents cells positive for Ki67.

### **Antibodies used in this thesis**

#### *Cyclin A*

The cyclin A2 (NCL-Cyclin A, 1:100, Novocastra Laboratories) antibody was applied at room temperature. The staining was evaluated by one more experienced (Cecilia Ahlin, CA) and one less experienced (Carina Forsare, CF) investigator. Numbers of positive cells were counted in the TMA-cores with the most positivity, and a minimum of 200 cells were counted. If there were not sufficient cells in the first core, additional cells in the second core were counted until 200 cells in total were counted. High cyclin A was predefined at the 7<sup>th</sup> decile of the cyclin A distribution, which corresponded to  $\geq 15\%$  (CA) and  $\geq 17\%$  (CF) positively stained tumor cells, respectively. The agreement between the investigators evaluations of cyclin A expression was good (as measured by kappa statistics, value of 0.71). In **Studies I** and **III**, we chose to focus on the results from the more experienced investigator (CA).

### *Ki67*

The analysis of Ki67 has been described elsewhere [61, 211]. In short, the MIB-1 antibody (DAKO, K5001, 1:500, Copenhagen, Denmark) was used. As predefined, cases above the 7<sup>th</sup> decile of the empirical Ki67 distribution (which corresponded to 20% positive cells) was used as cut-off.

### *PPH3/PHH3*

The anti-phosphohistone H3 antibody (ser 10 Upstate #06-570, Lake Placid, NY, USA) at a 1:1500 dilution was used. Previous assessments of PPH3 were performed on whole sections, starting at the periphery of the tumor in ten consecutive FOVs with a total area of 1.59 mm<sup>2</sup>. To mimic whole sections, the number of positively stained nuclei in one TMA-core was multiplied by 1.59 and divided by the total area of the TMA, 1.13 mm<sup>2</sup>. The cut-off value was predefined at the 7<sup>th</sup> decile, which corresponded to  $\geq 7$  positive cells.

### *MAI*

MAI was assessed by one experienced pathologist (JB) on hematoxylin-eosin whole sections according to the MMMCP 1987 protocol [212]. The area with the highest proliferation, in the periphery of the tumor, was chosen. In ten consecutive FOVs, structures that were mitotic figures were identified. The MAI was defined as the total number of mitoses in an area of 1.59 mm<sup>2</sup>. The same cut-off as previously described was chosen, with  $\geq 10$  mitoses defined as high risk [55].

### *Cyclin B1*

Slides were stained with the cyclin B1 antibody (cyclin B1 Y106, 1:200, Epitomics Inc., Burlingame, CA, USA). A minimum of 200 cells was counted. Cyclin B1 was assessed as previously described [129]. The cut-off value was predefined at the 7<sup>th</sup> decile of the cyclin B1 distribution, which corresponded to  $\geq 12.5\%$  positive cells.

### *ER, PgR, HER2, and histological grade*

ER, PgR, HER2, and histological grade were analyzed and evaluated as described elsewhere and previously defined cut-offs were used [126, 203, 206, 207, 213].

## Statistical methods and concepts

The statistical analysis software Stata (Stata Corp, College Station, TX, USA) was used for data analyses in all of the studies included in this thesis. In *Study I*, Stata 11.1 2010 was used and in *Studies II* and *III*, Stata 12.1 2012 was used. For analyses of the case-control study in *Study II*, SAS was used (SAS Institute, Inc.). In *Study IV*, Stata 14.1 2016 was used.

### Statistics used but not extensively described

Stepwise Cox analysis with backward elimination was used to fit the best model when highly correlated factors were included in the same multivariable model. An interaction term was used to test the null hypothesis of the effect of a factor in different subgroups, e.g., the effect of cyclin A in ER-positive vs. ER-negative breast cancer. Kappa statistics were used to evaluate the agreement between evaluators. Pearson's correlation coefficient, Pearson's  $\chi^2$  test, and Pearson's  $\chi^2$  test for trend were used for the analyses of associations between factors. Forest plots were used to visualize hazard ratios (HR:s) and 95% confidence intervals (CI:s) and the overall measurement of effect.

### The Kaplan-Meier method

In survival analysis, each studied subject has a study time. It could have begun when the subject entered the study or when a treatment was started. In our case, it was the date of diagnosis of breast cancer. The time ends when the endpoint is reached, i.e., when the event of interest occurs (for example death, recurrence, or distant metastasis): the study time also ends if the subject leaves the study due to a cause other than the event studied. If the time of the event is not reached (patients move away, refuse to continue in the study, or die of other causes), the study time is said to be censored. For patients who do not experience the event studied, the total survival time cannot be calculated. We do not know whether a censored patient would have experienced an event at a later time-point or not. The Kaplan-Meier method is a statistical procedure commonly used to calculate unbiased estimates of survival probabilities. The plot of these estimates, as a step-function of follow-up time, is known as a Kaplan-Meier graph. Such graphs were frequently used in the studies included in this thesis to illustrate differences in survival based on patterns of prognostic factors [214-216].

## **The log-rank test**

The log-rank test is used to compare survival in groups of patients. The null hypothesis to be tested is that there is no difference in survival between groups, and the alternative is either that the survival curves differ (the default test) or that they differ and are ordered. For two groups, these alternatives are equivalent, but for >2 ordered groups, the log-rank test for trend has greater power and should therefore be used. Briefly, the observed and expected numbers of events in each group at each event time are compared and combined into test statistics, which are compared to a chi-square distribution with appropriate numbers of degrees of freedom. It can only be used for one variable at a time [215, 216].

## **Cox regression**

The Cox proportional hazards regression model is commonly used to quantify relative effects, so-called hazard ratios (HR) of prognostic factors. A Cox regression model can be used to predict the hazard of the outcomes during the follow-up time. The relative effects of the factors in the model are assumed to be constant, i.e., independent of time, assumptions which must be checked. For instance, for a dichotomized prognostic factor with crossing survival curves, this model assumption is not fulfilled [215, 217].

Some of the advantages with Cox regression modeling, compared to the log-rank test, are that it provides effect measurements and not only *P*-values. Furthermore, variables measured on a continuous scale can be evaluated without categorization and adjustment for confounders is straightforward.

## **Schoenfeld's test**

In Cox proportional hazards regression models, the effects are assumed to be proportional over time, and with Schoenfeld's test, this non-proportional hazards assumption can be formally tested [218].

## **Stratification in Cox regression models**

Stratification allows for different mortality in different patient strata, e.g., studies, but equal effects of the factor relatively. It is used to ascertain that the estimated relative effect is a true effect and not an artifact due to the combination of patient series with different characteristics.

## ***P*-value**

A *P*-value is the probability of obtaining the outcome actually observed or an outcome that is even more extreme, assuming the null hypothesis to be true. If the *P*-value is less than a chosen critical value, the null hypothesis can be rejected, i.e., there is significant difference in predicted risk between the subgroups studied [215]. In this thesis, the typical applications of significance testing were to test whether survival curves were equal (log-rank) and to test factors effect on survival (HR = 1.00 means no effect). In this thesis, all of the *P*-values were two-sided and values less than 0.05 were considered significant.

## **C-index**

Different measurements of predictive performance and model fit have been suggested in the literature [219]. We chose Harrell's concordance index (*C*). Harrell's *C* is a generalization of the area under the ROC curve for survival data: therefore, it measures how well a model discriminates between different responses, i.e., is the predicted response low for patients not experiencing the event and high for patients experiencing the event? A Cox-model with good discrimination has low predicted hazards for patients with long follow-up not experiencing an event and high hazards for patients experiencing an event, especially if the event occurs early. The index is defined as the proportion of all evaluable pairs of patients, for which the patient with the highest predicted hazard also has the longest survival time. A pair of patients both of whom have censored survival times is not evaluable, whereas a pair is evaluable in whom one of the patients has a low predicted hazard and a survival time that is censored at a later time-point than the time for the event of the other patient. A model leading to perfect prediction yields  $C = 1.00$  whereas  $C = 0.50$  is not better than guessing [220].

## **Fractional polynomial**

Non-linear effects were modeled using fractional polynomial (FP) transformations [221, 222] in Cox regression models. Linear combinations of a series of 8 pre-defined power transformations ( $x^p$ ) of the predictors of interest as covariates were compared, and the best fitting transformation of each predictor were chosen. Following the standard definition of FPs, the powers  $p$  were chosen from the set  $(-2, -1, -\frac{1}{2}, 0, \frac{1}{2}, 1, 2, 3)$ , where  $x^0$  is defined as  $\ln(x)$ . A first degree FP is hence defined as  $\beta_0 + \beta_1 \cdot x^p$  and a second degree FP as a linear combination of two of the power transformations, e.g.,  $\beta_0 + \beta_1 \cdot x^{\frac{1}{2}} + \beta_2 \cdot x^2$ . First degree FPs will guarantee



monotonicity, whereas second degree FPs are more flexible, allowing for either a maximum or a minimum. Higher order FPs will be even more flexible, but the number of evaluated transformations grows exponentially, leading to severe multiple testing problems. To avoid over-fitting, a function selection procedure based on a closed test procedure was used, which added flexibility, i.e., extra polynomial terms, only if the model fit improved significantly at the chosen overall significance level after adjustment for multiple testing [222].

## **Multivariable FP**

The multivariable FP procedure (MFP), which is an extension of the function selection procedure based on FPs, was used to derive an FP-based prognostic index based on the originally continuous or integer valued predictors [222]. The MFP procedure applies a combination of backward and forward selection, starting from the full complex model, to find a parsimonious model, which is neither too simple nor too complex. It examines whether the effect of a continuous variable is better modeled by a non-linear member of the class of FP functions or by a linear function.

## **Restricted cubic splines**

Non-linear covariate effects were also modeled using restricted cubic splines (RCS). For each covariate,  $k$ , so-called knots, was chosen, which uniquely defines  $k-1$  polynomial transformations of the covariate. The definition of these transformations guaranteed that any linear combination of the  $k-1$  spline variable would be linear before the first knot, a piecewise cubic polynomial between the adjacent knots, and linear again after the last knot. To avoid over-fitting, we decided to use five knots located at the 5<sup>th</sup>, 27.5<sup>th</sup>, 50<sup>th</sup>, 72.5<sup>th</sup>, and 95<sup>th</sup> percentiles, respectively, as recommended by Harrell [223]. This definition worked for age and tumor size: however, for the number of positive lymph nodes (a variable with almost 40% zeros), we chose to place the five knots at 1, 2, 3, 4, and 10 positive nodes, respectively.

## **Endpoints**

In this thesis, distant disease-free survival (DDFS) was used as the endpoint in *Studies I* and *III*. In *Study IV*, we have called this endpoint DMFS (distant metastasis-free survival). Distant metastasis, but not locoregional recurrences was considered an event, and so was death by/of or with breast cancer. If a patient dies

due to breast cancer, most likely distant metastases existed: hence this is included in the endpoint. Contralateral breast cancer is not included as an event since we cannot know then whether a distant metastasis spread from the primary tumor or from the contralateral breast cancer and could thereby not be used to calculate time to endpoint. Death from other causes was not considered as an event.

For **Study II**, DDFS (as described above) was available for 1 305 of the patients. For 217 of these patients (one study cohort), we only had the definition breast cancer event: hence in **Study II**, all of these endpoints were compiled together and referred to as event-free survival. For the 332 patients in the case-control study included in **Study II**, the endpoint was breast cancer death.



# Results

## Study I

Cyclin A divided histological grade 2 tumors into two groups with significantly different DDFS (HR: 15, 95% CI: 4.3-52,  $P < 0.001$ ). Furthermore, it was only in the ER-positive subgroup that cyclin A was a prognostic factor (HR: 5.8, 95% CI: 2.2–16),  $P < 0.001$  vs. HR: 1.5, 95% CI: 0.55–3.9,  $P = 0.44$  for the ER-negative subgroup). A strong, but not significant, interaction of cyclin A between ER-positive and ER-negative cases was identified (HR: 3.9, 95% CI: 0.98–16,  $P = 0.054$ ).

Next we evaluated the combination variable CAGE, in which low CAGE consisted of all histological grade 1 cases and histological 2/ER-positive/low cyclin A cases. The high CAGE group was defined as histological grade 3, histological grade 2/ER-negative, or histological grade 2/ER-positive/high cyclin A. We found that CAGE was an independent prognostic factor for DDFS in multivariable analysis (HR: 4.1, 95% CI: 1.6-10,  $P = 0.002$ ), adjusted for HER2 and age. Low CAGE and HER2-normal identified 53% as low-risk patients with a 5-year DDFS of 95% (95% CI: 89–98%). Furthermore, a strong correlation between cyclin A and genetic grade, as defined by Sotiriou [158], was identified ( $P < 0.001$ ,  $\chi^2$ ).

## Study II

Since Ki67 is the generally accepted proliferation marker in clinical practice, Ki67 was used to validate the index defined in *Study I*. Comparably, the low KiGE-group was defined as histological grade 1 tumors and grade 2/ER-positive/low Ki67. High KiGE consisted of all of the other cases. Irrespective of menopausal status, lymph node status or endocrine therapy or not, KiGE was found to subdivide patients into groups with significantly different predicted risks of recurrence. Furthermore, we confirmed that the prognostic effect of proliferation was most pronounced in ER-positive and histological grade 2 tumors.

To evaluate the importance of KiGE in patients with the same characteristics as in **Study I**, subgroups were created, with *Set 1* corresponding to **Study I**. The other subsets (*Sets 2–5*) were created to evaluate whether KiGE was prognostic in a wide range of subsets:

*Set 1*: node-negative (N0), no adjuvant therapy,  $\leq 50$  years old at diagnosis ( $N = 169$ , 20 with events), HR: 4.4 (95% CI: 1.4–13),  $P = 0.010$ ;

*Set 2*: N0, no adjuvant therapy,  $>50$  years old at diagnosis ( $N = 488$ , 55 with events), HR: 4.0 (95% CI: 2.2–7.2),  $P < 0.001$ ;

*Set 3*: 1–3 positive lymph nodes (N1), no adjuvant therapy ( $N = 167$ , 39 with events), HR: 4.1 (95% CI: 1.9–8.6),  $P < 0.001$ ;

*Set 4*: N0, adjuvant endocrine therapy ( $N = 291$ , 39 with events), HR: 3.0 (95% CI: 1.5–6.0),  $P = 0.002$ ; and

*Set 5*: N1, adjuvant endocrine therapy ( $N = 407$ , 82 with events), HR: 4.1 (95% CI: 2.5–6.8),  $P < 0.001$ .

A significant association between event-free survival and high vs. low KiGE was found for all of the subsets and furthermore when including all 1 522 patients (HR: 3.9, 95% CI: 2.9–5.2,  $P < 0.001$ ). A case-control study ( $N = 166$  cases and 166 controls) was also included to corroborate the results further. For that study, breast cancer death was significantly associated with high vs. low KiGE (odds ratio: 2.7, 95% CI: 1.7–4.3,  $P < 0.0001$ ). The results were similar in multivariable analysis adjusted for age at diagnosis, tumor size, and adjuvant endocrine treatment, and as well as when adjusting for HER2.

Among the subgroup of patients in whom DDFS was available ( $N = 1 305$ ), KiGE identified a low risk group (constituting 53% of the patients) with a 5-year DDFS of 92% (95% CI: 89–93%).

## Study III

The purpose was to evaluate the prognostic value of MAI, PPH3, cyclin B1, cyclin A, and Ki67, alone and in combinations. We confirmed the strong prognostic value of all of the studied proliferation factors and that combining two factors improved the effects. The importance of proliferation, especially in ER-positive breast cancer, was thus further verified.

In univariable analysis, high vs. low MAI was the strongest prognostic proliferation factor for 10-year DDFS (HR 3.3, 95% CI: 1.8–6.1,  $P < 0.001$ ), followed by PPH3, cyclin A, Ki67, and cyclin B1. In the subgroup of ER-positive

tumors, a very strong prognostic effect of high MAI was found (HR: 7.0, 95% CI: 3.1–16,  $P < 0.001$ ). No prognostic effect was found in ER-negative patients. MAI added prognostic value in histological grade 2 (HR: 7.2, 95% CI: 3.1–22,  $P = 0.001$ ) and 1 (HR: 11, 95% CI: 2.3–55,  $P = 0.003$ ) but not in histological grade 3. Furthermore, because the strongest prognostic value was found for MAI alone, one factor at a time was combined with MAI. When combining MAI and/or high cyclin A, a stronger prognostic relationship was found (HR 4.2, 95% CI: 2.2–7.0,  $P < 0.001$ ) than for MAI in univariable analysis. Combinations of two proliferation factors were added to multivariable models, including age, ER-status, HER2-status, and adjuvant medical treatment. A high risk of developing distant recurrence was defined as having at least one of the two proliferation factors high. The combination of MAI and cyclin A showed a stronger prognostic effect on DDFS than when analyzing each factor separately (HR: 3.8, 95% CI: 1.6–8.7,  $P = 0.002$ ).

## Study IV

In the derivation set, risk stratification, based on models allowing for non-linear effects of continuous and integer-valued predictors, using FP models or RCS, was compared to stratification based on models for categorized predictors. Using FP transformations, univariable non-linear effects were detected for tumor size and the number of positive lymph nodes. For age, non-linear transformations did not improve the model fit significantly compared to the linear identity transformation chosen by the MFP procedure ( $C$ -index 0.513). The best fit for tumor size was a square root transformation ( $C$ -index 0.594), whereas for the number of positive lymph nodes, a combination of two polynomial terms provided the best fit ( $C$ -index 0.665). In multivariable analyses, categorization of each factor using two or three cut-points was found to improve prognostication compared to dichotomization ( $C$ -index 0.628 vs. 0.674). Modeling non-linear effects using MFP and RCS transformations modestly improved the  $C$ -index (0.695 vs. 0.696).

The model with categorized predictors generated 31 groups with different predicted relative hazards of distant metastases. In exploratory analyses, we investigated whether prognostication, within three homogenous risk groups according to the model with categorized predictors, could be improved using continuous predictors modeled with MFP transformations. As proof of principle, three groups with >100 patients and different levels of risk were chosen. A low-risk group (>50 years old, T1, and node-negative), an intermediate-risk group (>50 years old, T2, and 1–3 positive lymph nodes), and a high-risk group (>50 years old, T2, and at least ten positive lymph nodes) were identified. The estimated 10-

year DMFS was 92% for the patients within the low-risk group with the lowest predicted relative hazard, compared to 79% for the patients with the highest predicted relative hazard within this group. The corresponding 10-year DMFS based on categorized predictors was 88%.

The value of a prediction model is determined not by its performance in the dataset from which it was derived (the derivation set) but by its ability to perform well on independent validation data (the validation set). The Kalmar cohort ( $N = 1\ 132$ ) was therefore used to validate risk stratification based on FP and RCS transformed predictors from the derivation set. Four multivariable prediction models fitted in the derivation set were evaluated: dichotomized, categorized, MFP, and RCS models. For each model, the same predictor transformations as in the derivation set was applied and weights estimated in the derivation set were used to calculate the values for the prognostic indices (PIs) for the patients in the validation set. The validation *C*-index for the model with dichotomized predictors was 0.675, 0.700 for the model with categorized predictors, and 0.705 respective 0.701 for models with FP or RCS transformed predictors.

The prognostic discrimination was further analyzed by calculation of HR:s for four groups based on the 16<sup>th</sup>, 50<sup>th</sup>, and 84<sup>th</sup> percentile following the recommendation by Royston and Altman [219]. The HR:s after MFP transformation of continuous predictors for the derivation and validation set showed similar appearance. The calibration of the high-risk group was good between validation and derivation, but poorer for the lower risk groups.

# Discussion

More than 80% of breast cancer patients receive adjuvant treatment, although the majority is cured by surgery alone, leading to over-treatment [197]. Established clinical factors, such as hormone receptors, age at diagnosis, number of affected lymph nodes, tumor size, histological grade and HER2 are used in the clinic to sub-classify patients and to assign adequate treatment, but they could be improved for individual clinical decision-making. The advantage with new techniques, such as gene expression analysis, is the possibility of analyzing many genes at the same time and to finding gene patterns, instead of single gene expressions [63, 64, 158, 201, 224]. This ability might enable more individual-based treatments and large groups of patients could be divided into smaller and better-characterized groups that could benefit from individualized therapy. To identify tools to better guide the selection of patients for whom adjuvant chemotherapy might be avoided was number one of the top ten research questions in the web-based consultation performed by Dowsett et al., [200].

Before this thesis was initiated, it had been shown that gene expression profiles could be used to subdivide ER-positive breast cancer into groups with distinctively different prognosis and that the large proportion of tumors that are considered histological grade 2 (with intermediate risk for developing recurrence and hence difficulty in clinical decision making) using the Genomic Grade Index could be divided into one group with prognosis more similar to grade 1 and one group with prognosis more similar to grade 3 [158]. Furthermore, it was genes associated with proliferation that predominantly subdivided histological grade 2 [59, 63, 64, 158, 201]. It had also been shown that this subdivision of histological grade 2 could be performed using a single biomarker (Ki67) analyzed with IHC [61, 62].

We addressed these questions further in *Study I*. By combining cyclin A, histological grade, and ER into CAGE, and by creating an index based on CAGE and HER2, a subgroup of node-negative patients with low risk for recurrence (5-year DDFS of 95%, constituting 53% of the patients) for whom adjuvant chemotherapy might be avoided, was identified. The majority (95%) of the patients in the low-risk group were not administered adjuvant endocrine therapy, which was in accordance with standard procedure at that time of diagnosis (1991–1994). Today, the ER-positive patients in this low-risk group would have been recommended adjuvant endocrine therapy, which most likely would have



improved their 5-year DDFS even further. This low-risk group was characterized by low proliferation (low cyclin A), low histological grade (grade 1 and 2), and ER positivity, resembling luminal A breast cancer. The low recurrence rate for these patients suggests that adjuvant chemotherapy will have limited efficacy. In line with this suggestion, a recent study by Nielsen and co-workers concluded that patients with ‘luminal A-like’ tumors did not benefit from adjuvant cyclophosphamide-based chemotherapy [225]. With **Study I**, we corroborated the results that genes associated with proliferation could be used to subdivide ER-positive and histological grade 2 tumors into groups with different prognosis. Hence, our hypothesis that cyclin A could be used to subdivide histological grade 2 was fulfilled.

Although the staining of cyclin A was crisp and contrast-rich, we could not corroborate that evaluation of cyclin A was easier to perform than evaluation of Ki67 (kappa-value 0.71 for cyclin A compared to kappa-value >0.80 for Ki67) [61]. One explanation might be that the evaluation of cyclin A compared to Ki67 was performed by different persons with different levels of experience.

Based on the results of **Study I**, we wanted to confirm the importance of CAGE in a larger cohort, replacing cyclin A with the worldwide used factor Ki67 creating KiGE. In **Study II**, we evaluated this new index, KiGE, and showed that KiGE and CAGE performed equally well. Among node-negative patients, a low risk group constituting 57% of the patients had a 5-year DDFS of 92%, similar to the more expensive gene profile MammaPrint<sup>®</sup>, in which the primary objective of the study was to achieve a 95% CI with a lower boundary of at least 92% [58]. For patients with 1–3 positive lymph nodes and with highly ER-positive tumors, chemotherapy can be recommended if there are other risk factors (i.e., Ki67 or cyclin A): otherwise, endocrine therapy might be sufficient [226]. Similar to the results in **Study II**, one of the aims of the MINDACT trial and the primary aim of RxPONDER trial are that within the group of patients with 1–3 positive lymph nodes, there exist patients with low risk of developing recurrence, a group of patients who might be spared adjuvant chemotherapy [39, 40].

It should be mentioned that all patients in the MINDACT study were given endocrine treatment, compared to only 8 (4%) patients in **Study I** and 824 (54%) in **Study II** received endocrine therapy.

The strength of **Study I** was that the material was homogenous with regard to age at diagnosis (all premenopausal), and all were node-negative. Moreover, only 21 patients received adjuvant chemotherapy. At the time of **Study I**, it was one of few studies including untreated patients. One weakness was that the number of patients included was small. We circumvented this weakness in **Study II** with a larger cohort. A downside with **Study II** was the mixture of endpoints, and that many small retrospective studies were included. One large prospective study perhaps

would have been better. In contrast, the mixture of studies could also be considered as strength of the study, and hence, the robustness of the KiGE-index was strengthened by the factors being evaluated in different health care regions and by different persons using different cut-offs. And furthermore, the prognostic value of the KiGE-index could be demonstrated in several subsets. We used TMA-cores in our analyses, and it can be argued that TMA only captures a small fraction of the tumor. However, several studies have shown similar associations with survival for TMA-cores as for whole sections (including Ki67) [95, 123, 227-230].

The Cox proportional hazards model assumes proportional hazards over time. To avoid problems with non-proportional hazards, the time to follow-up can be restricted. In the first studies, we chose five years of follow-up since the risk of recurrence is greatest during the first five years (and MINDACT used five years). However, since many breast cancer patients experience recurrence beyond five years, we extended the follow-up to ten years in the other studies. The main prognostic value lay in predicting early recurrence. So far, there are no known single factors to predict long-term recurrence [231]. The EndoPredict score is a tool that can provide additional information in identifying late distant metastases in the ER-positive/HER2-negative subgroup and might thereby select patients who need prolonged endocrine therapy [163].

At the St Gallen conference 2013, two years after the publication of *Study I*, an index including ER, PgR, Ki67, and HER2 was presented [36]. Had we included HER2 in our CAGE-index, the indices would have been very similar. A recent study showed that both genomic grade and centrally reviewed Ki67 could be used to improve clinicopathological models [232].

In the present thesis, prognostic factors with a focus on proliferation were studied. There are many different techniques and factors that can be used to study proliferation. Some demand large amounts of fresh frozen tissue (SPF and older versions of gene expression analyses): others have poor reproducibility (Ki67). The advantage with IHC is that, unlike genetic profiling, it allows for the selection of malignant cells only, and the analysis can be performed in areas of the section with the highest proliferation. Sampling of tissue for genetic profiling inevitably includes normal cells.

Mitotic counting is a part of histological grading, which is performed in clinical practice. However, apoptotic cells can be mistaken for mitotic cells. Using PPH3 circumvents this problem because it does not stain apoptotic cells and can, in that respect, support MAI assessments [136]. There is an inconsistency in defining cut-offs and choice of antibody. Different numbers of positive cells have been counted. Should cells in hot spot areas be counted or should an average be calculated? One explanation for the poor reproducibility of Ki67 evaluations could be intratumoral heterogeneity, with cells in the periphery having higher expression

of Ki67. This uneven distribution within the section has an impact on the evaluation method to choose [233]. Aleskandarany et al., showed that the highest expression hot spots best reflect the outcomes with lymph node metastasis as the endpoint [234]. Others do not recommend hot spots [235]. There is however no standard method on evaluating Ki67. A recent study evaluated nine different ways of assessing Ki67 and reported that Ki67 assessed on hotspots and in the periphery of the tumor resulted in higher Ki67 median values compared to measuring average Ki67 (27.45% vs. 16.96%) [236].

Another study suggested that different cut-offs for Ki67 should be used for TMA vs. whole sections [230]. However, Ki67 is prognostic over a broad spectrum of cut-offs [237]. A common cut-off is  $\geq 20\%$  cells positive for Ki67; however, in Sweden, lab-specific cut-off values are used. The St Gallen 2015 conference recommends Ki67, with values 20–29% as cut-off, for discrimination between ‘luminal A-like’ and ‘luminal B-like’ [97]. Furthermore, recent results from our group showed that the value of Ki67 and PgR, for discrimination between luminal A- and B-like tumors seemed to be restricted to histological grade 2 [103].

To date, the evaluation of Ki67 has not been sufficiently stable to be included in the ASCO guidelines [37]. The utility of Ki67 will hopefully be improved by the introduction of standardized assessments and guidelines [96, 237, 238]. This is important since Ki67 is valuable, not only as a prognostic marker, but also for monitoring the response to neoadjuvant treatment [239].

The issues with reproducibility remain, although when applying national Swedish guidelines and recommendations for the evaluation of biomarkers, especially Ki67, good reproducibility can possibly be obtained [240]. To reduce interlaboratory and interobserver variations, repeated reproducibility studies are recommended [235, 238].

As mentioned above, prognostic factors have their pros and cons: nevertheless, studies have shown the prognostic value of proliferation regardless of the technique used. However, there is awareness that traditional biomarkers are insufficient in stratifying patients into appropriate risk groups for some of the reasons mentioned above. Using molecular profiles, a number of promising predictive models have been discovered: MammaPrint<sup>®</sup>, Oncotype DX<sup>®</sup>, Genomic Grade Index, and PAM50-ROR. These signatures can predict outcomes although they are based on different genes [58, 156, 158, 162]. This fact indicates that there exists an overlap of biological processes across gene patterns. Combining information from more than one gene signature would potentially increase the predictive power. Studies have shown that, by combining different genetic profiles, the prognostic potential can be increased [241, 242]. Other studies have shown the prognostic value of combining biomarkers [60, 243-247].

Using flow cytometry for analysis of the fraction of cells present in S-phase and, furthermore, combining the results with PgR-status and tumor size, members of our group showed, as early as 1990, a strong prognostic relationship by combining factors [87]. Furthermore, in 1999, Edén et al., showed that clinical markers performed as well as microarray gene expression profilers, and they suggested that a combination would improve prognosis [248]. The same year as the publication of **Study I**, Cuzick et al., published a combination of ER, PgR, Ki67 and HER2 into an IHC-score, which they called IHC4 [249]. According to them, IHC4 performed as well as the 21-gene recurrence score (i.e., *Oncotype DX*<sup>®</sup>) in predicting time to distant recurrence. The value in combining prognostic factors was also shown by Synnestvedt et al., who combined vascular invasion, histological grade, HER2, and Ki67. Similar to our results they found a large (61.4%) low-risk group with no unfavorable factors with only 3.6% distant relapses (median follow-up time was 86 months) who would not benefit from systemic adjuvant therapy [250].

Another combination-index is NPI+, which is a biomarker-based Nottingham prognostic index, as described by Rakha et al., and refined by Green et al., [251-253]. Regular NPI does not consider the biological heterogeneity. NPI+, which considers the IHC evaluation of ten biomarkers, classifies tumors into seven biological classes, similar to the intrinsic subtypes. These seven classes are then further classified into prognostic subgroups by adding information about prognostic clinicopathological variables (i.e., ER, PgR, cytokeratins, epidermal growth factor receptors, p53, and mucin), based on beta values generated in Cox regression analysis.

We hypothesized that, by combining two or more proliferation factors, the shortcomings previously mentioned could be substantially reduced. In **Study III**, we studied five factors for proliferation, separately and in combinations. We found that the strongest combination was MAI together with cyclin A. One possible explanation could be that they cover different phases of the cell cycle. MAI covers the M-phase, and cyclin A covers the S-phase. The results of **Study III** were based on a relatively small number of patients (~200 patients) and must be validated in a larger cohort: however, the results provide an indication that a combination of proliferation factors better predict survival. Due to a lack of power, we were not able to investigate whether a combination of more than two factors would have performed even better. In contrast, a combination of two factors for proliferation is very likely more practical in clinical routine than using three or more.

The results in **Study III** showed that MAI was a stronger prognostic factor than Ki67, especially when combined with cyclin A. Baak et al., reported similar results that MAI was the strongest predictor in node-negative breast cancer [55]. With the same focus on MAI and with standardized assessments and guidelines,

MAI might replace Ki67 as the prognostic marker for proliferation. Since MAI is already a part of histological grading, performed routinely on hematoxylin/eosin-slides, issues with choice of antibody, cut-off, hotspot or not, would be circumvented.

One way of improving the evaluation of Ki67 might be to use digital image analysis (DIA). The results have shown that DIA outperforms manual assessment [254]. In the luminal B subtype the results from DIA assessments of Ki67 were compared to manual assessments, and the DIA assessments performed the best. Others studies have shown similar result, with DIA outperforming manual scoring [255, 256].

To date, only a few molecular profiles/signatures have come into clinical use [257], and they have shed light on the inter-heterogeneity of breast cancer. The intrinsic subtypes have been refined [162, 258] and different combinations of profiles have been suggested [64, 259-262].

The first results from the MINDACT trial reported that 23.2% of patients (who did not receive chemotherapy) were assigned low genomic risk and high clinical risk (according to Adjuvant! Online) and had a 5-year DMFS of 94.7% (95% CI: 92.5 to 96.2). Thus, their primary objective of the study, to achieve a 95% CI with a lower boundary of at least 92%, was met [263]. Based on these results from the MINDACT study, patients classified as low genomic risk according to the 70-gene signature (i.e., MammaPrint<sup>®</sup>) could be recommended to forego chemotherapy. They had similar risk as those classified as low clinical and high genomic risk, hence MammaPrint<sup>®</sup> only added value to those classified as high clinical risk.

The TAILORx trial showed that the 21-gene recurrence score could select 16% of patients with low risk for metastasis (99% 5-year survival without distant metastasis) [40]. The results from chemotherapy vs. no chemotherapy in the intermediate group, as identified by the 21-gene assay, are still pending.

Nevertheless, these results are promising: however in countries with sparse economic resources, molecular profiling is still not an option and simpler and less expensive techniques, such as IHC, are especially important. Therefore, the St Gallen guidelines recommend ER, PgR, HER2, and Ki67 as IHC surrogate markers for intrinsic subtypes [36].

The use of prognostic factors in breast cancer has a long history dating back to the invention of the TNM classification system. Categorization of prognostic factors is intuitively appealing since the clinically relevant question is often to select between two (or a few) treatment options, but categorization of individual factors is not necessary for the construction of good prediction models [264]. Categorization is problematic since this will in general lead to information loss and hence lower power to detect true associations. Moreover, how should one

define the cut-offs? Should it be the median or some other percentile? Is it more correct to select the upper third of the patients as a cut-off, based on the argument that, in the long run, approximately one third of unselected breast cancer patients will develop recurrence? In addition, when applying a cut-off, patients on either side of the cut-off will be considered to have different predicted risks, rather than similar. Our aim was to show that prognostication could be improved by avoiding the categorization of risk factors and by allowing for non-linear effects using FP and RCS transformations. Sauerbei et al., evaluated the use of FP transformations in Cox modeling, comparing categorized with continuous prognostic factors, such as age, menopausal status, number of positive lymph nodes, ER, PgR, tumor size and grade. They concluded that important information could be extracted by allowing for FP transformations instead of traditional modeling [265]. Risk groups based on predictions from more flexible models of this type could be used to identify patients who could be spared adjuvant therapy and/or patients in need of additional treatment [265, 266].

In *Study IV*, more than 5 500 patients were compiled together and we observed that categorization into 3–4 groups, e.g., number of positive lymph nodes into N0, N1–3, N4–9, and N10+ instead of positive vs. negative, improved the prediction, as seen by increase in C-index. A multivariable model with age and tumor size in three categories and number of lymph nodes in four improved the C-index from 0.628, with the variables dichotomized in the model, to 0.674. Contradictory to our expectations, predictive performance was only moderately improved by using the predictors continuously. By increasing the number of cut-points, the possibly non-linear effects of the predictors seemed to be well captured. These findings strengthen the way age, tumor size, and number of lymph nodes are used in the clinic today.

Categorization is in general a more robust approach compared to FP or RCS transformations. However, using continuous values can have the advantage of making it possible to form risk groups of any size. Few publications so far have addressed this topic. Recently, Ejlersen et al., used FP transformations in developing a model for the prediction of excess mortality after adjuvant endocrine therapy [266]. Their model included age, tumor size, number of positive lymph nodes, and ER positivity in percentages. When using FP transformations instead of categorized variables, better identification of patients without excess mortality was found [266]. In contrast to our results, they showed that FP transformation performed better than models based on categorized variables. One explanation might be that their model included ER positivity in percentage and that their cohort was more homogenous (postmenopausal high-risk patients who all received five years of adjuvant endocrine therapy). In contrast to other studies, we did not see a non-linear effect for age [140-143]. This could perhaps be explained by, in our study, more than one third (23/69) of the patients younger than 35 years old

being treated with adjuvant chemotherapy, compared to 12% (511/4406) of the patients older than 35 years old. Furthermore, Fredholm et al., showed that differences in survival connected to age were primarily present in stage I and II breast cancers [140]. In *Study IV*, the majority of the patients were in stage II. Kroman et al., observed a similar age trend, with younger women having a higher risk of dying: however, this trend vanished in patients who received chemotherapy [143].

We modeled potentially non-linear relationships using both MFP and RCS transformations, and the results were strikingly similar. Both splines and FP transformation have their advantages and disadvantages [222]. FPs are more sensitive to outliers, which can be controlled for by restricting the degrees of freedom for each factor. On the other hand, RCS can lead to over-fitting of the data especially if many knots are used [222]. One advantage with the MFP analysis is that it stops itself from over-fitting the model. By incorporating prior knowledge into the statistical modeling strategy, it might be possible to avoid relationships that are not biologically true.

An alternative modeling strategy is artificial neural networks (ANNs). In a dataset, which to a large extent overlapped with the derivation set in *Study IV*, ANNs was applied. The results showed nearly identical performance between the ANN and Cox models [267]. The Cox and ANN models had almost the same *C*-indices, 0.71 vs. 0.70, which were comparable to the results in *Study IV*, with a *C*-index of 0.695 for the MFP-model.

As for *Study II*, a limitation with our study was that many different cohorts, instead of one large population-based cohort, were included. On the other hand, the results in the derivation set could be validated in another independent more population-based dataset. In the validation dataset, similar improvements in performance were obtained when more cut-points were used. The discrimination and calibration was found to be better for high-risk patients than for patients whose prognostic factors indicated lower risk. The relative effect estimates were found to be smaller when the models fitted in the derivation set were applied to the validation set. This could be explained by minor over-fitting to the derivation set.

In *Study IV*, only the three factors age at diagnosis, tumor size, and number of positive lymph nodes were included. A clinically applicable model should include all prognostic factors in use, i.e., according to current guidelines, also ER, PgR, HER2, Ki67, and histological grade [37, 97]. We did not have complete information for these additional factors: otherwise it might have been possible to create a nomogram that could be used for clinical decisions similar to the nomogram for DCIS or PREDICT, into which continuous values could be incorporated [42, 43, 268].

With the results from *Study IV*, the use of dichotomized factors in *Study I, II*, and *III* could be considered suboptimal. On the other hand, for *Study I* and *III* we did not have continuous values for all of the factors studied. Since *Study II* was a validation of the results in *Study I*, the factors were used accordingly.





# Conclusions

In *Studies I* and *II*, a combination of a marker for proliferation (cyclin A or Ki67), histological grade, and estrogen receptor could identify over 50% of the patients as low risk, with more than 90% of the patients having 5-year event-free survival.

In *Study III*, a combination of MAI and cyclin A improved prognostication compared to using the factors individually.

In *Study IV*, dichotomization of prognostic factors measured on a continuous or integer-valued scale led to considerable information loss and should thus be avoided. Models allowing for non-linear effects did not outperform models with predictors categorized into three or four groups.



# Future perspectives

Despite the use of single markers as surrogate makers for the intrinsic subtypes, the trend is toward the increased use of molecular profiles. My belief is that age at diagnosis, tumor size, and number of lymph nodes (the factors studied in *Study IV*) will be measured similarly in the near future, even after implementation of genetic profiles. The clinicopathological markers, however, might be replaced or combined with other techniques. One recent study showed that, when combining one gene predictor, i.e., PAM50-ROR, with established prognostic markers (tumor size and proliferation), the performance compared to pure molecular scores was improved [269]. Hence, a hybrid score, i.e., integrating a molecular signature such as PAM50-ROR (ProSigna) with clinicopathological factors, might increase the prognostic ability.

In Sweden, the South Sweden Cancerome Analysis Network – Breast (SCAN-B) consortium was launched in 2010 to address this challenge [270]. The aim is to establish infrastructure towards the implementation of genetic analyses in routine clinical practice. Tumors and blood (to date not paraffin-embedded material) are consecutively collected and molecularly classified. These classifications might in the future complement the clinicopathological factors and guide treatment decisions. A similar project is the Clinical Sequencing of Cancer in Sweden (Clinseq), which started in 2013 in Stockholm. The aim is to establish an infrastructure for genomic profiling and to perform classification of cancer. For both SCAN-B and Clinseq, the goal is to develop diagnostic tests based on molecular profiling, with the possibility of tailored treatment and hopefully thereby improving survival for all breast cancer patients. Furthermore, samples are collected in tumor banks for future projects.

Many of my projects would not have been possible if not for the large collection of tumor material and databases with patient characteristics and information about survival. For future research projects, collection of high quality tumor banks (eg., tissue, blood, paraffin blocks) and databases is greatly important. Moreover, to design studies in which the benefit of different new treatments for breast cancer can be evaluated is also important. Of additional importance is to design prospective studies in which a group of people is followed prior to developing breast cancer. Karma, the Karolinska Mammography Project for Risk Prediction of Breast Cancer, is one such study. Karma is a prevention study initiated in 2011

by researchers at the Karolinska Institute. The focus is primarily to identify high-risk women, based on lifestyle factors, genetic changes and mammographic morphology. More than 70 000 women have been included so far. If women at high risk for developing breast cancer could be identified in early stages, preventive treatments could be offered, and thereby the incidence and mortality in breast cancer could be reduced.

To identify markers that predict metastatic potential at an early stage is a challenge. Circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) in the blood are promising new markers in breast cancer. To analyze CTCs or ctDNA and relate this to molecular subtype could potentially provide information, which could guide individual treatment further.

# Populärvetenskaplig sammanfattning

Bröstcancer är en av de vanligaste cancersjukdomarna bland kvinnor. I Sverige diagnostiseras mer än 8000 kvinnor varje år. Det betyder att varje timme drabbas någon av bröstcancer. Tack vare hälsoundersökningar som mammografi är majoriteten av tumörerna idag små när de upptäcks och oftast utan spridning till lymfkörtlarna i armhålan. Därmed är chansen till bot större och idag lever nästan 90% av patienterna fem år efter diagnosen. Den mest effektiva behandlingsformen är kirurgi, vilken botar majoriteten av patienterna. För att minska risken för återfall ger man tilläggsbehandling, så kallad adjuvant behandling, men eftersom en stor grupp redan är botade innebär detta ibland att för mycket behandling ges. Hur kan man då identifiera de patienter som inte behöver extra behandling? Och trots att det ges tilläggsbehandling är det fortfarande drygt 1400 kvinnor som varje år dör av bröstcancer. Därför är det även viktigt att hitta den grupp av patienter som underbehandlas d.v.s. de som får återfall och som möjligen hade behövt ytterligare eller annan typ av behandling. Mycket forskning ägnas åt att identifiera faktorer och åt försök att kombinera befintliga faktorer, för att använda dem som hjälp för att ge rätt behandling. Målet är att på så sätt bättre definiera och behandla de patienter som har hög risk för återfall.

För att bestämma vilken ytterligare behandling som ska ges använder man prognostiska och behandlingsprediktiva faktorer. Dessa faktorer korrelerar till sjukdomens förlopp, aggressivitet och känslighet för behandling. De viktigaste som används rutinmässigt är ålder vid diagnos, tumörstorlek, spridning till lymfkörtlar i armhålan, histologisk grad, uttryck av olika biomarkörer som östrogenreceptorn (ER), progesteronreceptorn (PgR), faktorer för tillväxthastighet, så kallad proliferation (Ki67), och uttryck av tillväxtfaktor receptorn (HER2). Histologisk grad och proliferation avspeglar cellens förmåga till okontrollerad celledelning och tillväxt. Histologisk grad delas in i tre grupper där grad 3 har sämst och grad 1 bäst prognos. För grad 2, som är den stora mellangruppen, behöver man andra verktyg för att kunna välja rätt behandling.

Nyligen har profiler som beskriver genuttryck börjat användas som ett försök att bättre gruppera patienter i olika riskgrupper baserat på beräknad risk. När projekten i denna avhandling initierades hade bröstcancer med hjälp av genuttryck grupperats i ER-positiva och ER-negativa tumörer. Dessa grupper har dessutom olika prognos. ER-positiva, histologisk grad 2 tumörer kunde delas i en grupp med

prognos mer lik grad 1 och en grupp med prognos mer lik grad 3. Gener involverade i proliferation var särskilt viktiga för denna uppdelning. Användandet av genuttrycks-profiler ökar, framför allt i USA. De är dock tekniskt krävande och kostsamma och inte tillgängliga för majoriteten av patienter i andra delar av världen. Andra studier har visat att en liknande uppdelning gick att göra med enklare tekniker och vi ville studera detta vidare.

I **Study I**, fann vi att kombinationen av en markör för proliferation (cyclin A), histologisk grad och östrogenreceptorn (ER), i ett index vi kallade CAGE tillsammans med HER2 identifierade en relativt stor lågrisk grupp (53%), med liten risk för fjärrmetastasering (5-års fjärrmetastas-fri överlevnad 95%). Studien omfattade bara drygt 200 patienter. Nästa steg blev därför att validera indexet i ett oberoende och större material. Detta gjordes i **Study II**, i vilket Ki67, som är den proliferationsmarkör som används mest i kliniken, användes i stället för cyclin A. Vi kombinerade faktorerna på samma sätt som i **Study I** och skapade indexet KiGE. I **Study II** såg vi att KiGE, precis som CAGE i **Study I**, identifierade en stor grupp av patienter med liten risk för återfall, som troligen inte behöver erbjudas cellgifter. I en webb-baserad konsultation var den viktigaste frågan just att hitta de patienter som inte behöver ges cellgifter.

Alla tekniker och markörer har sina för- och nackdelar. I **Study III** undersökte vi om en kombination av flera markörer för proliferation kunde ge ett säkrare mått för tumörens tillväxt och därmed också säkrare bestämma prognosen. Vi fann bl.a. att kombinationen av cyklin A och MAI (som är ett mått på själva celledelningen) hade större prognostisk effekt än om man tittade på faktorerna var för sig.

Det finns idag ett begränsat antal behandlingsalternativ och för att bestämma om behfaktorer i två eller flera grupper. I statistiska modeller är detta inte nödvändigt. Problem med att dela in i grupper, kategorisera, är bland annat hur man då väljer gränsvärden. Värden på var sida om gränsen blir automatiskt olika (hög respektive låg risk) i stället för snarlika.

I **Study IV** undersökte vi om det gav mer prognostisk information att låta värdena vara kontinuerliga så länge som möjligt i modellen. Vi såg att det definitivt var bättre att dela faktorerna i fler kategorier än två. Däremot blev det bara marginell skillnad om man behöll dem kontinuerliga i analysen. För den enskilde patienten kan det dock fortfarande göra skillnad och målet i dagens sjukvård är att hitta en så väl individanpassad behandling som möjligt.

Sammantaget visar studierna i denna avhandling att flera olika markörer för proliferation kan användas för att förutsäga sjukdomsförlopp och att en kombination av dem förstärker det prognostiska värdet. Med dessa kombinationer är det möjligt att identifiera patienter med så låg risk för återfall att nyttan av cellgifter är tveksam. Vi såg också att det är bättre att dela prognostiska faktorer i

fler grupper än två, men bara marginellt bättre att behålla dem kontinuerliga. Däremot, om man behåller faktorerna kontinuerliga så länge som möjligt i analysen, ökar möjligheten att skapa subgrupper och då också möjligheten att göra individuella beräkningar om risk för återfall.





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