Proliferation, lymph vessel invasion and histologic type as prognostic factors in lymph node negative breast cancer

Einar Gudlaugsson, MD

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Scientific environment

The studies in this research project have been performed at the Department of Pathology, Stavanger University Hospital, Stavanger, the Gade Institute, University of Bergen, Bergen, Norway in collaboration with MD Anderson Cancer Center, Houston, Texas, USA and the Fudan University Shanghai Cancer Center, Shanghai, China (an MDACC Sister Institution).

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I am indebted to my co-supervisor dr. Emiel Janssen for his invaluable contribution and detailed and constructive comments on manuscripts. Emiel, what a journey this has been.

I am also grateful to dr. Ivar Skaland for the outstanding technical work on the immunohistochemical matters and the digital imaging.

I also want to thank Bianca van Diermen BSc and Eliza Janssen for their contribution.

I want to thank the Head of Department of Pathology, dr. Kjellevold and all my other colleagues and co-workers at the Department for the good working atmosphere.

_I dedicate this work to the many patients contributing to this thesis._
ABSTRACT

Summary

1. Mitotic Activity Index (MAI) <10 versus ≥10 is an accurate and well reproducible prognosticator in lymph node negative breast cancer in women <71 years and superior to both the online program Adjuvant! and the Norwegian Breast Cancer Group in prognostication in lymph node negative breast cancer in women <55 years.

2. In lobular invasive type breast cancers, a threshold of 5 is the most optimal threshold.

3. Lymph vessel invasion defined by D2-40 or D2-40/p63 have no additional prognostic value to the MAI or Phosphohistone-H3 (PPH3).

4. Ki67 assessed by interactive point-weighted morphometric analysis and especially digital image analysis, but not by subjective counts, is reproducible and prognostically strong. This casts serious doubt on guidelines advocating subjective counts for therapeutic decision making.

5. PPH3 is the strongest prognosticator in the node negative breast cancers studied; Ki67-6.5% by DIA has additional prognostic value in patients with a low PPH3<13. Ki67-6.5% by DIA also has additional prognostic value in patients with a MAI < 10, but not in patients with MAI ≥ 10.

6. In daily practice, both the MAI, PPH3 and Ki67 should be assessed and the results carefully compared for adequate quality control and most accurate prognosis prediction for lymph node negative breast cancer patients.

Breast cancer is a leading cause of cancer mortality among women in the western world, second only to lung cancer. Since the mammography screening program in Norway started in 1995, 70% and more of all new breast cancers diagnosed are small tumors ≤ 2cm in diameter without signs of regional or distant metastasis. Not surprisingly, the five year relative survival is very good (87.8%). Their improvement of absolute 10% survival with adjuvant systemic chemotherapy is less substantial than that for lymph node-positive patients. Both
the benefits and disadvantages of this 10% survival improvement must be considered when deliberating adjuvant systemic therapy (1, 2) and prognostic/predictive features are important in these decisions. Often, a combination of features is used in the decision making process for adjuvant systemic therapy, where tumour diameter, oestrogen receptor, grade and also age play a major role (3, 4). These guidelines identify 80% or more of all node-negative patients as high risk, although only ~15% of these patients die from metastatic disease without adjuvant treatment. This means that 65% of these patients are over-treated. On a world-wide basis, a more sensitive and specific prognosticator could therefore prevent enormous unnecessary suffering.

Proliferation markers such as the Mitotic Activity Index (MAI), Phosphohistone-H3 (PPH3) and Ki67 are prognostically stronger than classical prognosticators and multivariate guideline predictors, as shown in many prospective and retrospective (also multicenter) analyses (5-7). This is also true for the Norwegian national NBCG guidelines, as we have recently shown (8).

In the current PhD study we will address the following questions:

1. What are the criteria required for well reproducible assessment of the MAI?

2. What is the accuracy of MAI compared to other prognostic models such as the online program Adjuvant! and the guidelines from Norwegian Breast Cancer Group (NBCG)?

3. Is the prognostic threshold for proliferation in invasive breast cancers of the lobular invasive type the same as for ductal type invasive cancer?

4. Does lymph vessel invasion have additional prognostic value to the MAI?
5. Ki67 has recently been added to the national therapeutic decision making algorithm of the St Gallen and the Norwegian Breast Cancer Group guideline, but they do not give standard operating procedures how Ki67 must be determined. We therefore have analyzed the reproducibility and prognostic value of different methods to assess Ki67 expression.

6. Finally, we will compare the prognostic value of the MAI, PPH3 and Ki67 with each other and with other prognostic factors.

In the first article (Breast Cancer Res Treat. 2009 May; 115(2):241-54), we give a review of the literature, describing that proliferation of the primary tumour measured by the MAI is the strongest prognosticator. We also describe protocols, reproducibility and pitfalls in assessment of proliferation in node-negative breast cancer. In subsequent studies from our group (10), it was shown that Phosphohistone-H3 (PPH3) is a strong alternative for the MAI, and also can be measured by digital image analysis. This is a further extension of previous studies on automated counting of mitoses in tissue sections (11, 12). We also compare the prognostic value with alternative molecular prognostic markers. However, at the time of writing the review, the MAI was the most widely available and used method in the world to assess proliferation in breast cancer. Finally, we also describe possible error sources. When these factors are taken into account, the MAI can be well reproducible and has overriding prognostic significance (8, 13).
In the second article (J Clin Oncol 2010; 29(7):852-858), our aim was to assess the strength of MAI as compared with other models of prognostication in 516 lymph node negative breast cancer less than 55 years of age with a median follow-up of 118 months. Thresholds were set for MAI at <3, ≥3, Adjuvant! at <95%, ≥95% and NBCG features deciding adjuvant systemic treatment or not (lymph-node negative and ER positive with either: pT1, grad 1, all ages or: pT1a-b, grade 2-3, ≥35 years). All models were found to be prognostically significant, but with multivariable analysis, the MAI was prognostically stronger than both Adjuvant! and NBCG. Furthermore, stratification by MAI identified subgroups with different prognosis, indicating possible over- and undertreatment of T_{1-3}N_{0}M_{0} breast cancer patients <55 years.

More than 80% of invasive breast cancers are of the ductal histologic type. Proliferation has been evaluated in node-negative breast cancer patients in general. However, studies on lobular invasive cancers are rare. In the third article (Breast Cancer Res Treat. 2010 May; 121(1):35-40), we therefore evaluated prospectively the value of proliferation measured by the MAI compared with other classical prognostic factors in 121 lymph node negative invasive lobular cancers (ILCs) without systemic adjuvant treatment from the large Multicenter Morphometric Mammary Cancer Project (MMMCP). The 121 patients had 83 months median follow-up time. Cases were subtyped in accordance with WHO-2003 criteria, supplemented by E-cadherin immunohistochemical analysis in cases with non-classical morphology. We found that solid/alveolar ILCs subtype (n=17) had a worse 10-year survival (50%) than other ILCs subtypes (n=104; 83%, P<0.0001). Furthermore, the MAI (but not nuclear grade or tubule formation) with a threshold of 0-5 versus >5 was prognostic in the
lobular invasive cancers (contrasting MAI<10 versus ≥10 in breast cancers in general), 85 and 54% 10-year survival, respectively (P<0.0001).

In the fourth article (Mod Pathol. 2011 Apr; 24(4):502-11), we investigated whether PhosphoHistone-H3 (PPH3) assessed proliferation and lymph vessel invasion (LVI) by D2-40 are prognostic. Lymph vessel invasion has been propagated as a prognostic factor for many decades, but the assessment in conventional Hematoxylin-Eosin stained sections is poorly reproducible. D2-40 immunostaining can improve this, but myoepithelial D2-40-expression in small ducts completely filled by solid-pattern ductal carcinoma in situ can mimic lymphovascular invasion. As myoepithelial cells are also p63 positive, we have investigated whether LVI identified by combined D2-40/p63 is stronger prognostically than by D2-40 alone and whether it has independent prognostic value to PPH3. In 240 operable T1-2N0M0 node negative invasive breast cancer patients less than 71 years with long-term follow-up and without adjuvant systemic treatment, PPH3 was determined by quantitative immunohistochemistry and LVI by D2-40/p63 double immunohistochemical staining. Correlation analysis between the clinico-pathologic factors and LVI, and univariate and multivariate prognostic survival analysis using recurrence free (RFS) and distant metastases overall survival (DM-OS) were performed. With median 117 (range: 12-192) months follow-up, thirty-six patients (15%) developed and 28 (12%) died of distant metastases. Ten of the sixty-one patients (16%) with cancer cells surrounded by D2-40 were p63 positive and none of these “false LVI” recurred. D2-40+/p63- LVI occurred in 51/239 (21%) cases and correlated with grade, MAI, PPH3, oestrogen receptor (ER), cytokeratin 14 (CK14) and Herceptin (HER2/neu). D2-40+/p63- LVI was strongly prognostic, but far more in women
\[ \geq 55 \text{ than those } <55 \text{ years (P } <0.0001 \text{ and } 0.04). \text{ With multivariate analysis, PPH3 assessed} \]
proliferation was the strongest single prognosticator. LVI had additional prognostic value to
PPH3 but only in women \( \geq 55 \). For this group, where PPH3 \( \geq 13 \), patients without/with LVI
had 10 year survival rates of 83% and 50% respectively (Hazard Ratio LVI=HR-LVI=3.0,
P=0.04, HR-PPH3=6.9, P=0.002). Where age was <55, only PPH3 had independent
prognostic value. Combinations of other features had no additional value to PPH3. In
conclusion, in operable T_{1-2}N_{0}M_{0} invasive breast cancer patients \( \geq 55 \) years with PPH3 \( \geq 13 \),
D2-40+/p63- defined LVI identifies a subgroup with a very high risk of distant metastases.

**In the fifth article, submitted Histology September, 2011,** we set out to evaluate the
proliferation factor Ki67 in node negative breast cancer. Others have found that Ki67 is
prognostic, but immunohistochemical and measurement procedures differ between
laboratories. We compared the reproducibility and prognostic value of different Ki67
measurement methods. In 237 T_{1-2}N_{0}M_{0} breast cancers without adjuvant systemic treatment,
standardized and fully automated immunohistochemistry was used. The percentages of Ki67
positive nuclei were assessed using conventional counts and a “quick scan” rapid estimate by
independent pathologists, computerized interactive morphometry (CIM) and automated
digital image analysis (DIA). Reproducibility was studied by duplicate blinded assessments.
Using univariate and multivariate survival analyses, the prognostic value of widely accepted
Ki67 thresholds (10, 15%, 20%) and other objective thresholds indicated by Receiver
Operating Curve (ROC) analysis were studied. The Ki67 counts by two pathologists and
their corresponding optimal prognostic thresholds varied greatly (4% and 14%). Quick scan
rapid estimates were poorly reproducible and not prognostic. CIM-Ki67 were well and DIA-
Ki67 very well reproducible and strongly prognostic (P<0.0001). DIA-Ki67 with a ROC-
selected threshold of 6.5% was the strongest prognosticator. We conclude that in node negative breast cancer without adjuvant systemic treatment, Ki67% by digital image analysis but not subjective counts is reproducible and prognostically strong. This casts serious doubt on therapeutic guidelines using subjective counts of Ki67.

In the sixth article, manuscript October, 2011, we further analysed the reproducibility and prognostic value of Ki67 versus Mitotic Activity Index (MAI) and Phosphohistone-H3 (PPH3); these were compared with classical prognostic and predictive factors (tumour size, steroid receptors and HER2/neu). In total of 237 T1-2N0M0 breast cancers not undergoing systemic adjuvant treatment, formalized MAI assessment, strictly standardized and fully automated quantitative immunohistochemistry for Ki67 and PPH3 were used. The percentages of Ki67 positive nuclei were assessed independently by two pathologists using conventional counts, by computerized interactive morphometry (CIM) and by automated digital image analysis (DIA). Receiver Operating Curve (ROC) analysis was used to objectively assess the optimal threshold of continuous variable such as Ki67. Section thickness was measured to increase standardization of Ki67 IHC. Section thickness had a low coefficient of variation, indicating that this potential error source can be kept minimal. ROC analysis showed that a DIA-Ki67 threshold of 6.5% had optimal and strongest prognostic value and added prognostically to PPH3. None of the other biomarkers of clinicopathologic variables added prognostically to this PPH3/Ki67 combination. However, when PPH3 is replace by MAI the prognostic value is nearly the same. We conclude that in node negative breast cancer without adjuvant systemic treatment, Ki67 assessed by digital image analysis in the periphery of the tumour with a threshold of 6.5%, is prognostically
strong. The combination of either PPH3/Ki67 or MAI/Ki67 overshadowed the prognostic value of all other features.

**LIST OF PUBLICATIONS**


2. Lende TH, Janssen EAM, Gudlaugsson E, Voorhorst FJ, Smaaland R, van Diest P, Søiland H, Baak JPA. In patients younger than age 55 years with lymph node-negative breast cancer, proliferation by mitotic activity index is prognostically superior to Adjuvant! J Clin Oncol 2010; 29(7):852-858.


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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Adjuvant!</td>
<td><a href="http://www.adjuvantonline.com">www.adjuvantonline.com</a></td>
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<tr>
<td>A&amp;W</td>
<td>Alive and Well</td>
</tr>
<tr>
<td>AST</td>
<td>Adjuvant Systemic Therapy</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CIM</td>
<td>Computerized Interactive Morphometry</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical Intraepitelial Neoplasia</td>
</tr>
<tr>
<td>CK14</td>
<td>Cytokeratin 14</td>
</tr>
<tr>
<td>DIA</td>
<td>Digital Image Analysis</td>
</tr>
<tr>
<td>DM-OS</td>
<td>Distant Metastasis-Overall Survival</td>
</tr>
<tr>
<td>DOD</td>
<td>Dead Of Disease</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen Receptor</td>
</tr>
<tr>
<td>FDA</td>
<td>Federal Drug Administration (USA)</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin Fixed Parafin Embedded</td>
</tr>
<tr>
<td>FOV</td>
<td>Field Of Vision</td>
</tr>
<tr>
<td>HER2/neu</td>
<td>Herceptin</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard Ratio</td>
</tr>
<tr>
<td>IHC</td>
<td>ImmunoHistoChemistry</td>
</tr>
<tr>
<td>ILC</td>
<td>Invasive Lobular Carcinoma</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular Carcinoma In Situ</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Node</td>
</tr>
<tr>
<td>LVI</td>
<td>Lymph Vessel Invasion</td>
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<tr>
<td>MAI</td>
<td>Mitotic Activity Index</td>
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<tr>
<td>MMMCP</td>
<td>Multicenter Morphometric Mammary Cancer Project</td>
</tr>
<tr>
<td>NBCG</td>
<td>Norwegian Breast Cancer Group</td>
</tr>
<tr>
<td>PPH3</td>
<td>Phosphohistone H3</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>RS</td>
<td>Recurrence Score</td>
</tr>
<tr>
<td>TLI</td>
<td>Thymidine Labeling Index</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue MicroArray</td>
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<tr>
<td>TNP</td>
<td>Triple Negative Phenotype</td>
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1. INTRODUCTION

1.1 Breast cancer epidemiology

Breast cancer is a leading cause of cancer mortality among women in western world, second only to lung cancer. In Norway, the age-specific incidence rate of breast cancer has more than doubled from 37.3 to 76.7/100,000/year between 1955 and 2003 (Figure 1). The incidence increases by age and reaches a peak between 65 and 69 years. In 2008, 2753 new women were diagnosed with breast cancer in Norway and constituted 23% of all malignancies when all ages were grouped together. The cumulative risk for women to develop breast cancer is 8.1% and about one in twelve women in Norway will develop the disease by the age of 75 (14).

![Figure 1. Time trends for age-standardized incidence rate in Norway for selected cancers in women (from reference 14).](image-url)
Since the mammography screening program for women age (51-69 years) in Norway started in 1995, the incidence of breast cancer has sharply increased, but later leveled off. This is mainly due to the increased early detection of prognostically favorable tumours in the population screening material. As a result, 70% and more of all new breast cancers diagnosed in Norway since mammography screening started, are small tumours (i.e., ≤2cm in diameter) without signs of regional or distant metastasis. Not surprisingly, the five year relative survival is very good (87.8%) (14). On the other hand, an increased age adjusted mortality of 23% more than 20 years after diagnosis has been found for all stages of breast cancer (14). Whether mammography screening and early detection of breast cancer will result in reduced mortality, is still a matter of (sometimes hot) debate.

1.2 Proliferation and breast cancer

As mentioned, the prognosis of patients with lymph node-negative breast cancer is relatively good (20-30% die from recurrent disease). The improvement of survival of lymph node-positive patients due to adjuvant systemic therapy is about 25% absolute and 50-60% relative. However, for early lymph node negative breast cancer patients the survival improvement is much less substantial; the typical 15-year survival difference for treated and untreated patients is approximately 10% absolute and 15-30% relative. The benefits of this 10% absolute improvement in survival must be considered against the context of the disadvantages of adjuvant systemic therapy (1, 2) and prognostic/predictive features are important in these decisions.
Often, a combination of features is used in the decision making process for adjuvant systemic therapy, where tumour diameter, oestrogen receptor, grade and also age play a major role (3, 4). A simplified version of this is the Sankt Gallen guideline (15), which identifies 85% or more of all node-negative patients as high risk. As only ~25% of these patients die from metastatic disease without adjuvant treatment in the 20 years following the diagnosis, the remaining 60% of patients receiving adjuvant systemic treatment are overtreated as they would have remained alive and well anyway. On a world-wide basis, a more sensitive and specific prognosticator could therefore prevent enormous unnecessary suffering.

Proliferation markers such as the mitotic activity index (MAI) and Thymidine Labeling Index (TLI) are prognostically stronger than classical prognosticators and predictors, as shown in many prospective and retrospective (also multicenter) analyses (5-7). Moreover, while studies evaluating the role of individual genes regulating these processes have increased our knowledge of the complex process of proliferation, the functional end result is dividing cells. This is microscopically visible as mitotic figures which have remained the most important prognostic factor so far (16). Moreover, the MAI is widely available, easy to use, inexpensive, and highly reproducible (13, 17). A large, multicenter prospective long follow-up study (13) showed that lymph node negative patients with high proliferation (MAI ≥ 10) have the same poor outcome as women with 1-3 positive lymph nodes (Figure 2).
Figure 2. Results from the MMMCP study, showing that lymph node negative patients with high proliferation (MAI ≥ 10) have the same poor outcome as women with 1-3 positive lymph nodes (adapted from reference 13)

Moreover, in two independent studies, adjuvant chemotherapy was significantly beneficial to patients with rapidly proliferating tumours but not to patients with slowly proliferating tumours (18, 19) (Table 1).

Table 1. Adjuvant chemotherapy in node negative breast cancer patients is significantly beneficial to patients with rapidly proliferating tumours but not to patients with slowly proliferating tumours (modified from Janssen et al (18)).

<table>
<thead>
<tr>
<th>10-year survival (%)</th>
<th>Adjuvant Systemic Chemotherapy</th>
<th>Probability of no difference</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Low proliferation</td>
<td>92</td>
<td>94</td>
</tr>
<tr>
<td>(MAI &lt; 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High proliferation</td>
<td>87</td>
<td>75</td>
</tr>
<tr>
<td>(MAI ≥ 3)</td>
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Although proof of a crucial prognostic and predictive role of proliferation is thus very strong, two small studies did not confirm this (20, 21). Smaller studies inevitably carry the risk for selection bias, but other error sources should also be considered. In order to explain these discrepancies we have analyzed possible error sources together with potentially powerful immuno-histochemical alternatives for the MAI. Validation of a prognostic test in independent multicenter test-set studies is mandatory for a diagnostic or therapeutic clinical laboratory test (22, 23).

One objection against mitotic activity index (MAI) assessment is that it is subjective and not well reproducible. Much work has been done since the early 1980s regarding the factors causing lack of reproducibility and how to avoid pitfalls and these will be discussed in the first article. In the second article, we will compare the strength of MAI with other models of prognostication of T1-3N0M0 breast cancer patients and in the third article, we will study the prognostic significance of the MAI in a special subtype of invasive breast cancers: the lobular invasive cancers. In the other articles, the MAI and two other proliferation biomarkers (Phosphohistone-H3 and Ki67) will be studied as well.
2. Materials and Methods

2.1 Patients

The lymph node negative breast cancer and lobular invasive cancers described in the second and third article were from the prospective national Dutch Multicenter Morphometric Mammary Carcinoma Project and approved by the Ethics Committee of the Free University Medical Center (Amsterdam, the Netherlands) and all collaborating hospitals. In this study, 3,474 consecutive patients with primary breast cancer were diagnosed and treated in 34 collaborating hospitals between October 1987 and December 1989. Median follow-up time was 118 months (i.e., 9.8 years; range, 8 to 185 months). In the present PhD study, 516 patients with invasive T1-3 breast carcinoma (article 2) and 121 patients with invasive lobular carcinoma (article 3), all with LN-negative status, and age less than 55 years from the MMMCP were analyzed. All patients underwent mastectomy or breast-conserving surgery in addition to axillary LN dissection; the median number of LNs investigated was 14. Whole-breast radiotherapy was given to patients who underwent breast-conserving therapy. Because all patients were axillary node negative, they received radiotherapy to regional nodal fields only if they had medially localized tumours. Following the national guidelines during the enrollment period, none of these LN-negative patients received any form of adjuvant systemic chemotherapy.

The Stavanger material used in articles 4, 5 and 6 was a part of a large prospective multicenter international breast cancer study “Prognostic and predictive aspects of breast cancer”, started in 1975. The Stavanger part of this project was approved by the Regional Ethics Committee, The Norwegian Social Science Data Service, and the Norwegian Data
Inspectorate. In the studies described in articles 4, 5 and 6 we analyzed patients diagnosed with invasive, operable, lymph node negative (T\textsubscript{1-2}N\textsubscript{0}M\textsubscript{0}) breast cancer at the Stavanger University Hospital, between January 1, 1990 and December 31, 1997. None of these patients received adjuvant systemic chemotherapy.

2.2 Pathology

Post-surgical size of the tumor (pT) was measured in the fresh specimens; the tumors were cut into slices of 0.5 centimeter, fixed in buffered 4% formaldehyde and embedded in paraffin. Four micrometers thick paraffin sections were cut and stained with hematoxylin and eosin (the Norwegian breast cancer sections were also stained with safran). Histologic grade and type were assessed in the many participating centers and independently reviewed by three experienced breast pathologists. Invasive lobular cancers were defined using the World Health Organization (WHO) criteria (24) as cancers composed of non-cohesive cells individually dispersed or arranged in a single-file linear pattern (often referred to as ‘Indian filing’), often associated with lobular carcinoma in situ (LCIS), frequently with a diffuse growth pattern or cell infiltrate, and having a concentric “targetoid” pattern around normal ducts. Grade was assessed, again according to the WHO criteria using MAI 0-5=1, 6-10=2 and >10 as 3, nuclear atypia as mild=1, moderate=2 and marked=3, and tubule formation as much (>75%)=1, minimal (<10%)=3 and intermediate (10-75%)=2. A careful review of type in cases of adequate follow-up revealed that 1812 cancers were ductal; 113 ductal combined with other types (but non-lobular); 201 lobular invasive; 23 tubular; 22 colloid; 11 medullary, and 2 were papillary (total=2184). As the non-lobular non-ductal cancers were relatively rare and also had a much better prognosis than the ILCs and IDCs, they were not
further considered in the present studies. Of the 201 lobular invasive cancers, 121 were LNneg, and sub-typed independently by two of us (EG, JB). LCIS was characterized by extended lobules with a typical solid cell pattern without a clear lumen, consisting of cells with remarkably round and regular nuclei with regular spacing, often with somewhat more cytoplasm than in ductal cancers and frequently containing cytoplasmic vacuoles. The pleomorphic and solid variants may cause differential diagnostic problems with classical ("Indian filing") ILC on the one hand (for the pleomorphic ILCs) and ductal grade 3 cancers (for the solid ones) on the other. To avoid this, using the WHO 2003 criteria, we defined ILCs as pleomorphic if they had clearly large and round, rather than molded, small nuclei located in Indian files of one, or at maximum, focally two cells thickness. To avoid a false inclusion of ductal grade 3 cancers, the following characteristics of LCIS or Indian filing with targetoid growth pattern should be present to classify a cancer as an ILC, solid subtype. Presence of extensive necrosis occurred in one solid cancer which is unusual in solid ILCs, and therefore this case was excluded as well, also due to the E-cadherin staining result (see below). In all cases classified as pleomorphic or solid, E-cadherin staining was done using normal glands in the same sections as internal controls. The non-lobular ductal combinations were regarded as ductal but the ductulo-lobular cancers were regarded as a lobular subtype variant; they had the same survival as the ductal and the non-solid-non-pleomorphic lobular subtype cancers. The resection margins in the biopsies were evaluated as free or not free of tumour. Oestrogen receptor value (ER) was assessed in reference laboratories with the charcoal technique (cut off 10 fmol/mg protein). For the ER negative cases, immunohistochemical analysis was done.
2.3 Immunohistochemistry and measurement of section thickness

Antigen retrieval and antibody dilution were optimized prior to the onset of the studies. Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol. Antigen was retrieved with a highly stabilized retrieval system (ImmunoPrep, Instrumec, Oslo, Norway) using 10 mM TRIS/1 mM EDTA (pH 9.0) as the retrieval buffer. Sections were heated for 3 min at 110°C followed by 10 min at 95°C and cooled to 20°C. Rabbit polyclonal anti-phosphohistone H3 (ser 10) (Upstate #06-570; Lake Placid, NY) was used at a dilution of 1:1500. Ki67 (clone MIB-1, DAKO, Glostrup, Denmark) was used at dilution 1:100. ER (clone SP1, Neomarkers/LabVision, Fremont, CA, USA) was used at a dilution 1/400. PR (clone SP2, Neomarkers/LabVision) was used at a dilution of 1/1000. Anti-phosphohistone H3 was incubated for 60 min at 22°C. A primary antibody cocktail of p63 (DAKO, clone 4A4) and D2-40 (DAKO, clone D2-40) were used at final dilution of 1:1200 and 1:200, respectively. All other antibodies were incubated for 30 min at 22°C. For HER2 assessments, the HercepTest kit (DAKO) was used according to the manufacturer’s FDA-approved procedures. HercepTest 2+ and 3+ cases were retested with the PathVysion (Vysis, Downers Grove, IL, USA) assay following the manufacturer’s FDA-approved protocols. Only HER2 amplified cases were regarded as positive. Dako antibody diluent (S0809) being used. The EnVision™ Flex detection system (Dako, K8000) was used for visualization. Sections were incubated for 5 min with peroxidase-blocking reagent (SM801), 20 min with the EnVision™ FLEX/HRP Detection Reagent (SM802), 10 min with EnVision™ FLEX DAB+ Chromogen (DM827)/EnVision™ FLEX Substrate Buffer (SM803) mix and 5 min with EnVision™ FLEX Hematoxylin (K8008). The slides were then dehydrated and mounted. All immunohistochemical stainings were performed using Dako Autostainer Link 48 instrument and EnVision™ FLEX Wash Buffer (DM831).
The section thickness was measured in two different ways. First, in 10 randomly selected cases the distance of the lowest and highest focal plane of the bottom and the top of the Ki67 stained section was measured using a 100 times microscope objective (25). Secondly, in folds of the Ki67 sections, the section thickness could be measured as described by Elias et al (26). There was a reasonably good agreement between the two methods with a coefficient of variation of the section thickness being less than 7%.

For assessment of lymphatic invasion (LVI), the sections were evaluated with double staining (D2-40 (cytoplasmic) and p63 (nuclear)). D2-40-stained slide was assessed for LVI without knowledge of the LVI status based on the original H&E slide or originally reported findings. A lymph vessel that showed positive staining of the endothelium (cytoplasm) for D2-40 and surrounded the tumour cells was diagnosed as positive for LVI. p63 positive myoepithelial cell nuclei within such a structure excluded LVI. Interpretation of immunohistochemical results was made without knowledge of clinical outcome and the status of other prognostic variables.

Assessment of the percentages of Ki67- and PPH3-positive nuclei

The Ki67 counts were done as follows. First, the whole section was scanned with low magnification and in the invasive parts the “Hot Spots” of high Ki67 expression identified. Then, in each of the hot spots with the subjectively highest Ki67 expression, the percentage of epithelial cancer nuclei with any Ki67 positivity was assessed with an objective of 40x (numeric aperture 0.75) in an area at specimen level of 1.59 mm².
(following the counts for Mitotic Activity Index). The hot spot with the highest percentage Ki67 positive nuclei thus obtained was used for each cancer. Two pathologists independently did the Ki67 evaluations.

The PPH3 index was assessed by the same counting protocol as for the MAI in the same invasive peripheral epithelial cancer parts of the most PPH3-positive areas. Nuclei with fine granular PPH3 staining were not counted, as these cells are not in the G2 phase (27). PPH3-rich areas are usually localized in the periphery (i.e., growing zone) of the cancers. If the PPH3 counts of two observers differed by more than 3 figures, the count was repeated with a multi-head microscope and a consensus score was obtained.

2.4 Computer interactive morphometry

Quantitative assessments have been shown to be better reproducible, but still interobserver variation may be considerable. This can be due to differences between different observers in classifying an object as immunohistochemically positive, or a lack of reproducibility due to selection bias by the observer (28). Selection bias may be overcome by systematic random sampling, both in the fields of vision within a relevant area, and in the selection of certain objects. We therefore performed morphometric analysis of Ki67 by means of the semi-automatic interactive computerized QPRODIT system (Leica, Cambridge), as follows. At low magnification, the area with the subjectively highest Ki67 index where the counts had to be done was selected. This area was first marked with a black marker at the underside of the microscopic glass and then electronically demarcated. In that demarcated area, the QPRODIT system automatically selects at random 300 fields of vision (FOVs). Then, at higher magnification the nuclei in each FOV were projected on the monitor, and Ki67 immunoquantitation was performed at a final magnification of 400 (objective 40, numerical
2.5 Digital image analysis

In addition to performing subjective counts and computerized interactive morphometry, Ki67 and PPH3 expression was also evaluated using the fully automated VIS digital image analysis (DIA) system (Visiopharm, Hørsholm, Denmark), using similar image processing principles as described before (29). Depending on the tumour diameter, two-ten square areas of each 1.59 mm² with subjectively the highest Ki67 index were scanned at 20x magnification. A mask of tumour cells was semi-automatically created. Inside this mask blue (negative) and brown Ki67 positive nuclei were segmented using a Bayesian classifier. The Ki67 index was calculated using the areas of classified blue and brown nuclei. The square
with highest Ki67 index was used as the final result. Figure 3 illustrates the procedure.

![Image of Ki67 image analysis](image)

**Figure 3.** Examples of Ki67 image analysis. a) Overview image of the whole slide with 1.59 mm² squares manually placed in the areas that subjectively show the highest number of positive cells. b) Rescan at 20x magnification of one of the squares in (a) showing a semi-automated mask (dotted line) of tumour cells. c) Tumour cells inside the mask in (b) are classified using a Bayesian classifier into negative nuclei, blue label, Ki-67 positive nuclei, yellow label and cytoplasm, red label. d) and e) Small section of b and c showing more details. The Ki-67 percentage is calculated on the basis of the area of the blue and yellow label. Note that only epithelial tumour cells are analyzed. Normal epithelium, stroma cells and leucocytes are excluded by the mask. The square in (a) showing the highest percentage of Ki-67 positivity per 1.59 mm² is used as the final result.
Not surprisingly, the reproducibility of the DAI-Ki67 and PPH3 counts by the automated digital image analysis on different days by different observers on 10 randomly selected cases was close to perfect ($R^2=0.99$).
3. AIMS OF THE THESIS

Accurate and well reproducible prognostic factors are important for treatment decisions in breast cancer.

The aims of our studies therefore were:

1. Compare the prognostic power of Adjuvant! and the NBCG guidelines with the MAI.

2. To assess proliferation and other prognostic factors in lymph node negative invasive lobular carcinoma;

3. To clarify the prognostic value of D2-40 and D2-40/p63-defined lymph vessel invasion in lymph node negative breast carcinoma;

4. To assess the reproducibility and prognostic value of the proliferation marker Ki67 assessed by different determination methods and evaluated the multivariate independent value of Ki67, MAI and PPH3 in early (T1-2) lymph node negative breast carcinoma.
4. RESULTS

**Paper 1**


Independent studies have shown that in node negative breast cancer patients less than 71 years, the proliferation marker mitotic activity index (MAI) is the strongest, most well reproducible prognosticator and chemotherapy success predictor. The MAI should be determined in the periphery of the tumour. The MAI overshadows the prognostic value of tubule formation, nuclear atypia and thereby grade. An often used crude mitotic impression is much less prognostic than the MAI; strict adherence to the MAI protocol is therefore important. The prognostic value of the MAI is age dependent: although patients with a MAI $\geq 10$ always have a poor prognosis irrespective of age, a low MAI (<10) loses its favourable prognostic association in women $>70$ years. PPH3 counts are prognostically stronger than the MAI, and markers such as Cyclin-B and E2FR are promising, but must be validated.

*Compared with commercial prognostic gene expression signatures, the MAI is at least as strong prognostically, has far fewer false positive results and as such should be included as an independent feature in any lymph node negative breast cancer pathology report. The prognostic thresholds prescribed by the World Health Organization (WHO2003) of 0-5, 6-10 and $> 10$, are not correct for the node negative breast cancers. Rather, the most important prognostic threshold is $<10$ vs $\geq 10$. Patients with low proliferation (MAI<3) do not benefit from adjuvant chemotherapy.*
Lende TH, Janssen EAM, Gudlaugsson E, Voorhorst FJ, Smaaland R, van Diest P, Søiland H, Baak JPA. In patients younger than age 55 years with lymph node-negative breast cancer, proliferation by mitotic activity index is prognostically superior to Adjuvant!. J Clin Oncol 2010; 29(7):852-858.

In large multicenter prospective and retrospective studies, the MAI has proven to be a robust and reproducible prognostic factor in lymph node-negative patients younger than age 55 years and also predicts the effect of adjuvant systemic therapy in this age group (18). 516 lymph node-negative breast cancer patients age less than 55 years with a 118 months median follow-up were analyzed. The concordance between MAI on one hand and the online risk model/calculator Adjuvant! and the Norwegian breast cancer group (NBCG) guidelines on the other hand, was fair (κ = 0.35 and κ = 0.29, respectively). Adjuvant!, NBCG, and MAI were all prognostically significant (P≤.001). In the univariate analysis, the 10-year BCSS of MAI <3 versus ≥ 3 was 95% v 71%, respectively, with a hazard ratio of 7.0. In multivariable analysis, MAI was superior to Adjuvant! and NBCG. The 10-year survival of Adjuvant! ≥95% versus <95% was 91% v 74%, respectively, but stratification by MAI identified subgroups with different prognosis. Similar results occurred for NBCG and MAI. Adjuvant! and NBCG were not prognostic to each other.

We conclude that MAI is superior to Adjuvant! and NBCG in prognostication of patients with lymph node-negative breast cancer younger than age 55 years.
Paper 3


Histologic subtype and MAI have independent prognostic value in node negative invasive lobular cancers. With a median follow up time of 83 months (range: 19-181), thirty of the 121 (25%) ILC patients developed distant metastases and 27 (22%) died. None of the cases classified as solid/pleomorphic lobular were E-Cadherin or oestrogen receptor positive contrasting the other ILCs. The solid/ alveolar ILCs (n=17) had a worse survival (50%) than the other ILCs (n=104) (83%, P<0.0001). Mitotic Activity Index (MAI) (but not nuclear grade or tubule formation) was prognostic with a threshold 0-5 versus >5 (=MAI-5) (contrasting MAI<10 vs. ≥10 in breast cancers in general) (85% and 54% survival, P < 0.0001). With multivariate analysis, only ILC-subtype and MAI, and none of the other characteristics, had independent, prognostic value.

We conclude that histologic subtype of invasive lobular cancers and MAI have independent prognostic value in node negative invasive lobular cancers. However, the essential prognostic threshold is 5, not 10.
Gudlaugsson E, Skaland I, Undersrud E, Janssen EA, Søiland H, Baak JP. D2-40/p63 defined lymph vessel invasion has additional prognostic value in highly proliferating operable node negative breast cancer patients. Mod Pathol. 2011 Apr; 24(4):502-11

Phosphohistone H3 assessed proliferation has strong prognostic value. Lymph vessel invasion by D2-40 is also prognostic, but D2-40-positive myoepithelial expression in small ducts completely filled by solid-pattern ductal carcinoma in situ can mimic lymphovascular invasion. As myoepithelial cells are also p63-positive, we have investigated whether lymph vessel invasion identified by combined D2-40/p63 expression is stronger prognostically than by D2-40 expression alone, and whether it has independent prognostic value to phosphohistone H3. In 240 operable T1-2N0M0 node negative invasive breast cancer patients <71 years, phosphohistone H3 was determined by quantitative immunohistochemistry and lymph vessel invasion by D2-40/p63 double immunostaining. Correlation analysis between the clinico-pathologic factors and lymph vessel invasion, and univariate and multivariate prognostic survival analysis were performed. With median 117 (range: 12–192) months follow-up, 36 patients (15%) developed and 28 (12%) died of distant metastases. Ten of the 61 patients (16%) with cancer cells surrounded by D2-40 were p63-positive and none of these ‘false lymph vessel invasion’ recurred. D2-40-positive/ p63-negative lymph vessel invasion occurred in 51/239 (21%) cases and correlated with grade, mitotic activity index, phosphohistone H3, ER, cytokeratin14, and HER2. D2-40-positive/p63-negative lymph vessel invasion was strongly prognostic, but far more in women ≥55 than those <55 years (P<0.0001 and 0.04, respectively). With multivariate analysis, phosphohistone H3 proliferation was the strongest single prognosticator. Lymph vessel invasion had additional prognostic value to phosphohistone H3 only in women ≥55 years. This group of patients,
without/with lymph vessel invasion, had 10-year survival rates of 83 and 50%, respectively (hazard ratio-lymph vessel invasion = 3.0, P = 0.04; hazard ratio-phosphohistone H3 = 6.9, P = 0.002). Where age was <55 years, only phosphohistone H3 had independent prognostic value. Combinations of other features had no additional value.

We conclude that $T_{1-2}N_0M_0$ invasive breast cancer patients $\geq 55$ years with phosphohistone H3$\geq 13$, D2-40-positive/p63-negative defined lymph vessel invasion identifies a subgroup with a high risk of distant metastases. Lymph Vessel Invasion defined by D2-40 alone, gave many false positive lymph-vessel invasions and none of these ‘false lymph vessel invasion’ recurred.

Paper 5

Gudlaugsson, Skaland I, Janssen EAM, Feng W, Shao Z, Malpica A and Baak JP.

Automation of measurement of Ki67 proliferation is essential for reproducible and accurate prognosis prediction in $T_{1-2}N_0M_0$ breast cancer. Manuscript Sept, 2011

The proliferation factor Ki67 is prognostic in early breast cancer, but immunohistochemical determinations differ between laboratories, measurements not being standardized or automated. In 237 $T_{1-2}N_0M_0$ breast cancers without adjuvant systemic treatment, formalized MAI assessment, strictly standardized and fully automated quantitative Ki67 immunohistochemistry were used. The percentages of Ki67 positive nuclei were assessed independently by two pathologists using conventional counts, by computerized interactive morphometry (CIM) and by automated digital image analysis (DIA). Reproducibility was demonstrated by duplicate blinded assessments. Using univariate and multivariate survival
analyses, the prognostic value of widely accepted Ki67 thresholds (10%, 15%, 20%) and other thresholds indicated by Receiver Operating Curve analysis were studied. Quick scan rapid estimates of Ki67 were poorly reproducible and not prognostic. The Ki67 counts by two pathologists and their corresponding optimal prognostic thresholds varied greatly (4% and 14%). However, DIA-Ki67 and CIM-Ki67 were reproducible and highly prognostic (P<0.0001). DIA-Ki67 with a threshold of 6.5% was the strongest prognosticator.

We conclude that in node negative breast cancer without adjuvant systemic treatment, Ki67 in the periphery of the tumour assessed by digital image analysis, but not subjective counts, was reproducible and prognostically strong. This casts serious doubt on therapeutic guidelines using subjective counts of Ki67.

Paper 6

Gudlaugsson, Skaland I, Janssen EAM, Feng W, Shao Z, Malpica A and Baak JP. Multivariate comparison of the prognostic value of the proliferation markers Mitotic Activity Index, PhosphoHistone-3, Ki67, steroid receptors, HER2, basal cell type and classical prognostic factors in T_{1,2}N_{0}M_{0} breast cancer. Manuscript Oct, 2011

The reproducibility and prognostic value of Ki67 versus Mitotic Activity Index (MAI) and Phosphohistone-H3 (PPH3) is largely unknown and have not been studied. Further, these were compared with classical clinico-pathologic prognostic and predictive factors (tumour size, oestrogen receptor and HER2/neu). In 237 T_{1,2}N_{0}M_{0} breast cancers without systemic adjuvant treatment, formalized MAI assessment was performed. We used strictly standardized and fully automated quantitative immunohistochemistry for Ki67, PPH3, oestrogen (ER) and progesterone receptor (PR), HER2, cytokeratins 5 and 6, and automated
digital image analysis (DIA) for measuring PPH3 and Ki67. Receiver Operating Curve (ROC) analysis was used to objectively assess the optimal threshold of continuous variable such as Ki67. Section thickness was measured to increase standardization of Ki67 IHC. Univariate and multivariate survival analyses were used to compare the different proliferation and other well established clinico-pathologic prognostic factors.

Section thickness had a low coefficient of variation, indicating that this potential error source can be kept minimal. ROC analysis showed that a DIA-Ki67 threshold of 6.5% had optimal and strongest prognostic value and added prognostically to PPH3. None of the other biomarkers of clinicopathologic variables added prognostically to this PPH3/Ki67 combination. However, when PPH3 is replace by MAI the prognostic value is nearly the same.

*We conclude, that in node negative breast cancer without adjuvant systemic treatment, Ki67 assessed by digital image analysis with a threshold of 6.5%, is prognostically strong. The combination of either PPH3/Ki67 or MAI/Ki67 overshadowed the prognostic value of all other features.*
5. CONCLUSIONS OF THIS THESIS

On the basis of the literature studied and the research studies described in this PhD thesis, the following conclusions are drawn.

1. Mitotic Activity Index is an accurate prognostic factor in lymph node negative breast cancer in women under 71 years old. The MAI can be well reproducible when the MMMCP protocol for tissue processing, sectioning, staining and counting is carefully kept. The strongest prognostic threshold is $<10$ versus $\geq 10$, but patients with MAI $< 3$ still have a slightly better prognosis than those with $3 \geq$ MAI $\leq 10$.

2. The MAI is superior to Adjuvant! and NBCG in prognostication of patients with lymph node-negative breast cancer younger than age 55 years and identifies a high-risk subgroup of patients classified as low risk and a low-risk subgroup classified as high risk with conventional prognostication models.

3. The MAI is also prognostic in node negative breast cancers of the lobular invasive type, but the threshold of 10 is too high. A threshold of 5 is the most optimal threshold. The histologic subtype of invasive lobular cancer and the MAI have independent prognostic value.

4. Lymph vessel invasion defined by D2-40 is not specific enough; a double staining of D2-40 and p63 is better suited as it distinguishes ductal carcinoma in situ from real lymph vessel invasion. However, D2-40 and D2-40/p63 defined lymph vessel invasion have no additional prognostic value in node negative breast cancer patients under 71 years old.
5. In node negative breast cancer without adjuvant systemic treatment, Ki67 assessed by digital image analysis or interactive point-weighted morphometry, but not by subjective counts, was reproducible and prognostically strong. Digital image analysis assessed Ki67 expression gives the best reproducible results and is also prognostically strongest. The optimal Receiver Operating Curve (ROC) detected prognostic threshold is <6.5% versus ≥6.5%. The threshold of the Norwegian Breast Cancer Group of 15%, for node positive breast cancers, is prognostically much less strong than the threshold of 6.5 for the node negative patients.

6. CIM supported Ki67 evaluation is also strongly prognostic and well reproducible with a threshold of 9.5%

7. Subjective counts between different pathologists are not well reproducible. This casts a serious doubt on the NBCG guidelines of a fixed 15% Ki67-threshold, unless the measurements are done by DIA or CIM.

8. A quick global scan with a rapid Ki67 estimate is not reproducible at all, and not prognostic. The use of such strategy in routine surgical pathology must be regarded as a serious professional violation.

9. As single variables, the MAI or PPH3 are prognostically stronger than Ki67-6.5% by digital image analysis.

10. PPH3 is the strongest prognostic factor and Ki67-6.5% by DIA has additional prognostic value in patients with a low PPH3<13 per 1.59 mm² at specimen level. In daily practice, all three proliferation factors (MAI, PPH3 and Ki67) should be
assessed and the results carefully compared for adequate quality control and to get the most accurate prognosis and therapy effect prediction for node negative breast cancers.
6. GENERAL DISCUSSION AND FUTURE DIRECTIONS

6.1 General remarks

In patients under 71 years old with operable lymph node negative breast cancer, the current trend is to use increasingly integrated models of clinical and molecular quantitative pathological data for prognostic and predictive models and therapeutic decision making. The most accurate assessment of established markers should be key features for a choice of useful treatment.

6.2 Gene expression arrays

The development of sophisticated technologies, such as gene expression arrays, permitting simultaneous measurement of thousands of genes to create a molecular portrait of the tumour, has identified molecular signatures, such as the 21-gene recurrence score (RS, Oncotype Dx®) or the Amsterdam 70-gene prognostic profile (Mammaprint®), that augment conventional prognostic indicators in their ability to predict breast cancer outcome and response to treatment particularly in identifying good- and poor-risk tumours within ER-positive disease. The clinical value of these profiles for ER-negative disease is less clear (30) (Table 2).
Table 2. Characteristics of several well-known genomic profiles.

<table>
<thead>
<tr>
<th>Test</th>
<th>Name</th>
<th>Tissue source</th>
<th>Patient</th>
<th># genes</th>
<th>Validated for clinical use</th>
<th>Therapy</th>
<th>Certif</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prognostic</td>
<td>Mammaprint® (70-gene profile)</td>
<td>Fresh or Frozen</td>
<td>ER+/- LN-</td>
<td>70</td>
<td>Yes</td>
<td>No</td>
<td>Yes (FDA)</td>
<td>~1500$</td>
</tr>
<tr>
<td></td>
<td>Oncotype Dx®</td>
<td>FFPE</td>
<td>ER+</td>
<td>21</td>
<td>Yes</td>
<td>Yes TAM</td>
<td>No</td>
<td>~3500$</td>
</tr>
<tr>
<td>Prognostic and predictive</td>
<td>Rotterdam (76-gene signature)</td>
<td>Fresh or Frozen</td>
<td>ER+/- LN+/-</td>
<td>76</td>
<td>Yes</td>
<td>Perhaps TAM</td>
<td>No</td>
<td>Unknown</td>
</tr>
<tr>
<td>Two-gene ratio</td>
<td>FFPE</td>
<td>ER+</td>
<td>6</td>
<td>No</td>
<td>Yes TAM</td>
<td>No</td>
<td>~1400$</td>
<td></td>
</tr>
</tbody>
</table>

ER: Estrogen Receptor; LN: Lymph Node; FFPE: Formaldehyde Fixed Paraffin Embedded; FDA: Food and Drug Administration (USA); TAM: Tamoxifen; AST: Adjuvant Systemic Treatment; Certif: Certification

The Recurrence Score (RS) of Oncotype Dx® was developed using a different approach from the supervised analyses of gene expression arrays used by the 70-gene and 76-gene profile developers (discussed below). In this approach, the investigators started with the 250 most promising candidate genes selected from the literature. They used a reverse transcription polymerase chain reaction (RT-PCR)-based method for generating quantitative expression levels of these genes in fixed tissue from 447 patients collected from three largely hormone receptor-positive, node-negative datasets. The result is the RS, which is actually a mathematical formula that includes 16 genes (of which 10 are proliferation associated) weighted to optimize prediction of distant relapse despite tamoxifen therapy (31). It is among the best-validated prognostic assays, has some value in predicting chemotherapy response, and is relatively unique in that it can be used in fixed tissue. It is recommended by the
American Society of Clinical Oncology (ASCO) for use in women with node-negative, estrogen receptor (ER)-positive breast cancer (32).

The RS was validated in an independent dataset derived from 668 samples (from a total of 2617) collected in the tamoxifen-treated arm of National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14, a prospective randomized clinical trial examining the benefit of adjuvant tamoxifen in hormone receptor-positive, node-negative breast cancer (31). Study participants were largely postmenopausal (71 percent), stage I (62 percent), and with a good prognosis (85 percent free of distant metastasis at 10 years). Although this population had a generally good prognosis, reflecting the low stage and treatment with tamoxifen, the RS was able to distinguish prognostic groups: of those with low RS (<18), 93 percent were free of distant disease compared with only 70 percent of those with high RS (>31). Further analysis has shown the RS to be predictive of locoregional recurrence as well as distant recurrence (33). Similar findings have been reported with aromatase inhibitors in postmenopausal women (34), and it appears to identify relative benefit of chemotherapy across nonanthracycline, anthracycline, and anthracycline/taxane-based regimens (35-37). This usefulness is relatively clear at the extremes of the RS, but there is uncertainty with intermediate RS. The ongoing TAILORx trial (Figure 4) is expected to provide high-level evidence for the role of the RS in identifying those who may and those who may not benefit from chemotherapy (38).
Figure 4. Randomized trial design for TAILORx (Trial Assigning Individualized Options for Treatment Rx, from reference 38).

The Mammaprint® (Amsterdam 70-gene profile) was the first in-vitro diagnostic multivariate index assay to be approved by the US Food and Drug Administration (FDA) in 2007. It is an array-based prognosticator and classifies tumors as low-risk or high-risk for breast cancer recurrence. The test result, a binary score (low-risk and high-risk), is then derived through an algorithm based upon gene expression. A supervised analysis of the gene expression arrays on 98 breast tumor samples (80% were from lymph node-negative women younger than 55 years, of whom 44% had developed distant metastasis within five years), selected a 70-gene set with 83 percent accuracy at differentiating those with distant relapse versus those without (39).
The first validation study was a retrospective analysis from the same institution of 295 patients, who were also young (less than 53 years at diagnosis), with T1 and T2 tumors, node-negative (151 patients) or node-positive (144 patients), treated with heterogeneous adjuvant therapy, and followed for nearly seven years (40). This analysis found a strong correlation between the "good" or "bad" 70-gene signature and likelihood of distant recurrence or death in multivariable analysis; however, it was criticized because of the bias introduced by the inclusion in this study of patients from the original set used to create the profile.

A subsequent validation study used a truly independent patient cohort of 302 women; patients were under age 60, had node-negative T1 to T2 tumors, were treated without adjuvant systemic therapy, and were followed for over 10 years (41). The 70-gene signature performed independent of clinical variables in predicting time to distant metastasis (hazard ratio, HR 2.13) and overall survival (HR 2.63), but not disease-free survival (HR 1.36) (41). Patients in the gene signature high-risk group had a 10-year overall survival of 70 percent versus 90 percent for patients in the gene signature low-risk group.

The primary objectives of the MINDACT (Microarray In Node-negative and 1-3 positive lymph- node Disease may Avoid ChemoTherapy) trial is to better select \( T_{1-3}N_{0-3} \) breast cancer patients for adjuvant chemotherapy and hopefully thereby reduce the number of patients exposed to short and long term side effects of unnecessary cytotoxic treatments. It is a prospective multicentre randomized phase III study comparing the 70-gene signature with the common clinical-pathological criteria in selecting patients for adjuvant chemotherapy in breast cancer with 0-3 positive nodes (42).
The two other main objectives of the study address questions related to adjuvant treatment of breast cancer. MINDACT will compare anthracycline-based chemotherapy regimens to a docetaxel-capecitabine regimen and investigate the efficacy and safety of endocrine therapy with a 7 years single agent Letrozole to the sequential strategy of 2 years of Tamoxifen followed by 5 years of Letrozole (Randomisation-Endocrine therapy, see Figure 5).

The Rotterdam 76-gene prognostic signature was developed in a test set of 115 node-negative primary breast cancers from women who did not receive adjuvant therapy and had been followed for more than eight years (43). Recognizing the genetic heterogeneity of breast cancer, separate prognostic gene sets were developed for ER-negative (ER-, 16 genes)
and ER-positive (ER+, 60 genes) disease. The 76 prognostic genes were validated in an independent set of 171 mixed ER+ (75%) and ER- (25%) tumors, demonstrating 93 percent sensitivity and 48 percent specificity. In multivariate analysis of distant metastasis-free survival, the 76-gene prognostic indicator was independent of clinical variables (43).

In a 198-patient subset of the 302 node-negative patients used in a validation study of the 70-gene profile (Mammaprint®, described above), the 76-gene prognostic signature demonstrated independent value (44).

A major drawback of both the Mammaprint® and The Rotterdam signature is the need for fresh-frozen tissue. It is also noteworthy that the validation studies highlight the time dependence of these signatures, using early relapse as the endpoint; they predict early relapse far better than late relapse (>5 years). The biology of late relapse might differ from early relapse and has been inadequately studied.

A number of other signatures are under development. One of the most promising is the two-gene signature (HOX13:IL17BR). It was developed from 60 node-positive, hormone receptor-positive tumors and has been shown to predict breast cancer recurrence and endocrine therapy sensitivity (45). The antiapoptotic homeobox B13 (HOXB13) gene was associated with recurrence, while interleukin 17 receptor B (IL17BR) was associated with remaining disease free, making the ratio even more strongly associated with recurrence, with an adjusted odds ratio of approximately 7. However, various efforts to examine this signature in fixed tissue have yielded mixed results.

Gene expression assays are costly, require much more cancer tissue than the molecular quantitative data (like MAI, PPH3, Ki67, ER, PR, HER2 and CK5-6), the
prognosis prediction is not better than with MAI and PPH3 (Figure 6), and overtreatment is much worse (see Figure 7).

**Figure 6.** The Overall (disease specific) survival rates in the prospective study of the MammaPrint® 70-gene signature (41) (left) and from the prospective MMMCP study of the Mitotic Activity Index, adapted from reference 13 (right). Note the similarity in the survival rates.

**Figure 7.** a) The real deaths of metastatic disease in node-negative operable breast cancer patients (data of the prospective MMMCP study from reference 13). b) The number of patients classified as high risk by the Mitotic Activity Index, and c) the 70-gene signature mRNA MammaPrint® test (adapted from reference 41).
The Mammaprint® overtreats many more patients than the MAI with a threshold of 10 (see Table 3).

Table 3. Summary of survival rates of node-negative breast cancer according to gene expression signatures and Mitotic Activity Index.

<table>
<thead>
<tr>
<th></th>
<th>Mammaprint® 70-gene signature (from reference 41)</th>
<th>Mitotic Activity Index (MAI) (from reference 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>198</td>
<td>516</td>
</tr>
<tr>
<td><strong>Number and incidence (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good Signature/MAI&lt;10</td>
<td>55 (28%)</td>
<td>299 (58%)</td>
</tr>
<tr>
<td>Poor Signature/MAI≥10</td>
<td>143 (72%)</td>
<td>217 (42%)</td>
</tr>
<tr>
<td><strong>Overall Survival 10 years</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good Signature/MAI&lt;10</td>
<td>92%</td>
<td>92%</td>
</tr>
<tr>
<td>Poor Signature/MAI≥10</td>
<td>72%</td>
<td>65%</td>
</tr>
<tr>
<td>Difference Good and Poor signature/MAI&lt;10 and MAI≥10</td>
<td>20%</td>
<td>27%</td>
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The abovementioned data show that the Mammaprint® gene expression array for prognosis prediction in node negative breast cancer is less good than the MAI test with a threshold of 10. We therefore do not agree that appropriate integration of molecular assays (46) add power to the prognostic model obtained by the proliferation biomarkers described
by others (47), and also in the current thesis. Few studies have dealt with the methodological issues, such as sampling strategies, intratumour heterogeneity and reproducibility, as most studies are retrospective and thresholds vary.

However, that does not take away that gene expression arrays will not be important. Medical oncologists are increasingly interested in the effect of certain therapies, rather than prognosis in general. Is a tumour sensitive to a certain treatment? Such questions are increasingly asked to the pathologist and if the pathology laboratory does not give such answers, this role is taken over by other specialized laboratories. Pathology laboratories should equip themselves with adequate technology to give such answers, if they wish to survive rather than be marginalized.

6.3 Standardization, Automation, Validation

In our view, for breast cancer patients under 71 years, quantitative in situ proliferation markers MAI, PPH3 and Ki67, are the best predictors of outcome. The remaining problem is standardization. It has been shown that the MAI can be well reproducible when the MMMCP guidelines for tissue processing, staining, and MAI counts are strictly kept. However, this cannot be standardized and depends on the efforts of many technicians and pathologists worldwide.

As long as there are no (inter)national guidelines with external quality control programs, and penalties on violation, strictly controlled standards and automated machines for tissue processing, tissue section thickness measurement, antigen retrieval, staining, and automated assessment of the proliferation markers, there is little hope that the molecular quantitative pathological criteria will be accepted worldwide. The professional and medical organizations in the past 3 decades have failed to achieve these goals. Since certain
pharmaceutical companies have entered the pathology market in 2009, Ki67 has suddenly been accepted as a treatment criterion, in spite of lack of Quality Control and Assessment, or any guidelines for the assessment of the Ki67 expression.

In our laboratory, we assess MAI using the strict MMMCP protocol, and PPH3 and Ki67 by strictly protocolized automated immunohistochemistry and digital image processing. The quality of our IHC-assessments is continuously validated by participation in the NORDIQC external Quality Control program. However, validation studies between different pathology laboratories are largely lacking and mandatory before serious (inter)national conclusions can be drawn from the results of molecular pathology findings determined without such protocols. Unfortunately, such validations are largely lacking, both in Norway and outside. It is somewhat surprising that neither the Norwegian Pathology Society nor the European Society of Pathology have initiated such projects which are essential for the reliability of pathology determinations.
6.4 Elderly breast cancer patients

It seems that breast cancers in patients >70 years react differently from those in younger women. The biologic characteristics of breast cancers change as age increases. Tumors in older patients are more likely to be hormone receptor positive, HER-2 negative, lymph node negative, and p53 negative and have lower grade with lower proliferation indices (5, 48, 49) (Figure 8).

Figure 8. The prognostic value of proliferation in lymph node-negative breast cancer patients is age dependent (from reference 5).

However, very little serious research has been carried out, in spite of the fact that in the coming decades many more patients with breast cancer will be over 70 years of age at the time of diagnosis. Future studies should concentrate on unravelling the biology of breast cancers in this age group.
6.5 **Insulin and Insulin growth Factor Receptor**

There is increasing evidence that insulin, as an important effector of diet and lifestyle plays an important role in the development and progression of breast cancer (50). The findings are biologically plausible, circulating IGF1 concentration is associated with previously known risk factors for breast cancer for which the underlying physiological basis was unclear, such as height, age at menarche, and mammographic breast density, suggesting that these may be linked to risk, at least in part, because they act as surrogates for IGF1 concentration (51). Quantitative *in situ* analysis of Insulin and Insulin Growth Factor receptors seems utterly important.

6.6 **Lymph node positive patients**

Another important research aspect is patients with limited node positive breast cancer (1, 2 and 3 positive lymph nodes). These are increasingly important as patients with extensive nodal metastases are decreasing. It may be that patients with 1 positive lymph node, behave similar to node negative patients and may require less chemotherapy. The influence of the sentinel-node method on lymph node status should also be evaluated.

6.7 **microRNA and proteomics**

Finally, alternative techniques could be considered. We will discuss two methods: microRNA and Proteomics.
Recently an extra level of gene regulation was discovered: small non-coding RNA molecules called microRNA’s (miRNA). They play an important role in gene-silencing by binding directly and specifically to mRNA molecules. miRNAs are 19-25 nucleotides in length and compose the largest family of non-coding RNA’s involved in gene silencing. Their functions are exerted through translational inhibition of the targeted mRNA by binding to the 3’-UTR (imperfect match) and degradation of target mRNA (perfect match) (52) miRNAs are down-regulated in a number of different tumours (53, 54), and in some cases the re-introduction of these miRNAs has been shown to impair the viability of cancer cells (55).

We and others have recently shown that miRNA can be useful. In a recent study, Janssen et al (56) studied the miRNA pattern in 103 lymph node negative breast cancer and compared these profiles with different biologic characteristics and clinicopathologic features. Unsupervised hierarchical cluster analysis divides the patients in 4 main groups, of which the basal-like/TNP group is the most prominent (11% of all cases). The luminal A cancers containing the HER2 negative and ER/PR-positive tumours is the largest group (57%) and the group of luminal B (32%) is more heterogeneous and contains the HER2 positive/ER-negative patients as well. The highest overall classification values by ANOVA analysis followed by cross validation (leave one sample out and reselect genes) were found for CK5-6, TNP and ER, with 97%, 90% and 90% accuracy. MiR-106b is prominently present in all of these signatures and correlates strongest with high proliferation. Other interesting observations are the presence of several miRNAs (miR532-5p, miR-500, miR362-5p, and miR502-3p) located at Xp11.23 in cancers with a TNP signature and the upregulation of several miR-17 cluster members in ER-negative tumours. The study showed that ER negativity and CK5-6 expression are important and specific biologic processes in lymph
node negative breast cancer as the correlations with specific microRNAs are strongest in the ERα-negative and CK5-6 positive tumours. miRNA analysis in breast cancer therefore seem promising. However, topics such as test reproducibility, and sampling variations still have to be studied. In how far does tumour heterogeneity interfere with the results? Is there a difference between the center and the periphery, and what about small and large tumours? Are metastatic deposits different from the primary tumours? Such studies are largely lacking and must be undertaken before the methods can be taken into routine use.

*The proteome*, or protein composition, determines the functional state of a cancer. Expression of proteins between different diagnostic or prognostic groups can be detected by analysis of their proteins but may not be detectable by genomic analysis.

Proteomic analysis offers the possibility to compare protein abundance in almost any sample type. Proteomics has been applied to breast cancer for some time. Recent studies show promising results (57, 58).

SELDI-TOF mass spectrometry, where proteins are bound to a chromatographic chip and then analyzed, is a high-throughput method utilized to obtain protein profiles that can be used to compare sample groups. Unfortunately, the formaldehyde-fixed paraffin embedding (FFPE) procedure of biopsies destroys water-soluble proteins (59). Our group has recently described a method that can save and extract water soluble proteins from punch biopsies. It was shown that protein collection method for biopsy samples can further help to define the diagnosis and behavior of Cervical Intraepithelial Neoplasia (CIN) lesions. The fresh biopsies are placed in a RPMI1640 medium for 24 hours at 4°C, after which the biopsies were used for routine histological examination. The method resembles the one published by Celis et al (60). We have also shown that a panel of 3 peaks from SELDI-TOF protein
profiles can be used to differentiate normal tissue from CIN tissue samples and that a
discrimination of CIN2 and CIN3 lesions could be obtained using cytokeratin 2 (61).
Moreover, Zinc-finger protein 441 and Phospholipase D6 detected by proteomic LC-MS
(LTQ-Orbitrap) in supernatant samples from a water-soluble protein-saving punch biopsy
processing method can predict regression of CIN2-3 (46). It would be highly interesting to
evaluate the same method in breast cancer.
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