Biomarkers in breast cancer, with special focus on tumor cell proliferation

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Contents

Scientific environment

The *Tumor Biology Research Group* directed by Professor Lars A. Akslen has served as a fruitful environment for this thesis. This research group was established in 1995, and currently encompasses 30 members including senior researchers, postdoctoral fellows, PhD candidates and technicians. Projects are focused on characterization of the tumor microenvironment, tumor cell proliferation, markers of angiogenesis, and tumor-vascular interactions. Various cancers are being studied, including breast, prostate, endometrial, renal, lung cancer, and melanomas. Patient series with complete and long term follow-up are established. Methods involve the use of both fresh and formalin-fixed tumor samples. Also, animal models and cell lines are applied for translational purposes.

This group represents an integral part of the *Centre for Cancer Biomarkers (CCBIO)*, a Norwegian Centre of Excellence established in 2013 and directed by Professor Lars A. Akslen. CCBIO includes nine research groups devoted to research on novel cancer biomarkers and tailored therapy, with special attention drawn to the tumor microenvironment, matrix biology, angiogenesis, epithelial-mesenchymal transition and tumor cell plasticity in relation to metastatic spread. CCBIO has an extensive collaboration both at the national and international level.

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Abbreviations

ASCO	American Society of Clinical Oncology
ATM	Ataxia-telangiectasia mutated
BAD	Bcl-2 associated agonist of cell death
BAX	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma gene-2
BCT	Breast conserving therapy
BRCA1/2	Breast cancer susceptibility gene 1 and 2
BRIP1	BRCA1 interacting protein C-terminal Helicase
CAF	Cancer associated fibroblast
CAP	College of American Pathologists
CDH1	Cadherin 1, type 1 (E-cadherin gene)
CDK	Cyclin-dependent kinase
CHEK2	Checkpoint kinase 2
CK	Cytokeratin
CNB	Core needle biopsy
CS	Cold spot
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAB	Diaminobenzidine
DCIS	Ductal carcinoma in situ
DIA	Digital image analysis
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
FDA	Food and Drug Administration
FNAC	Fine needle aspiration cytology
FFPE	Formalin-fixed paraffin-embedded
FNAC	Fine needle aspiration cytology
H&E	Hematoxylin and eosin
HER2	Human epidermal growth factor 2
HIF	Hypoxia inducible factor
HPF	High power field
HR	Hormone receptor
HS	Hot spot
IHC	Immunohistochemistry
ISH	In situ hybridization
LBK1/STK11	Serine/threonine kinase gene

LCIS	Lobular carcinoma in situ
MAI	Mitotic activity index
MCM	Mini-chromosome maintenance protein
MET	Mesenchymal-epithelial transition
MIB-1	Molecular Immunology Borstel 1
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NBCG	Norwegian Breast Cancer Group
PALB2	Partner and localizer of BRCA2
PAM50	Prediction Analysis of Microarray 50
PCNA	Proliferating cell nuclear antigen
PD-1	Programmed Death 1 receptor
PHH3	Phosphohistone H3
PR	Progesterone receptor
PTEN	Phosphatase tensin homolog on chromosome 10
QSOX1	Quiescin sulfhydryl oxidase 1
R-point	Restriction point
RB	Retinoblastoma
RNA	Ribonucleic acid
SI	Staining index
SISH	Silver in situ hybridisation
SPF	S-phase fraction
SPSS	Statistical package for social sciences
STK11	Serine/threonine kinase 11
TCGA	The Cancer Genome Atlas
TMA	Tissue microarray
TNBC	Triple negative breast cancer
TNM	Tumor-nodes-metastasis
TP53	Tumor protein 53
TSP-1	Thrombospondin-1
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WS	Whole section

Abstract

Background: Breast cancer is a heterogeneous disease encompassing distinct subtypes that differ in incidence and prognosis. Better characterization of established biomarkers and exploration of novel biomarkers and possible treatment targets are important to improve prognostication and tailored therapy. A major challenge has been to predict which patients who are likely to suffer from recurrence and thus may benefit from adjuvant chemotherapy.

Objective: This study aimed to compare three proliferation markers across distinct tissue categories, with association patterns and survival as end-points. Also, we aimed to explore the protein expression and potential prognostic impact of the novel proliferation-related biomarker QSOX1.

Materials and methods: The thesis is based on three papers where a prospective, population-based series of breast cancer (n=534) was examined. In **Paper I**, the proliferation marker Ki67 was assessed by immunohistochemistry across matched samples of whole sections, WS (n=534), core needle biopsies, CNB (n=154) and tissue microarrays, TMA (n=459). In **Paper II**, mitotic count (mitoses per mm^2) was assessed on H&E sections and PHH3 was examined by immunohistochemistry across matched samples (WS, CNB, TMA), and compared with the Ki67 values. In **Paper III**, QSOX1 expression was assessed by immunohistochemistry on TMA sections (n=458).

Results: The proliferation markers (MC, Ki67, PHH3) showed significantly higher counts when assessed on WS as compared to CNB and TMA (**Paper I-II**). Tumor cell proliferation (MC, Ki67, PHH3) varied according to molecular subgroup with highest proliferation in the triple negative subgroup and lowest proliferation in the luminal category. In the luminal/HER2 negative subgroup, there were many discordant cases and only fair agreement when assessing luminal A and B on WS as compared to CNB and TMA (**Paper I-II**). Increased proliferation assessed by MC, Ki67 and PHH3 across all three sample categories showed significant associations

with high histologic grade and hormone receptor negativity (**Paper I-II**). In univariate survival analysis, the prognostic impact of MC, Ki67 and PHH3 were mostly retained across specimen categories. In multivariate Cox analysis, adjusting for age, tumor size, histologic grade and nodal status, mitotic count and Ki67 maintained their independent associations with prognosis, whereas PHH3 did not (**Paper II**). High expression of QSOX1 was associated with high histologic grade, hormone receptor negativity, increased proliferation (MC, Ki67), and HER2 positivity (**Paper III**). High QSOX1 expression was more common among HER2+ and triple negative subgroups. In univariate survival analysis, cases with high QSOX1 expression (SI=9) showed a 10 year survival probability of 67% compared to 89% for carcinomas with low QSOX1 levels (SI=0-6). QSOX1 expression showed independent prognostic impact in multivariate Cox models adjusting for age, histologic grade, tumor size and nodal status.

Conclusions: Assessment of proliferation markers on full sections, when available, should be regarded as current best practice (**Paper I-II)**. For assessment on core needle biopsies, specimen specific thresholds should be considered. TMA is less suited for assessment of proliferation in studies with potential clinical impact (**Paper I-II**). Mitotic count might be used for sub-classification of the luminal group of breast cancers (**Paper II**). High QSOX1 expression in tumor cells is a marker of more aggressive breast cancer (**Paper III**).

List of publications

The thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Knutsvik G**, Stefansson IM, Aziz S, Arnes J, Eide J, Collett K, Akslen LA. Evaluation of Ki67 expression across distinct categories of breast cancer specimens: A population-based study of matched surgical specimens, core needle biopsies and tissue microarrays. *PLoS One* 2014; 9: e112121.

II. **Knutsvik G**, Stefansson IM, Aziz S, Arnes J, Collett K, Akslen LA. Tumor cell proliferation by mitotic count can be used to subclassify hormone receptor positive breast cancer. *Manuscript*.

III. **Knutsvik G**, Collett K, Arnes J, Akslen LA, Stefansson IM. QSOX1 expression is associated with aggressive tumor features and reduced survival in breast carcinomas**.** *Mod Pathol 2016 (accepted for publication, minor revisions required).*

1. INTRODUCTION

1.1 Epidemiology

Breast cancer is the most common cancer type in women, accounting for 22% of all cancers, and with an estimated 1.7 million new cases worldwide in 2012.[1] In Norway, 3224 women were diagnosed with breast cancer in 2014.[2] Currently, the cumulative risk of developing breast cancer before the age of 75 is about 1 in 12.[2] Male breast cancer is rare with 24 new cases diagnosed in Norway in 2014, and will not be further considered in this thesis.

Breast cancer incidence rates have been increasing over decades, being highest in Western Europe and lowest in Eastern and Middle Africa (**Figure 1**).

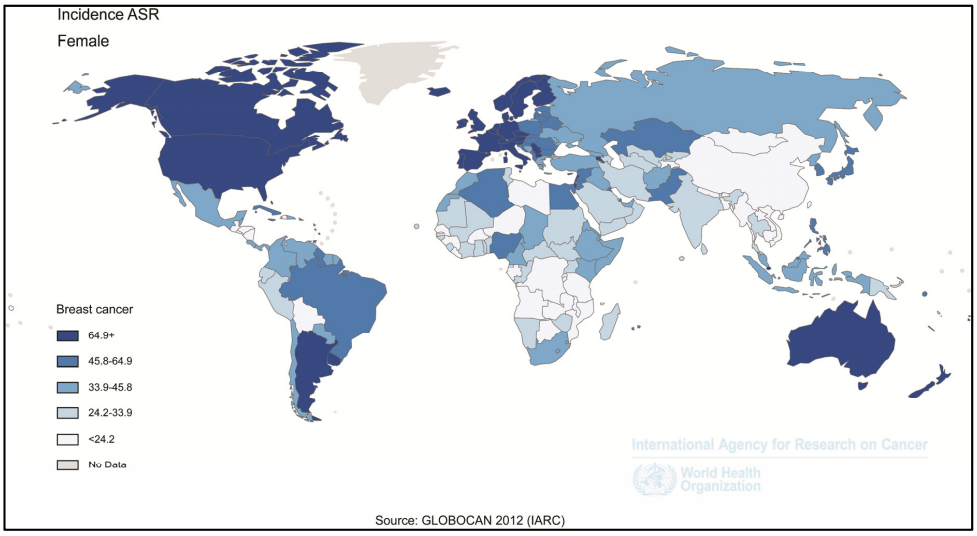


Figure 1. Estimated age-standardized incidence rate of breast cancer per 100 000 person-years, adapted from the Globocan report 2012.[3]

In Norway, the incidence rates have stabilized during the last 10 years, although with some fluctuations (**Figure 2**).[2]

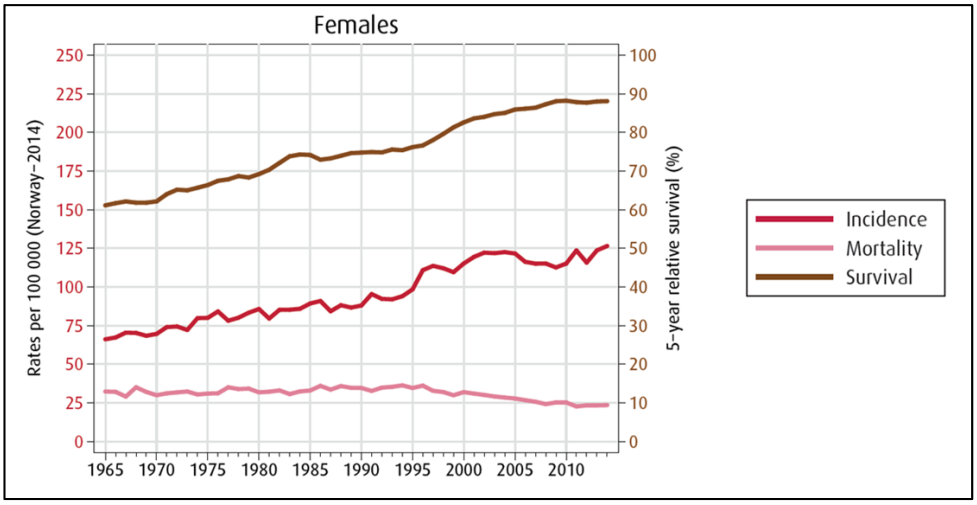


Figure 2. Trends in incidence, mortality and five-year relative survival rates in Norway (1965-2014), adapted from Cancer in Norway 2014.[2]

The incidence increases with age, reaching a peak at 65-69 years.[4] Over 50% of breast cancer occur in the age group 50-69 years,[5] and breast cancer under the age of 25 is uncommon.

In 2014, 663 women died of breast cancer in Norway.[2] The mortality rates were stable until late 1990s (**Figure 2**), after which there has been a decline reflecting earlier diagnosis and improved therapy.[6] Although the mortality rates have decreased, it is the second leading cause of cancer death among women in Norway, only surpassed by lung cancer.[2]

1.2 Etiology and risk factors

Cancer is considered a genetic disorder, meaning that mutations, both germline and somatic, are crucial etiological factors.[7] There are many factors that may contribute to the development of breast cancer, encompassing both reproductive and life-style related factors. The germline mutations reflect the hereditary predisposition to breast

cancer, and they will be considered in this section. The somatic mutations affecting the tumor cell genome will be referred to in chapter 1.4.1.

1.2.1 External, environmental factors and lifestyle exposures

Gender and age are the main risk factors for developing breast cancer.[8,9] Early menarche, late menopause,[8] nulliparity and late age at first childbirth are associated with increased breast cancer risk,[10] whereas early childbirth[11] and lactation may reduce the risk.[12] Also, the use of oral contraceptives and hormone replacement therapy exert an increased risk.[13-16]

Several life style factors have been associated with the development of breast cancer, including obesity,[17,18] lack of exercise,[19] excessive alcohol consumption,[20,21] tobacco smoking,[22-25] and a high dietary fat intake.[26]

Also, some benign breast diseases (usual ductal hyperplasia, sclerosing adenosis) are associated with a slight increase in breast cancer risk, whereas in lesions with atypia (atypical hyperplasia, DCIS, LCIS) the risk is more pronounced.[27-31]

1.2.2 Hereditary genetic factors

Although the majority of breast cancer cases are considered sporadic, about 10% may harbor predisposing germline mutations.[32] These mutations differ in their penetrance and associated breast cancer risk.[33]

About 25% of hereditary breast cancer is associated with mutations in highly penetrant genes including *BRCA1*, *BRCA2*, *TP53*, *PTEN*, *STK11* and *CDK1*.[34] These mutations are associated with a lifetime risk of developing breast cancer that exceeds 50%.[33] *BRCA1* and *BRCA2* are tumor suppressors involved in the DNA repair machinery where they restore double stranded DNA breaks.[35] Mutations in *BRCA1* and *BRCA2* are inherited in a dominant pattern and affect between 1 in 400 and 1 in 40 women, respectively.[36] Also, inherited cancer syndromes are associated with high

penetrance alleles. These include the Li-Fraumeni and Peutz-Jeghers syndromes caused by mutations in the tumor suppressors *TP53*[37] and *STK11*,[38] respectively.

Mutations in moderate-penetrance genes correspond to a ≥ 20% lifetime risk of developing breast cancer, and include mutations in *CHEK2*, *BRIP1*, and *PALB2*. These mutations are rare and may affect 1- 5% of breast cancer patients.[34,39,40] The Cowden syndrome involves germline mutations in the *PTEN* tumor suppressor gene.[41] Germline mutations of *CDH1*, the E-cadherin gene, convey an elevated risk of developing lobular breast carcinomas.[42] Also, hereditary ataxia-telangiectasia caused by mutations in *ATM*, is associated with an increased breast cancer risk.[34]

Mutations in low-penetrance genes are common and are associated with a 10-20% increase of lifetime risk.[33] Currently, little is known about the function of these genes, but they are proposed to affect DNA repair pathways, cell cycle regulation and apoptosis.

1.3 Classification of breast cancer

1.3.1 Morphological classification

The WHO classification of tumors of the breast was revised in 2012.[43] The terminology of infiltrating ductal carcinoma was changed to invasive breast carcinoma of no special type. This group comprises 40-80% of cases in population-based series.[43,44] A non-specialized pattern must affect over 50% of the tumor area. This subtype shows heterogeneity in terms of architecture, grade of glandular differentiation, and the appearance of individual carcinoma cells.

Invasive lobular carcinoma is composed of tumor cells individually distributed or arranged in single files surrounded by a fibrous stroma. This type accounts for 5-15% of breast carcinomas.[43] E-cadherin negativity, corresponding to the non-cohesive morphology, often helps to discriminate between lobular and ductal carcinomas, although 15% of otherwise typical lobular carcinomas are E-cadherin positive.[45]

Tubular, mucinous, and cribriform carcinomas are special subtypes associated with a better outcome compared to infiltrating ductal carcinomas.[46]

The WHO classification also includes several rare tumor types such as metaplastic, adenoid cystic, and papillary carcinomas, and carcinomas with medullary or neuroendocrine features. These will not be further considered.

1.3.2 Molecular classification

Although the current WHO classification of breast cancer relies on tumor morphology, molecular analyses of tumors have caused a paradigm shift. During the last decade, seminal microarray-based gene expression profiling studies by the Stanford group demonstrated the existence of distinct molecular subtypes. Originally, four subtypes were quoted; the luminal, HER2 enriched, basal-like, and normal breast-like.[47,48] Subsequently, the luminal group was subdivided into luminal A, B and C, although the existence of the luminal C subgroup has been questioned.[49] Further, it has been proposed that the normal-like subgroup mainly represents contamination of normal breast tissue in the original studies.[50]

These intrinsic subtypes have been reproduced across independent gene expression data sets,[51-53] and they have demonstrated differences in behavior and prognosis.[48,50,52] The luminal subclass is characterized by expression of ER, genes related to the ER pathway, and high expression of luminal cytokeratins (CK7, CK8, CK18, and CK19). The luminal A subgroup expresses higher levels of ER related genes and lower levels of proliferation related genes than luminal B tumors.[48] The HER2 subclass is associated with amplification of the *HER2* gene. The basal-like subgroup lacks expression of ER and HER2 related genes. These tumors express basal cytokeratins (CK 5, 6, 14, 15 and 17), and demonstrate high proliferative activity.[54]

The luminal A and B, HER2 enriched and basal-like subtypes were also confirmed by the Cancer Genome Atlas (TCGA) research network, although significant heterogeneity within these subclasses was underscored.[32]

In addition, three mainly ER-negative subtypes have been proposed, comprising the interferon-rich,[53] molecular apocrine,[55-57] and claudin-low subtype.[58,59] Further refinement of the triple negative subclass[60,61] and the HER2-enriched breast cancers have also been suggested.[32,62,63]

In 2012, a large global gene study (METABRIC) identified 10 distinct disease subgroups which further sub-classify both ER positive and ER negative tumors.[64]

Interestingly, unique genomic portraits of the morphologically defined lobular carcinomas have recently been demonstrated.[65-67]

Still, this is only the beginning of cancer genomics, and the definitive molecular classification of breast carcinomas is yet to be established.

1.4 Tumor biology

1.4.1 The development of cancer

During carcinogenesis, normal cells obtain a neoplastic phenotype through genetic alterations and epigenetic modifications, with contributions from the microenvironment and, in some instances, germline mutations.

In 2000, a seminal paper proposed six key features essential for progression from healthy cells to malignancy (**Figure 3**).[68,69] This transformation is driven by upregulation of oncogenes and inactivation of tumor suppressor genes causing a wide range of deregulated signaling pathways.[70] The core of these hallmarks is to trespass barriers against chronic cell proliferation.

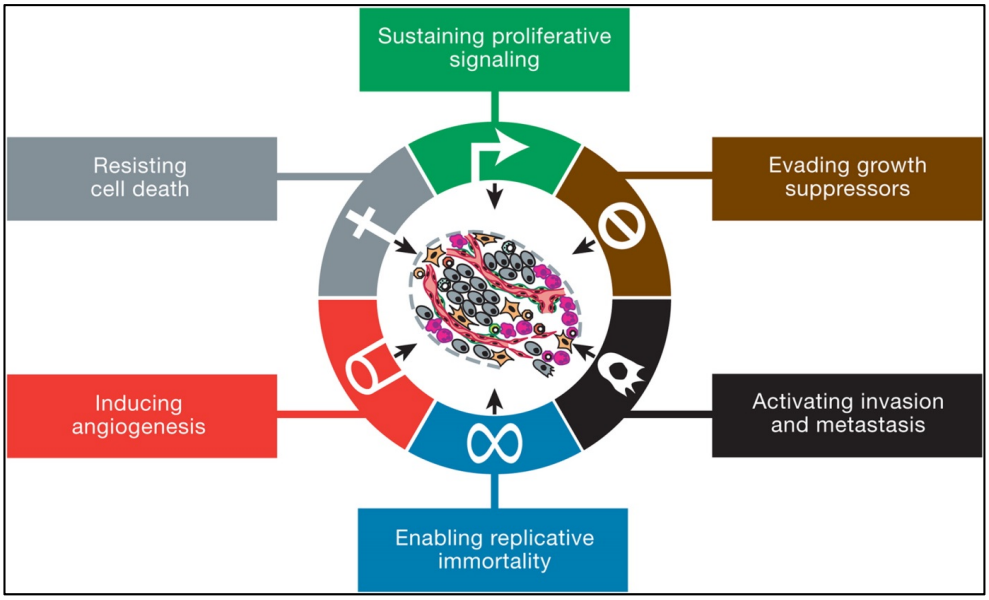


Figure 3. The hallmarks of cancer. Reprinted from Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-674. Copyright (2011), with permission from Elsevier.[69]

Tumor cells may acquire these hallmark capabilities through genome instability and mutations.[69] Further, epigenetic changes that modify gene expression without changing the DNA sequence, add to the heterogeneity and complexity of the disease process.

In 2011, two emerging hallmarks were proposed. One involves deregulating cellular energetics and the other escaping immune destruction.[69]

Somatic mutations arise in the genomes of normal cells as they pass through cell divisions as part of physiological maintenance of tissues. Cancer may develop if genome surveillance and DNA repair mechanisms fail.[39] The genomic instability of cancer cells causes a wide range of alterations including point mutations, insertions, deletions and chromosomal changes (translocations, loss and gain).[71] In breast cancer, mutations of *PIK3CA*, *TP53* and *GATA3* show an incidence of over 10%.[32]

1.4.2 Cell cycle regulation

In healthy tissue, the proliferative activity of cells is closely regulated to maintain tissue homeostasis and avoid neoplastic growth.[72] The four phases of the cell cycle are monitored by checkpoint controls and regulated by the cyclin proteins and the associated cyclin-dependent kinases.[39] (**Figure 4**)

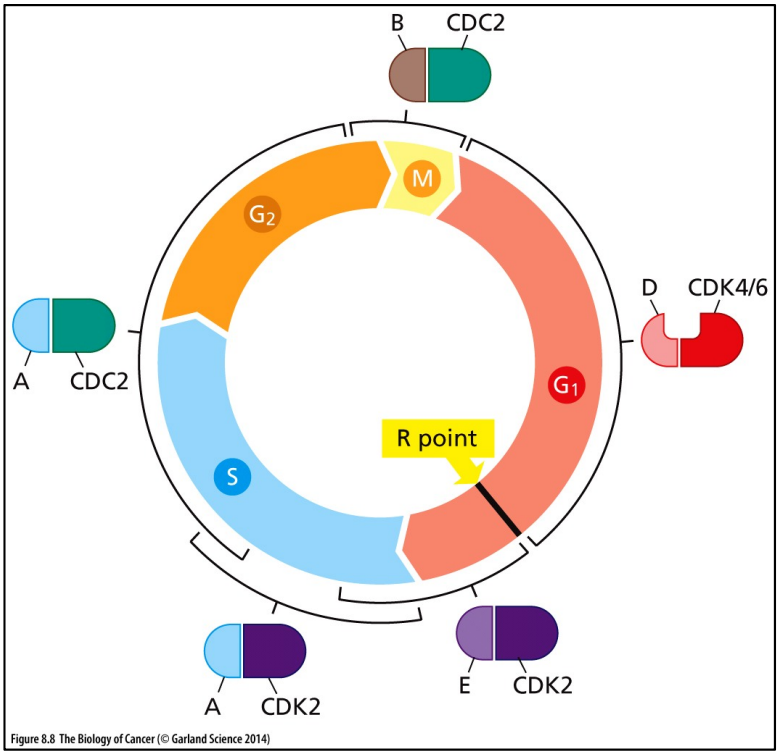
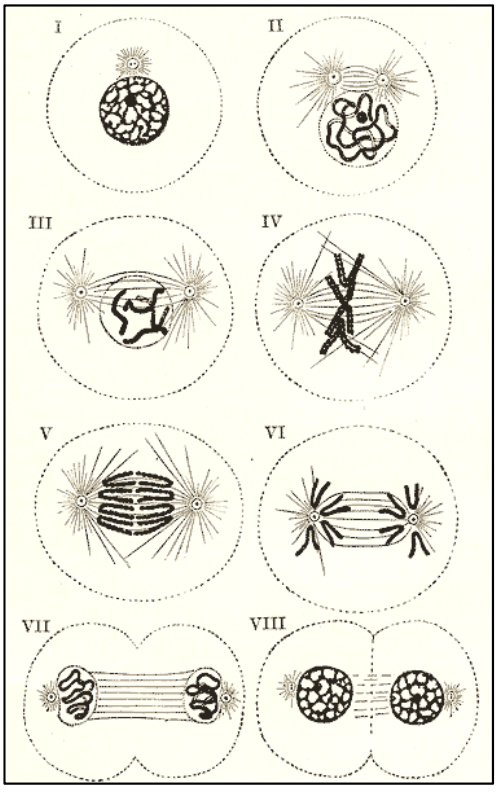


Figure 4. Pairing of cyclins with cyclin-dependent kinases, ©2014 from The Biology of Cancer by Weinberg.[39] Reproduced by permission of Garland Science/Taylor & Francis Group LLC.

Cells rely on external growth factors to exit the resting phase (G0) and enter the cell cycle. Towards the end of the first gap (G1) phase, a restriction point (R-point) occurs. At this time point, a cell has to decide whether to remain in G1 phase, withdraw into G0, or proceed into late G1-phase, and thus complete the entire cycle. This passage is governed by the retinoblastoma (RB) protein. Prior to the R-point, the cell responds to external stimuli, whereas the remaining phases are "pre-programmed". DNA replication occurs during the S (synthesis) phase, and during the second gap phase (G2), cells prepare for the mitotic phase. Irreparable DNA damage may arrest cells at the G2/M transition.

The M-phase is divided into the prophase, metaphase, anaphase and telophase (**Figure 5).**



The mitotic phase:

I-III, prophase

IV, metaphase

V-VI, anaphase

VII-VIII, telophase

Figure 5. The four phases of mitosis, adapted from Gray's anatomy of the human body, 1918.

Cancer cells need to overcome anti-proliferative signaling in order to prosper. The majority of these signals are funneled through the retinoblastoma (RB) pathway. The Cyclin Ds and their partners CDK4/6 complexes may inactivate the RB protein through hyper-phosphorylation, permitting passage of the R-point and progress through the cell cycle. In breast cancer, RB inactivation is associated with the luminal B and triple negative phenotype.[73] Perturbation of the RB pathway may be achieved through amplification of cyclin D1 (*CCND1*), *CDK4* and *CDK6*, loss of p16 (*CDKN2A*), and direct loss of *RB*.[74,75]

1.4.3 Apoptosis

The balance between cell proliferation and cell death is a barrier against cancer development.[69] Apoptosis is initiated in response to various stimuli such as DNA damage, hypoxia and deregulated growth signals. The apoptotic program is regulated through two pathways. The extrinsic pathway is initiated when external cellular stressors (tumor necrosis factor family) activate transmembrane death receptors at the cell surface. The intrinsic pathway is mediated through the tumor suppressor *TP53* which induces expression of pro-apoptotic target genes such as *BAX* and *BAD*.[76] Histologically, apoptosis is characterized by cell shrinkage, eosinophilic cytoplasm, chromatin condensation, collapse of the nucleus (pyknosis), nuclear fragmentation and finally, cell disintegration. The latter produces cellular fragments often called apoptotic bodies.[77] Apoptotic cells are sometimes confused with mitoses morphologically.

Cancer cells may escape apoptosis through loss of *TP53* function, and increased expression of anti-apoptotic factors such as Bcl-2. Loss of p53 is acquired through mutations, epigenetic silencing, or deregulations of pathways that affect p53. This loss inhibits checkpoint control of the cell cycle, permitting tumor cells to proliferate despite potential genomic aberrations, and also provides a fertile soil for acquisition of novel mutations.[78]

In breast cancer, about 30% of cases show somatic *TP53* mutations. The highest mutation frequency is found in basal-like and HER2+ tumors and the lowest frequency in luminal tumors.[32,79]

1.4.4 Angiogenesis

Tumors behave much in the same ways as normal tissues; they need nutrients and oxygen to survive, and also to get rid of metabolic wastes and carbon dioxide. In 1971, Judah Folkman suggested that tumors are dependent upon angiogenesis to survive and grow larger than 1-2 mm.[80] To fulfil these needs, tumors reactivate angiogenesis through an "angiogenic switch."[81] Several angiogenic regulators have been described, some of them with stimulatory (e.g. VEGF-A) and others with inhibitory effect (e.g. TSP-1). Hypoxia and oncogenic signaling are able of upregulating VEGF gene expression.[39,82] The vessels produced in tumors are abnormal, and anti-VEGF therapy (bevacizumab) may normalize the tumor vasculature facilitating delivery of other therapeutic agents (chemotherapy, immunotherapy).[83] Bevacizumab was initially FDA approved for treatment of metastatic breast cancer in 2008, but failed to demonstrate any increase in overall survival, and the approval was withdrawn in 2010.[84] Recent trials with other anti-VEGF agents have also been disappointing,[85] Clinical trials now assess combinations of anti-angiogenesis therapy and immunotherapy.[85]

1.4.5 Invasion and metastasis

Breast carcinomas do not usually affect survival prior to metastatic dissemination. However, metastases to brain, lungs or liver may rapidly disrupt vital functions and become threatening to life. Metastasis is a sign of inevitable progression of the disease, with currently only palliative therapy available.

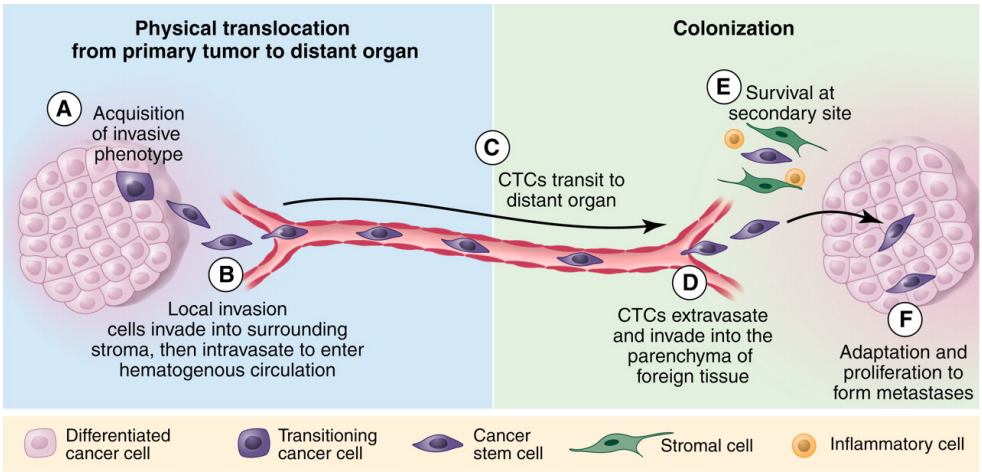


Figure 6. From Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. Science 2011; 331: 1559-1564.[86] Reprinted with permission from AAAS.

During the first step of the metastatic cascade, carcinoma cells have to breach the basement membrane to invade into the neighboring stroma.[87] To achieve this, cancer cells may exploit an embryological program used during organ morphogenesis, the epithelial-mesenchymal transition (EMT).[88,89] During this process the epithelial features of cells are replaced by mesenchymal properties characterized by loss of polarity and cell adhesion, and increased motility.[69,90] Through this dedifferentiation, cancer cells lose E-cadherin and upregulate N-cadherin.[91,92] The EMT passage is also suggested to provide carcinoma cells with stem-cell like features.[86] Epithelial cells harbor a remarkable plasticity with shifts between epithelial and mesenchymal states, also with partial EMT phenotypes.[93]

Of note, other modes of invasion exist, including collective infiltration and invasion as single cells. Currently, it is not clear whether these forms of invasion are associated with the EMT program.[69]

Second, tumor cells may enter the circulatory system (**Figure 6**), and some of these are able to colonize distant sites. Cancer cells that have completed their metastatic

escape may go through a reversed form of EMT termed mesenchymal-epithelial transition (MET).[94,95]

Stephen Paget was the first to notice that metastasis is not a random process, as proposed in his *seed and soil hypothesis* from 1889.[96] Thus, although cancer cells might be widely dispersed, they will only grow in certain microenvironments. In breast cancer, the molecular subtypes show different preferences of metastatic spread. Luminal tumors predominantly metastasize to bone with the highest risk shown for the luminal B subtype.[97] HER2 enriched cancers primarily metastasize to liver, lung and brain. Basal-like cancers metastasize to brain and lung, and show a lower rate of liver and bone metastases.[98]

1.4.6 Tumor microenvironment

The contributions of the tumor microenvironment in cancer progression have been increasingly appreciated.[68,99] Cancer cells exploit their neighborhood to facilitate local invasion, epithelial-mesenchymal transition and metastatic spread.[100] This microenvironment includes fibroblasts, immune cells and endothelial cells all embedded in the extracellular matrix. It functions as a supporting framework for the growing tumor providing growth factors and cytokines through reciprocal communication.

The extracellular matrix (ECM) is composed of different proteins including laminins, collagen, fibronectin and proteoglycans that are important for cell attachment.[101] Fibroblasts are involved in maintenance of the ECM, and are able to synthesize both components of the ECM and matrix-degrading enzymes (metalloproteases). Cancer-associated fibroblasts (CAF) take active part in remodeling of the ECM essential for tumor growth and invasion, and they promote angiogenesis and treatment resistance.[101-103] An increased number of peritumoral lymphocytes is associated with improved disease outcome, especially in the HER2 positive and triple negative subgroup.[104-106]

Also, the physical properties of the environment such as ECM stiffness and oxygen levels are of importance. Increased ECM stiffness may promote tumor cell proliferation and invasion.[107,108] Hypoxia leads to up-regulation of hypoxia inducible factors (HIF) that stimulate angiogenesis, provide a metabolic shift towards glycolysis, and promote invasion and metastasis through translation of HIF target genes.[109,110]

Microenvironmental-based therapy

The important role played by the tumor microenvironment in cancer progression makes it a promising treatment target.[111] Potential targets include immunoregulation, stromal cells, inflammation, angiogenesis, and the communication between tumor cells and the ECM.[112,113] Immune checkpoint blockade targets immunoregulation and includes PD-1, PD-L1 and CTLA-4 checkpoint inhibitors.[114] Advances have been made especially in treatment of melanoma, lung carcinoma and acute lymphocytic leukemia. In breast cancer, immunotherapy may be a promising target especially for subgroups enriched in mutations and genetic instability, such as triple negative and HER2+ cancer. Currently, around 50 clinical trials (phase I-III) are ongoing.[115]

1.4.7 QSOX1 and the secretory pathway in cancer

The secretory pathway is a complex network encompassing cell organelles that are responsible for maturation, folding and trafficking of both transmembrane and secreted proteins. The endoplasmic reticulum is the first component of the secretory pathway, and here proteins succumb to maturation steps such as folding, disulphide bond formation and glycosylation. The next compartment is the Golgi apparatus where proteins are packaged into vesicles destined for secretion.

The secretory pathway is important for interactions between tumors and their microenvironment. MMPs, extracellular matrix proteins and cytokines destined for the extracellular space are all transferred through this pathway.[116]

Quiescin sulfhydryl oxidase 1 (QSOX1/Quiescin Q6) is an enzyme that catalyzes disulphide bond formation in proteins with concurrent reduction of oxygen to hydrogen peroxide.[117] Although QSOX1 has been associated with the endoplasmic reticulum,[117] others report that human QSOX1 is mainly localized to Golgi structures.[118,119]

The human QSOX1 was initially assessed in an embryonal fibroblast cell line, where high QSOX1 gene expression was associated with exit of the cell cycle into G0 phase.[120] In 1999, the Thorpe and Coppock laboratories demonstrated that this protein was a member of a novel class of sulfhydryl oxidases, the quiescin sulfhydryl oxidases.[121] The QSOX1 gene is located on chromosome 1q24, and encodes two isoforms, QSOX1S (QSOX1B, short) and QSOX1L (QSOX1A, long), dependent on alternate RNA splicing.[122] The long isoform contains a transmembrane element. The short isoform is secreted into the extracellular space, and the long isoform may also be secreted after cleavage.[123] The highest expression of QSOX1 has been found in cells with a heavy secretory load.[124] The substrates of QSOX1 are currently unknown, but increased levels of QSOX1 mRNA and protein in tumor cells have been related to malignancy. Also, extracellular QSOX1 is necessary for proper laminin incorporation into the ECM, thus creating a pro-invasive environment.[119]

1.5 Prognostic factors

A prognostic factor may be defined as an assessable factor that can predict the outcome for patients, while a predictive factor reflects the possibility of a treatment effect.[125]

1.5.1 Prognostic patient characteristics

Breast cancer at young age is associated with worse prognosis and aggressive tumor features such as lack of hormone receptors and HER2 positivity.[126] This may reflect differences in subtype distribution; young age at diagnosis is associated with basal-like carcinomas,[127,128] whereas older patients more often develop luminal cancers.[129]

1.5.2 Prognostic tumor characteristics

Breast cancer stage

Tumor size, lymph node status and the presence of distant metastasis are important prognostic factors. The TNM (tumor size-nodes-metastasis) system arranges these factors into five tumor stages with significant differences in survival. Currently, five-year relative survival for stage I cancer is 99%, as opposed to 24% for stage IV.[2]

The main features of the current staging criteria (TNM staging manual version 7, 2010) are described in **Table 1**. pTNM classification is based upon histopathological evaluation.[130]

Table 1. pTNM grading of breast carcinoma, simplified version.

pT (Tumor)	
pTis	Carcinoma in situ (DCIS, LCIS, Pagets disease of the nipple)
pT1	≤ 2.0 cm.
pT2	> 2.0, ≤ 5.0 cm
pT3	> 5.0 cm
pT4	Involvement of skin, chest wall or inflammatory carcinoma

pN (Regional lymph nodes)	
pN0	No regional lymph node metastasis (> 0.2 mm) identified.
pN1 mi	Micrometastases (>0.2 mm and/ or more than 200 cells, ≤2.0 mm).
pN1	Metastases in 1-3 axillary lymph nodes, and/or metastases in internal mammary nodes detected by sentinel lymph nodes but not clinically detected.
pN2	Metastases in 4-9 axillary lymph nodes, or metastasis in ipsilateral internal mammary lymph nodes clinically/radiographic detected.
pN3	Metastases in ≥10 axillary lymph nodes, and/or metastases to ipsilateral infraclavicular/supraclavicular/internal mammary nodes.

M Distant metastasis	
M0	No clinical or radiographic evidence of distant metastases
M1	Distant detectable metastases

Table 2. Breast cancer stage grouping

Stage IA	T1	N0	M0
Stage IB	T0, T1	N1mi	M0
Stage IIA	T0, T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0, T1, T2	N2	M0
	T3	N1, N2	M0
Stage IIIB	T4	N0, N1, N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Histologic grade

The most widely used grading system is the Nottingham modification[131] of the Bloom-Richardson system.[132] Histologic grade reflects a combined score assessing gland formation, nuclear pleomorphism and mitosis counts. Each category provides a score of 1 to 3, resulting in a combined score of 3-9 categorized into 3 grades. Histologic grade is an important prognostic factor[133,134] guiding therapy in early breast cancer.[135,136] It is included in treatment algorithms such as the Nottingham Prognostic Index (NPI)[137,138] and Adjuvant Online.[139]

Mitotic count

The prognostic impact of mitotic count in breast cancer was first reported in 1925,[140] and it has been well documented.[141,142]

Mitotic count in H&E stained sections is the most simple and inexpensive method to evaluate proliferation. It is reproducible,[143] although strict standardization with adequate tissue fixation, section thickness, and quality of staining is necessary. Also, criteria for assessment of mitotic figures must be applied to avoid confusion with apoptotic cells.[144] Mitotic count is preferentially assessed on full sections by appreciating the highest proliferative area at the tumor periphery. In such hot-spots, mitoses are counted in 10 sequential HPFs (**Figure 7A**).

The predictive power of mitotic count has been assessed both in the adjuvant and neoadjuvant setting.[145-150]

Hormone receptors

ER and PR are nuclear receptors that function as transcription factors.[151,152] ER is an established biomarker in breast cancer; it provides prognostic information and predicts the sensitivity to endocrine treatment.[6,153] The PR gene is regulated by estrogen, thus PR positivity in the absence of ER is rare, although a recent study supports the existence of an ER negative/PR positive phenotype.[154]

ER and PR are routinely assessed by immunohistochemistry on all breast cancer cases. The St Gallen consensus 2009 proposed a 1% cut-off for defining ER positivity.[155] This threshold is currently recommended for clinical decision making by the ASCO/CAP guidelines, and by the Norwegian guidelines.[136,156] Further, cut-offs of 10% and 50% are used in treatment algorithms, based upon reports showing higher endocrine responsiveness with increasing hormone receptor values.[136,155] PR positivity is defined by a threshold of 10% positive cells.[136] In Norway, approximately 85% of breast carcinomas show ER-positivity by immunohistochemistry, and about 65% are PR positive.[157] PR negative breast tumors have been associated with poorer disease outcome,[158-160] although a large meta-analysis failed to demonstrate an independent role of PR in multivariate survival analysis.[161]

HER2

HER2 is one of four members of the human epidermal growth factor receptor family.[162] These are transmembrane receptor tyrosine kinases that receive extracellular signals and convert them to intracellular signaling.

HER2 is unable to bind ligands directly and is dependent on cooperation with the other HER family members.[163] Normal cells contain one copy of the HER2 gene on each chromosome, whereas breast cancer cells may have 25-50 copies.[164] This leads

to increased HER2 protein expression and increased number of HER2 receptors at the surface of tumor cells. HER2 gene amplification was first described in 1985.[165] It affects 15-20% of breast carcinomas,[166,167] and defines a distinct breast cancer subtype.[47] HER2 positivity is an adverse prognostic factor.[168,169] However, it also predicts response to trastuzumab (Herceptin®),[170] a monoclonal antibody introduced in 1998.[171] HER2 protein overexpression is associated with increased tumor cell proliferation, protection against apoptosis, and invasion.[164]

HER2 protein overexpression is routinely assessed in breast cancer. The equivocal 2+ cases by IHC are further analyzed for HER2 gene amplification using in situ hybridization. ISH assesses the number of HER2 copies in each nucleus using a DNA probe attached to either a chromogenic, fluorescent or silver (CISH, FISH, SISH) detection system.[172]

Ki67

Ki67 is a large, nuclear protein which is important for mitosis, but its function is almost unknown.[173,174] The Ki67 protein is encoded by the MKI67 gene on chromosome 10q26, and was first described in 1983.[175] It acts in all phases of the cell cycle except in G0, and can be detected by immunohistochemistry (**Figure 7C**).[176] During mitosis, it is phosphorylated and dephosphorylated.[177] Ki67 expression varies during the cell cycle, with lowest expression in the G1 and S-phase followed by a gradual increase until maximum is reached in the M-phase.[173] Although primarily regarded as a proliferation marker, Ki67 is also expressed in quiescent cells where it is associated with ribosomal RNA synthesis.[178,179] Several antibodies against the Ki67 antigen have been developed, of which Molecular Immunology Borstel 1 (MIB-1) is currently recommended for assessment of proliferation in breast cancer.[180]

Ki67 has been investigated both in the adjuvant and neo-adjuvant setting, and the prognostic impact is well-known.[142,173,181-183]

In contrast, Ki67 as a predictive biomarker in the adjuvant setting is controversial, and there are currently no prospective trials addressing chemotherapy benefit

according to the intrinsic subtypes.[184] Some retrospective studies have suggested treatment effect of adding taxanes to highly proliferative, luminal tumors,[185-189]although this was not confirmed by others.[190] Of note, the Oxford meta-analysis failed to find any factors (e.g. age, nodal status, tumor diameter, tumor differentiation or ER status), that predicted chemosensitivity.[191] Also, the use of Ki67 to withhold chemotherapy among node-positive patients is controversial.[192]

PHH3

Alternative mitotic markers have been explored, including phosphohistone H3 (PHH3). Chromatin is composed of a basic subunit called the nucleosome which consists of DNA wrapped around a core formed by histones.[193] Each histone protein has a tail which is subject to epigenetic, posttranslational modifications, including phosphorylation, methylation and acetylation.[194] Histone H3 is phosphorylated on Serine 10 and 28 in late G2 and M-phase of the cell cycle.[195] This phosphorylation is essential for appropriate chromosome condensation.[196] Dephosphorylation of PHH3 is initiated at late anaphase and is finalized by early telophase, preceding chromosome decondensation.[197,198]

A PHH3 (Ser10) antibody was introduced in 1997.[197] PHH3 expression (**Figure 7B**) has since been evaluated in a range of human cancers, including breast cancer.[199-202]

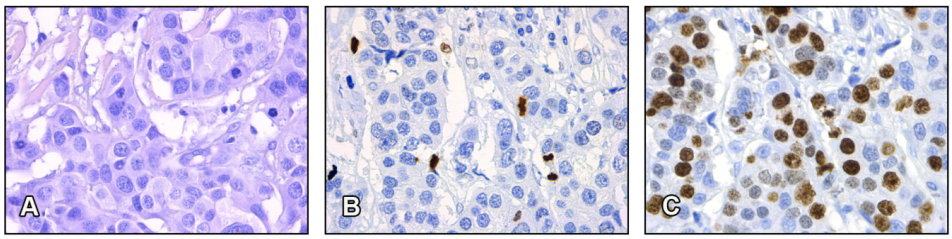


Figure 7. MC (A), PHH3 (B), and Ki67 (C) staining in breast cancer.

Additional proliferation markers

A wide range of prognostic biomarkers related to the cell cycle progress have been studied, some of these are assessed by immunohistochemistry, including Cyclin D1, Cyclin E, aurora kinases, mitosin, polo-like kinase 1, MCMs, geminin, and PCNA. Further, it is possible to assess the fraction of cycling cells that is in S-phase by flow cytometry.[203] Thymidine and Bromodeoxyuridine labeling index are methods that assess DNA synthesis (S-phase fraction) in tumor cells by evaluating thymidine uptake.[204]

Prognostic signatures

In parallel with the studies on molecular sub-classification of breast cancer, several microarray-based multigene prognostic classifiers have been established. Among these, the recent St Gallen and ASCO guidelines consider Oncotype DX®,[205-208] Endopredict®,[209,210] PAM-50 ROR® score,[47,211,212] Mammaprint®,[51,213-216] and Breast cancer index®[217] as useful prognostic indicators.[135,218]

These prognostic signatures stratify mainly ER-positive patients. They predict outcome and define high risk groups for which chemotherapy are recommended and low risk groups where chemotherapy may be withheld. However, the intermediate risk groups are still a challenge. Also, the high costs of these signatures are a limitation. Further, it has been shown that the prognostic signatures are mainly driven by proliferation genes.[219]

Immunohistochemical surrogate markers for molecular subtypes

An alternate approach to the gene expression analyses is to focus on well-characterized proteins that can be detected by antibodies applied on formalin-fixed, paraffin-embedded (FFPE) clinical samples. Immunohistochemical assessment of ER, PR, HER2, and Ki67 are currently used to guide treatment decisions. These four biomarkers constitute immunohistochemistry 4 (IHC4), a combined biomarker assay. They carry significant prognostic information on their own, and their combination has been shown to provide prognostic information comparable to Oncotype DX and PAM50.[220,221]

In the St. Gallen guidelines from 2009, two Ki67 thresholds were proposed for sub-classification of patients with ER+/HER2- disease (**Table 3**).[155]

Table 3. Chemoendocrine therapy in patients with ER positive, HER2 negative disease, adapted from St Gallen guidelines 2009.[155]

Clinicopathological features	Relative indications for chemoendocrine therapy	Factors not useful for decision	Relative indications for endocrine therapy alone
ER and PR	Lower ER and PR level		Higher ER and PR level
Histologic grade	Grade 3	Grade 2	Grade 1
Proliferation*	Ki67 >30%	Ki67 16-30%	Ki67 ≤15%
Nodes	≥ 4 positive nodes	1-3 positive nodes	Node negative
Vascular invasion	Extensive invasion		Absence of extensive invasion
Tumor size	> 5 cm	2.1-5 cm	≤ 2 cm
Gene signature	High score	Intermediate score	Low score

*Conventional measures of proliferation include assessment of Ki67-labelling index and pathological description of the frequency of mitoses

Of note, these Ki67 thresholds were based upon a single, small study of 265 breast cancer cases.[222] In that study, only mitotic count was an independent prognosticator in multivariate survival analysis.

In February 2010, a 15% Ki67 threshold was implemented by the Norwegian Breast Cancer Group (NBCG) in the treatment guidelines as a chemotherapy indication for luminal/HER2 negative tumors. This cut-off point was based upon a single institution study on TMA-samples.[223]

In 2011, the St. Gallen guidelines endorsed the immunohistochemical surrogates for intrinsic subtypes, with a 14% Ki67 threshold for sub-classification of the luminal subgroup (**Table 4**).[223,224]

Table 4. Immunohistochemical surrogates of intrinsic subtypes, adapted from St Gallen guidelines 2011.[224]

Intrinsic subtype	Immunohistochemical definition	Clinical management
Luminal A	ER+ and/or PR+ HER2- Ki67 low (<14%)	Endocrine therapy
Luminal B-HER2-	ER+ and/or PR+ HER2- Ki67 high (≥14%)	Endocrine Therapy Chemotherapy
Luminal B-HER2+	ER+ and/or PR+ HER2+ Any Ki67	Endocrine Therapy Chemotherapy Anti-HER2 therapy
HER2+	ER- and PR- HER2+	Chemotherapy Anti-HER2 therapy
Basal-like	ER- and PR- HER2-	Chemotherapy

However, guidelines for assessment of Ki67 in breast cancer were published in November 2011.[180] Here, the lack of reports on the correlation between Ki67 assessed on full sections and TMA sections was underscored. Although TMA is an important research tool, it should not be used for establishing Ki67 thresholds for clinical samples until studies comparing proliferation values on TMA and WS have been published.[180]

In Norway, the Ki67 threshold was adjusted to 30% in June 2013. This cut-off is based on "hot-spot" readings on full sections. Also, the St Gallen guidelines from 2013 suggested a Ki67 threshold of "at least" 20%, and added a PR-restriction to the definition of the luminal subgroup.[160,225] Further, due to discussions on the low validity of Ki67, a note was made on the use of local, laboratory specific cut-offs. Histologic grade 3 was still a relative indication for chemotherapy.

In the St Gallen 2015 guidelines, a Ki67 threshold of 20-29% was suggested to define luminal B disease.[135]

In the current ASCO guidelines,[218] Ki67 is not recommended for guidance on adjuvant therapy due to limited interlaboratory agreement. In contrast, the European Society for Medical Oncology (ESMO) recommends immunohistochemical subtyping of breast cancer based on the St Gallen guidelines.[135,226]

The triple negative phenotype, defined by ER-, PR-, and HER2 negativity by immunohistochemistry, is an approximation of the basal-like subgroup.

Several immunohistochemical biomarkers have been associated with the basal-like subtype of breast carcinoma. Among these, CK5/6 and P-cadherin have been used in this study.[52,227-230]

1.6 Diagnosis and treatment

1.6.1 Detection

Early breast cancer is often asymptomatic. Clinical signs of breast cancer include breast lumps, changes in breast shape or size, skin changes (edema, erythema or peau d'orange), Paget's disease, ulceration, nipple inversion, nipple discharge and presence of enlarged axillary lymph nodes.

Triple assessment based upon clinical breast examination, imaging (mammography and/or ultrasonography) combined with core needle biopsy (CNB) or fine needle aspiration cytology (FNAC) is currently the recommended diagnostic approach. With the invention of mammographic screening, breast cancer is often detected prior to development of symptoms.

In 1996, the Norwegian Breast Cancer Screening Program initiated screening as a four-year pilot project involving 4 out of 19 Norwegian counties. The screening became nationwide by 2005 and is administered by the Cancer Registry of Norway.[5]

This program invites all women between 50 and 69 years of age to biannual mammography. The incidence of breast cancer has increased after the introduction of

screening (but later tapered off), mainly due to small tumors being diagnosed early. Mammographic screening programs have led to an increased detection of early-stage, node-negative breast cancer. Whether mammographic screening leads to better survival has been heavily discussed in recent years. Based on four studies, the program has recently been assessed by The Research Council of Norway which estimated a mortality reduction in the range of 7-30%.[231]

1.6.2 Therapy

Treatment of breast cancer may include surgery, radiotherapy and systemic therapy. In Norway, clinicians depend upon the national guidelines published by the Norwegian Directorate of Health.[136] The treatment algorithms are complex and the following is a simplified version of the current guidelines (accessed April 2016) leaving out details and exceptions to the rules. Patients with locally advanced and metastatic disease receive individualized therapy; this will not be further considered here.

Surgical management

During the last three decades, a major shift has occurred from radical mastectomy to breast-conserving therapy (BCT), based upon results demonstrating comparable outcome.[232] Currently, 55% of breast cancer patients in Norway receive BCT.[157] Surgery is recommended for stage I-II tumors. Patients with T3 and T4 tumors receive neo-adjuvant therapy to down-stage the disease prior to surgery.

Sentinel node biopsy (SN) is indicated for T1 and T2 disease. Subsequent axillary dissection is not indicated for patients with negative SN or SN with small metastases (< 2 mm). Also, some patients with metastases > 2mm may avoid axillary dissection, as detailed in the guidelines.

Adjuvant radiotherapy

Radiotherapy reduces the risk of loco-regional relapse and improves survival.[233,234] With localized disease, postoperative radiotherapy is recommended to patients

receiving breast conserving therapy, large tumor size (> 5 cm), non-radical surgery and to patients with axillary lymph node metastasis.[136]

Adjuvant systemic therapy

Metastatic dissemination of cancer cells is often an early event,[235] and despite surgical removal of the primary tumor, some patients experience relapse. Adjuvant systemic therapy is directed towards eradicating these micrometastases to improve prognosis. Thus, patients at increased risk of developing metastasis may be offered chemotherapy, endocrine therapy and targeted therapy against HER2. Currently, adjuvant systemic therapy is decided according to age, nodal status, tumor size, histologic grade, hormone receptor status, HER2 and Ki67.

Chemotherapy

Adjuvant chemotherapy may reduce 10-year overall mortality by one-third.[191] Combination of agents has proven more effective than single agent therapy. Anthracycline-based regimens form the basis of adjuvant chemotherapy. Ki67 is used for decisions on adjuvant chemotherapy for HER2 negative disease, with currently two thresholds applied. High Ki67 (≥ 30%) is an indication for addition of taxanes for both luminal/HER2 negative cancer and for the triple negative subgroup. Luminal/HER2-cases with low Ki67 (< 15%) combined with high expression of hormone receptors (HR ≥ 50%), low histologic grade (1-2) and pN0-1 defines a group that usually can be spared chemotherapy. Taxanes might also be indicated for luminal/HER2 negative disease with Ki67 < 30% combined with histologic grade 3 and axillary metastasis (pN2-3). Patients with HER2 positive breast cancer receive chemotherapy independent of Ki67 status.

Endocrine therapy

Endocrine therapy is indicated for patients with ER-positive breast cancer (ER > 1%). Tamoxifen binds to the estrogen receptor, thus blocking transcription of ER responsive genes. Five years of tamoxifen treatment is currently recommended and has been shown to reduce mortality by one-third.[236] Moreover, a recent report suggested a benefit for prolonging tamoxifen treatment to 10 years.[237]

In post-menopausal women, the use of aromatase inhibitors is also considered. Aromatase inhibitors prevent the synthesis of estrogen in fat, and blocks aromatase activity in tumor tissue.[238,239]

Anti-HER2 therapy

Trastuzumab (Herceptin®) is a monoclonal antibody targeting the Her2/neu receptor. Patients with HER2 positive disease benefit from 1 year of trastuzumab.[170,240-242]

Novel targeted therapies

In breast cancer, over 6000 clinical trials are currently ongoing, several on targeted therapy, and a few examples will be provided here (accessed April 2016).

Luminal breast cancer is associated with increased activity of CDK4/6-cyclin D1.[243] In 2015, FDA approved the use of a CDK4/6 inhibitor (palbociclib) for treatment of metastatic luminal tumors,[239] and phase III studies are ongoing.[244] *PI3CA* is often mutated in ER positive breast cancer, and several PI3K inhibitors are in clinical trials (phase I-III).[245,246]

For HER2+ disease, simultaneous targeting of several HER receptors is a promising approach and might conquer development of resistance.[247,248] As an example, the dual kinase inhibitor lapatinib targeted against both EGFR and HER2, received FDA approval in 2010 for treatment of HR+/HER2+ metastatic breast cancer.

PARP inhibitors are novel targeted agents for breast cancer patients with triple negative disease and/or BRCA mutations. In 2014, olaparib received FDA approval for treatment of ovarian cancer with BRCA mutations (www.fda.gov). In breast cancer, clinical trials (phase I-III) are ongoing.[244,249]

2. BACKGROUND AND AIMS OF THE STUDY

2.1 Background

Breast cancer is a heterogeneous disease encompassing various subtypes showing differences in incidence, therapy response and prognosis. Precision oncology is dependent on characterization of prognostic subgroups. Thus, exploration of novel biomarkers and improved characterization of established factors are important to tailor therapy.

In breast cancer, traditional histopathological variables such as tumor size, histologic grade and nodal status are combined with ER, PR and HER2 to guide clinical management. Gene expression studies of breast cancer have led to a renewed focus on tumor cell proliferation. The high costs associated with gene signatures have enabled the use of surrogate immunohistochemical markers for molecular sub-classification. Thus, Ki67 was suggested as a marker to sub-classify luminal tumors and guide chemotherapy decisions. However, the use of this marker is heavily discussed, in terms of thresholds for patient stratification, interobserver variability in assessment, and the impact of intratumor heterogeneity on results.

Currently, there is no international consensus on the use of proliferation markers for therapy guidance, and the lack of comprehensive reports on the variation of proliferative markers across different specimens has been underscored.[180] Although mitotic count on HE-sections is routine procedure for assessment of histologic grade, the use of mitotic count for sub-classification of luminal tumors has not been focused.

2.2 General aim

The main purpose of the study was to examine and compare different proliferation markers in relation to specimen type, basic characteristics, molecular subtype and

prognosis. Also, we wanted to assess a novel, proliferation related biomarker addressing whether it could assist in disease subtyping and risk stratification.

2.3 Specific aims

1. To study the matched levels of proliferation by Ki67 in breast carcinomas using different tissue categories (whole sections, WS; core needle biopsies, CNB; tissue microarrays, TMA), and the associated prognostic value of these **(Paper I)**.

2. To examine the level of tumor cell proliferation by mitotic count, Ki67 and PHH3 across matched samples of three distinct tissue categories (WS, CNB, TMA) from breast carcinomas, and the concurrent prognostic impact of these markers **(Paper II)**.

3. To explore the protein expression, association patterns and potential prognostic impact of the novel biomarker QSOX1, also in relation to tumor cell proliferation and molecular breast cancer subtypes **(Paper III)**.

3. MATERIALS AND METHODS

3.1 Patient series

The patient series is described in **Paper I**. Shortly, we expanded a retrospective case-control series of 190 cases previously established by Dr. Karin Collett. We included all women between 50 and 69 years who had been diagnosed with primary breast cancer as part of the prospective, population-based Norwegian Breast Cancer Screening Program in Hordaland County during 1996-2003. The patient cohort is based upon records from the Cancer Registry of Norway.

In this thesis, we have used the TNM staging manual version 6 2002 edition.[250] TNM staging criteria were slightly modified in 2002; some nodal categories that previously were considered M1 (stage IV) were reclassified as N3 (stage III).

Patients with distant metastatic disease at time of diagnosis (stage IV) were not included. Nine patients were excluded due to lack of informed consent, 12 cases had technical inadequate material for proliferation assessment (Ki67, PHH3) and were later excluded, leaving 534 cases for further studies.

The patients received treatment according to standard protocols at that time in a single institution. In Norway, trastuzumab (Herceptin®) was introduced for treatment of metastatic disease in 2000, and as adjuvant treatment for early disease in 2005.

This study was approved by the Western Regional Committee for Medical and Health Research Ethics, REC West (REK 2012/1704).

3.2 Clinico-pathologic variables

Age at diagnosis, date of diagnosis, tumor diameter, histologic type, histologic grade, lymph node status, and hormonal receptor status were retrieved from the routine

histopathology reports, as detailed in **Paper I**. During this period, these specimens were reported by five experienced breast pathologists.

The assessment of mitotic count is described in **Paper II**. Briefly, mitoses were counted on H&E sections. At low power magnification (x100), the area with the highest mitotic activity (hot-spot, by subjective assessment), at the peripheral and most cellular part of the invasive tumor, was selected, corresponding to the approach used for histologic grading.[131] Care was taken to avoid areas of intense inflammation, necrosis, fibrosis and low cellularity.[144] Mitoses were counted in 10 consecutive HPFs at x400 magnification (Leica DMLB, field diameter 0.55 mm), and the number of mitoses per mm^2 was calculated.

3.3 Follow-up data

Follow-up information was provided by the Norwegian Cause of Death Registry. Last date of follow-up was December 31, 2011. Outcome data include survival status, survival time and cause of death. During the follow-up period, 79 patients (15%) died from breast carcinoma, and 62 (12%) died from other causes. No patients were lost to follow-up. Median follow-up time for survivors was 13 years (range 8-16 years), and a similar result was obtained through estimation of follow-up by the reverse Kaplan-Meier method.

3.4 Specimen characteristics

Paraffin-embedded breast cancer tissue samples were retrieved from the archives at the Department of Pathology, Haukeland University Hospital. Storage time of the archival formalin-fixed, paraffin-embedded tissue samples was up to 17 years.

3.4.1 Tissue microarray (TMA)

The TMA procedure is described in **Paper I**. Briefly, H&E stained slides were used for tumor verification. Triplicate cores (0.6 mm) were punched from the first 190

patients,[228,251] and 1.0 mm cores were used in the extended series. The cores were mounted into a recipient paraffin block using a semi-automated precision instrument (Minicore 3, Tissue Arrayer, Alphelys, France). Areas of high tumor purity, the tumor periphery and the highest histologic grade were included. Among the 534 cases with TMA available, 22 cases (4.1%) had tissue cores devoid of invasive tumor, 21 cases (3.9%) showed complete core loss and 32 cases (6.0%) showed fewer than 100 tumor cells on arrayed spots, leaving 459 cases (86%) available for assessment. TMA sections were used for mitotic count (**Paper II**), Ki67 (**Paper I-II**), PHH3 (**Paper II**), HER2, CK5/6, P-cadherin, and QSOX1 assessment (**Paper III**). For proliferation markers, TMA sections were used for comparative analyses with WS and CNB.

3.4.2 Core needle biopsies (CNB)

182 patients had undergone both preoperative core needle biopsy and subsequent surgical excision. Among these, 154 were eligible for analyses, as described in **Paper I**. 25 cases were excluded due to non-representative or inadequate material remaining for biomarker assessment. Three cases were excluded due to lack of informed consent. 310 cases were diagnosed by preoperative fine needle aspiration cytology (FNAC), this practice was according to national guidelines at the time. The remaining cases had either frozen sections, incisional or excisional biopsies performed. Sections from CNB were used for comparative analyses of mitotic count, Ki67 and PHH3 across tissue categories (**Paper I-II**).

3.5 Immunohistochemical methods

The tumor samples (WS, CNB, TMA) were cut into five µm sections by one highly trained person using one microtome and mounted onto Poly-lysine coated glass slides.

The sections were dewaxed with freshly prepared xylene/ethanol before proper target retrieval. Endogenous peroxidase activity was blocked in order to reduce background staining. Staining procedures were performed on a DAKO Autostainer® using the

commercial DAKO Envision detection systems. The EnVision™ is a two-step visualization system based on an HRP labelled polymer containing multiple secondary antibodies. Finally, DAB was used as chromogen for 10 minutes followed by brief counterstaining with hematoxylin.

For Ki67 and PHH3, sections from tonsils were used as positive controls as previously recommended.[252] For QSOX1, a breast carcinoma with known strong positivity was used as positive control. Negative controls were obtained by replacing the primary antibody with Tris-buffered saline. Controls were included in each staining run.

Table 5. Summary of immunohistochemical protocols used in the present studies.

Antibody	Company	Epitope retrieval	Dilution	Incubation
Ki67 MIB-1 (M7240)	DAKO	PC 10 min EDTA buffer (pH 9)	1:100	30 min
PHH3 (06-570)	Millipore	PC 10 min EDTA buffer (pH 9)	1:1500	60 min
CK5/6 (M7237)	DAKO	PC 10 min in TRS (pH 9)	1:200	30 min
P-cadherin (Clone 56)	BD transduction	PC 10 min EDTA buffer (pH 9)	1:400	60 min
QSOX1	Proteintech	MW 20 min TRS (pH 6)	1:100	60 min

PC: pressure cooker; MW: microwave oven; TRS: target retrieval solution

Evaluation of staining

The assessment of staining and scoring of proliferation markers is described in detail in **Paper I-II**. Briefly, for Ki67, the staining was recorded as percentage of tumor cells stained. For PHH3, mitotic figures were counted in 10 HPF, equivalent to the approach used for mitotic count.

For HER2, the established scoring system for DAKO Herceptest was used. HER2 SISH was performed in IHC 2+ cases (Ventana INFORM HER2 DNA probe

staining). The 2+ cases were considered HER2 positive if the HER2/Chr17 ratio by SISH was equal to or greater than 2.0.

For assessment of CK5/6, P-cadherin and QSOX1 (**Paper III**), we used a staining index (SI), a semi-quantitative and subjective scoring system which is well-established in our research group.[54,253,254] Shortly, the staining index (values, 0-9) is a product of staining intensity (0-3) and proportion of tumor cells showing a positive reaction (0= no staining, 1: <10%, 2: 10-50%, and 3: >50% of tumor cells). Staining of nucleus, cytoplasm and cell membrane were recorded as separate variables.

3.6 Statistical methods

Analyses were performed using the SPSS statistical package, version 18.0 (SPSS Inc., Chicago, IL) in **Paper I** and IBM SPSS Statistics version 21.0 (Armonk, NY: IBM Corp) in **Paper II-III**. Associations between categorical variables were evaluated by the Pearson's chi-square test. The Spearman's rank correlation test was used for correlation analyses between continuous variables. Ordinal and continuous variables not following the normal distribution were compared between two or more groups using Mann-Whitney U or Kruskal-Wallis H tests. Wilcoxon signed rank test and Bland and Altman analysis were used to compare related samples. Kappa statistics were used in analyses of intra- and interobserver agreement of categorical data. Receiver operated characteristic (ROC) analyses were also performed.

Univariate analyses of time to death due to breast carcinoma were performed using the product-limit procedure (Kaplan-Meier method), and differences between categories were estimated by the log-rank test, with date of diagnosis as the starting point. The breast cancer specific survival was defined as the time in months from the date of diagnosis to the date of death from breast cancer. Patients who died from causes other than breast carcinoma were censored. Median follow-up time was estimated by the reverse Kaplan-Meier method. The influence of covariates on patient survival was analyzed by the Cox' proportional hazards method, and tested by the

likelihood ratio. Covariates were examined by log-log plot to determine their ability to be incorporated in multivariate models. The proportional hazard assumptions were also assessed by studying the graphs of the Schoenfelds residuals. Interactions between the variables were tested by adding interaction terms if considered pertinent. A two-tailed p-value < 0.05 was considered significant.

4. MAIN RESULTS

Paper I

In this study, we assessed the concordance of Ki67 evaluated on full sections from surgical specimens (WS) as compared with preoperative core needle biopsies (CNB) and tissue microarrays (TMA). The Ki67 counts were significantly higher in WS as compared to CNB and TMA (median values 17% vs 13% vs 6%, paired cases, $P<0.001$ for each analysis). The Ki67 counts on WS were significantly correlated with the CNB and TMA counts. Elevated Ki67 values assessed on WS, CNB and TMA were all significantly associated with high histologic grade and hormone receptor negativity. We applied surrogate immunohistochemical markers for molecular subclassification based on the St Gallen 2011 guidelines. Ki67 counts varied according to molecular subgroup with highest proliferation in the HER2+ and triple negative subgroups. In the luminal/HER2 negative subgroup, there were many discordant cases and fair agreement only when assessing luminal A and B on WS as compared to CNB and TMA. Of note, in the luminal/HER2- subgroup, 21% of cases were upgraded from luminal A on CNB to luminal B on WS.

Univariate survival analysis showed that high Ki67 assessed on WS, CNB and TMA was associated with adverse outcome. We performed multivariate Cox analyses adjusting for basic prognostic factors age, tumor size, histologic grade and lymph node status, with separate models for WS, CNB and TMA. These models all showed independent prognostic impact of Ki67. In a separate model, Ki67 values from WS, CNB and TMA were included. Here, only Ki67 WS retained independent prognostic significance. Further, we assessed the prognostic significance of Ki67 in molecular subgroups, and found that Ki67 expression remained significant in the luminal subgroup only.

Paper II

Here, we compared mitotic count, PHH3, and Ki67 values across different types of specimens (WS, CNB, TMA) in terms of association patterns and prognosis. Proliferation assessed on CNB and TMA was significantly underestimated as compared with WS. We applied surrogate immunohistochemical markers for molecular subclassification based on the St Gallen 2013 guidelines. In the luminal/HER2 negative subgroup, there were many discordant cases when applying proliferation markers to WS, CNB and TMA for luminal sub-classification.

Tumor cell proliferation varied according to molecular subgroup with highest proliferation in the triple negative subgroup and lowest proliferation in the luminal subgroup. Increased proliferation assessed by MC, Ki67 and PHH3 across all three sample categories showed significant associations with high histologic grade and hormone receptor negativity. Based on receiver operating (ROC) curves, we proposed thresholds for mitotic count and PHH3 expression to separate luminal B from luminal A tumors. These cut-off points corresponded to a 20% Ki67 cut-off value.

In the complete cohort, univariate survival analysis showed that the prognostic impact of MC, Ki67 and PHH3 were mostly retained across specimen categories. In multivariate Cox analysis, adjusting for age, tumor size, histologic grade and nodal status, mitotic count and Ki67 maintained their independent associations with prognosis, whereas PHH3 did not. In the luminal subgroup, adjusting for standard prognostic parameters as above, only mitotic count retained independent prognostic impact in multivariate analysis.

Paper III

We assessed QSOX1 protein expression on tissue microarrays (n=458) with clinico-pathological association patterns and prognostic impact as end-points. We also assessed QSOX1 expression in relation to molecular subgroups and tumor cell

proliferation. The QSOX1 staining was cytoplasmic. Elevated QSOX1 expression was associated with high histologic grade, hormone receptor negativity, high proliferation, HER2 positivity and P-cadherin positivity. There was no significant association between QSOX1 and axillary nodal status. Increased QSOX1 expression was more common among HER2+ and triple negative subgroups. In univariate survival analysis, cases with high QSOX1 expression (SI=9) showed a 10 year survival probability of 67% compared to 89% for carcinomas with low QSOX1 expression (SI 0-6). Moreover, QSOX1 expression showed independent prognostic impact in multivariate Cox models adjusting for standard prognostic variables (HR=2.7, *P*=0.001). In an extended Cox model, also adjusting for luminal status, both QSOX1 and luminal status were independent prognostic indicators. There was no significant interaction between QSOX1 and luminal status.

5. DISCUSSION

5.1 Discussion of materials and methods

5.1.1 Patient series

We have used a population-based series in **Paper I-III**, consisting of all women in Hordaland County, Norway, diagnosed with primary breast carcinomas during 1996-2003. These patients were enrolled in the prospective, population-based mammographic screening program initiated in Hordaland County in 1996. This County represents about 10% of the total population of Norway and has a similar age-adjusted incidence rate of breast cancer as the whole Norwegian population.[4]

Data were retrieved from the Cancer Registry of Norway, a national registry established in 1951. In combination with the unique personal identification numbers used in Norway, this registry ensures accurate and complete information on all cancers.

This series is well characterized and with long term follow-up based on data from the Death Registry of Norway. Accurate and complete information from the Cancer Registry and the Death Registry of Norway is a strength of this study.

Population-based studies of cancer offer advantages in terms of complete data on incidence and outcome in a given cohort, and avoidance of sampling bias. Thus, conclusions drawn from such studies might be applicable to the general population of cancer patients.

Therapy decisions in this series were based upon traditional prognostic variables such as tumor diameter, histologic grade, lymph nodes and HR status, and the treatment protocols showed slight modifications during this period, as detailed in **Paper I.** We included 33 patients (6%) with locally advanced disease which were treated with neo-adjuvant chemotherapy. To reduce the impact of therapy on proliferation marker results, we assessed pre-therapy excisional biopsies. In addition, we performed

survival analyses after exclusion of these patients, with similar results. There were no changes of median proliferation values when excluding these cases.

We did not have information on the individual adjuvant treatment received, breast cancer recurrence or metastasis. However, this information is currently collected for future studies.

The current age group (50-69) which is the target group for mammographic screening might not be completely representative. Tumors from premenopausal patients show higher Ki67 values[255] as well as more frequent absence of hormone receptors.[256] Also, the incidence of luminal tumors increases with age, whereas the incidence of basal-like tumors decreases.[129,257]

Thus, an expansion of our patient series to include younger women (<50 years) is currently ongoing. Exclusion of patients aged over 70 years is often recommended in prognostic studies, due to differences in treatment protocols and comorbidity that may bias survival analysis.[258]

5.1.2 Use of archival tumor material

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were retrieved from the archives at the Department of Pathology, Haukeland University Hospital, Bergen, Norway. This archive represents a valuable resource of tissue material, and provides a substantial advantage in terms of simple, inexpensive construction of large, population-based patient series with long term follow-up.

Of 546 eligible cases, 12 cases (2.2%) were excluded due to missing tissue blocks or technical inadequate material for immunohistochemistry.

The use of archival material has some potential downsides. In retrospective studies, several pre-analytical factors may interfere with immunohistochemical results, including variation in tissue ischemia time, fixation time, type of fixative, temperature of the paraffin, processing, FFPE storage time and storage conditions. A previous study showed that prolonged storage of FFPE blocks may reduce biomarker

expression, also affecting Ki67 results.[259] In contrast, we found no effect of storage time on Ki67 expression despite storage for up to 17 years (**Paper I**).

It has been suggested that too short fixation or prolonged fixation may both lead to reduced Ki67 counts.[260] On the contrary, brief fixation did not affect the Ki67 results in another study.[261] Longer fixation time may lead to more extensive masking of antigens, although this might be compensated by thorough adjustment of the staining protocols. A previous report suggested severe loss of antigenicity for PHH3 (Serine 28) with improper or prolonged formalin fixation,[262] although it is not known whether this applies to PHH3 (Serine 10) used in our study. Also, loss of PHH3 (Ser10) antigenicity in older (>3-5 years) paraffin blocks has been reported, but proper staining of mitoses could be achieved through increased antibody concentration.[263]

In this study, slides were stored at 4ºC until further analyses. For proliferation markers, freshly cut sections (< 2 weeks) were used, as recommended.[180] For CK5/6, P-cadherin and QSOX1, slides had been stored for no longer than 2 months at 4ºC prior to staining.

It is controversial whether fixation delay influences mitotic count on H&E sections, but poor fixation might alter the morphologic appearance of mitotic figures, making assessment more difficult.[264-267]

5.1.3 Tissue microarrays (TMA)

The TMA technique was introduced in 1998[268] and has been validated for breast cancer studies.[269-271] The procedure is well established representing a cost-effective research method extensively used in our research group since 2000. Although the initial construction of TMA is labor intensive, this technique offers advantages in terms of saving tissue, laboratory reagents including antibodies, and time. Also, staining of all cases during one run reduces technical variation securing that every slide is treated the same way. Further, scoring of cases is easier and less time-consuming on TMA slides compared to WS.

Triplet cores are recommended to counteract possible bias introduced by tumor heterogeneity, and loss of cores during sectioning and antigen retrieval.[270,272] Of note, special subtypes like lobular carcinomas might require more extensive sampling.[272] In such cases we included up to six cores. Care was taken to sample different tumor components, including areas of highest histologic grade.

Random loss of cores during sectioning and antigen retrieval is of concern. To address this, we assessed different glass slides (Poly-lysine, Superfrost and Gold) and chose Poly-lysine, the one that resulted in least tissue detachment during processing.

Cases without TMA available for proliferation assessment (n=75) and the complete cohort (n=534), showed a comparable distribution with regards to age, tumor size, histologic grade and nodal status.

The size of the tissue cylinders in the 190 cases previously processed is smaller (0.6 mm) compared to the extended series (1.0 mm). Although the concordance rate between WS and TMA is acceptable by use of 0.6 mm triplicates,[270] the small area was a limitation in analyses of MC and PHH3 expression. In **Paper II**, we therefore restricted our analyses to cases with both CNB available and TMA with 1.0 mm cores. Thus, comparable areas (2.4 mm^2) could be assessed.

Herceptest and CISH were analyzed on TMA. Although good concordance has been shown for HER2 assessed on CNB and WS,[273] intratumoral heterogeneity for HER2 amplification has been reported.[274,275]

The impact of tumor heterogeneity on TMA results is further discussed in section 5.2.1.

5.1.4 Core needle biopsies (CNB)

Sections from core needle biopsies were included for comparative analyses of proliferation markers. 154 patients (29%) had CNB available. There were no differences in age, tumor size, nodal status, or HER2 status between patients diagnosed by FNAC and CNB. Patients diagnosed by FNAC showed a slightly higher frequency of ER negativity compared to CNB (17% versus 13%). Also, the CNB procedure was more frequent than FNAC between the years 2000-2003 compared to 1996-1999.

5.1.5 Immunohistochemical methods

Although molecular profiling has revolutionized cancer research, immunohistochemistry is still a valuable research tool. It is inexpensive, simple and makes it possible to assess the precise cell type and staining compartment of a specific protein.

For Ki67, we chose the monoclonal MIB-1 antibody which is currently recommended for assessment of proliferation in breast cancer.[180] For PHH3, we selected a polyclonal antibody (Millipore) previously studied in our research group,[276,277] and in several breast cancer cohorts.[200,278,279]

The polyclonal QSOX1 antibody (Proteintech) was chosen based on previous studies in breast cancer.[280,281] The immunohistochemical protocol chosen corresponded to a previous published protocol, aside from a small adjustment of antibody dilution.[280-283] For the remaining antibodies (CK5/6, P-cadherin), we used staining protocols as previously published.[228,284,285]

The staining protocols were optimized for each antibody by use of different heat induced retrieval systems, such as pressure cooker versus microwave oven, and retrieval buffers with different pH. Optimal titration of the antibodies was obtained through proper testing of dilutions. To optimize staining results, freshly prepared

xylene and alcohol were used for de-waxing. Although the use of microwave oven is recommended for epitope retrieval for Ki67,[180] a pressure cooker was preferred due to less tissue detachment.

All samples were scored blinded to patient characteristics and outcome as recommended.[286]

To ease the comparison of results between studies, guidelines for reporting of biomarker studies (REMARK) and checklists for retrospective histopathology studies have been published.[286-288]

For Ki67, there is currently no consensus on scoring method. For assessment of Ki67, published guidelines were roughly followed.[180] These guidelines recommend scoring of at least 500 tumor cells. With homogenous proliferation, three randomly selected HPFs should be scored. In case of proliferative heterogeneity with an increasing gradient towards the tumor periphery, three HPFs at the periphery should be scored. There is no consensus on the assessment of hot-spots.[180] Currently, assessment of the whole section with an overall Ki67 score is recommended. However, we decided to focus on hot-spot counting, in line with the approach used for mitotic count in histologic grading. This approach is also supported by studies showing highest prognostic impact of Ki67 when counted in hot-spot areas.[289,290]

Assessment of mitotic count is fairly well standardized, based upon guidelines for histologic grading, and a similar approach was used for PHH3. We did not apply any correction for tumor volume.[291] Tumors showing extensive fibrosis or larger cell size have less tumor cells per unit area. A previous study addressed this by comparing four different methods of mitotic assessment.[291] Mitotic activity was evaluated in hot-spots, in random HPFs with sampling across the whole tumor, and with two different modes of volume correction. Although all four methods provided prognostic information, the hot-spot approach was easier and less time-consuming, and there was no improvement of prognostic prediction by correction for tumor volume.

For QSOX1, P-cadherin and CK5/6, the scoring system used (SI) is well-established in our research group and has shown good interobserver agreement.[228,251,292] Although it is a subjective scoring system, it should be noted that alternative ways of scoring such as the modified Histochemical score (H-score) and the Allred score are equally subjective. Also, assessment of variables in clinical use (ER, PR and HER2) is subjective, but accepted by the clinical community.

5.1.6 Observer agreement

Intraobserver variability using Spearman's correlation and kappa statistics was assessed for all markers, with good agreement. Further, interobserver variability was evaluated for MC, Ki67, and QSOX1, also with good agreement (kappa-values 0.84, 0.77, 0.83). For PHH3, the agreement was moderate (kappa-value 0.52). Interobserver agreement for CK5/6 and P-cadherin has previously been assessed.[228]

In 2011, the International Ki67 in Breast Cancer Working Group published guidelines for Ki67 assessment.[180] On behalf of this group, international Ki67 reproducibility studies are currently ongoing. Interlaboratory reproducibility of Ki67 was initially questioned,[293] although a recent study showed that a high level of interlaboratory reproducibility for Ki67 scoring of TMA can be achieved with a common scoring method.[294] Results based on core needle biopsies and full sections are expected.

A previous study showed that Ki67 assessment of histologic grade 2 carcinomas is particularly affected by intra- and interobserver variation.[295] A recent study with Ki67 assessed on full sections, showed good intra- and interobserver agreement.[296] Also, a Swedish survey reported good interlaboratory agreement for Ki67 when using a standardized approach.[297]

We used manual Ki67 counting as compared to visual estimation. This has previously been shown to give the most consistent results,[293,298,299] and is the recommended approach.[180]

It has been suggested that the use of digital image analysis (DIA) may improve Ki67 reproducibility,[300] and mitoses counting via image analysis have also been proposed.[301] Studies comparing manual counting of Ki67 with DIA have shown good correlation between methods, but contradictory results in terms of prognostic superiority.[289,302-304] Thus, validation studies of image analysis are needed. Currently, image analysis is not recommended for proliferation assessment in breast cancer.[180]

Interobserver variability of mitotic count in breast cancer has previously been addressed,[131,143] and moderate to substantial agreement (kappa-values 0.45-0.82) have been reported.[305-312] This interobserver variation is accepted by the clinical community.

5.1.7 Cut-off point determination

Prior to the statistical analyses, biomarker frequency and distribution curves were explored. Proliferation markers were kept as continuous variables for selected analyses, but they were also dichotomized. Standardized or published thresholds were applied when available. Cut-off points were determined based on median and quartiles also taking into account the frequency distribution curves and the size of subgroups. Categorization by quartiles is recommended to avoid selection bias by multiple testing.[313] For survival analyses, the number of events in each subgroup was assessed prior to analysis. Subgroups with comparable outcome were merged.

For tumor diameter, a 20 mm cut-off was applied, corresponding to the segregation between stage T1 and T2, and representing the upper quartile in this series. For nodal status, we used presence or absence of metastasis.

In **Paper I**, we used a Ki67 cut-off point at the median value, specific for each specimen type. For luminal sub-classification, a 14% Ki67 threshold was applied.[223,224] In **Paper II**, we used a 20% Ki67 cut-off as suggested by the St Gallen 2013.[225] In **Paper III**, QSOX1 expression (SI) was kept as an ordinal variable for selected analyses. For association analyses, QSOX1 was categorized. For P-cadherin

and CK5/6, we applied cut-off points previously used and validated by our research group.[54,228]

5.1.8 Surrogate definitions of molecular subclasses of breast cancer

The molecular sub-classification of patients was based on the immunohistochemical surrogates endorsed by the St Gallen guidelines. The diagnostic shift from St Gallen 2011 to 2013 made it impossible to subclassify 11 carcinomas (2%) defined by ER negativity, PR positivity, and HER2 negativity. This subtype is uncommon, and it is currently not known whether it represents a mere technical artefact, or a clinically meaningful subgroup.[154,161,314]

ER and PR were considered positive if ≥ 10% of tumor cells stained positive, according to national guidelines at the time and consistent throughout the period. This may be regarded as a limitation given the currently widespread use of lower ER thresholds (1%). However, the low ER (1-9%) breast cancer is a small subgroup. In a cohort of 9639 patients, only 2.6% showed 1-9% ER positivity.[315] Also, low ER breast cancers (1-9%) may have more in common with ER-negative tumors (<1%) in terms of prognosis, endocrine resistance and chemotherapy response.[315,316]

It should be noted that the overlap between breast cancer subtypes defined by immunohistochemistry and gene expression is considerable, but not absolute.[317] Luminal B disease by PAM50 may show ER negativity by immunohistochemistry. Moreover, the triple negative phenotype, defined by ER-, PR-, and HER2 negativity by immunohistochemistry, is an approximation of the basal-like subgroup. There is around 70-90% overlap between the basal-like subgroup and the triple-negative category.[230]

5.2 Discussion of main findings

5.2.1 Proliferation markers across distinct specimen categories

In **Paper I and II**, our goal was to investigate the matched level of tumor cell proliferation by mitotic count, Ki67 and PHH3 across three distinct tissue categories (WS, CNB, TMA), and to compare the association patterns and prognostic impact of these markers.

In **Paper I**, the analyses were restricted to Ki67. Here, our results indicate that the proliferative activity is significantly underestimated on CNB and especially TMA compared to WS. In **Paper II**, we included mitotic count and PHH3. In line with the Ki67 results, underestimation of mitotic count and PHH3 was demonstrated on CNB and TMA specimens.

These are the first two studies, to our knowledge, to examine the matched level of Ki67, mitotic count and PHH3 across three distinct specimen categories. Our results support recent studies showing underestimation of Ki67 on CNB compared to WS.[318-321]

In contrast, there are reports indicating good agreement between Ki67 assessment on WS and CNB.[200,322-324] Two studies have shown higher Ki67 counts on CNB,[325,326] but Greer et al. compared Ki67 assessed in different laboratories with variation in antibody dilution, scoring method, and Ki67 thresholds.[325] Romero et al. assessed a limited number of cases.

Three small studies (20-86 cases) have shown good agreement between Ki67 in WS and TMA.[327-329]

For mitotic count, previous publications are broadly consistent, and our results are in line with these, showing underestimation of mitotic count on CNB.[200,330-332] Moreover, our findings are supported by reports demonstrating discrepant histologic grading mainly caused by an underestimation of mitotic count on CNB.[330,331,333-339]

A comparison between mitotic count and PHH3 on WS and TMA has previously not been published. Although we show significant underestimation on TMA, this study has limitations, as few TMA samples (n=101) were considered adequate for comparative analyses (see section 5.1.3).

Methodological aspects may, in part, explain the discrepant Ki67 and PHH3 values when comparing CNB and WS, and pre-analytical factors that may interfere with the results are discussed in section 5.1.2. Of note, the discrepancy between TMA and WS was more pronounced. Intratumor heterogeneity has to be considered, and is likely to affect the results of all proliferation marker studies. Small tumor samples, such as CNB and TMA may not reflect the entire tumor. Of note, heterogeneity may also affect histologic grading, and under-grading in CNB has been shown in 16-25% of cases.[334] For ER and HER2, concordance rates from 62-99% have been reported.[340]

Several risk factors have been associated with discrepant Ki67 levels between CNB and WS, including younger age, large tumor diameter, high histologic grade, and hormone receptor negativity.[321] The Ki67 increase from biopsy to surgical specimen has been associated with the HER2 positive subgroup,[319,341] triple negative tumors,[319] and ER-positive breast cancer as well.[342]

In WS, we found heterogeneous distribution of Ki67 in 23% of the cases by visual impression. Previous studies have reported Ki67 heterogeneity in 18-63% of the cases, although with varied definitions of heterogeneity.[343-345] Most studies have reported higher Ki67 counts at the periphery than in the central area. Higher counts at the tumor periphery have also been shown for mitotic count.[346]

For Ki67 scoring, we used a hot-spot approach. This method has previously been shown to provide higher prognostic impact,[289,290] and it is consistent with the approach used for mitotic count in histologic grading. Counting Ki67 average might reduce the impact of heterogeneity, and better concordance with overall mean counting than hot-spot counting has been shown.[320]

Our data support that assessment of proliferation markers on whole sections, when available, should be regarded current best practice. The results are of direct practical relevance showing that treatment decisions based on Ki67 thresholds from TMA may lead to overtreatment of some patients.

All three proliferation markers were significantly underestimated on CNB and especially TMA compared to WS. For assessment of proliferation markers on CNB, specimen specific proliferation thresholds should be considered. Although TMA is an invaluable research tool, tumor heterogeneity is a limiting factor. Thus, TMA is less suited for assessment of proliferation in studies with potential clinical impact.

5.2.2 Proliferation markers and association with clinico-pathological variables

Mitotic count, Ki67 and PHH3 assessed on WS were all associated with aggressive tumor features such as large tumor diameter, high histologic grade, nodal metastases, HR negativity and HER2 positivity (**Paper I-II**). Association patterns were mostly retained on CNB and TMA despite the significant variation in proliferation level across specimens.

5.2.3 Proliferation markers and association with prognosis

In **Paper I**, our results show that Ki67 is prognostic over a wide range of cut-off points.

In paired cases (WS, CNB and TMA), only Ki67 assessed on WS showed independent prognostic impact, in agreement with a previous report comparing Ki67 assessed on full sections with TMA.[347]

In **Paper I**, we found no association between Ki67 count and outcome in the non-luminal/HER2+ or triple negative subgroups, in line with a previous report.[348] However, this might have been caused by limited patient numbers in subgroups. Thus, prognostic impact of Ki67 in the triple negative subgroup has been reported.[349-352]

In **Paper II**, all three proliferation markers showed prognostic impact in univariate survival analyses across specimen categories, with the exception of PHH3 on TMA. In multivariate survival analysis on WS, mitotic count and Ki67 retained independent prognostic impact, whereas PHH3 did not.

In breast carcinomas, PHH3 has previously outperformed both mitotic count[278,353,354] and Ki67 expression[355] in survival analyses. Of note, these studies included a limited number of cases with few events. Our results indicate that PHH3 might not substitute MC as a mitotic marker. Indeed, for PHH3, several factors must be addressed. PHH3 counts were three times higher than mitotic count, in line with previous findings.[200,277,356,357] This discrepancy might have several explanations. One may speculate that hot-spots are easier to identify in PHH3 stained sections. PHH3 stains cells in late G2 and early prophase; whereas mitoses on H&E sections are not detectable prior to nuclear disintegration. Also, PHH3 might not be completely mitosis specific. We experienced weak to moderate granular staining of interphase nuclei, in line with previous reports.[197,199,276,277,358]

In addition, PHH3 might be related to apoptosis. Phosphorylation of histone H3 on serine 10 during apoptosis was reported in a cell line study, possibly related to chromatin condensation during apoptosis.[359] Whether this is applicable to immunohistochemical assessment of PHH3 is not known. Although PHH3 positive, non-mitotic nuclei have been described,[360,361] PHH3 positivity in apoptotic cells has previously not been reported.[199,278,362] Finally, we experienced PHH3 negative mitoses in 11% of the cases, despite thorough adjustment of antigen retrieval protocols and repeated staining. These results are in line with previous reports on melanomas.[277,363]

Thus, the specificity of PHH3 as a mitotic marker should be addressed in future studies prior to potential clinical implementation of this marker.

5.2.4 Cut-off points for proliferation markers

Ki67 thresholds

In **Paper I**, we showed that the 14% Ki67 threshold endorsed by the St. Gallen guidelines in 2011 for sub-classification of the luminal group, led to several discrepant cases when comparing WS with CNB and TMA. This supports the use of whole sections, when available.

In **Paper I**, we suggested a Ki67 cut-off point of 20% to separate luminal B from luminal A tumors. This threshold is in agreement both with the St Gallen 2013 and 2015 statements.[135,225] Also, a novel, large meta-analysis on the prognostic effect of Ki67, suggested a Ki67 threshold of at least 25%.[364]

Although direct comparison of studies is difficult due to variation in study population, analytical factors, scoring methods and Ki67 thresholds, the distribution of Ki67 counts in our series is in line with previous reports. We found a Ki67 median of 18% (WS) in the complete cohort (**Paper I**). This is consistent with reports showing a Ki67 median of 17-19%.[222,348,365-367] In contrast, lower median Ki67 counts (11% and 15%) have been reported.[255,368]

In the luminal/HER2- subgroup we found a Ki67 median of 15%. Previous studies have reported a Ki67 median of 11-18%.[348,367,369]

A limitation of our study is that the size of the luminal B subgroup (36%) was estimated based on immunohistochemical surrogates and subgroup sizes drawn from another study.[223] However, there are reports showing a comparable size of the luminal B subgroup (29-38%) by PAM50.[50,212,370]

Mitotic count

The use of mitotic count to segregate the luminal group has previously not been focused. In **Paper II**, we showed that mitotic count is not inferior to Ki67 in terms of prognostic impact. We indicate a MC threshold of 2.5 mm^2 for luminal sub-

classification. This is in line with a recent study suggesting that mitotic score may guide decisions on chemotherapy for the luminal subgroup.[309]

Comparison between studies is difficult due to differences in the reporting of mitotic counts. MC is usually reported per 10 HPF, equivalent to the approach used for histologic grading. However, the field diameter differs between microscopes and area adjustment should be applied, preferably with reporting of MC per mm^2.[371] Previously, the use of mitotic activity index (MAI) has been suggested.[143,144,372] MAI is the number of mitoses assessed in 10 HPF with a field diameter of 0.45 mm, and the total area assessed is 1.59 mm^2.

In our series, mitotic count (WS) showed a median of 2.1 mitoses per mm^2 (MAI 3.3), and the upper quartile was 5.5 mitoses per mm^2 (MAI 8.7). Previously published thresholds include MAI 3 (1.9 mitoses per mm^2) and MAI 9/10 (5.7-6.3 mitoses per mm^2). The distribution of MC in our series is fairly consistent with previous reports.[373-378]

In node-negative cancer, lower MC has been reported,[379] and higher MC has been shown in tumors from pre-menopausal,[380,381] node-positive[370] and locally advanced disease.[382] Also, there are breast cancer cohorts were higher median MC has been shown.[291,383,384]

To the best of our knowledge, this is the first study where the exact mitotic count has been used to segregate the luminal subgroup for prognostic purposes. Our results indicate that mitotic count might replace or support Ki67 as a marker for sub-classification of luminal breast cancer. Mitotic count is well established, and the interobserver variation is accepted by the clinical community. It is a simple, inexpensive method already being performed on all breast cancer cases. All the pre-analytical, analytical and scoring related downsides that may affect the Ki67 results are avoided. The only addition needed is to report the exact number of mitoses instead of the mitotic score used for histologic grading.

We suggest a cut-off point of at least 2.5 mm^2 to separate luminal A from luminal B tumors, but this threshold needs validation in separate breast cancer series from different institutions.

PHH3

For PHH3, comparisons between studies are challenging given the lack of consensus on immunohistochemistry and scoring methods. Different antibodies have been applied, and PHH3 has been assessed on TMA,[353,357,378] CNB,[200] and full sections.[278] PHH3 has been scored using staining indices (Allred)[385], and approaches corresponding to mitotic count in histologic grading.[278,354,378] Also, the number of PHH3 positive tumor cells has been reported as a percentage, in line with Ki67 reporting,[201,386] and digital image analysis has been applied.[355]

We assessed PHH3 with a similar approach as for MC, in line with other studies.[278,354,378] In **Paper II**, the frequency distribution of PHH3 with a median of 6.3 mitoses per mm^2 (15 mitoses per 10 HPF) corresponds to PHH3-MAI 10. This is comparable to a series of node-negative breast cancer (<71 years) where 65% of cases showed PHH3-MAI < 13.[354] In younger (<55 years) node-negative patients, a higher proliferation was shown, and 55% of patients had PHH3-MAI > 13.[278]

5.2.5 QSOX1 expression

In **Paper III**, we explored the prevalence, association pattern and potential prognostic impact of the novel biomarker QSOX1. Previously, QSOX1 was suggested as a luminal B specific marker,[280] thus, we also wanted to assess QSOX1 in relation to tumor cell proliferation and molecular breast cancer subtypes. We found that high QSOX1 expression is associated with increased Ki67 and MC, high histologic grade, hormone receptor negativity, and HER2 positivity. These findings concur with other reports that show associations between high QSOX1, increased Ki67 values and high histologic grade.[280,387]

Previous cell line studies have associated high QSOX1 expression with increased invasiveness in breast, pancreatic and prostate cancer.[280,283,388] QSOX1 has also been suggested as a target for HIF-1.[389]

Although QSOX1 has been associated with adverse tumor features in several reports, the prognostic impact of QSOX1 in breast cancer has been controversial.[280,281] To our knowledge, this is the first report addressing the prognostic impact of QSOX1 protein expression in breast cancer. We show that QSOX1 expression is an independent factor of poor prognosis in multivariate survival analysis. Consistent with this, high QSOX1 gene expression has been associated with poor prognosis.[280]
High QSOX1 expression was associated with increased tumor cell proliferation, but our findings did not support a study indicating QSOX1 as a luminal B specific marker.[280] Increased QSOX1 expression was more common among HER2+ and triple negative breast cancers, and we found no interaction between QSOX1 and luminal status.

We have assessed the intracellular compartment of QSOX1. It is currently unknown why subgroups of cancers overexpress QSOX1, and the role of this enzyme in the secretory pathway needs to be clarified. In the extracellular space, QSOX1 may play an important role providing proper incorporation of laminin into the ECM. This is a prerequisite for tumor cell invasion.[119]

This is a single cohort, exploratory study, and our results need validation in separate patient cohorts. Further studies on the role of QSOX1 in cancer progression are warranted.

6. CONCLUSIONS

1. The proliferative activity (mitotic count, Ki67, PHH3) was significantly underestimated on core needle biopsies (CNB) and especially tissue microarrays (TMA) compared to whole sections (WS). TMA is less suited for assessment of proliferation in studies with potential clinical impact (**Paper I-II**).

2. Assessment of proliferation markers on whole sections should be regarded current best practice (**Paper II**).

3. A Ki67 threshold of 20% seems appropriate to segregate the luminal subgroup based on WS specimens (**Paper I**).

4. Mitotic count and Ki67 provided independent prognostic information, and were superior to PHH3 as prognostic factors in breast cancer (**Paper II**).

5. Mitotic count using a threshold of 2.5 mitoses/mm^2 might potentially replace Ki67 in sub-classification of the luminal group (**Paper II**).

6. High QSOX1 expression by immunohistochemistry was associated with features of aggressive breast cancer and reduced survival by multivariate analysis (**Paper III**).

7. High QSOX1 expression was more common among HER2 positive and triple negative tumors. Our findings do not support QSOX1 as a luminal B specific marker (**Paper III**).

7. FUTURE PERSPECTIVES

The breast cancer series used in this thesis is currently under expansion to include women below 50 years of age. Novel survival data from the Norwegian Cause of Death Registry has been requested, and data on metastasis are collected. The subgroup of node-positive patients is being further characterized in a separate PhD project in which the proliferative rate (mitotic count, Ki67) is assessed in lymph nodes and compared with the primary tumor.[390] In another PhD project, assessment of proliferation markers (MC, Ki67) is planned in the subgroup of young patients (<50 years), and the results will be compared with the current series (50-69 years).

The main goal of the expanded project is an improved characterization of biological and clinico-pathological biomarkers in breast cancer subgroups to refine diagnostics and define potential treatment targets. The biomarkers will further be characterized according to molecular categories. A special attention will be drawn to the tumor microenvironment, angiogenesis and vascular invasion.

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9. PAPER I-III

I

Paper I

Evaluation of Ki67 expression across distinct categories of breast cancer specimens: A population-based study of matched surgical specimens, core needle biopsies and tissue microarrays

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Evaluation of Ki67 Expression across Distinct Categories of Breast Cancer Specimens: A Population-Based Study of Matched Surgical Specimens, Core Needle Biopsies and Tissue Microarrays

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Abstract

Introduction: Tumor cell proliferation in breast cancer is strongly prognostic and may also predict response to chemotherapy. However, there is no consensus on counting areas or cut-off values for patient stratification. Our aim was to assess the matched level of proliferation by Ki67 when using different tissue categories (whole sections, WS; core needle biopsies, CNB; tissue microarrays, TMA), and the corresponding prognostic value.

Methods: We examined a retrospective, population-based series of breast cancer ($n = 534$) from the Norwegian Breast Cancer Screening Program. The percentage of Ki67 positive nuclei was evaluated by visual counting on WS ($n = 534$), CNB ($n = 154$) and TMA ($n = 459$).

Results: The median percentage of Ki67 expression was 18% on WS (hot-spot areas), 13% on CNB, and 7% on TMA, and this difference was statistically significant in paired cases. Increased Ki67 expression by all evaluation methods was associated with aggressive tumor features (large tumor diameter, high histologic grade, ER negativity) and reduced patient survival.

Conclusion: There is a significant difference in tumor cell proliferation by Ki67 across different sample categories. Ki67 is prognostic over a wide range of cut-off points and for different sample types, although Ki67 results derived from TMA sections are lower compared with those obtained using specimens from a clinical setting. Our findings indicate that specimen specific cut-off values should be applied for practical use.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Introduction

Breast cancer is a heterogeneous disease. During the last decade, gene expression studies have identified distinct molecular subtypes, such as Luminal A, Luminal B, HER2-enriched, basal-like and normal breast-like, and these have markedly different behavior and prognosis [1,2]. Subsequent studies have introduced immunohistochemical surrogate markers for molecular classification, with a proposed Ki67 cut-point of 14% to separate Luminal A from Luminal B tumors [3,4]. Furthermore, the treatment effect of adding docetaxel to highly proliferative, luminal tumors has been demonstrated [5,6].

In 2011, the St Gallen International Expert Consensus included a Ki67 cut-off point of 14% in their recommendations for adjuvant therapy [7]. However, there is currently no agreement on specimen selection, technical protocols, evaluation methods or cut-off values [8,9], and the criteria for sub-classification of breast carcinomas by Ki67 has yet to be established. This area is controversial, and in the report from St Gallen 2013 recently published, the cut-off value has been changed [10].

On this background, we aimed to study the levels of tumor cell proliferation based on Ki67 expression according to specimen type such as whole sections (WS), core needle biopsies (CNB) and tissue microarrays (TMA) from a population-based series of breast cancers, and to study and compare the prognostic value of Ki67 in relation both to specimen type and molecular subgroups of breast cancer.

Materials and Methods

Patient series

This study was approved by the Western Regional Committee for Medical and Health Research Ethics, REC West (REK 2012/1704). We identified all women (50–69 years) who resided in Hordaland County, Norway, when diagnosed with primary invasive breast cancer as part of the population-based Norwegian Breast Cancer Screening Program during 1996-2003. Hordaland County has approximately 500,000 inhabitants, this represents about 10% of the total population of Norway.

Patients with distant metastatic disease at time of diagnosis (stage IV) were not included, leaving 555 potential cases. Written informed consent was not obtained from the patients, but in accordance with national ethics guidelines and procedures for such retrospective studies, all participants were contacted with written information on the study and asked to respond if they objected. In total, 9 patients (1.6%) did not approve participation. 12 cases had technical inadequate material for proliferation assessment (Ki67), leaving 534 cases for further studies. Patient records and information were anonymized and de-identified prior to analysis. The patients included had a median age of 60 years at diagnosis (factual range 49–72 years).

The patients received treatment according to standard national protocols in a single institution. Follow-up information was given by the Norwegian Cause of Death Registry, and can be considered accurate and complete. Last date of follow-up was December 31, 2011. Outcome data include survival status, survival time and cause of death. During the follow-up period, 79 patients (15%) died from breast carcinoma, and 62 (12%) died from other causes. The median survival of the censored patients was 12 years, and the median follow-up was 13 years calculated by the reverse Kaplan-Meier method. The 5-year breast cancer specific mortality was 9% (49/534).

Clinico-pathological variables

Patient's clinical history and tumor characteristics including age at diagnosis, largest tumor diameter, histologic type, histologic grade, lymph node status and hormonal receptor status were obtained from the clinical records and routine histopathology reports. Histologic type was assessed according to WHO criteria, whereas histologic grade was evaluated using the Nottingham modification [11] by five experienced breast pathologists (JE, JA, IMS, KC, LAA). Tumor size was assessed histologically (61%) and by macroscopic examination (29%). However, if pathologic tumor size was not available (as in patients with locally advanced or multifocal disease), the radiologic size estimate was included (10%). For immunohistochemical studies on whole sections, HE slides were re-examined, and representative slides (1–2 blocks) displaying both the peripheral and central parts of the tumor, as well as the most cellular and high-grade areas, were selected for further analyses. The corresponding FFPE block was also used for TMA construction.

Patient characteristics

Radical mastectomy was performed in 285 cases (53%), and breast conserving surgery in 245 cases (46%); four patients were represented with core needle biopsy only (three cases of locally advanced disease and one patient with surgery abroad). Adjuvant therapy was decided according to tumor size, histologic grade, hormone receptor status and nodal status. Treatment protocols showed slight modifications during the period. Chemotherapy was offered to patients below 55 years with stage I disease who had histologic grade 2 and 3 tumors, and to patients under 55 years with stage II disease. From 1998, chemotherapy was also recommended for patients between 55–65 years with stage I or II disease combined with hormone receptor negativity. 33 patients (6%) were treated with neo-adjuvant chemotherapy due to locally advanced disease.

Adjuvant radiation therapy was recommended for patients who received breast conserving surgery, had primary surgery without free resection margins, stage II disease with axillary metastasis, as well as stage III disease.

Specimen characteristics

The tumor samples were fixed in 4% buffered formaldehyde before processing and embedding in paraffin. Storage time of the archival formalin-fixed, paraffin embedded tissue samples (blocks) was up to 17 years. Five μm sections were cut by one person using the same microtome and mounted onto poly-lysine coated glass slides. Slides were stored for no longer than two weeks at 4°C until staining for Ki67 was performed.

Tissue microarray (TMA). H&E stained slides were used for tumor verification. Briefly, 1.0 mm cores in triplicate were punched and mounted into a recipient paraffin block using a semi-automated precision instrument (Minicore 3, Tissue Arrayer, Alphelys, France). Care was taken to select areas with high tumor purity and to include the periphery and areas of highest histologic grade. 190 cases had previously been processed [12,13]; from these cases three tissue cores with a diameter of 0.6 mm were obtained by a different instrument (Beecher Instruments, Silver Spring, MD, USA).

Among the 534 cases with TMA available, 22 cases had tissue cores devoid of invasive tumor, 21 cases had complete core loss and 32 cases showed fewer than 100 tumor cells on arrayed spots, leaving 459 cases (86%) available for proliferation assessment.

Preoperative core needle biopsies (CNB). 182 patients had undergone both preoperative core needle biopsy and subsequent primary surgical excision for breast carcinoma. Among these, 25 cases were excluded due to non-representative or inadequate material remaining for biomarker assessment. Three cases had previously been excluded due to lack of informed consent. In total, 310 cases received preoperative cytology only, and the remaining cases had either frozen sections, incisional or excisional biopsies performed; this practice was according to national guidelines at the time. The number of core biopsies taken ranged from 1 to 4 (mean = 2.4, median = 2). 92% of the cases had more than 1 core biopsy available.

Ki67 immunohistochemistry

Immunohistochemistry was performed on 5 μm slides of formalin-fixed and paraffin-embedded archival tumor tissue. The sections were de-waxed with xylene/ethanol before target retrieval in a pressure cooker (Decloaking Chamber Plus, Biocare Medical). Staining procedures were performed on a DAKO autostainer using the K4061/Envision Dual Link System (rabbit+mouse). Sections were incubated for 30 minutes at room temperature with a monoclonal rabbit antibody (M 7240, clone MIB-1, DAKO) at a 1:100 dilution. Finally, diaminobenzidine (DAB) as chromogen for 10 minutes was followed by haematoxylin as counterstain for 3 minutes. Sections from tonsils were used as positive controls; negative controls were obtained by replacing the primary antibody with Tris-buffered saline. Controls were included in each staining run.

Evaluation of staining

Hormone receptors. Results for estrogen and progesterone receptors were obtained from the routine pathology reports.

Tumors were considered ER or PR positive if ≥10% of tumor nuclei stained positive, according to national guidelines during the period.

HER2. The established scoring system for DAKO Herceptest was used. HER2 SISH was performed on IHC 2+ cases (Ventana INFORM HER2 DNA probe staining). The 2+ cases were considered HER2 positive if the HER2/Chr17 ratio by SISH was equal to or greater than 2.0.

Ki67 scoring. All slides were examined and scored by one pathologist (GK), blinded to patient characteristics and outcome. The slides were evaluated using light microscopy (Leica DMLB) with an eye-piece graticule for counting at x630 magnification, roughly following the approach used by Weidner et al. [14]. Care was taken to avoid areas of intense inflammation, fibrosis, necrosis, low cellularity or poor fixation. The slides were scanned at low magnification (x100) to identify and encircle the hot-spot (HS); this was defined as the area containing the highest density of Ki67-labelled tumor cells by visual impression. The hot-spot was usually situated at the periphery of the carcinoma. Further, the cold-spot (CS), the area with the lowest density of Ki67 positive tumor nuclei, was identified. Overall, 23% of all cases (WS) showed clearly heterogeneous proliferation. In these cases, 500 tumor cells in consecutive HPFs were counted in both hot and cold spots. For tumors with homogenous proliferation, or small areas of invasive tumor, 500 tumor cells at the peripheral part of the tumor were assessed, and a single figure for Ki67 expression was recorded. Only stained tumor cells crossing horizontal grid lines were counted. Any nuclear staining regardless of intensity was considered positive.

We did not find any correlation between Ki67 expression and years of storage of the tissue blocks (data not shown). Further, we found no difference in median Ki67 expression when comparing patients with 1–2 core biopsies (CNB) available versus 3–4 core biopsies (data not shown).

In a subset of 50 cases, the slides were evaluated at a different magnification (x400), with excellent correlation between the methods (Spearman's correlation coefficient (ρ) 0.96, kappa-value 0.79, $P<0.001$ for both tests).

Observer agreement for Ki67 counts. Intra-observer variability was evaluated by randomly rechecking 50 cases (WS) after a period of 6 months, with excellent correlation between the 2 counts (Spearman's ρ 0.99, kappa-value 0.88). Moreover, a separate researcher (SA) assessed 50 cases across all sample categories showing good inter-observer agreement: WS specimens: Spearman's ρ 0.95; kappa-value 0.71; CNB specimens: Spearman's ρ 0.93; kappa-value 0.80; TMA specimens: Spearman's ρ 0.88; kappa-value 0.74 ($P<0.001$ for each analysis).

For assessment of Ki67 on CNB, 500 tumor cells were counted by choosing the most proliferative region if possible. For assessment of Ki67 on TMA, all available cores were assessed, and the core with the highest Ki67 score was recorded. TMA samples with fewer than 100 tumor cells were considered not interpretable.

Furthermore, an "average" tumor cell proliferation was estimated as a mean of Ki67-HS and Ki67-CS in cases of heterogeneity. In a subgroup of 25 cases, the overall average score was also directly counted on the slides in addition to the estimated average. This was obtained by counting 200 cells in each of three representative tumor areas (hot-spot, intermediate area and cold-spot). There was a strong and positive correlation between the average score obtained by counting and the estimated mean (Spearman's ρ 0.86, $P<0.001$, kappa-value 0.62, $P=0.001$).

Definition of molecular classes of breast cancer

Molecular classes were defined as Luminal A (ER positive and/or PR positive, Ki67<14%), Luminal B (LuminalB-HER2 negative: ER positive and/or PR positive, Ki67≥14%; LuminalB-HER2 positive: HR positive and HER2 positive regardless of Ki67), HER2 enriched (ER and PR negative, HER2 positive), and triple negative (ER negative, PR negative, HER2 negative) based on published criteria [7].

Statistical methods

Analyses were performed using the SPSS statistical package, version 18.0 (SPSS Inc., Chicago, IL). Statistical significance was assessed at the two-sided 5% level. Non-parametric correlations were tested by the Spearman's rank coefficient. Bland and Altman analysis and Wilcoxon signed rank tests were used to compare related samples. Continuous variables not following the normal distribution were compared between two or more groups using the Mann-Whitney U or Kruskal-Wallis H-test. Continuous variables were categorized based on quartile limits, also considering the frequency distribution plot for each marker, as well as the number of events in subgroups. The Cohen's kappa measure was used to assess the agreement of two categorical scores.

For survival analyses, the end-point of interest was breast cancer specific survival (BCSS), defined as the time in months from the date of diagnosis to the date of death from breast cancer. Patients with missing data were excluded from analyses. Univariate survival analyses were performed using the product-limit procedure (Kaplan-Meier method), and differences between categories were estimated by the log-rank test, with date of diagnosis as the starting point. Patients who died from other causes were censored at the date of death. Multivariate survival analyses were conducted using Cox′ proportional hazards methods. Multivariate analyses adjusted for standard prognostic factors including tumor size, histologic grade, nodal status and age. Covariates were examined by log-log plot and by adding interaction terms to determine their ability to be incorporated in multivariate models. For continuous variables, the proportional hazard assumptions were also assessed by studying the graphs of Schoenfelds residuals.

Results

Clinico-pathologic characteristics of the patients

In the current study, median tumor size was 15 mm (range 3–110 mm). Table 1 gives an overview of clinico-pathological features of the complete series. See also table S1 in File S1 for a summary of clinico-pathologic characteristics in relation to molecular subclasses.

Among patients that underwent axillary node dissection, the median number of lymph nodes sampled was 11 (range 1–33).

Ki67 counts in relation to different specimen types

The following results are based upon hot-spot counts, unless otherwise is stated. The median percentages of Ki67 expression according to specimen types for both the complete series and paired cases are listed in Table 2, see also Figure S1-A. Ki67 counts were significantly higher in WS as compared to CNB (n = 154, Wilcoxon signed rank test, $P=0.001$), with a median absolute difference of 2.4% (range −44% to 42%), see Figure S1-B.

Ki67 counts were significantly higher in WS as compared to TMA (n = 459, Wilcoxon signed rank test, $P<0.001$), with a median absolute difference of 10% (range −6 to 76%). Furthermore, an increase in variability of the differences with increasing proliferation was shown (See Figure S1-C).

Table 1. Clinico-pathologic characteristics.

Characteristics	Complete series	
	N	(%)
Tumor diameter		
≤2 cm	405	75.8
>2 cm	129	24.2
Histologic grade		
1	218	40.8
2	226	42.3
3	90	16.9
Nodal status		
Negative	387	72.5
Positive	142	26.6
Missing	5	0.9
Histologic type		
Ductal	447	83.7
Lobular	55	10.3
Tubular	8	1.5
Mucinous	16	3.0
Medullary	4	0.7
Unclassified	4	0.7
ER		
Positive	451	84.5
Negative	83	15.5
PR		
Positive	377	70.6
Negative	157	29.4
HER2		
Negative	463	86.7
Positive	71	13.3

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In 137 cases with matched WS, CNB and TMA samples, the median percentages of Ki67 expression were significantly different with 17% (WS), 13% (CNB) and 6% (TMA), Wilcoxon signed rank test, $P<0.001$ for each analysis. Still, The Ki67 values obtained on WS were significantly correlated with both CNB (Spearman's ρ 0.56, $P = 0.001$) and TMA (Spearman's ρ 0.81, $P<0.001$). Further, Ki67 counts on CNB were significantly correlated with TMA (Spearman's ρ 0.49, $P<0.001$) (See Figure S2).

Using the 14% Ki67 threshold on the entire series (n = 534), based on WS specimens, 61% of tumors were classified as having high proliferation. In the CNB series (n = 154), 48% showed high Ki67 expression, as compared to 25% of the cases when using TMA specimens (n = 459), as illustrated in Figure S3.

Table 2. Ki67 counts according to tissue categories.

Complete series	*N*	Median	Range	Mean
Ki67-WS	534	18	1–94	24
Ki67-CNB	154	13	0.4–89	17
Ki67-TMA	459	7	0.2–83	12
Paired cases				
Ki67-WS	137	17	0.8–90	21
Ki67-CNB	137	13	0.4–89	18
Ki67-TMA	137	6	0.2–71	10

WS, whole sections; CNB, core needle biopsies; TMA, tissue micro arrays.

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Associations between Ki67 and clinico-pathological features

High Ki67 expression by all 3 classes of specimens was significantly associated with high histologic grade and hormone receptor negativity (Table 3). Furthermore, elevated Ki67 expression on WS and TMA was associated with large tumor size, lymph node metastasis, and HER2 positivity. No associations were found between high Ki67 and age or tumor type. For cold-spot counts on full sections, the associations between Ki67 and tumor size and HER2 status were not significant (See Table S2 in File S1).

Tumor cell proliferation in different molecular subgroups

Based on WS (complete series), the median expression of Ki67 in the Luminal subclass (including Luminal-HER2+) was 17% (Luminal A subclass 7%, Luminal B subclass 25%). In the HER2+ subclass (HR-, HER2+), the median expression of Ki67 was 35% whereas the triple negative subgroup demonstrated the highest Ki67 median of 62% (Kruskal-Wallis test, $P<0.001$, Figure 1). Assessment on CNB and TMA revealed the same pattern with highest proliferation shown for the triple negative group followed by the HER2+ subgroup. The lowest proliferation was observed in the Luminal subgroup.

We then applied the 14% cut-off point to WS, CNB and TMA. Among hormone receptor positive cases, excluding Luminal B/HER2+, the following figures for the frequency of cases having high proliferation were 52% (WS), 41% (CNB) and 14% (TMA), as illustrated in Figure 2. In the study by Cheang and colleagues, the Luminal B category comprised 36% of the HR+/HER2 negative cases [3]. By applying this frequency to our series, the following cut-off points for Ki67 would result in a similar size of the Luminal B (HER2 negative) subgroup: 20% (WS), 15% (CNB) and 8% (TMA).

We further applied the 14% cut-off point (St Gallen 2011) in the Luminal subgroup (excluding Luminal B/HER2+) and found classification agreement in 65% of the cases when comparing WS and CNB (n = 125, paired cases) as illustrated in Table 4 (kappa-value 0.29, $P<0.001$). Of note, 18 cases (14%) initially categorized as luminal B on CNB were downgraded on WS, whereas 26 cases (21%) categorized as luminal A on CNB were upgraded. We then compared the results between WS and TMA (n = 350, paired cases) and found concordance in 59% of cases (kappa-value 0.23, $P<0.001$). 143 cases (41%) categorized as luminal A on TMA were upgraded on WS, whereas only 1 case showed the opposite pattern.

Tumor cell proliferation and patient outcome

Univariate analyses displayed significant associations between Ki67-WS and patient survival using a cut-off at the median (Figure 3, see also table S3 in File S1). Further, significant influence of Ki67-WS counts was shown for all cut-points examined (10^{th}–90^{th} percentiles, Figure S4). Multivariate survival analyses, after adjustment for basic prognostic indicators including age, tumor size, histologic grade and lymph node status, showed that Ki67, tumor size and nodal stage were independent prognostic factors for breast cancer specific survival (Table 5).

Proliferation by Ki67-CS showed similar but weaker effects on BCSS in univariate analysis (Table S4 in File S1). We further performed survival analyses after excluding the 33 cases with locally advanced disease; the results were similar (data not shown).

Table 3. Associations between Ki67 expression and histopathological features.

	Ki67-WS (n = 534)		Ki67-CNB (n = 154)		Ki67-TMA (n = 459)	
Variables	**Median (%)**	***P*-value[a]**	**Median (%)**	***P*-value[a]**	**Median (%)**	***P*-value[a]**
Tumor diameter		<0.001		0.089		<0.001
≤2 cm	16.8		11.2		6.2	
>2 cm	28.0		16.8		11.2	
Histologic grade		<0.001		<0.001		<0.001
1	12.0		9.2		4.4	
2	19.5		14.1		7.0	
3	43.7		40.0		23.4	
Nodal status[b]		0.002		NS		0.002
Negative	16.8		11.9		6.2	
Positive	23.3		14.4		8.7	
ER		<0.001		<0.001		<0.001
Positive	16.6		11.0		6.0	
Negative	42.8		40.0		19.0	
PR		<0.001		0.005		<0.001
Positive	16.8		11.1		6.0	
Negative	26.2		19.3		12.0	
HER2		<0.001		0.088		<0.001
Negative	16.8		11.7		6.0	
Positive	32.4		18.4		15.2	

NS, not significant.

[a]Mann-Whitney or Kruskal-Wallis tests.

[b]5 cases (WS), 1 case (CNB) and 4 cases (TMA) with unknown lymph node status were excluded.

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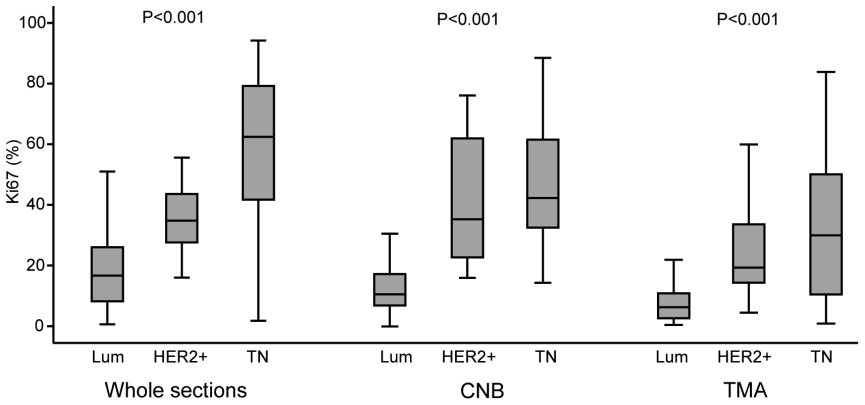

Figure 1. Box plots of tumor cell proliferation by Ki67 expression according to breast cancer molecular subgroups in different specimen categories. Horizontal lines inside the boxes represent the median value; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.
doi:10.1371/journal.pone.0112121.g001

Univariate survival analysis of Ki67 in CNB sections showed all examined cut-points above the 40th percentile to be prognostic. Multivariate analyses were performed, adjusting for age, tumor size, histologic grade and nodal status. In the final model, Ki67-CNB and nodal status retained prognostic significance.

For Ki67 in TMA sections, univariate survival analyses demonstrated all examined cut-points above the 10th percentile to be prognostic. In multivariate analysis, including the variables age, tumor size, histologic grade and nodal status, Ki67-TMA showed independent prognostic impact in addition to tumor size and nodal status.

Finally, Ki67 on WS, CNB and TMA (paired cases, n = 137) were included in a multivariate analysis. In this model, only Ki67-WS demonstrated independent prognostic significance. (HR 1.06; (1.02–1.10), $P = 0.006$, Ki67 included as a continuous variable).

Survival by Ki67 in different molecular subgroups

We also performed subgroup analyses on the complete series stratified by ER and HER2 status and based on Ki67-WS. In the luminal category (including Luminal-HER2+; n = 462), univariate survival analysis revealed a significant association between Ki67 and BCSS (HR 1.03, 95% CI = 1.02 to 1.04; $P<0.001$), also when using two categories with a defined cut-point of 14%, (HR 2.9, 95% CI = 1.5 to 5.5; $P = 0.001$; Figure 4). In multivariate analysis including Ki67 and the basic prognostic variables tumor size, histologic grade and lymph node status, Ki67 retained prognostic significance (together with nodal status and tumor size). Furthermore, by excluding the HER2+ cases and focusing on HR+ breast cancers (n = 412), similar results were obtained (data not shown). In contrast, univariate survival analysis revealed no significant association between Ki67 and outcome within the HER2+/HR- subgroup. By including the HER2+/HR+ cases, the analysis showed prognostic impact of Ki67 (HR 1.027, 95% CI 1.002–1.053; $P = 0.033$). Finally, univariate analysis demonstrated no association between Ki67 and survival in triple negative breast cancer.

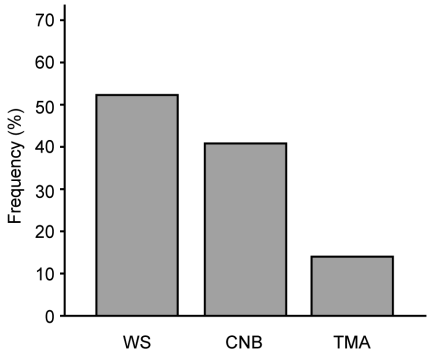

Figure 2. Frequency of cases in the Luminal/HER2- subgroup showing high proliferation when applying a Ki67 cutoff-point of 14% to different specimen categories. WS ($n = 415$), CNB ($n = 125$), TMA ($n = 350$).
doi:10.1371/journal.pone.0112121.g002

Discussion

It is well documented that tumor cell proliferation by Ki67 expression is strongly associated with breast cancer prognosis [15]. After the suggestion of Ki67 as a predictive marker for adjuvant chemotherapy, observer variation and methodological issues have been increasingly discussed [8,16]. Some recommendations for Ki67 assessment were presented in 2011, and the lack of systematic comparisons of Ki67 expression levels between tissue microarrays (TMA) and whole sections (WS) was noted [8]. As an example, the Ki67 cut-off point of 14% recommended for treatment decisions by the St Gallen 2011 guidelines was based on data from a series of tissue microarrays combined with gene expression analysis [3,7]. However, the clinical translation of these findings has not been well documented.

Here, we found a significant difference in proliferation level related to specimen type, with median Ki67 staining values of 18%, 13% and 7% for WS, CNB and TMA samples. These differences might in part be explained by intra-tumor heterogeneity, which is seen both at the morphological and molecular levels [17–25].

Studies based on CNB and TMA specimens are challenging as the amount of tissue examined is reduced compared with WS

Table 4. Ki67 concordance between WS, CNB and TMA in the luminal subgroup.

	WS		Agreement	Kappa	*P*-value
	LumA	**LumB**			
	N (%)	*N* (%)			
CNB					
LumA	48 (38)	26 (21)	65%	0.29	0.001
LumB	18 (14)	33 (26)			
TMA					
LumA	158 (45)	143 (41)	59%	0.23	<0.001
LumB	1 (0.3)	48 (14)			

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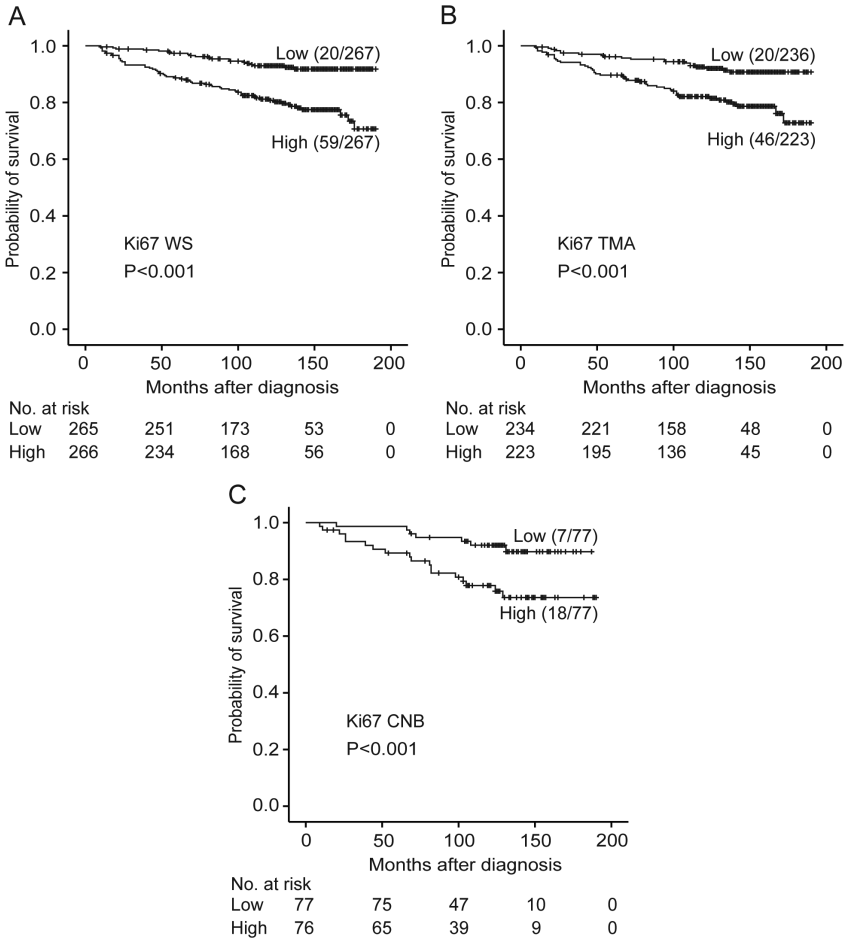

Figure 3. Breast cancer specific survival according to Ki67 expression. Survival curves (Kaplan-Meier) are shown for Ki67 expression on WS (A); TMA (B) and CNB (C). Cut-off points at the median were applied for all specimen categories. The number of events and total number of patients in each group are shown beside the description of each curve. Numbers at risk are presented below each curve.
doi:10.1371/journal.pone.0112121.g003

Table 5. Multivariate survival analysis (Cox' proportional hazards method) using different specimen categories.

Variables	*N*	HR	95% CI	*P*-value[a]
A. Whole sections (final model; n = 529)				
Tumor diameter				
≤2 cm	404			
>2 cm	125	2.3	1.4–3.7	0.001
Nodal status				
Negative	387			
Positive	142	3.3	2.0–5.3	<0.001
Ki67 count[b]				
Low, ≤18.3	265			
High,>18.3	264	2.4	1.4–4.1	0.001
B. Core needle biopsies (final model; n = 153)				
Nodal status				
Negative	112			
Positive	41	4.2	1.9–9.5	0.001
Ki67 count[b]				
Low, ≤12.8	77			
High,>12.8	76	2.8	1.1–6.7	0.024
C. TMAs (final model; n = 455)				
Tumor diameter				
≤2 cm	346			
>2 cm	109	2.0	1.2–3.5	0.009
Nodal status				
Negative	335			
Positive	120	3.5	2.0–6.0	<0.001
Ki67 count[b]				
Low, ≤7.0	236			
High,>7.0	219	2.2	1.3–3.7	0.005

HR, Hazard ratio; CI, confidence interval.
Final models after initial inclusion of age, tumor diameter, histologic grade, nodal status and Ki67.
5 cases (WS), 1 case (CNB) and 4 cases (TMA) were excluded due to missing information on lymph node status.
[a]Likelihood ratio.
[b]Cut-off point at the median.
doi:10.1371/journal.pone.0112121.t005

samples. Heterogeneity might especially affect studies using the hot-spot approach, since these areas are often small and might be missed on CNB and TMA sections. Still, prior studies of proliferation markers in breast tumors have shown good statistical correlation between TMA and full sections for Ki67 [26–28], and expected associations between Ki67 and clinico-pathologic and molecular features have been reproduced [29]. Also, the use of pre-surgical CNB has been validated for various biomarkers with significant correlation between methods [30]. Good to excellent agreement has been demonstrated for hormone receptors and HER2 status, whereas histologic grade has shown only modest concordance, mainly due to underestimation of mitotic count on CNB specimens [31–35]. Some studies on Ki67 have shown good concordance between CNB and WS tissues [36–39], whereas others have found only fair to moderate agreement [25,40,41]. Notably, even in studies demonstrating a good statistical correlation, there could be marked differences in scores on an individual basis [36]. In our study, a significant proportion of the cases are classified differently given a predetermined threshold and with potential consequences for patient treatment. Importantly, we found that 21% of Luminal A cases on CNB were upgraded to Luminal B on WS specimens, similar to other findings [42].

The subdivision of ER-positive tumors into Luminal A and Luminal B is based on the expression levels of proliferation-related genes among HER2 negative cases. Studies have revealed that proliferation levels are continuous, and sub-classification based on certain cut-points is therefore likely to be arbitrary [43,44]. Although the 14% cut-off point to separate Luminal A from Luminal B tumors was based on Ki67 expression in TMA samples and established against gene expression profiles, this cut-point showed only a modest sensitivity of 77% and a specificity of 78% in that study [3]. In spite of this, the 14% threshold has been used in research settings as well as in the St Gallen 2011 statement for clinical implementation. Interestingly, the size of the Luminal B subgroup has varied from 8% [45] to 66% [46] in published series. In our study, the 14% cut-off point results in an overestimation of the Luminal B subgroup based on WS specimens, whereas the TMA approach appears to underestimate same group. In the study by Cheang and colleagues, the Luminal B category represents 36% of the HR+/HER2 negative cases [3]. We applied

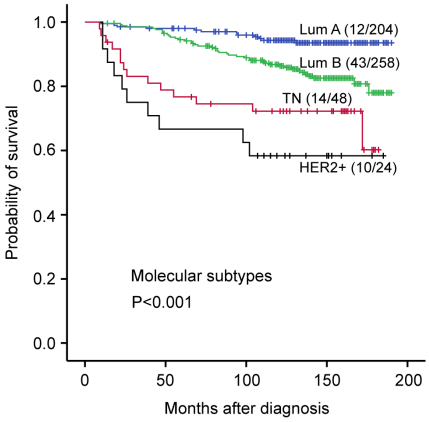

Figure 4. Breast cancer prognosis by molecular subtype. Survival curves (Kaplan-Meier) for breast carcinomas showing an association between molecular subtype and breast cancer specific survival. The Luminal B subgroup includes Luminal/HER2+ cases. For each category, the number of events is given followed by the number of patients.
doi:10.1371/journal.pone.0112121.g004

this frequency to our series, and found that the following cut-off points for Ki67 would result in a similar size of the Luminal B-HER2 negative subgroup: 20% (WS), 15% (CNB) and 8% (TMA). Thus, the importance of tissue-specific cut-off points must be considered, for instance when using core needle biopsies and when translating data from TMA-based research to a potential clinical use.

For prognostic purposes, there is no consensus regarding counting area or how many tumor cells should be scored [8]. Although a previous study showed that both the peripheral, central and average Ki67 rates were associated with overall survival [17], two recent studies have revealed that Ki67 has the strongest prognostic impact when counted in hot-spot areas [47,48]. Notably, using whole sections and hot-spot readings corresponds to what is done for mitotic activity as part of histologic grading. Since prognostic studies have indicated that disease progression is best predicted by Ki67 counted in hot-spot areas, a similar approach should probably be considered for predictive purposes. This must be assessed in carefully designed studies.

Regarding methodology, our study has some limitations, since pre-analytical and analytical variables can not be completely standardized in such retrospective studies [8]. Delayed formalin fixation may result in decreased expression of certain biomarkers [49], although a study of Ki67 found no decrease in expression after 180 minutes delay [50]. Of note, it has been shown that prolonged formalin fixation may cause more extensive masking of antigens, and that not all of this loss can be recovered by antigen retrieval [51,52]. Further, the TMA technique carries some drawbacks, such as sampling errors and loss of information due to missing tissue cores. Notably, false negative results have been reported for biomarkers studied on TMA sections [53], but it is not known whether this is applicable to Ki67. Regarding ER and PR expression, we used a threshold of 10% for molecular sub-classification according to national guidelines at the time, as compared to the 1% threshold recommended by the present St Gallen guidelines.

In conclusion, tumor cell proliferation as estimated by Ki67 is significantly dependent on specimen category, and our results indicate that specimen-specific cut-off values should be established and validated for clinical use. Furthermore, Ki67 is prognostic over a wide range of cut-off points. For practical purposes, whole sections should be preferred when available, in parallel to the assessment of mitotic count as an integral part of histologic grading. When using hot-spot readings on whole sections, a cut-off point of 20% as a minimum for Ki67 seems to be appropriate at least to predict disease progression. This is also in line with the recent St Gallen 2013 statement [10]. The value of Ki67 as a predictive marker needs to be further studied and validated.

Supporting Information

File S1 Supplementary tables. Table S1. Clinico-pathological features and associations with molecular subtypes of breast cancer. Table S2. Ki67 assessed in hot-spots and cold-spots on WS specimens and associations with histopathological variables. Table S3. Univariate survival analysis according to histopathological variables (Kaplan-Meier method). Table S4. Unadjusted Cox proportional hazards analysis used to estimate the prognostic value of Ki67 expression according to specimen category.
(PDF)

Figure S1 A. Ki67 expression scores across specimen category. The median and inter-quartile range of Ki67 is shown according to specimen type. B. Bland-Altman plot is shown for Ki67 expression on whole sections and core needle biopsies. Ki67 difference (WS-CNB) versus average of WS and CNB with 95% limits of agreement (LOA). The mean difference was 2.8% (95% LOA between -22 and 27; $P = 0.005$). **C.** Bland-Altman plot is shown for Ki67 expression on whole sections and TMA. Ki67 difference (WS-TMA) versus average of WS and TMA with 95% LOA. The mean difference was 10% (95% LOA between -10 and 36; $P<0.001$).
(TIF)

Figure S2 Scatter plots with line of equality illustrating the relationships between counts based on WS, CNB, and TMA specimens.
(TIF)

Figure S3 Frequency of cases showing high proliferation when applying a Ki67 cut-off point of 14% to different specimen categories, WS ($n = 534$), CNB ($n = 154$), TMA ($n = 459$).
(TIF)

Figure S4 Unadjusted Cox proportional hazards analysis used to estimate the prognostic value of possible Ki67 cut-off points. The hazard ratio (solid lines) including 95% CI (dashed lines) is shown in dependence of Ki67 cut-off points based on percentiles, with separate plots for WS (A), TMA (B), and CNB (C) specimens.
(TIF)

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Author Contributions

Conceived and designed the experiments: LAA IMS. Performed the experiments: GK IMS SA. Analyzed the data: GK IMS SA LAA. Contributed reagents/materials/analysis tools: JA JE KC. Wrote the paper: GK IMS SA JA JE KC LAA.

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Figure S1

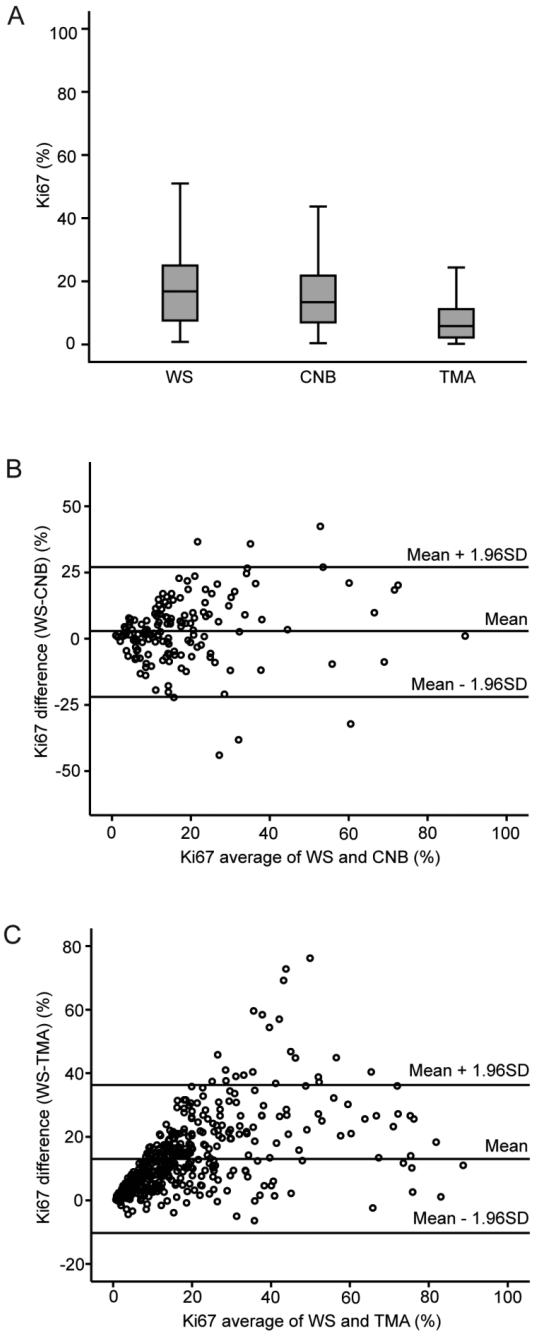
A
Ki67 (%)
100
80
60
40
20
0
WS
CNB
TMA
B
Ki67 difference (WS-CNB) (%)
50
25
0
-25
-50
Mean + 1.96SD
Mean
Mean - 1.96SD
0
20
40
60
80
100
Ki67 average of WS and CNB (%)
C
Ki67 difference (WS-TMA) (%)
80
60
40
20
0
-20
Mean + 1.96SD
Mean
Mean - 1.96SD
0
20
40
60
80
100
Ki67 average of WS and TMA (%)

Figure S2

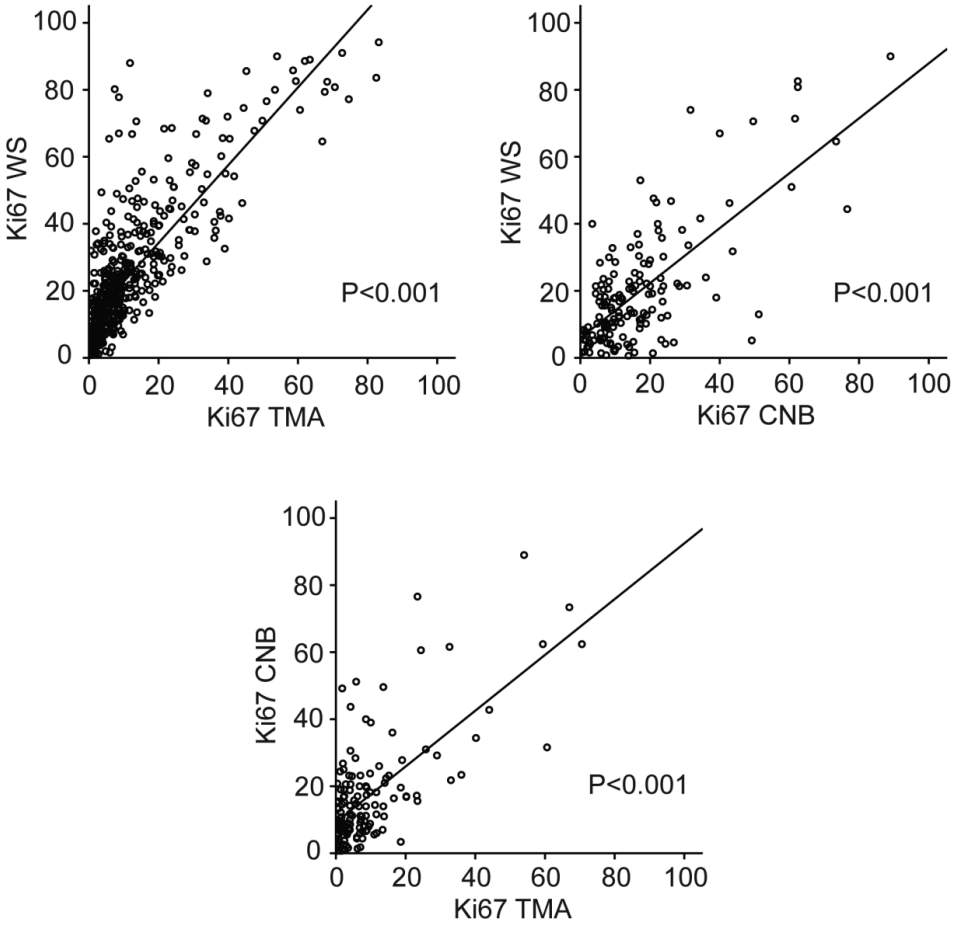
Ki67 WS
Ki67 TMA
P<0.001
Ki67 WS
Ki67 CNB
P<0.001
Ki67 CNB
Ki67 TMA
P<0.001

Figure S3

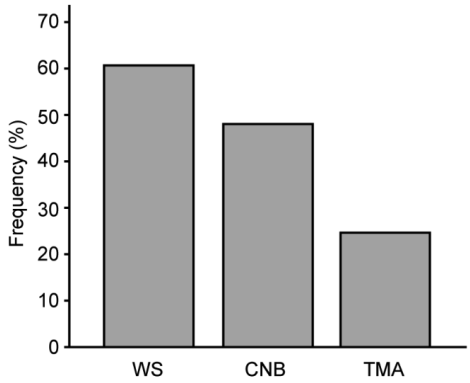

Figure S4

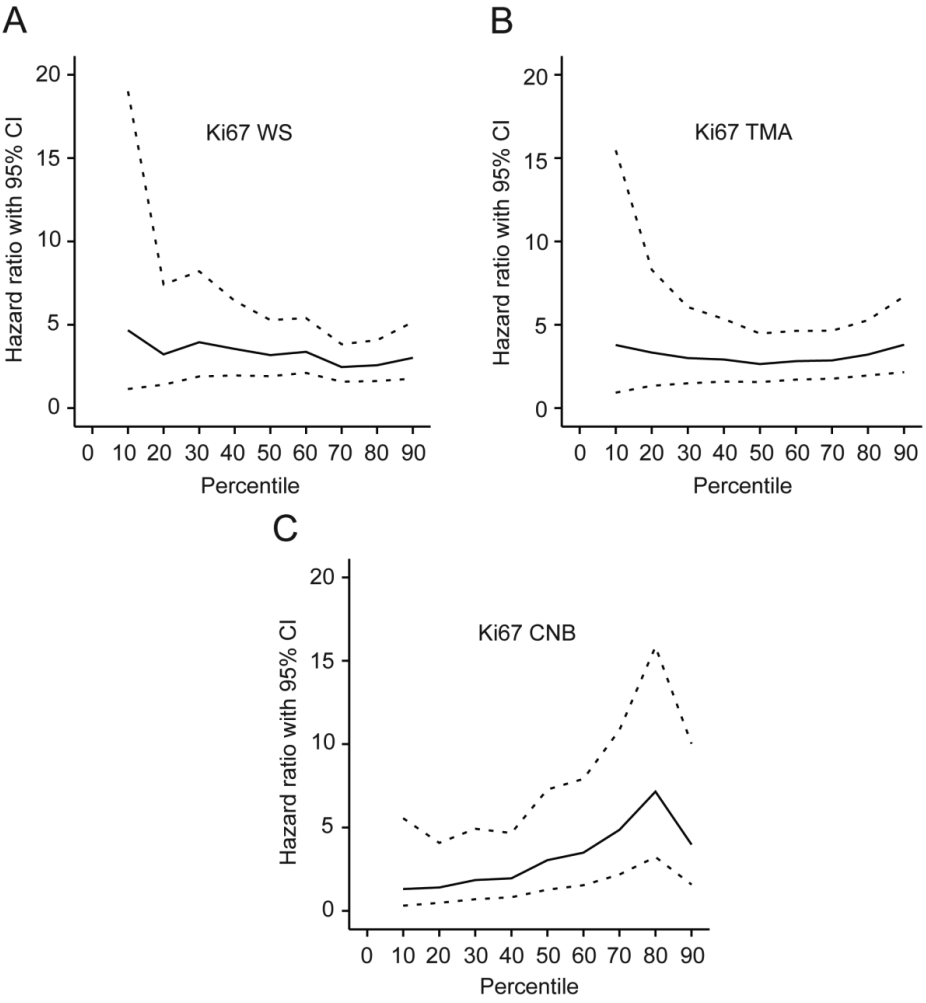
A
Ki67 WS
Hazard ratio with 95% CI
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90
Percentile
B
Ki67 TMA
Hazard ratio with 95% CI
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90
Percentile
C
Ki67 CNB
Hazard ratio with 95% CI
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90
Percentile

Table S1 Clinico-pathological features and associations with molecular subtypes of breast cancer

Characteristics	Luminal A[a]		Luminal B[b]		HER2 positive		Triple negative	
	No	(%)	No	(%)	No	(%)	No	(%)
Tumor diameter								
≤2 cm	172	84.3	192	74.4	12	50.0	29	60.4
>2 cm	32	15.7	66	25.6	12	50.0	19	39.6
Histologic grade								
1	129	63.2	85	32.9	3	12.5	1	2.1
2	70	34.3	134	51.9	7	29.2	15	31.3
3	5	2.5	39	15.1	14	58.3	32	66.7
Nodal status[c]								
Negative	163	80.3	178	69.5	10	45.5	36	75.0
Positive	40	19.7	78	30.5	12	54.5	12	25.0
Histologic type								
Ductal	159	77.9	227	88.0	20	83.3	41	85.4
Lobular	29	14.2	24	9.3	2	8.3		
Tubular	7	3.4	1	0.4				
Mucinous	9	4.4	6	2.3	1	4.2		
Medullary					1	4.2	3	6.3
Unclassified							4	8.3

[a]Cut-off point 14% used to separate Luminal A from Luminal B tumors
[b]Luminal B includes luminal/HER2+
[c]5 cases with missing information on lymph node status.

Table S2 Ki67 assessed in hot-spots and cold-spots on WS specimens and associations with histopathological variables

	Ki67 WS hot -spot		Ki67 WS cold -spot	
Variables	**Median (%)**	***P*-value[a]**	**Median (%)**	***P*-value[a]**
Tumor diameter		<0.001		0.146
≤ 2 cm	16.8		12.6	
> 2 cm	28.0		16.0	
Histologic grade		<0.001		<0.001
1	12.0		9.5	
2	19.5		13.3	
3	43.7		26.1	
Nodal status[b]		0.002		0.025
Negative	16.8		12.4	
Positive	23.3		16.2	
ER		<0.001		<0.001
Positive	16.6		11.6	
Negative	42.8		25.6	
PR		<0.001		0.005
Positive	16.8		11.6	
Negative	26.2		17.8	
HER2		<0.001		0.088
Negative	16.8		12.0	
Positive	32.4		24.2	

[a]Mann-Whitney or Kruskal-Wallis test

[b]5 cases with unknown lymph node status were excluded.

Table S3 Univariate survival analysis according to histopathological variables (Kaplan-Meier method)

Variables	*N*	Events	Estimated survival rates (%)		*P*-value[a]
			5 years	10 years	
Tumor diameter					
≤ 2 cm	405	37	96.0	91.8	
> 2 cm	129	42	83.6	72.1	< 0.001
Histologic grade					
1	218	19	96.3	92.5	
2	226	37	94.6	86.4	
3	90	23	81.1	75.4	< 0.001
Nodal status[b]					
Negative	387	31	96.3	93.0	
Positive	142	45	84.5	71.7	< 0.001
ER					
Positive	451	51	96.6	90.7	
Negative	83	28	73.2	66.9	< 0.001
PR					
Positive	377	41	96.8	91.2	
Negative	157	38	83.9	76.9	< 0.001
HER2					
Negative	463	62	94.6	88.6	
Positive	71	17	83.1	77.2	0.008
Ki67[c]					
Low ≤ 18.3	267	20	97.3	93.0	
High > 18.3	267	59	88.7	81.2	<0.001

[a] Log rank test

[b] 5 cases with unknown lymph node status were excluded.

[c] Ki67 assessed on WS; cut-off point at the median.

Table S4 Unadjusted Cox proportional hazards analysis used to estimate the prognostic value of Ki67 expression according to specimen category.

Variable	Hazard ratio	95% CI	*P*-value*
Ki67-HS whole section	3.2	1.9-5.3	<0.001
Ki67-CS whole section	1.8	1.2-2.9	0.009
Ki67 estimated average	2.8	1.7-4.5	<0.001
Ki67 CNB	3.0	1.3-7.3	0.013
Ki67 TMA	2.7	1.6-4.5	<0.001

Ki67-HS: Ki67 expression assessed in hot-spots; Ki67-CS: Ki67 expression assessed in cold-spots; Est. average: estimated average of Ki67-HS and Ki67-CS, CNB: core needle biopsy; TMA: tissue microarray.
*Likelihood ratio
Ki67 categorized at the median.