From the Department of Oncology and Pathology
Karolinska Institutet, Stockholm, Sweden

IMPROVING BIOMARKER ASSESSMENT IN BREAST PATHOLOGY

Stephanie Robertson

Karolinska Institutet

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Improving biomarker assessment in breast pathology

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Stephanie Robertson

Principal Supervisor:
Associate Professor Johan Hartman
Karolinska Institutet
Department of Oncology and Pathology

Co-supervisor:
Associate Professor Jana de Boniface
Karolinska Institutet
Department of Molecular Medicine and Surgery

Opponent:
Professor Mårten Fernö
Lund University
Department of Clinical Sciences Lund

Examination Board:
Associate Professor Anikó Kovács
University of Gothenburg
Sahlgrenska Cancer Center

Associate Professor Niklas Loman
Lund University
Department of Clinical Sciences Lund

Associate Professor Hanjing Xie
Karolinska Institutet
Department of Oncology and Pathology

The summit is not always the goal

ABSTRACT

The accuracy of prognostic and therapy-predictive biomarker assessment in breast tumours is crucial for management and therapy decision in patients with breast cancer. In this thesis, biomarkers used in clinical practice with emphasise on Ki67 and HER2 were studied using several methods including immunocytochemistry, immunohistochemistry, *in situ* hybridisation, gene expression assays and digital image analysis, with the overall aim to improve routine biomarker evaluation and clarify the prognostic potential in early breast cancer.

In paper I, we reported discordances in biomarker status from aspiration cytology and paired surgical specimens from breast tumours. The limited prognostic potential of immunocytochemistry-based Ki67 scoring demonstrated that immunohistochemistry on resected specimens is the superior method for Ki67 evaluation. In addition, neither of the methods were sufficient to predict molecular subtype. Following this in paper II, biomarker agreement between core needle biopsies and subsequent specimens was investigated, both in the adjuvant and neoadjuvant setting. Discordances in Ki67 and HER2 status between core biopsies and paired specimens suggested that these biomarkers should be re-tested on all surgical breast cancer specimens. In paper III, digital image analysis using a virtual double staining software was used to compare methods for assessment of proliferative activity, including mitotic counts, Ki67 and the alternative marker PHH3, in different tumour regions (hot spot, invasive edge and whole section). Digital image analysis using virtual double staining of hot spot Ki67 outperformed the alternative markers of proliferation, especially in discriminating luminal B from luminal A tumours. Replacing mitosis in histological grade with hot spot-scored Ki67 added significant prognostic information. Following these findings, the optimal definition of a hot spot for Ki67 scoring using virtual double staining in relation to molecular subtype and outcome was investigated in paper IV. With the growing evidence of global scoring as a superior method to improve reproducibility of Ki67 scoring, a different digital image analysis software (QuPath) was also used for comparison. Altogether, we found that automated global scoring of Ki67 using QuPath had independent prognostic potential compared to even the best virtual double staining hot spot algorithm, and is also a practical method for routine Ki67 scoring in breast pathology. In paper V, the clinical value of HER2 status was investigated in a unique trastuzumab-treated HER2-positive cohort, on the protein, mRNA and DNA levels. The results demonstrated that low levels of ERBB2 mRNA but neither HER2 copy numbers, HER2 ratio nor ER status, was associated with risk of recurrence among anti-HER2 treated breast cancer patients.

In conclusion, we have identified important clinical aspects of Ki67 and HER2 evaluation and provided methods to improve the prognostic potential of Ki67 using digital image analysis. In addition to protein expression of routine biomarkers, mRNA levels by targeted gene expression assays may add further prognostic value in early breast cancer.

LIST OF SCIENTIFIC PAPERS

I. **Stephanie Robertson**, Gustav Stålhammar, Eva Darai-Ramqvist, Mattias Rantalainen, Nicholas P Tobin, Jonas Bergh and Johan Hartman. Prognostic value of Ki67 analysed by cytology or histology in primary breast cancer. Journal of Clinical Pathology. 2018 Sept;71:787-794.

II. **Stephanie Robertson**, Caroline Rönnlund, Jana de Boniface and Johan Hartman. Re-testing of predictive biomarkers on surgical breast cancer specimens is clinically relevant. Breast Cancer Research and Treatment. 2019 Apr;174:795-805.

III. Gustav Stålhammar, **Stephanie Robertson**, Lena Wedlund, Michael Lippert, Mattias Rantalainen, Jonas Bergh and Johan Hartman. Digital image analysis of Ki67 in hot spots is superior to both manual Ki67 and mitotic counts in breast cancer. Histopathology. 2018 May;72:974-989.

IV. **Stephanie Robertson**, Balazs Acs, Michael Lippert and Johan Hartman. Prognostic potential of automated Ki67 evaluation in breast cancer: different hot spot definitions versus true global score. Breast Cancer Research and Treatment. 2020 Aug;183:161-175.

V. **Stephanie Robertson**[*], Caroline Rönnlund[*], Xinsong Chen, Josefin Bååth, Irma Fredriksson, Theodoros Foukakis and Johan Hartman. Detailed investigation and re-assessment of HER2 status in breast cancer patients treated with HER2-targeted therapy. [*]Co-authors with equal contribution.
Manuscript

LIST OF RELATED PUBLICATIONS

I. **Stephanie Robertson**, Hossein Azizpour, Kevin Smith and Johan Hartman. Digital image analysis in breast pathology-from image processing techniques to artificial intelligence. Translational Research. 2018 Apr;194:19-35.

II. Nobuyuki Tanaka, Shigeaki Kanatani, Dagmara Kaczynska, Keishiro Fukumoto, Lauri Louhivuori, Tomohiro Mizutani, Oded Kopper, Pauliina Kronqvist, **Stephanie Robertson**, Claes Lindh, Lorand Kis, Robin Pronk, Naoya Niwa, Kazuhiro Matsumoto, Mototsugu Oya, Ayako Miyakawa, Anna Falk, Johan Hartman, Cecilia Sahlgren, Hans Clevers, and Per Uhlén. Three-dimensional single-cell imaging for the analysis of RNA and protein expression in intact tumour biopsies. Nature Biomedical Engineering. 2020 Jun 29. doi: 10.1038/s41551-020-0576-z

III. Xinsong Chen, Emmanouil G. Sifakis, **Stephanie Robertson**, Shi Yong Neo, Seong-Hwan Jun, John Lövrot, Viktor Jovic, Jonas Bergh, Theodoros Foukakis, Jens Lagergren, Andreas Lundqvist, Ran Ma and Johan Hartman. Drug response profiling in a patient-derived breast cancer model retaining tumor-stromal interactions.
Manuscript submitted

IV. Yinxi Wang, Balazs Acs, **Stephanie Robertson**, Bojing Lui, Leslie Solorzano, Carolina Wählby, Johan Hartman* and Mattias Rantalainen*. Improved breast cancer histological grading using deep learning. *Co-authors with equal contribution.
Manuscript submitted

CONTENTS

LIST OF ABBREVIATIONS

AI	Artificial intelligence
AIs	Aromatase inhibitors
Akt	Protein kinase B
ASCO	American Association of Clinical Oncology
BCSS	Breast cancer-specific survival
CAP	College of American Pathologists
CDK	Cyclin-dependent kinase
CI	Confidence interval
CNB	Core needle biopsy
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DCIS	Ductal carcinoma *in situ*
DIA	Digital image analysis
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptor
ERBB2	Erb-B2 receptor tyrosine kinase 2
ERK	Extracellular signal-regulated kinase
FFPE	Formalin-fixed paraffin-embedded
FNAC	Fine needle aspiration cytology
HE	Haematoxylin and eosin
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IKWG	International Ki67 in Breast Cancer Working Group
ISH	*In situ* hybridisation
IVD	In vitro diagnostic
LCIS	Lobular carcinoma *in situ*
MAPK	Mitogen-activated protein kinase
NAC	Neoadjuvant chemotherapy
NGS	Next generation sequencing
NHG	Nottingham histological grade
NST	No special type
OS	Overall survival
PAM50	Prediction analysis of microarray 50
pCR	Pathological complete response
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1

PHH3	Phosphohistone H3
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PR	Progesterone receptor
RFS	Recurrence-free survival
RT-PCR	Reverse transcriptase polymerase chain reaction
TCGA	The Cancer Genome Atlas
TDLU	Terminal duct lobular unit
TILs	Tumour infiltrating lymphocytes
TNM	Tumour node metastasis
VEGF	Vascular endothelial growth factor
VIS	Visiopharm Integrator System
WHO	World Health Organisation

1 INTRODUCTION

1.1 DEVELOPMENT AND ANATOMY OF THE MAMMARY GLAND

1.1.1 The development of the mammary gland

The mammary glands, or breasts, are one of the characteristic features of mammals and are found in both sexes. During the embryologic development several glands develop along the mammary ridges, which are bilateral ectodermal thickenings, formed along the developing axilla extending to the inguinal region. In contrast to other mammals, only one pair of cell groups develop further in humans and form the rudimentary breast tissue on each side. At birth the mammary glands are rudimentary in both sexes but are to some extent responsive to maternal hormones. The ducts of the mammary tissue continue to elongate and branch. In females, this development is accelerated during puberty under the influence of hormones: Ovarian oestrogen and progesterone promotes the proliferation, differentiation and remodelling of the ducts and connective tissue to form the adult mammary glands.

The adult mammary glands are influenced by hormone levels during the menstrual cycle. Proliferation of the ductal epithelium occurs during the second half of the menstrual cycle, and sequential apoptosis follows with decreased levels of estrogen and progesterone at onset of menstruation. During pregnancy, the number of terminal ducts increases significantly and at this stage the mammary tissue mainly consists of lobular epithelium. During lactation, the terminal duct lobular units (TDLU) are enlarged, the epithelium becomes vacuolised, and the lumina of the ducts are distended by secretory material. The initiation of milk secretion is induced by prolactin and the ejection of milk is stimulated by oxytocin release from the adenohypophysis and neurohypophysis, respectively. Influenced by the altered hormonal environment at menopause, the TDLUs atrophy, but the intermediate and larger duct systems remain. Cystic dilation of residual ducts is frequently observed along with a decrease in interlobular fibrous connective tissue leading to an increase in the percentage of adipose tissue content.

1.1.2 The anatomy of the mammary gland

The breast is predominantly composed of adipose and glandular tissue along with connective tissue. It is covered by skin, rests on the pectoralis muscle and is posteriorly separated by a fascia. The mammary gland contains around 15-20 lobes with each lobe comprising of 20-40 TDLUs[1, 2]. The TDLU is the most important functional unit of the breast and consist of extralobular- and intralobular terminal ducts and acini (Figure 1). The ducts are composed of a double layer of epithelium: the innermost luminal epithelial cell layer, which is lined by a myoepithelial cell layer resting on a basal membrane[1]. The ductal system is surrounded by the stromal compartment, which comprises endothelial cells, fibroblasts, macrophages and inflammatory cells. The TDLUs are closely surrounded by a specialised, myxoid-like hormone-sensitive connective tissue with absence of elastic fibres. The foremost described development of the mammary gland is dependent on the interaction between the specialised

epithelium and its stroma. Surrounding larger ducts, continuous and well-developed elastic tissue is present with a smaller content of specialised stroma. The TDLUs are connected with larger ducts through subsegmental and segmental ducts that in turn lead to collecting ducts, which empty into the nipple[3]. Pathological changes in the TDLUs are believed to give rise to the majority of breast carcinomas and ductal carcinoma *in situ* (DCIS). The precursor lesions to invasive breast cancer are DCIS, which is a premalignant epithelial proliferation within the ducts, and lobular neoplasia confined to the lobules[4].

The mammary gland is well vascularised and supplied by the thoracic branches of the axillary artery, the internal thoracic artery and the anterior intercostal arteries[2]. The breast is supplied by sensory innervation from the second to sixth intercostal nerves and the supraclavicular nerve. The lymphatic system of the breast mainly drains to lower pectoralis axillary lymph nodes and further up to the supraclavicular nodes (75%)[2]. The axillary lymph nodes are divided into six group based on their relation to the pectoralis minor muscle, and recognised by surgeons. Lymphatic drainage also occurs to the parasternal lymph nodes, especially from the medial part of the breast. Furthermore, superficial lymphatic drainage may occur to the contralateral breast or the abdominal wall. In the case of direct metastasis to the supraclavicular lymph nodes, this indicates an advanced stage of disease. Lymphatic drainage of the breast tissue is the main route for breast cancer cells to metastasise.

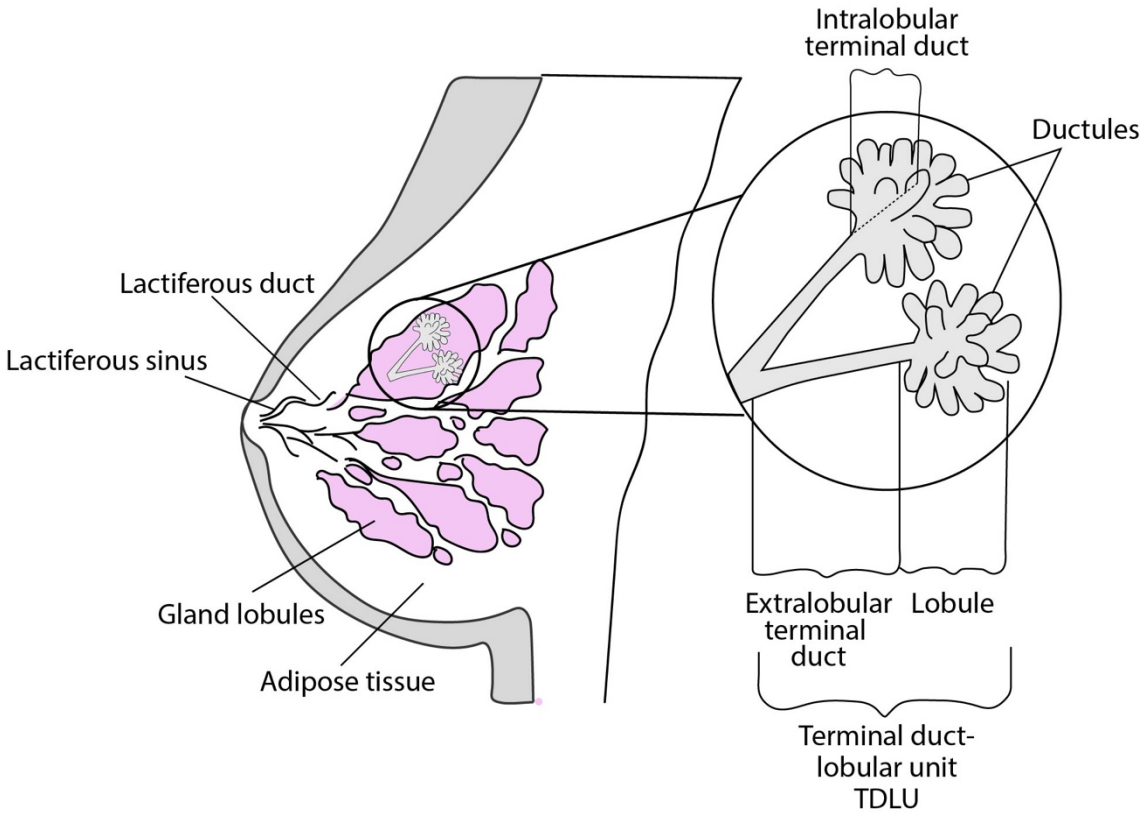

Figure 1. The anatomy of the mammary gland and the terminal duct lobular unit (TDLU).

1.2 BREAST CANCER - GENERAL BACKGROUND

The first description of cancer dates back to Egypt around 3000 BC and breast cancer is described in an ancient Egyptian textbook and furthermore mentioned by Hippocrates around 460 BC[5]. The modern era of surgical treatment for breast cancer dates back to 1894, when Halsted and Meyer introduced the radical mastectomy[6, 7]. This surgical removal of the tumour involved all breast tissue, the pectoralis muscles, all axillary lymph nodes and overlying skin, and led to postoperative complications. The current knowledge has led to the understanding that breast cancer is a heterogeneous disease consisting of several diverse entities with complex patterns of genetic and epigenetic alterations, presenting different morphological features, clinical behaviour, and response to treatment.

1.2.1 Epidemiology of breast cancer

Breast cancer is the most common malignant disease in women worldwide, with over 2 million new cases and 627,000 deaths in 2018[8, 9]. In the United States, more than 275,000 new cases and 42,000 deaths are expected in 2020[10]. In 2018, the United States had an age-standardised incidence rate of 84.8 per 100,000[9]. A similar incidence rate of 83.2 per 100,000 was seen in Sweden[11]. In 2016, the incidence of breast cancer in Sweden was 7,240 new cases[11]. Since the beginning of the 1960's, the incidence rate has almost doubled and at the same time there has been a steady decrease in the mortality rate in Sweden. The relative ten-year survival rate for breast cancer has increased from 50% to around 80% since the beginning of the 1960's, and the relative five-year survival reached 83% in 2019[12, 13]. The decrease in mortality is most likely a result of advances in treatment and earlier detection through screening programs[14, 15].

1.2.2 Risk factors and hereditary breast cancer

The aetiology of breast cancer is multifactorial and involves reproductive factors such as hormones and several epidemiologic factors. The most well-established risk factors for breast cancer are nulliparity, high age at first delivery, lack of breastfeeding, early menarche, late menopause, hormone replacement therapy, and mammographic density[8]. The association of several risk factors with breast cancer, e. g. parity and breastfeeding, are shown to vary across different molecular subtypes[16-18].

Hereditary breast cancer with known high-penetrance gene mutations is seen in 5-10% of all breast cancers. The most common mutations are found in the DNA repair genes *BRCA1* and *BRCA2*, which leads to a 70% lifetime risk of developing breast cancer. The prognosis of breast cancer is highly variable and dependent on several prognostic variables, which will be described below.

1.3 CARCINOGENESIS AND PATHOGENESIS OF BREAST CANCER

1.3.1 The hallmarks of cancer

Human tumour pathogenesis has been extensively described by Hanahan and Weinberg with the ten established hallmarks of cancer (Figure 2)[19, 20]. The core hallmarks for comprehensively

describing the neoplastic process include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis[19].

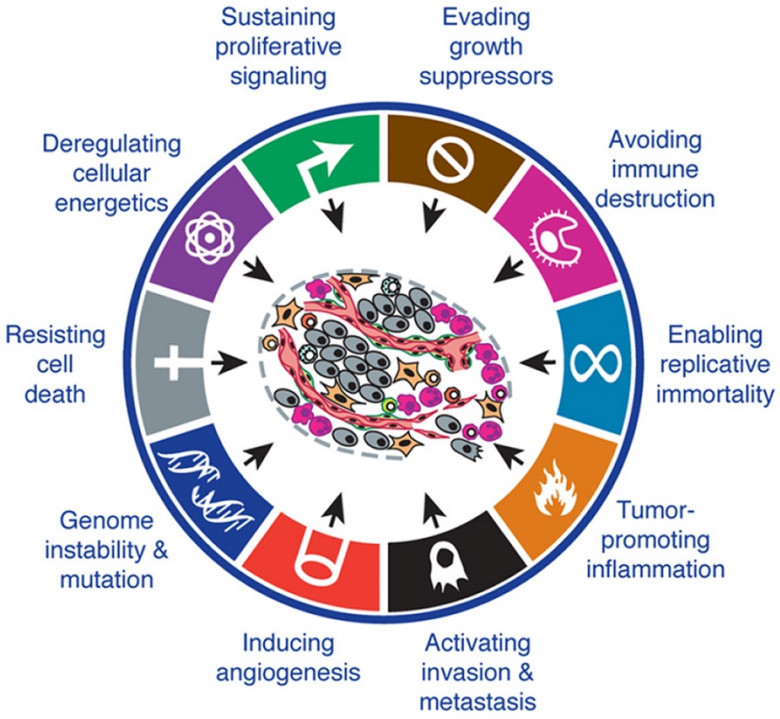

Figure 2. The hallmarks of cancer. Modified from Hanahan and Weinberg 2011[19], reproduced with permissions from Elsevier.

One of the foremost features of cancer cells is the capability to sustain chronic proliferation. Cancer cells overcome growth regulation by e.g. becoming self-sufficient in growth factors leading to overactive growth factor signalling or interacting with the tumour-associated stroma to produce growth factors. Thus, leading to uncontrolled proliferation by activating pathways for cell proliferation and inhibiting cell death. In breast cancer, amplification of the human epidermal growth factor receptor 2 (HER2) is an excellent example and a candidate for targeted therapy, such as the monoclonal antibody trastuzumab (see section 1.6.2). Apart from sustained proliferation, cancer cells have the ability to resist cell death e.g. by mutations in apoptosis genes, and also acquire the capability of replicative immortality, partly by upregulating telomerase. Another hallmark of cancer cells, is their ability to induce angiogenesis by releasing vascular endothelial growth factors (VEGF), which also can be targeted by the drug bevacizumab[21]. The complex process of activating invasion and metastasis in a stepwise cascade of events and acquired capabilities including an intricate crosstalk between cancer cells and the neoplastic stroma. The 'epithelial-mesenchymal transition' program is a broad regulator of the invasion-metastasis cascade[19]. Models of tumour progression are further described in section 1.3.2.3 and 1.3.2.4.

Mutations in key genes that control programs for the capabilities above briefly include oncogene-activation or loss of function in tumour suppressor genes. Genome instability and

mutations, along with tumour-promoting inflammation, are two more recently described enabling characteristics. Additionally, the evidence around reprogramming energy metabolism and evading immune destruction has emerged as hallmarks[19]. Avoiding immune destruction is one of the hallmarks that has gained huge attention lately. By binding to T cell surface proteins, such as programmed cell death protein 1 (PD-1), tumour cells can inhibit the immune response. Thereby with antibodies targeting immune cells, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and PD-1 or programmed death ligand 1 (PD-L1) on the tumour cell, the immune response can be reactivated. Several antibodies are available for immunotherapy in breast cancer (see section 1.6.6 and 1.11.6).

1.3.2 Pathogenesis of breast cancer

Armitage and Doll pioneered over 50 years ago by describing the exponential increase in cancer rates with age[22, 23], and thereby laid the fundaments of a multistage cancer model for tumour initiation, promotion and progression[24, 25]. Breast cancer incidence, however, does not follow an exponential curve, and in all of the early multistage cancer models a single age-specific incidence rate curve were a key feature[26-29]. A two-component cancer model was later introduced showing two distinct age-specific incidence rate curves; premenopausal early-onset oestrogen receptor (ER)-negative tumours and postmenopausal late-onset ER-positive tumours[30-34]. The complexity of this model incorporating gene expression profiling, biostatistical models and analytic epidemiology rather show a bimodal age distribution[35]. Based on gene expression profiling it has further been demonstrated that ER-positive and ER-negative tumours are fundamentally distinct diseases at the transcriptomic level[36]. The pathogenesis of breast cancer is thus described in multiple pathways and models.

1.3.2.1 The cell of origin of breast cancer

There are currently two leading models for breast carcinogenesis: the sporadic clonal evolution model and the cancer stem cell hypothesis[37-39]. Studies suggest that these two models may complement each other and neither of them provides the entire explanation[40]. The cancer stem cell hypothesis proposes that cancer stem cells, which have the capacity to indefinitely self-renew and differentiate, drive cancer initiation and progression. Cancer stem cells are considered to account for a minority of the tumour cell population and to give rise to metastatic disease and recurrence by spread and resistance to therapy[40].

In contrast, the sporadic clonal evolution model states that any normal cell may be the tumour initiator once multiple mutations occur providing natural selective growth advantages. This Darwinian approach suggests that the most fit and aggressive cells drive cancer progression. Over time, subpopulations of different cells develop, leading to tumour heterogeneity[40]. This model demonstrates that therapy resistance is acquired by the selection of cancer cell clones that survive therapy-induced microenvironmental pressure[41, 42]. However, sequencing of patient-derived cancer cells with stem cell properties and corresponding differentiated cells shows that conversion between the two states is possible[43].

1.3.2.2 Intratumoural heterogeneity in breast cancer

A common feature for all cancer types is the vast variety of the cancer cells constituting a single tumour[44]. Simplified, intratumoural heterogeneity is the results of the genetic and phenotypic evolution that occurs during tumour progression. Both the cancer stem cell hypothesis and the clonal evolution model provide explanations for tumour heterogeneity and are not mutually exclusive[45]. The concept of spatial heterogeneity is the heterogeneity across the subpopulations of cells in different regions of the tumour. On the other hand, temporal heterogeneity evolves between primary tumour and recurrence[46]. Tumour heterogeneity is not only observed on a genetic and molecular level, but also evident regarding morphological features such as histological grade, histological subtype components and phenotypic alterations[46, 47].

Breast cancer is a heterogeneous group of diseases and tumour heterogeneity is a complicating factor in clinical practice and cancer treatment, since tumour samples may not represent the entire tumour[41]. Thus, coexistence of several sub clones harbouring different molecular aberrations and drug sensitivities may lead to that the therapy is not effective against the whole tumour[47]. Furthermore, tumour heterogeneity is one of the underlying causes of emerging therapy resistance[47]. Most next generation sequencing (NGS) methods analyse the tumour as a bulk, but advances in single-cell-sequencing have provided new insights into intratumoural heterogeneity. E.g. Rye *et al*. recently demonstrated on a single-cell level that intratumoural heterogeneity for HER2 copy number was associated with worse outcome[48].

1.3.2.3 Models of breast cancer progression

With the advances in genomic and transcriptomic analyses that have now been performed on different stages of breast cancer, the complexity of the biological processes occurring in breast cancer progression is even more evident. The molecular subtypes of invasive carcinoma also seem to be present in non-invasive DCIS[49-51]. There are multiple proposed linear models for initiation, transformation and progression of breast cancer.

An historical model assumed that some pre-invasive lesions developed from either the ducts or from the lobules, and consequently the terminology of ductal carcinoma *in situ* and lobular carcinoma *in situ* (LCIS) arose[52]. Further on, Wellings *et al*. demonstrated that most of the pre-invasive lesions develop in the TDLUs[53]. This step-wise model described how normal epithelial cells in the TDLUs transformed into hyperplasia of usual type, then to atypical ductal hyperplasia that progressed into low-grade DCIS, and from here either acquire characteristics that enabled invasion or progression to high-grade DCIS. A similar lobular model proposed that normal cells in the TDLUs transformed into atypical lobular hyperplasia, LCIS and lastly invasive lobular carcinoma[54, 55]. Based on genomic and transcriptomic analyses, the validity of this model has been questioned, and it has instead been demonstrated that pre-invasive, *in situ* and invasive lesions of low- and high-grade cluster according to histological grade and not stage of tumour progression[56, 57]. Apart from histological grade as a main influencer on breast cancer evolution, molecular studies have proven that the expression of ER and activation of

ER-regulated genes form clusters and separate breast tumours into subtypes[58-60]. Thus, ER-signalling plays an important role in breast cancer progression.

The aforementioned models of breast cancer evolution are rather simplified versions of what we with the current available knowledge recognise as complex and multiple pathways leading to invasive breast cancer. Breast cancer precursor lesions are considered to comprise lesions with identical histological, immunohistochemical and molecular attributes with the corresponding invasive tumours. These are also referred to as non-obligate precursors, since they never have a 100% risk of developing into invasive tumours[52]. The low-grade precursor lesions comprise columnar cell lesions, flat epithelial atypia, atypical lobular neoplasia/classic LCIS, atypical ductal hyperplasia and low-grade DCIS. They are all characterised by low grade, expression of ER, lack of HER2 overexpression or gene amplification, and harbour genetic alterations such as deletions of 16q and gain of 1q[61, 62]. High grade precursor lesions include microglandular adenosis, pleomorphic LCIS and high-grade DCIS, and encompass an intricate pattern of genetic alterations[52].

1.3.2.4 From invasive cancer to metastasis

In breast cancer, death from the disease is a consequence of metastasis to other organs and rarely due to the primary tumour in the breast itself. The metastatic process occurs when neoplastic cells acquire the ability to not only invade the surrounding microenvironment but to pass through the endothelium linings of blood and lymphatic vessel walls. Once in the lymph and blood circulation, circulating tumour cells need to be programmed to extravasate at distant organ sites and further develop into a secondary tumour[63]. The seeding of tumour metastases is still not completely understood.

Axillary lymph nodes are the most frequent sites where locoregional metastases from breast cancer are found[64]. The most common organs for distant metastasis are lung, bone, liver, and non-axillary lymph nodes[65]. The metastatic routes from the primary tumor to distant organs either by hematogenous or lymphatic seeding, however, are not fully understood. In certain subtypes of breast cancer, such as triple-negative breast tumours, distant metastases may occur without the involvement of axillary lymph nodes[66]. At least one study indicates that metastatic cells can bypass the axillary lymph nodes and travel directly to distant organs[67]. Since the studies of the thesis only include early primary breast cancer, further details covering the metastatic process and tumour heterogeneity in metastases are out of the scope for this thesis.

1.4 BREAST CANCER DIAGNOSTICS

The triple diagnostic procedure of a suspicious breast lump includes clinical examination (palpation), radiological studies and pathological assessment.

1.4.1 Radiologic assessment

Since mammographic screening programs were introduced, breast tumours have been diagnosed at an earlier stage. At the same time, an increased number of both invasive and non-invasive lesions are detected. The screening programme in Sweden invites women of age 40-

74 with 2-year intervals. Mammographic screening has reduced breast cancer mortality[68, 69]. In Sweden, 65% of all breast tumours are detected through screening programs among eligible patients[13]. Mammography is the preferred modality for radiological evaluation of a suspicious breast lump in the majority of patients and has a sensitivity of 85-90%[12]. The development of digital mammography and digital breast tomosynthesis (3D-mammography) has improved the diagnostics of diffuse lesions but has not been introduced in general screening. For patients with dense breasts, however, and in those with findings on mammography, a complementary ultrasound examination is recommended. For women who are under 30 years, pregnant or breast feeding, ultrasound examination is the method of choice. In women with uncertain lesions, dense breast tissue, increased hereditary risk, or lobular carcinoma, magnetic resonance imaging can be used for detailed diagnostics and surgical planning.

1.4.2 Fine needle aspiration cytology

Fine needle aspiration cytology (FNAC) is a minimally invasive diagnostic method whereby a fine needle (27-22 gauge) is utilised for aspiration of cells from the suspected lesion. The aspirated material is immediately smeared on a glass slide (Figure 3) or used for immunocytochemistry (ICC). Aspiration cytology can be led by palpation or ultrasound for accurately targeting the lesion. FNAC is a rapid method that requires relatively sparse material for diagnosis of cancer. In Sweden, the use of FNAC for breast cancer diagnosis has been extensively used and developed since the beginning of the 1950's[70, 71]. If both clinical examination and radiological findings are conclusive for primary surgical removal of the tumour, FNAC may be sufficient for diagnosis. The disadvantage of FNAC samples is that the tumour material consists of a mixture of aspirated cells without organisation, and thereby, non-invasive tumours cannot be distinguished from invasive carcinoma. Certain morphological information cannot be acquired, such as growth patterns and histological grade. However, some anatomical locations may not be available for biopsy sampling other than with a fine needle, and in the metastatic setting, FNAC may sometimes be the only choice for diagnosis.

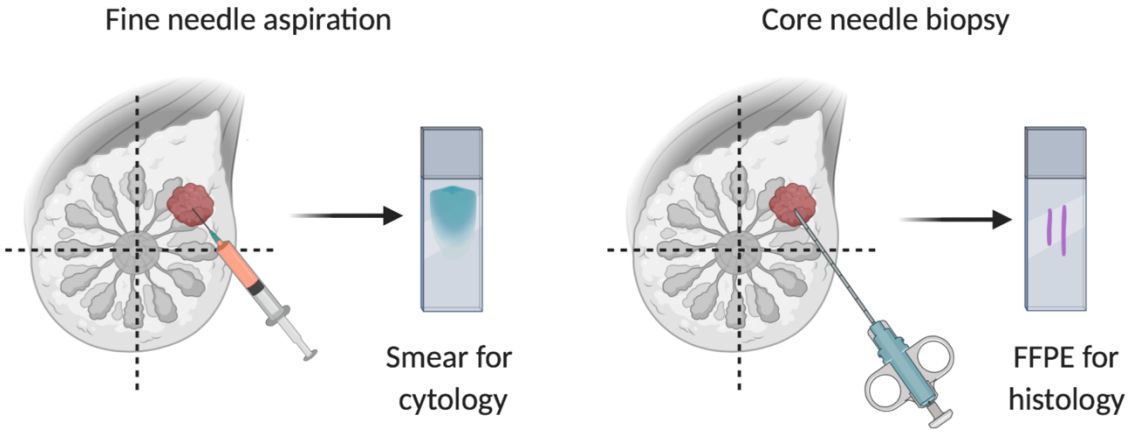

Figure 3. Fine needle aspiration for aspiration cytology versus a core needle biopsy prepared for formalin-fixed paraffin-embedded (FFPE) tissue sections. Created with BioRender.com.

1.4.3 Core needle biopsy

With the core needle biopsy technique, a tissue biopsy is punched out and retracted with a 14-12 gauge needle. If larger tissue biopsies are required vacuum aspiration (needle of 8 gauge) is an alternative method. In the pathology department laboratory, the tissue biopsy is prepared by formalin fixation and paraffin embedding after which sections are mounted on glass slides (Figure 3). The sections are routinely stained with haematoxylin and eosin (HE) for pathological assessment. The advantage of core biopsies is that the tissue and its architecture are preserved, which allows for morphological assessment of tumour type and histological grade, and for distinguishing *in situ* from invasive carcinoma.

Additional immunohistochemistry staining can be performed on biopsy samples, and for all breast cancers, a panel of biomarkers (ER, progesterone receptor (PR), HER2, and depending on country also Ki67) is routinely assessed. According to international guidelines, biomarker evaluation should be performed on either core biopsy or resection specimen, and not solely on cytology material[12, 72-74].

With the increasing evidence for the benefits of neoadjuvant therapy, a preoperative biopsy is required for diagnosis and biomarker evaluation, which helps to predict the potential benefit of neoadjuvant treatment and guide the choice of therapy. For patients with tumours that respond well to neoadjuvant therapy, complete response may be reached and no residual tumour cells found in the surgical specimen. In this case, the preoperative biopsy with the assessment of biomarkers will be the only tumour information provided for deciding on adjuvant therapy. If the biomarker evaluations are not accurate, the patient risks either to receive potentially harmful therapy without added benefit or miss out on life-saving therapy such as HER2-targeted therapy.

Circulating tumour cells in the blood can be detected in some early cancers and the majority of advanced cancers through analysis of so-called liquid biopsies. Two conceptually different approaches can be applied; circulating tumor cells or circulating tumor DNA[75]. Although several prospective investigations have been performed, there is not yet evidence for implementation in clinical practice[76].

1.4.4 Surgical specimen

The majority of surgical breast cancer specimens are partial resections from breast-conserving surgery or mastectomies (surgical techniques are further presented in section 1.11.1). Surgical specimens containing breast tumours are directly sent fresh from surgery to the pathology department without prior formalin fixation. The resection margins are inked in order to evaluate tumour-free margins by microscopy. The specimen is cut, either in the frontal or sagittal plane depending on local traditions, enabling macroscopic assessment of the tumour and its extent. This procedure prior to fixation enables biobanking, in which small tumour samples are snap-frozen and stored in a biobank collection. The stored fresh-frozen tumour material can later be used for molecular analysis or for research purposes. Axillary lymph node resections and

sentinel node biopsies are included in the surgical samples received for pathological assessment (see section 1.11.1).

1.4.5 Pathological assessment

Apart from diagnosis and morphological classification including histological grade described in detail in section 1.5, the histopathological assessment of specimens from breast tumours and axillary content includes evaluation of tumour size, focality, distance to peripheral margins, presence of lymphovascular invasion, along with the presence, number and size of lymph node metastases. All breast cancer diagnoses are discussed in pre- and postoperative multi-disciplinary team conferences consisting of a surgical pathologist, radiologist, breast surgeon and oncologist as well as team nurses[77].

1.5 MORPHOLOGICAL CLASSIFICATION OF BREAST CANCER

1.5.1 Non-invasive breast cancer

DCIS is characterised by intraductal proliferation of malignant epithelium. Large multifocal and high-grade DCIS lesions do not generally pose any diagnostic concern. However, small low-grade lesion may be difficult to distinguish from atypical ductal hyperplasia and consequently lead to a large interobserver variability with both under- and overinterpretations[78]. These lesions are frequently detected by mammographic screening and pose a diagnostic challenge[78].

LCIS is the proliferation of neoplastic cells originating from the TDLUs and that expand the acini. The loss of E-cadherin or impaired function thereof, caused by *CDH1* inactivation, is a characteristic feature of lobular lesions[79]. Impaired E-cadherin leads to loss of cell-cell adhesion and augmented proliferation, as well as the architectural alterations morphologically recognised as lobular neoplasia[80]. As mentioned in section 1.3.2.3, LCIS is a non-obligate precursor lesion to invasive cancer with a low risk of progression to invasive lobular carcinoma[81-84], and is not classified as pre-malignant and does not require surgical excision.

The classification of non-invasive intraductal proliferative breast lesions is continuously evolving as new insights and molecular data refine the concept. We can probably expect to see changes in the traditional classification along with emerging data on molecular characteristics that will support clinical implications.

1.5.2 Histological subtypes of invasive breast carcinoma

Invasive breast carcinomas comprise a wide range of morphological phenotypes and are categorised into different histological subtypes according to the World Health Organisation (WHO)[85]. The largest group of invasive breast cancers is classified as invasive carcinoma of no special type (NST), previously referred to as infiltrating ductal carcinoma or invasive ductal carcinoma not otherwise specified. Pure special tumour types that exhibit a special histological pattern in ≥90% of the tumour, include e.g. lobular, mucinous and tubular carcinoma. Invasive lobular carcinoma is the second most common group and together with invasive carcinoma

NST, these groups constitute approximately 95% of all breast tumours. Breast tumours showing characteristic features are furthermore classified into e.g. cribriform carcinoma, apocrine adenocarcinoma, invasive micropapillary carcinoma and metaplastic carcinoma. The WHO has classified several other categories of breast tumours with characteristic morphology[85]. Papillary neoplasms, including encapsulated papillary carcinoma, solid papillary carcinoma and intraductal papillary carcinoma, are classified separately and have a complex histopathological appearance and an indolent clinical behaviour[85].

1.5.3 Histological grade

Morphologic assessment and tumour grading are part of the histopathological diagnostic work-up of invasive breast carcinomas. A tumour's histological grade is based on the assessment of tubule formation (glandular differentiation), nuclear pleomorphism and mitotic counts. The first described method for tumour grading by Bloom and Richardson was later modified by Elston and Ellis[86, 87]. Tumour grade is a powerful prognostic factor in invasive breast cancer[88, 89] and is routinely assessed for all invasive breast tumours by manual counting mitoses that together with tubule formation and nuclear pleomorphism gives the tumour Nottingham histological grade (NHG). Mitotic activity is assessed by counting mitoses; until recently in 10 high-power fields, but since the latest WHO recommendations of 2019, mitoses are counted within a defined area in mm^2 [85]. Accurate mitotic counts rely on optimal tissue fixation and preparation. Each of the three characteristics is given a numerical score of 1 to 3. These three scores are added to a total score (3 to 9) and final tumour grading of 1 to 3. A higher grade reflects a more aggressive and poorly differentiated tumour. The reproducibility of low (NHG 1) versus high grade (NHG 3) is relatively sufficient. However, the challenges in classification lie within the intermediate grade (NHG 2), comprising up to 50% of tumours. NHG 2 tumours are likely a include a mixture of low- and high-grade tumours and provide limited clinical significance[90, 91]. In several other tumour types, such as squamous intraepithelial neoplasia of the lower anogenital tract and gastrointestinal adenoma, the three-tier classification of grading has been omitted and tumours are dichotomised by a two-tier grading into low- and high-grade dysplasia[92, 93]. Furthermore, gene expression studies have shown that grade better reflects "the molecular make-up of breast cancer" than lymph-node status or tumour size[94, 95].

1.5.4 TNM classification system and tumor stage

Apart from patient age, tumour grade, histological subtype, margin status, and lymphovascular invasion, the most important prognostic factor is still the stage of the disease, which is captured in the TNM classification system of tumours of the breast and comprises primary tumour size (T), regional lymph node involvement (N), and spread to distant metastatic sites (M). The T, N and M are combined into stages 0-IV[96, 97]. Both clinical TNM (cTNM) and pathological TNM (pTNM) are evaluated for each breast cancer. Clinical stage is based on physical examination and radiological studies (T, N, M), whereas pathological stage is based on histopathological examination of surgical tumour specimen (T) and lymph nodes (N). However, M is generally determined by radiology findings and in some cases by FNAC or CNB diagnosis. Only pTNM will be discussed below. Tumour size is classified into T1 (≤2cm), T2 (>2cm but ≤5cm), T3

(>5cm), and T4 (tumour extending into chest wall and/or skin). Nodal involvement is divided into N0 (no regional lymph node metastasis or isolated tumour cells), N1 (micrometastasis or metastasis in 1-3 ipsilateral lymph nodes), N2 (metastasis in 4-9 ipsilateral lymph nodes) and N3 (metastasis in ≥10 lymph nodes). The presence of distant metastasis is classified as M1 and their absence of metastases as M0; most breast cancers may be defined as Mx (distant metastasis status unknown) since organ imaging for distant metastasis is not included in the work-up of most breast cancer cases today.

Up to 50% of breast cancer patients present as stage I and only 5% as stage IV disease[98]. This can be reflected in the component of stage, where tumour size and nodal involvement are tightly correlated; e.g. 19% of pT1 tumours present with lymph node metastasis and 1% with distant metastasis, whereas pT3 tumours are associated with more frequent nodal (40%) and distant (10%) metastasis. In the recent AJCC TNM classification system, anatomical TNM information is combined with tumour-intrinsic biology into a 'breast cancer prognostic stage'[97]. Apart from T, N and M, predictive biomarkers (ER, PR and HER2, see section 1.6) and grade are included for the stratification of outcome prediction.

1.5.4.1 Staging of residual tumour after neoadjuvant therapy

Similarly, classification of neoadjuvantly treated tumours is described using ypTNM, e.g. a resection of a primary tumour following neoadjuvant chemotherapy with a residual tumour size of 25 mm and two residual positive axillary lymph nodes without known distant metastases would be classified as ypT2N1M0.

Pathological staging of residual tumour burden is crucial for the evaluation of the effect of neoadjuvant therapy. Pathological complete response (pCR) has been used extensively as an endpoint in neoadjuvant trials, and is associated with better prognosis[99], especially for HER2-positive and triple-negative tumours[100, 101]. pCR is defined as no residual invasive tumour in the breast and axillary lymph nodes (ypT0/tisN0)[102]. However, when complete response is not achieved, the residual tumour burden may vary from no change in tumour cellularity to a marked disappearance with only remaining clusters or dispersed tumour cells, which has until recently been classified as partial pathological response according to the Miller-Payne grading system, which estimates the decrease in cellularity[102]. The current WHO classification of breast tumours recommends the standardised evaluation of residual cancer burden (RCB), which considers the size of the tumour bed, the percentage of residual invasive cancer or DCIS, the number of residual positive lymph nodes and the size of the largest metastasis[85, 103]. These parameters are used to calculate an RCB index, and one of the four risk scores (RCB-0 to RCB-III) is determined, which provides prognostic information across different biological breast cancer subtypes[104].

1.6 PREDICTIVE AND PROGNOSTIC BIOMARKERS

Biomarkers defined as measurable indicators of normal physiological processes, specific disease states or pharmacological responses, are widely used in oncology[105]. A prognostic biomarker provides information on the likelihood of a clinical event, e. g. recurrence or

progression of disease, regardless of therapy or after standard therapy[106, 107]. A predictive biomarker correlates to the likelihood of benefit from a specific clinical intervention, or the differential outcomes of several interventions, also including toxicity. Such a biomarker can also be used as a target for therapy[107]. Several biomarkers may provide both prognostic and predictive information.

In breast cancer, established prognostic clinicopathological factors are age, tumour size, lymph node status, disease stage, histological grade, ER, PR, HER2 and the proliferation-associated nuclear protein Ki67. Of these, the predictive single-molecule biomarkers ER, PR and HER2 have been used to identify patients eligible for targeted therapy for the past decades[108]. Ki67, however, is still one of the most controversial biomarkers in breast cancer (see section 1.6.3). Furthermore, several multigene expression assays have entered clinical practice and provide additional prognostic and predictive information (see section 1.7.7).

As patient demand for personalised breast cancer therapy grows, we face an urgent need for more precise biomarker assessment and more accurate histopathologic breast cancer diagnosis to make better therapy decisions. In routine management of invasive breast cancer, analysis of the prognostic and therapy-predictive biomarkers ER, PR, HER2 and in some countries Ki67, is paramount[77, 109-113]. Today's immunohistochemistry (IHC)-based methods for biomarker assessment, however, struggle with intra- and interobserver variability, which hampers its reproducibility. This is especially evident for Ki67[114, 115]. Since treatment decisions are based on these IHC-determined biomarkers, accurate assessment is essential. According to international guidelines ER, PR, HER2 and Ki67 should be analysed on the surgical tumour specimen or on a preoperative core biopsy[12, 72, 74].

1.6.1 Oestrogen and progesterone receptors

The correlation between hormones and breast cancer development was first recognised more than a century ago[116]. Oestrogen receptors belong to the nuclear receptor superfamily[117]. The first main receptor for oestrogen was discovered in the 1950's by Jensen and colleagues[118, 119] and was later named ERα, which was cloned in 1986[120, 121]. The second receptor for oestrogen, ERβ, was discovered and cloned in 1996 by Gustafsson and colleagues[122]. ERα is encoded by the gene *ESR1* located on chromosome 6q, whereas ERβ is encoded by *ESR2* on chromosome 14q[123, 124]. The structure of the two receptors shows several similarities, and both ERα and ERβ bind to oestrogen with similar affinity.

The central hub in oestrogen signalling and the target for endocrine therapy is ER, a ligand-activated transcription factor that binds and activates hundreds of target genes in response to ligand activation or growth factor signalling[125]. In the normal breast, ER signalling stimulates the growth of epithelial cells. Approximately 80% of all breast tumours express ERα, which is the clinically used biomarker predicting response to endocrine therapy[126-128]. Furthermore, co-expression of PR through the ER-induced gene *PGR* is considered a positive response factor[129]. The prognostic and predictive role of ERβ in breast cancer is still unclear[130, 131].

PR is foremost considered a prognostic marker, independent of ER expression, and not predictive[132]. The predictive role of PR status is not fully understood; there are studies demonstrating that the role of PR may not be of as high importance as ER[133, 134], and others showing that PR alone provides additional value[135].

Tumour expression of ERα, here referred to as only ER, and PR is determined by IHC staining, and scored through manual estimation of the percentage of positively stained nuclei across the whole tumour section. ER and PR status should be determined on all invasive breast tumours and recurrences. According to international guidelines, tumours with ≥1% ER and PR are considered positive, and these patients should be considered for endocrine therapy. Tumours expressing less than 1% of tumour cells staining for ER or PR have internationally been considered negative, lacking benefit from endocrine therapy[136]. The percentage level, however, may provide valuable predictive and prognostic information regarding treatment strategies. In recent guidelines tumours expressing ER 1-10% (low-positive) have been highlighted[74, 137]. Tumours with ER low-positive expression are a heterogeneous group that have shown molecular features and clinical outcome more resembling triple-negative breast cancer[138-140]. Limited data on benefit from endocrine therapy in ER low-positive tumours render clinical challenges[74]. In clinical practice, the Swedish guidelines have for several years considered ER positivity as ≥10% ER expression indicating a benefit from endocrine therapy[12, 109].

1.6.2 Human epidermal growth factor receptor 2

Human epidermal growth factor receptor 2 (HER2), encoded by the proto-oncogene *ERBB2* (also referred to as *HER2*) located on chromosome 17, is a tyrosine kinase receptor located on the cell surface[141, 142]. The *neu* oncogene is the rodent homologue to *HER2*[143, 144]. HER2 is a member of the HER (ErbB) family of epidermal growth factor receptors (EGFRs) which is involved in normal cell growth, proliferation and survival[145, 146]. Receptor activation involves a ligand-induced receptor dimerisation (homo- or heterodimerisation) and subsequent activation of the intrinsic tyrosine kinase leading to initiation of downstream signalling pathways through the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) proliferation pathway and/or the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) prosurvival pathway[147-150]. Apart from HER2 gene amplification and overexpression of the protein, the HER2 protein can heterodimerise with other members of the HER family (HER1, HER3 and HER4)[149], illustrated in Figure 4.

Amplification of the HER2 oncogene in breast cancer was first described by Slamon and colleagues in 1987[151]. HER2 gene amplification and/or receptor overexpression occurs in 10-15% of breast cancers, and is associated with poor prognosis and a more aggressive cancer phenotype if untreated[151-155]. The monoclonal antibody trastuzumab, which targets the HER2 protein was introduced almost two decades ago[156] and since then the prognosis for HER2-positive breast cancer has dramatically improved[157, 158]. HER2 is thereby a prognostic marker for aggressive tumours and a predictive biomarker for therapeutic response. HER2 overexpressing and/or gene amplified tumours is also found among subsets of gastro-oesophageal and endometrial cancers and associated with poor prognosis[159-161].

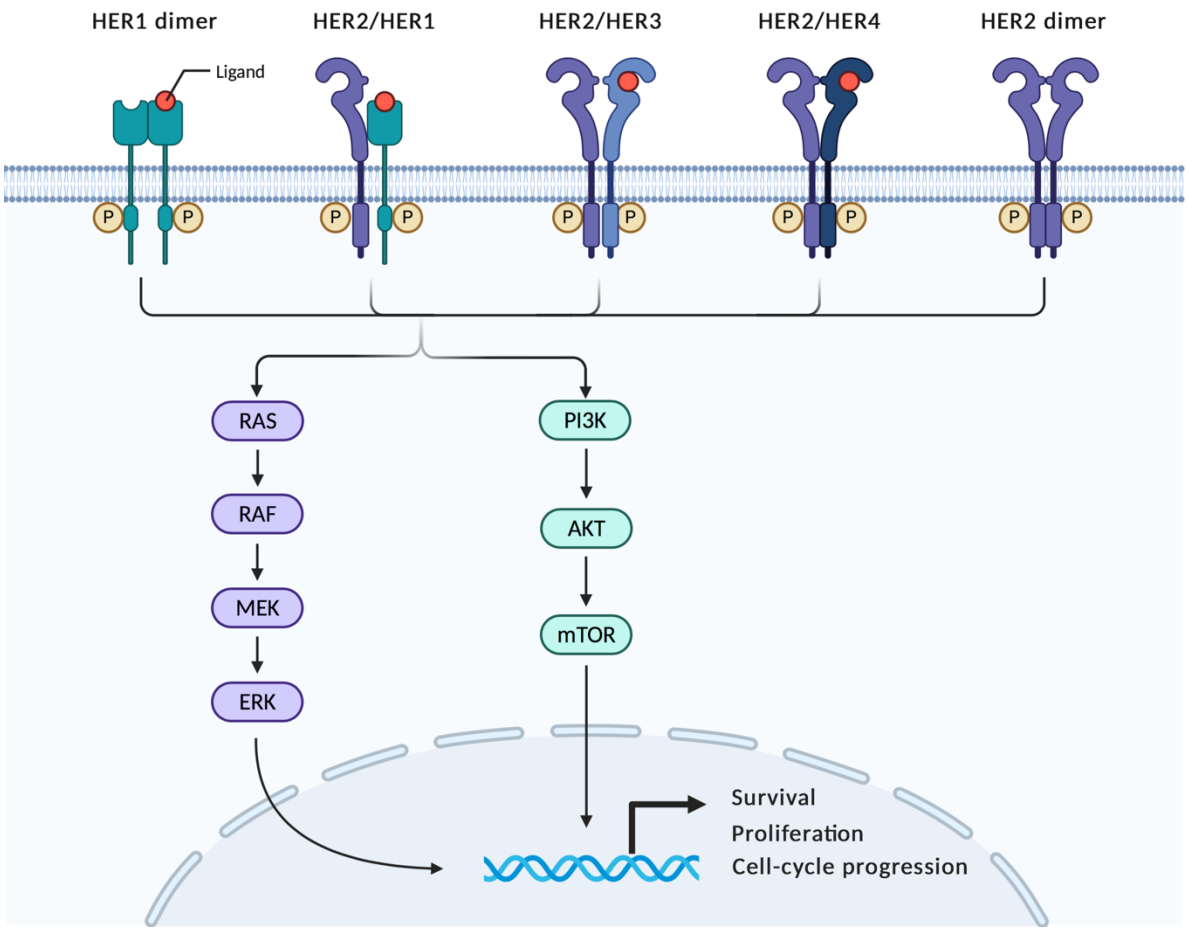

Figure 4. The HER family and HER2 signalling pathways. Receptor activation involves a ligand-induced receptor dimerisation (homo- or heterodimerisation) and subsequent activation of the intrinsic tyrosine kinase leading to initiation of downstream signalling pathways through the MAPK/ERK proliferation pathway, and/or the PI3K/Akt prosurvival pathway. HER2 receptor activation occurs through homodimerisation or heterodimerisation with HER1, HER3 or HER4. Created with Biorender.com.

Pathological assessment of HER2 status is a crucial part of breast cancer diagnostics and determination of HER2 status is recommended for all stages of invasive breast cancer, as well as at disease recurrence[72, 73, 155, 162]. There are several available methods for determining HER2 status at a protein, RNA and DNA level[163]. In routine pathology, overexpression of the HER2 protein is evaluated by the membranous IHC staining of tumour cells and the scoring is based on the degree of circumferential or only partial membrane staining and its intensity. According to guidelines[73, 162], an IHC score is given between 0 (no staining) to 3+ (strong staining). IHC scores 0-1+ are referred to as negative HER2 status, whereas score 3+ is considered positive. An IHC score of 2+ is considered equivocal and additional *in situ* hybridisation (ISH) should be performed to confirm amplification. HER2 gene amplification is determined as HER2 copy numbers by a DNA probe integrated to an ISH detection platform. Fluorescent and chromogenic ISH have been used during different time periods and lately mostly replaced by silver-enhanced ISH. The American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) HER2 testing guideline currently recommends the use of dual-probe ISH assays instead of singe-probe assays[162].

In dual-probe ISH assays, the locus-specific probe for HER2 detects *ERBB2* and the chromosome enumeration probe 17 (CEP17) detects the centromeric region of chromosome 17[164]. The HER2/CEP17 ratio is determined by assessment of copy numbers for HER2 and CEP17, respectively. In rare cases, chromosome 17 polysomy may occur, where the tumour cells have increased number of chromosome 17 regardless of HER2 amplification[165-167]. The ASCO/CAP HER2 guidelines for breast cancer were first published in 2007, revised in 2013 and included significant updates in 2018 especially for the groups of less common ISH results[73, 162, 168]. Tumours with HER2 IHC score 2+ require reflex testing with ISH. The diagnostic algorithm for evaluation of HER2 ISH presented by ASCO/CAP 2018 guidelines divides tumours into five groups (Group 1-5) based on HER2/CEP17 ratio and average HER2 copy numbers[162]. Tumours with a ratio ≥2.0 and average HER2 copies/cell of ≥4.0 (group 1) are defined as ISH positive whereas those with a ratio <2.0 and average copies/cell <4.0 (group 5) are ISH negative. These two groups comprise up to 95% of HER2 tested cases in a population[162]. The less common scenarios include tumours with a ratio ≥2.0 and <4.0 copies/cell (group 2), ratio <2.0 with ≥6.0 copies/cell (group 3) or with ≥4.0 but <6.0 copies/cell (group 4). Group 2-4 required additional work-up and correlation to IHC. In summary, only tumours with HER2 IHC 3+, or 2+ along with ISH group 1 or 3 are determined HER2 positive. The clinical significance of HER2 "equivocal" variants remains, however, unclear.

1.6.3 Proliferation-associated marker Ki67

The tumour's ability to sustain proliferative activity is one of the hallmarks for cancer and the most fundamental trait of cancer[19, 20]. Apart from mitotic count, the proliferation-associated nuclear protein Ki67 is assessed by IHC for evaluation of a tumour's proliferative activity. Ki67 is expressed in all proliferating cells and is not specific for breast tissue. Ki67 is predominantly expressed in all active phases of the cell cycle other than G_0, including apoptosis, and is therefore a rather unspecific marker for mitotic activity[169]. The exact function of Ki67 is not completely understood, although studies suggests that Ki67 plays a crucial role in ribosomal RNA synthesis[170, 171].

IHC assessment of Ki67 is the most widely used method for proliferation in clinical practice for breast cancer (Figure 5). Ki67 has been demonstrated to be a prognostic biomarker in early breast cancer, but there are still uncertainties regarding its role in clinical breast cancer management[172-174]. There is strong evidence for a biological relationship between Ki67 and prognosis, but the cut-off to distinguish high from low proliferation shows variation from 1% to 28.6% in larger studies, which limits the clinical applicability[175]. Furthermore, Ki67 has shown prognostic value in patients with NHG 2 tumours, thus dividing these tumours into two different prognostic groups[176, 177].

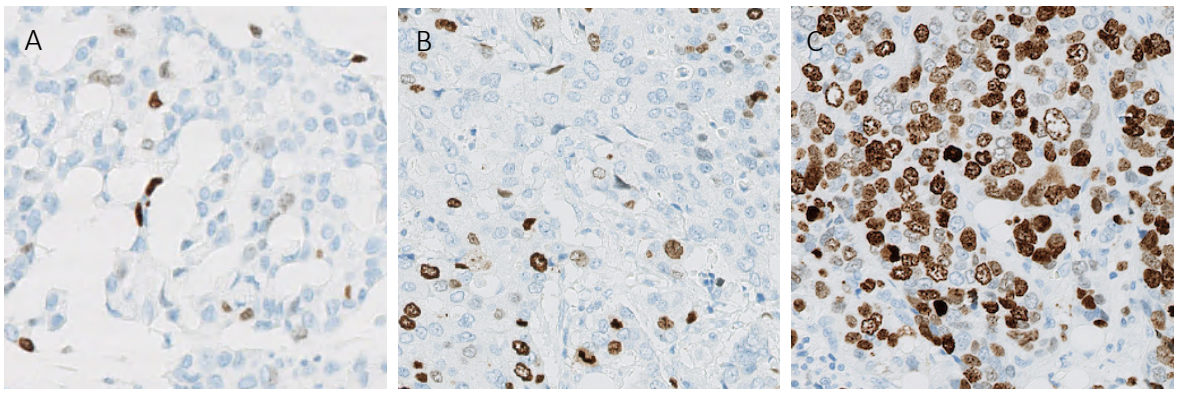

Figure 5. Breast tumours with low (**a**), intermediate (**b**) and high proliferation (**c**) by Ki67 immunohistochemistry staining (clone 30-9).

Inconsistent Ki67 cut-offs result in varying distributions of luminal A- and B-like tumours (described in section 1.7 and 1.8) in different pathology laboratories thus effects the decisions for chemotherapy[77]. The International Ki67 in Breast Cancer Working Group (IKWG) agreed that IHC of Ki67 was the current assay of choice for proliferation assessment in pathology specimen, although recognising the poor agreement on its specific clinical use. The working group proposed a concept to standardise the interpretation of Ki67, including number of cells, tumour region of interest and analytical validity[178]. However, Ki67 is prone to inter- and intraobserver variability, further hampering its reproducibility[114, 115]. In addition, different assessment methods are still being used, from eyeball estimation, counting up to 1000 tumour cells to automated digital image analysis[172]. Furthermore, the application of Ki67 and its cut-off for decision making is considered unreliable outside experienced laboratories with own reference data[178]. With interlaboratory discordance and lack of standardised protocols, neither the IKWG nor the St Gallen expert deliberations have settled recommendations for Ki67 in routine oncology[110, 178, 179]. There is no consensus protocol regarding what tumour region to assess for highest biological or clinical importance. In international recommendations 1000 cells with a minimum of 500 cells should be counted[77, 178], whereas the Swedish guidelines recommend Ki67 scoring of 200 cells in a hot spot region[12]. The definition of a hot spot is an area in which Ki67 nuclear staining is particularly prevalent, but this approach varies across studies[178]. In addition, the ASCO guidelines recommend against the use of Ki67 for guiding therapy due to insufficient evidence[180]. Digital image analysis (DIA) is suggested to improve reproducibility[181-183]. Recently, the IKWG suggested automated scoring methods for Ki67 based on reproducibility but sill acknowledges the need for standardisation and assessment of clinical validity[182, 184].

1.6.4 Phosphohistone H3

Moreover, in contrast to the proliferation-associated biomarker Ki67, phosphorylation of histone H3 (PHH3) occurs exclusively in late G_2 and M-phase. PHH3 is involved in chromatin condensation and crucial for entering mitosis, and a more recently described marker for proliferation in breast cancer. Thus, it is both in theory and practice a very specific marker for

mitotic activity, and high PHH3 has been associated with poor prognosis[185-188]. This approach, however, has not been introduced in clinical practice.

1.6.5 Tumour-infiltrating lymphocytes

Tumour-infiltrating lymphocytes (TILs) are mononucleated lymphoid cells, which infiltrate the tumour and its surrounding stroma, reflecting the host immune response. Evaluation of tumour immune response is gaining importance as prognostic and predictive markers in several solid tumour types as the implications for immunotherapies are expanding[189]. Increasing data has shown that TILs are associated with increased response to neoadjuvant therapy and improved outcome after adjuvant chemotherapy in triple-negative and HER2-positive breast cancer[190-193]. Routine assessment of TILs in triple-negative tumours has now been incorporated in the most recent guidelines[12, 194].

Visual TIL assessment on HE sections is a prognostic marker in breast cancer, and efforts have been made to increase reproducibility and standardisation[195]. Standardised methods for quantification of TILs have been proposed by Salgado *et al*. from the International TILs Working Group[196]. Stromal TILs are scored by estimating all mononuclear lymphocytes within the stroma between the areas of invasive tumour as the percentage of the stromal area[196]. Computer-aided diagnosis will provide an important tool for increased reproducibility. In a study on melanoma, automated TIL scoring showed robust and independent prognostic value[197]. An automated deep learning approach for TIL scoring in breast cancer has recently demonstrated correlations with gene expression data and survival outcome[198].

1.6.6 Programmed death-ligand 1 (PD-L1)

PD-L1 also known as B7-H1, is a ligand to the inhibitory checkpoint molecule PD-1 present on activated T cells, B cells and myeloid cells. Upregulation of PD-L1 on tumour cells is associated with aggressiveness and evasion from the host immune system. Agents targeted at blocking PD-1/PD-L1, so called check-point inhibitors such as pembrolizumab and atezolizumab, have shown promising results in several solid tumours including metastatic triple-negative breast cancer[199-202]. In the ongoing I-SPY2 phase 2 trial, data recently showed that the addition of pembrolizumab to neoadjuvant chemotherapy doubled the estimated pathological complete response rates for triple-negative and ER-positive/HER2-negative early breast cancer[203]. In triple-negative breast tumours, PD-L1 expression is mainly present on tumour-infiltrating immune cells, and not on tumour cells, with the ability to inhibit anti-tumour immune responses[204]. Recent results from the IMpassion130 trial demonstrated that PD-L1 expression on >1% of tumour-infiltrating immune cells was predictive of improved progression-free and overall survival in metastatic triple-negative breast cancer treated with atezolizumab combined with chemotherapy (nab-paclitaxel)[204, 205].

Therefore, IHC assessment of PD-L1 is required prior to determine eligibility to treatment with anti-PD-L1 antibodies. Of importance is that approved antibodies are used with corresponding scoring algorithms, so called companion diagnostics, which may vary depending on organ site and manufacturer. The only currently approved assay for breast cancer is the PD-L1 SP142

assay (VENTANA, Roche Diagnostics, Rotkreuz, Switzerland)[204]. The indications for check-point inhibitors are rapidly evolving, and several new assays will most certainly emerge in clinical practice in the near future.

1.6.7 Quality assurance of biomarkers

Tumour biomarkers are not only used for prognosis and prediction, but have wide utility for differential diagnosis, monitoring and risk stratification. Regardless of use, quality assurance is of utmost importance. Analytic validity, clinical validity and clinical utility all need to be considered when recommending a biomarker test to guide treatment decisions[206]. The accuracy, reliability and reproducibility of a biomarker assay are parts of analytical validity. Clinical validity is the ability of a biomarker test to distinguish biologically or clinically different groups, e.g. if a positive test is associated with worse prognosis. If a biomarker test is judged to have clinical utility, it is unlikely that it would lack clinical validity[206].

Internal quality assurance of biomarker assays includes positive and negative control samples on the glass slide to assure accurate performance of the IHC staining, as well as accreditation of each laboratory performing the assay. The results from a biomarker test should be identical regardless of in which laboratory the assay was performed. To ensure this, participation in external quality assurance programmes are mandatory, such as the Nordic immunohistochemical Quality Control (NordicQC)[207], the UK National External Quality Assessment Scheme[155, 208] and CAP[209]. National cancer registries, such as the National quality registry for breast cancer database also provide continuous data of biomarker results across hospitals in Sweden[13].

As described above, the controversies regarding Ki67, are mainly due to concerns in analytical validity, and especially reproducibility[114, 115, 179]. Regarding HER2 status, both local and regional variations in HER2 positivity rates are evident, and continuous improvement strategies are important to limit false-negative and false-positive results[13, 210, 211]. As for other biomarkers, HER2 testing is influenced by pre-analytical and analytical factors, but also tumour features such as tumour heterogeneity[211].

1.7 MOLECULAR SUBTYPES OF BREAST CANCER

Over the past decade, it has been established that breast cancer comprises a heterogeneous group of diseases with distinct molecular features[212]. Gene expression profiling of breast cancer has provided additional prognostic information to the standard clinicopathological assessment[59, 213-215]. The recognition of gene expression signatures and intrinsic subtypes based on global mRNA expression, as first described by Perou *et al.* in 2000, has provided promising alternatives for stratification of invasive breast tumours[58, 59]. Based on DNA microarray and hierarchical clustering, the intrinsic subtypes luminal A, luminal B, HER2-enriched, basal-like and normal-like have been widely investigated[58-60, 214, 216, 217]. Other rare subtypes include the claudin-low, interferon-rich and molecular apocrine subtype[60, 214, 218, 219]. The prediction analysis of microarray (PAM) 50 classifies breast tumours into four major intrinsic subtypes: luminal A and B, HER2 enriched and basal-like[214]. These molecular subtypes of breast cancer

and the correlation to clinicopathological features are illustrated in Figure 6. Several commercial multiparameter molecular marker assays have been developed for subtype classification and estimated risk of recurrence (described in section 1.7.7).

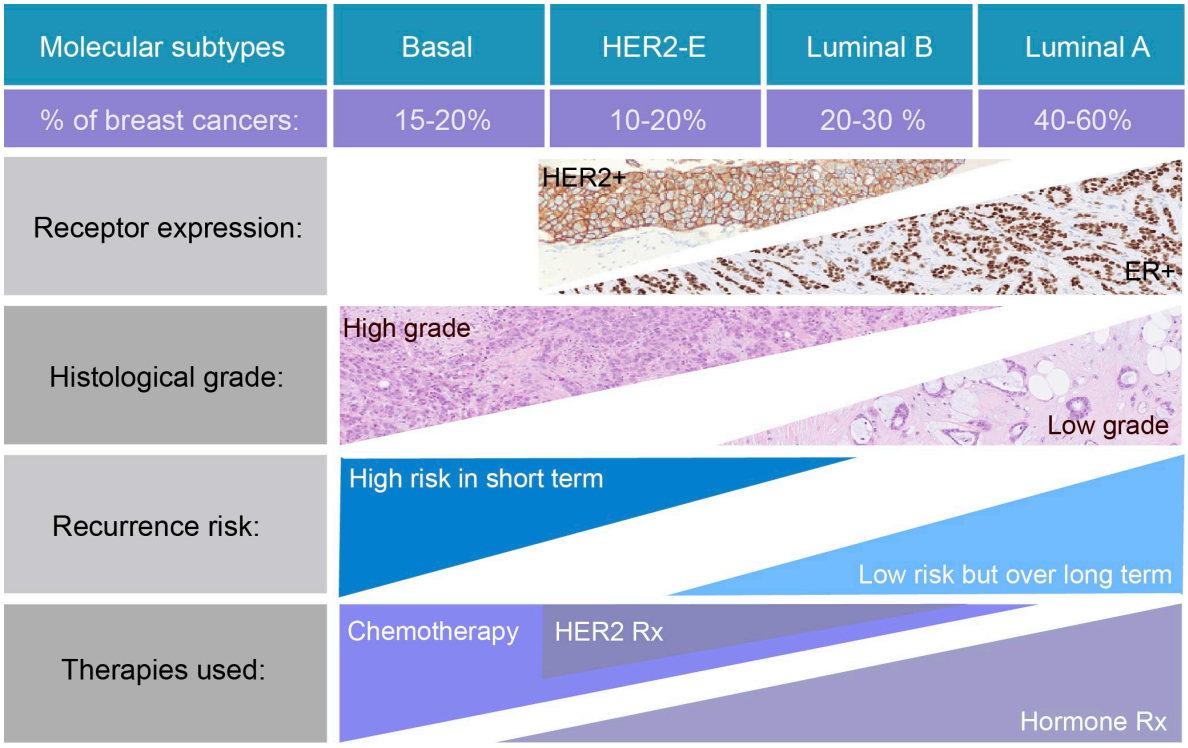

Figure 6. Molecular subtypes of breast cancer and the correlation to clinicopathological features. HER2-E, HER2-enriched; HER2 Rx, HER2-targeted therapy; Hormone Rx, endocrine therapy. Adapted from WHO Classification of tumours 2019[85].

1.7.1 Luminal A

The luminal A and B subtypes of breast cancer are the most heterogeneous subtypes characterised by the expression of ER-associated genes, and together account for 70% of all breast tumours[220]. Luminal A tumours show a high expression of luminal epithelial genes, low expression of proliferation/cell-cycle related genes (e.g. *MKi67*) and have a good prognosis[58-60, 221]. At a protein level, these tumours show high expression of ER and PR, but low Ki67 and HER2. The Luminal A subtype is a heterogeneous group of tumours and the most frequent somatic mutations include *PIK3CA, GATA3, MAP3K1 and TP53* [220].

1.7.2 Luminal B

The Luminal B subtype corresponds to approximately 20% of all breast cancers[220]. Luminal B tumours show higher expression of proliferation/cell-cycle related genes, have poorer prognosis and are less sensitive to endocrine therapy than luminal A tumours[222]. The major difference between luminal A and B subtypes is the expression of proliferation-associated genes, and thus Ki67 has been suggested to act as a surrogate marker for discrimination between the two subtypes[223]. Furthermore, luminal B tumours have been associated with high tumour grade (NHG 2-3), whereas luminal A tumours rarely are NHG 3[60]. Luminal B tumours

exhibit a higher number of mutations including *TP53*, but similar *GATA3* mutations and less *PIK3CA* and *MAP3K1* mutations as compared with luminal A[212].

1.7.3 HER2-enriched

HER2-enriched tumours are characterised by a high level of *ERBB2* gene expression, *ERBB2* amplicon genes (e.g. *GRB7*) and receptor tyrosine kinases (e.g. *FGFR4* and *EGFR*), and a low expression of luminal genes[58, 224]. The HER2-enriched subtype clinically best corresponds to ER-negative, HER2-positive tumours, effectively targeted with anti-HER2 therapies[60, 225]. The highest numbers of mutations are found within this tumour subtype, and include both *TP53* and *PIK3CA*. However, the HER2-enriched subtype is not uniquely found among HER2 overexpressing or *ERBB2* gene-amplified tumours, but is also present within HER2-negative tumours[226]. Furthermore, it was recently shown that HER2-enriched tumours are more frequently found to have a high mRNA expression of *ERBB2* and are associated with increased response to HER2-targeted therapy compared with those with low *ERBB2* expression[224]. In addition, tumours with low *ERBB2* expression comprised a variety of all molecular subtypes[224].

1.7.4 Basal-like

The basal-like subtype has the most distinct genomic profile containing genes (e.g. *c-KIT, FOXC1 and P-cadherin*) characteristic of basal epithelium; on the DNA level, it shows a high prevalence of *TP*53 mutations[60]. Basal-like tumours mainly correspond to the triple-negative phenotype, although a small proportion of ER-positive tumours may also display a basal-like phenotype[58, 59, 217, 221]. In addition, 2-17% of basal-like tumours feature HER2/*ERBB2* overexpression or amplification[226]. Rare histological subtypes such as medullary and adenoid cystic carcinoma can be found among basal-like tumours.

Triple-negative tumours, lacking ER, PR and HER2 expression, are often high-grade tumours with poor prognosis[227]. Unsupervised analysis of triple-negative tumours revealed several subtypes, such as luminal androgen receptor, mesenchymal, basal-like immunosuppressed and basal-like immune-activated, each with different prognoses[228, 229]. These distinct molecular subgroups may reveal future targets for precision medicine and need further investigation.

1.7.5 Normal-like

The normal-like subtype comprises about 5-10% of all tumours, including both ER-positive and ER-negative tumours. The classification of normal-like is thought to be a mixture of normal breast tissue and tumour or a group of basal-like tumours without expression of proliferation associated genes[214, 217, 218]. The clinical significance of this subtype is not fully understood.

1.7.6 Claudin-low

The biological and clinical significance of the more recently identified subtype of claudin-low tumours is still somewhat uncertain[218]. The claudin-low subtype is a heterogeneous group and recent findings have identified three distinct subgroups within the claudin-low subtype each of which emerge from different cells of origin[230]. These tumours show a characteristically low

gene expression of tight junction proteins (claudin 3,4 and 7) and E-cadherin[231]. The claudin-low subtype has a low expression of luminal and proliferation-related genes, but overexpresses immune response genes, which suggests tumours with high immune infiltration[231]. They are also enriched in epithelial-to-mesenchymal transition and cancer stem cell features. The claudin-low subtype is associated with poor prognosis and is over-represented among metaplastic and medullary carcinoma[231]. Claudin-low together with basal-like tumours constitute the majority of triple-negative breast tumours[230].

1.7.7 Prognostic multigene signatures

With the increasing demand for personalised precision medicine, multiple commercial gene expression-based assays and risk stratification analyses have been developed and are now available. Gene expression analysis can improve stratification of patients and select patients who may actually benefit from cytotoxic chemotherapy. For patients >50 years with ER-positive HER2-negative and lymph node-negative breast cancer, valuable prognostic information can be attained from gene expression analysis for risk categorisation of the tumour prior to choice of chemotherapy[206]. Several multigene signature assays show robust prognostic clinical utility in identifying low-risk node-negative early breast cancer without benefit of chemotherapy[232-235]. Similar prognostic results have been demonstrated for patients with limited lymph node positivity (1-3 lymph node metastases) with Prosigna and EndoPredict[236-239]. With the collected evidence, the current recommendation is to omit chemotherapy in ER-positive/HER2-negative cases with low-risk multigene signatures and limited lymph node involvement[234, 240, 241]. The following section will cover the evidence-based gene expression assays available for clinical use in Europe. A principal overview of multigene signature assay is illustrated in Figure 8.

1.7.7.1 Oncotype DX recurrence score

Oncotype DX Breast Recurrence Score® (Exact Sciences Corp., Madison, USA) is a quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)-based 21-gene signature assay that generates a recurrence score between 0 and 100, indicating the likelihood of distant metastasis[232, 235]. There is a large amount of data supporting the prognostic value of Oncotype DX, and it is the most validated multigene signature test[241-243]. The prospective trial TAILORx was specifically designed to investigate its clinical utility in ER-positive/HER2-negative and node-negative breast cancer[240]. Secondary data analysis showed that only premenopausal patients with a recurrence score of 16-25 had a decreased risk for distant metastasis when treated with chemotherapy in addition to endocrine therapy, compared with endocrine therapy alone[244]. There is, however, limited evidence for the use of Oncotype DX recurrence score to identify low-risk cases among lymph node-positive patients, who could be spared chemotherapy[245]. Results from the ongoing randomised trial RxPONDER may further clarify this (ClinicalTrials.gov Identifier: NCT01272037).

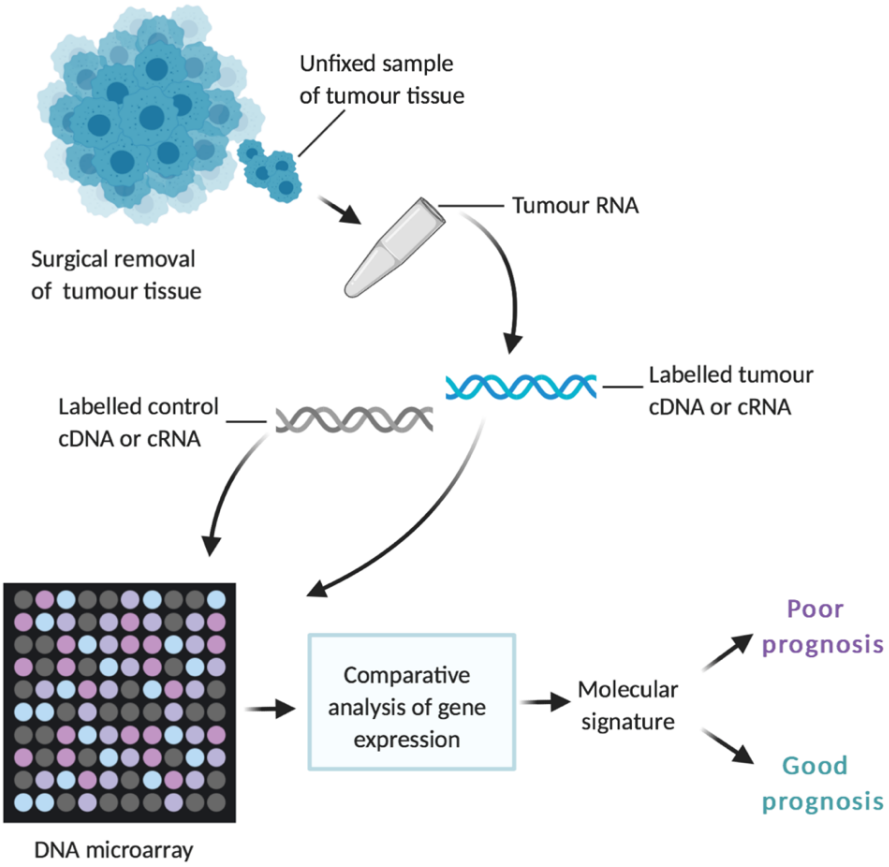

Figure 7. Principal overview of molecular gene expression signature assays. Adapted from van't Veer *et al*. 2005[247]. Created with Biorender.com.

1.7.7.2 PAM50-based Prosigna risk of recurrence score

As mentioned above, the PAM50 assay classifies tumours into luminal A, luminal B, HER2-enriched and basal-like. The PCR-based PAM50 signature was combined with clinical information into a model that provides a prognostic risk of recurrence score[214]. Based on this, Prosigna® (NanoString Technologies, Seattle, USA) was developed, which is a PAM50-based classifier of risk of recurrence score that is optimised for formalin-fixed paraffin-embedded (FFPE) material and utilises the NanoString nCounter DX analysis system instead of PCR analysis for improved accuracy of mRNA expression[238]. The PAM50-based risk of recurrence score has demonstrated robust prognostic value in ER-positive/HER2-negative breast cancer, and is currently the only multigene signature assay that provides molecular subtypes[214, 236, 239]. Sestak *et al*. recently compared six multigene signatures among node-negative ER-positive/HER2-negative patients and found that PAM50 risk of recurrence, followed by Breast Cancer Index and EndoPredict provided the strongest prognostic value for overall and distant recurrence[246]. The PAM50-based Prosigna assay and risk of recurrence score are currently used in clinical practice in Scandinavia.

1.7.7.3 MammaPrint

MammaPrint® (Agendia, Amsterdam, the Netherlands) is based on the Amsterdam 70-gene signature and utilises both FFPE and fresh-frozen tumour material. Based on microarray

expression profiling, the tumour is classified as posing a low or high risk of recurrence[213, 234, 247]. The prognostic value of MammaPrint has been validated, although not as extensively as Oncotype DX[242]. The clinical utility of MammaPrint was further investigated in the MINDACT trial, but no predictive value for choice of chemotherapy could not be demonstrated[234].

1.7.7.4 EndoPredict

EndoPredict® (Myriad Genetics, Salt Lake City, USA) is a 12-gene expression assay that provides a score from 0 to 15 along with low or high risk for early and late distant recurrence in ER-positive/HER2-negative early breast cancer[248, 249]. The prognostic value of EndoPredict has been demonstrated in both node-negative and node-positive ER-positive/HER2-negative breast cancer[237, 248, 250, 251].

1.8 IMMUNOHISTOCHEMICAL SURROGATE SUBTYPES

With reduced costs, gene expression profiling is becoming more integrated in routine diagnostics of breast cancer. However, such assays are not universally accessible, especially not in low-income countries. The molecular intrinsic subtypes can be recapitulated using the IHC measurements of ER, PR, HER2 and Ki67[77, 110, 111, 221]. Surrogate subtypes are defined as luminal A-like, luminal B-like (HER2 negative), luminal B-like (HER2 positive) and triple negative.

1.8.1 Surrogate subtype classification

1.8.1.1 The international St Gallen consensus recommendations

The initial Ki67 cut-off set to 14% to dichotomise luminal B from luminal A tumours was established by Cheang *et al.*[223]. Furthermore, the 2011 St Gallen International Expert Consensus adopted the gene expression profiling subtypes and proposed IHC-based surrogates, which included Ki67 (14% cut-off) to distinguish luminal B-like from luminal A-like tumours[111]. With increasing evidence, the 2013 St Gallen panel presented clinicopathologic surrogate definitions of the intrinsic subtypes, with emphasis on the luminal subtypes and their definitions[77], suggesting that either high Ki67 or low PR may distinguish between luminal A-like and luminal B-like (HER2 negative) tumours. However, the controversies regarding Ki67 cut-offs between high and low values remained, and laboratory specific values were recommended, although a ≥20% threshold was suggested for high Ki67 status. Furthermore, Prat *et al*. demonstrated the prognostic value of PR and the use of a PR cut-off of ≥20% to define luminal A-like cancer[132]. In addition, the St Gallen recommendations suggested type of systemic treatment for each defined surrogate subtype in 2013. The surrogate subtype definitions of the St Gallen 2013 were as follows: Luminal A-like: ER positive, PR positive, HER2 negative and Ki67 low; Luminal B-like (HER2 negative): ER positive, HER2 negative and Ki67 high or PR negative; Luminal B-like (HER2 positive): ER positive, HER2 positive and any Ki67 or PR; HER2 positive (non-luminal): HER2 positive and ER and PR absent; Triple negative: ER and PR absent and HER2 negative (Table 1)[77, 132].

In the 2015 St Gallen expert consensus, a Ki67 cut-off within the range of 20-29% was accepted to distinguish luminal B-like disease[110]. In recent recommendations, tumour grade in addition to Ki67 was suggested to distinguish between luminal A and B-like tumours[252]. Adding NHG to St Gallen 2013 surrogate subtype classification of ER-positive/HER2-negative tumours has demonstrated independent prognostic information in Swedish cohorts[253]; ER-positive/HER2-negative tumours of NHG 1 had similar prognosis as luminal A-like tumours, whereas NHG 3 tumours had prognosis similar to luminal B-like tumours[253].

The current European Society for Medical Oncology guidelines, however, refer to the St Gallen 2013 surrogate definitions of intrinsic subtypes[72]. The expert consensus regarding early breast cancer from the 16th St Gallen International Breast Cancer Conference held in 2019 recommends gene expression assays for distinguishing luminal A from B tumours, and raises concerns regarding the lack of validity for basing treatment decisions (adjuvant chemotherapy) on only IHC-based surrogate subtypes[194].

Table 1. Surrogate subtype classification adapted from the St Gallen 2013 consensus[77, 132].

Intrinsic surrogate subtype	Clinicopathologic surrogate definition
Luminal A-like	ER positive (≥1%) and PR positive (≥20%; Prat 2013) *and* HER2 negative *and* Ki67 low (<20%; panel consensus)
Luminal B-like (HER2 negative)	ER positive (≥1%) HER2 negative *and at least one of:* Ki67 high (≥20%; panel consensus) PR negative or low (<20%; Prat 2013)
Luminal B-like (HER2 positive)	ER positive (≥1%) HER2 over-expressed or amplified *any* Ki67/PR
HER2 positive (non-luminal)	HER2 over-expressed or amplified ER and PR absent (<1%)
Triple negative (ductal)	ER and PR absent (<1%) HER2 negative

1.8.1.2 Swedish national guideline recommendations

Congruence between IHC-based surrogate subtypes and the gene expression-based intrinsic subtypes is of utmost importance with regard to clinical implementations. In 2014 Maisonneuve and colleagues introduced an intermediate Ki67 group and demonstrated the prognostic value of PR in this group[254]. The most recent Swedish national guidelines adopted

this work and recommend a three-tier classification of Ki67 into low, intermediate and high proliferation for ER-positive/HER2-negative tumours, with the use of laboratory-specific Ki67 cut-offs (Figure 8)[12]. In addition, tumours showing intermediate Ki67 levels, PR positivity ≥20% is required for luminal A-like disease. The major impact of these distinctions between luminal A and B-like tumours is the clinical implication for utility of adjuvant chemotherapy[254]. As mentioned in section 1.6.1, the Swedish classification for ER positivity is ≥10% as opposed to ≥1%, which is generally considered positive internationally[136].

The concordance rate between PAM50 molecular subtype and IHC-based surrogate classifications by St Gallen 2013 is rather poor (62%, kappa 0.30) regarding ER-positive/HER2-negative tumours but can be somewhat improved by applying the Maisonneuve classifications (66%, kappa 0.35)[254], and as shown in a Swedish population-based cohort even further improved by using a grade-based classification for distinguishing luminal A from luminal B tumours (70%, kappa 0.41)[255]. Furthermore, by only classifying NHG 1-2 as luminal A-like and NHG 3 tumours as luminal B-like, the agreement with PAM50 subtype (luminal A and B) could be even increased (80%, kappa 0.46)[255]. Apart from grade the three-tier Ki67 groups are incorporated into the current Swedish national guidelines, as illustrated in Figure 8 and Table 2.

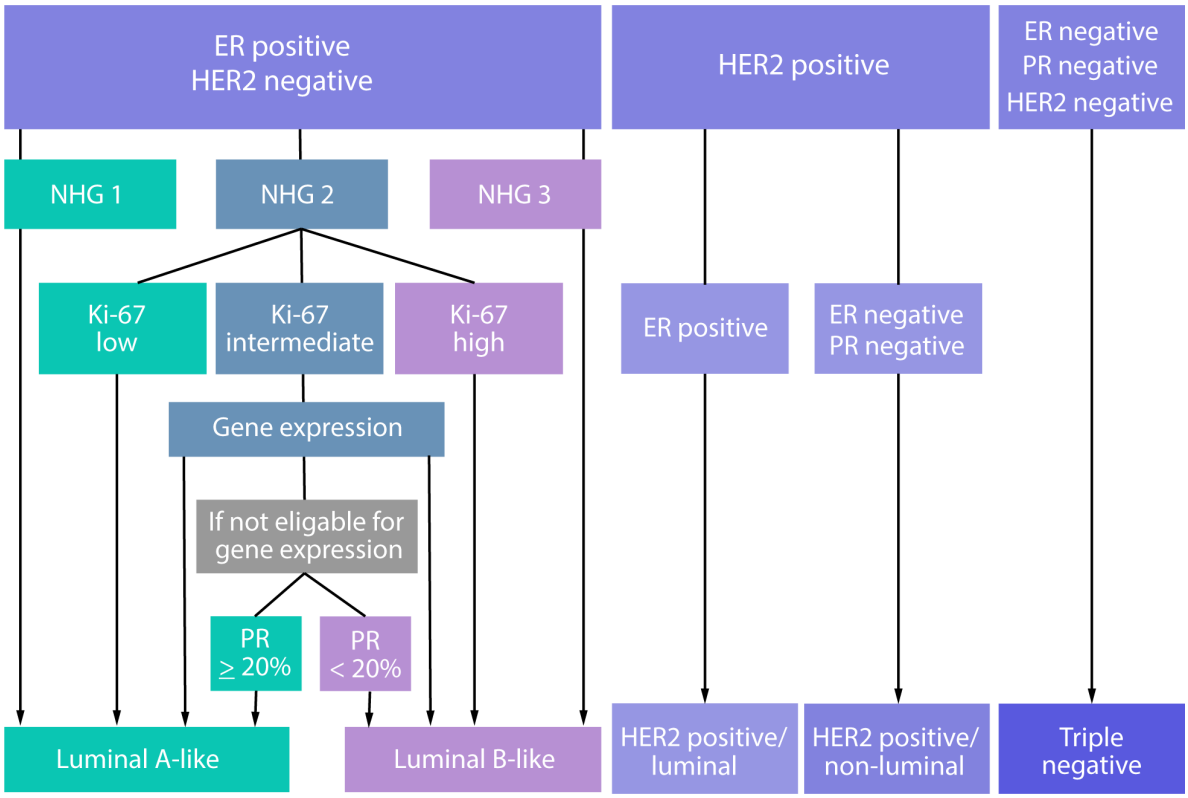

Figure 8. Flow diagram for surrogate subtype classification according to the Swedish guideline recommendations. ER-positivity defined as ER ≥10%. Laboratory-specific cut-offs are used for determiningnlow, intermediate and high Ki67 groups. Gene expression analysis is recommended for patients aged > 50 years when surrogate subtype will have effect on treatment decisions. Adapted from the 2020 National care program for breast cancer[12].

Table 2. Surrogate subtype classification adapted from the 2020 Swedish national guidelines[12].

Intrinsic surrogate subtype	Clinicopathologic surrogate definition
Luminal A-like	ER positive (≥10%) HER2 negative *and* Ki67 low° and NHG 1-2* *or* Ki67 intermediate and PR ≥20% and NHG 1-2*
Luminal B-like (HER2 negative)	ER positive (≥10%) HER2 negative *and* Ki67 high° and NHG 2-3* *or* Ki67 intermediate° and PR <20% and NHG 2-3*
Luminal B-like (HER2 positive)	ER positive (≥10%) HER2 over-expressed or amplified *any* Ki67/PR/NHG
HER2 positive (non-luminal)	HER2 over-expressed or amplified ER and PR negative
Triple negative (ductal)	ER negative (<10%) and PR negative (<10%) HER2 negative

°Ki67 scoring according to laboratory-specific cut-offs for low, intermediate and high Ki67.
*Tumours with e.g. NHG 1 and high Ki67 or NHG 3 and low Ki67 should warrant re-evaluation.

1.9 GENOMIC PROFILING OF BREAST CANCER

Evidently, there is an increasing demand for personalised cancer diagnostics and treatment. DNA and RNA sequencing-based molecular profiling of tumours holds potential to provide patients and clinicians with information on tumour characterisation and genomically matched therapy[256, 257]. High-throughput massively parallel NGS has rapidly changed the content and throughput of sequencing-based diagnostics. RNA sequencing-based molecular profiling of breast cancer could be used to predict the current routine biomarkers ER, PR and HER2. One of the great advantages provided by sequencing-based diagnostics is the improvement of models for patient stratification, such as a transcriptomic grade model, and additional information on targetable somatic alterations[258]. Sequencing of single genes such as *EGFR* and *KRAS* in non-small cell lung cancer is now part of routine pathology. However, a broader approach is needed to identify therapy-predictive mutations and structural variants. Several university hospitals, Karolinska University Hospital included, are on their way to design in-house gene panels for pan-cancer testing for targeted drugs. The nation-wide initiative Genomic Medicine Sweden is developing a national Swedish gene panel for genomic profiling.

With the advances in PI3K inhibitors, clinical practice will require PIK3CA mutation analysis, and sequencing panels need to be available (see section 1.11.6).

Zehir *et al*. recently demonstrated the application of prospective clinical sequencing of solid tumours using MSK-IMPACT, a hybridisation capture-based NGS panel, in order to guide the use of genomically matched therapies[259]. Notably, in the SHIVA trial, investigating the use of molecularly targeted "personalised" agents outside their indications and based on tumour molecular profiling, they could not confirm any improved patient outcome compared to routine treatment of choice[260]. To date, off-label use of molecularly targeted agents is discouraged. The true clinical utility of molecular profiling is still uncertain and further clinical trials are required to prove the efficacy of molecular targeted therapies.

1.10 DIGITAL IMAGE ANALYSIS

Computerised image analysis in histopathology of breast tumours holds promise to improve breast cancer diagnosis, including reproducibility for biomarkers and novel methods for precision pathology[261]. However, there is controversy about how imaging should be implemented[110, 262]. The development of systems of digital image analysis (DIA) has shown excellent reproducibility and accuracy, though so far in subsets with individual biomarkers or smaller populations[263-266]. There is an expanding industry and competition for digital pathology image analysis solutions, and several available software solutions for breast pathology have emerged. The majority of the commercial solutions operate on input from scanned whole-slide images. The focus of DIA has until recently been on quantifying biomarkers by IHC. Apart from biomarker analysis, automated image analysis has e.g. been demonstrated to eliminate negative metastasis-free sentinel node biopsy images stained by IHC pancytokeratins, as a screening method[267]. The most exciting advances in digital pathology have been reached using artificial intelligence (AI) and machine learning approaches[261, 268] (see section 1.10.4).

Several pathology departments are adopting a digital work flow with whole-slide image scanning as an integrated part of the histology laboratory workflow, and the demand for advanced image analysis software is steadily increasing. The U.S. Food and Drug Administration recently approved the first whole-slide imaging system for digital surgical pathology[269]. Apart from routine diagnosis, digital pathology enables remote consultation and telepathology, interactive presentations of tumour features in multidisciplinary team conferences, enhanced histopathology education and research approaches.

1.10.1 Image data acquisition

To enable any kind of DIA, histopathological glass slides need to be digitised into an image file. Whole-slide scanners capture tissue slide images tile by tile or in a line-scanning manner, and assemble the tiles or lines to create a digital image of the tissue[270]. Modern scanners utilise incorporated tissue recognition to allow for efficient scanning and focus points that operate by continuous automatic refocusing. In parallel to a brightfield microscope, scanning can be performed in several magnifications. For most purposes scanning at x20 magnification is the standard for HE and IHC images. However, x40 magnification provides in-depth details

required for e.g. ISH analysis or for training advanced machine learning models. Modern scanners have the capacity to load up to 400 slides and depending on magnification and scanner, scanning times vary from 30 seconds to minutes per slide [271]. Regarding digitisation of cytological slides special considerations are needed to improve digital cytopathology[272]. Cytological slides are composed of three-dimensional cell groups, and for this, optimal z-axis scanning is required for accurate focus. The variety of cytological specimens result in direct smears, liquid-based cytology and cell blocks, all of which require different approaches[272]. With the rapid technological development, high-quality cytopathological whole-slide imaging is in the near future[273].

High-resolution viewing of digital slides, or whole-slide images, is both dependent on the resolution of the scanner as well as the resolution of the monitor. An image scanner at x40 generally has a resolution of 0.25 µm/pixel and a 24-bit colour depth[270]. The file size of whole-slide images often exceeds 1 GB, which poses challenges for storage capacity. In the digitisation of routine pathology workflows, not only the storage is an issue but also the time for archiving digital images. This needs to be both standardised and regulated across countries, and must follow patient data protection regulations.

1.10.2 Image processing techniques

A variety of image processing methods are used for pre-processing, nuclei detection, segmentation, separation and classification[274]. Whole-slide imaging reflects variations in tissue staining intensities and colours even better than glass slides. Colour differences may occur when the same tissue slide is digitised with different scanners or presented in different viewers and displays[270] but there are several colour normalisation methods developed for both HE and IHC staining[274], and colour deconvolution is one of the methodologies for normalisation[275, 276]. Other pre-processing steps that can be used to adjust for adverse conditions across several images (batches) are illumination normalisation, noise reduction and region of interest detection.

For many histopathological applications, such as IHC counting and mitosis detection, optimal nuclei detection methods are important. For tumour grading, the quality of nuclei segmentation has profound impact. Nuclear heterogeneity and overlapping can be handled with nuclei separation methods. Furthermore, with the previous methods computed, nuclei features and classification generate more information required for e.g. tumour detection[274].

1.10.3 Software

Several scanner systems also provide image viewing software. The basic features of viewers include efficient image overview with zoom features, annotation tools, and selections of regions for snapshots and exporting images. More advanced image analysis features are usually provided in locked pre-defined packages from vendors and can be integrated into the workflow. A freely available alternative is the ImageJ software, developed by the National Institute of Health, which thus pioneered the field of open source image analysis algorithms[277]. In 2017, the open source platform QuPath was developed, specifically designed to handle whole-slide

images, and also allowing researchers access to advanced open source algorithms[278]. In the following sections, two different software platforms, which were used in this thesis will be described in more detail.

1.10.3.1 Visiopharm Integrator System

Within the Visiopharm Integrator System (VIS) provided by Visiopharm A/S (Hoersholm, Denmark), a virtual double staining technique has been developed where a pancytokeratin-stained tumour section is aligned with a parallel section IHC-stained for the biomarker of interest, e.g. Ki67 or PHH3. The Conformité Européenne (CE) In Vitro Diagnostic (IVD) approved algorithm application (so called APP) for tumour detection (PCK VDS APP) enables automatic precise biomarker analysis of tumour cells, omitting proliferating stromal cells and artefacts. Hereby, only epithelial cells are eligible for image analysis to determine the percentage of stained tumour cells expressing the specific biomarker (Figure 9). Depending on tissue and task, individual APPs are developed and then run for each image of biomarker[279]. Using the VIS with virtual double staining, Stålhammar *et al*., showed the advantages in congruence to gene expression assays and the prognostic power of automated image analysis compared to current manual methods of biomarker assessments[280]. Further APPs for specific tumour regions of interest have been developed and investigated on proliferation-associated biomarkers such as Ki67 and PHH3. Using the VIS and these applications, findings presented within my thesis project show that digital image analysis of Ki67 in hot spots outperforms the alternative proliferation-associated markers including mitotic count, when compared to other tumour regions and manual assessments, as well as in the discrimination of good versus poor outcome among breast cancer patients (described further in **paper III)**[281].

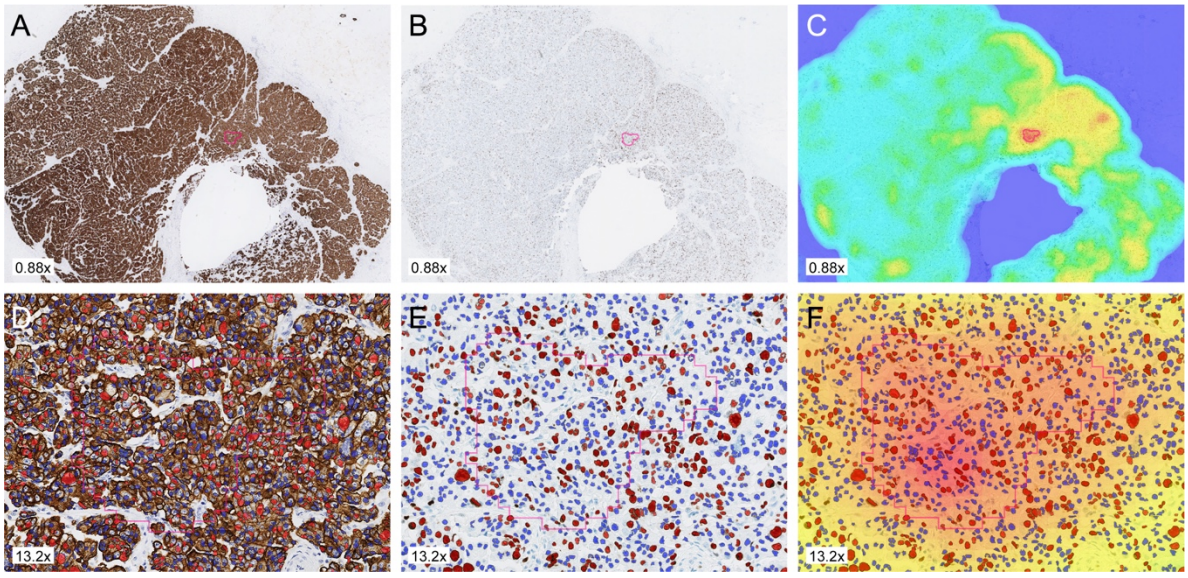

Figure 9. Virtual dual staining with Visiopharm Integrator System hot spot APP. Aligned CKMNF116 (**a**) and Ki67 (**b**) parallel IHC stained tumour sections. Hot spot (pink circle) identified using the heatmap feature (**c**). High magnification (**d-f**) of the hot spot area in **a-c**, respectively. Modified from Robertson *et al*. 2020[282], reprinted under a creative common license.

The CE IVD hot spot APP has a number of configurable parameters, which enable adaption of the hot spot to the user's objectives. Recently, automated Ki67 assessment using a virtual triple staining (pancytokeratin, p63, Ki67) method and the hot spot APP in the VIS showed high concordance with the pathologist review and prognostic utility[283]. Apart from APPs focusing on IHC biomarker evaluation, Visiopharm recently launched a CE IVD approved APP for detection of lymph node metastasis using an AI approach, and also provides entire software systems including Hamamatsu scanners for a digital work flow.

1.10.3.2 QuPath software

QuPath is an open source software platform[278] for bioimaging designed for digital pathology image analysis. The QuPath platform is user friendly and incorporates a broad spectrum of annotation and visualisation tools. It is written as a cross-platform java application and apart from ready-made algorithms, allows the user to develop custom workflows and add extensions. Built-in workflows range from tissue microarray analysis to whole-slide image processing. Cell segmentation algorithms allow for object detection across an entire whole-slide image for measuring biomarker IHC expression and morphology. This object feature further allows for object classification and trainable cell classification (Figure 10). The QuPath software utilises machine learning methods such as colour deconvolution, cell segmentation algorithms and supervised classifiers[275, 284, 285]. QuPath has previously demonstrated high reproducibility for Ki67 scoring[181]. In addition, an algorithm for scoring of TILs in malignant melanoma on HE sections using QuPath recently demonstrated prognostic potential[197].

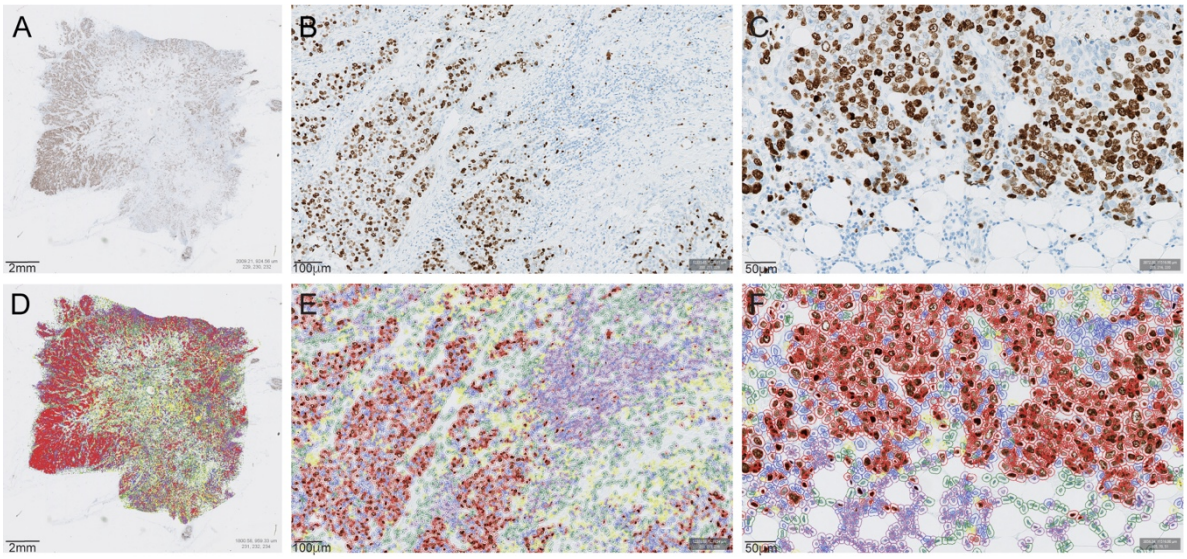

Figure 10. A QuPath cell classification algorithm for Ki67 scoring in breast cancer. The cell classifier correctly identifies different cell types (**d-f**) in the Ki67 stained tumour slide (**a-c**). Ki67-positive tumour cells in red, Ki67-negative tumour cells in blue, stromal cells in green, immune cells in purple and other cells in yellow. Modified from Robertson *et al*. 2020[282], reprinted under a creative common license.

1.10.4 Machine learning

The exploding interest and recent breakthroughs in AI may revolutionise the way cancer, including breast cancer, is detected and treated in the near future. Machine learning is a subfield

of AI that has the ability to learn from data and recognise patterns without human instruction. Supervised machine learning models require exposure to a properly labelled training data (e.g. images with clinical classification, outcome, annotations) but without the need for specific instructions, and can be used for automated pattern recognition and prediction. Unsupervised machine learning approaches learn from a data set without labels available. Both methods need to be tested on a ground truth test set after the initial training step.

Deep learning is a recent machine learning approach that has produced ground-breaking results in e.g. image classification and speech recognition and uses biologically inspired networks, so called neural networks that requires minimal processing on input or output values[286]. By an end-to-end approach to learning it takes raw images (input) and learns a model to produce the desired output (Figure 11).

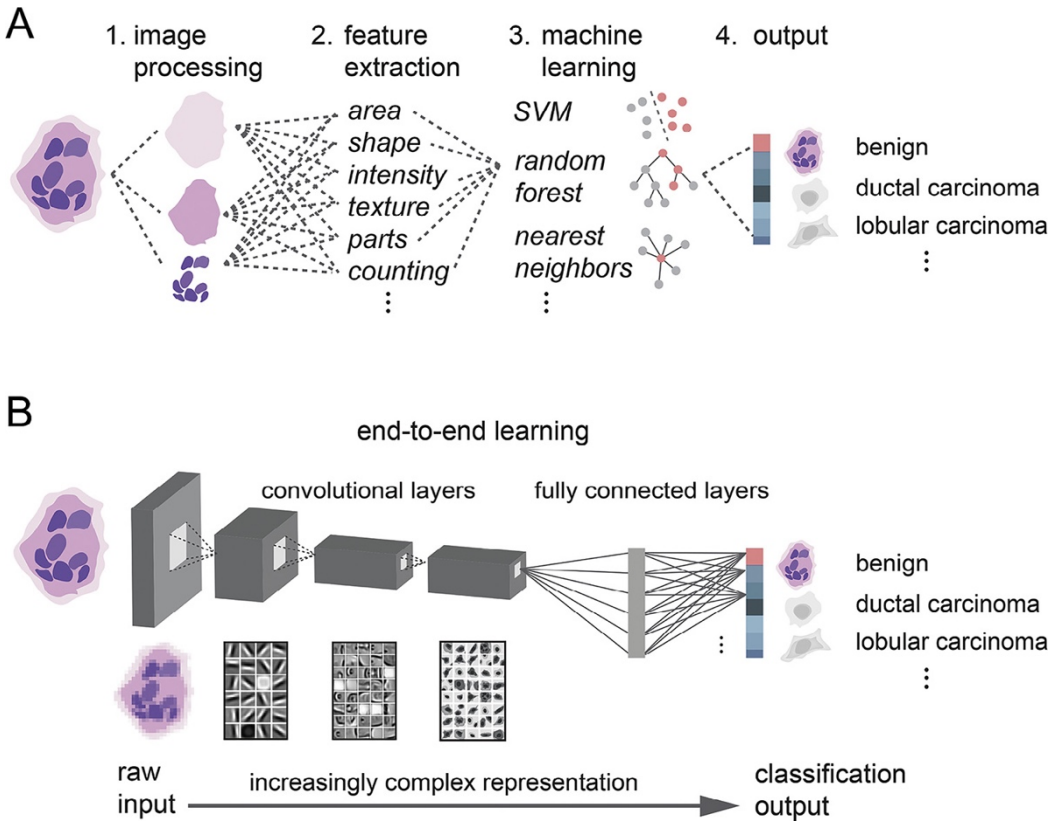

Figure 11. Deep learning versus traditional machine learning. Traditional machine learning with steps requiring human expert knowledge to recognise tumour in images (**a**). Deep learning as an end-to-end learning approach that utilises convolutional neural networks (**b**). Reprinted from Robertson *et al.* 2018[268], with permission from Elsevier (author's material).

Deep learning algorithms are tackling complex pattern recognition tasks in histopathology apart from providing computer-aided diagnosis[287] and has been applied for detection of tubular formation[288], nuclear pleomorphism[288, 289], tumour grading[288-290], as well as for mitosis detection[287, 291-296]. Convolutional neural networks have shown to facilitate classification of benign versus malignant lesions[297] and invasive tumor detection[298, 299] in breast cancer (Figure 11).

Machine learning algorithms are being applied for computer-aided prognostic models based on histologic features, gene expression profiling and outcome data combined to distinguish patients with more aggressive disease. Although deep learning has demonstrated promising results, further studies are needed for validation and to assess their use before models are implemented in clinical decision making[261, 268].

1.11 BREAST CANCER TREATMENT

1.11.1 Surgery

While surgical removal of the tumour is the established main treatment for early breast cancer, surgical approaches have improved considerably over the last decades. There is strong evidence from multiple randomised trials with long-term follow up that breast-conserving surgery followed by radiotherapy provides survival outcomes equivalent to mastectomy for early breast cancer[300-302]. Recent studies, however, have reported increased breast cancer-specific and overall survival after breast-conserving surgery with whole-breast radiotherapy compared to mastectomy without radiotherapy[303-305]. Breast-conserving surgery is recommended as long as tumour-free margins can be obtained with an acceptable cosmetic result. Free margins are by histopathological examination determined when there is "no tumour in ink"[194]. For large tumours, where primary surgery cannot provide these criteria, the patient may be treated with preoperative (neoadjuvant) therapy in order to achieve tumour shrinkage and subsequently allow for breast-conserving surgery (see section 1.11.3 for neoadjuvant chemotherapy). Given free resection margins, the choice of breast-conserving surgery or mastectomy does not affect survival outcomes in patients treated with neoadjuvant chemotherapy (NAC)[306]. When surgery cannot provide margins free of tumour with acceptable cosmetics, in cases of increased hereditary risk or patient preference, mastectomy with or without reconstruction is performed. For patients with distant metastases at diagnosis (stage IV), treatment is palliative with modified individual surgical strategies.

Axillary lymph node status is one of the most important prognostic factors, additionally providing information for therapy decisions[307, 308]. For clinically node-negative patients, axillary staging is performed by sentinel node biopsy. The sentinel node is the first lymph node receiving lymphatic drainage from the tumour and is identified with an injected radioactive isotope. It is a well-established and reproducible method[309-311]. Lymph nodes from sentinel node biopsies are serial sectioned, stained with HE and examined with immunohistochemistry for pan-cytokeratins, in order to detect even single isolated tumour cells. A negative sentinel node harbours no or up to 200 isolated tumour cells or single tumour cell clusters within an area of ≤0.2mm[85, 96]. A positive sentinel node biopsy may contain micrometastasis (N1mic; largest tumour cell group >0.2mm and ≤2.0mm) or macrometastasis (largest cell group >2.0mm). Completion of axillary clearance after detection of sentinel node micrometastasis in patients treated with breast-conserving surgery and subsequent radiotherapy does not increase survival and is not routinely performed[312, 313]. According to some evidence, the same is true for patients with a limited amount of sentinel node macrometastases[314]. Furthermore, axillary radiotherapy may yield equivalent locoregional control and survival outcomes as completion

axillary clearance after positive sentinel node biopsy, but results in about half the incidence of postoperative lymphedema[315]. Initiated in Sweden, the prospective randomised SENOMAC trial (ClinicalTrials.gov identifier NCT02240472) is currently validating the omission of axillary clearance also in patients with 1-2 sentinel nodes with macrometastasis, including even the thus far strongly under-represented group of patients undergoing mastectomy[316].

1.11.2 Radiotherapy

Radiotherapy is the use of ionising radiation for management of malignant disease with the aim to eradicate residual microscopic tumour while sparing the surrounding normal tissue. The normal tissue can be spared by delivering the irradiation dose in multiple fractions. Ionising radiation exposure causes cell cycle arrest, mutation induction, transformation and cell death[317]. The target for radiotherapy is the DNA, and radiation results in DNA double strand breaks, single strand breaks and modification[318]. The radiation sensitivity of cells varies depending on which cell cycle phase the cell is in with the mitosis phase being the most radiation sensitive. Radiation induces aberrations in the cell and may either be lethal or non-lethal to the cell[317].

Data from large meta-analyses conclude that adjuvant radiotherapy reduces the risk of local recurrence and breast cancer-related mortality[319]. Adjuvant radiotherapy of the breast following breast-conserving surgery is standard of care[194] and reduces the risk of recurrence with 50% after 10 years (rate ratio 0.52) and the risk of breast cancer mortality by a sixth after 15 years (rate ratio 0.82)[320]. Omitting radiotherapy after breast-conserving surgery for early-stage, low-risk, ER-positive breast cancer is a safe alternative in older patients[321].

For node-positive patients, post-mastectomy locoregional radiotherapy reduces the 10-year recurrence risk by three quarters (rate ratio 0.75) and 20-year risk of breast cancer specific mortality by a sixth (rate ratio 0.84)[322]. For node-negative patients, locoregional radiotherapy is not generally recommended after mastectomy[322]; however, tumours >50 mm may benefit from adjuvant locoregional radiotherapy. Adjuvant locoregional radiotherapy after mastectomy can also be spared patients with only one lymph node metastasis and low grade (NHG 1) tumours[322].

1.11.3 Endocrine therapy

The majority of breast tumours express ER and/or PR and are thus treated with adjuvant endocrine therapy for 5-10 years[323]. For ER-positive breast cancer, several randomized trials have demonstrated substantially reduced recurrence and death rates up to 15 years after 5 years of adjuvant tamoxifen treatment[126]. Sensitivity to endocrine therapy is correlated to estrogen receptor positivity by IHC[126].

In premenopausal patients who have significant ovarian oestrogenic activity, 5 years of tamoxifen is the standard treatment. In premenopausal patients with a high recurrence risk, the addition of ovarian suppression through a gonadotropin-releasing hormone agonist to tamoxifen or aromatase inhibitors (AIs) improves disease outcomes[324, 325].

In postmenopausal patients both tamoxifen and AIs are treatment options as monotherapy or in sequence[326]. In a meta-analysis by the Early Breast Cancer Trialists' Collaborative Group in 2015, studies comparing 5 years of AIs versus 5 years of tamoxifen showed a significant reduction of recurrence with AIs[326]. Specifically, 5 years of AIs reduced 10-year breast cancer related mortality rates by 15% (rate ratio 0.85) and reduced recurrence rates by 30% during the first years with different treatment (rate ratio 0.70).

Both pre- and postmenopausal women with low recurrence risk are recommended tamoxifen for 5 years, whereas those with high risk of recurrence (N+ or T3-4) may benefit from prolonged endocrine therapy up to 10 years[327, 328]. The MA.17 trial concluded that 5 years of tamoxifen followed by extended AIs therapy improved disease-free survival[329, 330]. The recommendation for postmenopausal patients with high recurrence risk is prolonged therapy with AIs. Recent results from the NSABP B-42 trial showed that after the completion of 5 years of endocrine treatment, the addition of 5 years of AIs did not increase disease-free or overall survival as compared to placebo[331]. However, the evidence for 5 years of endocrine therapy followed by prolonged AIs treatment is hard to evaluate due to limited follow-up, statistical power and heterogeneity in randomisation, including fewer side effects among patients with prolonged treatment. When considering prolonged endocrine therapy, individual risk assessment should be carefully balanced, and multigene assays for risk of recurrence prediction may be valuable (see also section 1.7.7)[332]. Genomic signatures are now included in the international recommendations for treatment options for ER-positive early breast cancer with limited lymph node involvement (see section 1.7.7)[194].

1.11.3.1 Selective oestrogen receptor modulators

Selective ER modulators (SERMs), a category of therapeutic agents including tamoxifen, act on the oestrogen receptor as competitive partial agonists and/or antagonists depending on target tissue. In breast tissue, tamoxifen acts as an oestrogen antagonist, whereas in bone, liver, cardiovascular system and uterus it exerts oestrogen agonist activity[333]. Tamoxifen is a non-steroidal triphenyl ethylene derivate, which was first approved for breast cancer therapy in 1977.

1.11.3.2 Selective oestrogen receptor degraders

Fulvestrant belongs to the category of selective ER degraders (SERDs). It is a pure oestrogen antagonist that bind and destabilises the ER, which is thereby degraded, and exhibits antagonistic effects in all tissues[333, 334]. Fulvestrant is approved for treatment of postmenopausal patients with advanced hormone receptor-positive breast cancer with progression following routine endocrine therapy[335].

1.11.3.3 Aromatase inhibitors

Oestrogen biosynthesis starts with cholesterol being converted to progesterone and corticoids, then further to androgens (androstenedione or testosterone) and finally to oestrogens (oestrone or oestradiol), each step catalysed by different enzymes[336]. Importantly, the last step is

catalysed by the enzyme aromatase. Aromatase inhibitors (AIs) do not block ER but instead act by inhibiting the enzyme aromatase. Thus, they reduce the oestrogen production and supresses plasma oestradiol, oestrone and oestrone sulphate levels. AIs do not affect ovarian oestrogen production, which is the main source of oestrogen in premenopausal women[337]. In postmenopausal women, on the other hand, oestrogen production mostly takes place in peripheral tissue such as adipose stroma and muscle[336].

For breast cancer treatment, there are two types of selective AIs with different mechanisms of action; irreversible steroidal inhibitors (e.g. exemestane) and nonsteroidal inhibitors (e.g. anastrozole and letrozole). The first bind permanently to aromatase and deactivates the enzyme, whereas nonsteroidal inhibitors inhibit the synthesis of oestrogen through reversible competition[336].

1.11.3.4 Resistance to endocrine therapy

Approximately 20% of patients with ER-positive primary tumours will later develop endocrine-resistant recurrences despite adjuvant therapy[338, 339]. Endocrine therapy resistance includes both *de novo* and acquired resistance, and the mechanism behind endocrine resistance is still elusive and complex. In the majority of tumours, ER expression is retained but the tumor cells do nevertheless not respond to endocrine therapy; the tumor has now progressed into a hormone refractory state[340]. This may be driven by ligand-independent ER reactivation, such as aberrant activation of receptor tyrosine kinases by mutation or amplification, or activation via Ras, which enhances PI3K and MAPK signalling[341].

Acquired mutations in *ESR1*, encoding ERα, are present in approximately 20% of ER-positive recurrences after long-term endocrine therapy[342]. For ER-positive/HER2-positive tumours, endocrine treatment is combined with HER2-targeted therapy. This is a crucial combination, since HER2 amplification is known to reduce sensitivity of endocrine agents, mainly by activating alternative survival pathways such as PI3K-Akt and MAPK pathways[343].

ER reactivation via MAPK pathways conveys a sensitive target for cyclin-dependent kinase (CDK) 4/6 inhibition[344], and the addition of CDK4/6 inhibitors (e.g. palbociclib) to anti-oestrogens in metastatic ER-positive breast cancer has shown promising results. Mutations in *PIK3CA,* encoding PI3Kα, are frequent (up to 40% in ER+/HER2-) in ER-positive tumours but without observed differences in rates among primary or resistant recurrences[345]. In PIK3CA-mutated ER-positive advanced breast cancer, however, the addition of the PI3Kα inhibitor alpelisib to fulvestrant renders prolonged progression-free survival[346].

Other reported mechanisms comprise epigenetic alterations and tumour microenvironmental changes[341]. Intratumoural heterogeneity of the before-mentioned alterations leading to endocrine resistance is a major challenge in the clinical management of metastatic breast cancer. Preclinical and clinical approaches including molecular profiling and NGS are currently probed for the discovery of novel mechanisms of resistance. Overall, endocrine

resistance results in escape of resistant cancer clones, disease progression with need for toxic chemotherapy and eventually death in progressive metastatic disease.

1.11.4 Chemotherapy

In an early meta-analysis from the Early Breast Cancer Trialists' Collaborative Group, results showed that chemotherapy increased 5-year survival rates and that polychemotherapy was more efficient than single-agent chemotherapy[347]. By 2005, EBCTCG concluded that mortality rates could approximately be halved by 6 months of anthracycline-based chemotherapy followed by 5 years of tamoxifen[338]. In an individual patient data meta-analysis, it was further demonstrated that combined taxane and anthracycline regimens or high-dose anthracycline regimens reduced breast cancer mortality by up to one third[348]. Today, chemotherapy is recommended for HER2-positive, triple-negative, and luminal B-like tumours[72]. Luminal A-like tumours seldom require adjuvant chemotherapy, except if tumour burden is high (≥T3 or ≥N2). Generally, ER-negative tumours respond best to chemotherapy[349]. When indications for adjuvant chemotherapy are uncertain, gene expression assays can be used.

For early breast cancer, anthracycline-based (e.g. epirubicin, doxorubicin) and taxane-based (e.g. docetaxel or paclitaxel) therapies comprise the standard regimens for chemotherapy, and sequential single therapy is generally recommended[12, 72]. Sequential treatment reduces the risk for development of resistance and for cumulative side effects. Anthracyclines combined with cyclophosphamide are more effective than cyclophosphamide alone, and are today part of the standard therapy (e.g. epirubicin-cyclophosphamide or fluorouracil-epirubicin-cyclophosphamide). Anthracyclines can lead to severe side effects such as cardiac mortality, severe bone marrow suppression, myelodysplastic syndrome and induced leukemia[348]. The combination of taxanes and antracyclinebased regimens (e.g. docetaxel-doxorubicin-cyclophosphamide) has a higher risk of bone marrow toxicity. Results from multiple trials suggest that non-antracycline based regimens (docetaxel/cyclophosphamide) could be an alternative in ER-positive, HER2-negative and triple-negative breast cancer[245, 350, 351]. Furthermore, in early triple-negative breast cancer, addition of capecitabin improves disease-free and overall survival[352, 353]. Systemic therapies for metastatic breast cancer will not be further covered in this thesis.

1.11.4.1 Neoadjuvant chemotherapy

Today we see that an increasing number of patients presenting with stage II and III breast cancer receive primary systemic therapy, referred to as neoadjuvant therapy. This is also evident for triple-negative and HER2-positive breast cancer of any stage[194]. Neoadjuvant therapy allows for real-time evaluation of the tumour response to given therapy and for tailored approaches in HER2-positive and triple-negative breast cancer that may improve survival outcomes[353, 354]. Neoadjuvant chemotherapy (NAC) compared with adjuvant chemotherapy does, however, not improve distant recurrence-free or overall survival, but is associated with increased risk of local recurrence[355]. Highest benefit of neoadjuvant chemotherapy is seen for

HER2-positive and triple-negative tumours. pCR in the surgical specimen after neoadjuvant therapy has prognostic value and is associated with improved survival[99].

1.11.5 HER2-targeted therapy

The advances in HER2-targeted therapies have dramatically improved the prognosis for HER2-positive disease and nowadays patients with metastatic disease treated with trastuzumab have been shown to have better prognosis than those with HER2-negative disease[158]. In 1998 the humanised anti-HER2 monoclonal antibody trastuzumab received approved by the Food and Drug Administration and was the first agent targeting HER2 in breast cancer[356, 357]. Shortly thereafter studies demonstrated prolonged survival in metastatic breast cancer treated with trastuzumab and chemotherapy and later with single-agent therapy in metastatic HER2-positive breast cancer[156, 358]. Since then several studies with long-term follow up have demonstrated improved overall survival and disease-free survival in early HER2-positive breast cancer[359]. The HERA trial showed that prolonged trastuzumab treatment (two years) did not improve survival outcome compared to one year[360]. Trastuzumab is currently the therapy of choice both in the adjuvant and neoadjuvant setting for early HER2-positive breast cancer[72, 194, 361]. Trastuzumab is generally recommended together with taxane-based chemotherapy but anthracyclines are to be avoided due to heart toxicity[362].

1.11.5.1 Monoclonal anti-HER2 antibodies

Trastuzumab binds the extracellular juxtamembrane domain IV of HER2, which inhibits HER2 signalling through several mechanisms including prevention of homodimerisation, increased destruction of the receptor and activation of antibody-dependent cell-mediated cytotoxicity[363]. This is, however, not the only way to inhibit HER2-signalling and a handful of HER2-targeted agents have received approval for HER2-positive breast cancer. These advances have led to several novel agents currently in clinical trial investigations[150].

The monoclonal antibody pertuzumab binds to a different domain of HER2 compared to trastuzumab and prevents heterodimerisation of HER2 with EGFR (HER1) and especially HER3. Thus, preventing activation of PI3K pathway signalling through HER2-HER3 dimers[157]. Clinical benefit of pertuzumab added to trastuzumab was shown in HER2-positive metastatic breast cancer after progression on trastuzumab[364]. In the adjuvant setting, dual HER2 blockade with pertuzumab combined with trastuzumab and chemotherapy was shown to improve disease-free survival, especially among node-positive patients but long-term results are pending[365]. This dual HER2-targeted therapy also showed to increase pCR rates when administered together with neoadjuvant chemotherapy[366, 367]. The benefit in relation to costs of adjuvant pertuzumab is currently limited to selected high-risk HER2-positive patients.

1.11.5.2 Small-molecule tyrosine kinase inhibitors

The small-molecule tyrosine kinase inhibitors lapatinib and neratinib are clinically approved for HER2-positive breast cancer[150]. Addition of the oral HER1 and HER2 tyrosine kinase inhibitor lapatinib, demonstrated increased outcomes in metastatic breast cancer and increased

pCR when added to trastuzumab in the neoadjuvant setting[368, 369]. However, in the adjuvant setting, lapatinib failed to improve disease-free survival and added toxicity compared to trastuzumab alone[370]. Neratinib is an irreversible pan-HER tyrosine kinase inhibitor targeting HER1, HER2 and HER4, and is orally administrated[371]. Recent data suggests that prolonged anti-HER2 therapy with neratinib, after one year of adjuvant trastuzumab lowers the risk of recurrence[372].

1.11.5.3 Antibody-drug conjugates

The antibody-drug conjugate trastuzumab emtansine combines trastuzumab with a cytotoxic maytansine derivate and microtubule inhibitor[373]. Thus, this drug inhibits HER2 and delivers cytotoxic effects intracellularly. Trastuzumab emtansine was clinically approved for HER2-positive pre-treated metastatic breast cancer[374, 375], and is also recommended for HER2-positive residual tumour after neoadjuvant treatment[194, 354]. Several antibody-drug conjugates are investigated in clinical trials[150].

1.11.5.4 Risk stratification for HER2-positive breast cancer

Biomarker analysis in routine cancer diagnosis requires considerable resources and is hampered by insufficient specificity, which is also evident for HER2 analysis. There are currently no molecular assays for risk stratification of HER2-positive breast cancer. Thus, therapy decisions are based on HER2 testing along with ER status and disease stage. Several studies have demonstrated the relationship of the HER2 receptor with hormone dependency and the association between HER2 amplification and hormone receptor negativity[376]. This is supported by the occurrence of HER2-positive tumours not responding to endocrine therapy[377].

Deeper knowledge of the HER2 expression patterns and their correlation to outcome after adjuvant anti-HER2 therapy could be a key to selecting a personalised HER2-targeting therapy. Alternative methods for HER2 assessment have potential to improve stratification of patients into therapy-responsive subgroups. A clinical important difficulty is distinguishing patients who will derive substantial benefit from escalating therapy from those who instead would do as well with de-escalating therapy.

1.11.5.5 Resistance mechanisms to HER2-targeted therapy

Despite the established therapy predictive role of HER2 testing methods they lack accuracy to distinguish therapy-resistant tumours. Unfortunately, some HER2-positive tumours progress on HER2-targeted therapy. Several mechanisms of intrinsic and acquired resistance to HER2-targeted therapies have been described[150]. Incomplete inhibition of the HER family, could potentially be overcome by combinations of targeted therapies. The presence or acquisition of genetic, epigenetic or post-translational of HER2 can thus impair effective inhibition. The understanding of and the clinical importance of e.g. *ERBB2* mutations at progression after prior HER2-targeted therapy is continuously evolving. In the case of effective inhibition of HER2, three main mechanisms of resistance have been described: dysregulation of downstream signalling pathways (e.g. via *PIK3CA* mutations), bidirectional crosstalk with ER and upregulation of escape pathways[150]. In recent years, it has been discovered that the immune

system may be involved in the response to anti-HER2 therapies[190, 378], since immune infiltration is associated with better prognosis in HER2-positive breast cancer[190, 196, 379].

1.11.6 Emerging targeted therapies

The emerging spectrum of targeted therapies in clinical trials for breast cancer treatment cannot be covered in this thesis, but they are generally investigated in the metastatic setting. As mentioned in section 1.11.3, several agents targeting the PI3K-Akt-mTOR pathway have been developed to enhance the effect of endocrine therapy in ER-positive breast cancer[341]. The specific PI3Kα inhibitor alpelisib, was recently approved for PIK3CA-mutated ER-positive advanced breast cancer in combination with fulvestrant[346]. There are also several mTOR inhibitors, such as everolimus, which is approved for metastatic breast cancer in combination with AI regardless of PIK3CA status[380]. Furthermore, several inhibitors targeting Akt are under investigation[341].

CDKs are central player in cell-cycle regulation, and CDK4/6 inhibition demonstrated reversing endocrine resistance in advanced ER-positive breast cancer[381]. The most studied CDK4/6 inhibitor is palbociclib, which demonstrated improved outcome for both endocrine treatment naïve and pre-treated patients with advanced disease[382, 383]. There is however, no conclusive evidence for appropriate biomarkers for the current CDK4/6 inhibitors[384].

The emerging field of immunotherapies for cancer treatment has gained large interest in recent years. Immune checkpoint blockade using monoclonal antibodies against CTLA-4, PD-1 and PD-L1 have shown promising results across several tumour types[385-389]. In breast cancer, check point inhibitors targeting PDL-1/PD-1, such as atezolizumab, have been approved for metastatic triple-negative breast cancer[204, 205] and is further discussed in section 1.6.6.

The use of angiogenesis inhibiting drugs in breast cancer is not conclusive. In selected patients with advanced breast cancer the monoclonal antibody bevacizumab targeting VEGF, could potentially be added to chemotherapy[390]. Furthermore, for BRCA-mutated advanced breast cancer, Poly (adenosine diphosphate-ribose) polymerase inhibitors show promise[391, 392]. Further therapies available for advanced breast cancer will however, not be covered in this thesis.

2 AIMS OF THE THESIS

The overall objective was to improving the accuracy for assessment and prognostic value of prognostic and predictive biomarkers in routine breast pathology; to this end, we compared standard methods with the prognostic potential of digital image analysis techniques and gene expression assays.

Specific aims:

Paper I

- To investigate the concordance of biomarker assessment, surrogate subtypes and molecular subtypes on preoperative fine-needle aspiration cytology versus corresponding resected breast tumours.

Paper II

- To estimate the value of re-testing biomarkers from core needle biopsies in subsequently surgically resected breast tumours, with emphasise on HER2 and surrogate subtype concordance, in the adjuvant and neoadjuvant setting.

Paper III

- To compare the clinical relevance of mitotic counts, Ki67 and PHH3 in early breast cancer, and clarify which proliferation marker is better in terms of prognostic potential, sensitivity and specificity fin distinguishing molecular subtypes luminal B and A, and transcriptomic grade.
- To investigate differences in scoring proliferative activity in hot spots, invasive tumour margins and across the entire tumour, in relation to outcome.

Paper IV

- To investigate different configurable parameters for defining a digital hot spot for Ki67 scoring with regards to prognostic potential.
- To compare the prognostic potential for Ki67 hot spot scoring and global scoring using different digital image analysis platforms in ER+/HER2- breast tumors.

Paper V

- To investigate the prognostic significance of HER2 copy numbers, HER2/CEP17 ratio, and *ERBB2* mRNA gene expression levels in a HER2-positive breast cancer cohort treated with HER2-targeted therapy.

3 MATERIALS AND METHODS

3.1 PATIENT COHORTS

3.1.1 The immunocytochemistry versus immunohistochemistry cohort

In **paper I** we designed a retrospective cohort consisting of patients with primary breast cancer diagnosed by FNAC during 2005 and 2006 at the Karolinska University Laboratory or Capio St Göran's Hospital, Stockholm, Sweden. A total of 301 tumours with biomarker evaluations both from cytology and the corresponding surgical specimen were included. Exclusion criteria were as follows: neoadjuvant therapy, previous breast cancer within the past 5 years and missing Ki67 value. Clinicopathological data, including biomarker status, were retrieved from the laboratory information system and medical records, with end of follow-up in July 2016. Overall and breast cancer-specific survival were the measured outcomes. The cohort had a median follow-up time of 10.3 years.

3.1.2 The core biopsy versus surgical specimen cohort

In **paper II**, a retrospective study cohort was designed, consisting of 716 patients with primary breast cancer diagnosed at the Karolinska University Laboratory during 2016 and 2017, and available biomarker evaluations both on CNBs and paired surgical specimens. Two cohorts were created: a primary surgery cohort (n = 526) without NAC, and a NAC cohort (n = 190) with NAC based on biomarkers from CNBs. Patients with pCR after NAC and those with neoadjuvant endocrine therapy alone were excluded. Clinicopathological data were retrieved from routine pathology reports and medical records, with end of follow-up in March 2018.

3.1.3 The Clinseq cohort

The Clinseq study cohort of primary breast cancers comprises patients diagnosed in 2002-2010 at the Karolinska University Hospital and in 2012 at the Stockholm South General Hospital, respectively and included in the retrospective Libro1 and prospective KARMA tissue banks. The Clinseq study cohort contained 307 patients with fresh-frozen tumour tissue and germline DNA from blood, apart from routine FFPE tumour tissue. The study cohort has previously been described in detail[258]. Apart from PAM50 subtype classification (described in section 3.5), each tumour had been assigned a 'transcriptomic grade' based on RNA sequencing data[393]. No new gene expression data was performed in the studies of this thesis.

In **paper I**, we identified 84 tumours with corresponding preoperative cytology and ICC-assessed biomarkers from the Clinseq cohort that were used for biomarker and PAM50 subtype comparisons. 'Transcriptomic grade' was used for comparisons in **paper III**. Here, whole tumour sections of 204 tumours from 196 patients were stained for Ki67, PHH3 and CKMNF116, and digitised at x20 with a NanoZoomer 2.0 HT (Hamamatsu Photonics K.K., Japan) for DIA. All tumours had available PAM50 subtype and clinicopathological data. Out of these tumours, 139 ER-positive/HER2-negatve tumours were included in **paper IV** for DIA of Ki67 scoring methods.

3.1.4 The Stockholm cohort

The population-based so called 'Stockholm cohort' consists of 524 patients with primary breast cancer who underwent surgery at the Karolinska University Hospital during 1994 to 1996. Patients were identified in and clinicopathological data was retrieved from the National Breast Cancer Register and medical records. This cohort included 5- and 10-year overall survival data. The study cohort has been described and published previously[280, 394, 395]. After exclusions (only FFPE tissue, insufficient material for RNA expression arrays, neoadjuvant therapy, stage IV), fresh-frozen tumour material for RNA expression profiling was available for 159 tumours. Molecular subtype classification had been performed (see section 3.5) and histological grade re-evaluated by an experienced pathologist.

In **paper III**, whole tumour sections of 90 tumours from 84 patients were stained for Ki67, PHH3 and CKMNF116, and digitised at x20 with a NanoZoomer 2.0 HT for DIA. All tumours had available molecular subtype and clinicopathological data.

3.1.5 The Stockholm HER2 cohort

For **paper V,** we designed a study cohort comprising patients with HER2-positive primary breast cancer who were treated with HER2-targeted therapy (trastuzumab). This cohort is referred to as the 'Stockholm HER2 cohort' and consists of 591 patients diagnosed at the Karolinska University Laboratory, Stockholm, Sweden, 2006-2014. Only patients with available detailed HER2 status from either CNB or surgical specimen were included. Clinicopathological data were retrieved from laboratory information system and medical records, with follow-up until June, 2020. The following exclusion criteria were applied: stage IV disease, previous ipsilateral breast cancer, bilateral breast cancer, lack of follow-up data, HER2 negativity, lack of HER2-targeted therapy. Archived FFPE tumour tissue blocks were retrieved from 460 cases, from which parallel sections were stained for HE, HER2 IHC and HER2 ISH. All stained sections were scanned at x40 with a NanoZoomer XR (Hamamatsu Photonics K.K., Japan) and digitized for pathological assessment by experienced pathologists. Additional macro-dissected tumour material from parallel sections was used for RNA extraction protocols including STRAT4 gene expression analysis (described in section 3.6).

3.2 TUMOUR TISSUE SAMPLES

Tumour tissue from core biopsies or surgical resections (described in section 1.4.3 and 1.4.4) were fixed using 4% formaldehyde upon arrival to the pathology department. The entire biopsy or cut tumour pieces was paraffin-embedded into tissue blocks after dehydration. The tissue block is sectioned in 3-4 µm thin sections and mounted on glass slides. The whole tumour tissue sections were stained with HE and additional immunohistochemical stains or ISH as described below. All clinical FFPE tissue blocks and glass slides were stored in archives as valuable resource that enables not only diagnostic review but also tissue for research use. From surgical resections, small samples of fresh tumour tissue were obtained and snap-frozen for storage in the medical biobanks prior to fixation. This enables important opportunities for

studies of preserved DNA and RNA for high quality sequencing, both for clinical purposes and research.

In contrast, fine needle aspiration-based cytological assessments from breast tumours were performed by smear cytological evaluations and additional air-dried smears for immunocytochemical analysis of biomarkers (ER, PR and Ki67). As described in section 1.4.2, FNAC is an established method for breast cancer diagnosis at the Karolinska University Hospital. Liquid-based cytology techniques are mainly used for cervical cancer screening, but could be adapted for FNAC[396]. Cell block preparation is a technique where cytological material is processed, sectioned and stained as a histological sample, and thus allows for IHC staining and ISH[397].

3.3 IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is a technique for detection of antigens (proteins) in tissue by specific antibodies, and immunocytochemistry (ICC) is the same principal procedure used for cytological material[398]. IHC visualises the protein of interest in the tissue and provides information on its location in specific cellular compartments, such as the nucleus, the cytoplasm or the cell membrane. In the early 1990's heat-induced epitope retrieval methods revolutionised the practice of pathology by enhancing retrieval in FFPE tissue and revealing a large variety of proteins[399]. In today's pathology departments, IHC staining has advanced from previously being a complex manual process to a fully automated process. Automated staining platforms include steps as slide labelling, baking, de-paraffinisation, antigen retrieval, staining, cover-slipping, and in some cases even digital image analysis.

Rabbit and mouse are the most common hosts for antibody production. The host is injected with the antigen of interest whereby polyclonal antibodies are produced by several different plasma cell lineages as a response and antiserum containing polyclonal antibodies is obtained. In contrast, monoclonal antibodies are produced using *in vitro* tissue culture techniques; immune cell tissue (e.g. spleen) is removed from the host immunised with the specific antigen and immortalised cells are cultured to continue producing monoclonal antibodies. Importantly, polyclonal antibodies bind to multiple epitopes on a specific protein leading to high sensitivity but less specificity, while a monoclonal antibody only binds to one specific epitope, rendering high specificity. An epitope can consist of only a few amino acids. In the staining procedure, secondary antibodies that bind to the primary antibody are used to visualise the protein expression by either a chromogen, e.g. diaminobenzidine that is converted to a brown product by horseradish peroxidase tagged to the secondary antibody, or in the case of immunofluorescence a fluorophore.

Throughout the papers presented within this thesis, IHC staining was performed using the following antibodies: monoclonal rabbit anti-Ki67 (Ventana Medical Systems, USA: clone 30-9), anti-PHH3 (Biocare Medical, USA: clone BC37) and anti-HER-2/*neu* (Ventana Medical Systems, USA: clone 4B5) antibodies, and the mouse monoclonal anti-cytokeratin (Agilent Dako, USA: clone MNF116) antibody. The retrospective biomarker evaluations retrieved from

pathology reports had all been ICC and IHC stained using current routine protocols (monoclonal rabbit anti-ER (clone SP1), anti-PR (clone 1E2), anti-Ki67 (clone 30-9) and anti-HER2/neu (clone 4B5) antibodies or mouse monoclonal anti-Ki67 (clone MIB-1; for ICC) antibody) at the accredited department of Pathology/Cytology, Karolinska University Laboratory, Stockholm, Sweden. The ICC and IHC protocols used in the papers included in this thesis are described in the 'materials and methods' sections of each paper.

3.4 IN SITU HYBRIDISATION

In situ hybridisation (ISH) is a technique widely used to visualise gene amplifications, deletions, translocations and chromosomal copy number alterations in cells. ISH testing uses a labelled probe that recognises and hybridises to the target gene, and thereby enumerates the targeted gene copy numbers within the cells[164]. The DNA probe can be coupled to a fluorescent, chromogenic or silver detection system, or combination thereof for detection. Fluorescent ISH required a fluorescence microscope for evaluation, whereas an advantage of chromogenic and silver ISH is that they are evaluated in a brightfield microscope.

Fluorescent ISH signals fade over time and for **paper V,** re-testing of HER2 was performed by IHC and silver ISH on archived FFPE tumours. The HER2 dual-probe ISH staining VENTANA HER2 Dual ISH DNA Probe Cocktail assay (Roche Diagnostics, Rotkreutz, Switzerland) together with VENTANA Silver ISH DNP Detection kit and VENTANA Red ISH DIG detection kit was utilized according to the manufacturer's instructions (BenchMark ULTRA IHC/ISH Staining Module, Ventana Medical Systems, Arizona, USA). HER2 gene status was determined by brightfield light microscopy and on digitalised images. Details on HER2 ISH evaluations are described in detail in section 1.6.2.

3.5 PAM50 - GENE EXPRESSION PROFILING

Originating from microarray gene expression data Perou *et al*. identified the four breast cancer intrinsic subtypes with clinical implications[58], and later Parker *et al*. developed a 50-gene single sample predictor classifier for subtype assignment[214]. As described in section 1.7.7, NanoString's Prosigna test was approved. Research-based PAM50 subtyping is widely used. The PAM50 classifier makes calls based on the 50-gene centroid correlation distance to subtype-specific centroids[214].

3.5.1 RNA sequencing

In the papers within this thesis (**paper I, III-IV**), RNA extraction (Qiagen AllPrep kit, Germany) from snap-frozen tumour tissue in the 'Clinseq cohort' had previously been performed and used for PAM50 subtype classification. In brief, stranded RNAseq libraries were constructed (TruSeq Stranded Total RNA Library prep kit, Illumina, USA). Gene-level expression estimates were calculated (HTSeq count version 0.6.1)[400] and normalised (TMM method[401] in edgeR package[402]) using Python and R frameworks for analysis of high-throughput sequencing data. Unaligned RNAseq data from the Cancer Genome Atlas (TCGA) breast cancer data set[212] was run in parallel as reference. Molecular subtypes (luminal A,

luminal B, HER2-enriched and basal-like) had been assigned using the Nearest Shrunken Centroid classifier and the PAM50 gene set[214] using parameters from the TCGA dataset[258]. The ‘Clinseq’’ and TCGA datasets were pre-processed using the same bioinformatic pipeline to reduce potential batch differences.

3.5.2 Microarray gene expression

In **paper III**, molecular subtype classification of the ‘Stockholm cohort’ had been performed using DNA microarray profiling[394, 395]. In brief, RNA was extracted (RNeasy mini protocol, Qiagen, Germany) from snap-frozen tumour tissue. Microarray profiling was performed using protocols of Affymetrix HG-U133AB gene-chip arrays (Affymetrix, USA) for preparation of *in vitro* transcription products and oligonucleotide array hybridisation, and scanning. Raw expression data were normalised using a global mean method[403]. For microarray data analysis, genes were selected based on the ‘intrinsic’ gene list described by Sorlie *et al*[217]. Gene expression data from Affymetrix chips was matched with the publicly available reference dataset used by Sorlie and colleagues. Genes were median-centred and molecular subtypes (basal-like, ERBB2, luminal A, luminal B and normal-like) were defined by a hierarchical clustering of the reference dataset[394].

3.6 STRAT4 GENE EXPRESSION ASSAY

STRAT4 is a closed-system real-time quantitative RT-PCR (RT-qPCR) assay (XPERT® Breast Cancer STRAT4 Assay, Cepheid, Sunnyvale, USA). It is a CE-IVD RT-qPCR test that involves primers and probes to measure ERBB2 (HER2), ESR1, PGR and MKi67 transcripts together with an endogenous control gene, Cytoplasmic FMR1-Interacting Protein 1 (CYFIP1), and was used in **paper V**. Tumour tissue lysate aliquots were added to the GeneXpert cartridge containing the assay reagents, sample-preparation and STRAT4 RT-qPCR assay, which utilises a specific computer system and the GeneXpert Instrument system (Cepheid, Sunnyvale, USA). This semi-automated targeted gene expression assay provided transcript results within 2.5 h with limited manual handling.

ESR1, PGR, ERBB2 and MKi67 target gene cycle threshold (Ct) measurements by the system are individually normalised against the Ct of the reference gene, CYFIP1, resulting in delta Ct values (dCt). The target mRNA dCt measurements were classified as positive or negative based on pre-defined dCt detection cut-offs for each target mRNA based on previous studies[404, 405].

3.7 DIGITAL IMAGE ANALYSIS

3.7.1 Visiopharm Integrator System

The Visiopharm Integrator System (VIS) provided by Visiopharm A/S (Hoersholm, Denmark) was used in **paper III** and **IV**, in academic collaborations. See section 1.10.3.1 for general descriptions of the VIS software. In **paper III** and **IV**, DIA methods were applied for investigation of IHC Ki67 and PHH3 scoring. The VIS virtual double staining method operates by virtually aligning a pan-cytokeratin section with the biomarker section (e.g. Ki67 or PHH3), and thereby excludes non-epithelial cells from analysis. However, this method cannot

distinguish *in situ* from invasive lesions, and manual review to eliminate regions of DCIS by annotations from a pathologist is required. The different steps in image analysis in VIS is run by specific APPs (PCK APP, Ki67 APP, VDS APP, hot spot APP etc) designed for breast cancer. The details for annotations of invasive edges and average scoring across full sections are described in 'material and methods' section of **paper III**. The details of all configurable hot spot APP parameters are described in the 'materials and methods' section in **paper IV**.

3.7.2 QuPath

QuPath is an open source bioimage analysis software designed for image analysis of whole-slide images in pathology[278] and is described in section 1.10.3.2. A key feature is the hierarchical object-based data model, where the object is a structure or region in the image that is detected or annotated. This system allows for classification of objects and maintain relationships between objects. In **paper IV**, QuPath was used for global scoring of Ki67. Algorithms for Ki67 scoring were based on a cell classifier identifying tumour cells from stromal cells, immune cells and others using machine learning methods. The Ki67 score was calculated as the percentage of all Ki67-stained tumour cells out of all tumour cells on the whole-slide image. Larger areas of DCIS were eliminated from the analysis by manual annotations by a pathologist. Further details are described in the 'materials and methods' section in **paper IV**.

3.8 STATISTICS

The statistical methods used in this thesis is described in "materials and methods" of each paper (**paper I-V**). Only the main statistical methods are summarised here. All statistical tests were two-sided and significance considered at a $p<0.05$ level.

Normal distribution was tested with Kolmogorov-Smirnov test of normality. Comparison of proportions with categorical outcome was performed with the Chi-squared test and Fisher's exact test. The Mann-Whitney U test was applied as non-parametric test for independent samples with continuous variables.

Non-parametric tests for two dependent samples were as follows: McNemar's test or Fisher's exact test for paired samples with two categories and the Marginal Homogeneity test for paired samples with more than two categories. Related-samples Wilcoxon signed rank test was applied to continuous outcome data.

Pearson's correlations and Spearman's rank order coefficients were calculated for continuous variables. Cohen's κ statistics were evaluated for the level of agreement between categories. The Landis and Loch 1977 agreement categories for κ-values were applied as follows: 0.21-0.4 as fair, 0.41-0.6 as moderate, 0.61-0.8 as substantial and 0.81-1 as almost perfect agreement. The agreement between scoring methods in **paper IV** was assessed in a Bland-Altman plot.

In **paper II** we defined 'numbers needed to re-classify' (NNRC) as 1/risk of re-classification, in analogy with 'numbers needed to treat'. Receiver operating characteristics (ROC) and area

under the curve (AUC) methods were performed for determining new cut-offs, with equal emphasis on sensitivity and specificity (**paper I and III**). The intraclass correlation coefficient was used to test reproducibility in **paper IV** using log-transformed Ki67 values.

The Kaplan-Meier survival estimates were calculated for overall survival (OS), breast cancer-specific survival (BCSS) and recurrence-free survival (RFS). Cox regression likelihood ratio (LR) χ^2 and change in LR (LR - $\Delta\chi^2$) were used to test individual and relative prognostic values (**paper I**). The Cox proportional hazards regressions models for univariate and multivariate analysis was used to estimate the hazard ratios (HR) for recurrence or death.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Prognostic value of Ki67 analysed by cytology or histology in primary breast cancer

Accurate biomarker assessment is crucial for breast cancer management and therapy decisions. International guideline recommendations state that ER, PR, HER2 and Ki67 should be analysed from preoperative biopsies or surgical resections[72-74]. Since tumour progression may affect biomarker expression, re-analysis from metastatic sites is important, and may in some cases only be accessible for fine-needle aspiration. However, biomarkers, especially Ki67 are prone to both interobserver and intraobserver variability[114, 115]. The accuracy and concordance of biomarker evaluations across different tissue sample types has clinical implications. The IHC-based biomarkers are used to classify tumours into surrogate subtypes, which act as clinical surrogates for molecular subtyping. In the local clinical setting at the Karolinska University Hospital, preoperative ICC biomarker assessments were performed even though re-testing by IHC followed on all invasive breast tumours. This dual biomarker analysis provided a unique possibility to investigate the diagnostic efficacy and prognostic value of the two methods.

In **paper I**, we investigated the concordance of original biomarker evaluations from FNAC and consecutive surgical specimens in two combined cohorts comprising 385 tumours. Overall, the median Ki67 was higher in surgical resections compared with aspiration cytology. The concordance rate for Ki67 was 66% (κ 0.35) for paired samples, meaning that 34% of the tumours changed from low to high Ki67 score or vice versa between the two methods. High concordance was observed for ER (97%, κ 0.88).

In addition, we used surrogate subtype classifications and PAM50 subtypes to investigate the concordance between both methods. The concordance rate for surrogate subtypes based on ICC versus IHC reached 65%. Almost 50% of the tumours classified as luminal A-like on cytology were reclassified to luminal B-like after surgical resections. In the subset of tumours with available PAM50 subtype, cytology-based surrogate subtypes showed a 60% concordance rate (κ 0.23) and similarly, histology-based surrogate subtype classification had a 64% concordance rate (κ 0.31). Thus, neither of the two methods was excellent nor superior for agreement with gene expression-based subtypes.

Kaplan-Meier survival curves demonstrated that high Ki67 using IHC from resections was significantly associated with worse outcome in terms of an increased HR for overall death compared with low Ki67 tumours. No significant difference in overall survival between low and high Ki67 groups from cytology was observed. The results were similar when adjusted cut-offs were used. The individual prognostic values of ICC and IHC Ki67 scores were tested and showed that IHC but not ICC contributed with significant prognostic information.

In summary, we found a considerable difference in prognostic value of Ki67 scoring depending on diagnostic method. We demonstrate the prognostic significance of IHC assessment of Ki67 from surgical specimens compared to ICC from FNAC in terms of predicting lymph node metastasis and survival outcome. Regarding subtype classification, both ICC and IHC-based methods are suboptimal in predicting true molecular subtypes.

4.2 PAPER II

Re-testing of predictive biomarkers on surgical breast cancer specimens is clinically relevant

After **paper I**, the next step was to investigate the concordance of the four biomarkers and surrogate subtypes from CNBs and paired surgical specimens. The accuracy of biomarker evaluation in breast cancer is not only paramount in the adjuvant setting but even more so in the neoadjuvant setting, where treatment decisions are based on preoperative CNB results. Among patients with ER-positive/HER2-negative tumours, high Ki67 aids to distinguish the more aggressive luminal B-like tumours from luminal A-like, and is of clinical importance for decision regarding the benefit of neoadjuvant or adjuvant chemotherapy. In **paper II**, the agreement between preoperative CNB and paired tumour specimens regarding biomarker status and surrogate subtypes was evaluated in 526 adjuvantly and 190 neoadjuvantly treated patients.

In the adjuvantly treated primary surgery cohort, there were significant differences in ER, PR and Ki67 scores between CNB and paired resection. ER status had an almost perfect agreement between samples (99% concordance rate) and PR status a substantial agreement (89% concordance rate). The concordance rate for Ki67 was 79%, thus moderate agreement (κ 0.53), and only five tumours needed to be assessed on CNB before one was re-classified on resection (NNRC=5). Accordingly, ER status had the highest NNRC, meaning that 73 tumours needed to be evaluated on CNB for one to be re-classified on resection specimen. In the NAC cohort, similar results were observed for ER and PR. As expected, Ki67 was decreased in surgical specimens after NAC, and thus only slight agreement was observed.

The concordance for HER2 IHC was 75% with moderate agreement (κ 0.46), which was further improved when IHC was combined with ISH for HER2 status. NNRC for HER2 status was 28. However, 3.6% of the cases had discordant HER2 status. In the NAC cohort, HER2 status had a 94% concordance rate with substantial agreement.

To further investigate the clinical application of these findings, IHC-based surrogate subtypes according to the 2013 St Gallen consensus[77] and current Swedish guidelines[109] were compared between CNB and surgical resections. The concordance rates applying these to subtype classifications were 78% (κ 0.63) and 73% (κ 0.59), respectively. With both classifications, 38% luminal A-like tumours on CNB were re-classified as luminal B-like on the surgical resection.

In summary, we demonstrate that the agreement of Ki67 and HER2 between CNBs and surgical specimens is insufficient in primary tumours among patients with or without NAC. In addition, the limited agreement of surrogate subtype classifications indicates the clinical importance of re-testing biomarkers on surgical specimens.

4.3 PAPER III

Digital image analysis of Ki67 in hot spots is superior to both manual Ki67 and mitotic counts in breast cancer

Evaluation of tumor proliferation, one of the hallmarks of cancer, is currently included in the routine biomarker assessment for breast tumours by manual counting of mitosis and IHC scoring of Ki67 protein expression. Despite the established prognostic and predictive value of Ki67 in early breast cancer[172], it remains one of the most criticised and controversial biomarkers. For patients with luminal A-like and luminal B-like tumours, Ki67 is an important surrogate marker to distinguish between patients who may benefit from adjuvant chemotherapy (luminal B-like) and those who will instead be over-treated while potentially suffering adverse effects (luminal A-like). The international recommendations for the use of Ki67 to guide clinical decisions regarding chemotherapy are conflicting due to the lack of reproducibility and standardisation. Large efforts have been made to improve the intra- and interlaboratory variability of manual Ki67 assessment. Over the past years, the recommendations for Ki67 cut-offs have changed repeatedly, and there is still no established international consensus on which cut-off to adopt for dichotomising low and high proliferation. Apart from cut-offs, neither has a standardised method for scoring Ki67 been established. To overcome parts of the reproducibility concerns, DIA methods have been suggested to improve reproducibility and provide methods for standardisation[181-183].

In **paper III**, we investigated the clinical relevance of mitoses, Ki67 and PHH3 in the combined Clinseq and Stockholm cohorts comprising 294 primary breast tumours. In addition, we sought to identify the best tumour region for assessment of proliferation (hot spots, invasive front and across entire tumour section) in relation to outcome. All DIA was performed using the VIS Apps.

Among luminal A and B tumours, AUC derived from ROC analysis for the tested methods showed that regardless of the tumour region DIA of Ki67 outperformed the other markers (mitotic rate, PHH3 mitotic rate, manual Ki67, and DIA PHH3) in sensitivity and specificity for luminal B subtype.

Cut-offs for each marker were selected based on AUC-ROC scores in relation to OS and RFS. However, no meaningful cut-off could be found for DIA of PHH3. Based on these cut-offs, Cox regression HRs for all-cause mortality were significantly increased for high versus low proliferation groups, using DIA of Ki67 in hot spots (HR 2.97) and full tumour section (HR 2.19) as well as PHH3 counts/10 high-power-fields (HR 2.19). Kaplan-Meier curves also showed that DIA of Ki67 regardless of region was superior to mitotic counts, manual Ki67 and PHH3 in separating patients into poor versus relatively good overall survival groups.

Histological grading based on automated Ki67 scores instead of mitoses increased the differences in overall survival between grade 1 and 3, and added significant prognostic information.

In summary, this study showed that DIA scoring of Ki67 in hot spots outperformed the alternative markers, most importantly in discriminating prognostic groups. Histological grading based on automated Ki67 scores showed increased prognostic potential. Altogether these findings suggest that when using the VIS virtual double staining method, DIA of Ki67 in hot spots should be the marker of choice for assessment of proliferative activity in breast cancer.

4.4 PAPER IV

Prognostic potential of automated Ki67 evaluation in breast cancer: different hot spot definitions versus true global score

As concluded in **paper III**, hot spot Ki67 scoring by DIA virtual double staining is the suggested marker of choice for assessment of proliferation in primary breast cancer. In Sweden, Ki67 counting in the hottest area of the tumour containing 200 cells, a so-called hot spot has been adopted for manual scoring, whereas other countries recommend counting an average Ki67 score across the entire tumour. With the rapid development of DIA systems, average scoring of >1000 cells can efficiently be performed.

Based on the results from **paper III**, we first sought to further investigate the prognostic influence of different parameters defining the hot spot for DIA evaluation of Ki67. The Clinseq cohort of 139 ER-positive/HER2-negative primary breast tumours with digitised whole-slide images of Ki67 and CKMNF116 was used for DIA scoring of Ki67. Across 19 different algorithms (apps) for hot spot scoring, those with higher scored number of cells had greater reproducibility in terms of intraclass correlation coefficients. Among the configurable hot spot apps, APP24 had the highest risk for recurrence in high versus low Ki67 cases and was selected for further comparisons. This app included 400 cells, 40x field of view and a heatmap-shaped hot spot. Regarding risk for overall death, a similar app including 1200 cells had slightly higher HR than APP24.

The prognostic value of the selected hot spot app was thereafter compared against true global (average) Ki67 scoring using a different DIA platform (QuPath). The global scoring had lower median Ki67 values than all hot spot apps, which has been reported previously[184]. Hot spot APP24 had twice as high HR for recurrence as global scoring among high versus low Ki67 cases. Regarding risk for overall death, global scoring was however superior, which was also seen among node-negative cases.

We further investigated the prognostic significance in adjusted multivariate Cox regression models estimating the HR associated with RFS or OS among high versus low Ki67 cases. Importantly, we found that only global Ki67 scoring added independent prognostic information to the model associated to both RFS and OS.

Molecular subtypes based on PAM50 algorithm showed that luminal B cases had an increased risk associated with both RFS and OS as opposed to luminal A cases. This is in line with the established data on the prognostic value of molecular subtypes as discussed in section 1.7. We then applied IHC-based surrogate subtype classifications to distinguish luminal A-like from luminal B-like based on Ki67 values from each method. The global scoring provided higher concordance with PAM50 subtype than hot spot scoring. Further, the global method also showed increased HR for OS in luminal B-like versus luminal A-like cases.

In summary, we showed similar outcome prediction using DIA hot spot in VIS and global Ki67 scoring in QuPath, but only the global method had independent prognostic value associated to both RFS and OS. In addition, the global method is a more practical method that could be adopted in a digital work flow for automated Ki67 assessment in breast pathology. Prior to clinical implementation, these findings need to be confirmed in a larger independent cohort.

4.5 PAPER V

Detailed investigation and re-assessment of HER2 status in breast cancer patients treated with HER2-targeted therapy

Advances in HER2-targeted therapy, pioneered by trastuzumab, have dramatically improved survival for patients with HER2-positive breast cancer. Unfortunately, some patients progress despite HER2-targeted therapy. Assessment of HER2 status by IHC and ISH has been the standard method since the introduction of trastuzumab, but is however, insufficient to identify patients with therapy-resistant disease.

In a retrospective cohort of 591 patients with HER2-positive breast cancer treated with trastuzumab, we investigated the prognostic value of HER2 status on the protein, DNA and targeted mRNA level. By performing HER2 ISH re-testing on 460 tumours, we examined the association of HER2 copy numbers and HER2/CEP17 ratio with RFS and BCSS. Measurement of ERBB2 transcript mRNA levels was performed with the STRAT4 assay.

Neither HER2 copy numbers (≥6.0 vs <6.0), HER2/CEP17 ratio (≥2.0 vs <2.0) nor ER status provided any prognostic value in terms of RFS or BCSS. Higher HER2 copy numbers and ratio was identified among HER2-positive/ER-negative tumours compared to ER-positive, but no difference in prognosis was observed.

Results from the STRAT4 assay revealed that 85% had transcript mRNA levels scored as ERBB2 ‘positive’ and 15% did not reach the pre-defined cut-off, thus scored ERBB2 ‘negative’. However, among the ERBB2 ‘negative’ tumours almost 50% were HER2 protein overexpressing (IHC 3+) and gene amplified by ISH. Interestingly, survival analysis and Cox regression analysis showed that ERBB2 ‘negative’ tumours were associated with disease recurrence, and ERBB2 remained an independent prognostic factor in multivariate analysis adjusted for lymph node status. Approximately 20% of the ER-positive tumours were ERBB2 ‘negative’. For ER-positive tumours, ‘negative’ ERBB2 levels were significantly associated with risk of recurrence.

Furthermore, when we analysed patients presenting with recurrent disease (12%), there was no significant difference in HER2 copy numbers or ratio compared to those without recurrence. However, when divided by ER status, ER-negative tumours had significantly higher HER2 copy numbers, ratio and ERBB2 levels.

In summary, our findings showed that routine clinicopathological information is insufficient to discriminate those patients who are or will become HER2-resistant and progress despite trastuzumab treatment. We demonstrate that neither HER2 copy numbers nor HER2/CEP17 ratio or ER status, but low levels of ERBB2 mRNA, which may more accurately indicate the HER2 protein levels, were associated with risk of recurrence.

4.6 CONCLUSIONS

The findings presented in **paper I-V** within this thesis can be summarised as follows:

- Discordances in biomarker status from aspiration cytology and paired surgical resections from breast tumours, and the limited prognostic potential of ICC-based Ki67 scoring demonstrates that IHC is the superior method for Ki67 evaluation.
- Discordances in Ki67 and HER2 status between CNB and paired resection demonstrates that these biomarkers should be re-tested on all surgical breast cancer specimens.
- Digital image analysis using virtual double staining in VIS of hot spot Ki67 outperforms alternative markers of proliferation in breast cancer.
- Automated global scoring of Ki67 in QuPath has independent prognostic potential compared to hot spots in VIS, and is a practical method for routine Ki67 scoring in breast pathology.
- Low levels of ERBB2 mRNA, but neither HER2 copy numbers, HER2 ratio nor ER status, are associated with risk of recurrence among anti-HER2 treated breast cancer patients.

4.7 GENERAL DISCUSSION

The accuracy of prognostic and predictive biomarker assessment in breast tumours is crucial for management and therapy decision in patients with breast cancer. In the papers presented within this thesis, biomarkers used in clinical practice with emphasise on Ki67 and HER2 were studied using several methods. These studies cover methods including ICC and IHC, ISH, gene expression assays and DIA, with the overall aim to improve routine biomarker evaluation and clarify the prognostic potential.

ER, PR, HER2 and Ki67 are routinely evaluated for all invasive breast tumours and hold therapy-predictive and prognostic information that can be combined to represent molecular

subtypes (see section 1.8 for details). Multigene signature assays for risk stratification are available for selected patient groups but are not implemented in the routine management of breast cancer in Sweden due to high costs. Therefore, since IHC-based biomarker evaluations are used for clinical surrogate subtype classification, biomarker scores need to be accurate. Among patients with luminal A- and luminal B-like tumours, Ki67 together with grade and PR help distinguish the high-proliferative luminal B-like tumours with benefit from added chemotherapy from the low-proliferative luminal A-like tumours with good prognosis on only endocrine therapy.

The discordance between Ki67 scoring from FNAC and corresponding surgical specimens presented in **paper I** could partly be explained by tumour heterogeneity and tumour material representing different areas of the tumour, as well as the two different methods used. By using fine-needle aspiration-derived cell blocks for IHC, HER2 testing on cytological material is possible, and is also suggested to improve biomarker concordance[406, 407]. Ki67 was scored in a selected hot spot area on whole-slide sections from surgical specimens, whereas ICC-based scoring from FNAC represents non-specific small portions of the tumour. As opposed to ER, both Ki67 and PR are more heterogeneously expressed across the tumour[136, 408]. From our findings in **paper I**, it was concluded that ICC-scored Ki67 from aspiration cytology cannot be regarded as a reliable prognostic measure for overall survival outcome. However, FNAC-based ICC of Ki67 was previously demonstrated to be a prognosticator of disease recurrence-free interval[409]. We also showed that both surrogate subtype classification (St Gallen 2013) based on ICC and IHC had limited agreement with molecular subtype, which regarding ICH is in line with previous concordance rates[255]. Despite the lack of prognostic value of Ki67 from cytology in our study, FNAC is still a valuable method for diagnosing malignancy. We compared cytology with re-evaluated Ki67 and ER scores in surgical specimens of primary tumours; however, in the metastatic setting FNAC may be the only method of choice for diagnosis and biomarker assessment. Our findings in primary tumours suggest that ER scoring is reliable on cytological material but Ki67 has limited value, which might reflect on the metastatic setting. HER2 status was generally not assessed from cytology in our study data, which however, along with ER is the clinically most important biomarker for patients with metastatic disease. When a tumour already has progressed to a metastatic state, tumour proliferation may add limited information for therapy decisions.

Ki67 along with HER2 status discordance between CNB and surgical specimens when evaluating the same tumours were demonstrated in **paper II**. Although a 3.6% discordance rate for HER2 status is seemingly low, for the individual patient approximately 1 in 25 could potentially receive the wrong therapy, leading to serious implications for patients not receiving HER2-targeted therapies. The use of NAC has increased, and up to 17% of patients with early breast cancer were treated with neoadjuvant therapy in Sweden during 2019[13]. Neoadjuvant treatment provides an opportunity to histologically evaluate the effect of given therapies, and to plan adjuvant therapies accordingly. From our findings in **paper II**, we reported on the importance to re-test biomarkers on all surgical specimens. The observed discordances could mainly be due to the intratumoural heterogeneity of biomarker expression and the difference

in tumour representation in a CNB compared with surgical excisions. The 2013 ASCO/CAP guidelines for HER2 testing state that tumours with initially negative HER2 status on CNBs must be re-tested on the specimen[73]. However, the latest 2018 ASCO/CAP guidelines do not require re-testing of HER2 on surgical specimens with initially negative status; only in cases of NHG 3 and equivocal HER2 results a new test *may* be performed[162]. Furthermore, it is still debatable whether resistance to chemotherapy is caused by selection of clones or through acquisition of new genomic alterations. Single-cell sequencing of triple-negative breast tumours prior to, during and after NAC illustrated both the presence of pre-existing genomic alterations and induced transcriptional reprogramming of chemo-resistant signatures[410].

In **paper III,** the Visiopharm VIS double staining method was applied for investigating the optimal region of Ki67 scoring, and cut-offs for each method were adjusted by AUC-ROC for OS and RFS (all cause-mortality). This rendered a cut-off at 36% for hot spots, 17% for invasive edges and 12% for full sections. Thus, different cut-offs for each method were used in the Cox regression analysis. Further, AUC-ROC for discriminating luminal B versus luminal A yielded a cut-off at 26% for hot spots, 23.5% for invasive edges and 13.5% for full section. The intention of this study was to find the optimal scoring for proliferative activity by comparing several markers both by manual and digital virtual double staining algorithms. The digital full section scoring was performed by the software sampling 25% of the tumour area, as opposed to counting all cells (for global scoring in **paper IV**). The finding that DIA scoring of Ki67 using the virtual double staining-based hot spot app was the superior method, led to the attempt to try and define an optimal hot spot in **paper IV**. The Ki67 hot spot app in VIS provided the opportunity to investigate the prognostic value of different configurable parameters such as cell counts, shape and how the algorithm detected the hot spot. However, the virtual double staining method is not an efficient method for routine use in breast pathology since it requires an additional parallel cytokeratin-stained section. The emerging evidence for average (global) Ki67 scoring by DIA, especially in terms of reproducibility[182], suggested to compare our best hot spot app with an automated global scoring using QuPath. Since the QuPath algorithm was trained to score Ki67 based on Ki67 IHC sections alone, its clinical application is more feasible. In this setting, we used a cut-off at 20% for both methods. As our results showed, global scoring in QuPath showed independent prognostic potential. However, before clinical implementations of a global scoring method, our findings need to be validated in a separate cohort. The current cut-offs in Sweden apply for manual hot spot scoring, and adjusted cut-offs for automated global scoring need to be investigated.

The controversies around Ki67 mainly concern the reproducibility of the biomarker. Apart from scoring methodology, e.g. hot spot versus global or manual versus automated scoring, which was investigated extensively in **paper III** and **IV**, there are several other factors that influence Ki67 assessments. Fixation times, antibody clone, antigen retrieval heating times and staining techniques are all parameters that may affect Ki67 evaluation[108]. In a Swedish cohort there was no difference in reproducibility or prognostic value between the rabbit monoclonal antibody SP6 and the mouse monoclonal antibody MIB1[411]. For image analysis, SP6 is substantially better suited than MIB1, due to reduced background[412]. In addition, Focke *et al*.

showed that the interlaboratory variability of Ki67 and luminal A-like classifications remains even when the same antibody clones (30-9, MIB1 and SP6) are used[413]. It is therefore important that each pathology department has standardised protocols and internal monitoring of Ki67. In addition, external monitoring increases quality assurance. In the papers of this thesis, rabbit anti-Ki67 monoclonal antibody clone 30-9 (Ventana Medical Systems) was used repeatedly. The alternative marker for proliferation that we studied in **paper IV**, PHH3, did not prove to provide significant prognostic value. Worth mentioning is that prior to routine use of Ki67, several other markers of proliferative activity have been used in different Swedish pathology departments, such as DNA flow cytometry determination of S phase[414] and cell cycle regulating cyclins[112].

Histological grade has unquestionable clinical value[88, 89], but there is a variability in scoring. Preliminary results from an ongoing study revealed variability in biomarker status across Swedish pathology departments, especially for grade and Ki67, despite using the same guidelines as well as and internal and external quality assurance programmes (abstract from SABCS 2019)[415]. Histological grading can be improved with DIA by combining DIA-scored Ki67 as shown in **paper III**. Instead of the subjective measure of mitoses and nuclear pleomorphism, grading by computerised methods provides clear reproducibility and thus more robust histological grade. Since NHG 2 tumours provide limited clinical information, efforts have also been made to dichotomise these into low and high transcriptomic grade based on gene- and isoform-level expression data from RNA sequencing[393]. Furthermore, trained AI models are promising for dichotomising NHG 2 tumours into either good or bad prognostic groups, resembling a two-tier grading system (Wang *et al.*, unpublished).

With the complexity and heterogeneity of breast cancer, the prognostic and predictive information in each biomarker can no longer be analysed separately but needs to be considered together with grade, lymph node status and molecular subtypes. Based on the findings in **paper IV**, it would be of interest to include grade and the proposed three-tier classification of Ki67 (low-intermediate-high) by Maisonneuve *et al.*[254], which is also adopted by the Swedish recommendations, in future validation studies of computer-aided scoring. Determining a single cut-off value for dichotomising tumours into luminal A-like and luminal B-like has not proven to be clinically applicable. The clearly low proliferative or highly proliferative tumours are not the cause of the controversies, since clinical decisions regarding therapeutic choices for these groups are well studied. Instead, the challenge concerns the intermediate Ki67 values around 15-25%, and identifying those patients who would actually benefit from added chemotherapy from those who instead would risk suffering from cytotoxic side effects with no added survival benefit.

Although HER2 is an established predictive biomarker for the benefit of HER2-targeted therapy, it is insufficient to predict disease recurrence. In **paper V** we demonstrate HER2 investigations on a DNA, RNA and protein level; additional RNA level data e.g. on molecular subtypes would be of interest to distinguish the luminal HER2-positive tumours from the true HER2-enriched tumours. As we could show, routine HER2 IHC and ISH status was not

associated with risk of recurrence. On the contrary, we found low ERBB2 levels associated with increased recurrence risk. The correlation of STRAT4 ERBB2 levels, especially those cases with low ERBB2, and HER2-enriched subtypes would be interesting to investigate in this HER2-positive cohort. In addition, differences in intensities of HER2 IHC 2+ among ERBB2 'negative' and 'positive' tumours would be valuable to see if the mRNA levels can be well reflected by IHC. In **paper V**, pre-set cut-offs[404] were used to discriminate ERBB2 'positive' versus 'negative' tumours. Since other antibodies as well as fluorescent ISH, were used to set these pre-defined cut-offs and without outcome correlation, it would be important to investigate prognostic cut-points for risk of recurrence in our HER2-positive trastuzumab-treated cohort. HER2 mRNA measurements as a continuous value may better reflect the HER2 status than IHC/ISH. Apart from this, especially in resource-limited settings, a quick closed-system targeted gene expression assay for routine biomarkers, has potential to provide diagnostic information that may otherwise not be available.

The tumour microenvironment and stromal factors have proven to play an important role in breast cancer progression. In HER2-positive breast cancer, the presence of TILs is correlated to improved outcomes in the neoadjuvant setting[416]. Apart from studying HER2 expression and gene amplification, we speculate that assessment of TILs in our adjuvant trastuzumab-treated HER2-positive cohort may provide important prognostic information. The results from **paper V** also highlight the need for more detailed molecular analyses to further understand the biology behind resistance to HER2-targeted therapy. Thus, we speculate that a future molecular model would incorporate image analysis of protein expression staining intensities and heterogeneity, molecular subtypes and PI3K pathway status in order to predict sensitivity to targeted therapies.

5 FUTURE PERSPECTIVES

The field of surgical pathology and breast pathology specifically is rapidly evolving with digitised work flows and molecular techniques, moving towards comprehensive precision pathology. With all emerging targeted therapies and companion diagnostics that follows, the pathological evaluation will need to be continuously updated for accurate assessment. Some future perspectives for improving biomarker evaluation are presented in the following sections.

5.1 CELL-BASED METHODS

Cytology is a minimally invasive method that provides unique possibilities to retrieve tumour material from organ sites not available for biopsy sampling, e.g. distant visceral metastases. Advances in image analysis have shown promising results also for cytology and AI algorithms may further improve accuracy. In addition, cytological tumour material derived from fine-needle aspirations are also perfect for NGS and a valuable source for molecular analysis of metastatic lesions[417].

Liquid biopsies, either circulating tumour cells or circulating tumour DNA is a rapidly emerging field in precision oncology. Still there are uncertainties regarding clinical validity and clinical utility, especially in early-stage disease[76, 418]. Tumour cells in liquid biopsies may potentially act as surrogates for the tissue-based tumour and provides an opportunity to monitor tumour biological changes[419]. Molecular characterisation and biomarker status (e.g. ER, HER2, Ki67) can be measured in circulating tumour cells[420, 421], but whether or not these predict clinical response or outcome remains uncertain[46]. For example, HER2 positivity was not predictive of response to lapatinib[422].

5.2 TUMOUR HETEROGENEITY AND BIOMARKER CONCORDANCE

The tumour heterogeneity is a complicating factor in clinical practice and cancer treatment, since tumour samples may not represent the entire tumour and therefore the evaluation may vary both between sampling methods and areas within the tumour[41]. Rye *et al*. recently demonstrated on a single-cell level that intratumoural heterogeneity for HER2 copy number was associated with worse outcome[48]. Sampling from several areas of the tumour may provide insights into spatial heterogeneity.

As demonstrated in several of the papers in this thesis, there were discordances between IHC-based subtypes and PAM50 intrinsic subtypes, which has also been reported by others[226, 255]. Since molecular subtyping covers a large number of genes that together cluster and define the intrinsic subtype, one cannot assume that IHC-based subtyping of only ER, PR, HER2 and Ki67 would provide the same classification; it is therefore important to acknowledge that IHC-based subtypes act as surrogates. However, there is rather limited research to increase the consistency of PAM50- and IHC-based subtypes. The clinically approved molecular signatures assays will most likely become part of routine pathology for assigning not only molecular subtype but also for risk stratification.

5.3 OVERCOMING KI67 CONTROVERSIES

With over a decade of controversies regarding Ki67 scoring, we will probably leave hot spot methods and see a general acceptance of computerised global counting of Ki67. Since current cut-offs for Ki67 scoring in Sweden is based on hot spots, new cut-offs need to be investigated in larger cohorts prior to implementation. Clearly high and low proliferation by Ki67 provides prognostic value but for the intermediate group additional methods are required. Over the last few years, the use of gene expression signature assays has been more widely adopted for clinical practice. In the latest Swedish national guidelines, gene expression assays are recommended for patients with ER-positive/HER2-negative NHG 2 tumours with intermediate Ki67 in order to distinguish luminal A from luminal B subtypes in those patients were therapy decisions could be affected by subtype classification. Furthermore, IHC of Ki67 could altogether be replaced by a whole group of proliferation genes on mRNA level from e.g. PAM50 assays.

5.4 FUTURE HER2 TESTING

Evaluation of HER2 ISH is both time-consuming and to some extent hampered by reproducibility issues. Automated machine learning approaches are under development for HER2 ISH scoring by e.g. Roche and will most certainly provide improved reproducibility and more efficient assessment. DIA methods such as QuPath could provide opportunities to further investigate the association of IHC 2+ staining intensities and the level of mRNA expression in our trastuzumab treated HER2-positive cohort. In addition to tissue-based assessment, HER2 status can be detected in liquid biopsies both using serum (HER2 levels) and in circulating tumour cells[420].

Genomic profiling of solid tumours including breast cancer is becoming a part of precision medicine. In the United States, FoundationOne CDx by Roche Foundation Medicine was the first FDA approved companion diagnostic for genomic profiling. In Sweden, FoundationOne CDx is available on request for selected patients. Genomic profiling provides opportunities to investigate and analyse gene copy numbers by DNA sequencing and mutations in the HER2 gene.

One can speculate that not only ERBB2/HER2 mRNA levels but also levels of the ESR1, PGR and MKi67 are important to discriminate therapy responsive patients. Based on gene expression analysis, further studies are ongoing to subgroup patients into intrinsic molecular subtypes and HER2 expression may have different prognostic value in different subtypes. Hence, the aim is to proceed with an anti-HER2 response prediction algorithm based on all four quantitative biomarkers within the trastuzumab-treated HER2-positive cohort. Regarding sequencing of HER2, there are two possibilities: either to analyse HER2 copies by RNA sequencing or to count gene copies on DNA level by DNA sequencing. However, RT-qPCR assays for measuring mRNA levels, which is an accessible and rapid method not relying on specialised pathologists may have large potential for improved biomarker assessment in resource-limited settings.

5.5 NOVEL BIOMARKERS AND COMPANION DIAGNOSTICS

Apart from the four routine biomarkers, ER, PR, HER2 and Ki67, in breast pathology that have been investigated in the work of this thesis, several novel biomarkers and companion diagnostics are entering the diagnostic spectrum of breast pathology. As mentioned, the tumour-immune cell interaction plays an evident role in tumour progression[423-425]. TILs predict clinical outcome in breast cancer[426-428] and are currently included in the diagnostic evaluation of triple-negative breast cancer[72, 194]. Scoring of TILs has demonstrated to be a predictor of complete response to chemotherapy in patients with HER2-positive disease and associated with benefit of trastuzumab but further evidence of the clinical relevance in the adjuvant setting is needed[192, 427-429]. TILs scoring from HE may potentially be a simple method for identifying patients with excellent prognosis and play a role in guiding therapy de-escalation and for designing clinical trials.

Despite targeted therapies, the development of therapy resistance is a complicating factor in breast cancer management. Gene expression analysis could be used to potentially detect altered expression in key pathways, e.g. PI3K or MAPK overexpression that could indicate therapy resistance as discussed in section 1.11.

5.6 AI APPROCHES FOR PRECISION PATHOLOGY

During the last decade we have seen ground-breaking advances in AI and computerised pathology, which today also includes aspects of precision pathology.

AI models can be trained to detect and grade prostate cancer with expert-level performance[430], and similar AI approaches are ongoing for grading of breast cancer. Breast tumours of NHG 2, e.g., provide limited clinical information, and trained AI models can dichotomised these into either good or bad prognostic groups, resembling a two-tier grading system (Wang *et al*, manuscript). Apart from aiding the diagnostic process and fairly simple tasks such as biomarker scoring, AI models hold promise to capture prognostic and predictive information from HE-stained tumour images, which the human eye cannot interpret through the bright field microscope. Instead of using IHC stained images for Ki67 scoring, we speculate that deep learning models could be applied to predict proliferational activity directly from HE. In the recent work by Kather *et al*., deep learning models could detect high-proliferation versus low-proliferation signatures in lung and gastric cancer[431]. The demand for molecular profiling of tumours for selecting patients eligible for targeted or biomarker-based therapies is increasing. However, the turnaround time and costs for these assays are considerable, and applying AI models for this task is an efficient yet challenging alternative. Deep learning-based AI models have shown promising abilities to detect gene mutations in lung cancer[432], prostate cancer[433] and uveal melanoma[434], as well as predict molecular subtypes in breast cancer[435]. Furthermore, spatial transcriptomics and histological features can be combined to predict gene expression in breast cancer[436]. Two recent studies demonstrated that deep learning can predict genomic alterations based on morphological features from HE images across several cancer types[431, 437]. Apart from detecting molecular subtypes, the models could predict mutation status of several

genes across different cancer types, including *MAP3K1, TP53, PIK3CA, FOXA1* and *MAP2K4* in breast cancer. In addition, pan-cancer models showed better prediction of prognosis than using grade and subtype[437]. In summary, AI-based methods for image analysis will not only transform the way pathologists diagnose cancer but also change the way how clinicians interpret diagnostic data. Most likely, in the next few years, we will see the first AI methods get introduces into routine pathology.

To summarise, the complexity of tumour biology and emerging combinations of targeted therapies pose diagnostic challenges. Future diagnostic methods for therapy-prediction and prognostication might involve advanced deep learning algorithms combining morphology, grade and perhaps IHC-stained biomarkers to select patients for whom additional sequencing of the tumour would actually be beneficial.

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