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Validation of biomarkers and digital image analysis in breast pathology

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Det finns inget sant som aldrig också är ljug

Och ingen skillnad därmed, för den som är slug

Det man bör betvivla är det huggna i sten

För när sanningen är full står den på vingliga ben

ABSTRACT

For women worldwide, the risk of developing breast cancer is second only to that of non-melanoma skin cancer. Significant improvements have been made in survival over the past decades and today about 80 % of the patients survive 10 years or more after their breast cancer diagnosis. Still, far from all patients enjoy the relatively good survival indicated by statistics on breast cancer patients as one homogenous group. Improving prognostication of aggressive vs. less aggressive disease, and to separate tumors based on genetic differences for optimal treatment strategies, is therefore the focus of intensive research, including this thesis.

In **paper I**, we compared if tumor characteristics differ depending on what method of sampling the tumor that have been used for analysis. We compared routine immunohistochemistry on surgically resected breast specimens, including stains of the Estrogen receptor alpha (ER), the Progesterone receptor (PR), Human Epidermal growth factor receptor 2 (HER2) and the proliferation-associated protein Ki67, with analysis of the same stains done on material obtained from fine needle aspiration (immuno*cyto*chemistry). We found that there were substantial differences in the expression of these biomarkers between the two methods. Thus, the same rules for interpretation of biomarkers cannot be used for immunohistochemistry and immunocytochemistry, and consequently, validation of each method should be performed individually.

In **paper II**, we explored the scope of digital image analysis in biomarker evaluations. We scored ER, PR, HER2 and Ki67 status in several different regions of breast tumors by both manual methods and digital image analysis. The outcomes of the scoring of these biomarkers were then combined into IHC surrogate subtypes and compared to PAM50 gene expression-based subtypes as well as patient survival. All tested methods of automated digital image analysis of Ki67 outperformed manual scores in terms of sensitivity and specificity for the Luminal B subtype. Comparing digital versus manual testing concordance to all breast cancer subtypes as determined by PAM50 assays, the digital approach was superior to the manual method. The manual and digital image analysis methods matched each other in hazard ratio for all-cause mortality of patients with tumors with a “high” vs “low” Ki67 index. Manual assessments of the biomarkers ER, PR, HER2 and Ki67 were in most aspects less precise than digital image analysis.

In **paper III**, we evolved the concept of paper I with an evaluation of the concordance of consecutive Ki67 assessments performed on fine needle aspiration cytology versus resected tumor specimens. We investigated how a status of Ki67 “low” and “high” as determined by immunohistochemistry and immunocytochemistry corresponded to overall survival, respectively. Again, Ki67-index varied when the two methods were used on the same tumors, and was prone to switch the classification between low and high proliferation. ER evaluations were discordant in 5.3 % of the tumors, which in the clinical setting would mean that 1 in 20 patients would risk being left out of beneficial endocrine treatment or being given it without benefit. Ki67 “high”, as determined by immunohistochemistry, defined as a

proportion of Ki67-positive cells above the 67th percentile of the material, was significantly associated with poor overall survival and a significantly higher probability of axillary lymph node metastasis. This could not be reproduced for immunocytochemistry. In summary, this study adds to the results of paper I, in which we showed discordance between the methods. By including survival data, we now conclude that not merely are the methods discordant, but immunocytochemistry fails to provide prognostic information. Consequently, immunohistochemistry should be regarded as the superior method.

In **paper IV**, we focused on proliferation comparing the results in the tumors' hot spot, in the tumor periphery, and as the average proportion of Ki67-positive cells across the whole tumor section. Both manual and digital scores of Ki67 and the recently described marker for mitotic activity, PHH3, were evaluated along with mitotic counts. Their sensitivity and specificity for the gene expression based Luminal B versus A breast cancer subtypes, for the high versus low transcriptomic grade, for axillary lymph node status as well as for their prognostic value for breast cancer specific and overall survival were analyzed. Digital image analysis of Ki67 in hot spots outperformed the other markers in sensitivity and specificity both for gene expression subtypes and transcriptomic grade. In contrast to mitotic counts, tumors with high expression of Ki67, as defined by digital image analysis and high numbers of PHH3-positive cells, had significantly increased HR for all-cause mortality at 10 years from diagnosis. When we replaced the manual mitotic counts with digital image analysis of Ki67 in hot spots as the marker for proliferation when determining histological grade, the differences in estimated mean overall survival between the highest and lowest grades increased. It also added significantly more prognostic information to the classic Nottingham combined histological grade. We conclude that digital image analysis of Ki67 in hot spots might be suggested as the marker of choice for proliferative activity in breast cancer.

LIST OF SCIENTIFIC PAPERS

I. Stålhammar G, Rosin G, Fredriksson I, Bergh J, Hartman J. Low concordance of biomarkers in histopathological and cytological material from breast cancer. Histopathology 2014;64(7):971-980.

II. Stålhammar G, Fuentes Martinez N, Lippert M, Tobin NP, Mølholm I, Kis L, Rosin G, Rantalainen M, Pedersen L, Bergh J, Grunkin M, Hartman J. Digital image analysis outperforms manual biomarker assessment in breast cancer. Modern Pathology 2016;29(4):318-329.

III. Robertson S, **Stålhammar G,** Darai-Ramqvist E, Rantalainen M, Tobin NP, Hartman J. Biomarker assessment in cytology and corresponding resected breast tumors—correlation to molecular subtypes and outcome in primary breast cancer. *Manuscript submitted May 2017.*

IV. Stålhammar G, Robertson S, Wedlund L, Gholizadeh S, Lippert M, Rantalainen M, Bergh J, Hartman J. Digital image analysis of Ki67 in hot spots is superior to manual Ki67, phosphohistone H3 and mitotic counts in breast cancer. *Manuscript submitted August 2017.*

LIST OF RELATED MANUSCRIPTS

V. Govindasamy KM, Rantalainen M, **Stålhammar G**, Lövrot J, Ullah I, Ma R *et al*. Intra-tumor heterogeneity in breast cancer has limited impact on transcriptomic-based molecular profiling. *Manuscript submitted.*

VI. Ullah I, Govindasamy KM, Alkodsi A, Kjällquist U, **Stålhammar G**, Lövrot J *et al*. Genomic analyses of primary breast cancer and matched metastases reveal both linear and parallel progression with minimal seeding from axillary lymph node metastasis. *Manuscript submitted.*

VII. Stålhammar G, Farrajota P, Olsson A, Silva C, Hartman J, Elmberger G. Gene protein detection platform – a comparison of a new human epidermal growth factor receptor 2 assay with conventional immunohistochemistry and fluorescence in situ hybridization platforms. Annals of Diagnostic Pathology 2015;19(4):203-210.

CONTENTS

LIST OF ABBREVIATIONS

AI Artificial intelligence
AIs Aromatase inhibitors
ALDH1 Aldehyde dehydrogenase 1
AREG Amphiregulin
BCS Breast conserving surgery
BCSC Breast cancer stem cells
BMP4 Bone morphogenetic protein 4
DIA Digital image analysis
DCIS Ductal cancer in situ
EGF Epidermal growth factor
EMT Epithelial-to-mesenchymal transition
ERα or ER Estrogen receptor alpha
ERβ Estrogen receptor beta
FFPE Formalin fixed paraffin embedded
FGF Fibroblast growth factor
FGFR2 Fibroblast growth factor receptor 2
FNAC Fine needle aspiration cytology
GH Growth hormone
ICC Immunocytochemistry
IF Immunofluorescence
IGF-1 Insulin like growth factor 1
IHC Immunohistochemistry
HER2 Human epidermal growth factor receptor 2
HR (Cox regression) Hazard ratio
Ki67 Kiel, clone 67 proliferation-associated protein
LBD Ligand binding domain
LCIS Lobular cancer in situ
LR Likelihood ratio
LR χ^2 Likelihood ratio chi-square
LR -$\Delta\chi^2$ Likelihood ratio chi-square change
MSC Mammary stem cells
NGS Next generation sequencing
PCR Polymerase chain reaction
PHH3 Phosphohistone H3
RT-PCR Real time polymerase chain reaction
PR or PgR Progesterone receptor
SERM Selective estrogen receptor modulator
Sn Sensitivity
Sp Specificity
SSP Single sample predictors
SLN Sentinel lymph node
TDLU Terminal ductal lobular unit
TGFβ Transforming growth factor beta
TIL Tumor infiltrating lymphocytes
TMA Tissue microarray

1. INTRODUCTION

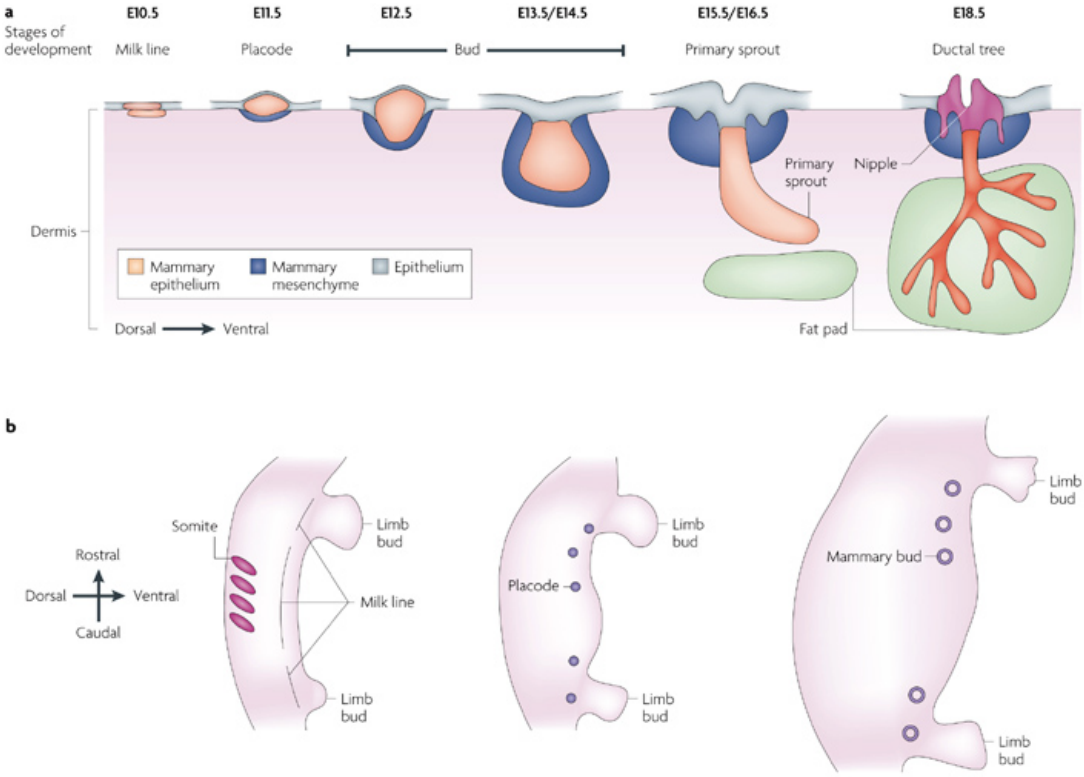
1.1 THE MAMMARY GLAND

1.1.1 DEVELOPMENT AND PHYSIOLOGY

Along with distinct features such as the neocortex, viviparity and a skin at least partially covered by hair, the mammary gland is at the very core of mammalian life and evolution. This exocrine gland defines and enables the unique concept of offspring being nutritionally attached to the parent even after their mechanical separation, in turn a driver for advanced forms of social and communicative behavior. It gave early mammals the advantage of relatively fast juvenile growth rates and young fertility. As it reduces the dependence of different food supplies for young and old, it also facilitated adaptive behavior to the varying, and to the Dinosaurs overly challenging, ecological niches at the end of the Mesozoic (1,2)

On the individual level, the development and organization of epithelial-, mesenchymal-, immune- and endothelial cells that together form the mammary gland starts during embryogenesis and continues through adolescence and pregnancy until menopause, after which a degree of involution will occur (3).

Most stages of signaling pathways in embryogenesis overlap between different mammals, currently amounting to >5 000 species. Cells from the ectoderm layer, guided by the Wnt-signaling pathway, form the epithelial actively secreting component of the mammary gland. Cells from the mesoderm layer, guided by the fibroblast growth factor (FGF) pathway, form the stromal elements, which have a supporting role to the epithelium in both a mechanical, nutritional and functional sense (2,4).



	Milk line	Placode	Mammary bud epithelium	Mammary bud mesenchyme
Secreted molecules and receptors	FGF10/FGFR2	WNTs	PTHLH	PTHR1
	WNT10b,6	FGFs	IGF1	BMP4/BMPR1A
	NRG3/ERBB2,4	BMP4	BMP4	
Transcription factors	TBX2,3	TBX2,3	LEF1	MSX2
	GLI3	LEF1	MSX1,2	
			TBX3	
			GLI3	

Figure 1. Schematic representation of the mammary gland development in mouse. After embryonic day 10 (E10), the milk line (orange) breaks up into individual placodes (orange). Starting on embryonic day 15 (E15), the primary mammary epithelial sprout pushes through the mammary mesenchyme towards the fat pad (green). On E18, the duct has grown into the fat pad and has branched into a small ductal system. Modified from Robinson GW (5). Reprinted with permission from Nature Publishing Group.

Ectodermal cells will form a milk line, and together with underlying mesenchymal cells a breast bud from which several primary sprouts project (Figure 1). These primary sprouts then elongate and branch, creating a ductal tree with thin end buds and open lumina. Paracrine communication between epithelial and mesenchymal cells via parathyroid hormone-related protein (PTHrP), and secreting factors such as insulin like growth factor 1 (IGF1) and the growth- and differentiation factor bone morphogenetic protein 4 (BMP4) plays crucial roles in this branching process in both mice and humans. Simultaneously, the epidermal layers of the skin form the nipple through thickening and suppression of the formation hair follicles. Before partus, a full mammary anlage is present for further development during childhood and puberty (2,5,6).

In the childhood years, with an increased rate towards puberty, the hormonal changes in the female body lead to proliferational and functional stimulation of both stroma and epithelium of the mammary gland. In fact, the mammary gland is the only organ that undergoes most of its development postnatally (7). This leads to further elongation and branching of the end bud structures, thereby forming the functional unit of the mammary gland - the terminal ductal lobular units (TDLU) (3).

Apart from PTHrP and BMP4, growth hormone (GH), progesterone, prolactin and estrogen is increasingly important in this phase of mammary gland maturation (2,5,7). GH stimulates increased paracrine signaling of IGF1 from the local breast stroma as well as from its classic hepatic expression site (8,9). Estrogen is mainly produced in the ovaries and adipocytes while progesterone is produced in the corpus luteum and the adrenal glands. Both hormones are also produced in the placenta during pregnancy (7,9). Estrogen exerts its effect by binding to intracellular estrogen receptors (ER), of which two main subtypes exist: ERα and ERβ (10). During postnatal development, neither ER subtype is however significantly expressed in proliferating mammary epithelium, as the stimulatory effect of estrogen is rather produced through paracrine secretion, uptake and indirect ERα-activation. This has been illustrated in ER knockout mice, where only a few transplanted cells expressing ER is sufficient to rescue normal mammary growth (11). Members of the epidermal growth factor family (EGF) located in the stromal tissue, such as Amphiregulin (AREG), have been suggested as the active mediator in this paracrine secretion (2,12) Consequently, AREG is believed to promote much of the proliferation seen by estrogen stimulation (13,14). It is strongly induced in mammary tissue during puberty, and knocking out AREG or ERα in mice leads to similar phenotypes. Other candidates are the fibroblast growth factor (FGF) and one of its receptors (FGFR2) and the transforming growth factor β (TGFβ) with its receptor (TGFβR). Binding of the former induces the epithelium to elongate the glandular ducts, while binding of the latter inhibits the very same process and decreases duct density. Both are essential, as the ducts form the framework for alveolar outgrowth during pregnancy but an overly dense network of ducts would encroach on the inter-ductal space needed to form enough alveoli for milk production (Figure 2) (15-19). All of this perhaps serves as an illustration of the intimate relationship between the different cell types in the mammary gland and their molecular cross talk in the development and maturation of the organ during puberty. Estrogen signaling and ER, as well as progesterone receptors (PR) will be described in further detail in section 1.3, as they are two of the biomarkers of central interest in this thesis.

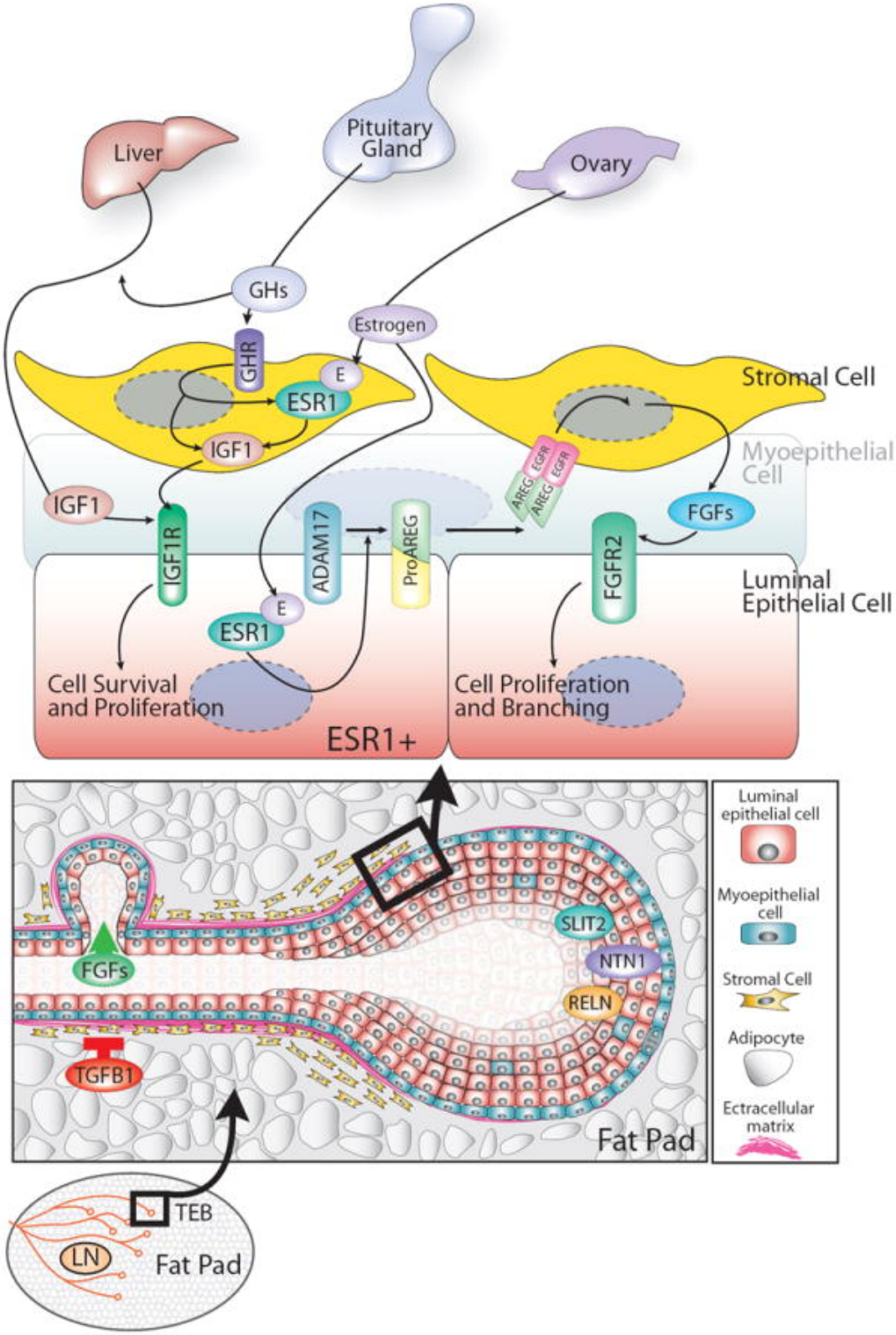


Figure 2. Overview of the events occurring during pubertal breast development. GH promotes cell proliferation by inducing the expression of IGF1 in both the liver and the mammary stroma. IGF1 acts, together with estrogen secreted from the ovary, to induce epithelial cell proliferation. Estrogen signaling through its receptor (ER) acts via a paracrine signaling to stimulate the release AREG, which proceeds to bind its receptor on stromal cells and induce expression of FGFs, which in turn stimulate luminal cell proliferation. Other factors

contribute to mammary architecture by either positively or negatively regulating cell proliferation or maintaining cell-to-cell interactions. From Macias H *et al* 2012 (2). Reprinted with permission from Nature Publishing Group.

During pregnancy, the mammary gland undergoes further changes to fully prepare for lactation. Stimulation from prolactin, human placental lactogen (HPL) and increased secretion of estrogen and progesterone, leads to budding of alveoli from the TDLUs in a process known as alveologenesis. While progesterone is not essential in pubertal or prepubertal mammary development, it is vital for alveologenesis (20-22). Absence of progesterone leads to hypoplasia of the TDLU while over expression of progesterone and PR, leads to abundant alveolar proliferation (24). The PR-positive cells lining the ducts do not proliferate at an increased rate during progesterone stimulation. Instead, the progesterone seems to promote proliferation in surrounding cells through paracrine signaling, similar to that of estrogen (21,23).

At partum, the delivery of the placenta results in a sudden drop in blood levels of progesterone, estrogen and HPL, which induces secretary activation and a sudden profuse milk production. Suckling by the offspring then triggers milk ejection through the nipple via release of oxytocin by the posterior pituitary, in turn leading to contraction of the myoepithelium -a smooth muscle layer of band-like cells surrounding the alveoli. The first milk released, the colostrum, is especially rich in white blood cells and IgA that helps protect an offspring with an immature immune system. Continued suckling over time, and thereby continuous prolactin secretion, maintain the production of milk in galactopoiesis. This also disrupts the pulsatile release of gonadotropin releasing hormone (GnRH) from the hypothalamus and hence luteinizing hormone (LH) from the pituitary, thereby preventing a new pregnancy (2, 21-26).

The involution of the mammary gland upon weaning of the offspring is a two-step procedure. First, there is a vast apoptosis of the alveoli and TDLU. Second, the gland is remodeled into a structure very much resembling that of a nullipara, except the number of branches of ducts distal to the TDLU which remain close to that of the pregnancy (Figure 3) (2,3).

After menopause, further involution of the mammary gland takes place. Leading to epithelial structures and interlobular connective tissue being replaced by adipocytes and some degree of fibrotic connective tissue (1).

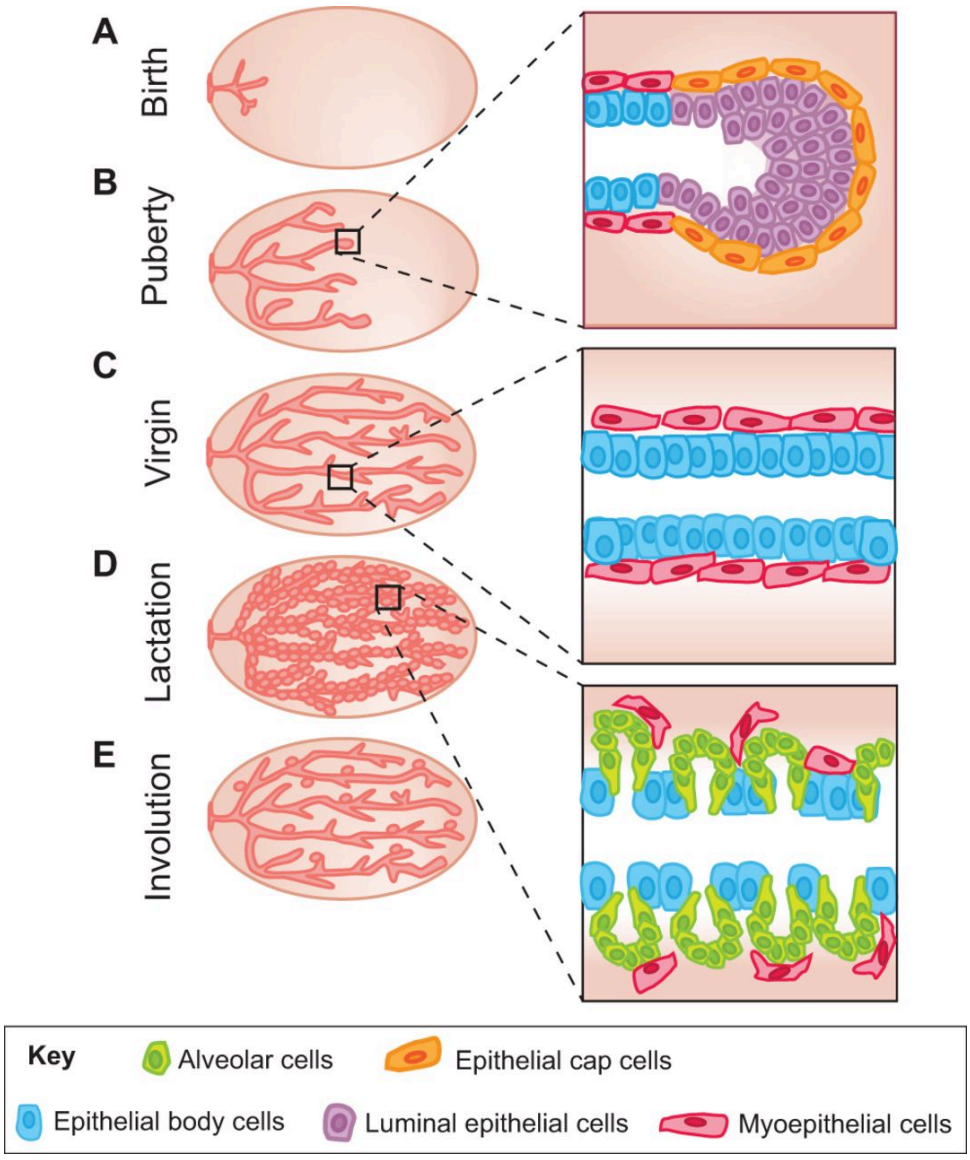


Figure 3. The mammary gland development from birth to involution. A: The mammary anlage is present at birth but remains inactive until puberty. B: During puberty, the epithelial ductal cells grow into the mammary fat pad, led by highly proliferative multilayered terminal end buds (inset 1). The multilayered epithelial body cells are surrounded by a single layer of epithelial cap cells. C: The mammary gland of a postpubertal nullipara is filled with mature epithelial branching structures. The ducts of this structure (inset 2) contain an outer layer of myoepithelial cells and an inner layer of luminal epithelial cells. D: Pregnancy induces hormonal changes that promote an expansion of alveolar cells, in turn evolving to milk-secreting alveoli. The alveoli (inset 3) expand from the ducts now filling the major part of the fat pad. E: Upon weaning, involution proceeds through cell death and ECM remodeling, giving rise to a state that resembles the resting adult mammary gland. From Inman *et al* (27). Reproduced with permission from the Company of Biologists.

1.1.2 HISTOLOGY AND ANATOMY

The human female breast consists of several types of tissue (Figure 4): Fat tissue, the pectoral muscles and the anterior chest wall will not be further elaborated on here as they are of relatively low interest in the perspectives of both physiological function and breast cancer.

The glandular tissue is organized into 15-20 lobes, arranged like petals in the frontal section. Each lobe consists of 20-40 lobules with 10-100 alveoli each, together known as the TDLU. Each alveolus is made up of epithelial cells, surrounded by myoepithelial cells and the basal membrane (3). The TDLUs drains into lactiferous ducts that gradually converge towards the nipple, where approximately 25 main ducts empty (3,26).

Both lobules and ducts are lined with a two-layered epithelium. The layer closest to the lumen of these structures consists of cuboidal milk producing cells, also known as luminal cells. The outer layer consists of contractile myoepithelial cells that helps push the milk produced in the TDLUs in the direction of the nipple. On the level of the myoepithelium are also mammary stem cells that can mature to either of the two epithelial cell types. These stem cells should not necessarily be confused with breast cancer stem cells, which will be discussed below. Myoepithelial cells and mammary stem cells rest on a basal membrane, in turn surrounded by stromal tissue (3,27)

The stromal tissue consists of extracellular matrix, peripheral nerves, blood- and lymphatic vessels. Among these are interspersed fibroblast, adipocytes, dendritic cells, macrophages and lymphocytes. At regular intervals, the stroma is organized into fibrous connective tissue septa, or suspensory ligaments, known as Cooper's ligaments that help maintain structural integrity of the breast. Lymphatic vessels drain the mammary tissues to the axillary lymph nodes, in some individuals via intramammary lymph nodes (3).

Blood to the mammary gland is supplied through branches of the internal mammary- and the lateral thoracic artery. Starting during pregnancy and peaking during lactation, the blood flow increases to meet the demand of nutrients and oxygen of the mammary tissue. Naturally, this also increases the supply of immune cells and antibodies to the milk (3,26).

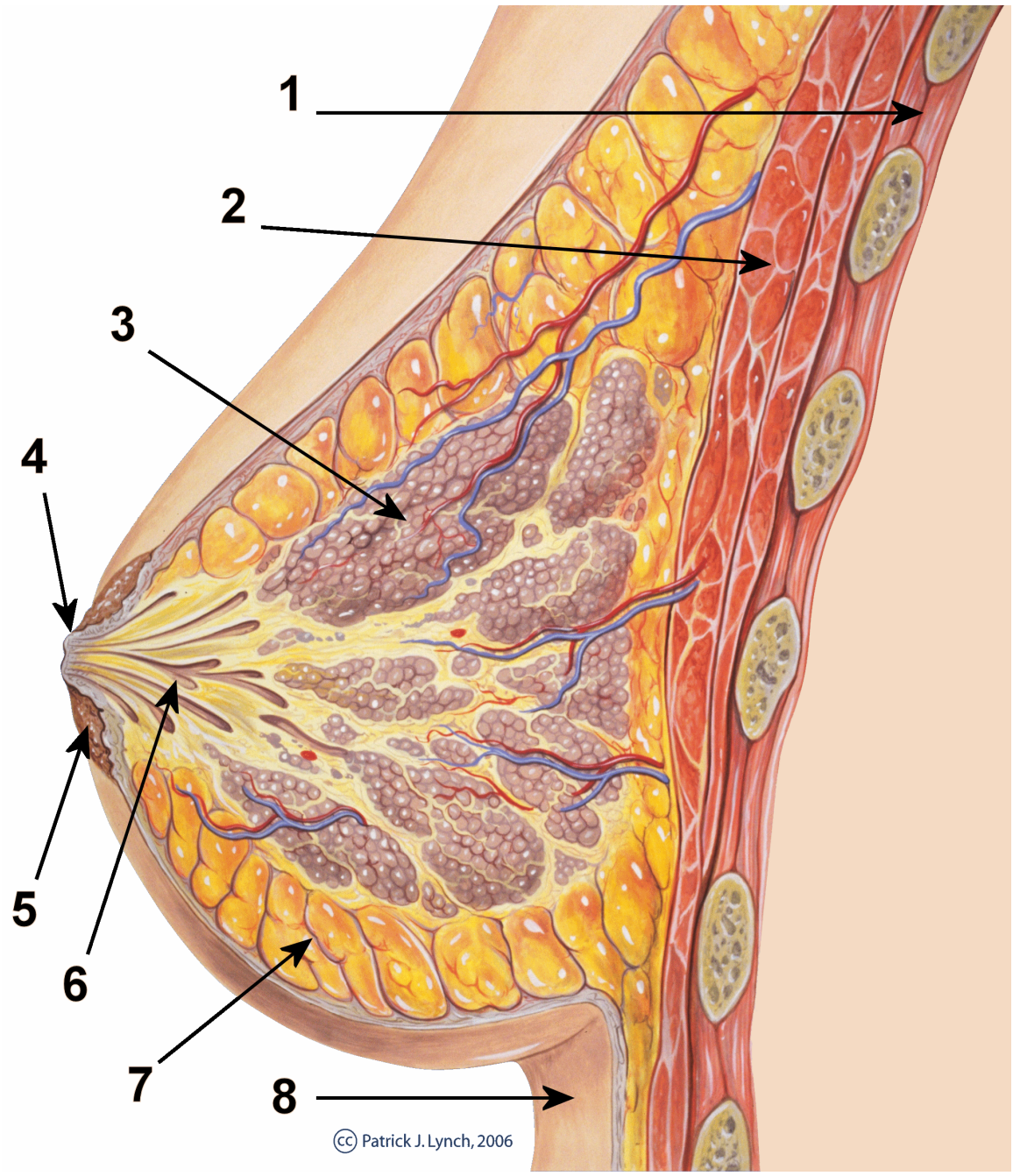


Figure 4. The different tissues of the human mammary gland. 1: Chest wall including ribs. 2: Pectoral muscles. 3. Breast lobes surrounded by stroma. 4: Nipple surface. 5: Areola. 6: Lactiferous ducts. 7: Adipose tissue. 8: Skin. Reproduced under a creative commons license.

1.2 BREAST CANCER, BACKGROUND

1.2.1 EPIDEMIOLOGY

Excluding non-melanoma skin cancer, breast cancer is by far the most common cancer and surpassed only by lung cancer as the most common cancer death in women worldwide (28). In 2012, there were an estimated 1.7 million new breast cancer patients and 0.5 million breast cancer specific deaths globally. The number of new cases per capita is still up to several times higher in developed countries, while mortality is higher in poorer countries (Figure 5) (29). The lower incidence in developing countries can chiefly be explain by a generally lower use of hormone replacement therapy, younger age at first child, higher number of children, older age at menarche, higher physical activity and less obesity. The relatively higher mortality on the other hand, is partially explained by lower access to screening programs and worse detection rates as well as less efficient healthcare for affected women, but genetically induced differences in risk cannot be ruled out (30-31). In concrete figures, 249 000 new invasive breast cancers (approx. 160 cases/100 000 women), 61 000 carcinomas in situ and 41 000 breast cancer-related deaths (26 deaths/100 000 women), were expected in the U.S. in 2016 (28). The figures in Western Europe including Sweden are similar on a per-capita basis, implying a female life time risk of obtaining the disease of 12.1 % (31). This can be compared to an incidence ranging from 11 to 45/100 000 women in Africa and mortality rates in the range of 10 to 35/100 000 women (33,34). In a historical perspective, survival has increased in both developing and developed countries. Since the 1970s, age-standardized mortality rates have been decreasing by roughly 1 % annually in the western world; ten-year relative survival has increased from around 40 % to at least 80 % today. Additionally, many western countries including Sweden have actually managed to decrease breast cancer incidence, or at least reduce it to a steady state, for women aged 50-64 years in the very last decade. This is attributed to reduced use of hormone replacement therapy for menopausal symtoms: current users of Oestrogen-progestagen combinations have a doubled relative risk for the disease (32), and perhaps increased awareness of liftestyle- and hereditary risks including susceptibility genes like *BRCA1* and *BRCA2* (28,29,31).

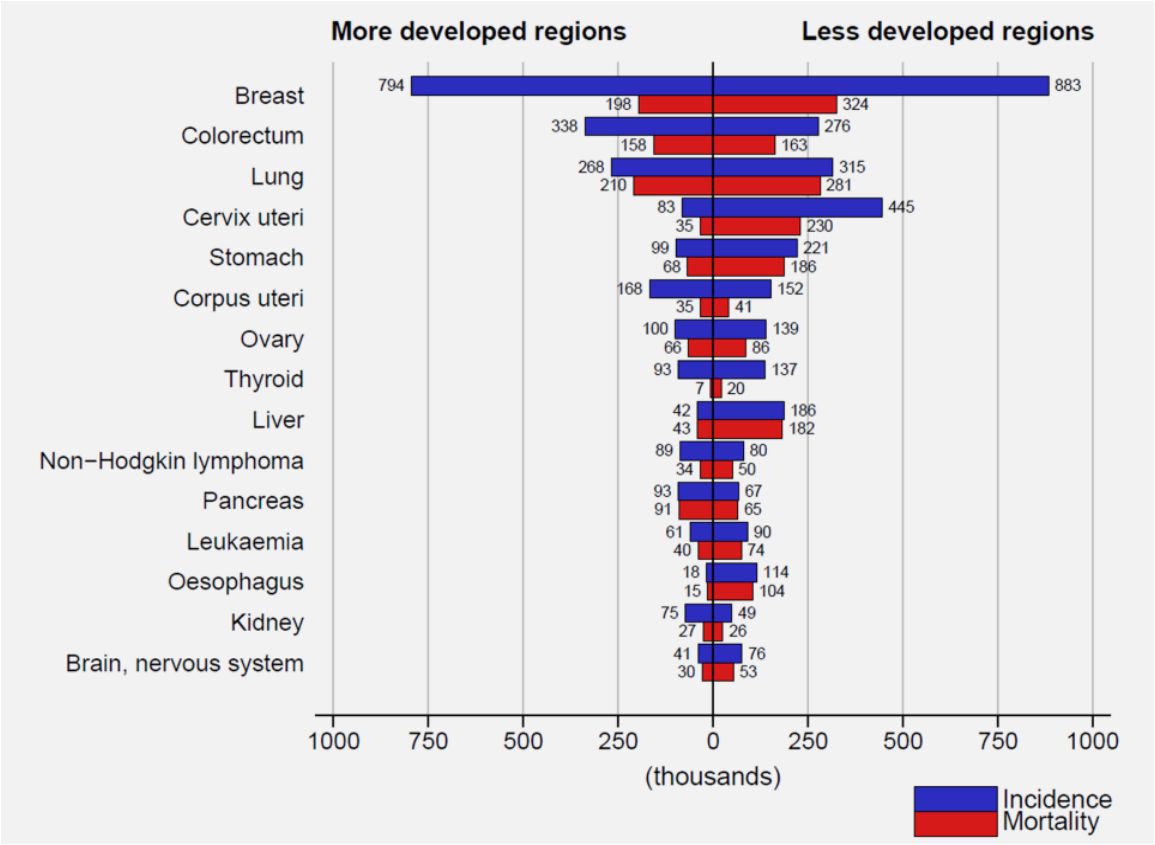


Figure 5. Estimated numbers (in thousands) of new cancer cases and mortality in women in more developed (left) and less developed (right) regions of the world in 2012. Modified from Ferlay *et al* (29). Reprinted with permission from John Wiley and Sons.

1.2.2 THE HALLMARKS OF CANCER

Cancer development is a complicated process, and even though remarkable progress toward understanding its mechanistic underpinnings has been made in the last decades, all details are yet to be understood. Cells acquire multiple changes on multiple levels from the DNA nucleotide sequence to the proteome, each driving them a step on their way towards malignancy. Naturally, the random and unspecific distribution of such changes implicate that most of them will lead to severe cell damage and apoptosis. Through natural selection, only the changes that happen to prolong cell survival, increase proliferation, induce invasiveness etc. will be accumulated throughout the tumorigenic process.

To enable comprehension of such a vital and faceted subject, systematization and some degree of simplification is warranted. The concept of "Hallmarks of cancer", introduced by Hanahan and Weinberg in 2000 and updated in 2011, offers a simple and conceptual model of these changes, or capabilities, that a normal cell will have to acquire in the process of becoming malignant (Figure 6) (35,36).

Figure 6. The Hallmarks of cancer. As normal cells evolve progressively to a neoplastic state, they acquire a succession of these capabilities:

1. Sustaining proliferative signaling
2. Evading growth suppressors
3. Activating invasion and metastasis
4. Enabling replicative immortality
5. Inducing angiogenesis
6. Resisting cell death
7. Genome instability and mutation (enabling characteristic)
8. Tumor-promoting inflammation (enabling characteristic)
9. Reprogramming energy metabolism
10. Evading immune destruction

Modified from Hanahan and Weinberg (36). Reprinted with permission from Elsevier.

Evidently, these hallmarks are in no way mutually exclusive. Genomic instability for example, can be viewed as a prerequisite for generating enough genomic events to induce the other changes and is therefore identified as an enabling characteristic. Evading growth suppressors and resisting cell death are in some aspects overlapping entities, not least by the fact that many growth suppressors act to induce apoptosis.

Point by point:

1. Sustaining proliferative signaling

The ability to sustain chronic proliferation is very central to the research papers included in this dissertation, and presented by Hanahan and Weinberg as the most fundamental trait of cancer. In normal cells, the production and release of growth promoting signals is under careful control to maintain the tissue architecture. Cancer cells are however independent of this control, usually by activation of cell surface receptors containing intracellular tyrosine kinase domains. The activation can be triggered through autocrine secretion, an increased number of receptors or alterations in the receptor itself. Activation of the receptor in turn trigger branched intracellular signaling pathways that ultimately increase the cell's progression through the cell cycle. Most of the *proto-oncogenes*, pathologically activated by point mutations, boosted promoter region activity, gene amplification, increased RNA or protein stability and/or a chromosomal translocation into *oncogenes* coding for oncoproteins, are involved in proliferative signaling by definition. In the context of breast cancer, 15-20 % of tumors profit from amplification of the *ERBB2* gene, which leads to an increased expression of HER2 growth factor receptor and thereby increased proliferation (37). Further, the *PIK3CA* gene that transcribes the PI3K protein kinase is often constitutionally activated by mutations resulting in increased proliferation (38). The HER2 receptor will be elaborated on further below.

2. Evading growth suppressors

Cancer cells must circumvent extensive reactions to regulate growth. *Tumor suppressor genes* encode proteins that effectively block pathological proliferation, usually by activating programs for senescence or apoptosis. These operate as control nodes, in which metabolic stress, damage to the genome, suboptimal growth factor signaling etc. is discovered and acted upon. Many of these tumor suppressors function in larger networks leading to a degree of redundancy. The classic prototypes for these suppressors are the Tumor protein 53 (*TP53*) and retinoblastoma (*RB1*) genes. While the protein product of *TP53* receives input from intracellular systems, the retinoblastoma protein integrates signals from diverse extracellular and intracellular sources. If abnormalities are discovered, both then work to inhibit the growth-and-division cycle. Evasion of these suppressors is thereby a prerequisite for the formation of many malignancies. This ability is normally acquired by loss-of-function mutations, deletions or downregulation of protein expression. In a two-hit model, loss of function of both alleles of *RB1* is required for tumor formation. Indeed, children with a constitutional deactivating *RB1* mutation need only a second somatic hit to develop retinoblastoma and other tumors, and have a relative risk of >40 000 of doing so (36,39).

3. Activating invasion and metastasis

Much like the overall concept of cancer hallmarks, tumor cells' invasion and formation of metastases is not a single event but rather a multistep procedure. This encompasses a succession of cell changes, beginning with the capability to invade local tissues, then intravasation into nearby blood and lymphatic vessels and transit to distant sites, followed by extravasation, the formation of micro metastases and finally growth into macroscopic metastases. In the initial steps, many epithelial cancers have impaired cell-to-cell adhesion by

loss of the E-cadherin adhesion molecules. In the last decade, increased interest has been paid to this loss as a part of a larger cascade dubbed the epithelial-mesenchymal transition (EMT), in which a plethora of transcriptional factors including Snail, Slug, Twist, and Zeb1/2 have been identified. These drive the cells towards mimicking the machinery intended for wound healing and cell migration during embryogenesis. Further, crosstalk with stromal cells and macrophages surrounding the preinvasive epithelium has been found to prime these cell lines before invasion occur. The final step of adaption to a new environment and macroscopic detectable metastasis can take several years (36,40).

4. Enabling replicative immortality

With the exclusion of stem cells, normal cells can only perform a limited number of mitoses. Central to this limit are the telomeres that cap the ends of each chromosome. They consist of multiple hexanucleotide repeats and are successively shortened with each mitosis, ultimately leading to failure of protecting the chromosomal ends from end-to-end fusions, which usually triggers senescence or cell apoptosis. The length of these telomeres is thereby an indication of the number of divisions a cell can manage. Tumor cells obtain immortality by expression of the telomerase enzyme or by a closely related recombination mechanism, that both serves to maintain telomere length. In contrast to normal mature epithelium where no expression of the telomerase gene *TERT* is detectable, it is expressed in more than 90 % of invasive breast cancers (36,41).

5. Inducing angiogenesis

The increased proliferation, cell turnover and metabolic rate in solid tumors make for a high demand on nutrients and oxygen, as well as a mechanism for evacuation of accumulated waste and carbon dioxide. Naturally, recruitment of a rich vascular network is necessary before any tumor can grow into a macroscopically detectable lesion. Following an embryological model of angiogenesis otherwise only present in wound healing and the endometrial proliferative phase in the menstrual cycle, the tumor cells flip the balance of inducing and opposing factors to promote sprouting of new vessels. Factors secreted include the vascular endothelial growth factor (VEGF) and members of the fibroblast growth factor (FGF) family. Further, the presence of a premalignant lesion attracts macrophages, neutrophils, mast cells and myeloid progenitors that orchestrate an inflammatory reaction that contributes to the angiogenesis (35,36).

6. Resisting cell death

As mentioned previously, normal cells enjoy several parallel systems that inhibit excessive proliferation and accumulation of genetic damage. In addition to this, specific death-inducing signals can be administered through both extracellular and intracellular mechanisms in response to severe deviations from the normal state. The Fas ligand, belonging to the tumor necrosis factor (TNF) superfamily, is the archetype for extracellular death signals. In the intracellular domain, Bax and Bak dissolve the outer mitochondrial membrane and release cytochrome c into the cytoplasm. Both pathways culminate in a proteolytic cascade, triggered

by the release of caspases and the start of the apoptotic program. Tumor cells, including breast cancer cells, can avoid these death signals by one or several processes: Increased expression of antiapoptotic BCL2 proteins, increased expression of survival signals like IGF1 or by downregulation or deactivating mutation of apoptotic mediators including the Fas ligand receptor and *TP53* (35,36,42).

7. Genome instability and mutation (enabling characteristics)

An unstable genome is the prerequisite for enough genomic events to make the other abilities possible. Tumor progression can be viewed as a succession of clonal expansions, of which each profit from chance acquisitions of enabling changes to the genotype. This is not the result of a will of the malignant or premalignant cells to become more aggressive, but rather a consequence of natural selection that eliminates all but the changes of the genome that increase survival and proliferation. Cancer cells eventually increase the rate of mutation by becoming more sensitive to mutagenic agents or by a breakdown in the genomic maintenance machinery like *TP53* or DNA repair agents. *BRCA1* and *BRCA2* are DNA repair agents that protect the integrity of the genome. Having a germline mutation in either of these genes results in a greatly increased risk of developing breast cancer: 55 - 65 % of women who inherit a *BRCA1* mutation and around 45 % who inherit a *BRCA2* mutation will develop breast cancer by the age of 70 years (36,42,43).

8. Tumor-promoting inflammation (enabling characteristics)

The presence of an inflammatory response to virtually every neoplastic lesion is an established fact. Initially thought to represent an attempt to eradicate the tumor, these inflammatory responses are now seen as intimate partners and in more than a few instances promoters of the disease. Thus, the concept of tumor promoting inflammation has been coined. In this aspect, the inflammatory response mainly operates by supplying bioactive molecules to the tumor and its closest environment. This includes reactive oxygen species that break down cell membranes and are actively mutagenic, growth factors, proangiogenic factors, survival factors and enzymes that modify the extracellular matrix to aid invasion and metastasis (36).

9. Reprogramming energy metabolism

The increased rate of proliferation in cancer naturally requires an increased supply of energy. At first sight, it is therefore somewhat confusing that many cancers have a solid drive towards glycolysis even under aerobic conditions, with its 18-fold lower efficiency of ATP production. Glucose transporters like GLUT1 and the glycolysis promoting transcription factors HIF1α and HIF2α can be upregulated by both activated oncogenes like *RAS* and *MYC* and mutated tumor suppressors like *TP53*. The rationale from the cancer's perspective is that it renders the tumor cells somewhat resistant to environmental conditions that would otherwise be very limiting to tumor growth. Hypoxia is common in the central regions of a rapidly expanding solid tumor. Further, glycolytic metabolites allow for synthesis of nucleosides, amino acids and the macromolecules and organelles required for the assembly of

new cells. In some tumors, a symbiosis has been found between glycolytic cells that secrete lactate and cells that import the lactate for use in the citric acid cycle, rendering the tumor somewhat self-sufficient in energy (36).

10. Evading immune destruction

Both the innate and adaptive arms of the immune system contribute to the defense against tumor formation, including tumors of non-viral etiology. Consequently, tumors will have to avoid or cooperate with the immune system to prosper. An example of this is that colon and ovarian tumors with dense infiltrations of cytotoxic T lymphocytes and natural killer cells have better prognosis than tumors that avoid such infiltrates (36). As mentioned in the "Resisting cell death" and "Tumor promoting inflammation" points above, other evidence suggests that tumors in other aspects can benefit from the interplay with the immune system. This serves as an illustration that compensatory mechanisms exist, that help cancer cells not only to avoid immune destruction but in some instances even prosper from the interaction. The molecular mechanisms behind this are not fully understood, but include recruitment of regulatory T cells and myeloid derives suppressor cells, secretion of immunosuppressive factors such as TGF-β and activation of the inhibitory CTLA-4 receptor on T-cells (36).

1.2.3 BREAST CANCER GENESIS AND HETEROGENEITY

The invasion of epithelial cells from the TDLUs through the myoepithelial layer and basal membrane into the surrounding stroma is the very definition of malignant breast cancer. The molecular steps behind this progression are not established in full detail. In many instances, the actual invasion is preceded by a morphological carcinoma in situ or hyperplastic stage where events that gradually change the genotype of both epithelial and myoepithelial cells are accumulated (44,45).

However, in contrast to a historic morphological theory of a linear progression through several increasingly severe premalignant stages before actual invasive disease, later evidence points to the fact that not all premalignant lesions lead to malignancy, and that not all malignancies are preceded by the premalignant morphological stages (40,44,45). Additionally, it is increasingly clear that breast cancer is not one single disease, but rather several different diseases originating from the same organ, with different prognosis and optimal treatments. These different diseases do not share all molecular characteristics, and thereby not necessarily the same molecular or morphological precursor states.

The discovery of cancer stem cells has further challenged the idea of a successive progression of changes to mature epithelium. The cancer stem cell theory points out that cancer cannot be seen exclusively as a homogenous clonal expansion in which any cell has equal probability of driving further tumor development and proliferation. Indeed, the first potential candidate for breast cancer stem cells (BCSC) identified was over 50 times more tumorigenic than a

random population of its matured peers. This entails that a malignant or premalignant morphological state such as DCIS could be interpreted as the result of proliferation of a minor subpopulation of stem cells initiated by a founding stem-like cell, or at least a less differentiated cell, rather than the result of an initiating event to the entire region of the epithelium (46-49).

Note that BCSC should not necessarily be confused with the normal mammary stem cells that can be found in the deep layer of the epithelium. Their slow proliferation rate would make them quite unsusceptible to transcriptional errors and genomic instability. On the other hand, their potential for unlimited divisions and the lack of other candidates with stem cell-like properties still make the mammary stem cells worth mentioning in this context.

The stochastic plasticity model of the origin of breast cancer, interweaves with the concept of BCSC and theorizes that differentiated breast cancer epithelial cells have the potential to de-differentiate to a stem cell-like state, to form a clone that then drives further tumor growth and progression. This de-differentiation mechanism thereby signifies that the BCSC have acquired a stem cell-like phenotype, rather than being a clone with a stem cell-like genotype. The de-differentiation is thought to occur very much like the epithelial-mesenchymal transition previously described as one of the hallmarks of cancer (50-53).

Several immunohistochemical markers for the BCSC have been proposed, among which Cd44, Cd24, ALDH1, PKH26, DLL1 and DNER can be mentioned. Isolation of BCSC is further aided by their ability to form and proliferate in rounded clusters of cells called mammospheres without being inhibited or destined for apoptosis by loss of cell-to-cell contact. However, none of these techniques have complete sensitivity and specificity for all BCSC candidates, which still fuels some controversy over their existence (54-58).

Studies have shown that the concentration of BCSC is different in different breast cancer subtypes, and that they are less susceptible to conventional chemo- and radiotherapy. Several mechanisms have been proposed for the latter. For instance, the expression of detoxifying enzymes, efflux pumps and repair enzymes have been found to be upregulated, and the expression of death receptors to be downregulated in BCSC. The therapeutic targeting of these cells is thereby elusive. The DLL4 receptor, part of the Notch-signaling pathway, has been proposed to be such a target. Others are the interleukin 8 receptor and intracellular enzymes downstream in the JAK/STAT pathway. Considering the possibility of a switch between a differentiated and de-differentiated state however, specific targeting might only promote the state not targeted (59-69). Our own group have identified ERβ as a mediator of estrogen stimulation of BCSC but not differentiated breast cancer cell lines. Consequently, the ERβ-selective antagonist 4-[2-Phenyl-5,7bis (trifluoromethyl) pyrazolo[1,5-α]pyrimidin-3-yl]phenol (PHTPP) is a potent inhibitor of BCSC proliferation. Furthermore, inhibition of the mTOR pathway with agents such as rapamycin and everolimus significantly reduced mammospheres formation (70,71).

At first sight, these findings are seemingly contradicted by the clonal evolution model, which offers a different perspective of cancer genesis and progression. In short, the model states that tumors progress by natural selection of the subpopulations of cells with the most advantageous characteristics at any given time. As such, this offers some explanation to the significant heterogeneity found within a tumor, which is a subject that defines a whole scientific field in itself. In fact, concept intratumor heterogeneity offers a way to reconcile the two seemingly contradictory concepts of cancer stem cells and clonal evolution. Given the increasing amount of research into tumor heterogeneity, both concepts can be valid.

Intratumor heterogeneity acknowledges the existence of different cell subpopulations within a tumor, or between a primary tumor and its metastasis, regardless if their differences are measured at the morphological, gene expression, protein expression or mutational load level. For instance, HER2 and Ki67-status may vary significantly between primary tumors and metastases, and both markers may also vary between different regions within the same tumor. Massively parallel sequencing has shown that both spatial heterogeneity, which signifies clonal differences across geographically separated regions of a tumor, and temporal heterogeneity, in which tumor tissue varies over time or with disease progression, are indeed common phenomena. Consequently, the differences within a tumor can be the result of both hierarchically arranged subpopulations founded by BCSC and clones that have evolved through natural selection (53, see also section 4.5)

1.2.4 THE METASTATIC PROCESS

Metastasis is a complex and only partially understood process. To be able to colonize a foreign anatomic site, the cancer cell must overcome a series of obstacles: these typically include separation from the original tumor and its surrounding tissue, invasion through barriers such as vessel walls, fasciae and basal membranes, intravasation and survival in blood- or lymphatic vessels, extravasation from these vessels, implantation and survival in a new microenvironment. Further, this needs to be followed by proliferation, angiogenesis, metabolic adaptation and avoidance of the immune system before these cells can generate a clinically detectable metastasis (35,36).

Nevertheless, metastasis is a common clinical issue, as breast cancer mortality is closely associated with disseminated disease but very rarely with the presence of a primary tumor only. In other words, patients with tumors restricted to the breast have a much better prognosis regardless of the other characteristics of that tumor. Recent investigations suggest that metastasis can be an early event, and that 60-70 % of patients have cancer cells that have undergone at least the early steps in metastasis at the time of discovery of the primary tumor. Further, up to one third of women without axillary lymph node metastases will still develop distant site metastases at a later point in time (72). Further insights into this process are therefore essential for improved diagnosis and treatment.

Several models have been suggested for the metastatic process:

The *progression model* is the classic theory that prevailed for decades, and is still regarded as valid in many aspects. It suggests that a metastasis is the result of a series of sequential mutational events to one or several clones of cells in the primary tumor, usually driving them towards a less differentiated state and eventually allowing for selection of a small fraction of cells with full metastatic potential.

The *dynamic heterogeneity* and the closely related *extended transient metastatic compartment* models were proposed as an explanation to the fact that disseminated cells in the circulation and metastases do not always have higher metastatic potential than their primary tumors. If dissemination and metastasis is always the result of a series of mutations that successively increase the metastatic potential, disseminated cells and metastasis would always be more inclined to generate further metastases than the primary tumor. However, this is not always the case. As a solution to this paradox, these models suggest that all cells in a primary tumor eventually acquire metastatic potential, but only a small fraction will be at the right location and in the right environment to be able to actually spread. Consequently, the cells that eventually form a distant metastasis might not be the clone with the very highest metastatic potential at a later point in time.

The *horizontal gene transfer* or *genometastasis* model suggests that metastatic growth can be induced not only by seeding of cells themselves from the primary to the distant site, but through uptake of circulating tumor DNA by cells with stem-cell like properties at the distant site. In this model, metastases could consequently be viewed as de novo tumors that have been induced by a form of genetic signaling from the primary tumor (72).

The *early oncogenesis* model applies findings of gene expression arrays pointing to the existence of several different gene signature profiles associated with risk for metastasis, both in the sense of intratumor heterogeneity within a bulk tumor and intertumor heterogeneity between different but in other aspects similar breast tumors. Consequently, this model gives some support to the original progression model, in that subsets of cells or tumors possess an inherent metastatic potential. This subset might however be so small in relation to the other populations of cells within a tumor that it might not always be possible to detect with tissue sampling (72).

As demonstrated by these brief accounts for several different models of the metastatic process, none gives a complete answer for all situations. Again, the different models are increasingly regarded as not mutually exclusive, but complementary and all needed for a thorough appreciation of the process. Quite possibly, all of them are correct at least in part, supporting the notion that there are several ways in which cancer cells can overcome the obstacles for metastasis presented here (see also subsection 1.2.2 on the hallmarks of cancer).

1.3 PREDICTIVE AND PROGNOSTIC FACTORS IN BREAST CANCER

1.3.1 BACKGROUND -THE DIAGNOSTIC PROCEDURE

After discovery of a suspicious breast lump through palpatory findings by the patient herself, by a doctor or through routine mammography, cells or tissue from that lump is usually sampled by a fine needle aspiration or core biopsy. Based on the pathologist's findings in this cell or tissue analysis, and other relevant information from clinical and imaging examinations, the diagnosis of breast cancer and a plan for treatment is set. To an increasing extent, the diagnosis of breast cancer and the planning of treatment is the shared responsibility of a multidisciplinary team including pathologists, breast surgeons, oncologists and radiologists.

Several fundamentally distinct treatment modalities exist, including surgery, radiotherapy, cytotoxic chemotherapy, endocrine treatment and anti-HER2 therapy.

Neoadjuvant treatment, i.e. therapy given before surgical resection, can be considered in many clinical contexts: Generally, the aim is to reduce tumor size and axillary lymph node tumor burden and thereby downstage the disease. In some cases this allows for previously inoperable tumors to be radically resected. Cytotoxic chemotherapy is typically given for any high grade, large, axillary lymph node metastasized, ER negative, triple negative (ER, PR and HER2-negative), highly proliferative or HER2 overexpressing tumor. For HER2 overexpressing tumors, the clinical routine is to use dual blockade with HER2-monoclonal antibodies trastuzumab and pertuzumab on a chemotherapy backbone, usually in the form of taxanes followed by anthracyclines. In the neoadjuvant and metastatic setting, lapatinib can be used instead of pertuzumab (in combination with chemotherapy and trastuzumab) with an increased ratio of pathological complete response. The side effects of lapatinib, mainly diarrhea and nausea, is however clearly higher with this regimen. For hormone receptor positive tumors, endocrine treatment is typically added (for further details, see section 1.5 on breast cancer treatment).

After surgical removal, the specimen is measured and weighed. Several gross samples from the tumor and surrounding tissue are then embedded in one block of paraffin each. These blocks are then sectioned, stained immunohistochemically as well as with haematoxylin and eosin and put on histopathological glass slides for examination under the microscope by the pathologist.

Historically, breast cancer has been classified according to its histological appearance. Still, the World Health Organization (WHO) suggests a largely morphological classification of this heterogeneous disease, which remains a very important part in current clinicopathological routine. Here, carcinoma characterized as "no special type", also known as ductal carcinoma

no special type or invasive ductal carcinoma, constitute the majority of invasive breast cancers (≈70 %). The designation comprises a heterogeneous group of tumors without the specific morphological characteristics that would classify them into one of the "special" subtypes. Hence, this is more or less a default diagnosis of invasive breast cancer. The most common of the special subtypes are lobular carcinoma, tubular carcinoma, mucinous carcinoma, carcinoma with medullary and apocrine features, micro papillary carcinomas, papillary carcinomas and metaplastic carcinomas (73).

A quite significant overlap in karyotype between these histological subtypes exists. Generally, a lower number of genetic aberrations have been found in lobular cancer compared to carcinoma no special type, which may reflect a generally lower histological grade of lobular cancer (further elaborated on in subsection 1.3.3).

Categorization according to the four gene expression-based 'intrinsic' subtypes "Luminal A", "Luminal B", "HER2-enriched" and "Basal-like" is a more novel and perhaps viable method of choice for prognostic and predictive value (subsection 1.3.4). A fifth frequently mentioned "Normal-like" subtype is excluded from many major documents, not least because it has been suggested to represent an artifact of contamination of tumor RNA with RNA from normal breast cells (Figure 7) (74-83).

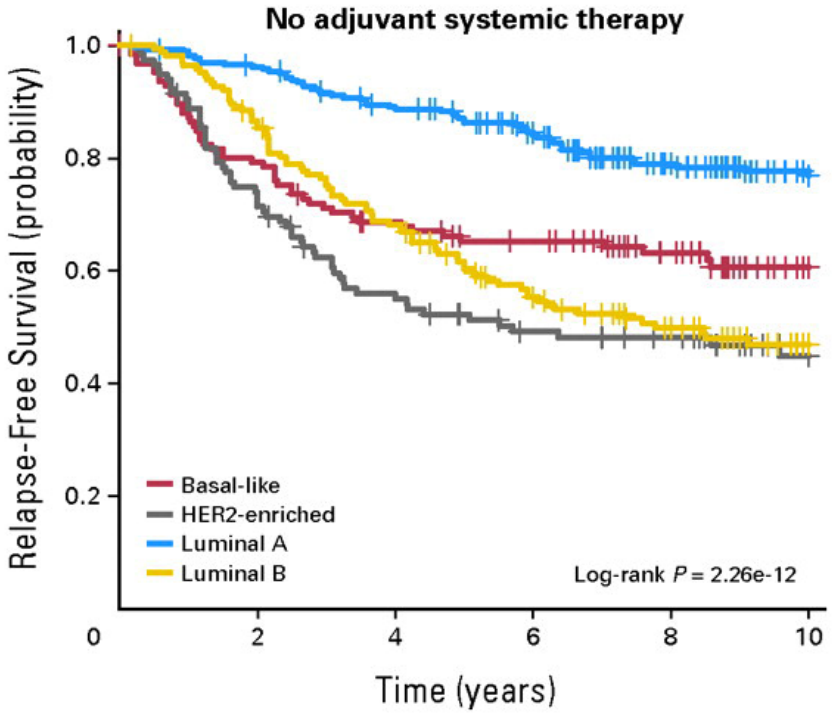


Figure 7. Relapse free survival for patients without adjuvant systemic therapy including HER2-targeted therapy across gene the expression based PAM50 intrinsic subtypes of breast cancer: Luminal A, Luminal B, HER2-enriched and Basal-like. Modified from Parker *et al* (78). Reprinted with permission from the American Society of Clinical Oncology.

However, gene expression tests are still expensive and time consuming, and expected to remain beyond the financial and practical boundaries of clinical practice for a few more years. This has created a demand for the cheaper and more readily accessible immunohistochemical (IHC) stains to act as surrogate biomarkers for the gene expression-based subtypes. International expert consensus recommend primarily four such biomarkers to be evaluated during routine pathological work-up of resected or biopsied breast cancer tissues (74-76): the human epidermal growth factor receptor 2 (HER2), the estrogen receptor α (ER) and the progesterone receptor (PR) and the proliferation-associated nuclear protein Ki67. The latter has however not seen widespread use in the United States (these biomarkers are further described in subsections 1.3.5 to 1.3.8). Based on the status of the respective surrogate biomarker, conclusions can be drawn about the biological behavior, prognosis and surrogate subtype of the individual tumor, which in turn guide the treatment strategy (76,84-88) (Table 1).

Intrinsic subtype	Surrogate IHC classification
Luminal A	ER ≥ 1 % and/or PR ≥ 20 % and HER2 “negative” and Ki67 “low”
Luminal B	1. ER ≥ 1 % and/or PR ≥ 20 % and HER2 “negative” and Ki67 “high”, or:
	2. ER ≥ 1 % and PR < 20 % and HER2 “negative”. Any Ki67, or:
	3. ER ≥ 1 % and/or PR ≥ 1 % and HER2 “positive”. Any Ki67.
HER2-enriched	ER < 1 % and PR < 1 %. HER2 “positive”. Any Ki67.
Basal-like	ER < 1 % and PR < 1 %. HER2 “negative”. Any Ki67.

Table 1. Gene expression based “intrinsic” subtypes of breast cancer and their surrogate classification based on immunohistochemical (IHC) stains of ER, PR, HER2 and Ki67. % = Proportion of tumor cells stained with the respective biomarker to the total number of tumor cells counted. ”Positive”, ”negative” = As defined by the American Society of Clinical Oncology and College of American Pathologists recommendations for human epidermal growth factor receptor 2-testing in breast cancer. “High”, “low” = Proportion of Ki67 above or below a threshold that should be predefined according to each laboratory’s own reference data. This threshold is generally in the range of 14-29 %. Adapted from international guidelines and other relevant publications (74-77, 85, 89-91).

Consequently, it is very important that evaluations of biomarker status is sufficiently concordant with gene expression tests. Any dissimilarities in subtype classification between the two methods are associated with a risk of dissimilar conclusions of prognosis and divergent treatment decisions. If a specific therapy is indicated for patients with Luminal tumors as defined by gene expression tests, it might not be given to patients with tumors wrongly classified as non-luminal (Basal-like or HER2-enriched) with IHC. Conversely,

treatments with severe side effects such as cytotoxic chemotherapy might unnecessarily be given to patients if the IHC status suggests a more aggressive phenotype than gene expression tests. Unfortunately, evaluations of biomarker status do struggle with significant intra- and interobserver variability, as well as repeatedly shown dissimilarity with the gene expression tests (92). This is highlighted in the evaluation of Ki67, for which there is no general consensus on what number of cells to score in which tumor region, or even what threshold for the number of Ki67-positive cells (Ki67-index) that distinguish highly from lowly proliferative tumors (93-101). Although interobserver concordance have reached 99 % (κ 0.95), 85 % (κ 0.85), 85 % (κ 0.70) and 85 % (κ 0.64) for ER, PR, Ki67 and HER2 IHC, respectively, with strict adherence to guidelines (95), thresholds and general definitions are considered unreliable outside individual laboratories' own reference data (74,77,96).

A threshold proportion of Ki67-positive cells to the total number of assessed tumor cells in the range of 20 to 29 % have been suggested as one of the criteria to distinguish the more proliferative 'Luminal B-like' disease from the less proliferative 'Luminal A-like" disease. More specifically, a cutoff of ≥ 20 % for highly proliferative tumors is commonly used (75,76,97). The 2015 version of St. Gallen International Expert Consensus mention that the uncertainty and variability of IHC testing may be reduced by Image Analysis, but provide no concrete suggestions or details on how to apply this in practice (76). Improvements to the biomarkers' prognostic value and congruence to gene expression tests are therefore a major aim of this thesis.

According to the National Institutes of Health biomarkers definitions working group, a biomarker, or biological marker, is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (102). In other words, a biomarker is an objective sign of medical state that can be observed and measured from the outside of the patient. Examples of biomarkers include anything from blood pressure and visual acuity to laboratory tests on blood samples, immunohistochemistry and gene expression assays. Again, the immunohistochemical biomarkers ER, PR, HER2 and Ki67 provide surrogate value in both a therapy predictive and prognostic sense. 'Therapy predictive' denotes a factor that identifies an outcome of a specific therapeutic intervention. E.g. a hormone-receptor (ER and/or PR) positive breast cancer is expected to respond to treatment with an ER-antagonist like tamoxifen, cytotoxic chemotherapy is mainly effective on highly proliferative (high Ki67) and/or > stage I disease, and trastuzumab is expected to be effective for HER2 overexpressing tumors. 'Prognostic' denotes the biomarker's ability to forecast the outcome for the patient, unrelated to given therapy. E.g. tumor size, histological grade and lymph node metastases. In this sense, HER2 and Ki67 are both therapy predictive and prognostic biomarkers since HER2 over expression and high concentrations of Ki67-positive cells in the tumor tissue implicate a poor prognosis (Table 2) (101,103-105).

Further details on Ki67, gene expression assays and other relevant biomarkers in breast cancer will be given along with the classic clinicopathological parameters below.

Therapy predictive biomarkers Correlate with outcome of a specific therapeutic intervention.		**Prognostic biomarkers** Correlate with patient prognosis
ER Ki67 HER2	Endocrine treatment (1.5.3) Cytotoxic chemotherapy (1.5.4) Anti-HER2 therapy (1.5.5)	Tumor size (1.3.2) Lymph node metastases (1.3.2) Histological grade (1.3.3) PR (1.3.5) HER2 (1.3.6) Ki67 (1.3.7)

Table 2. Examples of therapy predictive and prognostic biomarkers relevant to this thesis, as well as basic treatment regimens directly suggested by the former. Note that some biomarkers are both therapy predictive and prognostic. The correlation between biomarker and therapy response and prognosis is not necessarily positive. E.g. a higher proportion of Ki67-positive cells, but a lower proportion of PR-positive cells, indicate a worse prognosis. In several publications, PR has been regarded as a therapy predictive biomarker for intact signaling pathways of ER and thereby sensitivity to endocrine treatment, indicating that the distinction is not clear cut (24,136). Numbers in parentheses indicate subsections in which further details can be found.

1.3.2 BREAST CANCER STAGE

The 5-tiered stage of breast cancer (0 to IV) is determined by primary tumor size (T), presence of metastasis in loco-regional lymph nodes (N) or at distant sites (M), very much like most other solid tumors in the TNM classification system (103,104).

Stage 0 signifies that the tumor is non-invasive, i.e. a carcinoma in situ, commonly of the ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) type. These tumors have the very best prognosis. Stage I breast cancer signifies the earliest invasive stage with a 5-year overall survival (OS) well over 90 %. Stages II-III signify a gradually more fulminant disease for which therapy is generally intended to be curative. Stage IV indicates the presence of one or several distant metastases with a mean 5-year OS of only 12 % (103,104). Patients with stage IV disease can generally only be offered palliative treatment.

Disseminated cancer cells usually spread intravascularly in blood- or lymphatic vessels, and the presence of axillary lymph node metastases is indeed the very strongest prognostic factor in breast cancer. For the time being, any identified axillary lymph node metastasis suggest surgical removal of all detectable axillary lymph nodes with or without macroscopic metastases, which has been shown to reduce the risk of axillary recurrence. Whether it reduces the risk for distant metastases remains to be proven. The presence of one or several axillary lymph node metastases is a strong indication for systemic chemotherapy and extended radiotherapy. An extensive resection of 10-20 out of the total 30-40 lymph nodes in the axilla is however not done arbitrarily, as side effects in terms of lymphedema, pain and limited arch of movement can be quite substantial (106-116).

To avoid unnecessary axillary dissections, the sentinel lymph node (SLN) technique with intraoperative lymphatic mapping in clinically node negative women has been generally accepted since the 1990s (106). A radiolabeled colloid is injected along with a dye in the peritumoral and periareolar area of the breast prior to surgery. The SLN is then detected using a gamma-ray detection probe in the axilla. At many institutions, the histopathological examination of the excised SLN is done intraoperatively with a frozen section evaluation for immediate feedback to the surgeon, who then can decide whether to proceed with a complete axillary dissection or not. If the SLN is free from metastasis, the risk for metastases in other locoregional nodes is very low. There is no evidence that axillary dissections increase survival in women without metastasis in the SLN (SLN-negative) upon histological examination (106-113).

In SLN-negative patients, the largest diameter of the primary tumor is the most important prognostic factor. The 5-year OS for primary tumors <10 mm is nearly 99 %, but only 86 % with a largest diameter of 30-50 mm (103-105). The introduction of mammography screening programs have decreased the average size of detected tumors to <20 mm in many western countries (105,108).

Naturally, both largest tumor diameter and presence of lymph node metastasis is included in the criteria for anatomic stage groups (Table 3a and 3b) (102,103). Newer versions of the AJCC Cancer Staging Manual also include extensive criteria for prognostic stage groups, where many of the prognostic and predictive biomarkers beyond TNM are included. The different factors described in this section should thereby not be seen as alternative (Table 4, at the end of this chapter) or interchangeable tests, but rather as parts of an extensive diagnostic work-up of breast cancer specimens that each contribute with parts to the full picture.

Primary tumor		Regional lymph nodes		Distant metastasis	
Category	**Criteria**	**Category**	**Criteria**	**Category**	**Criteria**
pTX	Cannot be assessed	pNX	Cannot be assessed	M0	No evidence of distant metastases
pT0	No evidence of primary tumor	pN0	No lymph node metastasis	cM0(i+)	Micrometastases detected microscopically or by molecular techniques.
pTis	Carcinoma in situ	pN0(i+)	ITC only	M1	Distant metastases
pT1mi	≤ 1 mm	pN0(mol+)	Molecular findings only		
pT1a	1 ≤ 5 mm	pN1mi	Micrometastasis only (0.2-2 mm)		
pT1b	5 ≤ 10 mm	pN1a	1-3		
pT1c	10 ≤ 20 mm	pN1b	Metastases in internal mammary nodes, microscopically detected		
pT2	20 ≤ 50 mm	pN1c	pN1a + pN1b		
pT3	> 50 mm	pN2a	4-9		
pT4a	Extension to chest wall	pN2b	Metastases in internal mammary nodes, macroscopically detected		
pT4b	Skin ulceration or edema	pN3a	≥ 10, or metastases in infraclavicular nodes		
pT4c	T4a + T4b	pN3b	pN1a or pN2a + metastases in internal mammary nodes. Or pN2a + pN1b		
pT4d	Inflammatory carcinoma	pN3c	Metastases in ipsilateral supraclavicular nodes		

Table 3a. Definitions of pathological TNM categories for primary tumor (pT), regional lymph nodes (pN) and distant metastases (M). Measurements indicate greatest dimensions. ITC = isolated tumor cells. Numbers in criteria for pN indicate number of node metastases. Modified from AJCC Cancer staging manual 8th Edition 2017 (104). Reprinted with permission from Springer International Publishing.

ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 3b. AJCC anatomic stage groups, combined from the TNM stages defined in Table 3a. From AJCC Cancer staging manual 7th Edition 2010 (103). Reprinted with permission from Springer International Publishing.

1.3.3 HISTOLOGICAL GRADE

A tumor's grade is defined by how abnormal the cells and tissue look under a microscope. It is an indicator of how aggressive the tumor is, how quickly it grows and the risk for dissemination, whereas the tumor's anatomic stage indicates how far this progress has gone without regard to the time frame or the way of doing so. The major method for defining a breast cancer's grade is the Nottingham combined histological grading system, popularly also known as the Elston-Ellis grade (117,118). This dictates examination and quantification of 1) the remaining tendency for tubular formation, 2) the nuclear atypia and 3) the number of mitotic figures. Each parameter is given a score of 1 to 3, which is then combined into a total score of up to 9 points (117). All tumors can then be separated into one of three grades. Grade 1 tumors have a total score of 3 to 5 points, grade 2 tumors a total score of 6 or 7 points and grade 3 tumors a total score of 8 or 9 points. Grade 1 tumors have the best prognosis and grade 3 tumors the worst (118,119). The intermediate histological grade, to which roughly

half of all invasive breast cancers are categorized, are associated with an intermediate risk of recurrence and limited value in clinical decision-making (120). In some reports, interobserver concordance in the grading of breast cancer have been disappointing: in a study where 93 invasive breast cancers were evaluated at 7 different pathology departments, only 31 % of the tumors obtained the same histologic grade across all departments (overall mean kappa (κ) 0.54) (121). Further, gene expression and transcriptome patterns of grade 2 tumors does seem to be a mix of either grade 1 or grade 3 tumors, rather than a distinct entity of its own (120,122). Analyses of chromosomal and genetic aberrations in different tumor grades have also shown that evolution from lower to a higher grade is quite rare, challenging the concept of a gradual progression from lowly to highly aggressive cancer in the fashion of tumor stage (52). An example of this is that loss of 16q and gain of 1q is common in grade 1 tumors but rare in grade 3 tumors, especially evident in luminal tumors (39,40,44). If a progression of an individual tumor from a lower to a higher grade was truly the case, these losses and gains would likely be retained, with additions of further changes from grade 1 to grade 2 and from grade 2 to grade 3. Conversely, grade 3 tumors commonly have aberrations of other chromosomes and relatively higher rates of HER2 amplification and lower rates of ERα and PR expression (45,52,65,120).

1.3.4 INTRINSIC SUBTYPES

For decades, classification of breast cancer was exclusively based on the histological appearance and clinical parameters described above. The diagnostic criteria for characterization and differentiation of each possible morphological appearance under the microscope (ductal carcinoma no special type, lobular carcinoma, tubular carcinoma, mucinous carcinoma, carcinoma with medullary and apocrine features, micro papillary carcinomas, papillary carcinomas and metaplastic carcinomas) are however far from clear-cut, reducing its usefulness for clinical decision making (73).

Over the last two decades, gene expression studies have revealed that breast cancer is a heterogeneous group of diseases rather than one disease with several presentations. Perou *et al*. defined four different 'intrinsic' subtypes in 2000 (81): Luminal, Basal-like, HER2-enriched and Normal breast-like. Later studies have not established consensus that the Normal breast-like subtype is an actual form of breast cancer, as some propose it is rather a contamination of non-malignant cells to the breast cancer samples. Further, the Luminal group of cancers have been subdivided into a prognostically more favorable Luminal A subtype, and a less favorable Luminal B subtype. Other findings suggest additional but less often used intrinsic subtypes, such as interferon-rich, claudin-low and molecular apocrine (77,81).

The number of mutated genes have a positive correlation both with histological grade and intrinsic subtype, in the sense that the aggressive subtypes Basal-

like and HER2- enriched have more mutations in *TP53*, *NCOR1*, *NF1*, *PTPRD* and *RB1.* As expected, this mutational burden is also associated with a worse prognosis (79).

Since individual tumors cannot be subtyped by hierarchical clustering, single sample predictors (SSP) have been developed. This allows for classification of individual samples based on their gene expression profiles. Later, the SSP has been developed further and the number of measured gene expression levels reduced to include panels of 7-70 genes (described below).

Since the early 2000s, these gene expression-based arrays have seen increased clinical use. After analysis of the expression levels of thousands of genes, panels of 7 (Breast cancer index®) to 70 (Mammaprint®) genes that differentiate specific subclasses of breast cancer have been identified (77,78,80,81,84). The subclasses are in turn used for accurate prognostication in terms of the likelihood of relapse and distant metastasis (e.g. Breast cancer index®, OncotypeDx®, PAM50 and Mammaprint®), to identify a subset of hormone-positive tumors with excellent prognosis with endocrine therapy only (e.g. Endopredict®) and to predict response to different therapies. By the PAM50 assay, in which the level of mRNA from 50 selected genes are measured, tumors can be assigned one of the "Luminal A", "Luminal B", "HER2-enriched" or "Basal-like" intrinsic subtypes, each with a different expected relapse free survival (Figure 7, Table 1) (74-77, 80-85).

The major watershed in the clustering of subtypes is the level of expression of hormonal receptors. Tumors with high expression of these, of which ERα and PR are the most important, are classified as luminal tumors. The name is derived from the gene signature of the ducts' inner layer of luminal epithelial cells, which are the closest healthy relatives in the TDLU. Luminal A tumors have higher expression of cytokeratins 8 and 18 and hormone receptors, and lower expression of growth factor receptors and genes associated with proliferation, and thereby naturally a longer relapse free survival compared to Luminal B tumors (78). Among the hormone receptor-negative tumors, the Basal-like subtype express genes common in the outer lining of myoepithelial cells, like cytokeratins 5 and 14. HER2-enriched tumors overexpress HER2 and genes associated to this receptor. Additionally, other classes such as the Claudin-low subtype with similar traits to mammary Cd44+/Cd24- stem cells and a Normal-like subtype with similar traits to normal mammary tissue have been proposed. As mentioned however, these are not included in major guideline documents and the latter has been suggested to represent an artifact of contamination of tumor RNA with RNA from normal breast cells (74-77, 80-85).

Even with the feasibility to run RT-PCR on a minor number of selected genes from FFPE, which has reduced cost and time consumption significantly, gene expression tests are still relatively expensive and time consuming compared to routine immunohistochemical stains. As the latter indicate the expression of certain genes on a protein level, which is not

necessarily an exact reflection of RNA levels and thereby not a measurement of the same parameter, they are referred to as surrogate markers for the intrinsic subtypes (76,77,102).

1.3.5 HORMONE RECEPTORS

Estrogen receptors belong to a nuclear receptor superfamily with nearly 50 members that show large structural similarities (123). This indicates that they stem from a common ancestor that later has developed minor variations inducing different physiological functions. Nuclear receptors are generally transcription factors that alter the transcription of select genes when activated (123-125). The first estrogen receptor (ERα) was discovered by Elwood V. Jensen in the 1950s while the second (ERβ) was discovered by Gustafsson and colleagues in the 1990s (126). Both are expressed in normal breast tissue and bind estrogen with equal affinity, which triggers proliferation of mammary epithelium. The gene for the 595 amino acids long ERα *ESR1* is located on chromosome 6q while the gene for the 530 amino acids long ERβ *ESR2* is located on chromosome 14q (127,128)

Approximately 70 % of all primary breast cancers are ER positive (129). Without regard to tumor stage, grade or intrinsic subtype in detail, hormone receptor positive tumors have a 5-year OS of 92 %, compared to 82 % for ER negative tumors (129). This difference in survival between luminal and non-luminal tumors tend to decrease slightly with longer follow up. Hence, it has been suggested that ER and PR expression should be used as a marker for a slower progression to metastasis and death, rather than a marker for the absence of it (105).

ER expression is also used as a predictive marker (129). Adjuvant endocrine therapy is the most important systemic treatment for hormone receptor positive tumors, and multiple endocrine therapies are currently available. The different classes include selective estrogen receptor modulators (SERMs) like tamoxifen, aromatase inhibitors (AIs) and the estrogen antagonist fulvestrant. In summary, these aim at estrogen deprivation, which has been shown to be anti-proliferative and improve prognosis even in tumors with miniscule proportions of cells immunohistochemically positive for ER (129-135).

ER and PR expression is strongly interlinked, where the latter is sometimes regarded as a marker of intact signaling pathways of the former. Conversely, the *PR* gene is a target of nuclear ERα activation by several upstream estrogen receptor-binding sites, or estrogen response elements (ERE). Therefore, it is believed that PR is a predictive indicator for endocrine treatment (24,136). However, there are currently no treatments specifically targeting PR.

A large retrospective study referred to by the 2015 version of St. Gallen International Expert Consensus found that tumors with a proportion of 1-9 % of cells immunohistochemically positive for ER and PR constitute a subgroup with recurrence free survival more closely tracking hormone negative tumors than tumors with higher ER and PR

positivity (76). Prat *et al* have pointed out a prognostic disadvantage of a PR positivity of < 20 % within the Luminal spectrum of tumors (89).

Consequently, small differences in the results of ER and PR testing induces dissimilar conclusions about prognosis and predicted response to therapy. The most thorough guidelines for the IHC testing of ER and PR are provided by the American Society of Clinical Oncology and the College of American Pathologists. Among several important statements, these stress that up to 20 % of current IHC determinations of ER and PR testing worldwide may be inaccurate. Further, all tumor containing areas of a tissue section should be evaluated under the microscope, and at least 100 tumor cells counted for an average positivity across the tumor section. All tumors with ≥1 % of cell nuclei immunoreactive for ER or PR should be considered "positive" (133-138).

1.3.6 HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2

15-20 % of breast cancers have amplification of the *ERBB2* gene on chromosome 17, from which the protein constituting the transmembrane human epidermal growth factor receptor 2 (HER2) is transcribed (139). It is functionally and structurally a tyrosine kinase that drives proliferation (37). Thus, *ERBB2* amplification and HER2 over expression is strongly associated with poor prognosis (105,140,141). Further, increased numbers of HER2 on the cell surface, with or without aberrations in the protein structure, can itself lead to ligand-independent receptor activation (141-144).

With the advent of treatments targeting HER2, like the humanized monoclonal antibody trastuzumab in 1998, the relapse rate and overall prognosis for these patients have improved dramatically (see also subsection 1.5.5 on anti-HER2 therapy) (143). Thereby, testing of the expression of HER2 or *ERBB2* amplification has become predictive for the response to this therapy.

Although a rather time consuming and complex procedure with an equivocal finding or positivity on HER2 IHC being followed by a mandatory determination of *ERBB2* amplification by HER2 Fluorescence *in situ* hybridization (FISH), the testing of HER2 status might be considered as one of the least controversial biomarker assessments in breast cancer. Again, the American Society of Clinical Oncology and the College of American Pathologists provides specific guidelines for the testing (91). Recently, a combined gene-amplification and protein over expression assay for assessment of HER2 status was made commercially available. This assay was also found to enable simplified and faster analysis without impairment of precision compared to separate IHC and FISH assays (145,146).

1.3.7 Ki67

In addition to a count of mitoses when determining the histological grade, proliferative activity in breast cancer is commonly assessed by immunohistochemical staining of the nuclear Ki67 protein (74-76,147,148). Its name is derived from the city of Kiel, Germany, where the original clone was first discovered in position 67 of a 96-well plate. Just recently, it was discovered that Ki67 act as a biological surfactant that mediates the process in which chromosomes undergo reorganization into separate bodies to prepare for mitosis. Without this surfactant, they would simply collapse into a single cluster without potential for successful replication and division (149). Consequently, a large fraction of Ki67-positive cells indicates a large fraction of proliferating cells, or in other words: a fast growing tissue. It should however be noted that the *MKI67* gene is actively transcribed in all phases of the cell cycle except G_0 including in cells destined for apoptosis, possibly entailing high false positive rates (147).

Clinically, the proportion of Ki67-positive cells (usually expressed in percent) is important when deciding on which patients with hormone receptor positive tumors to give cytotoxic chemotherapy. High proportions of positive cells predict a good response to such treatment, while it also is an indication of a high risk for tumor relapse and distant metastasis. In terms of intrinsic subtypes, hormone receptor positive tumors with high proportions of Ki67-positive cells would classically imply a Luminal B designation and poor prognosis, while a low proportion would imply a Luminal A designation (74-76).

The value of Ki67 for determination of proliferative activity is supported by several studies, but so far, there is a lack of consensus on what tumor region and number of cells to score for a Ki67 index with highest clinical relevance. Further, while clearly high and clearly low values are reproducible, it has been very hard to establish a general threshold for dichotomization of these two (99,150,151). International collaborations and expert consensus have sought to define specific guidelines for the testing of Ki67. Currently, these describe three different approaches to what tumor region to assess under the microscope, depending on the distribution of proliferative activity across the tumor section: If the staining of Ki67 is homogenous without concentration of immunoreactive cells at the tumor periphery or in hot spots, ≥ 3 random high-power (×40 objective) fields should be assessed for a total of ≥ 1000 cells (500 cells absolute minimum). If the staining of Ki67 is heterogeneous with increasing intensity towards tumor edge, ≥ 3 high-power fields at tumor periphery should be assessed for the same number of cells. If the staining of Ki67 is heterogeneous with increased intensity in hot spots, the whole tumor section should be assessed for an average Ki67 index. Local threshold values for distinction of Ki67 “high” and “low” should be defined using each laboratory’s own reference data (74,77,97). Currently, this threshold is commonly in the range of ≥ 14-29 % for Ki67 “high”, derived from the number of Ki67-positive tumor cells divided by total number of counted tumor cells (Figure 8) (74-77,97,100,101).

1.3.8 PHOSPHO-HISTONE H3

Histone H3 is one of the five main highly alkaline histone proteins involved in the structure of the chromatin in eukaryotic cells. Together with the DNA strand and eight units of such histone proteins they form the nucleosome, the basic unit of DNA packaging (152). Recent studies with an antibody against Ser-10 phosphorylated H3 (PHH3) demonstrate a tight correlation between PHH3 and mitotic chromatin condensation (153).

In contrast to Ki67 that is expressed in all phases of the cell cycle except for G_0 (154), phosphorylation of histone H3 occurs exclusively in late G_2 and M-phase and does not occur in cells undergoing apoptosis. Thus, it is both in theory and practice a very specific marker for mitotic activity. Studies on immunohistochemical staining of the histones have been showing promising results throughout almost a decade, but this approach has so far not been introduced in clinical practice nor included in guideline documents (Figure 8) (76,152,153,155).

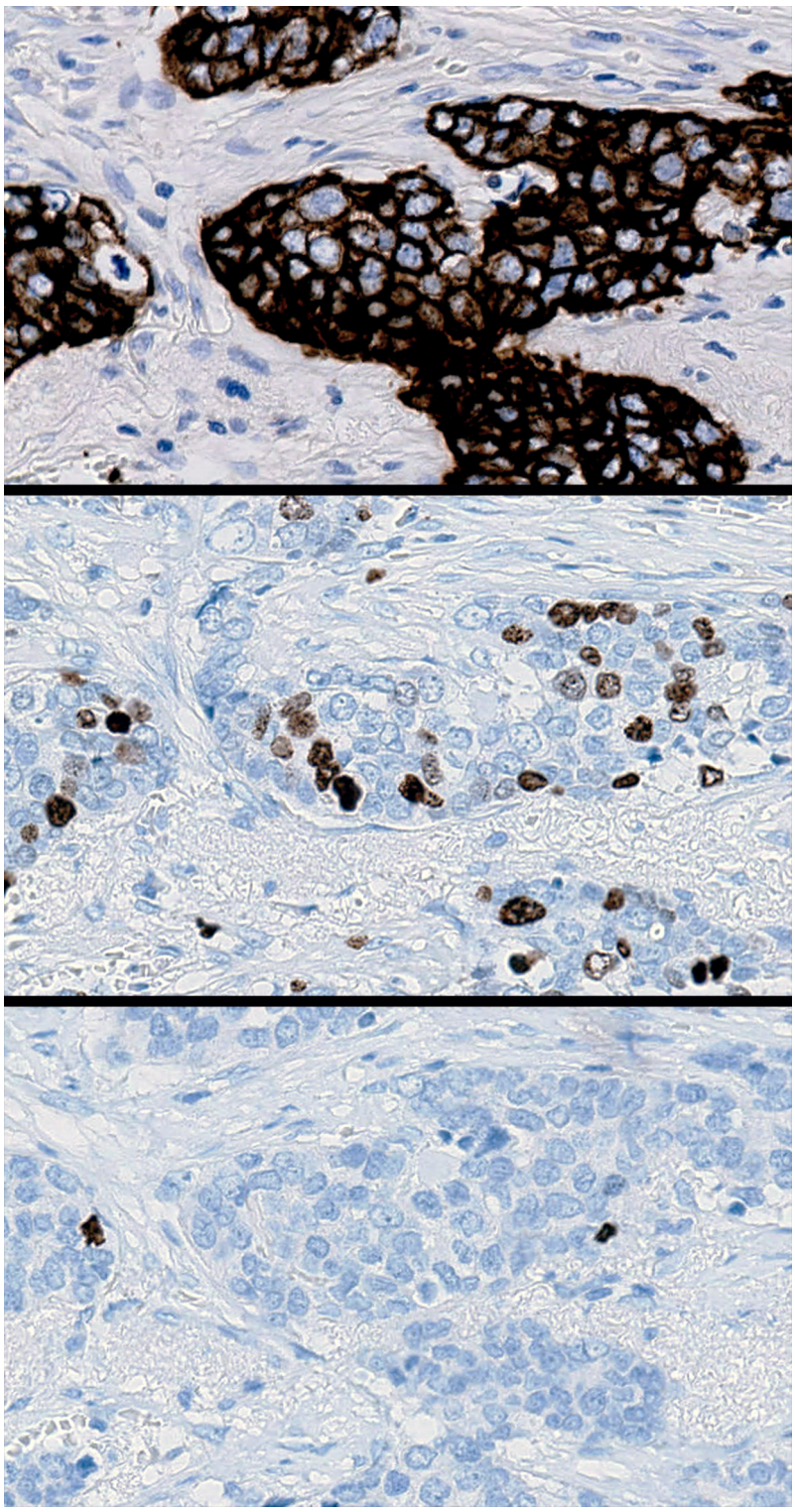


Figure 8. Comparison of immunohistochemical stains of the pancytokeratin marker CkMNF116 (top) that is positive in all epithelial (tumor) cells but negative in the surrounding stroma, Ki67 (middle) and PHH3 (bottom) in the same region of a breast cancer. Note the relatively small fraction of tumor cells being stained by PHH3 (2 out of 259 tumor cells = 0.8 %) compared to Ki67 (55 out of 259 tumor cells = 21 %), which exemplifies their different presence during the cell cycle. In contrast to Ki67 that is expressed in all phases of the cell cycle except for G_0 including cells underway to apoptosis, phosphorylation of histone H3

occurs exclusively in late G_2 and M-phase and does not occur in cells undergoing apoptosis. Modified from the author's own material.

1.3.9 NATIONAL VS. INTERNATIONAL GUIDELINES FOR BIOMARKER TESTING

The Swedish Quality- and Standardization Committee (KVAST) propose regularly updated national guidelines for biomarker testing in breast cancer (98). Generally, these closely track international guidelines on biomarker testing that have been mentioned for each factor in subsection 1.3.5 through 1.3.8 (Figure 9). All analyzes are to be validated by both test- and population level quality controls such as NordiQC (nordiqc.org)/UK Neqas (ukneqas.org.uk) and SweQA/Equalis, respectively. External controls from endometrium, cervix or cell lines are to be included on each tumor slide. Positive staining is defined as any brown stain in the nucleus above background. As PHH3 is not used in clinical breast cancer diagnostics, no guidelines have been developed. In paper IV of this thesis, PHH3 was scored according to the recommendations for mitotic counts.

In some details however, the national guidelines differ from the international guidelines on biomarker testing, in that they:

1. Exclusively focus on tumor regions with the highest proliferative activity (hot spots) for Ki67 assessment (97,98).
2. Recommend a different number of cells to be counted in assessment of Ki67 index (Swedish guidelines: ≥ 200 cells. International: ≥ 1000 cells with 500 cells as an absolute minimum) (97,98).
3. Have a higher threshold for classification of a tumor as ER and PR positive. In the Swedish guidelines, this cutoff is actually not spelled out, but a proportion of 10 % stained cells is generally used, although endocrine treatment can be considered for patients with lower positivity. This is also the parameter (proportion of cases with ER >10 %) recommended to be reported to the Swedish National quality register. The 2015 version of St. Gallen International Expert Consensus (76) define this threshold as 1 %, but add that ER values between 1% and 9% are equivocal and that patients with these values cannot rely on endocrine therapy alone. In clinical practice, the outcome of the Swedish vs. international guidelines on ER should be very similar.
4. Have a lower threshold for amplification of the HER2-gene. According to the Swedish guidelines, a single-probe average HER2 copy number of ≥ 4/cell defines gene amplification and an indication for trastuzumab. The U.S. guidelines define an average copy number of 4 to 6/cell as "equivocal" with a recommendation of reflex testing, and ≥ 6 as the threshold for amplification (91,98).
5. Emphasize that the pathologist informs the referring clinician on what clones of antibodies and ISH probes, what lab equipment and makes of machinery including names of its suppliers have been used in each assessment of a biomarker. Neither the necessity for this nor the clinical demand for it is underlined in international guidelines (98,75-77).

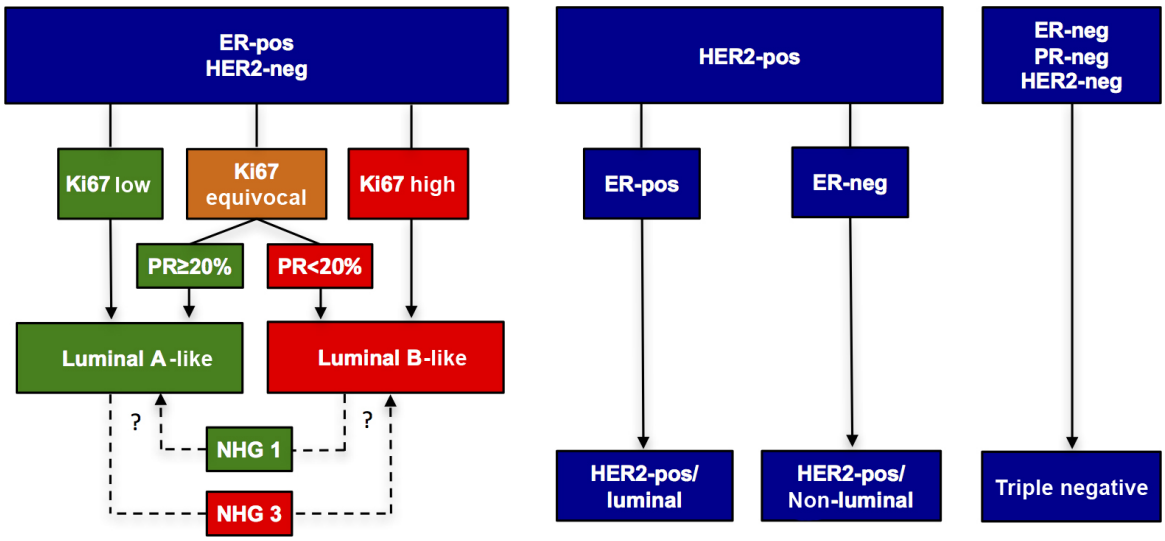


Figure 9. Flow chart for the classification of surrogate subtypes of breast cancer based on protein expression assessed by immunohistochemistry. Modified from the Swedish Quality- and standardization document for breast tumors, Ekholm *et al* (98) with permission from the author. "pos" and "neg" = immunohistochemically positive and negative, respectively. Threshold for ER not specified, generally 10 %. HER2 positive if IHC 3+, IHC 2+ and ISH HER2 copy to C17 ratio ≥ 2 or IHC 2+ and HER2 copy number >4. Ki67 is divided into three groups, where all cases below the 34th percentile in each laboratory's own reference data are classified as low, all cases between the 34th and the 58th percentile as equivocal, and all cases above the 58th percentile as high. NHG1 and NHG3: If ER, PR, Ki67 and HER2-status indicate a Luminal A-like subtype but the Nottingham combined histologic grade is 3, a new assessment of both the biomarkers and grading is indicated. If the NHG is still 3, the tumor should be classified as Luminal B-like. Vice versa: If the biomarkers indicate a Luminal B-like tumor but the NHG is 1, and this is confirmed in a new assessment, the tumor should be classified as Luminal A-like. These guidelines differ in some details, but not in general from the definitions for surrogate subclassification used in the papers presented in this thesis (Table 1).

1.4 DIGITAL IMAGE ANALYSIS

1.4.1 SOFTWARE

In its most basic definition, image analysis is the extraction of information from images (156). In this sense, all examinations of tissue since the dawn of the light microscope in the 17th century, and all conclusions drawn from the visual appearance of these tissues, would classify as image analysis. Where the analysis previously has been the exclusive task for a human observer however, it can now to an increasing extent be assigned to digital image processing techniques. Hence the term computer image analysis or digital image analysis (DIA). The latter will be used in this thesis and the included scientific papers.

Originally developed in the fields of artificial intelligence and robotics, the first versions of software capable of pattern and geometry recognition and processing were presented in the 1970s. It is now a scientific field of its own with a plethora of private sector spin-offs and both free-to-use and commercially available software available (156-159). In medicine, these can be applied to anything from the estimation of tumor volumes in mammography and differential leukocyte counts to the assessment of immunohistochemically stained cells in tumor sections. The evolution of this wide variety of software has been compared to a natural selection, where only a portion of gradual changes over the last 40 years have been retained based on unsentimental evaluations of what's being used and functional, and what's not (157). Currently, most algorithms incorporate all of pattern recognition, texture analysis, densitometry and digital signal processing that basically compare and analyze patterns, contrast and colors of the pixels making up the image. Having previously only been feasible to run on super computers or clusters of several smaller computers, these algorithms are now simple enough to run on off-the shelf laptop or desktop computers. In addition to the challenge of image size and efficient logistics mentioned below, they still have to overcome the challenge of varying file formats as each scanner manufacturer tend to use its own proprietary image compression. For example, Aperio scanners, sold by Leica Biosystems, Wetzlar, Germany, store their images as .SVS-files, based on a standard pyramid tagged image file format (TIFF) and use a red, green, blue, alpha color model, while NanoZoomer scanners, sold by Hamamatsu Photonics, Hamamatsu, Japan, store their images as .NDPI-files, based on a stripped TIFF format that save colors in a different order: Blue, green and red. This serves to exemplify that thresholding, i.e. determining whether a group of pixels with a relatively intense color signal constitutes a positively stained cell nucleus or not, is merely one of several end-games of DIA. Between slide scanning and actual analysis, several preparatory steps will have to be successfully completed (156).

The validation of DIA will henceforth be limited to its application for immunohistochemical stains of ER, PR, HER2, Ki67 and PHH3 in their predictive and prognostic functions and as surrogates for gene expression profiles.

As mentioned in subsection 1.3.1, recent international guidelines state that the uncertainty and variability in the testing of these biomarkers in breast cancer may be reduced by Image Analysis (76). The emerging plethora of DIA-systems have shown excellent reproducibility and accuracy, though so far in subsets with individual biomarkers or smaller populations (160-164).

Modern software for DIA in pathology distinguish between tumor and non-tumor tissue, requires relatively few manual commands before analysis and present the data, i.e. the fraction of Ki67-positive tumor cells, in a quick, systematic and comprehensible way. Costs ranges from free open source-solutions where users can add, change and develop new applications, like the public domain, Java-based software ImageJ, originally developed at the National Institutes of Health, to advanced licensed software for which the user pay in excess of 20 $ per tumor slide (Figure 10) (165,166).

Note that software for DIA should not be confused with picture archiving and communication systems (PACS), that are aimed at providing storage, convenient access and facilitating workflow in radiology and digital pathology. These are generally not involved in actual analysis of images, but are often offered as parallel systems to systematically handle the large volumes of images and data generated when DIA is used in clinical routine. In many cases, DIA applications and functions can be incorporated into PACS (167).

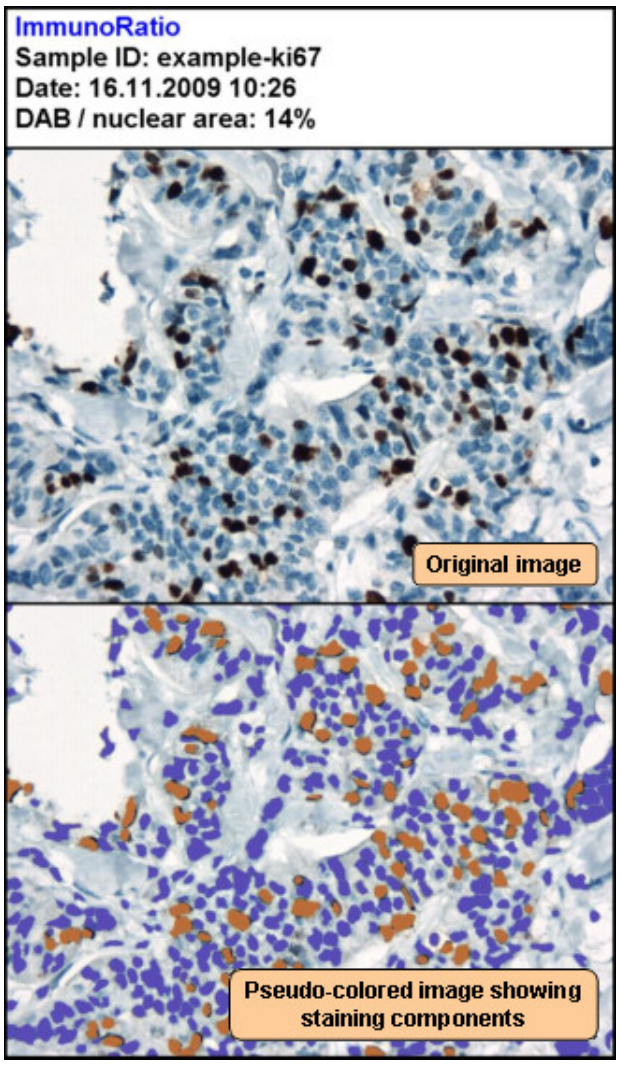


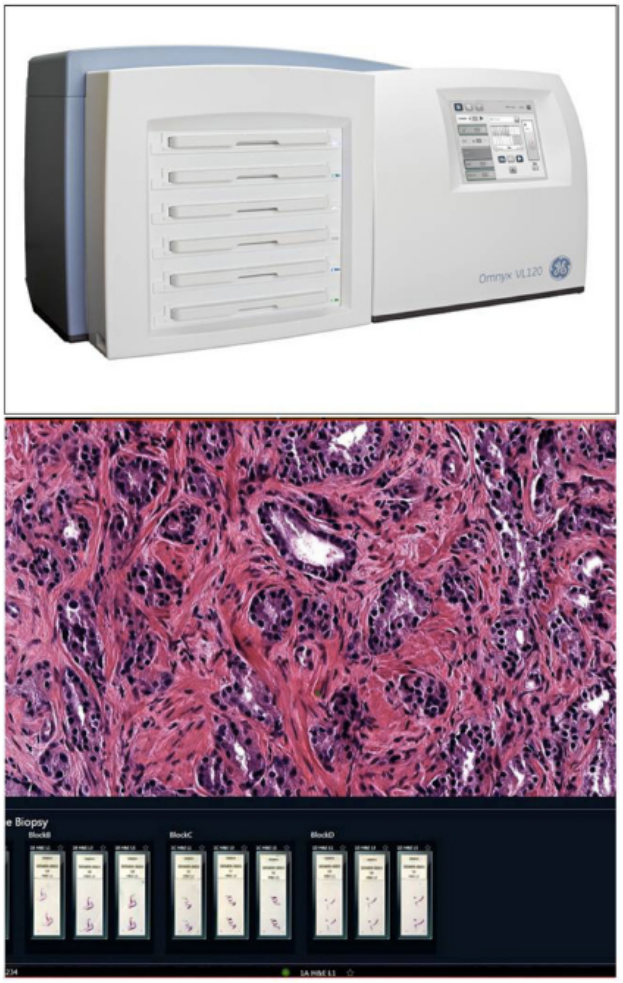
Figure 10. Example of a result image from a free web application for automated image analysis of ER, PR and Ki67 immunohistochemically stained and digitally scanned tissue sections. The result image includes a sample identifier, the analysis date, the labeling index (percentage of Ki67-stained tumor nuclei to total number of tumor nuclei), the original image (top), and a pseudo-colored image showing the segmented staining components (bottom). Positive cells = orange. Negative cells = blue. Reprinted from Tuominen et al (165) under a creative commons license.

1.4.2 SLIDE SCANNING

Any image analysis, manual or digital, requires a properly lighted, focused, sized, projected and formatted image. In addition to the nearly 200 years old prerequisites of proper fixation, dehydration, embedding, sectioning and staining of tumor tissue for the production of histopathological glass slides, DIA requires that a high quality image be generated through digital scanning (156,166-169).

The first step in this process is to insert the glass slide in a digital scanner, either in single units or multiple on a tray. Currently most scanners are adopted for the standard 75

× 25 mm (3″×1″) size glass slides. Approx. 300 of these slides can be loaded into high throughput scanners (Figure 11).



Vendor	WSI scanner model
3DHistech	Pannoramic SCAN II, 250 Flash
DigiPath	PathScope
Hamamatsu	NanoZoomer RS, HT, and XR
Huron	TISSUEscope 4000, 4000XT, HS
Leica*	ScanScope AT, AT2, CS, FL, SCN400
Mikroscan	D2
Olympus	VS120-SL
Omnyx	VL4, VL120
PerkinElmer	Lamina
Philips	Ultra-Fast Scanner
Sakura Finetek	VisionTek
Unic	Precice 500, Precice 600x
Ventana**	iScan Coreo, iScan HT
Zeiss	Axio Scan.Z1

Notes: *Leica was formerly known and operated as Aperio; **Ventana was formerly known and operated as Bioimagene.

Figure 11. Left: Example of a whole slide imaging scanner (top) and PACS from the same manufacturer (Omnyx®, bottom). Virtual slide composed of multiple image aqcuisitions from a physical glass slide. Right: List of currently available commercial whole slide scanners. Modified from Farahani *et al* (170), reprinted under a creative commons license.

The second step of the digitization process is then to decide on what area or region of the slide to scan. Virtually all scanners offer a pre-visualization tool, which projects overview images. From these, the tumor or region of interest can be outlined to avoid unnecessary data generation or disturbing artifacts (169).

The third step is to adjust the focus point and focus depth for the selected region and adjust image settings, such as white balance, contrast, scanning magnification etc. Naturally, most manufacturers offer the possibility to have the scanner automatically identify regions of interest, multiple point focus depths and image settings. Further, there is usually an option to scan the entire glass slide or a predefined subset of it without regard to the location and orientation of the tissue on an individual slide.

The fourth step is the actual scan. Several objectives, each focused on a different field, or one moving objective delivers images to a digital camera. The most common solution is acquisition of the microscopic fields square-by-square, from the slide's upper left corner to the lower right (tile-based method). Alternatively, the moving objective travels over the slide in a straight line, moving in the Y-axis only after reaching the edges of the scan area (line-based method, Figure 12) (169,170). The small individual images are then adjoined to create a seamless virtual slide. As the objectives are generally scanning at 20 – 60x, each generating an acquisition with a field diameter of approx. 0.9 to 0.3 mm, the virtual slide is a mosaic that can consist of several hundred individual images, allowing for free movement from an overview magnification of the whole slide down to individual cells (156).

If a monochromatic camera is used, three sequential scanning rounds are required (for red, green and blue), thereby tripling the amount of data per acquisition. The total size of a whole slide scanned on a modern scanner is generally in the range of 100 megabytes to several gigabytes, and can contain more than a billion pixels (156,169).

After the scan process is finished, the images are imported as individual files to folders on a receiving computer, to a PACS for archiving or to the DIA software for analysis.

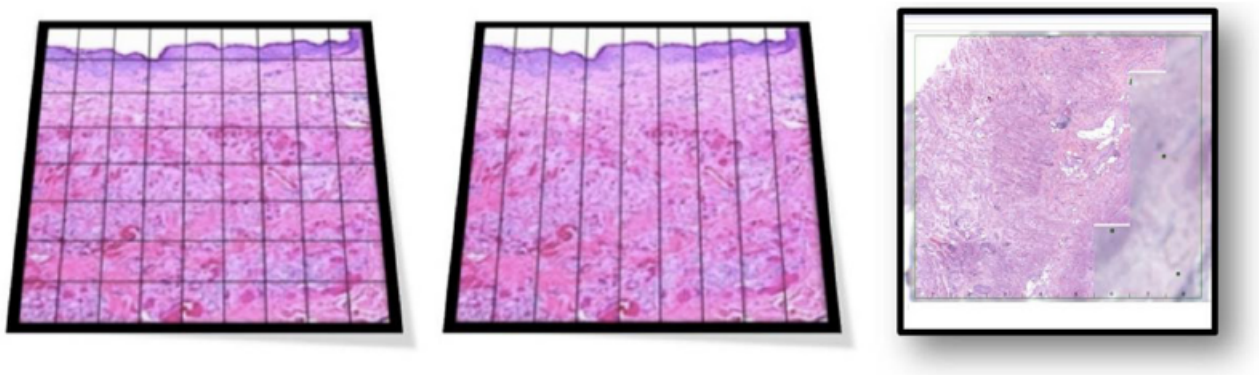


Figure 12. Illustration of (left) a tile-based and (middle) line-based scanning method. (Right) Line-based scanning of an actual glass slide in progress. Modified from Farahani *et al* (170), reprinted under a creative commons license.

1.5 BREAST CANCER TREATMENT

1.5.1 SURGERY

Surgical removal of tumorous tissue is the main pillar of breast cancer treatment. For a majority of patients, the procedure is curative. For small primary tumors (<5 cm, anatomic stage I or II, Table 3a and 3b), the removal of the tumor along with all mammary tissue (mastectomy), and breast conserving surgery (BCS) where the cancerous lump is removed with an attempted surgical margin of 1 cm are equally safe in terms of prevention of recurrence and metastasis (130,171,172). In other words: extensive removal of tissue does not prolong survival in these patients. All patients, except lymph node negative T1-T2 tumors treated with mastectomy, benefit from postoperative radiotherapy (130). For women without clinically detectable axillary lymph node metastases, the sentinel lymph node technique with intraoperative lymphatic mapping has earlier been considered gold standard, but is now seeing decreased use in many institutions in favor of FFPE tissue (105) (see also subsection 1.3.2 on breast cancer stage). If the SLN is free from metastasis, the risk for metastases in other locoregional nodes is < 7.5 % (106). There is no evidence that axillary dissections increase survival in women without metastasis in the SLN (SLN-negative) upon histological examination (106). The 5-year OS for patients with SLN-negative disease is 83 %. If 1-3, 4-12 and ≥13 axillary lymph nodes are positive for metastatic growth, the 5-year OS is 73, 46 and 29 %, respectively (106-113). If distant metastases are present (stage IV), surgery is less favorable and generally aimed at reducing tumor mass and symptoms.

1.5.2 RADIOTHERAPY

Radiotherapy is commonly directed at the surgical bed to reduce the risk of recurrence and distant metastasis. In this setting, repeated fractions of small doses, e.g. 2 Gy/day for 25 days, or more recently in more intense “hypo fractions” of >2 Gy/day for a fewer number of days, are given to reduce side effects (172,173). After BCS, radiotherapy to the conserved breast halves recurrence rates and reduces the breast cancer death rate by a sixth (172). Further, meta-analyses have shown that postoperative radiotherapy improves survival not only after BCS, but also after mastectomies (174,175). Expert consensus recommend post-mastectomy radiotherapy for patients with axillary lymph node metastasis, for tumors >5 cm regardless of nodal status and after resections with positive deep margins (76). Swedish guidelines recommend postoperative radiotherapy to all patients except lymph node negative T1-T2 tumors treated with mastectomy, and patients with T1-T2 tumors and micro metastasis (0.2-2.0 mm) in 1 lymph node only that have undergone mastectomy and adequate axillary dissection of ≥ 10 lymph nodes (130).

The benefit of radiotherapy increases with markers for aggressive disease, like histological grade and proliferational rate. This is an indication of its mechanism of action:

Photons or charged particles causes direct or indirect ionization of the atoms that make up the DNA chain, causing double-stranded breaks which in turn causes relatively more harm to tissues consisting of highly proliferational cells with malfunctioning DNA repair mechanisms (176,177). A degree of damage to surrounding healthy tissues is however unavoidable, and radiotherapy is consequentially associated with significant morbidity, such as pleuritis, lymph edema, neural damage and skin rashes (178,179).

1.5.3 ENDOCRINE TREATMENT

Antagonists to ERα, or inhibition of estrogen production, is beneficial for both pre- and postmenopausal patients with hormone receptor positive tumors (180,181). The metabolites of tamoxifen - the most widely used and tested drug in breast cancer, compete with estrogen for the binding of ER. Thereby, the transcription of estrogen-responsive genes is reduced (180). Treatment for 5 or 10 years after surgery reduces the risk of recurrence by nearly 50 % and significantly improves overall survival (181).

Aromatase enzymes convert androgens into estrogens. Blockage of this conversion with AIs leads to lower levels of estrogens available for hormone receptor positive tumors (182,183). Like the regulation of most endogenous hormones however, reduction of the downstream effector is compensated for by increased production of the substrates. Decreased concentrations of estrogen will activate the hypothalamus and pituitary to secrete gonadotropin, which in turn will stimulate aromatase promoters and competent ovaries to increase androgen production. The effect of AIs is thereby counteracted. Consequently, AIs like anastrozole and exemestane is only used in postmenopausal women, where estrogen is mainly produced in peripheral adipose tissue (183,184). AIs are slightly more effective in preventing recurrence than tamoxifen, and comes with a lower risk of thromboembolic events and endometrial cancer. Osteoporosis and muscular pain is however more common in patients treated with AIs (185-187).

Despite both tamoxifen and AIs being effective treatments for hormone receptor positive tumors, hormone receptor positive relapses do occur. Significant efforts have been made to understand the mechanisms behind this. Among many discoveries, it has been shown that a shift from estrogen dependent- to estrogen independent growth has occurred in as many as 20 % of tumor relapses (188,189). Other mechanisms behind relapses are constitutional phosphorylation of both nuclear and membrane-bound ERα, leading to ligand independent activation, truncated variants that activate transcription even in the absence of hormone and a switch in the ratio of coactivators versus corepressors (189-192). Another obstacle to fully successful endocrine treatment is compliance. Taking one pill a day for 5 or even 10 years with adverse side effects and no instant benefit means that as many as 31 % of patients do not adhere to treatment recommendations (193,194).

1.5.4 CYTOTOXIC CHEMOTHERAPY

Cytotoxic treatment regimens are indicated for a broad group of both pre and postmenopausal breast cancer patients. A combination of drugs such as fluorouracil, epirubicin, cyclophosphamide (FEC) followed by a taxane such as docetaxel or paclitaxel is beneficial even for early stage ER-positive disease without axillary lymph node metastases but with at least one high risk feature such as a high histological grade 3, high Ki67 expression, or other-than-Luminal A subtype (130). For stage III and IV disease, chemotherapy is nearly always recommended (75,76). In stage I-III patients, chemotherapy reduces mortality and risk of relapse significantly. Relative risk reductions of 22 to 36 % have been reported (195,196).

The most common combinations of drugs are fluorouracil, epirubicin, cyclophosphamide (FEC), cyclophosphamide, methotrexate and fluorouracil (CMF), fluorouracil, doxorubicin (formerly Adriamycin) and cyclophosphamide (FAC), epirubicin and cyclophosphamide only (EC) and doxorubicin and cyclophosphamide only (AC) sometimes followed by paclitaxel (76,195-197). All of these target vital elements in cellular division, often inducing severe genetic damage that targets the cell for apoptosis. This illustrates the relatively higher static and toxic effect in highly proliferative tumors. Consequently, side effects to normal highly proliferative tissues like the gastrointestinal tract, hair follicles, skin and immune system is quite severe. Other side effects of cytotoxic chemotherapy include nausea, infection, phlebitis, thromboembolism and a risk for secondary malignancies (196,197).

The rising concept of neoadjuvant treatment, often with combinations of AC or FEC that can be followed by docetaxel, paclitaxel or carboplatin, to reduce tumor size before surgical resection, is recommended for patients with locally advanced or inoperable tumors (130). This includes stage III and IV disease, axillary node fixation and supraclavicular or parasternal metastases. This aims to reduce tumor size and axillary lymph node tumor burden and thereby downstage the disease. In some cases this allows for previously inoperable tumors to be radically resected. In a large Cochrane review, even primarily operable patients could be offered neoadjuvant treatment without decreased survival versus adjuvant regimens, but with the benefit of reducing the frequency of mastectomies in favor of BCS (196). For HER2 overexpressing tumors, chemotherapy can be combined with dual HER2 blockade in the form of trastuzumab plus pertuzumab in both the neoadjuvant and metastatic setting (further details in subsection 1.5.5. on anti-HER2 therapy) (76,130,198-200).

1.5.5 ANTI-HER2 THERAPY

ERBB or Human epidermal growth factor receptor (HER) -signaling is a receptor-ligand system of four receptors and more than eleven ligands. Receptor expression generally occurs in epithelial tissue, whereas the ligands are synthesized in the stroma. The four different types of HER are rarely expressed alone or without their co-receptor ERBB2. The system as a whole is frequently over-activated in proliferative diseases, including breast cancer, psoriasis

and arteriosclerotic plaques. It is functionally and structurally a tyrosine kinase, and functions as an oncogene. Consequently, it has gained much interest as a therapeutic target (201-203).

As described in subsection 1.3.6, 15-20 % of breast cancers have amplification of the *ERBB2* gene on chromosome 17, from which the protein constituting HER2 is transcribed (139). *ERBB2* amplification and/or HER2 over expression is associated with poor prognosis (105,139). Further, increased numbers of HER2 on the cell surface, with or without aberrations in the protein structure, can itself lead dimerization and ligand-independent activation of the receptor (139-142).

The humanized monoclonal IgG1-antibody trastuzumab was developed in the 1990s. It has high affinity for the juxtamembranous domain IV of the extracellular receptor 2 for HER2. Binding leads to blockage, downregulation and internalization of HER2, antigen-dependent cellular toxicity by the adaptive immune system, induction of apoptosis and reduced angiogenesis (201-202). When trastuzumab is administered to patients with HER2 over expression, relapse rate and overall prognosis improve dramatically (143). If given in adjunct to chemotherapy such as a taxane followed by anthracyclines, it reduces the relative mortality with as much as 34 % (204-208). Resistance to trastuzumab can appear in both the adjuvant and metastatic setting. Early evidence points to an escape-mechanism through alterations of the PI3 kinase pathway. Several new drugs have therefore been introduced in the last decade, including the tyrosine kinase inhibitor lapatinib that inhibits the ATP-binding pocket of HER2 and prevents self-phosphorylation and activation of signal pathways, with some effect even in single non-HER2 overexpressing tumors by IHC, and the HER2-HER3 dimerization antibody pertuzumab (203). In clinical routine, HER2 over expression is therefore targeted with a dual blockade, where trastuzumab is combined with pertuzumab. In the neoadjuvant and metastatic setting, lapatinib can be added instead, with a slightly increased ratio of pathological complete response. Nonetheless, mutations in the intracellular kinase domain of HER2, in addition to other escape-mechanisms, can render even the intensified HER2-directed therapy ineffective in the long term (139,203). Major side effects of anti-HER2 therapy are flu-like symptoms and cardiac dysfunction in as many as 2-7 % of patients with trastuzumab (209). The latter is caused by the drug's downregulation of neuregulin-1 (NRG-1), which is essential for survival pathways in cardiomyocytes. Therefore, the left ventricle should be examined both before and during treatment (205). More than 25 % of patients treated with lapatinib suffer from diarrhea and nausea (130,203). Less common side effects of pertuzumab are diarrhea, upper respiratory tract infections, rashes, headache, joint pain and fatigue (130,203).

The Epidermal growth factor receptor (EGFR) is an alternative pathway to target with monoclonal antibody "EGFR inhibitors". E.g. gefitinib, erlotinib, afatinib, brigatinib in lung cancer, and cetuximab in colon cancer. These antibodies block the receptors' extracellular ligand binding domain. Consequently, the receptors cannot longer bind the ligand to activate the intracellular tyrosine kinase. Disruption of EGFR can also be accomplished by direct action at the tyrosine kinase itself. Compounds such as quinazolines

compete with ATP, which is needed for tyrosine kinase activation. Without access to ATP, its activity will thereby decrease. Although the EGFR inhibitors are routinely used in other cancers, and the quinazolines have been effective and well tolerated in trials, none of them have been introduced for the treatment of breast cancer (37,139).

1.5.6 TREATMENT OF HEREDITARY BREAST CANCER

Most breast cancers are sporadic in the sense that they are caused by de novo genetic events during cellular replication, with or without influence of known environmental or lifestyle factors. All the same, the hereditary tendency is well documented in breast cancer, either through polygenetic alleles with unpredictable levels of risk, or through monogenetic syndromes with known major increases in the risk for the disease. Today, 5 to 10 % of all breast cancers are caused by known hereditary genetic aberrations (39,210).

Genes and loci associated with risk of developing breast cancer can be divided into groups with high, intermediate and low penetrance. The distribution of these in the population is inverse to their degree of penetrance: High penetrance genes are responsible for approximately 25 % of all hereditary breast cancer, while the intermediate and low penetrance genes are responsible for the remaining 75 % (210-213).

The most well-known high penetrance genes are *BRCA1* and *BRCA2* (see also subsection 1.5.6 on future and experimental treatments). Alone, they cause 5 % of all breast cancer cases. *BRCA1* and *BRCA2* are DNA repair agents that protect the integrity of the genome. Having a deactivating germline mutation in either of these genes results in a risk of developing breast cancer before the age of 70 of 55 - 65 % for carriers of *BRCA1* and 45 % for carriers of *BRCA2* (39,43,214). Therefore, presymptomatic genetic testing is commonly offered to patients in well-known high-risk families, and in the case of a mutation to either *BRCA1* or *BRCA2* prophylactic bilateral mastectomy (215). Other singular genes responsible for familiar breast cancer, including *PTEN*, *TP53* and *CDH1*, are less common in the population and have lower penetrance (211).

Carriage of mutations in medium penetrance genes, like *PALB2, CHEK2*, *ATM* or *BRIP1* mutants confer moderately increased risk for breast cancer (216-219). These mutations are generally not screened for in the clinic, and thereby not treated. This also holds true for the vast majority of low penetrance genes. Among the latter, *FGFR2* and *MAP3K1* can be mentioned. These encodes for the Fibroblast growth factor receptor 2 - a member of the tyrosine kinase receptor family that promotes proliferation, cell motility and angiogenesis and the main protein in the mitogenic MAP-kinase signaling pathway. Several thousand more remain to be discovered. Most of the hitherto unexplained hereditary risk is thought to be caused by these low penetrance variants, together with structural differences, gene-gene interactions and gene-environment interactions (216-222).

1.5.7 FUTURE AND EXPERIMENTAL TREATMENTS

Despite being the earliest developed and most frequently used targeted therapy in breast cancer, endocrine treatment still has potential for improvement and future use in situations beyond the current standard practice.

Paradoxically, loss of ER expression does not account for most cases of tumor recurrence in patients on endocrine treatment. As the vast majority of breast cancers are ER positive at diagnosis, the mechanism of this endocrine resistance is currently at the focus of intensive preclinical and clinical research including characterization of induced mutations of the ER gene through deep genome sequencing. Novel drug candidates target potentially upregulated cascades downstream ER, such as the PIK3CA/mTOR and the proliferation-associated cyclin pathways (70,71,222,223)

Further, several randomized trials have investigated the role for tamoxifen as a preventive agent. Indeed, reduction of relative breast cancer incidence with up to one third versus placebo has been shown for up to 10 years after administration of tamoxifen during a period of 5 to 8 years. Similarly, AIs such as emestane and anastrozole have reduced the risk of invasive breast cancer with up to two thirds. However, many clinicians fruitfully point out the low tolerance for potentially grave side effects like thromboembolism, endometrial cancer, cataracts and hot flashes in healthy women, and that the absolute risk reduction is very limited at 2-3 % (223). In a large cohort of Swedish women (n=70877), Hall and colleagues currently seeks to identify a subgroup of women with favorable risk-reward profile for preventive tamoxifen treatment. The risk factors studied so far (high mammographic density, high mass and microcalcifications) have been identified to increase the risk of breast cancer nine fold (224).

Immune therapy shows great promise in breast cancer, as in other solid tumors. It spans a wide range of conceptually distinct approaches, including checkpoint inhibitors, antibodies (see also subsection 1.5.5. on anti-HER2 therapy), vaccinations, antibody-drug conjugates and targets in the tumor microenvironment. As these treatment modalities evolves further and become readily available in the arsenal of oncologists, the demand on the diagnostic process and on biomarker analysis to guide patient selection will increase. Immune checkpoints refers to inhibitory signals to the T cell receptor. Normally, these exists to prevent excessive T cell activation and collateral damage to self tissues when fighting pathogens. Tumors can avoid destruction by the immune system by exploitation of checkpoints through a plethora of these inhibitory signals. The Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD1) are two of the most frequently studied T cell receptors in this context. Activation of CTLA-4 blocks the response of CD8-positive cells, suppresses T helper activation and increases the amount of regulatory T-cells, which suppresses immune response. Tumor infiltrating lymphocytes (TIL) in breast cancer largely consists of regulatory T-cells, thus potentially aiding the tumor escape immune response (225,226). Other studies however, shows a clear survival advantage of tumor infiltrating lymphocytes in triple negative breast cancer in terms of reduced risk of relapse and death. In HER2 overexpressing tumors, the presence of a lymphocytic infiltration in the tumor tissue and stroma has been shown to predict a significantly treatment response with docetaxel added to anthracycline (225). CTLA-4 can be targeted with the monoclonal IgG1 antibody ipilimumab and with the IgG2 antibody tremelimumab. The former has been FDA-approved for the treatment of skin Melanoma since 2011. The latter is showing

promising results in trials with increased immune responses against the tumors, but is not yet approved for clinical use. PD1 is constitutively activated in many different tumor types, resulting in reduced T cell, B cell and natural killer cell activity. Nivolumab, pembrolizumab and atezolizumab are relatively well established antibodies with inhibitory action on PD1. Although used in other tumors such as renal cell carcinoma, non-small cell lung cancer and melanoma, it is not in current clinical use for breast cancer. Phase 1 trials have shown overall response rates in the region of 20 %, with 75 % of the responses in triple negative breast cancer, and other trials are ongoing (225).

Tumor vaccines are aimed at stimulating the adaptive immune system to generate memory T cells with potential long-term protection. The biggest obstacle for their use is that there are very few antigens that are expressed exclusively in tumor tissue and not at all in normal tissue. Further, even in cases where actual tumor specific antigens have been identified in individual tumors, they do not seem to be recurring in any larger groups of breast cancer patients. Vaccines with HER2-derived peptides have been shown to increase disease-free survival from 80.2 to 89.7 % at 5 years, at least for patients with low HER2 expression (IHC 1 and 2) in a phase I trial. In a phase II trial, a similar vaccine reduced breast cancer recurrence in a group of patients with earlier treatment for node-positive or high risk node-negative disease with more than 50 % (225).

Antibody-drug conjugates offer an improved way to deliver drugs with high effect but low specificity for tumor tissue, such as cytotoxic chemotherapeutic agents. Two complexes have made it through at least phase II trials: T-DM1 consists of the HER2-antibody trastuzumab coupled with the cytotoxic anti-microtubule agent emtansine. The latter will thereby be delivered in increased concentration to tissues with increased HER2 expression, i.e. HER2 over expressing breast cancer. So far, the compound has been shown to somewhat reduce side effects due to the lower systemic concentration needed and prolong overall survival in heavily pre-treated patients as a second-line after treatment with trastuzumab and a taxane, compared to lapatinib and capecitabine. Other randomized trials have not been able to reproduce this superiority over current standard treatment. MM-302 is another agent, also coupled with trastuzumab, in which doxorubicin is delivered in liposomes to HER2 expressing cells. Early results indicate overall response rates of 12 % and increased progression-free survival when compared to cyclophosphamide (225).

A substantial amount of preclinical and clinical evidence suggests that angiogenesis is central to breast cancer growth (as described in subsection 1.2.2), and that increased concentrations of the vascular endothelial growth factor (VEGF) is associated with worse outcome. Targeting VEGF therefore makes sense. Indeed, in early clinical trials, progression-free survival doubled when weekly paclitaxel was combined with the anti-VEGF agent bevacizumab. However, with longer follow-up, these early trials as well as latter larger well-powered, placebo-controlled international trials including most subgroups of breast cancer, have not been able to reproduce the increased progression-free survival or show increased overall survival in patients treated with anti-VEGF including bevacizumab and its later followers. Several mechanisms of resistance to anti-VEGF therapy has since been discovered, including activation of alternative angiogenic pathways, cell de-differentiation and increased aggressiveness from hypoxia and increased numbers of cancer stem cells (208,223,227).

T	N	M	G	HER2	ER	PR	Prognostic stage group
Tis	N0	M0	1–3	A	A	A	0
T1	N0	M0	1	P	A	A	IA
T1	N0	M0	1–2	N	P	P	IA
T1	N0	M0	2	P	P	P	IA
T1	N0	M0	3	P	P	A	IA
T0–1	N1mi	M0	1	P	A	A	IA
T0–1	N1mi	M0	1–2	N	P	P	IA
T0–1	N1mi	M0	2	P	P	P	IA
T0–1	N1mi	M0	3	P	P	A	IA
T1–2	N0	M0	1–3	N	P	A	IA
T1	N0	M0	1	N	N	N	IB
T1	N0	M0	1	N	P	P	IB
T1	N0	M0	2	P	N	N	IB
T1	N0	M0	2	P	N	A	IB
T1	N0	M0	2	N	N	P	IB
T1	N0	M0	3	P	P	A	IB
T1	N0	M0	3	N	P	P	IB
T0–1	N1mi	M0	1	N	N	N	IB
T0–1	N1mi	M0	1	N	P	P	IB
T0–1	N1mi	M0	2	P	N	N	IB
T0–1	N1mi	M0	2	P	N	A	IB
T0–1	N1mi	M0	2	N	N	P	IB
T0–1	N1mi	M0	3	P	P	A	IB
T0–1	N1mi	M0	3	N	P	P	IB
T2	N0	M0	1–3	P	P	P	IB
T2	N0	M0	1,2	N	P	P	IB
T1	N1	M0	1–3	P	P	P	IB
T1	N1	M0	1–2	N	P	P	IB
T2	N1	M0	1	N	P	P	IB***
T2	N1	M0	2	P	P	P	IB***
T0–2	N2	M0	1–2	P	P	P	IB***
T3	N1–2	M0	1	P	P	P	IB***
T3	N1–2	M0	2	P	P	P	IB***
T1	N0	M0	1	N	N	N	IIA***
T1	N0	M0	2	N	N	N	IIA***
T1	N0	M0	3	N	P	N	IIA***
T1	N0	M0	3	N	N	P	IIA***
T1	N0	M0	3	N	N	N	IIA***
T0–1	N1mi	M0	1	N	N	N	IIA
T0–1	N1mi	M0	2	N	N	N	IIA
T0–1	N1mi	M0	3	N	P	N	IIA
T0–1	N1mi	M0	3	N	N	P	IIA
T0–1	N1mi	M0	3	N	N	N	IIA
T0–1	N1	M0	1	P	P	N	IIA
T0–1	N1	M0	1–2	P	N	A	IIA
T0–1	N1	M0	1	N	P	N	IIA
T0–1	N1	M0	1	N	N	P	IIA
T0–1	N1	M0	3	N	P	P	IIA
T2	N0	M0	1	P	P	N	IIA
T2	N0	M0	1–2	P	N	A	IIA
T2	N0	M0	1	N	P	N	IIA
T2	N0	M0	1	N	N	P	IIA
T2	N0	M0	3	N	P	P	IIA
T0–2	N2	M0	1	N	P	P	IIA***
T3	N1–2	M0	1	N	P	P	IIA
T0–1	N1	M0	1	N	N	N	IIB
T0–1	N1	M0	2	P	P	N	IIB
T0–1	N1	M0	2	N	P	N	IIB
T0–1	N1	M0	2	N	N	P	IIB
T0–1	N1	M0	3	P	P	N	IIB
T0–1	N1	M0	3	P	N	A	IIB
T2	N0	M0	1	N	N	N	IIB
T2	N0	M0	2	P	P	N	IIB
T2	N0	M0	2	N	P	N	IIB

T2	N0	M0	2	N	N	P	IIB
T2	N0	M0	3	P	P	N	IIB
T2	N0	M0	3	P	N	A	IIB
T2	N1	M0	1	P	A	A	IIB
T2	N1	M0	1	N	N	P	IIB
T0–2	N2	M0	2	N	P	P	IIB
T0–2	N2	M0	3	P	P	P	IIB
T3	N1–2	M0	2	N	P	P	IIB
T3	N1–2	M0	3	P	P	P	IIB
T0–1	N1	M0	2	N	N	N	IIIA***
T0–1	N1	M0	3	N	P	N	IIIA
T0–1	N1	M0	3	N	N	A	IIIA
T2	N0	M0	2	N	N	N	IIIA***
T2	N0	M0	3	N	P	N	IIIA***
T2	N0	M0	3	N	N	A	IIIA***
T2	N1	M0	1	N	P	N	IIIA
T2	N1	M0	2	P	N	N	IIIA
T2	N1	M0	2	N	P	N	IIIA
T2	N1	M0	3	P	P	N	IIIA
T2	N1	M0	3	P	N	N	IIIA
T3	N0	M0	1	N	P	N	IIIA
T3	N0	M0	2	P	N	N	IIIA
T3	N0	M0	2	N	P	N	IIIA
T3	N0	M0	3	P	P	N	IIIA
T3	N0	M0	3	P	N	N	IIIA
T0–2	N2	M0	1	P	P	N	IIIA
T0–2	N2	M0	1	P	N	A	IIIA
T0–2	N2	M0	1	N	P	N	IIIA
T0–2	N2	M0	1	N	N	P	IIIA
T0–2	N2	M0	2	P	P	N	IIIA
T0–2	N2	M0	2	P	N	A	IIIA
T3	N1–2	M0	1	P	P	N	IIIA
T3	N1–2	M0	1	P	N	A	IIIA
T3	N1–2	M0	1	N	P	N	IIIA
T3	N1–2	M0	1	N	N	P	IIIA
T3	N1–2	M0	2	P	P	N	IIIA
T3	N1–2	M0	2	P	N	A	IIIA
T4	N0–2	M0	1	N	P	P	IIIA
A	N3	M0	1	N	P	P	IIIA***
T2	N1	M0	1–2	N	N	N	IIIB***
T2	N1	M0	3	N	P	N	IIIB***
T3	N0	M0	1–2	N	N	N	IIIB
T3	N0	M0	3	N	P	N	IIIB
T0–2	N2	M0	2	N	P	N	IIIB
T0–2	N2	M0	2	N	N	P	IIIB
T0–2	N2	M0	3	P	P	N	IIIB
T0–2	N2	M0	3	P	N	A	IIIB
T0–2	N2	M0	3	N	P	P	IIIB
T3	N1–2	M0	2	N	P	N	IIIB
T3	N1–2	M0	2	N	N	P	IIIB
T3	N1–2	M0	3	P	P	N	IIIB
T3	N1–2	M0	3	P	N	A	IIIB
T3	N1–2	M0	3	N	P	P	IIIB
T4	N0–2	M0	1	P	A	A	IIIB
T4	N0–2	M0	2	P	P	P	IIIB
T4	N0–2	M0	2	N	P	P	IIIB
T4	N0–2	M0	3	P	P	P	IIIB
A	N3	M0	1	P	A	A	IIIB
A	N3	M0	2	P	P	P	IIIB
A	N3	M0	2	N	P	P	IIIB
A	N3	M0	3	P	P	P	IIIB
T2	N1	M0	3	N	N	A	IIIC***
T3	N0	M0	3	N	N	A	IIIC
T0–2	N2	M0	2	N	N	N	IIIC***
T0–2	N2	M0	3	N	P	N	IIIC***
T0–2	N2	M0	3	N	N	A	IIIC***

T3	N1–2	M0	2	N	N	N	IIIC***
T3	N1–2	M0	3	N	P	N	IIIC***
T3	N1–2	M0	3	N	N	A	IIIC***
T4	N0–2	M0	1	N	P	N	IIIC
T4	N0–2	M0	1	N	N	A	IIIC
T4	N0–2	M0	2	P	P	N	IIIC
T4	N0–2	M0	2	P	N	A	IIIC
T4	N0–2	M0	2	N	P	N	IIIC
T4	N0–2	M0	2	N	N	A	IIIC
T4	N0–2	M0	3	P	P	N	IIIC
T4	N0–2	M0	3	P	N	A	IIIC
T4	N0–2	M0	3	N	A	A	IIIC
A	N3	M0	1	N	P	N	IIIC
A	N3	M0	1	N	N	A	IIIC
A	N3	M0	2	P	P	N	IIIC
A	N3	M0	2	P	N	A	IIIC
A	N3	M0	2	N	P	N	IIIC
A	N3	M0	2	N	N	A	IIIC
A	N3	M0	3	P	P	N	IIIC
A	N3	M0	3	P	N	A	IIIC
A	N3	M0	3	N	A	A	IIIC
A	A	M1	1–3	A	A	A	IV

Table 4. AJCC prognostic stage groups. T = Primary tumor T category. N = regional lymph node category. M = Distant metastasis category (see also Table 3a). HER2 = Immunohistochemical, FISH or CISH testing of Human Epidermal growth factor receptor 2 according to guidelines by the American Society of Clinical Oncology and the College of American pathologists. ER, PR = Immunohistochemical testing of Estrogen receptor (ER) and Progesterone receptor (PR) according to guidelines by the American Society of Clinical Oncology and the College of American pathologists. P = Positive. N = Negative. A = Any. *** = Stage group for which the use of grade and prognostic factors changed the group more than one stage group from the anatomic stage group (Table 3b). Modified from AJCC Cancer staging manual 8th Edition 2017 (104). Reprinted with permission from Springer International Publishing.

2. AIMS OF THE THESIS

The aim of this thesis is to evaluate immunohistochemical biomarkers currently used in the diagnostic process of breast cancer and potential solutions for its improvement. Special emphasis is placed on the utility of digital image analysis, Ki67 and other markers for proliferative activity.

Aim of paper I: To evaluate the concordance of immunocytochemistry and immunohistochemistry in the expression pattern of ERα, PR and Ki67 between fine needle aspiration cytology and surgical resections from the same tumors.

Aim of paper II: To evaluate the concordance of biomarker analyses between currently used manual methods and digital image analysis in surgical resections from the same tumors, and their correlation to overall survival and gene expression profiles.

Aim of paper III: To evaluate concordance of immunocytochemistry and immunohistochemistry in the expression pattern of ERα, PR, HER2 and Ki67 between fine needle aspiration cytology and surgical resections from the same tumors, its impact on surrogate subtype classification and correlation to survival.

Aim of paper IV: To compare the prognostic relevance of proliferational activity as defined by the count of mitoses and manually as well as DIA-defined proportions of phosphohistone H3- and Ki67-positive cells in different tumor regions.

3. MATERIALS AND METHODS

3.1. PATIENT COHORTS

3.1.1. IMMUNOCHEMISTRY CONCORDANCE COHORT 1

The first immunochemistry concordance cohort, used in paper I, consists of all patients diagnosed with invasive ductal carcinoma NOS or invasive lobular carcinoma of the breast during routine work-up of surgical pathology at the Department of Clinical Pathology at the Karolinska University Hospital in the year 2011. In total, 454 patients (360 ductal and 94 lobular carcinomas) were identified. Patients who had received neoadjuvant chemotherapy, had not undergone fine needle aspiration cytology (FNAC) or had a delay of longer than 100 days between FNAC and surgical removal were excluded, resulting in 346 patients. Pathological data was then retrospectively extracted from the patient's digitalized medical records. As biomarker assessment was not performed on FNA (immunocytochemistry) in all patients, data from 133 patients on ERα status, from 80 patients on PR status and from Ki67 on 131 patients remained for analysis.

3.1.2. IMMUNOCHEMISTRY CONCORDANCE COHORT 2

The second immunochemistry concordance cohort, used in paper III, consists of breast cancer patients that have performed FNAC at the Department of Clinical Pathology at the Karolinska University Hospital during 2005 and 2006. In total, 1671 patients were identified. After exclusion of benign lesions, relapses and cases without Ki67 assessment, 517 patients remained. The consecutive surgical resections including Ki67 assessment were analyzed at the Department of Clinical Pathology, Karolinska University Hospital or at the Department of Clinical Pathology, Capio S:t Göran Hospital. Of 392 cases with both Ki67 evaluations by ICC from aspiration cytology and by IHC from resection specimen, 301 were included for Ki67 assessment. Reasons for exclusion were: Neoadjuvant treatment (*n*=43), no numeric Ki67-value (*n*=39), previous breast cancer diagnosis within 5 years (*n*=9), patients with stage IV disease (*n*=0). In total, 299 patients remained with 301 invasive tumors of the breast (256 invasive carcinoma of no special type/ductal carcinoma, 22 invasive lobular carcinoma, 23 other or missing data). Further data on adjuvant treatment, relapse or distant metastasis and survival for the included patients was obtained from the digitalized patient medical record system. All events of breast cancer recurrence, loco regional- and distant metastasis as well as date and cause of death were gathered from detailed clinical follow-up history and the cause of death certificate, when available. Overall survival was defined as time from date of diagnosis to death or end of follow-up and breast cancer specific survival was defined as patients who had not died from breast cancer disease by end of follow-up.

3.1.3. UPPSALA COHORT

The Uppsala breast cancer cohort, used in paper II and previous publications (83,228-230), consists of altogether 315 patients diagnosed with invasive breast cancer in Uppsala County, Sweden, between the years 1987 and 1989. This represents 65 % of all patients diagnosed with breast cancer in Uppsala during the period. The clinical data and pathological characteristics of the tumors were collected from the patients' medical records. Using registries, follow up has been updated several times by examining the survival status of the patients together with the cause of death. Global gene expression analysis was performed using Affymetrix microarray chips on 260 of the patients within the cohort. The analysis was performed on all the patients which had sufficient and high enough quality mRNA. The tumors were then classified into the intrinsic subtypes Luminal A, Luminal B, Basal-like and HER2-enriched. Due to the construction of TMAs from the original formalin fixed paraffin embedded (FFPE) tumor tissues, the cohort can still today be used to examine the expression levels of potential novel cancer biomarkers using immunohistochemistry (IHC) techniques.

3.1.4. STOCKHOLM COHORT

The Stockholm breast cancer cohort, used in studies II and IV and previous publications (83,231), consists of patients from Stockholm and Gotland counties, Sweden, with invasive breast cancer that was surgically removed between January 1st 1994 to December 31st 1996. 280 of these had available FFPE tumor tissue. Clinical and pathological data for tumor size, lymph node status, hormone receptor status, treatment, date and site of relapse and cause of death, were collected from the Stockholm-Gotland breast cancer registry. The histological grade was re-examined by an experienced pathologist. 159 remaining tumors were examined using global gene expression microarray chips from Affymetrix. Reasons for exclusion from the gene expression analysis were lack of available frozen tumor tissue, emigration abroad or refusal to participate, low quality or low amounts of extracted RNA, or that the patient had received neoadjuvant therapy. The patients excluded because of lack of frozen tissue had on average smaller tumor size, fewer affected lymph nodes and less recurrences. However, the patients excluded due to other reasons did not differ from the patients included in on the microarray analysis.

3.1.5. CLINSEQ COHORT

The Clinseq ("Clinical sequencing of cancer in Sweden") breast cancer cohort was used in paper II, III and IV. It consists of both fresh frozen and paraffin-embedded breast cancer tissue from patients who underwent surgery at the Karolinska University Hospital from November 1st 2002 to December 31st 2010. The patients were identified in the Stockholm–Gotland breast cancer registry and digitalized patients' medical records, along with clinical data and results from manual immunohistochemical evaluations as well as HER2 FISH.

Haematoxylin and eosin-stained slides were used for selection of invasive tumor areas without carcinoma in situ, intense inflammation, fibrosis, necrosis, or poor fixation. 4–8 tissue cores (Ø 0.8 mm) per patient were then mounted into a tissue microarray using a semi-automated instrument (Minicore 3, Tissue Arrayer, Alphelys, France). These tissue microarrays were stained with ER, PR, and HER2. In contrast, full sections were used for the staining of Ki67, considering the heterogeneous distribution of this marker. A total of 195 patients remained for analysis after exclusions of patients with incomplete PAM50 gene assay data and/or clinical immunohistochemical data, tissue microarray cores with <100 tumor cells, failed digital scanning, and errors in software operation. For paper IV, survival data of up to 14 years was added to the cohort.

3.2. TISSUE SAMPLES AND LABORATORY METHODS

3.2.1. FORMALIN FIXED PARAFFIN EMBEDDED TUMOR TISSUE

Archives of formalin fixed paraffin embedded (FFPE) tissue blocks are very important sources of material and data in the fields of oncology and pathology. After surgical removal of a tumor, the tissue is placed in 4 % formaldehyde for fixation. It is then cut into smaller pieces that can be embedded in paraffin blocks. In many cases, this step is performed by pathologists for selection of preferred and representative regions of the tissue. The paraffin embedded tissue blocks are cut into sections of 3-4 μm and mounted on a glass slide. These thin sections are then finally stained for pathological evaluation and diagnosis under the microscope. Hundreds of different stains are currently in clinical use. By routine, most specimens are stained with Haematoxylin and eosin. In breast cancer, immunohistochemical stains of ER, PR, HER2 and Ki67 are commonly added (76,98).

After diagnosis, most clinical pathology units archive the remaining FFPE to enable further tests and future re-analysis. With approval from an ethical review board, these can also be used for research purposes. Commonly, they are used to examine the tumors' expression of selected proteins using methods such as immunohistochemistry (IHC) or immunofluorescence (IF). The advent of next generation sequencing (NGS) techniques and the refinement of gene expression arrays have made it possible to use FFPE for examinations of its genetic profile on a DNA and RNA level (81,84). The benefits using these FFPE collections instead of fresh material is that it allows for long follow-up times and large numbers of available tumors. To some degree, the quality of the RNA and DNA that can be extracted from the FFPE is however still inferior to the quality that can be achieved with the gold standard fresh frozen tissue.

3.2.2. IMMUNOHISTOCHEMISTRY AND IMMUNOCYTOCHEMISTRY

In simple terms, DNA is the recipe for proteins. What specific proteins a cell produce, thereby defining the nature and functions of that cell, is determined by what mRNA is translated. In other words, far from all genes is actively expressed in all cells.

In cancer, the expression of proteins is often dysregulated. Both abundance and lack of certain proteins is a common feature. This can be used in both the clinical and research setting, as the abnormal presence of a protein can signal the presence of cancer and the biological characteristic of that cancer. In a way, dysregulated protein expression is also a signal to the immune system, in many cases triggering an eradicating response (36). Protein expression can be directly illustrated through the similar techniques of immunohistochemistry (IHC) and immunocytochemistry (ICC) (94,232,233). IHC is performed on histological tissue sections whereas ICC is performed on aspirated cytological material without intact

anatomical tissue structure. When the latter is performed as a direct smear, the evaluation of cells and protein expression can be completed within minutes from aspiration from the patient, whereas IHC typically requires a couple of days for fixation, paraffin embedment, cutting and staining. An advantage of IHC is the spatial information on where within a tumor or other tissue a protein is expressed. This allows for visual assessment of the site of expression, in contrast to other methods such as ICC, flow cytometry or Western blotting. Both IHC and ICC are based on the ability of the adaptive immune system to produce antibodies with specific affinities to different proteins. These antibodies are produced in two principally different ways:

Many clones used in clinical routine have been formed in animal hosts, like rabbits or mice that have been inoculated with a target protein such as the human estrogen receptor. The ER protein is identified as foreign, which triggers immune responses and intense secretion of antibodies that can be harvested for use on human tissues. In the case of monoclonal antibodies, a specific sample of immune tissue is isolated and used for creating immortalized cell lines that keep producing antibodies with high specificity for a single antigen. Polyclonal antibodies are sampled from the whole serum of the inoculated animal. Thereby, higher sensitivity but less specificity is achieved. Once the antibodies are isolated, they are usually conjugated to an enzyme that can catalyze a color-producing reaction that is visually detectable in the light microscope. In this example, cells expressing ER are easily detected by a brown color, contrasting to the usually light bluish haematoxylin counterstain. Note that this method of producing diagnostic antibodies is not far from the method used for creating monoclonal therapeutic antibodies like trastuzumab. In order not to trigger an immune response against the mouse antibody protein in the patient, trastuzumab is humanized. This is a process in which proteins are modified to increase their similarity to antibody variants produced naturally in humans. Usually, this is done by using recombinant DNA injected in immortalized cells grown in a bioreactor, only using mouse DNA for the complementarity determining region segments responsible for the ability of the antibody to bind to its target antigen (232,233).

The alternative method for IHC/ICC antibody production uses such immortalized bioreactor cells that have been injected with gene fragments corresponding to a specific epitope. This allows for production of monoclonal antibodies that ideally target only one specific region of the protein of interest. There is however always some degree of cross reactivity, resulting in false positive results (232,233).

Several procedures can be performed to assure oneself of the specificity of the antibodies. Before using antibodies to detect proteins by IHC or ICC, all nonspecific epitopes on the tissue sample should be blocked in a blocking buffer to prevent nonspecific binding of the antibodies. Otherwise, the antibodies or other detection reagents may bind to any epitopes on the sample, independent of specificity. One should also expect diminished staining of the antibody in a culture or tissue where the protein has been knocked out. Other biochemical methods, such as Western blotting, are commonly used for validation.

One of the main challenges of IHC and ICC is to turn the vast and complex visual information on protein expression in a tissue section into standardized, quantified and reproducible data. Evaluation of IHC and ICC is by routine a manual task, requiring meticulous adherence to protocols. For evaluation of Ki67 expression in breast cancer, ≥ 1000 cells should be counted, and what region to score depends on the distribution of positively stained cells. Even if two pathologists would agree on an exact proportion of Ki67-positive to total tumor cells, e.g. 17 %, no undisputable consensus exists to help determine if this constitutes a "high" or "low" proliferational rate (76, 94-97). As mentioned previously, digital image analysis has been suggested as a way to improve the evaluation of IHC, and to a lesser extent ICC (76).

3.2.3. VISIOPHARM INTEGRATOR SYSTEM

The DIA software used in papers II and IV is the Visiopharm Integrator System (VIS), supplied under the non commercial terms of a collaboration with Visiopharm A/S, Hoersholm, Denmark. This software can be made into an illustrating example of the current state of the field: VIS utilizes a method for tissue classification based on virtual double staining, which automatically distinguish epithelial- from stromal tissue. In short, after being digitally scanned at x20, each biomarker slide is aligned with an adjacent 3 μm slide stained immunohistochemically for a pancytokeratin marker such as CkMNF 116. This enables automatic exclusion of non-epithelial cells that potentially express the biomarker in question, i.e. proliferating Ki67-positive lymphocytes. Thus, only cells that express cytokeratin are eligible for detection of positivity or negativity for a second immunohistochemical stain, like ER, PR, KI67, HER2 or PHH3. Individual applications (Apps) for each of these then run the scoring of positive and negative cells itself, with sub-cellular resolution (234). Growing evidence points to the advantages in congruence to gene expression assays, sensitivity and specificity for the Luminal B subtype and prognostic power of DIA compared to current manual methods of biomarker assessments (159-162, see also section 1.4).

3.2.4. PAM50 GENE EXPRESSION ASSAY

Tissue from the tumors in paper II and IV were snap frozen after surgical resection. These tissues were then used for extraction of RNA (DNA/RNA/protein mini kit, Qiagen, Hilden, Germany). The obtained RNA was then assessed to ensure sufficient quality by a standardized protocol (RNA integrity number). 1 μg of RNA was used for rRNA depletion using a removal method (Ribo-Zero removal kit, Illumina, San Diego, CA, USA). Libraries of stranded RNA sequences were then constructed (TruSeq Stranded Total RNA Library Prep Kit, Illumina). Gene-level expression estimates were calculated and normalized using HTSeq count version 0.6.1 and edgeR, which are packages for the Python and R programming languages that provides infrastructure to process data from high-throughput sequencing assays (235,236). A data library from the "Cancer Genome Atlas" by the National Cancer

Institute and National Human Genome Research Institute (n = 1073) were run in parallel for reference (234). Further, the reference data was used to train a nearest shrunken centroid classifier for each gene in the PAM50 gene set (78). Variances between our material and the data from the "Cancer Genome Atlas" were then mean-centered and scaled to unit variance. Last, each tumor in our material was classified into one of the intrinsic subtypes Luminal A, Luminal B, HER2-enriched or basal-like.

This procedure is in accordance with the method of assigning intrinsic subtype based on the PAM50 gene set used elsewhere (77,78,80-88,237,238) (see also subsection 1.3.4).

3.3. STATISTICS

Several statistical methods and tests have been performed during the work with the different papers in this thesis.

Repeatedly, Student's T-tests have been performed to determine whether two sets of normal distributed data differ significantly from each other. Where the data has not followed a normal distribution, the similar Mann-Whitney U test has been performed.

For a determination of suitable threshold for a continuous variable in relation to a binary classifier system, e.g. what percentage of Ki67 or PHH3-stained cells that has the best specificity and sensitivity for PAM50 Luminal B versus PAM50 Luminal A status, the points on receiver operating characteristics curves method has been used. In all papers, equal emphasis were put on achieving best possible sensitivity and specificity without one taking precedence over the other.

For comparisons of classification concordance between two different methods, such as manual and DIA surrogate subclassification to PAM50 gene expression assays, Cohen's kappa statistics and percentages of concordant cases were computed.

Medical research in Sweden is facilitated to a large extent by the existence of personal identification numbers. These are attached to each individual and will not vary in relation to insurance companies, health care providers, banks or other authorities. Thereby, registers are usually easily cross-referenced once a researcher obtains access to them (with approval from an ethical review board). Consequently, data on diagnosis, treatment and survival is usually of excellent quality. In this context, the data can be used to examine prognostic values of novel biomarkers, or of older biomarkers tested with novel techniques.

The Kaplan-Meier method has been repeatedly used to estimate and visualize survival across different subgroups of our breast cancer patients. The Cox proportional hazard model has been used to test survival differences between different groups and calculate hazard ratios.

To compare individual methods ability to discriminate between different prognostic groups, the likelihood ratio test (LR χ^2) was performed. In short, this is a test of the sufficiency of a smaller model versus a more complex model. The higher the χ^2 statistic, the higher the prognostic information contained in the model. The null hypothesis of the test states that the smaller model provides as good a fit for the data as the larger model. If the null hypothesis is rejected, then the alternative, larger model provides a significant improvement over the smaller model. Likelihood ratio chi-square changes (LR -$\Delta\chi^2$) were computed for a test of the relative amount of prognostic information in the different methods in an attempt to answer questions like: Will significantly more prognostic information be obtained if a new method is added to an old?

Where survival data wasn’t available, Spearman's rank-order correlations were run to determine the relationship between biomarkers and other characteristics that are known to correlate with survival, i.e. the Nottingham combined histologic grade, primary tumor diameter and axillary lymph node status. Throughout all papers, differences with a $p < 0.05$ were considered significant, and all p were two-sided.

4. RESULTS AND DISCUSSION

4.1. PAPER I

"Low concordance of biomarkers in histopathological and cytological material from breast cancer"

Stringent evaluation of biomarkers such as ER, PR and Ki67 is essential for the correct treatment of breast cancer patients. In this study, we collected retrospective data from paired ICC and IHC evaluations of 346 tumors (Immunochemistry concordance cohort 1, as described in subsection 3.1.1). As an early step in the diagnosis of a breast lump, detected by palpation or mammography, fine needle aspiration cytology is commonly performed. This material is then sometimes stained using ICC of selected biomarkers. Perhaps only intended to aid the cytologist's decision on whether the lump is a breast cancer or not, the results of the ICC also has some degree of influence on decisions of treatment. This is especially manifest in the metastatic setting, where fine needle aspiration cytology is often the only available source of material. IHC is still considered the gold standard when defining surrogate subtypes, and also the material used in the vast majority of the literature on evaluation of immunochemical markers in breast cancer. Consequently, it is very important that there is a high correlation between ICC and IHC.

Therefore, we compared the concordance of ICC and IHC regarding the results of ER, PR and Ki67 evaluations. In total, we had access to data from both IHC and ICC on 133 patients for ER, 80 patients for PR and 131 patients for Ki67. We found that in average, the proportion of ER-positive cells using ICC was 10.6 percentage points lower than when the same tumor was evaluated using IHC. When comparing tumor status as either "ER positive" or "ER negative", a major predictive and prognostic difference as it distinguish luminal tumors with expected response to endocrine treatment from non-luminal tumors (see also subsection 1.5.3 on endocrine treatment), using first 1 % and the 10 % positive cells as a threshold, 9.0 % and 10.5 % of the tumors, respectively, switched status from ICC to IHC. Similarly when evaluating PR, the expression was in average 13.6 percentage points lower using ICC than IHC. Using a 1 % or 10 % threshold to discriminate "PR positive" from "PR negative", 7.5 % and 11.3 % of the tumors switched status from ICC to IHC, respectively.

When comparing the scoring of Ki67 expression, the expression was in average 7.9 percentage points lower by ICC than by IHC. Because there is no consensus threshold for labeling a tumor's Ki67 positivity or proliferational rate as either "high" or "low", we used the two different thresholds of 14 and 20 %, that also have been used by previous authors (74-76). When using the 14 % cutoff, 32.8 % of the tumors changed proliferation classification from "low" to "high" or vice versa from ICC to IHC. With a threshold of 20 %,

29.8 % of the tumors were reclassified. We also showed that by adjusting the cutoffs used for ICC classification of Ki67 from 14 % to 10 %, the number of patients that were reclassified was slightly decreased.

We observed both over- and underestimation of ER, PR and Ki67 when comparing ICC to IHC on individual cases. On average although, ICC systemically under-classified all three biomarkers. There could be several reasons for this, where one is the difference in the time point from when the samples were collected as FNAC can precede the surgery by months. Another reason is that sampling using FNAC represents a random small area of the tumor compared to the complete tumor evaluation by IHC. This is especially apparent for Ki67, since the recommendation is to evaluate the expression in different areas based on the pattern of expression across the tumor section. Using ICC it is impossible to know if the sample has been extracted from an area of relatively high or relatively low expression within the tumor. In addition differences in fixation and preparation process are common between ICC and IHC and have been shown to cause variances in the intensity of staining. This is mainly believed to happen through differences in the deterioration rate of the biomarker.

When evaluating the data, we observed that a disproportional high number of the ER and PR evaluations were concentrated in the vicinity of 10 % positive cells. This was seen in both ICC and IHC evaluations, but was more common for ICC. The reason for this may perhaps be explained by a will of the cytologist and pathologist to not under-diagnose patients. This bias could be introduced unconsciously, to not withhold patients from endocrine treatment that is considered both efficient and safe. Additionally, it cannot be excluded that since the ICC evaluation is usually followed by an IHC evaluation, which the treatment should be based upon, the perceived need for precision of ICC evaluation decreases.

Novel techniques of liquid and paraffin based ICC has been shown to improve concordance with IHC evaluations. It is therefore important that pathological and cytological labs consider implementing these techniques. Evaluation of the correlation between ICC and IHC is important to decrease the variability of biomarker assessment, especially considering the limited alternatives in the metastatic setting.

4.2. PAPER II

"Digital image analysis outperforms manual biomarker assessment in breast cancer"

In order to suggest concrete methods of improving surrogate IHC-based concordance to gene expression tests, we evaluated DIA as an alternative to the currently used manual methods of biomarker scoring. In this study, we used the Stockholm and Clinseq cohorts (as described in subsections 3.1.4. and 3.1.5, respectively) and the TMA-based Uppsala cohort (subsection 3.1.3.) for a total n = 436. For these patients, we had access to up to 28 years of survival data. The tumors were scored for Ki67, ER, PR, and HER2 status manually and by DIA. The results were then compared for their concordance to PAM50 assays in subtype classification and prognostic power in terms of Cox regression likelihood ratios (LR $\chi 2$) as well as sensitivity and specificity for the Luminal B subtype. The DIA system used was the Visiopharm Integrator System (subsection 3.2.3).

All tested DIA methods of scoring Ki67 outperformed even our most accurate pathologist's manual scores in terms of sensitivity (Sn) and specificity (Sp) for the Luminal B subtype (DIA Ki67 hot spot Sn 86 % Sp 77 % vs. manual Ki67 Sn 74 % Sp 75 %). When comparing DIA versus manual immunohistochemical surrogate concordance with PAM50 gene expression assays, all tested DIA methods were superior to the manual method. The hazard ratios for all-cause mortality in tumors with a 'high' vs 'low' Ki67 index as defined by manual methods and DIA were equivalent. With regard to tumor region, Spearman's rank-order correlations showed a positive and significant correlation with histological grade for both manual and DIA methods, with the strongest correlation for the DIA method giving an automatic representation of the average Ki67 positivity across the full tumor cross-section ($r_s = 0.575$, $p < 0.001$) and the weakest for the manual scores ($r_s=0.459$, $p < 0.001$). When the prognostic value of a Ki67 index determined by each of the manual and DIA scoring methods was tested, all contributed with significant information on overall survival in the PAM50 Luminal A and B subtype tumors, with the highest LR $\chi 2$ for DIA of Ki67 in hot spots (LR $\chi 2 = 7.22$, $p = 0.007$). Furthermore, DIA of Ki67 in hot spots added significantly more prognostic information than the manual scoring method in the same subgroup. This was however not the case when we included all PAM50 subtypes, confirming that the prognostic role for Ki67 is mainly related to the Luminal A and B subtypes. The expression of Ki67 was indeed different in different tumor regions. This did not induce any major differences in performance of subclassification or prognostication as long as the thresholds for "high" vs. "low" proliferation was adjusted accordingly, except for DIA of Ki67 in 'hot spots' that had a slightly better prognostic value.

In summary of this study, manual assessments of the biomarkers HER2, ER, PR and Ki67 with an emphasis on the latter, was in most aspects an inferior alternative to digital image analysis. This implicates that in current clinical routine, an avoidable high

proportion of patients risks being treated with potentially harmful agents such as cytotoxic chemotherapy without benefit, or being excluded from the beneficial treatments the DIA method would indicate.

Accordingly, we concluded that DIA is a viable and competitive alternative for biomarker testing in breast cancer. We strongly encourage further studies to confirm the results found here in larger populations, to provide data on cost-benefit ratios, to facilitate implementation and to evaluate the performance of digital image analysis in prospective clinical use.

4.3. PAPER III

"Biomarker assessment in cytology and corresponding resected breast tumors—correlation to molecular subtypes and outcome in primary breast cancer"

In this study, we evolved the concept of study I with an evaluation of the concordance of consecutive ICC and IHC assessments performed on paired fine needle aspirations and resected tumor specimens, respectively. Furthermore, we investigated how a status of Ki67 "low" and "high" with ICC and IHC corresponded to overall survival. The immunochemistry concordance cohort 2 and Clinseq cohorts were used (see subsections 3.1.2. and 3.1.5) for a total n = 495. Ki67 assessments, as well as ER, PR and HER2 when available, were analyzed. Clinicopathological data, including up to 10-year overall survival, was retrospectively gathered from patient medical record systems. PAM50 subtype data was used to correlate surrogate subtypes to gene expression subtypes.

Ki67-indices varied between ICC and IHC, and were prone to switch between low- and high proliferation within the same tumor. ER evaluations were discordant in 5.3 % of the tumors, which in the clinical setting would mean that 1/20 patients would risk being left out of beneficial endocrine treatment or being given it without benefit. Discordance with PAM50 subtypes was seen in 40 % of the cases with ICC, and in 36 % with IHC. Patients with IHC Ki67 "high", in this study defined as a proportion of Ki67-positive cell above the 67^{th} percentile of the material, had significantly worse survival than patients with IHC Ki67 below this threshold, and a significantly higher hazard for all-cause mortality at 10 years (HR 1.75, 95% CI 1.01-2.80, p = 0.02). Additionally, these patients had a significantly higher probability for axillary lymph node metastasis. No significant differences in survival or risk for axillary lymph node metastasis could be shown between ICC Ki67 "high" and "low".

In summary, this study adds to the results of paper I, in which we showed a discordance of ICC and IHC. By including survival data, we now conclude that not merely are the methods discordant, but ICC fails to provide prognostic information. Consequently, IHC is the superior method of Ki67 assessment. Although FNAC can still have an important role in distinguishing malignant from benign epithelium, these findings consequently do not support the use of direct smear ICC Ki67 to draw other conclusions than diagnosis when IHC is available in the clinical setting.

4.4. PAPER IV

"Digital image analysis of Ki67 in hot spots is superior to manual Ki67, phosphohistone H3 and mitotic counts in breast cancer"

In this paper, we compared the prognostic value of mitoses, Ki67 and the more recently described Phosphohistone H3, when evaluated by manual methods and DIA in two cohorts of primary breast cancer specimens (total n=294).

Proliferative activity is one of the most important prognostic parameters in cancer. By international standards, it is routinely evaluated by a count of mitoses during the pathological examination of breast tumors. Adding immunohistochemical stains of the nuclear protein Ki67 provides extra prognostic and predictive information. However, the currently used methods for both of these evaluations have some substantial limitations, primarily in terms of reproducibility. Importantly, it is still unclear whether analysis of Ki67 should be performed in the tumors' hot spot – i.e. the area where the biomarker is expressed in the relatively highest proportion of tumor cells, in the tumor periphery – i.e. the border region formed by the invasive tumor cells and the surrounding stroma, or as the average proportion of Ki67-positive cells across the whole tumor section.

In this paper, both manual and DIA scores of Ki67 and PHH3 were evaluated along with mitotic counts for their sensitivity and specificity for the gene expression based Luminal B versus A breast cancer subtypes, for the high versus low transcriptomic grade based on gene- and isoform-level expression data from RNA-sequencing (121), for axillary lymph node status as well as for their prognostic value in terms of Cox regression hazard ratios (HR) and prediction of breast cancer specific and overall survival.

Especially evident in the tumors' hot spots, digital image analysis of Ki67 outperformed the other markers in sensitivity and specificity for gene expression Luminal A and B subtypes (DIA Ki67 hot spot Sn 81.5 % Sp 65.6 %. PHH3 Sn 68.5 % Sp 71.2 %. Mitotic counts Sn 77.8 % Sp 45.2 %) as well as in sensitivity and specificity for the transcriptomic grades. In contrast to mitotic counts, tumors with high expression of Ki67 as defined by digital image analysis, and high numbers of Phosphohistone H3-positive cells, had significantly increased HR for all-cause mortality within 10 years from diagnosis (HR 2.93, 95 % CI 1.61–5.31 for DIA of Ki67 in hot spots vs. HR 1.89, 95 % CI 0.66–5.46 for manual assessment of the number of mitoses in 10 high power microscopic fields). Further, DIA of Ki67 was superior to manual Ki67 and PHH3 evaluations as well as to mitotic counts in terms of separation of patients with poor versus relatively good overall survival.

Finally, we replaced the manual mitotic counts with digital image analysis of Ki67 in hot spots as the marker for proliferation when determining histological grade. This increased differences in estimated mean overall survival between the highest and lowest grades (3.4 years vs. 2.1 years between the classic Nottingham combined histological grades 1 and 3) and added significantly more prognostic information. We conclude that digital image analysis of Ki67 in hot spots might be suggested as the marker of choice for proliferative activity in breast cancer.

4.5. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The diagnostics and treatment of breast cancer has improved drastically in the last several decades or even the last century. This is especially evident for early breast cancer given neoadjuvant or adjuvant treatment, where targeted therapies like trastuzumab have led to a dramatic reduction of recurrences. Most, but not all, patients now have a good prognosis. Accurate and reliable identification of the different subtypes of the disease, including the most aggressive ones, is therefore key in further advancing the field.

Improved evaluations of markers for these different subtypes and predicted therapy responses holds promise in serving this end, regardless if these markers are based on gene expression profiles, immunohistochemistry or other techniques. Fine needle aspirations are safe, reliable and cost efficient in identifying malignant cells. In certain situations they are also the only practical way of obtaining material for analysis: As it has been shown that tumor characteristics transform during disease progression and that biomarker profiles frequently change from primary tumor to metastasis, one cannot rely on findings from immunohistochemistry of the primary tumor to draw conclusion on the metastasis (239). Therefore, the metastasis will have to be sampled separately and the biomarkers reassessed, ideally with immunohistochemistry (240). But due to anatomic location, size and accessibility of the metastatis, fine needle aspirations are sometimes the only available option. Consequently, it is crucial that the results of immunocytochemistry and immunohistochemistry are concordant. In this context, it is important to highlight that the findings of two of the papers presented in this thesis suggest that outcomes of the two methods actually do differ. Tumors classified as negative for the Estrogen receptor with one method might be classified as positive with the other, resulting in an altogether different treatment strategy. When it is possible to choose between fine needle aspirations and obtaining material for immunohistochemistry through a core needle or incisional biopsy, our results indicate that one should choose the latter. This is not indicating an opinion on our behalf that fine needle aspirations are obsolete per se. In the event future developments allow for sequencing of all tumors on the RNA or DNA level to fine tune treatment (“precision medicine”), the fine needle might prove to be a reliable source of material provided enough can be aspirated.

Regarding digital image analysis, one might argue that it is only complicating a fairly straightforward task of counting stained and unstained cells, and that it is not user friendly enough in comparison with eyeballing a glass slide under the light microscope. Its potential is indeed fairly limited, as even a perfect immunohistochemical test still would be nothing but a surrogate for the gene expression assays in many clinical applications. Simply put: You can never obtain perfect understanding of something by measuring something else. Substantial investments in digital scanning capacity, data storage, software, and training are

required at each institution before effective use of the technology can be expected. And with an excessive automation, DIA could withdraw some degree of control over the biomarker assessments, potentially leading to dire consequences to patients. Furthermore, DIA may in itself be a source of variance. Different DIA approaches will inherently classify tumor, nuclei, and membranes differently, and poor performance of the algorithm's identification of tumor versus non-tumor tissue as well as cellular components would be a significant source of error.

To minimize the variance contributed by the digital image analysis software, and to reassure the medical field of the validity of its results, the industry must make sure they do not deliver "black boxes" to the clinical end users. Strict industry standards, perhaps a comprehensible and easily verifiable version of the rules governing medicinal products in the European Union (EUDRALEX) should be agreed upon (241). Further, each manufacturer should strive for the maximum possible degree of transparency in how their product handle and analyze the tissues, and towards producing interoperable hard- and software and standardized file formats, such as the DICOM standard for medical imaging (242).

When interpreting the results of any method's concordance to gene expression assays, one should also note that any tumor's subtype is based on the average gene expression profile in the very piece of tumor tissue from which RNA was extracted. Thus, presence of substantial intratumor heterogeneity could potentially lead to uncertainty in subtype assignment and consequentially impact the immunohistochemical versus PAM50 subtype concordance. In an ongoing study we seek to shed clarity to this subject (unpublished). So far, our preliminary data indicates that intratumor heterogeneity in terms of PAM50 subtype is quite limited and not a common occurrence. Simultaneously, one should keep in mind that the gene expression panels frequently mentioned here should not be viewed as the final truth on breast cancer subclasses. In the near future, whole exome or even whole genome sequencing might be standard practice in clinical breast cancer diagnostics (243,244). Moreover, manual versus digital image analysis immunohistochemical subtype concordance to PAM50 assays would be influenced to a similar degree by a presence of intratumor heterogeneity, why we believe that it is not likely to affect the results and conclusions of this study in any major way.

And after all, digital image analysis is in many ways already an accessible, reliable and simple option with superior reproducibility (160-163). The industry has left the early, experimental days behind and can now offer several mature systems for immediate introduction in clinical routine. A growing number of applications are offered on the market, including the one tested in two of the papers presented this thesis. Combined with increasingly efficient and affordable digital glass slide scanners, digital pathology is now challenging manual biomarker scoring for the method of choice. In addition to its competitive performance, digital image analysis provides an opportunity for strained healthcare institutions to reduce time consumption for pathologists and to allocate precious resources to more qualified tasks. When digital image analysis operations are fully automatized, manual input and thereby the sampling bias can be reduced to a minimum. This

could potentially allow for biomedical scientists or other laboratory personnel with only a basic understanding of histopathology and immunohistochemistry to manage biomarker testing, including surrogate immunohistochemical subclassification in breast cancer.

Although unexplored, a near future development to be expected is the introduction of artificial intelligence (AI) and deep learning algorithms for the interpretation of digitalized tissue and tumor images. In contrast to the software tested in this thesis that does no more or no less than what it's originally told, AI relies on computational methods that allows for a degree of self-programming by learning from an initial set of examples that demonstrates the desired behavior. Recently, Webster and colleagues, sponsored by Google Inc., trained such an algorithm to identify diabetic retinopathy on a retrospective set of more than 100 000 retinal images. When this algorithm was applied on 2 separate cohorts, it operated with a sensitivity and specificity well over 90 %, and an area under the receiver operating curve of 0.991 (245).

Finally, it should be noted that the results of AI-based image interpretation, DIA and any other visual method might be viewed as more than mere surrogates for other more sophisticated tests, such as gene expression assays. It is not a law of nature that subclassification, prognostication and therapy selection must be based on RNA or DNA profiles. Depending on future developments of this field, any of a large number of methods might see a share of diagnostic use, and immunohistochemistry is set to retain a place when assessing protein expression.

In conclusion, we acknowledge the objections that might be raised against digital image analysis and recommend pathologists and laboratories to proceed with reasonable deliberation when acquiring equipment and selecting software. In this sense, the introduction of digital image analysis should not differ from the general level of precaution used when introducing any novel technique. If anything, the results of the papers presented in this thesis gives us confidence to recommend an automated analysis as a solution to some of the long standing problems of tumor classification based on immunohistochemical stains.

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