Clinical significance of immunohistochemistry in breast cancer diagnostics

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Cover illustration: “The breast cancer puzzle” by Wilma Nymark.

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Everything happens for a reason

To Olivia, my everything

“What does not kill you makes you stronger”

Friedrich Nietzsche

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ABSTRACT

For patients with breast cancer, modern patient-tailored treatment depends on tumor-specific characteristics, i.e., estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor 2 (HER2), and the proliferation marker Ki67. These biomarkers are used in different combinations to classify breast cancer into subtypes on which treatment recommendations are based. Although modern multi-gene tests are available, the cornerstone for assessing these biomarkers remains immunohistochemistry (IHC). This thesis is aimed at investigating various clinical aspects of IHC and breast cancer subtypes in breast cancer diagnostics and treatment.

In Paper I, the mRNA-based assessment tool STRAT4 was compared with IHC, and the potential changes in adjuvant treatment recommendations based on the differences between tests were compared. The results indicated that adjuvant treatment decisions based on STRAT4 rather than IHC were more aggressive.

Paper II investigated whether IHC assessment on more foci than only the largest focus in patients with multifocal breast cancer would affect adjuvant treatment recommendations. The results suggest that all detected foci within a breast specimen should be assessed with IHC.

Because guidelines do not recommend IHC assessment of lymph node metastasis (LNM), Paper III investigated whether treatment recommendations might differ if the biomarker status in the LNMs were known. Although both biomarker and subtype discordances were observed, no additional treatment was recommended according to these changes.

Paper IV, a national population-based registry study, investigated the effects of chemotherapy on survival outcomes in women ≥70 years of age with a breast cancer subtype lacking ER, PR, and HER2 biomarker expression, i.e., those with triple-negative breast cancer. Statistically significant survival benefits were shown for women treated with adjuvant chemotherapy, thus highlighting the importance of considering chemotherapy in this group of older patients.

Key words: breast cancer biomarkers, immunohistochemistry, surrogate subtype, RT-qPCR, multifocal breast cancer, lymph node metastasis, triple-negative breast cancer, older women

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SAMMANFATTNING PÅ SVENSKA

Trots att bröstcancer (BC) är den vanligaste cancerdiagnosen och den vanligaste orsaken till cancerrelaterad död bland kvinnor i världen, är det en cancerform med väldigt bra överlevnad. Det finns många olika typer av BC och dagens moderna behandling skräddarsys för den enskilda patienten. Behandlingen är en kombination av kirurgi, läkemedel och strålbehandling. En av de viktigaste pusselbitarna för att få rätt behandling är information om brösttumörens natur på cellnivå. Denna fås traditionsenligt med hjälp av s.k. immunhistokemisk färgning (IHC) av tumören, där man får information om de olika bröstcancermarkörerna ER (östrogenreceptorn), PR (progesteronreceptorn), HER2 (som gör att cancercellerna delar sig oftare) och Ki67 (som mäter delningshastigheten i cancercellerna). Dessa biomarkörer i olika kombinationer utgör sedan en panel som översätts till de olika bröstcancertyperna, som i sin tur utgör grunden för vilken behandling patienten rekommenderas: om uttryck finns av ER och PR erbjuds patienten antihormonell behandling, om det finns uttryck av HER2 rekommenderas målinriktad antikroppsbehandling och om det finns höga nivåer av Ki67 så kan patienten bli erbjuden kemoterapi (cellgifter). Syftet med denna avhandling är att undersöka olika kliniska aspekter av IHC i bröstcancerdiagnostik.

I **delarbete I** jämfördes en ny metod, STRAT4 med IHC för de olika bröstcancermarkörerna. Därefter utvärderades huruvida behandlingsrekommendationerna hade ändrats om bröstcancertypen hade bestämts med hjälp av STRAT4 i stället för IHC. Överensstämmelsen mellan de två metoderna för varje enskild biomarkör var god, men när de översatts till bröstcancertyper skulle 18 av 98 patienter teoretiskt ha rekommenderats en annan behandling om STRAT4 var den metod som hade bestämt bröstcancertypen.

När flera tumörer (multifokal BC) upptäcks i bröstet, görs IHC analysen rutinmässigt endast på den största, såvida inte den/de mindre tumörerna skiljer sig avsevärt från den största. I **delarbete II** undersöktes förekomsten av multifokal BC hos patienter som genomgått operation för BC vid Sahlgrenska Universitetssjukhuset mellan åren 2012 och 2017 och hur stor andel av dessa multifokala tumörer som skilde sig åt beträffande bröstcancermarkörer och bröstcancertyp. Vidare undersöktes om dessa skillnader hade konsekvenser för patienterna i besluten om den efterföljande behandlingen. Av de 180 patienterna som var med i studien fick 6,1% av patienterna en ändrad behandling på grund av skillnaden i bröstcancertyp mellan de olika tumörerna. Dessa resultat talar för att man bör titta närmare på rutinerna för IHC analyser

av multifokal BC och överväga rutinmässig analys av alla tumörer som hittas i ett bröstprov, så att dessa patienter får optimal behandling.

Det är analysen av brösttumören som är vägledande för den individanpassade behandlingsrekommendation patienten får. När bröstcancern hunnit sprida sig till lymfkörteln/körtlarna i armhålan, analyseras dessa lymfkörtlar inte rutinmässigt med IHC. I **delarbete III** undersöktes huruvida behandlingsrekommendationen hade ändrats om patienter med samtidig lymfkörtelspridning hade fått sina sjuka lymfkörtlar analyserade med IHC. IHC utfördes på 94 patienters lymfkörtelmetastaser. Dessa patienter hade blivit opererade för bröstcancer på Sahlgrenska Universitetssjukhuset under 2018. Det observerades skillnader i bröstcancertyp mellan brösttumör och lymfkörtelmetastas hos 28,7% av patienterna, dock hade dessa skillnader inte lett till ytterligare/ändrade behandlingar, vilket talar för att IHC-analys på lymfkörtelmetastaser inte tillför ytterligare avgörande information. Dessa resultat behöver utforskas ytterligare i större studier för att bringa klarhet i frågan om huruvida ytterligare analyser skall göras på lymfkörtelmetastaser eller inte.

Den förväntade livslängden hos befolkningen har ökat och beräknas fortsätta öka. Bröstcancer blir vanligare med åldern, men stora randomiserade studier (studier där patienter slumpas till olika behandlingar) inkluderar sällan patienter som är 70 år eller äldre. En aggressiv bröstcancertyp är den så kallade trippel-negativa bröstcancern, som saknar uttryck av bröstmarkörerna ER, PR och HER2. Kemoterapi, som är en tuff behandling, är det behandlingsalternativ som vi har att tillgå för att förbättra överlevnaden för patienter med denna bröstcancertyp. De nationella behandlingsriktlinjerna följs inte alltid för den äldre patienten, ofta på grund av samsjuklighet, som gör att man bedömer att de inte klarar behandlingen. I **delarbete IV**, som är en nationell registerstudie, studerades kvinnor, 70 år och äldre, som fått diagnosen trippel-negativ BC i Sverige under åren 2009–2016. Vi jämförde överlevnaden på de kvinnor som hade fått kemoterapi med de kvinnor som inte hade fått kemoterapi efter att de blivit opererad för bröstcancern. Det visade sig att de kvinnor som fått kemoterapi hade betydande bättre överlevnadssiffror än de kvinnor som inte hade fått kemoterapi. Dessa resultat understryker vikten av att överväga denna behandling även till de äldre patienterna.

Det görs många och snabba framsteg inom bröstcancerforskningen. Det är därför extra viktigt att samarbetet mellan, de nyckelpersoner inom hälso- och sjukvården som omgärdar bröstcancerpatienten, är välfungerande och att all ny kunskap som kommer kan tillämpas.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Janeva S**, Parris TZ, Nasic S, De Lara S, Larsson K, Audisio RA, Olofsson Bagge R, Kovács A. Comparison of breast cancer surrogate subtyping using a closed-system RT-qPCR breast cancer assay and immunohistochemistry on 100 core needle biopsies with matching surgical specimens. *BMC Cancer* 2021 Apr 21;21(1):439.

II. **Janeva S,** Krabbe E, Parris TZ, Nasic, S, Sundquist M, Karlsson P, Audisio RA, Olofsson Bagge R, Kovács A. Clinical evaluation of molecular surrogate subtypes in patients with ipsilateral multifocal primary breast cancer. *Breast Cancer Research* 2023 Apr 6;25(1):36.

III. **Janeva S**, Parris TZ, Krabbe E, Sundquist M, Karlsson P, Audisio RA, Olofsson Bagge R, Kovács A. Clinical relevance of biomarker discordance between primary breast cancers and synchronous axillary lymph node metastases. *Submitted, under revision*

IV. **Janeva S**, Zhang C, Kovács A, Parris TZ, Crozier JA, Pezzi CM, Linderholm B, Audisio RA, Olofsson Bagge R. Adjuvant chemotherapy and survival in women aged 70 years and older with triple-negative breast cancer: a Swedish population-based propensity score-matched analysis. *Lancet Healthy Longevity* 2020 Dec;1(3):e117-e124.

CONTENT

ABBREVIATIONS

ALND	axillary lymph node dissection
BCSS	breast cancer specific survival
cDNA	complementary DNA
CNB	core needle biopsy
ER	estrogen receptor
FFPE	formalin-fixed and paraffin-embedded
FNAC	fine needle aspiration cytology
H&E	hematoxylin-eosin
HER2	human epidermal growth factor2
HR	hormone receptor
IHC	immunohistochemistry
ISH	*in situ* hybridization
OS	overall survival
PR	progesterone receptor
RT-qPCR	Real-time quantitative polymerase chain reaction
TNBC	triple-negative breast cancer
WHO	World Health Organization

DEFINITIONS IN SHORT

Adjuvant treatment	Treatment given after surgery
c-DNA	Complementary DNA which is translated from single-stranded RNA
Neoadjuvant treatment	Treatment given before surgery

1 INTRODUCTION

1.1 Breast cancer

Breast cancer is the most commonly diagnosed form of cancer in women worldwide, with 2.3 million new patients diagnosed annually. This disease is also the leading cause of cancer-associated death in women, accounting for 685,000 deaths worldwide (1, 2). However, breast cancer has a relatively favorable prognosis, and the 10-year survival rates in Western countries have improved and currently exceed 85%, probably because of the availability of more effective treatments and earlier detection through mammography screening (2). In 2021, 8,486 new patients were diagnosed with breast cancer, and 1,326 deaths from the disease were reported in Sweden. The median age at the time of diagnosis is 65 years (3, 4) **(Figure 1**).

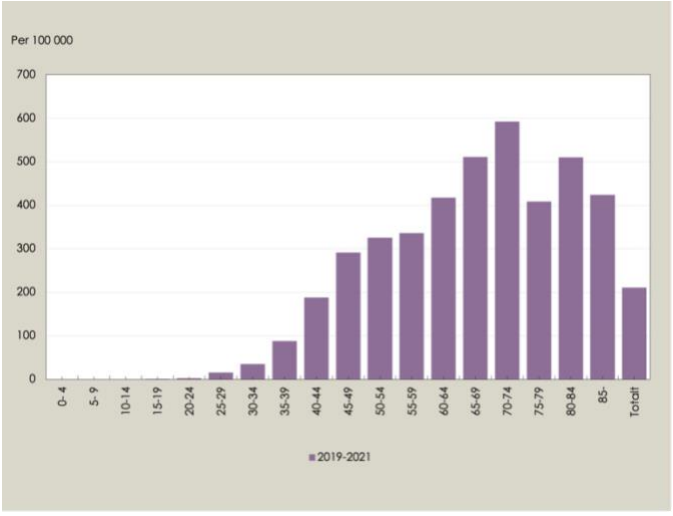

Figure 1. *Breast cancer incidence presented in age categories in women in Sweden for 2019-2021 (retrieved from socialstyrelsen.se) (3)*

Although most breast cancers are sporadic, approximately 5–10% have a hereditary component (5). Certain gene mutations have been associated with elevated risk of developing breast cancer; the most common of these mutations occur in the *BRCA1* and *BRCA2* genes, which have roles in the DNA repair system.

The diagnosis of breast cancer involves a triple-diagnostic approach including clinical examination, imaging (e.g., mammography and ultrasound), and assessment of the tumor tissue (preferably through core needle biopsy; CNB). Treatment recommendations are tailored to individual patients and are recommended by multidisciplinary teams (MDTs), which consider both patient and tumor characteristics. The available treatments for breast cancer include surgery (which involves removal of the tumor from the breast and staging in the axilla), systemic therapy either before (neoadjuvant) or after (adjuvant), and radiotherapy.

1.2 Detection and diagnostics

1.2.1 Mammography

Breast cancer can be detected in different ways. Patients may seek medical attention after discovering a palpable mass or other clinical symptoms, such as redness, edema, bloody nipple discharge, skin retraction, or ulceration. In addition, mammography screening can detect breast cancer before the onset of clinical symptoms. The proportion of breast cancers detected with screening significantly varies among countries and is dependent on the availability of organized mammographic screening programs (1, 6, 7). In Sweden, mammography screening is recommended for women 40–74 years of age, and approximately 65% of all breast cancers are detected through screening (4). Mammography screening has been demonstrated to decrease breast cancer mortality rates by enabling early detection (8, 9). Regardless of whether mammography imaging is conducted for clinical or screening purposes, a biopsy of the tumor and additional clinical evaluation are necessary to confirm the diagnosis. This comprehensive evaluation, also known as the "triple assessment" is standard of care and an important quality measure in medical practice (10).

1.2.2 Specimen examination

The field of pathology underwent a major transformation with the emergence of microscopy during the 17th century. This breakthrough paved the way to better understanding of disease and the development of innovative diagnostic techniques.

1.2.2.1 Fine needle aspiration cytology

Fine needle aspiration cytology (FNAC) is a rapid and low-risk method for diagnosing breast cancer. A small sample of breast cells or fluid is extracted from a suspicious area with a thin needle and is examined for the presence of cancer cells. This procedure can be performed under clinical guidance if the tumor is palpable or under ultrasound guidance. However, the drawback of FNAC is that the analyzed cells are not organized, thus hindering determination of the tumor's growth patterns and distinguishing invasive from non-invasive tumors. Consequently, CNB has become the preferred method for assessing breast tumors, whereas FNAC remains the method of choice for evaluating suspicious axillary lymph nodes.

1.2.2.2 Core needle biopsy

CNB is a widely used preoperative diagnostic technique for evaluating breast lesions (11, 12). This procedure involves using a hollow 12–14 gauge needle to extract tissue samples from suspicious areas in the breast. CNB can be performed under clinical or ultrasound guidance. Unlike FNAC, CNB preserves the tissue architecture, thereby enabling morphological and histological assessment after preparation (13). This information is particularly critical in patients in whom neoadjuvant treatment is the preferred option because of specific clinical features such as large tumor size, lymph node involvement, locally advanced breast cancer, or breast cancer subtypes, e.g., triple negative or HER2+ (14, 15).

1.2.2.3 Surgical specimens

After a breast specimen is surgically excised (through partial mastectomy or mastectomy), it is then transported to the pathology department for further assessment of the tumor, and formalin-fixed, paraffin-embedded (FFPE) blocks are created. Slides are then prepared from theses FFPE blocks and subjected to histological staining (Section 1.2.3) and immunohistological staining (Section 1.2.4) for pathological evaluation. The assessment of simultaneously extracted lymph nodes follows the same steps except for the immunohistochemistry (IHC) staining step. The surgical margins of the breast specimen are critical. According to current international consensus (16, 17), "no tumor at ink" (i.e., negative margin), wherein one tumor-free cell layer is sufficient, is considered an adequate margin.

1.2.3 Morphological classification

Between the late 19th and early 20th centuries, the textile dyeing industry inspired tissue processing. Initially, natural dyes such as carmine and indigo were used by microscopists, but synthetic dyes, such as eosin and van Gieson, were soon developed thereafter. The use of histochemical staining allowed for the recognition of various cell types and tissues, thereby providing crucial insights into the patterns, shapes, and structures of cells. Hematoxylin/eosin (H&E) is currently the histochemical dye most commonly used for tissues in general, including in breast cancer diagnostics.

Invasive breast cancers are morphologically categorized on the basis of two factors: 1) their growth patterns or histological subtype, and 2) their degree of differentiation or histological grade.

1.2.3.1 Histological subtypes

Invasive breast cancer can be classified into various histological subtypes, including special and no special type (18). The most common histological subtype is the no special type, previously known as invasive ductal carcinoma, which accounts for approximately 75%–80% of all invasive breast cancers (18). This type of cancer is characterized by invasion through the outer myoepithelial cell layer of the breast duct. The most common special type is lobular breast cancer, which accounts for 5%–15% of all breast cancers (18). Owing to the loss of the E-cadherin gene, this histologic type tends to have a more diffuse pattern of spread within the breast in characteristic single-cell file patterns, thus making them difficult to diagnose clinically and radiologically. They are often multifocal, and the tumor size can be underestimated (19). Other special types, such as tubular, adenoid cystic, and mucinous (colloid) types, generally have very good prognosis (**Figure 2**).

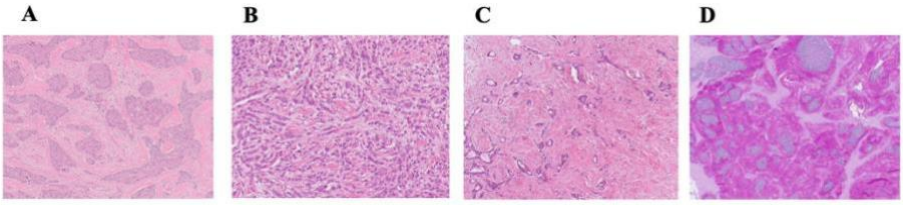

Figure 2. *Different histological breast cancer subtypes.* ***A****= invasive cancer of no special type, formerly termed invasive ductal carcinoma (H&E staining),* ***B****= Invasive lobular cancer (H&E staining),* ***C****= Invasive tubular cancer (H&E staining),* ***D****= Invasive mucinous cancer (PAS staining).*

1.2.3.2 Histological grade

Histological grade, also known as the degree of differentiation, reflects how closely the neoplastic cells resemble normal breast epithelial cells. The grade is determined by assessment of three morphological features: 1) the degree of tubule or gland formation, 2) nuclear pleomorphism, and 3) mitotic count. The tubule or gland formation indicates how closely the tumor resembles normal ductal structure; the nuclear pleomorphism measures the extent to which the nuclei of the cancer cells diverge from a normal nuclear size; and the mitotic count indicates how rapidly the cells are dividing or replicating. Each of these three characteristics is given a score of 1–3, thus resulting in a total Nottingham histological grade score. A score of 3–5 is classified as grade 1 (well differentiated), a score of 6–7 is classified as grade 2 (moderately differentiated), and a score of 8–9 is classified as grade 3 (poorly differentiated). The tumor grade is considered a prognostic factor: high-grade breast cancer (grade 3) is associated with a higher risk of recurrence. Radiation therapy has been shown to decrease this risk (20).

The accuracy of grading relies on tissue preservation, and suboptimal fixation can lead to a disrupted cell structure and inaccurate mitotic assessment, thus resulting in deceptive Nottingham histological grading (21). Histological grading may show interobserver variability, thereby decreasing reproducibility. To improve interobserver agreement, several guidelines have been implemented at both national and international levels. These guidelines include a range of variables including tissue handling, fixation, and preparation, as well as grading methods. The College of American Pathologists, the World Health Organization, and the Swedish Society of Pathology are among the organizations providing these guidelines.

1.2.3.3 TNM staging

Staging of breast cancer is performed with the TNM classification system, in which T refers to tumor size; N refers to regional lymph node involvement; and M refers to distant metastasis **(Table 1)**. This system is used for both clinical staging, which is based on clinical and radiological findings before surgery, and pathologic staging, which is based on histopathological assessment of breast and axillary specimens after surgery. Treatment recommendations in MDT meetings are based on the TNM stage.

Table 1. *Staging and TNM classification of breast cancer. The table is simplified and adapted from American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 8th edition (22).*

Stage	T[1]	N[2]	M[3]
0	Tis	N0	M0
IA	T1	N0	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1 or N2	M0
IIIB	T4	Any N	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

[1] Size of the primary tumor: Tis= Ductal carcinoma in situ or Page's disease, T1 ≤ 20 mm, T2 >20 mm ≤ 50 mm, T3 >50 mm, T4 any size with direct extension to the chest wall and/or to the skin.
[2] Regional lymph nodes: N0= no positive axillary lymph nodes, N1= 1-3 positive axillary lymph nodes, N2= 4-9 positive axillary lymph nodes, N3 ≥ 10 positive axillary lymph nodes.
[3] Distant detectable metastases (clinical, radiographic, or histologically proven).

1.2.4 Immunohistochemistry

IHC, which emerged in the 1980s, enables semiquantitative assessment of protein expression levels on histological slides by using antibodies (markers) to detect specific proteins (antigens) present on the surfaces of or within cancer cells. It is used to assess the main breast cancer biomarkers of hormone receptors (HR) estrogen receptor (ER) and progesterone receptor (PR), human epidermal growth factor 2 (HER2), and the proliferation marker Ki67. The HercepTest is used for determination of HER2 protein expression. *In situ* hybridization techniques were developed enabling detection of RNA or DNA sequences rather than proteins in cancer cells. The two main labeling and detection methods are 1) radio-isotope labeling, which is detected with X-ray film or emulsion autoradiography, and 2) non-isotope labeling, such as fluorescein and silver, which is visualized with IHC (23). In Sweden, silver

(SISH) is the most commonly used labeling method for detecting HER2 amplification.

However, IHC has limitations of inter- and intraobserver variability: discrepancies have been reported to be as high as 20% for ER, PR, and HER2 (24, 25). Ki67, in particular, is a challenging biomarker, as it helps clinicians distinguish between the luminal A and luminal B subtypes (Section 1.2.6), which each have different therapeutic recommendations (26). Although the intralaboratory concordance is good, the interobserver agreement remains unsatisfactory for Ki67, because of factors such as tumor region selection, counting methods, and subjective assessment of staining positivity (27). International and national efforts to standardize Ki67 scoring have led to improvements, but the interobserver agreement remains unsatisfactory (28, 29). Despite these limitations, IHC remains a valuable tool for assessing breast cancer biomarkers.

If more than one tumor is detected in a breast specimen (i.e., multifocal breast cancer), IHC is assessed on only the largest tumor unless other foci differ from the largest focus in grade and morphology. IHC is also not performed on synchronous lymph node metastases (LNMs).

1.2.5 Gene assays

Multi-gene assays such as Oncotype DX, PAM50/Prosigna, and MammaPrint have been accessible to healthcare professionals since 2004 and have been used to guide adjuvant systemic therapy for women with early-stage invasive breast cancer. In Sweden, gene expression analysis using Prosigna or Oncotype DX is suggested specifically for postmenopausal women with HR+, HER2- breast cancer with one to three lymph node metastases, for whom the potential benefits of chemotherapy are unclear. Both Prosigna and Oncotype DX have been evaluated and found to be cost-effective (4).

The need for fast, reliable, and reproducible methods to standardize the assessment of the four breast cancer biomarkers has led to the development of new assays for determining the expression of these biomarkers according to their mRNA levels (30, 31, 32). RNA extracted from routine clinical FFPE samples (CNBs or surgical specimens) enables mRNA-based surrogate subtyping. Although several studies have demonstrated good agreement between these new assays and IHC, limitations such as cut-off values and sample handling must be considered (30, 33, 34). One notable advantage of these new assays is the decreased bias in routine Ki67 assessment, thus preventing the results from being affected by subjective interpretation (35).

1.2.6 Breast cancer subtypes

In 2001, Sorlie *et al.* (36) achieved a major milestone in breast cancer diagnostics by demonstrating distinct gene expression patterns in breast carcinoma, thereby enabling classification into different molecular intrinsic (cDNA-based) subtypes. The authors identified ER, PR, HER2, and Ki67, as four key genes that could be assessed using IHC in clinical practice, thus, introducing the molecular (protein-based) surrogate subtypes: luminal A, luminal B HER2-, luminal B HER2+, non-luminal HER2+, and triple-negative breast cancer (TNBC). The subtype is the basis for MDT recommendations for patient treatment. Although gene expression assays are more frequently used, IHC-based surrogate subtyping with the breast cancer biomarkers and tumor grade currently remains the routine method of choice.

The luminal A and B subtypes apply to approximately 65%–70% of all breast cancers, HER2+ subtypes accounts for 15%–20%, and the TNBC subtype accounts for approximately 10–20% (37, 38, 39). Different therapies are recommended according to subtype (15, 40, 41). Endocrine therapy is recommended for patients with luminal A and B tumors, which strongly express HR. Moreover, luminal B HER2- tumors, which exhibit higher expression of proliferation-associated genes, benefit more from chemotherapy in both the neoadjuvant and adjuvant settings. Anti-HER2 agents are recommended for HER2+ tumors in both the neoadjuvant and adjuvant settings. Due to the lack of biomarkers, systemic therapy for patients with TNBC is limited to chemotherapy. **(Figure 3)**.

Although breast cancer generally has very good survival outcomes, the outcomes differ among subtypes: the luminal (HR+) subtypes have the best survival rates, and TNBC has the poorest survival rates (37, 42). Howlander *et al.* (37) have reported 4-year survival rates of 92.5% for patients with the HR+/HER2- subtype, 90.3% for patients with the HR+/HER2+ subtype, 82.7% for patients with the HR-/HER2+ subtype, and 77.0% for patients with the TNBC subtype (**Figure 4**). TNBC also exhibits a higher incidence of distant metastatic recurrence than other breast cancer subtypes and is more prevalent among younger women (43, 44).

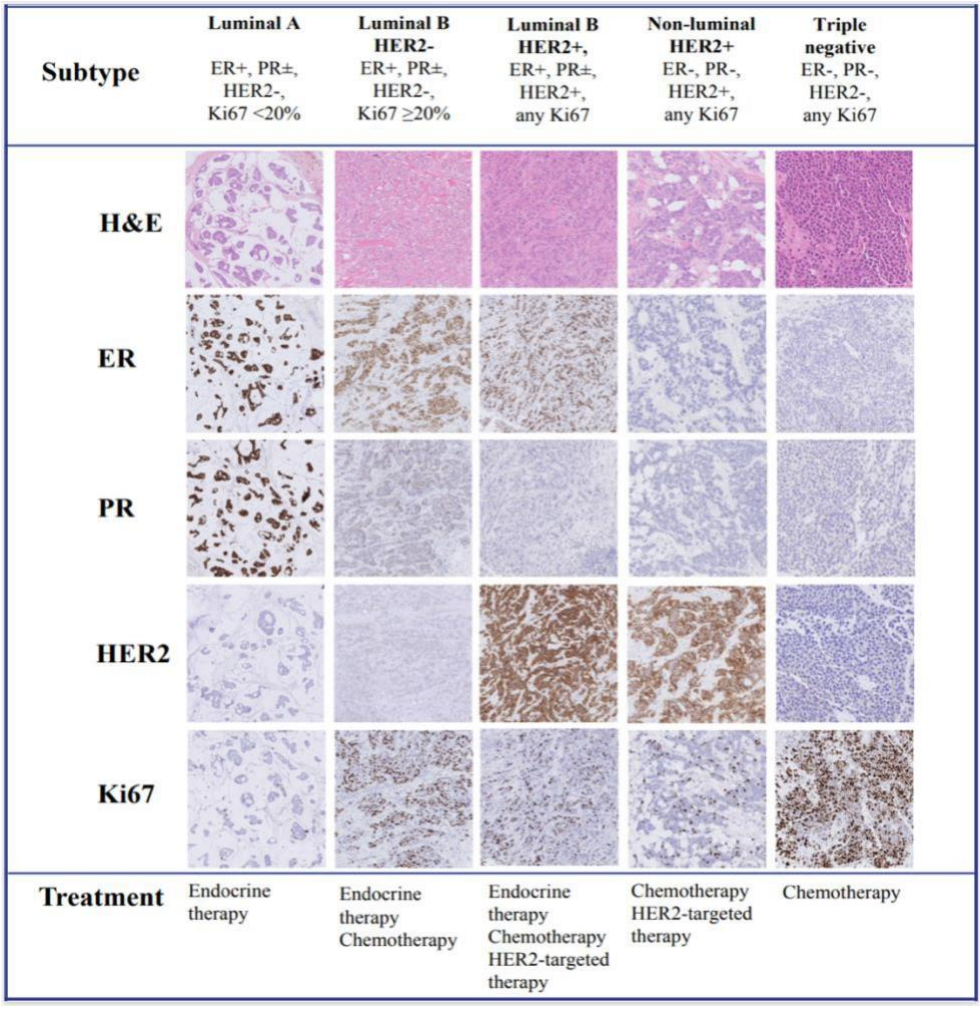

Figure 3. *The molecular surrogate breast cancer subtypes based on breast cancer biomarker expression and treatment options.*

ER= estrogen receptor, PR= progesterone receptor, H&E= hematoxylin/eosin, HER2= human epidermal growth factor 2

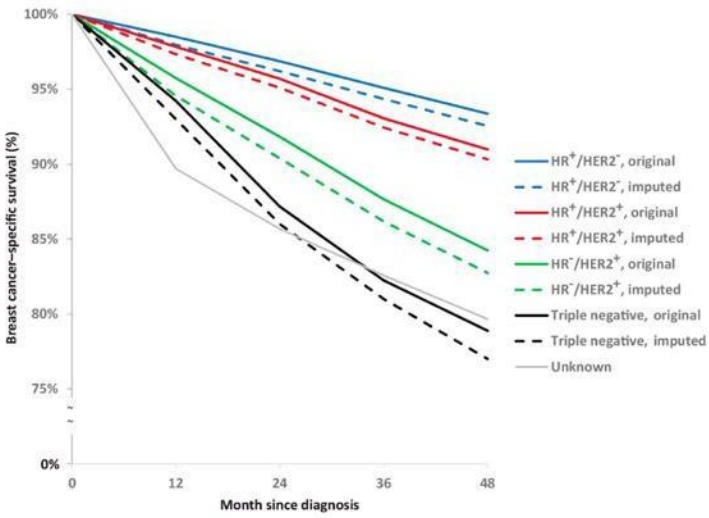

Figure 4. *Four-year breast cancer-specific survival by molecular subtypes in the United States. Source Howlander et al.(37).*

1.2.6.1 Predictive and prognostic factors

Established clinicopathological factors for early breast cancer prognosis and predictive factors/markers include patient age, disease stage, tumor type, margin status, and lymphovascular status (18). Additionally, biological factors such as the expression of ER, PR, HER2, and Ki67 are strongly associated with prognosis and treatment response, as recognized by the World Health Organization and American Joint Committee on Cancer (AJCC) guidelines. Notably, the presence or absence of axillary lymph node metastasis is a strong predictor of breast cancer recurrence. Carter *et al.* (45) have reported a 5-year overall survival of 92% for node negative patients, 81% for patients with one to three positive lymph nodes, and 57% for patients with more than four involved nodes. Tumor size is another independent prognostic factor: the 5-year relative overall survival rates are close to 99% for tumors less than 1 cm, as compared with 89% and 86% for tumors of 1–3 cm and 3–5 cm, respectively (45, 46). Longer follow-up studies have confirmed these findings (47, 48).

1.2.7 Debated issues in breast cancer diagnostics

Guidelines suggest that in patients in whom multiple tumors are discovered in the breast, i.e., those with multifocal breast cancer, only the largest tumor is

considered for assessment and treatment recommendations, unless the other focus/foci differ in morphology and grade (18, 22). However, this recommendation has been a topic of debate. Studies have produced conflicting results: whereas some have indicated differences in survival and treatment outcomes when considering all tumors found in a specimen, others have supported the current guidelines (49, 50, 51, 52). In managing the axilla (either sentinel lymph node biopsy or axillary lymph node dissection), the tissue processing is the same as that for surgical breast specimens with the exception of the immunohistochemical staining step. IHC is not routinely performed on axillary lymph nodes when metastasis is found. Studies have indicated inconsistencies in IHC-assessed breast cancer biomarkers between the primary breast cancer and the LNM, thus indicating a potential need for changing treatment recommendations (53, 54, 55). Further exploration is required in both these areas to ensure that each patient receives the most beneficial treatment recommendations.

1.3 Surgical treatment

Breast cancer treatment with surgery has evolved from pure description of the disease in ancient Egyptian papyrus rolls, to more or less amputative surgical techniques. Surgical oncologists now consider not only the removal of the tumor but also long-time cosmesis. Similarly, axillary lymph node dissection has de-escalated and been replaced with the sentinel lymph node biopsy technique for disease staging.

1.3.1 Breast surgery

Mastectomies have become less common in favor of the breast conserving approach, which is now the standard of care surgery and provides better quality of life for patients (15). The establishment of breast conserving surgery dates to the 1980s, when Umberto Veronesi and Bernhard Fisher conducted randomized trials indicating no difference in overall survival between patients who underwent breast conserving surgery with additional radiotherapy and those who underwent mastectomy (56, 57, 58, 59). Over the past few decades, the breast conserving approach has evolved and incorporated modern oncoplastic techniques influenced by plastic surgery. These techniques enable larger excisions while also improving breast shape and cosmesis (60).

Despite the preference for breast conserving surgery, mastectomy remains recommended in certain situations. For instance, mastectomy is advised if

radiotherapy to the breast is not feasible, the patient has been diagnosed with inflammatory breast cancer, or risk reduction surgery is planned because of hereditary factors. Additionally, mastectomy may be recommended if satisfactory aesthetics cannot be achieved because of the ratio of tumor size to breast size or the presence of tumors in multiple areas of the breast (multicentricity). Finally, patients may choose to undergo mastectomy according to their personal preferences and desires.

1.3.2 Surgery in the axilla

The status of the lymph nodes, which is used for disease staging, is a critical prognostic factor in breast cancer, (45, 61, 62). Knowledge of axillary lymph node status is essential in the decision-making process for adjuvant treatment. In the past, the preferred surgical method for patients with breast cancer was traditional axillary lymph node clearance. This method is associated with pain, impaired movement, numbness, arm swelling (lymphedema), and a resultant decline in quality of life in a considerable percentage of patients (20%–40%) (63, 64). In contrast, the sentinel lymph node technique, which detects the first lymph node(s) draining the breast, is associated with lower morbidity and has become the standard method for staging the axilla in patients who are clinically node-negative (63, 65, 66). The ACOSOG Z0011 trial has indicated that sentinel lymph node biopsies are sufficient, and no additional lymph node clearance is necessary for patients with one or two positive sentinel lymph nodes: short- and long-term follow-up data support this finding (67, 68). Nonetheless, axillary lymph node clearance is recommended for patients with clinically node-positive breast cancer.

Several methods are available for detecting the sentinel node. The combination of a radioactive tracer and a vital blue dye has been the preferred method, but in recent years, use of alternative substances such as superparamagnetic iron oxide combined with a magnetic probe has become increasingly common. At Sahlgrenska University Hospital, this method has been the preferred option for the past several years.

1.4 Systemic therapy and radiotherapy

Information on the expression of biomarkers in breast cancer has been used to identify patients eligible for targeted therapy: patients with expression of ER/PR are recommended endocrine therapy, whereas HER2-targeted therapies (antibody treatment) such as trastuzumab are recommended for patients with

HER2-amplified breast cancers. For patients with high histological grade, and/or elevated Ki67, addition of chemotherapy is recommended, mainly because of relatively poorer prognosis in these patients.

1.4.1 Systemic therapy

The goal of administering systemic therapy such as chemotherapy, endocrine therapy, targeted therapies, immunotherapy, is to eradicate any potential micro metastatic disease. For TNBC and HER2+ breast cancers >2 cm, modern treatment has shifted from adjuvant to neoadjuvant therapy. These cancers frequently demonstrate excellent pathological complete response, which is considered a favorable prognostic factor (69, 70).

1.4.1.1 Chemotherapy

Chemotherapy was first introduced in the 1970s, initially for patients with node-positive breast cancer (71, 72). Currently, modern treatment strategies use chemotherapy either before or after surgery, guided by the subtype and stage of breast cancer. The aim is to prevent metastatic recurrence, even in node-negative patients. Several chemotherapy regimens have been demonstrated to be effective in improving breast cancer outcomes, including cyclophosphamide, methotrexate, and 5-fluoracil (CMF); anthracycline-based regimens; and taxane-based regimens (73, 74, 75). In recent years, neoadjuvant therapy has become a standard of care not only for inoperable or locally advanced cases but also for certain subtypes of smaller, operable tumors, such as TNBC and HER2+ tumors (76). Neoadjuvant treatment has also enabled a more complex breast-conserving approach for patients in whom mastectomies were previously the preferred surgery. Modern gene assays, such as Oncotype Dx, Mammaprint, and Prosigna, further aid in making individually tailored decisions about patients' potential benefit from the addition of chemotherapy in comparison with endocrine treatment alone among patients with ER+/HER2- breast cancer. These assays are primarily used to determine if certain patient groups will experience improved survival if chemotherapy is administered.

1.4.1.2 Endocrine therapy

The correlation of hormones with breast cancer development and growth has been known since the late 1800s. In 1882, Thomas William Nunn reported the case of a woman with breast cancer whose disease regressed 6 months after she entered menopause. Later, in the early 1900s, George Beatson discovered that removing the ovaries of premenopausal women with advanced breast cancer significantly decreased tumor size and improved prognosis. Although

oophorectomy did not benefit all patients with breast cancer, it remained the standard of care for a considerable time.

In the 1960s, the discovery of ERs on breast cancer cells and the development of tamoxifen marked a major milestone in antihormonal treatment for women with ER+ breast cancer. The goal of antihormonal treatment is to suppress the growth-stimulating effect of estrogen on breast cancer cells (77, 78). Tamoxifen was initially used to treat metastatic breast cancer, but randomized controlled trials also showed beneficial survival effects in the adjuvant setting (73, 79, 80). Tamoxifen is classified as a selective estrogen receptor modulator, acting as both an ER agonist and antagonist depending on the target tissue. It has anti-estrogenic effects on breast tissue, and also has pro-estrogenic effects on bone and the uterus.

In premenopausal women, the primary source of estrogen is the ovaries, whereas in postmenopausal women, estrogen production is limited to peripheral tissue such as fat tissue, muscle tissue, and adrenal glands. Through a process called aromatization, androgens (such as testosterone) in the body are converted into estrogen by the enzyme aromatase. Aromatase inhibitor drugs decrease the production of estrogen in postmenopausal women by inhibiting the activity of the aromatase enzyme. Aromatase inhibitors, compared with tamoxifen, have been found to achieve slightly superior survival outcomes in postmenopausal women with ER+ breast cancer and are now considered the preferred treatment option for these women (41, 81, 82). The usual duration of endocrine therapy is 5 years, but extended therapy for as many as to 10 years is recommended for patients at high risk (83).

Several combinations of ovarian function suppression (OFS) and endocrine therapy (tamoxifen and aromatase inhibitors) have been studied for HR+ breast cancers in premenopausal patients. The primary purpose of OFS is to inhibit ovarian function and decrease estrogen levels in the body. A recent meta-analysis from four randomized trials conducted by the Early Breast Cancer Trialists' Collaborative Group has compared aromatase inhibitors (anastrozole, exemestane, or letrozole) to tamoxifen for 3 or 5 years in premenopausal women with ER+ breast cancer receiving ovarian suppression (goserelin or triptorelin) or ablation (surgical removal of ovaries) (84). This meta-analysis indicated that treatment with an aromatase inhibitor instead of tamoxifen in patients receiving OFS, decreases the risk of breast cancer recurrence. However, further follow-up is needed to assess the effects on breast cancer mortality.

1.4.1.3 Targeted therapies and immunotherapy

If the first major advancement was the introduction of tamoxifen, the second was the discovery of the human epidermal growth factor gene, which is overexpressed in the HER2+ subset of breast cancers. In normal cells, the HER2 pathway promotes cell growth, but overexpressed HER2 in neoplastic cells results in rapid cell growth and proliferation. Consequently, the monoclonal antibody trastuzumab, which blocks the signaling pathway for growth and proliferation, was developed (85). Trastuzumab was initially used in combination with chemotherapy as a first-line treatment for HER2+ metastatic breast cancer. In 2001, Slamon *et al*. (86) demonstrated significant survival benefits for patients with early-stage HER2+ breast cancer treated with a combination of trastuzumab and chemotherapy. Several studies have investigated survival benefits in the adjuvant setting, with different treatment durations, and sequential versus concurrent administration with chemotherapy. The standard of care treatment is one year of trastuzumab concurrently with paclitaxel (87, 88, 89). In the neoadjuvant setting, patients with HER2+ breast cancer are recommended to receive double HER2 targeting antibodies with trastuzumab and pertuzumab, another monoclonal antibody blocking this pathway (90, 91, 92). The discovery of the HER2 gene enabled HR+ tumors to be divided into luminal HER2+ and luminal HER2- subtypes and the HR- tumors into non-luminal HER2+ and TNBC subtypes.

A new entity of breast cancer has been identified called HER2-low, which has lower expression of HER2 than observed in HER2-amplified tumors but higher expression than observed in HER2 negative (HercepTest score 0) tumors. Patients with HER2-low tumors might respond to anti-HER2-antibody-drug conjugates (anti-HER2-ADC)—a new treatment strategy combining HER2 directed antibody with a cytotoxic agent, in anti HER2 therapy (93, 94). Agents targeting the PIK3CA/mTOR pathway, an intracellular signaling pathway with an important role in regulating the cell cycle, have been developed to enhance the effects of endocrine therapy in ER+ breast cancer. CDK4/6 inhibitors, which are central players in cell-cycle regulation, have been reported to reverse endocrine resistance in advanced ER+ breast cancer (95, 96). PARP inhibitors have demonstrated efficacy in treating metastatic breast cancer in patients with BRCA1/2 mutations (97, 98) by inhibiting the activity of PARP, which normally facilitates the repair of DNA breaks in damaged cells, thus ultimately resulting in cancer cell death.

Promising results have also been demonstrated for monoclonal antibodies targeting immune checkpoint proteins such as PD-1/PDL-1, which have a key function as a “brake” in the immune system. Inhibiting these proteins enables

the immune system itself to kill cancer cells more effectively and has shown promising results in TNBC (99).

1.4.2 Radiotherapy

Radiotherapy to the breast is administered to patients who have undergone breast conserving surgery to prevent local recurrence (100, 101). Patients with tumors larger than 5 cm are recommended to undergo local radiotherapy to the chest wall, whereas those with a high axillary tumor burden may require regional lymph node irradiation. Use of oncoplastic techniques in breast conserving surgery, involving volume replacement and displacement approaches, presents a challenge in radiation planning, and requires close collaboration between the radiation oncologist and the surgeon.

1.5 The crucial collaboration

As a result of continued progress in research and clinical developments across various fields associated with breast cancer, the management of patients with breast cancer has become increasingly complex. In the current era of personalized patient care, effective collaboration among radiologists, surgical oncologists, medical oncologists, radiation oncologists, and pathologists has become essential. Their collective expertise and services, together with those of clinical geneticists, breast nurses, counselors, physiotherapists, and other healthcare professionals, are crucial elements in caring for and supporting patients with breast cancer.

1.6 Areas of focus in this thesis

This thesis sheds light on several areas of controversy in breast cancer. First, it compares a new diagnostic method to IHC for assessing ER, PR, HER2, and Ki67 expression, and translating the findings into molecular surrogate subtypes. Second, it investigates whether additional IHC assessment is necessary in multifocal breast cancer and breast cancer with synchronous LNM. Finally, it examines survival outcomes after adjuvant chemotherapy in older ($\geq$70) patients with TNBC, as older patients are rarely included in randomized controlled clinical trials.

2 AIMS

The primary aim of this thesis was to examine various clinical aspects of IHC in the context of breast cancer diagnostics. To achieve this aim, the specific objectives were as follows:

Paper I

To (1) determine the concordance of breast cancer subtypes assessed by IHC versus real-time quantitative polymerase chain reaction (RT-qPCR), and (2) evaluate the effects of the two methods on adjuvant treatment recommendations.

Paper II

To (1) determine the concordance of breast cancer subtypes between different foci in patients with multifocal breast cancer, and (2) investigate the clinical implications of discordance in subtype on adjuvant treatment recommendations.

Paper III

To (1) determine the concordance of breast cancer subtypes between the primary breast cancer and synchronous LNM, and (2) investigate the effects of discordance in subtype on adjuvant treatment recommendations.

Paper IV

To examine the effects of adjuvant chemotherapy on survival outcomes for patients 70 years of age or older undergoing surgical treatment for TNBC.

3 METHODS

3.1 Patient population

Because several aspects of the clinical significance of IHC in breast cancer diagnostics were investigated, a single uniform patient cohort could not be used. Paper I was a validation study comprising 100 CNBs and matching surgical specimens from 98 patients who had undergone primary surgery for breast cancer at Sahlgrenska University Hospital (Gothenburg, Sweden) between January and May of 2017. Papers II and III were both observational retrospective cohort studies. Paper II included 180 patients with ipsilateral multifocal breast cancer who were treated at Sahlgrenska University Hospital between 2012 and 2017. The inclusion criteria required patients to have at least two invasive tumors evaluated with IHC. In Paper III, 98 consecutive patients with unifocal primary breast cancer and synchronous LNM who had undergone surgical treatment at Sahlgrenska University Hospital in 2018 were included. IHC was performed on the LNM. Paper IV was a population-based registry study, in which data from various Swedish registries were used to retrieve information on all women 70 years of age or older who had undergone surgical treatment for TNBC in Sweden between 2009 and 2016.

3.2 Local hospital records and national registries

Patient data for Papers II and III were obtained from both the Swedish National Breast Cancer Register and local hospital records from Sahlgrenska University Hospital (in the Sympathy and Melior systems). Local pathology reports and follow-up information including the date of recurrence, assessment of recurrence/metastasis, and date of death for Paper III were accessed through Sympathy, which is linked to the Swedish population register. Patient characteristics such as age and co-morbidities, as well as surgical and medical treatments were accessed through Melior. In Paper I, patients were identified through Sympathy, and patient characteristics and treatment were accessed through Melior. All FFPE samples used herein were obtained from the Department of Clinical Pathology at Sahlgrenska University Hospital.

For Paper IV, data were collected from the Swedish National Breast Cancer Register, the Swedish Patient Register, and the Swedish Cause of Death Register. These registries have almost complete coverage of the Swedish population and are both mandatory and validated (102, 103). The personal

identification number assigned to each Swedish resident allows for matching of data across registries. Comorbidities were retrieved from the Swedish Patient Register and converted into a Charlson-Deyo comorbidity index, which was then used as a proxy for patient fitness.

3.3 Sample handling and immunohistochemistry

After surgical removal, fresh breast specimens are transported to the pathology department, whereas the lymph node samples from the axilla and CNBs are fixed in formalin at the time of removal during surgery or examination at the radiology department. In Paper III, additional IHC staining and *in situ* hybridization were performed on archived FFPE LNM samples with the antibodies presented in **Table 2**. The work was performed at the Department of Clinical Pathology, Sahlgrenska University Hospital.

Table 2. *Antibodies used for immunohistochemistry*.

Biomarker	Host for antibody production	Manufacturer	Clone	Dilution	Antigen retrieval
ER	rabbit	DAKO IR084	EP1	Ready to use	TRS high, pH9.0
PR	mouse	DAKO IR068	636	Ready to use	TRS high, pH9.0
Ki-67	mouse	DAKO IR626	MIB-1	Ready to use	TRS low, pH6.0
HercepTest[1]	rabbit	DAKO SK001	poly	Ready to use	TRS low, pH6.0

ER= estrogen receptor, PR= progesterone receptor, TRS= target retrieval solution; high refers to high pH and low refers to low pH of the solution

[1] Samples with HercepTest scores of 2+ or 3+ were additionally assessed with Ventana dual SISH test (silver *in situ* hybridization) for possible HER2 amplification.

3.4 RT-qPCR breast cancer assays

The Xpert® Breast Cancer STRAT4 Assay (STRAT4) described in Paper I uses a cartridge-based RT-qPCR method. This assay has specific qualitative cut-off values for mRNA expression of *ESR1* (ER), *PGR* (PR), *ERBB2* (HER2), and *MKi67* (Ki67), which are normalized to values for a reference gene (*CYFIP1*). The study analyzed FFPE tissues from CNBs and

corresponding surgical specimens. The total time required for the STRAT4 assay, including hands-on time was less than 2 hours. The paper provides detailed protocols for mRNA extraction from FFPE slides of CNBs and breast cancer specimens for assaying the four breast cancer biomarkers. The laboratory work was conducted at the Department of Clinical Pathology at Sahlgrenska University Hospital. The manufacturer Cepheid contributed materials and the cartridge-based STRAT4 assay used in the study.

3.5 Surrogate subtyping

In Papers II–III, receptor-based molecular surrogate subtyping was conducted with ER, PR, HER2, and Ki67, in compliance with relevant national guidelines in the respective years (**Table 3**) (104). The cut-off values for Paper I differed from the national guidelines for ER and PR but were consistent for HER2 and Ki67. In Paper I, the cut-off for the HRs was in line with the St. Gallen guidelines, in which immunopositivity is considered positive if ≥1% staining is observed in neoplastic cells (40).

Table 3. *Cut-off values for 2012-2018 according to the Swedish Quality and Standardizing Committee for breast pathology (KVAST-group). % indicates percentage of immunostaining in neoplastic cells.*

Year	2012	2013	2014	2015	2016	2017	2018
Estrogen receptor							
positive	≥10%	≥10%	≥10%	≥10%	≥10%	≥10%	≥10%
Progesterone receptor							
positive	≥10%	≥10%	≥10%	≥10%	≥10%	≥10%	≥10%
HER2	†	†	#	#	#	#	#
positive	*	*	*	*	*	*	*
Ki67							
high	≥14%	≥30%	≥30%	≥30%	≥30%	≥20%	≥20%

† Membrane staining on HercepTest needs to be expressed in at least 30% of neoplastic cells
# Membrane staining on HercepTest needs to be expressed in at least 10% of neoplastic cells
⋆ HER2 was considered positive if 1) the HercepTest on immunohistochemistry was 3+ or 2) the HercepTest was 2+ with ratio ≥2 (number of HER2 copies/copies of chromosome 17). If the ratio was <2, the average of HER2 copies needed to be ≥4 for HER2 positivity.

3.6 Multidisciplinary team meeting

This thesis included a clinical component aimed at theoretical reevaluation of the possibility of treatment changes or additional treatment for biomarker/subtyping discordances identified in Papers I and III. Because of the retrospective nature of these studies, the number of MDT members involved was limited. No reassessments involved the radiology team. In Paper I, only the surgical oncologist and the medical oncologist evaluated the results with respect to the recommended treatments. In Papers II–III, the team responsible for reassessing the treatments included a surgical oncologist, a medical oncologist, and a board-certified pathologist.

For Papers I and III, patient characteristics such as age and co-morbidities were available, but the MDT participants were blinded to patient treatments (both recommended and received) as well as tumor biomarker status and subtypes at the time of treatment. After the new results were obtained, treatment recommendations were made, and the MDT was unblinded to previous subtypes and recommendations, thereby enabling comparison of treatment recommendations. Patient and tumor characteristics were available for the MDT participants in paper II.

3.7 Statistics

The statistical tests used in this thesis were two-sided with a significance level of $p < 0.05$. Descriptive statistics for continuous variables are presented as medians and quartiles, whereas categorical variables are presented as frequencies and percentages.

In Papers I–III, the concordance between categorical variables was explored through two-way crosstabulation with the chi-square test. Comparisons of numerical variables were evaluated with non-parametric tests, such as Wilcoxon signed rank-test for related measurements, whereas comparisons between independent groups were performed with the Mann-Whitney U test. To estimate the level of agreement between categories, the Cohen kappa statistic with a 95% two-sided confidence interval (CI) was used.

Kaplan-Meier survival analyses were conducted to estimate overall survival (OS) in Papers II–IV, disease-free survival in Papers II–III, and breast cancer specific survival (BCSS) in Paper IV. Cox regression analysis was used to determine survival outcomes for chemotherapy treatment versus no

chemotherapy treatment. Hazard ratios with 95% CIs were estimated from the Cox regression analyses in Paper IV. Additionally, a propensity score-matched model was created with the 1:1 nearest neighbor method without replacement to match patients who received chemotherapy with those who did not. Absolute values greater than 0.2 were considered unbalanced in the analysis in Paper IV.

4 RESULTS AND DISCUSSION

4.1 Paper I

Comparison of two methods for assessing breast cancer biomarkers and surrogate subtyping for clinical treatment decisions

Although multi-gene assays are currently available, "protein-based" IHC of the breast cancer biomarkers (ER, PR, HER2, and Ki67) remains the standard used by MDTs to make patient treatment decisions. Newer, more rapid methods for assessing these breast cancer biomarkers have been developed, wherein mRNA is extracted from the tumor area and analyzed. In this study the cartridge-based STRAT4 assay was used and compared with routine IHC staining. The STRAT4 system uses pre-specified cut-offs to determine positivity or negativity for different biomarkers.

In the current dataset, 100 matching CNBs and surgical specimens were assessed with STRAT4 and compared with IHC. We found good agreement for both sample types when each biomarker was investigated separately. Cohen´s kappa values were used for comparisons between methods, and ER was found to be the most consistent, with a kappa value of 0.87 (95% CI 0.73–1.00) for CNBs and 0.82 (95% CI 0.65–0.99) for surgical specimens. In contrast, Ki67 was least consistent between methods, with a kappa value of 0.54 (95% CI 0.39–0.69) for CNBs and 0.56 (95% CI 0.40–0.72) for surgical specimens. In the investigation of the concordance for two or more biomarkers in surgical specimens, the overall percentage agreement was best for ER and HER2 combined at 87% (95% CI 78.8–93.0), and worst for the four biomarkers (ER, PR, HER2 and Ki67) combined with an overall percentage agreement of 66% (95% CI 55.8–75.2) (**Table 4**).

During the MDT, translation of biomarkers into surrogate subtypes was performed with the STRAT4 results to compare the hypothetical treatment (which would have been recommended if the STRAT4 results had been used instead of IHC) and the treatment actually administered to the patients according to IHC-based surrogate subtyping. In total, 74 (74%) specimens had concordant breast cancer subtypes between methods. Discordant subtyping was found in 26 patients because of changes in the status of ER (n=5), HER2 (n=9), and Ki67 (n=12). If the STRAT4 results had been used to select treatment instead of the IHC results, different treatment decisions would have been made in 18 patients: 16 patients would have received more, and 2 patients would have received less treatment (**Figure 5**).

Table 4. *Concordance between STRAT4 and IHC for two or more biomarkers in the surgical specimen. Table adapted from Janeva et al.(34).*

	OPA% (95% CI)
2-ways[1] STRAT4 vs 2-ways[1] IHC (ER and HER2)	87 (78.8–93.0)
3-ways[1] STRAT4 vs 3-ways[1] IHC (ER, HER2 and Ki67)	70 (60.0–78.8)
4-ways[1] STRAT4 vs 4-ways[1] IHC (ER, PR, HER2 and Ki67)	66 (55.8–75.2)

OPA= overall percentage agreement. [1]Agreement with respect to 2-, 3-, and 4-ways analysis to the bracketed biomarkers

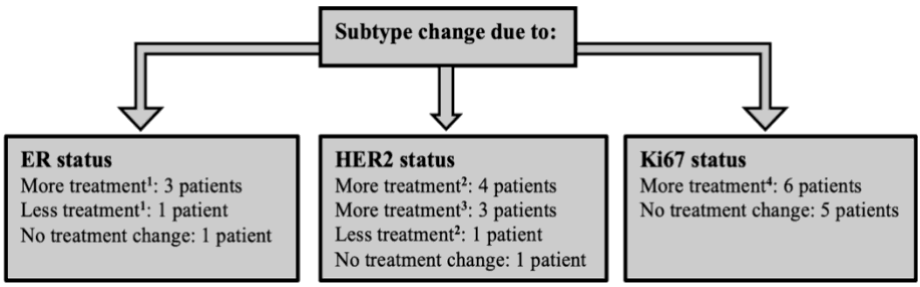

Figure 5. *Changed treatment recommendations due to STRAT4 in patients with discordant surrogate subtyping compared to IHC. Figure adapted from Janeva et al.(34).*

[1] Treatment refers to endocrine therapy, [2] Treatment refers to chemotherapy and trastuzumab, [3]Treatment refers to trastuzumab, [4] Treatment refers to chemotherapy.

The relatively good concordance between STRAT4 and IHC for the individual biomarkers (ER, PR, HER2, and Ki67) was in line with previous findings (31, 105). However, in practice, treatment recommendations in clinical settings do not consider the biomarkers separately; instead, a four-marker panel is translated into a surrogate subtype to guide decision-making. In this study, the results for such translation were less favorable and would theoretically have led to patients being recommended more adjuvant treatment (primarily with chemotherapy and trastuzumab). With a correct diagnosis, these treatments would provide an overall survival benefit, but they also would need to be

administered with careful consideration to avoid unnecessary suffering and potentially irreversible adverse effects without additional survival benefits.

When a pathologist examines IHC slides, the focus is on neoplastic cells. In the STRAT4 protocol, a single FFPE section adjacent to the section used for the H&E staining was assessed. This section can include both normal breast cells and ductal cancer *in situ*, a non-invasive form of breast cancer. Normal breast cells express ER/PR, thus potentially resulting in the misclassification of ER- tumors as ER+. Ductal cancer *in situ* can also include components expressing HER2, thus potentially resulting in altered HER2 results when tumors are assessed with STRAT4. Although macrodissection (i.e., use of whole FFPE sections) has been suggested to be sufficient (106), recommending microdissection, wherein the pathologist marks the invasive area for the histotechnician to dissect, might yield potential improvements.

We cannot state that the results of our comparison between the "protein"-based IHC assessment and mRNA based STRAT4-assessment favor IHC assessment. Therefore, further large trials (either prospective or retrospective analysis of completed prospective trials) are needed, wherein large numbers of tumors are assessed with additional multi-gene tests providing data on the intrinsic nature of the tumor.

4.2 Papers II–III

Is further specimen assessment needed for more accurate treatment decisions?

Several aspects of IHC assessment of primary breast cancers have been debated. According to both international (18, 22) and national guidelines (29), IHC assessment of ER, PR, HER2, and Ki67 is mandatory only for invasive breast carcinoma. For patients with multiple tumors detected in breast specimens, IHC is assessed on only the largest focus unless other foci differ in morphology and grade. Whether assessing only the largest tumor is sufficient remains unclear. For patients with synchronous LNMs, a question is whether IHC should also be performed on the LNM. In Papers II and III, these questions were addressed, on the basis of the hypothesis that current guidelines might perhaps be insufficient, and that patients with multifocal breast cancer and patients with synchronous LNMs might possibly be undertreated.

Paper II investigated multifocal breast cancer specimens with two or more foci that had previously been assessed with IHC (**Figure 6**). A total of 347 specimens (from 342 patients) were multifocal, and 183 specimens (180 patients) were included in the study.

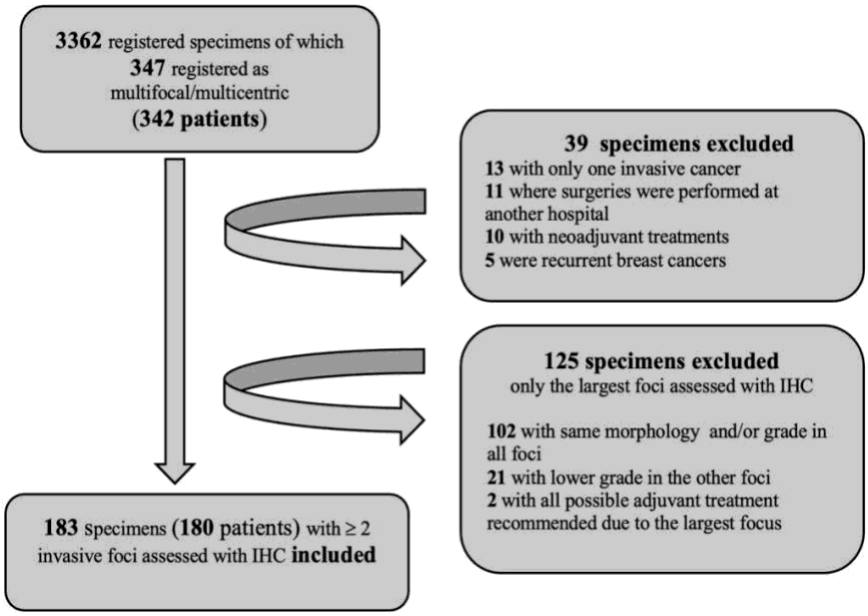

Figure 6. *Flow-chart of patients surgically treated for multifocal breast cancer at Sahlgrenska University Hospital (Gothenburg, Sweden) 2012-2017. Figure adapted from Janeva et al.(107).*

Concordance was investigated between two foci: primary tumor 1 (PT1), which was the largest focus, and primary tumor 2 (PT2), which was the second largest focus in most patients. The molecular surrogate subtypes were concordant for 135/183 (73.8%) specimens and discordant for 48/183 (26.2%) specimens. The luminal A group had the highest concordance (PT1 luminal A/PT2 luminal A), with 102 of 135 (75.6.8%) concordant tumors, whereas the luminal B HER2- group (PT1 luminal B HER2-/PT2 luminal A) was the most discordant, with 21 of 48 (43.8.%) discordant samples (**Table 5**).

A new MDT was convened to reevaluate the previous patient treatment recommendations for the 48 discordant specimens (from 48 patients). Of the 48 patients, 18 (37.5%) had a more aggressive subtype in PT2 than PT1. PT1 luminal A/PT2 luminal B HER2- was the most common discordance and was observed in 9 of 48 (18.6%) patients, whereas 30 (62.5%) had a less aggressive

subtype in PT2 compared with PT1. Molecular surrogate subtype changes between PT1 and PT2 that had the potential to result in changes in treatment recommendations were found in 20 patients (41.7% of the discordant group and 11.1% of the study cohort), but only 11 (22.9% of the discordant group and 6.1% of the study cohort) were recommended to undergo therapy changes because of the subtype of PT2: two patients received endocrine therapy because of their ER status, six patients received combined trastuzumab and chemotherapy because of their HER2 status, and three patients received additional chemotherapy because of Ki67 status or a change in tumor grade (**Table 5**).

Table 5. *Concordance and discordance in molecular surrogate subtypes in multifocal breast cancer. Table adapted from Janeva et al. (107).*

		PT2						
		Luminal A	**Luminal B HER2-**	**Luminal B HER2+**	**Non-luminal HER2+**	**TNBC**	Total[d]	
PT1	**Luminal A**	102	9 (2)[a,c]	5 (4)[a,c]	0	1 (1)[a,c]	15 (7) 102	117
	Luminal B HER2-	21[b]	17	2 (2)[a,c]	0	0	23 (2) 17	40
	Luminal B HER2+	4[b]	2[b]	7	1[a]	0	7 7	14
	Non-luminal HER2+	1[b,c]	0	0	3	0	1 3	4
	TNBC	1 (1)[b,c]	1 (1)[b,c]	0	0	6	2 (2) 6	8
	Total[d]	27 (1) 102	12 (3) 17	7 (6) 7	1 3	1 (1) 6	48 (11) 135	183

Data are presented as the number of patients with concordant subtypes (green boxes) and discordant subtypes (nude boxes). Within parentheses are the number of patients where therapy was added due to discordance in PT2.

[a] more aggressive molecular surrogate subtype changes in PT2 compared to PT1.

[b] less aggressive molecular surrogate subtype changes in PT2 compared to PT1.

[c] patients with potential therapy changes.

[d] total number of patients (white boxes) divided into nude boxes for discordant subtypes and green boxes for concordant subtypes.

Further analysis of the study cohort indicated that 103 specimens (from 103 patients) among the 183 included specimens (from 180 patients) shared morphology and grade between the assessed foci. This analysis demonstrated that 15 of the 103 patients (14.6%) had discordant molecular subtyping despite the two foci sharing the same morphology and grade, and 4 of the 15 patients received different/additional treatment (**Table 6**).

Table 6. *Subgroup analysis of 103 patients within the study cohort with matching histologic type and grade (NHG) between the largest primary tumor (PT1) and second primary tumor (PT2). Table adapted from Janeva et al. (107).*

Histology/Grade shared between foci	Number of patients	Number of patients with discordant molecular subtyping PT1/PT2	Number of patients with additional treatment
Ductal/Grade2	52	12	4
Ductal/Grade 3	18	1	0
Lobular/Grade 2	11	0	0
Ductal/Grade 1	9	0	0
Tubular/Grade 1	8	0	0
Tubulolobular/Grade 1	3	1	0
Mixed/ Grade 2	2	1	0

Decision-making for targeted treatments is based not only on the biomarker characteristics of the primary breast tumor but also on the lymph node status. In Paper III, the prevalence and possible treatment consequences of biomarker and surrogate subtype discordance between the primary breast cancer and synchronous LNM were investigated. A total of 94 patients who underwent primary surgery for unilateral invasive breast cancer at Sahlgrenska University Hospital in 2018, with synchronous LNM (pre- or postoperatively verified), were included. Their LNMs were assessed with IHC and classified according to the molecular surrogate subtypes (**Table 3**).

The concordance rates for ER, PR, HER2, and Ki67 between the breast cancer and LNM were highest for ER (98.9%) and lowest for Ki67 (72.3%). Ki67 changed from high/low (breast cancer/LNM) in 22 patients (23.4%) and from low/high (breast cancer/LNM)in four (4.3%) patients. No changes were observed from ER- or HER2- to ER+ or HER2+ in the breast cancer/LNM (**Table 7**).

***Table 7**. Discordance between the breast cancer and lymph node metastasis considering immunohistochemical expression of the breast cancer biomarkers.*

	ER	PR	HER2	Ki67
n (%)	1 (1.2)	10 (10.6)	4 (4.3)	26 (27.7)
BC+ / LNM -	1 (1.2)	7 (7.4)	4 (4.3)	22 (23.4)
BC- / LNM+	0 (0.0)	3 (3.2)	0 (0.0)	4 (4.3)

n (%) refers to number (%) of the total cohort of 94 patients, BC= breast cancer, LNM= lymph node metastases, ER= estrogen receptor, PR= progesterone receptor, HER2= human epidermal growth factor 2

ER and PR are considered positive with ≥ 10% immunostaining in neoplastic cells.
Ki67 is considered high (+) with ≥ 20% immunostaining in neoplastic cells.
HER2 is considered positive with HercepTest scored 3+ or confirmed HER2 amplification using SISH (silver *in situ* hybridization) testing.

Investigation of surrogate subtype concordance indicated that 67 (71.3%) of the breast cancer/LNM pairs were concordant, and 27 (28.7%) were discordant. The change was to a more favorable surrogate subtype in 22 of the 27 discordant pairs: 18 changed from luminal B HER2- to luminal A, three changed from luminal B HER2+ to luminal A, and one changed from luminal B HER2+ to luminal B HER2-. No change was detected in which ER- or HER2- in the breast cancer changed to ER+ or HER2+ in the LNM (**Figure 7**). The subsequently convened MDT did not recommend any treatment changes after considering surrogate subtyping for the LNM.

Current international and national guidelines do not support assessing IHC on all detected foci (unless differences in morphology and grade exist among different foci) or synchronous LNM (22, 29). For multifocal breast cancer, studies have shown mixed results regarding the necessity for further assessment of all detected foci with IHC (51, 52, 108, 109, 110). It was shown in Paper II that a total of 11 (6.1%) patients were recommended to undergo additional treatment because of the additional IHC assessment performed on foci other than the largest focus, most of whom received combined trastuzumab and chemotherapy because of HER2+ tumors. Notably, 4 of the 11 patients had primary breast cancers that shared morphology and grade; if the guidelines were followed, IHC assessment would not normally have been performed.

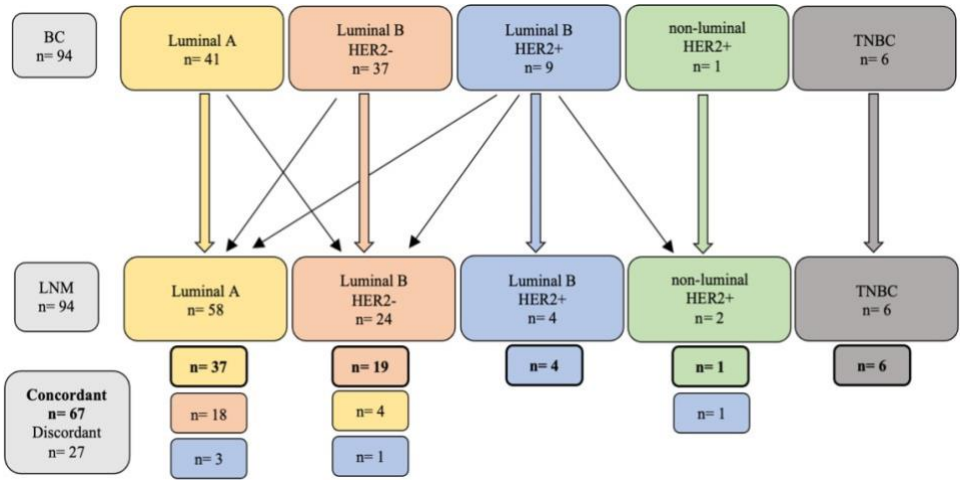

Figure 7*. Changes in subtypes between the breast cancer and the lymph node metastasis.*

BC= breast cancer, LNM= lymph node metastasis
Bold numbers represent the concordant surrogate matches in the BC/LNM pairs.

A weakness in Paper II is that not all multifocal breast cancers with shared morphology and grade were assessed with IHC. Of the 103 patients included in the study who shared morphological features between foci, a comparable number (102 patients) with similar features were excluded because only one focus was assessed with IHC (**Figure 6**). This finding suggests that only approximately 50% of all tumors with shared features were evaluated, thus resulting in selection bias. Nevertheless, discussions within the Swedish Quality and Standardizing Committee for breast pathology (KVAST group) are ongoing regarding the incorporation of routine IHC assessment on all tumors found within breast specimens. We acknowledge that the changes in adjuvant treatment recommendations for the 6.1% of the patients with multifocal breast cancers might have been due to selection bias. Therefore, larger studies are needed to verify these results. Those studies should assess all detected foci regardless of shared histology or grade.

For synchronous LNM, studies have shown discrepancies and discordance rates in breast cancer/LNM pairs ranging from 3% to 46% for both breast cancer biomarkers and surrogate subtypes (111, 112, 113, 114). In Paper III, the results for the individual biomarkers with discrepancies for ER (1.1%), PR

(10.6%), HER2 (4.2%), and Ki67 (27.7%) are in line with those from studies with lower ranges of discrepancy (54, 115). With surrogate subtyping, we observed a discordance in 28.7% of the breast cancer/LNM pairs. The discordance resulted predominantly in more favorable subtyping in the LNM than the breast cancer samples. No changes from breast cancer ER- to LNM ER+ or breast cancer HER- to LNM HER2+ were detected. The most common change was from breast cancer luminal B HER2- to LNM luminal A. The results support the findings of Bonin *et al.* (114) and Mandó *et al.* (116), but contrast with those from several reports indicating more aggressive subtype changes in the LNM (117, 118, 119). Nevertheless, no relevant treatment additions (e.g., endocrine therapy or HER2 targeted therapy) were recommended because of subtype changes in breast cancer/LNM pairs in the current study.

In Paper III, most mismatches between breast cancer/LNM suggested a less aggressive nature, thus casting some doubt over the presumption that the most aggressive breast cancer cell clones find their way to lymph nodes. The recommendations for chemotherapy were largely based on the presence of LNMs, thus making interpretation of cases with less aggressive subtyping in the LNM difficult. The current findings cautiously support that assessing synchronous LNMs with IHC provides no additional clinical value. Further studies must be conducted to determine the clinical value of performing IHC staining on synchronous LNMs and the resultant effects on adjuvant treatment decision-making.

4.3 Paper IV

Investigating the benefit of chemotherapy treatment in older women with the breast cancer surrogate subtype that lacks biomarker expression: are we doing enough?

TNBC lacks ER, PR, and HER2 expression. It accounts for 10%–20% of all invasive breast cancers worldwide and affects primarily younger patients (44). Moreover, this aggressive subtype is associated with poor survival. Because of the lack of biomarker expression, adjuvant medical treatment is limited to chemotherapy. Randomized clinical trials investigating the use of adjuvant chemotherapy have rarely included patients older than 70 years. The aim of Paper IV was to investigate the effects of chemotherapy on survival outcomes in older women with early TNBC.

Of 4,818 patients who underwent surgical treatment for TNBC during 2009–2016, 1,418 were 70 years of age or older, and 1,130 met the inclusion criteria. In total, 368 (32.6%) patients received adjuvant chemotherapy, whereas 717 (63.5%) did not receive adjuvant chemotherapy. Additionally, 45 (4.0%) patients received neoadjuvant treatment. An approximate 10-year difference in median age was found between groups of patients receiving versus not receiving chemotherapy. Most patients had a Charlson-Deyo index of 0, thus indicating that this group was very fit despite higher age (**Table 8**).

Table 8. *Patient characteristics for women ≥ 70 years diagnosed with TNBC. Table adapted from Janeva et al. (120).*

	Adjuvant chemotherapy	No chemotherapy	Neoadjuvant chemotherapy
Total number of patients	368	717	45
Age, years (median, [IQR])	73 (71–76)	82 (78–8)	74 (71–76)
Charlson/Deyo index (%)			
0	255 (69.3)	366 (51.0)	34 (75.6)
1	75 (20.4)	163 (22.7)	4 (8.9)
2	20 (5.4)	86 (12.0)	5 (11.1)
3	9 (2.4)	59 (8·.)	1 (2.2)
4	7 (1.9)	20 (2.8)	0 (0.0)
5	2 (0.5)	11 (1.5)	1 (2.2)
6	0 (0.0)	9 (1.3)	0 (0.0)
7	0 (0.0)	2 (0.3)	0 (0.0)
8	0 (0.0)	0 (0.0)	0 (0.0)
9	0 (0.0)	1 (0.1)	0 (0.0)
Follow-up, years (median, [IQR])	4.2 (3.0–5.9)	3.4 (1.9–5.5)	2.8 (1.7–3.9)

Data are presented as number of patients (%).
IQR= interquartile range

Significantly better 5-year OS and 5-year BCSS were observed among patients who received adjuvant treatment than patients who did not (79% [95% CI 75–84]) vs 49% [95% CI 45–53], p<0.0001 for 5-year OS; 85% [95% CI 81–89] vs 68% [95% CI 64–72], p<0.0001 for 5-year BCSS; **Figure 8**).

The significant differences between patients receiving adjuvant chemotherapy versus no adjuvant chemotherapy persisted in the adjusted propensity score-matched model analysis, wherein 406 patients were successfully randomly matched between patients receiving and not receiving adjuvant chemotherapy (75% [95% CI 69–82] vs 63% [95% CI 57–71], p=0.029 for 5-year OS; 83% [95% CI 78–89] vs 73% [95% CI 67–80], p=0.014 for 5-year BCSS; **Figure 9**).

Our results are in line with those from a recently published study by Crozier *et al.* (121), who collected data from the National Cancer Database in the USA between 2004 and 2014, for 16,062 patients ≥ 70 years of age, who were surgically treated for TNBC. Similarly to the findings in Paper IV, the 5-year OS was better for patients who received adjuvant chemotherapy (68.5% [95% CI 66.4–70.6]) than for patients for whom chemotherapy was recommended but not given (61.1% [95% CI 59.0–63.2]), and for patients who were neither recommended nor given chemotherapy (53.7% [95% CI 51.8–55.8]; pooled log rank p<0.001).

Studies have shown that patients ≥ 70 years of age less frequently undergo adequate surgery and adjuvant medical treatments than younger patients, although survival benefits have been reported (122). A cutoff age of 70 years has been reported to be associated with decline in physiological reserves (123). In the present study, the Charlson-Deyo index was 0–1 for most patients in the different treatment groups: 89.7% for patients who received adjuvant treatment, 84,5% for patients who received neoadjuvant treatment, and 73.7% for patients who did not receive treatment. These findings demonstrate that the patients in all treatment groups were fairly fit and—together with the increased life expectancy in recent decades and the significantly improved OS and BCSS—highlight the importance of considering adjuvant chemotherapy treatment in patients ≥ 70 years of age.

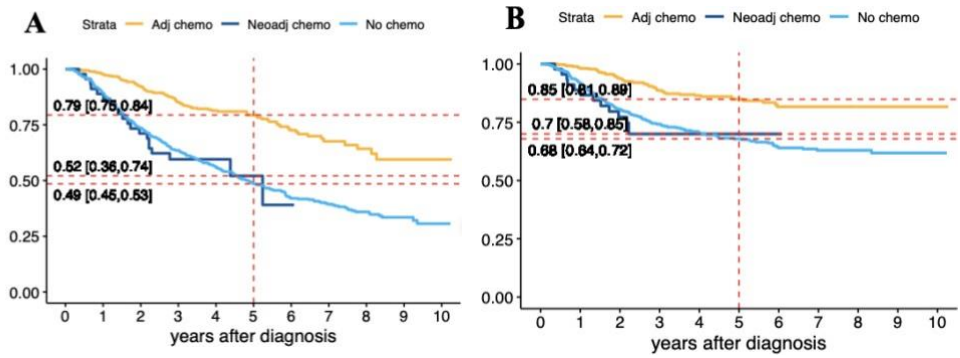

Figure 8. (A) *5-year overall survival (OS) and* ***(B)*** *5-year breast cancer specific survival (BCSS) comparing patients ≥70 years with TNBC that received adjuvant chemotherapy, no adjuvant chemotherapy and neoadjuvant chemotherapy. Figure adapted from Janeva et al.(120).*

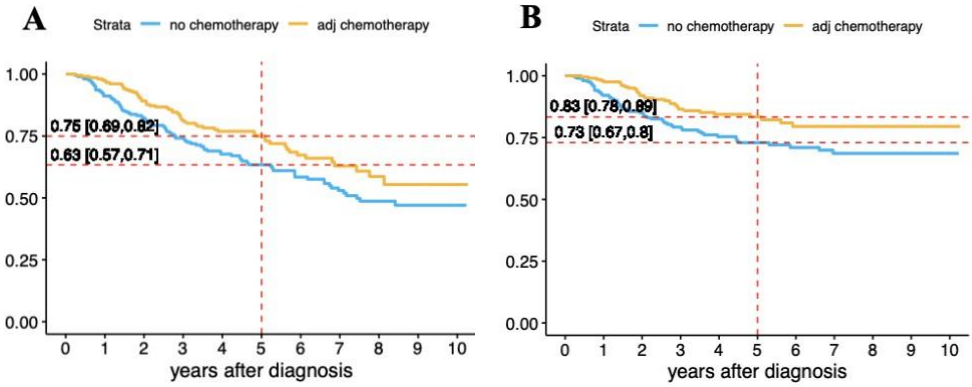

Figure 9. (A) *5-year overall survival and* ***(B)*** *5-year breast cancer specific survival (BCSS) after propensity score matching patients ≥70 years with TNBC that received and did not receive chemotherapy as adjuvant treatment. Figure adapted from Janeva et al. (120).*

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Accurate breast cancer diagnostics is crucial because patient-tailored treatment depends on tumor-specific characteristics.

- In Paper I, a new mRNA-based assessment tool (STRAT4) was compared with IHC. If adjuvant treatment decisions had been based on this new method rather than IHC, more aggressive treatment would have been recommended.

- In Paper II, we investigated whether IHC assessment of each tumor focus in multifocal breast cancer, rather than only the largest focus, would affect adjuvant treatment. The results suggested that all detected foci within a breast specimen should be assessed with IHC, because additional treatments were recommended on the basis of this more extensive analysis.

- In Paper III, we investigated whether IHC performed on synchronous lymph node metastases affected adjuvant treatment recommendations (endocrine or HER2-targeted therapies). We did not observe any additional value in performing IHC on the lymph node metastases.

- In Paper IV, a national observational registry study was conducted to examine the effects of chemotherapy on survival outcomes in women 70 years or older who were surgically treated for TNBC. Significant survival benefits of treatment with adjuvant chemotherapy were shown, thus underscoring the importance of this treatment in older patients.

IHC in breast cancer diagnostics has been the cornerstone of pathological analysis for decades and has served as an important assessment tool for decision-making in patient-tailored treatment. Breast cancer diagnostics is continually developing and must be in step with emerging targeted therapies that are currently under development. Continued updating of current diagnostic tools will therefore be needed to ensure accurate assessment of the disease.

Genomic profiling with multigene assays has become more common for breast cancer. However, the cost and availability of such methods are limiting, particularly in under-developed countries. Therefore, more cost-effective multi-gene assays revealing the intrinsic breast cancer subtypes must be developed and validated. Large comparative studies, preferably involving prospective, or at least retrospective analysis of completed prospective trials, will be necessary to determine whether new methods are more accurate than IHC in classifying breast cancer according to intrinsic subtypes.

Research questions in science should be based on clinically important issues that are relevant for the patient. This also goes for the hypotheses studied in this thesis, e.g., about whether the surgical specimens are assessed adequately though the guidelines are followed. Conflicting results could potentially be further investigated through large multicenter studies. Although discrepancies in breast cancer biomarkers are detected between different foci in multifocal breast cancer, and between the primary tumor and the LNM, these discrepancies were found to affect clinical outcomes, (e.g., different treatment recommendations) in only a small percentage of patients. Again, to address these discrepancies, larger studies incorporating multi-gene testing for subtyping must be conducted. With longer life expectancy comes a responsibility to include the growing population of older patients in research and randomized controlled trials, particularly given the emergence of novel targeted therapies.

The limitations concerning IHC have been addressed in various ways. Digital pathology, wherein full-faced histopathology slides are scanned to generate digital images, is increasingly being incorporated into the daily operations of pathology departments worldwide. This method enables objective histopathological assessment as well as application of various image analysis and artificial intelligence based algorithms. Promising results have indicated that deep learning-based models can predict molecular subtypes in breast cancer (124) and have the potential to enhance the interpretation of mammography screening findings (125).

Precision medicine research aims to select patients with breast cancer who are eligible for emerging target- and biomarker-based therapies, thus increasing the demand for molecular profiling. As the field of breast cancer research continues to evolve, “non-clinical” specialists, such as molecular biologists, are likely to become increasingly active key players in MDTs working toward solving the complex puzzle of breast cancer.

6 ETHICAL CONSIDERATIONS AND FUNDING

The four studies presented in this thesis were conducted in accordance with the principles outlined in the Declaration of Helsinki. Additionally, Paper I was approved by the Regional Ethical Review Board in Gothenburg, Sweden (678–18), and Papers II–III were approved by both the Regional Ethical Review Board in Gothenburg (479–18) and the Regional Ethical Review Board in Linköping, Sweden (2016/387–31). Paper IV was approved by the Ethical Review Board in Gothenburg, Sweden (633–18). All four studies were retrospective and did not result in changes in any treatment decisions for the included patients. Therefore, the requirement for informed consent was waived by the ethical review board.

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REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209-49.
2. Lima SM, Kehm RD, Terry MB. Global breast cancer incidence and mortality trends by region, age-groups, and fertility patterns. EClinicalMedicine. 2021;38:100985.
3. Socialstyrelsen (Swedish National Board of Health and Welfare). Statistik om nyupptäckta cancerfall 2021 [Available from: http://socaialstryrelsen.se.
4. Regional Cancer Centres in Collaboration. National Quality Register for Breast Cancer: Nationellt kvalitetsregister för Bröstcancer (NKBC) 2023 [Available from: https://statistik.incanet.se/bröstcancer.
5. Economopoulou P, Dimitriadis G, Psyrri A. Beyond BRCA: new hereditary breast cancer susceptibility genes. Cancer Treat Rev. 2015;41(1):1-8.
6. Tabar L, Dean PB, Chen TH, Yen AM, Chen SL, Fann JC, et al. The incidence of fatal breast cancer measures the increased effectiveness of therapy in women participating in mammography screening. Cancer. 2019;125(4):515-23.
7. Oeffinger KC, Fontham ET, Etzioni R, Herzig A, Michaelson JS, Shih YC, et al. Breast Cancer Screening for Women at Average Risk: 2015 Guideline Update From the American Cancer Society. JAMA. 2015;314(15):1599-614.
8. Broeders M, Moss S, Nystrom L, Njor S, Jonsson H, Paap E, et al. The impact of mammographic screening on breast cancer mortality in Europe: a review of observational studies. J Med Screen. 2012;19 Suppl 1:14-25.
9. Njor S, Nystrom L, Moss S, Paci E, Broeders M, Segnan N, et al. Breast cancer mortality in mammographic screening in Europe: a review of incidence-based mortality studies. J Med Screen. 2012;19 Suppl 1:33-41.
10. Kaufman Z, Shpitz B, Shapiro M, Rona R, Lew S, Dinbar A. Triple approach in the diagnosis of dominant breast masses: combined physical examination, mammography, and fine-needle aspiration. J Surg Oncol. 1994;56(4):254-7.
11. Tamaki K, Sasano H, Ishida T, Miyashita M, Takeda M, Amari M, et al. Comparison of core needle biopsy (CNB) and surgical specimens for accurate preoperative evaluation of ER, PgR and HER2 status of breast cancer patients. Cancer Sci. 2010;101(9):2074-9.

12. You K, Park S, Ryu JM, Kim I, Lee SK, Yu J, et al. Comparison of Core Needle Biopsy and Surgical Specimens in Determining Intrinsic Biological Subtypes of Breast Cancer with Immunohistochemistry. J Breast Cancer. 2017;20(3):297-303.
13. Kooistra B, Wauters C, Strobbe L, Wobbes T. Preoperative cytological and histological diagnosis of breast lesions: A critical review. Eur J Surg Oncol. 2010;36(10):934-40.
14. Burstein HJ, Curigliano G, Loibl S, Dubsky P, Gnant M, Poortmans P, et al. Estimating the benefits of therapy for early-stage breast cancer: the St. Gallen International Consensus Guidelines for the primary therapy of early breast cancer 2019. Ann Oncol. 2019;30(10):1541-57.
15. Burstein HJ, Curigliano G, Thurlimann B, Weber WP, Poortmans P, Regan MM, et al. Customizing local and systemic therapies for women with early breast cancer: the St. Gallen International Consensus Guidelines for treatment of early breast cancer 2021. Ann Oncol. 2021;32(10):1216-35.
16. Houssami N, Macaskill P, Marinovich ML, Dixon JM, Irwig L, Brennan ME, et al. Meta-analysis of the impact of surgical margins on local recurrence in women with early-stage invasive breast cancer treated with breast-conserving therapy. Eur J Cancer. 2010;46(18):3219-32.
17. Pilewskie M, Morrow M. Margins in breast cancer: How much is enough? Cancer. 2018;124(7):1335-41.
18. Breast Tumours, WHO Classification of Tumours, 5th Edition2019. 82-101 p.
19. Fortunato L, Mascaro A, Poccia I, Andrich R, Amini M, Costarelli L, et al. Lobular breast cancer: same survival and local control compared with ductal cancer, but should both be treated the same way? analysis of an institutional database over a 10-year period. Ann Surg Oncol. 2012;19(4):1107-14.
20. Rakha EA, Reis-Filho JS, Baehner F, Dabbs DJ, Decker T, Eusebi V, et al. Breast cancer prognostic classification in the molecular era: the role of histological grade. Breast Cancer Res. 2010;12(4):207.
21. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology. 1991;19(5):403-10.
22. AJCC Cancer Staging Manual. Eight Edtion ed: Springer; 2017. 616-7 p.
23. Jin L, Lloyd RV. In situ hybridization: methods and applications. J Clin Lab Anal. 1997;11(1):2-9.
24. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Oncol. 2010;28(16):2784-95.

25. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol. 2013;31(31):3997-4013.
26. Yerushalmi R, Woods R, Ravdin PM, Hayes MM, Gelmon KA. Ki67 in breast cancer: prognostic and predictive potential. Lancet Oncol. 2010;11(2):174-83.
27. Polley MY, Leung SC, McShane LM, Gao D, Hugh JC, Mastropasqua MG, et al. An international Ki67 reproducibility study. J Natl Cancer Inst. 2013;105(24):1897-906.
28. Nielsen TO, Leung SCY, Rimm DL, Dodson A, Acs B, Badve S, et al. Assessment of Ki67 in Breast Cancer: Updated Recommendations From the International Ki67 in Breast Cancer Working Group. J Natl Cancer Inst. 2021;113(7):808-19.
29. KVAST-gruppen Swedish Society of Pathology. KVAST dokument brösttumörer. https://medlemforeningssupportse/foreningar/svfpse-brostpatologi.
30. Finsterbusch K, Decker T, van Diest PJ, Focke CM. Luminal A versus luminal B breast cancer: MammaTyper mRNA versus immunohistochemical subtyping with an emphasis on standardised Ki67 labelling-based or mitotic activity index-based proliferation assessment. Histopathology. 2020;76(5):650-60.
31. Wu NC, Wong W, Ho KE, Chu VC, Rizo A, Davenport S, et al. Comparison of central laboratory assessments of ER, PR, HER2, and Ki67 by IHC/FISH and the corresponding mRNAs (ESR1, PGR, ERBB2, and MKi67) by RT-qPCR on an automated, broadly deployed diagnostic platform. Breast Cancer Res Treat. 2018;172(2):327-38.
32. Wilson TR, Xiao Y, Spoerke JM, Fridlyand J, Koeppen H, Fuentes E, et al. Development of a robust RNA-based classifier to accurately determine ER, PR, and HER2 status in breast cancer clinical samples. Breast Cancer Res Treat. 2014;148(2):315-25.
33. Sinn HP, Schneeweiss A, Keller M, Schlombs K, Laible M, Seitz J, et al. Comparison of immunohistochemistry with PCR for assessment of ER, PR, and Ki-67 and prediction of pathological complete response in breast cancer. BMC Cancer. 2017;17(1):124.
34. Janeva S, Parris TZ, Nasic S, De Lara S, Larsson K, Audisio RA, et al. Comparison of breast cancer surrogate subtyping using a closed-system RT-qPCR breast cancer assay and immunohistochemistry on 100 core needle biopsies with matching surgical specimens. BMC Cancer. 2021;21(1):439.
35. Noske A, Loibl S, Darb-Esfahani S, Roller M, Kronenwett R, Muller BM, et al. Comparison of different approaches for assessment of HER2 expression on protein and mRNA level: prediction of chemotherapy response

in the neoadjuvant GeparTrio trial (NCT00544765). Breast Cancer Res Treat. 2011;126(1):109-17.
36. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98(19):10869-74.
37. Howlader N, Cronin KA, Kurian AW, Andridge R. Differences in Breast Cancer Survival by Molecular Subtypes in the United States. Cancer Epidemiol Biomarkers Prev. 2018;27(6):619-26.
38. Ciriello G, Sinha R, Hoadley KA, Jacobsen AS, Reva B, Perou CM, et al. The molecular diversity of Luminal A breast tumors. Breast Cancer Res Treat. 2013;141(3):409-20.
39. Kumar P, Aggarwal R. An overview of triple-negative breast cancer. Arch Gynecol Obstet. 2016;293(2):247-69.
40. Curigliano G, Burstein HJ, Winer EP, Gnant M, Dubsky P, Loibl S, et al. De-escalating and escalating treatments for early-stage breast cancer: the St. Gallen International Expert Consensus Conference on the Primary Therapy of Early Breast Cancer 2017. Ann Oncol. 2017;28(8):1700-12.
41. Ruhstaller T, Giobbie-Hurder A, Colleoni M, Jensen MB, Ejlertsen B, de Azambuja E, et al. Adjuvant Letrozole and Tamoxifen Alone or Sequentially for Postmenopausal Women With Hormone Receptor-Positive Breast Cancer: Long-Term Follow-Up of the BIG 1-98 Trial. J Clin Oncol. 2019;37(2):105-14.
42. Carey LA, Perou CM, Livasy CA, Dressler LG, Cowan D, Conway K, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA. 2006;295(21):2492-502.
43. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007;13(15 Pt 1):4429-34.
44. Newman LA, Reis-Filho JS, Morrow M, Carey LA, King TA. The 2014 Society of Surgical Oncology Susan G. Komen for the Cure Symposium: triple-negative breast cancer. Ann Surg Oncol. 2015;22(3):874-82.
45. Carter CL, Allen C, Henson DE. Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases. Cancer. 1989;63(1):181-7.
46. Hayes DF, Isaacs C, Stearns V. Prognostic factors in breast cancer: current and new predictors of metastasis. J Mammary Gland Biol Neoplasia. 2001;6(4):375-92.
47. Rosen PP, Groshen S, Kinne DW, Norton L. Factors influencing prognosis in node-negative breast carcinoma: analysis of 767 T1N0M0/T2N0M0 patients with long-term follow-up. J Clin Oncol. 1993;11(11):2090-100.

48. Quiet CA, Ferguson DJ, Weichselbaum RR, Hellman S. Natural history of node-negative breast cancer: a study of 826 patients with long-term follow-up. J Clin Oncol. 1995;13(5):1144-51.
49. Coombs NJ, Boyages J. Multifocal and multicentric breast cancer: does each focus matter? J Clin Oncol. 2005;23(30):7497-502.
50. Vera-Badillo FE, Napoleone M, Ocana A, Templeton AJ, Seruga B, Al-Mubarak M, et al. Effect of multifocality and multicentricity on outcome in early stage breast cancer: a systematic review and meta-analysis. Breast Cancer Res Treat. 2014;146(2):235-44.
51. Pedersen L, Gunnarsdottir KA, Rasmussen BB, Moeller S, Lanng C. The prognostic influence of multifocality in breast cancer patients. Breast. 2004;13(3):188-93.
52. Joergensen LE, Gunnarsdottir KA, Lanng C, Moeller S, Rasmussen BB. Multifocality as a prognostic factor in breast cancer patients registered in Danish Breast Cancer Cooperative Group (DBCG) 1996-2001. Breast. 2008;17(6):587-91.
53. Ataseven B, Gologan D, Gunesch A, Kehl V, Hoegel B, Beer M, et al. HER2/neu, Topoisomerase 2a, Estrogen and Progesterone Receptors: Discordance between Primary Breast Cancer and Metastatic Axillary Lymph Node in Expression and Amplification Characteristics. Breast Care (Basel). 2012;7(6):465-70.
54. Kinoe H, Yamanouchi K, Kuba S, Morita M, Sakimura C, Kanetaka K, et al. Discordance of hormone receptor, human epidermal growth factor receptor-2, and Ki-67 between primary breast cancer and synchronous axillary lymph node metastasis. J BUON. 2018;23(7):60-6.
55. Zhao S, Xu L, Liu W, Lv C, Zhang K, Gao H, et al. Comparison of the expression of prognostic biomarkers between primary tumor and axillary lymph node metastases in breast cancer. Int J Clin Exp Pathol. 2015;8(5):5744-8.
56. Veronesi U, Saccozzi R, Del Vecchio M, Banfi A, Clemente C, De Lena M, et al. Comparing radical mastectomy with quadrantectomy, axillary dissection, and radiotherapy in patients with small cancers of the breast. N Engl J Med. 1981;305(1):6-11.
57. Fisher B, Bauer M, Margolese R, Poisson R, Pilch Y, Redmond C, et al. Five-year results of a randomized clinical trial comparing total mastectomy and segmental mastectomy with or without radiation in the treatment of breast cancer. N Engl J Med. 1985;312(11):665-73.
58. Veronesi U, Cascinelli N, Mariani L, Greco M, Saccozzi R, Luini A, et al. Twenty-year follow-up of a randomized study comparing breast-conserving surgery with radical mastectomy for early breast cancer. N Engl J Med. 2002;347(16):1227-32.
59. Fisher B, Redmond C, Fisher ER, Bauer M, Wolmark N, Wickerham DL, et al. Ten-year results of a randomized clinical trial comparing radical mastectomy and total mastectomy with or without radiation. N Engl J Med. 1985;312(11):674-81.

60. Clough KB, Kaufman GJ, Nos C, Buccimazza I, Sarfati IM. Improving breast cancer surgery: a classification and quadrant per quadrant atlas for oncoplastic surgery. Ann Surg Oncol. 2010;17(5):1375-91.
61. Nemoto T, Vana J, Bedwani RN, Baker HW, McGregor FH, Murphy GP. Management and survival of female breast cancer: results of a national survey by the American College of Surgeons. Cancer. 1980;45(12):2917-24.
62. Beenken SW, Urist MM, Zhang Y, Desmond R, Krontiras H, Medina H, et al. Axillary lymph node status, but not tumor size, predicts locoregional recurrence and overall survival after mastectomy for breast cancer. Ann Surg. 2003;237(5):732-8; discussion 8-9.
63. DiSipio T, Rye S, Newman B, Hayes S. Incidence of unilateral arm lymphoedema after breast cancer: a systematic review and meta-analysis. Lancet Oncol. 2013;14(6):500-15.
64. Norman SA, Localio AR, Potashnik SL, Simoes Torpey HA, Kallan MJ, Weber AL, et al. Lymphedema in breast cancer survivors: incidence, degree, time course, treatment, and symptoms. J Clin Oncol. 2009;27(3):390-7.
65. Wilke LG, McCall LM, Posther KE, Whitworth PW, Reintgen DS, Leitch AM, et al. Surgical complications associated with sentinel lymph node biopsy: results from a prospective international cooperative group trial. Ann Surg Oncol. 2006;13(4):491-500.
66. McLaughlin SA, Wright MJ, Morris KT, Sampson MR, Brockway JP, Hurley KE, et al. Prevalence of lymphedema in women with breast cancer 5 years after sentinel lymph node biopsy or axillary dissection: patient perceptions and precautionary behaviors. J Clin Oncol. 2008;26(32):5220-6.
67. Giuliano AE, Hunt KK, Ballman KV, Beitsch PD, Whitworth PW, Blumencranz PW, et al. Axillary dissection vs no axillary dissection in women with invasive breast cancer and sentinel node metastasis: a randomized clinical trial. JAMA. 2011;305(6):569-75.
68. Giuliano AE, Ballman KV, McCall L, Beitsch PD, Brennan MB, Kelemen PR, et al. Effect of Axillary Dissection vs No Axillary Dissection on 10-Year Overall Survival Among Women With Invasive Breast Cancer and Sentinel Node Metastasis: The ACOSOG Z0011 (Alliance) Randomized Clinical Trial. JAMA. 2017;318(10):918-26.
69. Liedtke C, Mazouni C, Hess KR, Andre F, Tordai A, Mejia JA, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol. 2008;26(8):1275-81.
70. Loibl S, Gianni L. HER2-positive breast cancer. Lancet. 2017;389(10087):2415-29.
71. Bonadonna G, Brusamolino E, Valagussa P, Rossi A, Brugnatelli L, Brambilla C, et al. Combination chemotherapy as an adjuvant treatment in operable breast cancer. N Engl J Med. 1976;294(8):405-10.

72. Fisher B, Carbone P, Economou SG, Frelick R, Glass A, Lerner H, et al. 1-Phenylalanine mustard (L-PAM) in the management of primary breast cancer. A report of early findings. N Engl J Med. 1975;292(3):117-22.
73. Early Breast Cancer Trialists' Collaborative G. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005;365(9472):1687-717.
74. Trudeau M, Charbonneau F, Gelmon K, Laing K, Latreille J, Mackey J, et al. Selection of adjuvant chemotherapy for treatment of node-positive breast cancer. Lancet Oncol. 2005;6(11):886-98.
75. Nowak AK, Wilcken NR, Stockler MR, Hamilton A, Ghersi D. Systematic review of taxane-containing versus non-taxane-containing regimens for adjuvant and neoadjuvant treatment of early breast cancer. Lancet Oncol. 2004;5(6):372-80.
76. Kaufmann M, von Minckwitz G, Mamounas EP, Cameron D, Carey LA, Cristofanilli M, et al. Recommendations from an international consensus conference on the current status and future of neoadjuvant systemic therapy in primary breast cancer. Ann Surg Oncol. 2012;19(5):1508-16.
77. Jensen EV, Block GE, Smith S, Kyser K, DeSombre ER. Estrogen receptors and breast cancer response to adrenalectomy. Natl Cancer Inst Monogr. 1971;34:55-70.
78. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. Clin Cancer Res. 2003;9(6):1980-9.
79. International Breast Cancer Study G, Colleoni M, Gelber S, Goldhirsch A, Aebi S, Castiglione-Gertsch M, et al. Tamoxifen after adjuvant chemotherapy for premenopausal women with lymph node-positive breast cancer: International Breast Cancer Study Group Trial 13-93. J Clin Oncol. 2006;24(9):1332-41.
80. Early Breast Cancer Trialists' Collaborative G, Davies C, Godwin J, Gray R, Clarke M, Cutter D, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet. 2011;378(9793):771-84.
81. Early Breast Cancer Trialists' Collaborative G. Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials. Lancet. 2015;386(10001):1341-52.
82. Smith IE, Dowsett M. Aromatase inhibitors in breast cancer. N Engl J Med. 2003;348(24):2431-42.
83. Mamounas EP, Jeong JH, Wickerham DL, Smith RE, Ganz PA, Land SR, et al. Benefit from exemestane as extended adjuvant therapy after 5 years of adjuvant tamoxifen: intention-to-treat analysis of the National Surgical Adjuvant Breast And Bowel Project B-33 trial. J Clin Oncol. 2008;26(12):1965-71.
84. Early Breast Cancer Trialists' Collaborative G. Aromatase inhibitors versus tamoxifen in premenopausal women with oestrogen receptor-

positive early-stage breast cancer treated with ovarian suppression: a patient-level meta-analysis of 7030 women from four randomised trials. Lancet Oncol. 2022;23(3):382-92.
85. Harbeck N, Beckmann MW, Rody A, Schneeweiss A, Muller V, Fehm T, et al. HER2 Dimerization Inhibitor Pertuzumab - Mode of Action and Clinical Data in Breast Cancer. Breast Care (Basel). 2013;8(1):49-55.
86. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001;344(11):783-92.
87. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med. 2005;353(16):1659-72.
88. Cameron D, Piccart-Gebhart MJ, Gelber RD, Procter M, Goldhirsch A, de Azambuja E, et al. 11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin Adjuvant (HERA) trial. Lancet. 2017;389(10075):1195-205.
89. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Jr., Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med. 2005;353(16):1673-84.
90. Gianni L, Pienkowski T, Im YH, Roman L, Tseng LM, Liu MC, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. Lancet Oncol. 2012;13(1):25-32.
91. von Minckwitz G, Procter M, de Azambuja E, Zardavas D, Benyunes M, Viale G, et al. Adjuvant Pertuzumab and Trastuzumab in Early HER2-Positive Breast Cancer. N Engl J Med. 2017;377(2):122-31.
92. Piccart M, Procter M, Fumagalli D, de Azambuja E, Clark E, Ewer MS, et al. Adjuvant Pertuzumab and Trastuzumab in Early HER2-Positive Breast Cancer in the APHINITY Trial: 6 Years' Follow-Up. J Clin Oncol. 2021;39(13):1448-57.
93. Banerji U, van Herpen CML, Saura C, Thistlethwaite F, Lord S, Moreno V, et al. Trastuzumab duocarmazine in locally advanced and metastatic solid tumours and HER2-expressing breast cancer: a phase 1 dose-escalation and dose-expansion study. Lancet Oncol. 2019;20(8):1124-35.
94. Nakada T, Sugihara K, Jikoh T, Abe Y, Agatsuma T. The Latest Research and Development into the Antibody-Drug Conjugate, [fam-] Trastuzumab Deruxtecan (DS-8201a), for HER2 Cancer Therapy. Chem Pharm Bull (Tokyo). 2019;67(3):173-85.
95. Hartkopf AD, Grischke EM, Brucker SY. Endocrine-Resistant Breast Cancer: Mechanisms and Treatment. Breast Care (Basel). 2020;15(4):347-54.
96. Finn RS, Crown JP, Lang I, Boer K, Bondarenko IM, Kulyk SO, et al. The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination

with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. Lancet Oncol. 2015;16(1):25-35.
97. Underhill C, Toulmonde M, Bonnefoi H. A review of PARP inhibitors: from bench to bedside. Ann Oncol. 2011;22(2):268-79.
98. Robson M, Im SA, Senkus E, Xu B, Domchek SM, Masuda N, et al. Olaparib for Metastatic Breast Cancer in Patients with a Germline BRCA Mutation. N Engl J Med. 2017;377(6):523-33.
99. Schmid P, Cortes J, Pusztai L, McArthur H, Kummel S, Bergh J, et al. Pembrolizumab for Early Triple-Negative Breast Cancer. N Engl J Med. 2020;382(9):810-21.
100. Early Breast Cancer Trialists' Collaborative G, Darby S, McGale P, Correa C, Taylor C, Arriagada R, et al. Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10,801 women in 17 randomised trials. Lancet. 2011;378(9804):1707-16.
101. Clarke M, Collins R, Darby S, Davies C, Elphinstone P, Evans V, et al. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005;366(9503):2087-106.
102. Lofgren L, Eloranta S, Krawiec K, Asterkvist A, Lonnqvist C, Sandelin K, et al. Validation of data quality in the Swedish National Register for Breast Cancer. BMC Public Health. 2019;19(1):495.
103. Socialstyrelsen. Bortfall och kvalitet i patientregistret 2020 [Available from: https://www.socialstyrelsen.se/statistik-och-data/register/alla-register/patientregistret/bortfall-och-kvalitet/.
104. KVAST-group SSoP. KVAST-document for breast tumors [updated 2014-05-08. 3.1.3:[Available from: https://svfp.se/kvast/brostpatologi/.
105. Wasserman BE, Carvajal-Hausdorf DE, Ho K, Wong W, Wu N, Chu VC, et al. High concordance of a closed-system, RT-qPCR breast cancer assay for HER2 mRNA, compared to clinically determined immunohistochemistry, fluorescence in situ hybridization, and quantitative immunofluorescence. Lab Invest. 2017;97(12):1521-6.
106. Gupta S, Mani NR, Carvajal-Hausdorf DE, Bossuyt V, Ho K, Weidler J, et al. Macrodissection prior to closed system RT-qPCR is not necessary for estrogen receptor and HER2 concordance with IHC/FISH in breast cancer. Lab Invest. 2018;98(8):1076-83.
107. Janeva S, Krabbe E, Parris TZ, Nasic S, Sundquist M, Karlsson P, et al. Clinical evaluation of molecular surrogate subtypes in patients with ipsilateral multifocal primary breast cancer. Breast Cancer Research. 2023;25(1):36.
108. Li S, Wu J, Huang O, He J, Chen W, Li Y, et al. Association of Molecular Biomarker Heterogeneity With Treatment Pattern and Disease

Outcomes in Multifocal or Multicentric Breast Cancer. Front Oncol. 2022;12:833093.
109. Choi Y, Kim EJ, Seol H, Lee HE, Jang MJ, Kim SM, et al. The hormone receptor, human epidermal growth factor receptor 2, and molecular subtype status of individual tumor foci in multifocal/multicentric invasive ductal carcinoma of breast. Hum Pathol. 2012;43(1):48-55.
110. Middleton LP, Vlastos G, Mirza NQ, Eva S, Sahin AA. Multicentric mammary carcinoma: evidence of monoclonal proliferation. Cancer. 2002;94(7):1910-6.
111. Falck AK, Ferno M, Bendahl PO, Ryden L. Does analysis of biomarkers in tumor cells in lymph node metastases give additional prognostic information in primary breast cancer? World J Surg. 2010;34(7):1434-41.
112. Dikicioglu E, Barutca S, Meydan N, Meteoglu I. Biological characteristics of breast cancer at the primary tumour and the involved lymph nodes. Int J Clin Pract. 2005;59(9):1039-44.
113. Aitken SJ, Thomas JS, Langdon SP, Harrison DJ, Faratian D. Quantitative analysis of changes in ER, PR and HER2 expression in primary breast cancer and paired nodal metastases. Ann Oncol. 2010;21(6):1254-61.
114. Bonin S, Pracella D, Barbazza R, Sulfaro S, Stanta G. In stage II/III lymph node-positive breast cancer patients less than 55 years of age, keratin 8 expression in lymph node metastases but not in the primary tumour is an indicator of better survival. Virchows Arch. 2015;466(5):571-80.
115. D'Andrea MR, Limiti MR, Bari M, Zambenedetti P, Montagutti A, Ricci F, et al. Correlation between genetic and biological aspects in primary non-metastatic breast cancers and corresponding synchronous axillary lymph node metastasis. Breast Cancer Res Treat. 2007;101(3):279-84.
116. Mando P, Rizzo M, de la Puente CP, Maino M, Ponce C, Pombo MT, et al. High Histologic Grade and High Ki-67 Expression Predict Phenotypic Alterations in Node Metastasis in Primary Breast Cancers. J Breast Cancer. 2017;20(2):170-5.
117. Kimbung S, Kovacs A, Danielsson A, Bendahl PO, Lovgren K, Frostvik Stolt M, et al. Contrasting breast cancer molecular subtypes across serial tumor progression stages: biological and prognostic implications. Oncotarget. 2015;6(32):33306-18.
118. Falck AK, Ferno M, Bendahl PO, Ryden L. St Gallen molecular subtypes in primary breast cancer and matched lymph node metastases--aspects on distribution and prognosis for patients with luminal A tumours: results from a prospective randomised trial. BMC Cancer. 2013;13:558.
119. Cabibi D, Mustacchio V, Martorana A, Tripodo C, Campione M, Calascibetta A, et al. Lymph node metastases displaying lower Ki-67 immunostaining activity than the primary breast cancer. Anticancer Res. 2006;26(6B):4357-60.
120. Janeva S, Zhang C, Kovacs A, Parris TZ, Crozier JA, Pezzi CM, et al. Adjuvant chemotherapy and survival in women aged 70 years and older

with triple-negative breast cancer: a Swedish population-based propensity score-matched analysis. Lancet Healthy Longev. 2020;1(3):e117-e24.
121. Crozier JA, Pezzi TA, Hodge C, Janeva S, Lesnikoski BA, Samiian L, et al. Addition of chemotherapy to local therapy in women aged 70 years or older with triple-negative breast cancer: a propensity-matched analysis. Lancet Oncol. 2020;21(12):1611-9.
122. Inwald EC, Ortmann O, Koller M, Zeman F, Hofstadter F, Evert M, et al. Screening-relevant age threshold of 70 years and older is a stronger determinant for the choice of adjuvant treatment in breast cancer patients than tumor biology. Breast Cancer Res Treat. 2017;163(1):119-30.
123. Balducci L, Extermann M. Management of cancer in the older person: a practical approach. Oncologist. 2000;5(3):224-37.
124. Jaber MI, Song B, Taylor C, Vaske CJ, Benz SC, Rabizadeh S, et al. A deep learning image-based intrinsic molecular subtype classifier of breast tumors reveals tumor heterogeneity that may affect survival. Breast Cancer Res. 2020;22(1):12.
125. Schaffter T, Buist DSM, Lee CI, Nikulin Y, Ribli D, Guan Y, et al. Evaluation of Combined Artificial Intelligence and Radiologist Assessment to Interpret Screening Mammograms. JAMA Netw Open. 2020;3(3):e200265.