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# UNRAVELING PREDICTIVE INDICATORS FOR THERAPEUTIC RESPONSE IN HER2-POSITIVE BREAST CANCER

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# UNRAVELING PREDICTIVE INDICATORS FOR THERAPEUTIC RESPONSE IN HER2-POSITIVE BREAST CANCER

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

**Yajing Zhu**

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– To my beloved family and friends–

# ABSTRACT

Breast cancer is a highly heterogeneous disease, exhibiting significant diversity both in its biological subtypes and clinical manifestations. Biologically directed therapies have revolutionized the treatment landscape for breast cancer in the past three decades, offering more targeted and personalized approaches and have significantly changed the treatment paradigm. This shift in treatment conception has indeed resulted in improved outcomes for patients, driven by extensive research efforts aimed at personalized biomarker exploration.

The aim of my thesis was to identify biomarkers for optimizing therapy selection in HER2-positive breast cancer.

In **paper I**, we concentrated on current evidence regarding the dynamics of tumor-infiltrating lymphocytes (TILs) during neoadjuvant treatment and examined the fluctuating patterns of TILs, correlating them with treatment prediction and survival outcomes. We observed a consistent decrease in TILs levels after neoadjuvant therapy (NAT) across all breast cancer subtypes, with a numerically larger decrease noted in HER2-positive breast cancer. In TNBC patients, increased TILs during treatment were associated with better disease-free survival (DFS) or recurrence-free survival (RFS), as indicated by pooled hazard ratios from univariate analyses of four eligible studies. However, due to insufficient studies, this analysis was limited to TNBC. Additionally, we identified eight studies reporting on-treatment TILs counts, which was uniformly increased compared to baseline levels. Increased on-treatment TILs compared to bassline were positively associated with pathological complete response (pCR) status in seven out of total eight studies, but no pooled analysis was done due to data heterogeneity. These findings suggest that dynamic monitoring of TILs may serve as a flexible and economical biomarker for treatment de-escalation and future trial design, particularly in HER2-positive and TNBC patients.

In **paper II**, a comprehensive analysis was conducted to assess the predictive and prognostic significance of baseline and serial levels of serum thymidine kinase (sTK1) in patients with HER2-positive early breast cancer enrolled in the PREDIX HER2 trial. At baseline, no association was found between serum thymidine kinase 1 (sTK1) levels and clinicopathological characteristics such as age, tumor grade, and Ki-67 status. We observed a dramatic increase in TK1 activity in all patients after two cycles of treatment, although neither baseline sTK1 levels nor sTK1 levels at subsequent on-treatment time points were associated with pathological complete response (pCR) status. Furthermore, there was no significant effect of baseline or cycle 2 sTK1 activity on event-free survival (EFS). For patients with residual disease (non-pCR), a higher sTK1 activity at the end of treatment visit appeared to be linked with longer survival time, though the association did not reach statistical significance. Our study provides evidence of sTK1 activity dynamics in a prospective phase II trial, although no prognostic association was identified.

In **paper III,** we profiled intrinsic molecular subtypes in longitudinally collected tissue material obtained from patients enrolled in the PREDIX HER2 trial and explored their

association with treatment response and long-term outcomes. The PAM50 intrinsic subtypes were determined using an SSP-based method. The results revealed that the majority of patients at baseline were classified as HER2-enriched (HER2-E) subtype (55%), as expected. Approximately 40% of patients were categorized as Luminal A or Luminal B types, while the remaining were classified as basal-like (BL) subtype. The baseline HER2-E subtype showed a significant association with better pCR and EFS. Under treatment, intrinsic subtypes exhibited temporal plasticity, with the majority of patients experiencing a subtype switch. Specifically, 71 out of 93 HER2-E patients transitioned to a non-HER2-E subtype from baseline to on-treatment. The present study highlights the potential utility of PAM50 intrinsic molecular subtypes for prognostication in HER2-positive breast cancer, and a prospective validation clinical trial is ongoing. Further exploration of the clinical implications associated with subtype switching during treatment is needed.

In summary, our research provides a comprehensive biomarker exploration aimed at predicting treatment response and adding prognostic value in patients with early HER2-positive breast cancer. Through these investigations, we aim to enhance our understanding of breast cancer heterogeneity and improve treatment escalation and de-escalation strategies for achieving better patient outcomes.

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*Manuscript*

# LIST OF ABBREVIATIONS

| | |
|---|---|
| BC | Breast cancer |
| eBC | Early breast cancer |
| mBC | Metastatic breast cancer |
| TNBC | Triple negative breast cancer |
| TILs | Tumor-infiltrating lymphocytes |
| sTILs | Stroma tumor-infiltrating lymphocytes |
| NAT | Neoadjuvant therapy |
| NACT | Neoadjuvant chemotherapy |
| HER2 | Human epidermal growth factor receptor 2 |
| ASCO | American Society of Clinical Oncology |
| IHC | Immunohistochemistry |
| mIHC | Multiplex immunohistochemistry |
| OS | Overall survival |
| PAM50 | Prediction analysis of microarray 50 |
| PIK3CA | Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic |
| SOC | Standard of care College of American Pathologists |
| CAP | College of American Pathologists |
| PI3K | Phosphoinositide 3-kinase |
| FDA | Food and Drug Administration |
| CEP | Chromosome enumeration probe |
| CPS | Combined positive score |
| pCR | Pathologic complete response |
| FISH | Fluorescence in situ hybridization |
| ISH | In situ hybridization |
| HR | hormone receptor |
| HRs | Hazard ratios |
| ADC | Antibody drug conjugate |
| T-DM1 | Ado-trastuzumab emtansine |
| DM1 | Emtansine |
| MCC | N-maleimidomethyl cyclohexane-1-carboxylate |

| | |
|---|---|
| CTneoBC | Collaborative Trials in Neoadjuvant Breast Cancer |
| EFS | Event-free survival |
| CDx | Companion diagnostic |
| ctDNA | Circulating tumor DNA |
| NSCLC | Non-small-cell lung cancer |
| RPPA | Reverse Phase Protein Microarray |
| HER2-E | HER2-enriched |
| RD | Residual disease |
| AIMs | Absolute Intrinsic Molecular Subtyping |
| CTC | Circulating tumor cell |
| TRAR | TRAstuzumab Risk |
| AI | Artificial intrelligence |
| RFS | Relapse-free survival |
| miRNA | microRNA |
| WHO | World Health Organization |
| scRNA | Single-cell RNA |
| scDNA | Single-cell DNA |
| SNS | Single-nucleus sequencing |
| CyTOF | Molecular profiling with mass cytometry |
| MIBI | Multiplexed ion beam imaging |
| CODEX | Co-detection by indexing |
| IMC | Image mass cytometry |
| PD-1 | Programmed Cell Death Protein 1 |
| PDL-1 | Programmed Cell Death Ligand 1 |
| sTK1 | Serum Thymidine kinase-1 |
| dTMP | deoxythymidine monophosphate |
| PLK1 | Polo-like kinase 1 |
| CDK | Cyclin-dependent kinases |
| DHP | Docetaxel, trastuzumab and pertuzumab |
| MRI | Magnetic Resonance Imaging |
| PET/CT | Positron Emission Tomography/Computerised Tomography |

| | |
|---|---|
| HRQoL | Health-related quality of life |
| BR | Broad range |
| HS | High sensitivity |
| USA | United States of America |
| DIN | DNA Integrity Number |
| RIN | RNA Integrity Number |
| QC | Quality control |
| DNA | Deoxyribonucleic acid |
| RNA | Ribonucleic acid |
| IVT | In vitro transcription |
| TSA | Tyramide Signal Amplification |
| CI | confidence interval |
| ORs | Odd ratios |
| SMD | Standardized mean differences |
| ICIs | Immune checkpoint inhibitors |
| BL | Basal like |
| NBL | Normal breast like |
| SSPs | Single sample predictors |
| H&E | hematoxylin and eosin |
| KI | Karolinska Institute |
| TIM-3 | T cell Ig- and mucin-domain-containing molecule-3 |
| CTLA-4 | cytotoxic T-lymphocyte–associated antigen 4 |
| HLA-DR | Human Leukocyte Antigen-DR isotype |
| SUV | Standardized uptake value |
| FDG | F-fluorodeoxyglucose |
| BRCA1 | BReast CAncer gene 1 |
| BRCA2 | BReast CAncer gene 2 |
| TME | Tumor microenvironment |
| ICIs | Immune checkpoint inhibitors |
| TMA | Tissue microarray |

# 1 INTRODUCTION

Breast disorders and diseases are prevalent and can affect women across all age groups. While benign breast conditions outnumber malignant diseases, breast cancer remains a significant concern due to its potentially life-threatening nature. Research in breast cancer holds crucial significance in advancing our understanding, treatment options, and ultimately, the well-being of individuals affected by this formidable disease.

## 1.1 EPIDEMIOLOGY, RISK FACTORS, HEREDITARY AND PATHOGENESIS OF BREAST CANCER

Breast cancer stands as the most prevalent malignant disease affecting women worldwide, with most recently published data showing 313,510 estimated new cases and 42,780 deaths in 2024[1]. In Sweden, breast cancer exhibits the highest five-year prevalence among women, reaching 36,296 in 2022 [2]. Despite an increase in incidence, mortality has shown a gradual decline among Swedish women from 1970 to 2022[2]. The decline in mortality is likely attributed to advancements in treatment and early detection facilitated by screening programs, access to high-quality prevention, and treatment services.

Breast tumors typically originate from ductal hyperproliferation, progressing to benign or precancerous lesions, invasive carcinoma, and eventually metastatic cancer due to continuous stimulation by various carcinogenic factors. The etiology of breast cancer is multifactorial, encompassing reproductive factors such as hormones and various epidemiologic influences. The most well-established risk factors encompass a range of factors including those related to pregnancy, lifestyle choices, hormone replacement therapy, and mammographic density. [3]. Importantly, the association of certain risk factors, such as parity and breastfeeding, has been observed to differ among distinct molecular subtypes of breast cancer[4].

About 10% of all breast cancers are attributed to hereditary factors involving high-penetrance gene mutations. The most common mutations women carrying are pathogenic *BReast CAncer gene 1* (*BRCA1)* and *BReast CAncer gene 2 (BRCA2)*. Mutations in the *BRCA1/2* genes elevate the risk of breast cancer to 45% to 65% by the age of 70 years [5]. The prognosis of breast cancer exhibits significant variability and relies on various prognostic variables.

## 1.2 MOLECULAR SUBTYPING OF BREAST CANCER, SURROGATE SUBTYPES AND TREATMENT PRINCIPLES

Breast cancer encompasses a diverse array of diseases characterized by distinct molecular features [6-8]. As techniques advance, gene expression profiling of breast cancer has offered alternative prognostic information beyond standard clinicopathological features[9, 10]. Through the adoption of cluster analysis utilizing an 'intrinsic' gene subset consisting of 496 genes on the DNA microarray data, researchers have successfully identified the intrinsic subtypes: luminal A, luminal B, HER2-enriched (HER2-E), basal-like (BL), and normal-like subgroup (NBL). Following the initial breakthrough, other rare subtypes have been identified, including the claudin-low, interferon-rich, and molecular apocrine subtypes[8]. In 2009, a 50-

gene set based identifier named PAM50 was further developed and validated for the genomic classification of intrinsic subtypes. IHC measurements of ER, PR, HER2 and Ki67 were used as surrogate markers to define clinical subtypes of luminal A-like, luminal B-like (HER2 negative), luminal B-like (HER2 positive) and triple negative. Further adding to the complexity of breast cancer classification, immunohistochemistry-based subgroups such as ER low and HER2 low with their prognostic and therapeutic implications have emerged[11, 12].

Standard treatments for breast cancer patients encompass a range of options, including surgical procedures, radiation therapy, chemotherapy, endocrine therapy, targeted therapies, and, more recently, immunotherapy.

For early BC, the main therapeutic goals are eradicating tumor from the breast and regional lymph nodes, along with averting metastatic recurrence. Local therapy typically involves surgical removal of the tumor along with sampling or complete removal of nearby axillary lymph nodes. This is often followed by postoperative radiation therapy. Systemic therapy may be given preoperatively (neoadjuvant), postoperatively (adjuvant), or both. Neoadjuvant therapy (NAT) aims to treat distant micro-metastases, reduce tumor burden, improve operability, and even increase the likelihood of breast- conserving surgery in patients with locally advanced and inflammatory cancers. Most importantly, NAT can offer a distinct advantage in assessing the response of breast cancer patients to various treatments. Adequate patient samples taken before and after various treatments can be evaluated through both non-invasive and invasive methods and provide prognostic implications. For ER-HER2+ early breast cancer(eBC), the preferred primary treatment is TCHP, as indicated by the TRYPHAENA trial where patients treated with TCHP demonstrated the highest pathological complete response (pCR) rate at 66.2% and clinically manageable toxicity[13], which have also been validated in a large network metanalysis[14]. The clinical use of 21-gene assay (Oncotype Dx) has been validated for predicting the benefit of adding adjuvant treatment to further reduce the risk of recurrence in hormone receptor-positive (HR+), human epidermal growth factor receptor-2-negative (HER2-) breast cancer(HR+HER2- BC) patients[15-17]. For high-risk (T1cN1-2 or T2-4N0-2) early TNBC patients, the preferred regimen involves pembrolizumab combined with chemotherapy given preoperatively. Following surgery, pembrolizumab is continued as a single agent for adjuvant treatment. [18].

## 1.3 HER2-POSITIVE BREAST CANCER

### 1.3.1 Evolution of HER2 testing: historical perspectives and definition changes over time

The utilization of HER2-targeted agents has significantly enhanced clinical outcomes in individuals diagnosed with HER2-positive breast cancer, spanning across both metastatic and adjuvant treatment settings. Consequently, precise determination of HER2 status is crucial for optimizing clinical outcomes in breast cancer patients. The most common methods for

assessing HER2 status are immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH).

In 2007, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) jointly developed guidelines with the goal of standardizing the conduct and interpretation of HER2 testing. This initiative aimed to minimize potential inaccuracies in HER2 testing results across different laboratories. This guideline adopted a higher cutoff of 30% for HER2 IHC positivity (3+), deviating from the previous FDA recommendation of a 10% cutoff. The guidelines considered the HER2 gene as amplified if the HER2/chromosome enumeration probe 17 (CEP17) ratio exceeded 2.2 in a dual-probe assay (contrasting with the previous recommendation of 2.0) or HER2 gene copy exceeded 6 signals per cell in single-probe assays[19].

The 2013 ASCO-CAP guideline aimed at addressing both false-negative and false-positive HER2 assessments with focus on optimizing the identification of patients who could benefit from HER2-targeted therapy while minimizing the administration of potentially toxic, costly, and ineffective treatments. This iteration of guidelines introduced changes of the expansion of the range for HER2 IHC equivocal cases (2+), and redefined HER2 IHC 3+ using a cutoff value of 10% instead of 30%. In the context of a validated dual-probe in situ hybridization (ISH) assay, positivity was defined as HER2/CEP17 ratio $\geqslant$2.0 or HER2/CEP17 ratio <2.0 and an average HER2 copy number $\geqslant$6.0. Notably, both the 2007 and 2013 ASCO/CAP guidelines addressed ISH equivocal results, a challenge for clinical decision-making[20].

The updated 2018 ASCO-CAP HER2 testing guideline addressed certain controversial aspect aspects of the 2013 guidelines. The clarification of the IHC 2+category reverted to definition of weak to moderate complete membrane staining in >10% of tumor cells. And in light of extensive clinical experience confirming high concordance in HER2 testing between core and excisional biopsies, a mandatory repeated HER2 testing of resected tumors with a prior negative HER2 test on the needle core biopsy is now viewed as discretionary. Special attention is also warranted in less common clinical scenarios, which account for approximately 5% of cases. These scenarios are identified when using a dual-probe ISH assay categorized as ISH group 2, 3, and 4 in the guideline.[21].

Recently, the concept of HER2-low breast cancer has garnered renewed attention in the breast cancer landscape. This subtype is defined by immunohistochemistry (IHC) scores of 1+ or 2+ and ISH results. These cases are typically reported as HER2 negative, categorized as either triple-negative breast cancer (TNBC) or luminal-like if hormone receptors (HRs) are expressed. Previous trials have not demonstrated any clinical benefit from agents that disrupt the HER2 pathway in these subset of patients[22]. More recently, it was demonstrated that this subgroup could derive benefit from targeting HER2 with the novel antibody-drug conjugate(ADC) drug trastuzumab deruxtecan [23]. In contrast with HER2-overexpressing tumors, the benefit of novel ADC drugs in HER2-low patients might be driven through additional pharmacological mechanisms. These mechanisms could include the penetration

not only of HER2-expressing cells but also neighboring cells, resulting in a local bystander effect[24]. Additionally, these drugs may be evenly distributed in both the HER2+ and HER2- regions of the tumors, as visualized using fluorescent nanoparticles[25]. The clinical development of novel anti-HER2 agents for HER2-low breast cancer has the potential to shift the paradigm of HER2-expressing tumors. A significant proportion of patients who were previously not considered candidates for HER2-targeted therapy may now benefit from these advancements.

The 2023 updated guideline also highlights the challenges associated with adopting the HER2-Low terminology in the HER2 IHC reporting as current IHC2+ are reported as equivocal for protein overexpression, with reflex in situ hybridization (ISH) testing required to determine gene amplification status. As a result, the final IHC result category, distinguishing between HER2-Low and HER2-positive, could not be reported until reflex ISH results are available[26]. Furthermore, given that the DESTINY-Breast04 trial adhered to the prevailing standard IHC scoring definitions (0, 1+, 2+, and 3+), there is presently inadequate evidence to advocate for altering these definitions.

### 1.3.2 Escalation and de-escalation of HER2-targeted therapies

Since the first approval of the monoclonal antibody trastuzumab for HER2-positive breast cancer nearly three decades ago, numerous drugs with diverse mechanisms of action and safety profiles have been sanctioned for use in both early-stage and metastatic settings. Further details on these medications are outlined and deliberated below.

In the metastatic setting, a milestone study in 2001 started the era of HER2-targeted therapy for metastatic BC (mBC), showing that trastuzumab combined with conventional paclitaxel or docetaxel improved median overall survival (OS) to about 25 months. Dual blockade of HER2 with trastuzumab and pertuzumab in addition to docetaxel resulted in a prolonged median overall survival (OS) of 56.5 months in the CLEOPATRA trial. This regimen remains the preferred first-line treatment option for patients with HER2-positive metastatic breast cancer[27].

Most recently advances in targeting HER2 are focusing on further exploitation on HER2-targeted ADCs drugs. ADC drugs were initially designed to target specific tumor cells, thereby reducing the cytotoxic effects of chemotherapy. ADCs are composed of a tumor antigen-specific antibody conjugated to a cytotoxic drug (payload) via a synthetic linker. Ado-trastuzumab emtansine (T-DM1) is an ADC composed of three key components: (1) trastuzumab, (2) the cytotoxic agent emtansine (DM1), which is a potent microtubule polymerization inhibitor derived from maytansinoids, and (3) a non-cleavable N-maleimidomethyl cyclohexane-1-carboxylate (MCC) linker. This linker is deliberately designed to maintain stability both in the bloodstream and within the tumor microenvironment (TME). T-DM1 has its unique mechanism of action of retaining trastuzumab activity while providing intracellular delivery of DM1 to HER2-overexpressing cells. T-DM1 was approved by FDA firstly in 2013 as a single-agent treatment option for

HER2+ mBC patients who had previously received trastuzumab and a taxane mainly based on the phase III EMILIA trial [28]. T-DM1 also showed significantly prolonged PFS and OS and positive safely profile for heavily pretreated mBC patients in the TH3RESA trial[29].

As the association between pathological complete response (pCR) at surgery and improved disease-free survival and overall survival has been highlighted in the Collaborative Trials in Neoadjuvant Breast Cancer (CTNeoBC) pooled analysis, the neoadjuvant approach is a standard therapy option for operable HER2-positive BC. Dual anti-HER2 blockade of trastuzumab and pertuzumab plus chemotherapy has shown significant improvements in pCR rates when compared with chemotherapy and trastuzumab in the preoperative setting. The phase II TRYPHAENA (NCT00976989) trial investigated neoadjuvant dual trastuzumab and pertuzumab followed by FEC and docetaxel versus FEC followed by docetaxel and dual blockade versus TCHP. The study confirmed the best efficacy achieved by anthracycline-free approaches in terms of pCR and 3-year DFS[30]. A large network meta-analysis also indicates that dual anti-HER2 blockade in conjunction with non-anthracycline-based chemotherapy containing carboplatin is favored for achieving improvements in pCR, event-free survival(EFS), and OS[14]. However, approximately 15% of patients will still experience disease relapse or mortality within a span of 3 to 5 years.

## 1.4 EXPLORATION ON DRUG RESISTANCE AND BIOMARKERS FOR HER2-TARGETED THERAPY

### 1.4.1 Biology of resistance

The clinical effectiveness of HER2-directed treatments in combination with chemotherapy still shows significant variation among patients, despite the availability of various therapeutic options. While some individuals achieve complete tumor regression with HER2-targeted agents alone, others derive no benefit. The diverse response to HER2-targeted drugs has spurred extensive research into the mechanisms governing both response and resistance to trastuzumab. The goal is to identify biomarkers that can aid in selecting the most suitable treatment based on individual tumor characteristics. Several resistance mechanisms have been identified thus far, including HER2 evasion of targeted drugs and the activation of alternative signaling pathways. More recently, innate and adaptive immune mechanisms have emerged as significant contributors to the modulation of HER2-targeted drug effects.

### 1.4.2 Biomarker definition and validation

In the management of breast cancer patients, biomarkers play an indispensable role. Biomarkers are quantifiable traits that serve as indicators of normal biological processes, pathological developments, or responses to various treatments or exposures. A robust biomarker should demonstrate analytical validity, robustness, reproducibility, and clinical utility. Additionally, for practical integration into routine practice, a good biomarker should also be cost-effective and highly accessible to healthcare centers worldwide.

The validation of biomarker assays typically involves several stages: evaluating fundamental assay performance (analytical validation), assessing the assay's performance in its intended clinical context (clinical validation), and confirming its robustness in clinical trials according to predefined criteria (fit-for-purpose validation). This approach enables the establishment of clear acceptance criteria for clinical application (validation of clinical utility). The fit-for-purpose strategy in biomarker development and validation underscores the importance of customizing validation processes to suit the specific intended use of the biomarker[31]. The biomarkers under current exploration for HER2+ diseases are described in detail below.

### 1.4.3 Genomic alterations

Phosphatidylinositol-3 kinase (PI3K) acts as a downstream effector of HER2 signaling. *PIK3CA* mutations can lead to the constitutive activation of PI3K, which occurs independently of HER2 signaling. The activation of *PTEN/PI3K/AKT/mTOR* pathway (i.e. activating mutations) has been reported to associate with resistance to HER2-targeted therapy in preclinical models and in retrospective analysis of randomized clinical studies, with controversial results[32].

Sequencing of tumor DNA in blood samples, often referred to as liquid biopsy or circulating tumor DNA (ctDNA), provides convenient access to tumor-based genetic information at any given timepoint. In some instances, this approach may serve as a viable alternative to traditional tumor tissue biopsy, helping to avoid the delays and potential complications associated with invasive procedures. ctDNA represents a larger portion of the tumor burden compared to circulating tumor cells (CTC)s and might exhibit superior diagnostic sensitivity and specificity for detecting certain gene mutations compared to CTC testing[33, 34]. Though this is particularly relevant in the context of metastatic cancer where acquiring tissue samples can be challenging. ctDNA sequencing has been prospectively employed to identify HER2-mutated non-amplified metastatic breast cancer patients for assessing the efficacy of Neratinib in this population[35]. However, for early breast cancer, dynamic ctDNA monitoring might help identify early metastatic recurrence and track treatment response, especially for those with residual disease (RD). In the ISPY-2 trial, the absence of ctDNA clearance, as detected by using a previously validated personalized ctDNA test containing a panel of up to 16 most clonal somatic variants present in the pretreatment tumor, has been shown to be a significant indicator of inadequate NAC response and heightened risk of metastatic recurrence, whereas its clearance correlated with enhanced survival outcomes, even among patients who did not attain pathological complete response (pCR), regardless of HER2 statue[36].

### 1.4.4 mRNA and protein expression data

HER2 is often heterogeneously expressed, with around 6-20% of BC patients exhibiting varying expression or amplification status within the same tumor (intra-tumoral, spatial) or across tumors from different locations or time points for the same patient (temporal) [37-39]. A recent phase II trial showed a significant association between HER2 heterogeneity (defined

in the study as the presence of ERBB2 amplification in more than 5% but less than 50% of tumor cells within an region or a HER2-negative area identified by FISH on two spatially distinct biopsies) and pCR for patients receiving neoadjuvant T-DM1 plus pertuzumab [37]. In addition, quantitative measurement of HER2 protein and activated EGFR by Reverse Phase Protein Microarray (RPPA) in I-SPY2 trial are also shown to have a positive correlation with response to T-DM1+pertuzumab [40]. Biomarker analysis from EMILIA suggested that HER2 mRNA levels associate with TDM1 efficacy (OS 34.1 months vs 26.5 months in high vs low groups).

### 1.4.5 T-DM1 specific markers

For T-DM1 to exert its antitumor effects, it must be internalized into cancer cells. Inefficient endocytosis of T-DM1 through non-predominant internalization route might lead to a reduced response to the drug[41]. In a preclinical study, it was also found that T-DM1 induced G2-M cell-cycle arrest in sensitive breast cancer cells. However, this effect was not observed in resistant cells. The resistance was found to be dependent on CDK1/cyclinB1, indicating that defective Cyclin B1 induction is involved in acquired resistance to T-DM1 [42]. Also, upregulation of the ATP binding cassette (ABC) transporters acting as efflux pumps might contribute to T-DM1 resistance based on T-DM1 resistant breast cancer cell line studies [43]. As DM1 binds to tubulin, mutations in β-tubulin as well as aberrant expression of different β-tubulin isotypes can be associated with resistance to tubulin-binding agents [44].

### 1.4.6 Multi-gene signatures

First introduced in 2009, PAM50 is a clinically relevant gene expression-based test that offers an intrinsic subtype diagnosis specifically for breast cancer. The assay was based on the expression of 50 genes and demonstrates a high accuracy in identifying diverse intrinsic subtypes compared to original genomic classifications which was relied on approximately 1,900 intrinsic genes.[10]. The Prosigna™ (Veracyte, South San Francisco, USA) assay derived from intrinsic subtypes, has been Food and Drug Administration (FDA)-approved for clinical use since 2013. It has demonstrated reliable analytical performance on formalin-fixed paraffin-embedded (FFPE) breast tumor blocks[45].

All the main intrinsic molecular subtypes of breast cancer (Luminal A, Luminal B, HER2-enriched [HER2-E], Basal-like) can be identified in clinical HER2+ BC, indicating the biologically heterogeneous nature of the disease. HER2-E subtype is highly addicted to the HER2/EGFR signaling pathway. Research findings suggest that intrinsic subtypes within HER2+ breast cancer hold predictive value. Specifically, the prevalent HER2-E subtype, constituting 50-60% of cases, demonstrates a higher probability of achieving a pCR following anti-HER2-based neoadjuvant treatments, with or without chemotherapy, compared to other subtypes[46]. The PAMELA phase II neoadjuvant clinical trial was designed to prospectively validate the predictive value. Patients with stage I-III HER2+ disease were enrolled to receive neoadjuvant trastuzumab and lapatinib. Rates of pCR among

patients with HER2-E subtype was 41% versus 10.0% in non-HER2-E tumors, indicating that a subset of patients might omit chemotherapy [47].

The limitations of research-based PAM50 schemes include the necessity to address batch effects and ensure comparability between gene expression levels across patients. This requirement arises from the application of normalization procedures and gene-centering techniques. Various normalization procedures have been adapted for the method, resulting in different subtype classifications for patients. New bioinformatics approaches, such as Absolute Intrinsic Molecular Subtyping (AIMS) [48], and recent RNA-sequencing-tailored AIMS-based models [49] have been developed to estimate patient subtypes.

It's worth noting that the PAM50 subtype can exhibit variations across different racial groups. A recent comprehensive genomic study revealed that in Asian HR+HER2+ patients, HER2 amplification is more prevalent compared to white race counterparts[50]. These findings underscore the importance of considering such differences in future clinical trial design to ensure that treatment strategies are appropriately tailored to specific patient populations. Another noteworthy aspect is the rapid phenotypic changes observed in response to treatment. Multiple studies have demonstrated that HER2-E tumors can transition into Luminal A type following HER2-targeted chemotherapy and the change is rapid and reversable [47, 51]. This phenomenon underscores the complicated dynamic nature of breast cancer subtypes. Additionally, for patients with residual disease, PAM50 subtypes and PAM50-derived ROR scores assessed on surgical samples may help identify individuals who require treatment escalation, highlighting the importance of future validation studies in this area[51].

Association between immune-related gene expression signatures and response to anti-HER2 treatment has been observed in multiple neoadjuvant [52] and adjuvant trials [53]. In a recent study comparing the prognostic and predictive capabilities of patients with early-stage ERBB2/HER2-positive breast cancer treated in two clinical trials (CALGB 40601 and PAMELA), it was discovered that multiple B-cell-related signatures exhibited stronger associations with pathological complete response (pCR) and event-free survival (EFS) compared to tumor-infiltrating lymphocytes (TILs), which primarily represent T cells. This suggests that when both TILs and gene expression data are available, immune-related signatures demonstrate superior prognostic value [54].

First developed and introduced in 2020 and subsequently updated in 2022, HER2DX genomic assay is a 27-gene assay for early-stage HER2-positive breast cancer, which incorporates clinical data and evaluates the expression of four signatures: immune, proliferation, luminal, and HER2. HER2DX has emerged as a useful tool for predicting both long-term prognosis and likelihood of pCR in patients with HER2-positive early breast cancer [55, 56]. HER2DX has demonstrated robust concordance both within individual laboratories and across different facilities, as demonstrated in an analytical validation study [57]. Two HER2DX scores were developed independently: a prognostic HER2DX risk score and a HER2DX pCR likelihood score. The HER2DX risk score was trained using data from 434 patients enrolled in the phase III adjuvant Short-HER trial, while the HER2DX pCR

likelihood score was trained on a cohort of 116 patients at the Hospital Clinic of Barcelona. HER2DX risk score has been shown to provide independent prognostic information in 1341 patients across five datasets[56]. A collective analysis of seven prospective studies validated that the HER2DX pCR likelihood score exhibited a significant association with pCR in all patients, regardless of the presence or absence of dual HER2 blockade [58]. Only one study has assessed longitudinal HER2DX scores, revealing significant changes in patients before and after TPH treatment[59]. The Spanish clinical guidelines for early-stage breast cancer have adopted HER2DX as useful tool for escalation and de- escalation treatment strategies (IIA recommendation). However, future prospective validation to assess clinical utility is warranted.

The 41-gene TRAstuzumab Risk (TRAR) prediction model is another genomic tool designed to predict the benefit of adjuvant trastuzumab. It was trained using data from 53 patients treated with adjuvant chemotherapy plus trastuzumab. However, further external validation on a larger scale is required for this model [60].

### 1.4.7 CTC/ct miRNA

Using non-invasive procedures like liquid biopsy to identify predictive biomarkers has been a promising area of development for decades.

In breast cancer patients, disseminated tumor cells were first identified in the bone marrow and were found to be significantly correlated with poor prognosis[61]. Due to the invasive nature of bone marrow aspirations procedures, blood-based CTC monitoring tools are more acceptable and can be repeated for dynamic monitoring. Researchers detected and characterized CTCs before and after neoadjuvant HER2-targeted chemotherapy in the GeparQuattro trial, showing the CTC abundance is often low in primary breast cancer tumors. While the decline in CTC incidence during treatment did not correlate with clinical characteristics or primary tumor response, the HER2 status detected on the CTCs could be valuable for stratifying and monitoring HER2-directed therapies[62].

Circulating microRNAs (miRNAs) are a unique group of endogenous non-coding small RNA molecules secreted into the circulation. They have been shown to correlate with the degree of tumor progression and exhibit different expression patterns at various stages of cancer. Circulating miRNAs identified at the end of neoadjuvant treatment in NeoALTTO study is reported to be prognostic and might perform a role for salvage adjuvant therapy[63].

### 1.4.8 Imaging-derived markers

The Positron Emission Tomography/Computerised Tomography (PET/CT) imaging has gained recognition as a versatile staging, prognostic and predictive tool in the past few years. By assessing metabolic response using The 18F- F-fluorodeoxyglucose(FDG) PET/CT, it offers valuable insights into the likelihood of response to HER2-based therapy combined with chemotherapy. Initial findings from the NeoALLTO trial highlight its potential to differentiate patients with high and low probabilities of response, indicating its promising role as a pharmacodynamic biomarker[64]. The maximum standardized uptake value (SUVmax)

changes after short-term exposure to treatment are strongly prognostic for survival, highlighting the potential of PET/CT for patient selection for treatment de-escalation [65, 66]. $^{89}$Zr-trastuzumab or $^{89}$Zr-pertuzumab PET/CT are PET radiotracers that allow visualization of HER2-positive lesions. The utilization of probes holds significant potential in monitoring the response to HER2-targeted treatments [67].

### 1.4.9 Multimodality Machine Learning modeling

The integration of Artificial Intelligence (AI) into scientific discovery is progressively growing, serving to enhance and expedite research. An innovative multi-omic machine learning model was built on digital pathology, genomic and transcriptomic features extracted from clinical data of 168 patients treated with neoadjuvant chemotherapy, either combined with or without HER2-targeted therapy [68]. It is worth noting that the model can be tailored to incorporate additional features and applied to other types of cancer. Validation in larger cohorts is necessary, with potential challenges including data quality and stewardship.

## 1.5 THE IMMUNOLOGY OF HER2-POSITIVE BC AND EMERGING BIOMARKERS

Apart from potential resistance mechanisms mentioned above, the interaction between tumor cells and tumor microenvironment components in the stroma, especially innate and adaptive immune cells, also plays a vital role in resistance to HER2-targeting.

Relative to melanoma and lung cancer, breast cancer is often characterized as immunologically “cold” tumor. However, the immune landscape of breast cancers is dynamic and heterogeneous. HER2-positive BC in particular is mostly characterized by the wound healing (marked by elevated expression of angiogenic genes, high proliferation rate and Th2 cell bias in the adaptive immune infiltrate) and the IFN-γ dominant subtype (distinguished by the highest CD8+ T cell presence, the most significant M1/M2 macrophage polarization, and greatest TCR diversity) immunogenomic subtypes [69].

The current methods used to study the TME are briefly described hereunder:

### 1.5.1 TILs counting on H&E slides

First introduced by Denkert in 2010, histopathologic analysis of TILs on HE stained sections showed robust correlation with the mRNA expression levels of lymphocyte markers, and TILs assessed at diagnosis are significantly associated with a more favorable response response to NACT [70]. Subsequent research has confirmed the positive predictive and prognostic significance of TILs in both neoadjuvant and adjuvant settings, particularly notable in TNBC and HER2+ patients[71]. Emerging data on pre- and post-treatment TILs in paired tissues during neoadjuvant chemotherapy have reported on the prognostic indication with TILs change. In 57 patients receiving FEC (fluorouracil, epirubicin and cyclophosphamide) with trastuzumab+/- lapatinib in CherLOB trial, no intertumoral TILs (iTILs) or stromal TILs change was observed [72]. In another French cohort of 175 patients, median TILs decreased in most cases and the variation of TIL levels was strongly associated

with pCR[73]. Change of TILs level before and after NAT was also accessed in a retrospective study of HER2+ and TNBC patients, low pre-NAT TILs were associated with RFS in TNBC but not HER2+ disease[74]. Methodological Post-NAC TILs enumeration in residual cancer and pCR specimens needs standardization. Digital counting, cut-offs exploration and validation in prospective trials are future research directions.

### 1.5.2 Immunohistochemistry

The "Immunoscore" scoring system quantifying the densities of CD3+ and CD8+ T-cell effectors within the tumor center and its invasive margin has been validated to predict survival of early and advanced stage colorectal cancer patients and has been integrated as important diagnostic criteria for colorectal cancer according to the 5$^{th}$ edition of World Health Organization (WHO) classification [75]. In a small cohort including 18 HER2-positive BC patient receiving NACT, those with higher Immunoscore tended to achieve pCR [76].

Presence of CD8+ T cells[77] and absence of FOXP+ Tregs [78] are associated with a significant reduction in the relative risk of death in HER2+ BC. Cautions must be taken when interpreting the prognostic role of single IHC markers in retrospective studies, as the marker can be associated with other prognostic factors. The reproducibility of methods used regarding tissue types and antibodies etc. also needs further validation.

### 1.5.3 Genomic and Transcriptomic Profiling

The analysis of genetic and transcriptomic characteristics of bulk tumor tissues has greatly increased our understanding of the various types of immune cell phenotypes involved in the breast cancer biology. The CIBERSORT tool was used to analyze bulk gene expression profiles of 10,988 breast tumors from 56 publicly available datasets [79]. Activation of specific immune subsets was found to be associated with favorable outcome in ER−/HER2+ patients [80]. Bulk sequencing is restricted to data from genome- transcriptome- wide molecular assays and lacking cell phenotype distribution information.

### 1.5.4 Single-cell analysis

Single-cell RNA (scRNA) and DNA (scDNA) sequencing methods have enabled precise cell mapping and provide individual immune cell subtyping and spatial information. Single-nucleus sequencing (SNS) together with bulk sequencing were used to study adaptive clonal evolution and acquired transcriptional reprogramming in response to NAC in TNBC [81]. Another study revealed that conventional HER2+ tumors might be classified as Luminal B type by individual tumor cell characteristics, and distinct immune system status with different activating and suppressive gene expression signatures can be identified in different patients[82].

Molecular profiling with mass cytometry (CyTOF) has also been used to identify immune checkpoint molecules associated with high Programmed Cell Death Protein 1 (PD-1) expressions in T cell populations [83], for example PD-1$^{high}$CD8+ T cells expressed TIM-3,

CTLA-4 , HLA-DR and CD38, which is a phenotype associated with T cell exhaustion and anti-PD-1 therapy response previously in melanoma.

### 1.5.5 Multiplex Tissue Analysis

Different platforms and assays such as OPAL multiplex immunohistochemistry (mIHC), GeoMx Digital Spatial Profiling (DSP),multiplexed ion beam imaging (MIBI), co-detection by indexing (CODEX), and image mass cytometry (IMC) have been used to investigate the tissue architecture and evaluate the relationships of single cells in a spatial context [84]. These analyses are not limited to the immune microenvironment, but also include other components thought to promote cancer progression, such as the tumor vasculature and fibroblasts. Studies have suggested that stroma TILs(sTILs) that reside close to the tumor cells may exhibit a distinct molecular phenotype compared to sTILs situated farther away from the primary tumor bulk[85]. In the DAISY trial, a significant decrease of PDL1 expression was detected by multiplex immunofluorescence in HER2-positive mBC and Following treatment with T-DXd as a single agent, the reduction in PDL1-positive cells was linked to objective response, whereas there were no changes observed in other intratumoral lymphocytes. [86]. A DSP multiplex proteomic assay employing a panel of up to 40 markers, including markers of immune response, HER2 pathway members, and other cancer-related markers, was utilized to investigate the temporal and spatial heterogeneity of tissue from baseline, on-treatment, and surgical samples obtained from 28 HER2+ breast cancer patients enrolled in the neoadjuvant TRIO-US B07 clinical trial. Single marker of on-treatment CD45 A single marker, CD45, identified during on-treatment assessment, was discovered and subsequently validated to exhibit strong performance in predicting pathological complete response (pCR) with high accuracy. Measuring the percentage of CD45-positive cells via on-treatment immunohistochemistry could represent a cost-efficient method for predicting HER2-targeted therapy treatment response[87]. Another study, utilizing 64 protein targets on the GSP platform, demonstrates temporal heterogeneity in primary and metastatic HER2+ tumors. It reveals that stromal and tumor-localized immune cells in the tumor microenvironment are more active in primary disease compared to metastatic disease[88].

## 1.6 PROLIFERATION AS A CANCER HALLMARK

In early breast cancer, assessing proliferation alongside tumor size, grade, nodal status, and receptor status can serve as a prognostic indicator[89]. Current knowledge about the role of proliferation markers in breast cancer is presented below.

### 1.6.1 Mitotic count

Among the three components used for grading breast cancer, mitotic count has demonstrated the highest prognostic value, indicative of tumor growth rate[90]. The traditional method of mitotic count involves examining tumor tissue under light microscopy within ten high-power fields, focusing on areas with the highest density of mitotic activity, known as mitotic hotspots. Mitotic counts are then categorized using specific cut-offs into scores of 1, 2, or 3. Due to the subjectivity of defining mitotic hotspot, mitosis appearance variance and slides'

quality, substantial interobserver-variability can exist. Also, the process is quite time-consuming and requires trained personnel. Recently, artificial intelligence (AI) based tools have been developed as an alternative for reliable large-scale analyses on digitally scanned slides (whole-slide images; WSI) [91].

In a retrospective cohort of 298 TNBC patients, both manual and automatic assessments of mitotic count were assessed, with none of the cut-off values shown to be prognostic [92]. Similar non-significant value of automated mitotic count was seen in HER2+ BC, while in HR+HER2- BC, mitotic count was found to be prognostic for OS and RFS both when analyzed as a continuous variable or when applying cut-offs based on the Nottingham criteria or median values. [93].

### 1.6.2 Ki67 index

The nuclear antigen Ki67 is commonly employed as a marker of proliferation in breast cancer. The Ki67 protein is predominantly expressed during G1, S, G2 and M phases, but not during G0 phase. A large number of studies have demonstrated the prognostic significance of baseline Ki-67 as a biomarker, indicating its utility in predicting response and clinical outcomes[94]. Additionally, longitudinal assessment of Ki67 was used as a pharmaco-dynamic marker to elucidate potential benefit of neoadjuvant lapatinib in the phase II MAPLE Trial in HER2-positive and HER2-negative patients [95]. Ki67 in residual disease also has prognostic value for patients after neoadjuvant chemotherapy [96]. As a result, ongoing trials (such as PHOENIX [NCT03740893]) have adopted Ki67 as an endpoint for a novel DNA damage response inhibition drug for neoadjuvant chemotherapy-resistant patients. Nevertheless, standardization of tissue handling and processing is still necessary to enhance the reliability and clinical utility of Ki-67 testing[94]. Currently, Ki67 measurement at central laboratories involves using a validated assay that has been demonstrated to be highly reproducible across different pathologists and laboratories. This assay utilizes an automated staining protocol and standardized scoring method [97] or through artificial intelligence-aided strategies might potentially help overcome the challenge [98]. Like other markers, the importance of spatial heterogeneity (intratumoral and intertumoral) should be further investigated.

### 1.6.3 Thymidine kinase-1 (TK1)

TK1 is an enzyme involved in the pyrimidine salvage pathway, which catalyses the phosphorylation of thymidine to thymidine monophosphate, and plays a vital role in DNA synthesis. TK1 is low or undetectable in resting cells, increasing during G1/S transcription and peaking at late S-phase in proliferating cells. Circulating TK1 is increased in BC patients compared to healthy subjects [99]. Multiple studies have linked higher baseline and on-treatment serum TK1 activity to high-risk clinicopathological features and poorer outcomes of early BC patients [100, 101]. Conventional radiolabel-based assays have limited reliability and reproducibility for the quantification of TK1 expression levels and activity. TK1 immunoassays, enzyme activity assays and aptamer-based sandwich assays have also been

developed. Enzyme activity-based assays are based on use of different TK1 substrate and the detection of labelled deoxythymidine monophosphate (dTMP) derivatives. These technical advances have made TK1 a more feasible target for studies.

TK1 kinetics act as a marker for long-term prognosis and a pharmacodynamic predictive marker for current drugs used in BC are currently under investigation. In TNBC patients receiving NACT, greater increase in TK1 activity after two cycles of chemotherapy was associated with improved event-free survival [102]. TK1 serum enzymatic activity change in response to palbociclib can be used to predict tumor Ki-67 response [103]. Recently, a prospective monitoring trial is ongoing to investigate whether serial TK1 monitoring within first 48-weeks of 1st line ET+CDK4/6 treatment will affect physician-reported intended change in imaging testing interval in HR+HER2- MBC patients (NCT04968964). Future studies to further elucidate the interplay of TK1 and other proliferation makers as well as prospective validations are needed. Data regarding predictive or prognostic role of TK1 kinetics in early HER2+ BC are limited.

### 1.6.4 Proliferation-associated gene signatures and cell-cycle-regulated genes

Distinct groups of genes exhibiting high expression levels was identified in highly proliferative breast tumor cells as compared to samples from normal breast tissue as early as 1999. Multiple proliferation gene expression signatures have been developed using different platforms, gene selection algorithms, sample types, and patient datasets. However, most of these signatures lack external validation, and their clinical applicability remains controversial. Proliferation-associated genes and cell-cycle related genes are often overlapping but not all correlated [104].

### 1.6.5 Other approaches

Other nuclear antigens such as proliferating cell nuclear antigen (PCNA), Cyclins and cyclin-dependent kinases (CDKs) assessed by immunohistochemistry, argyrophilic nucleolar organiser regions(AgNORs) assessed and quantified by silver staining techniques on tumour tissues have been developed to evaluate the proliferation status in breast cancer [105].

# 2 AIM OF THE THESIS

The overall objective of this thesis was to explore potential predictive and prognostic biomarkers for HER2-positive breast cancers.

In **paper I**, our objective was to explore the dynamics of TILs following neoadjuvant therapy. We assessed the extent and direction of TILs changes and their association with therapy response prediction and survival outcomes.

In **paper II**, we aimed to investigate the potential predictive and prognostic value of baseline and the dynamic changes of serum TK1 activity levels in patients with HER2+ early BC patients.

In **paper III**, we aimed to validate the predictive and prognostic value of PAM50 intrinsic subtypes and explore the subtype conversion during neoadjuvant HER2-targeted treatment.

# 3 MATERIALS AND METHODS

The detailed methods are specified individually in each publication. Here, we provide an overview of the most used methods in the present thesis.

## 3.1 PATIENT COHORT

The PREDIX HER2 clinical trial is an academic, multicenter, phase II, randomized clinical trial (principal investigator Assoc. Prof. Thomas Hatschek). The study is registered at Clinicaltrials.gov, Identifier: NCT02568839. Eligible patients had a newly diagnosed HER2+ BC, stage II or III (tumor >2cm and/or axillary node metastasis) and were randomized (1:1) to 6 three-weekly cycles of the combination of trastuzumab, pertuzumab and docetaxel (DHP, Arm A) or trastuzumab-emtansine (T-DM1, Arm B) as neoadjuvant (preoperative) therapy. Patients who did not respond to treatment after 2 cycles switched to the other study arm. Post-operative anthracycline-based chemotherapy was given in both groups. The study has a strong translational focus; blood samples and core tumor biopsies were obtained from all patients at baseline, after 2 cycles and at surgery and the response to treatment was monitored with radiological assessments (mammography, ultrasound or Magnetic Resonance Imaging [MRI]) every 2 cycles as well as with FDG-PET at baseline and after 2 cycles. A total of 202 patients were randomized and 197 patients comprise the intention-to-treat population. Accrual was completed in Q4 2018, and follow-up is ongoing. The primary endpoint of the trial is the rate of pathological complete response (pCR), defined as ypT0 or Tis ypN0. pCR has the advantage of being readily available at the time of surgery without need of extended follow-up and has been demonstrated to be a good surrogate of long-term outcomes in HER2+ BC[106]. Primary efficacy analysis, 5-year survival outcomes, health-related quality of life (HRQoL) results, exploratory analysis of TILs and longitudinal PET evaluation results have been previously published[66, 107].

## 3.2 TISSUE AND BLOOD

RNA, DNA and peptides were extracted from fresh frozen tumors from baseline, on-treatment and surgery using AllPrep DNA/RNA/Protein mini kit (Cat. No. 80004, QIAGEN, Germany). Germline DNA was extracted from patients' peripheral blood samples using the FlexiGene DNA kit (Cat No. 51206, QIAGEN, Germany). Quality control (QC) was performed for the estimated the yield and integrity of the extracted RNA and DNA. Concentration and the A260/A280 and A260/230 absorbance ratios (purity estimation) were obtained using the spectrophotometer NanoDrop ND-1000 (Saveen Werner, Sweden). To further estimate RNA and DNA concentration, the Qubit™ dsDNA BR (Broad Range) Assay kit (Cat No Q32853, Invitrogen, USA) or dsDNA high sensitivity (HS) Assay kit (Cat No Q32854, Invitrogen, USA) for DNA, and QubitTM RNA BR (Broad Range) Assay kit (Cat No 10211, Invitrogen, USA) or RNA high sensitivity (HS) Assay kit (Cat No Q32855, Invitrogen, USA) for RNA respectively, were performed using the Qubit® 3.0 Fluorometer (ThermoFisher Scientific, USA). The integrity of RNA and DNA was estimated based on RNA Integrity Number (RIN) and DNA Integrity Number (DIN) values, using the Agilent

Tapestation 2200 System (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions.

### 3.3 RNA SEQUENCING

RNA sequencing (RNA-seq) libraries were created using the Illumina Stranded Total RNA library preparation kit with Ribo-Zero Plus treatment (catalog numbers 20040525/20040529, Illumina Inc.), starting from 100ng of total RNA whenever available. We employed unique dual indexes (catalog numbers 20040553/20040554, Illumina Inc.) for sample identification. The RNA-seq library preparation procedure followed the manufacturer's protocol (reference number: 1000000124514) and was sequenced on the NovaSeq6000 system (v1.5 reagents, S4 flowcell) with paired-end reads of 150bp. To mitigate potential confounding between batch and biological effects, all samples were randomized into different batches (i.e., sequencing lanes and runs). Seven samples encountered issues (low concentration) during library preparation, leading to their exclusion from downstream analysis.

### 3.4 DATA PROCESSING

We employed the nf-core/rnaseq pipeline (v3.3)[108] to analyze RNA-seq data, adhering to best practices. This pipeline, built on Nextflow (v21.04.3)[109], facilitates reproducible analysis. Briefly, the raw sequencing data were subjected to quality control (QC) with FastQC (v0.11.9). TrimGalore (v0.6.6) was used for adapter and quality trimming with the following parameters --clip_r1 1, --clip_r2 1, --three_prime_clip_r1 1, and --three_prime_clip_r2 1 . The QC-passed and trimmed reads were mapped by STAR (v2.6.1d)[110] to the NCBI GRCh38 genome, as provided by iGenomes. Transcript expression levels (measured in Fragments Per Kilobase per Million reads, FPKM) were estimated using Stringtie (v2.1.7)[111]. Finally, quantified transcripts were summarized to the gene level using the R/Bioconductor package tximport (v1.30.0)[112].

### 3.5 PAM50 GENE EXPRESSION ANALYSIS

The Prediction Analysis of Microarray with the 50-gene classifier (PAM50) subtype prediction classifier was utilized alongside benchmark RNA-seq-based SSP models (sspbc-subtype and sspbc-PAM50 models), developed from a comprehensive population-based cohort comprising nearly 8000 patients[113]. The proliferation score by mean expression of the 11-proliferation-related genes in the PAM50 assay as a continuous variable were also evaluated at baseline.

### 3.6 STATISTICS

The statistical methods employed in this thesis are elaborated upon in the "Materials and Methods" sections of each paper.

In paper I, to compare time-to-event variables based on the direction of tumor-infiltrating lymphocytes (TILs) changes, a meta-analysis was conducted. Initially, Hazard ratios (HRs) and their errors were transformed into their logarithmic counterparts, followed by inverse variance method for reconversion back into the HR scale. If adequate data on time-to-event variables were not directly available from primary studies, data extraction followed the method described by Tierney et al. A pooled analysis was conducted only when data from at least three primary studies were available in sufficient quantity for analysis.

Statistical heterogeneity among the studies was assessed using Q statistics, with the magnitude of heterogeneity determined using the I2 statistic. A p-value < 0.10 or an I2 value greater than 50% indicated substantial statistical heterogeneity. Given the significant clinical diversity among eligible studies, all meta-analyses, except those with time-to-event variables as the outcome of interest, were performed using random-effects models.

Publication bias was evaluated qualitatively using a funnel plot.

In paper II, violin plots were generated to display serum thymidine kinase 1 (TK1) activity by time point in all patients. Undetectable TK1 activity (< 45 DuA) at baseline and extreme high TK1 activity (> 3081 DuA) were represented as 45 and 3081, respectively, in the analysis descripting TK1 levels over time. Line plots illustrated the levels of serum TK1 activity by time point in all patients and by treatment groups.

The distribution of TK1 levels in standard clinicopathological subgroups as categorical variables was compared using either the Chi-square test or Fisher's exact test. For continuous variables, differences in means or medians between groups were assessed using the t-student test or ANOVA (parametric) or the Mann-Whitney test or Kruskal-Wallis test (non-parametric) as appropriate.

Univariate and multivariate logistic regression and Cox regression analyses were employed to assess the association between sTK1 activity and pCR, EFS, and DFS. Factors that were statistically significant in the univariate analyses and/or clinically relevant were included in the multivariate model. Kaplan-Meier survival estimates were calculated for EFS, DFS, and recurrence-free survival (RFS).

In Paper III, the association of PAM50 molecular subtypes with pCR, EFS, and RFS was examined using univariate logistic regression and Cox regression. Multivariable analyses, including clinically relevant factors such as treatment arm, tumor size, ER status, and node status, were conducted to assess adjusted odds ratios and hazard ratios.

All statistical tests were two-sided, and the significance level was set to <0.05.

The statistical analyses, both descriptive and inferential, were performed using R version 4.2.1 software (R Foundation for Statistical Computing, Vienna, Austria) or GraphPad Prism version 8.0 (GraphPad Prism, San Diego, CA, USA).

### 3.7 META-ANALYSIS SPECIFIC STATISTICS

For the analysis of pooled expression of tumor-infiltrating lymphocytes (TILs) in matched breast cancer patients, studies presenting TILs as a categorical variable were included. A random-effects model was employed to calculate the pooled high-level TILs and corresponding 95% confidence interval (CI) for sTILs before and after treatment for different breast cancer subtypes (HER2-positive, triple-negative breast cancer [TNBC], luminal, and not specified [containing studies that recruited all breast cancer patients without limitation of molecular subtype]).

Subsequently, we calculated an overall effect estimate using the Odds Ratio (OR) with a 95% confidence interval (CI), employing the DerSimonian and Laird method[114].

When TILs were presented as continuous variables, pooled analyses comparing TILs expression pre- vs. post-treatment were conducted using standardized mean differences (SMD) with 95% confidence intervals (CI) for each study. Subsequently, these values were pooled to determine the effect size of the difference in TILs between pre- and post-treatment groups.

# 4 RESULTS AND DISCUSSION

## 4.1 PAPER I

In paper I, we investigated the dynamics of tumor-infiltrating lymphocytes (TILs) during neoadjuvant therapy (NAT) in matched paired breast cancer tissues. Additionally, we explored the prognostic value of ΔTILs, defined as the change in median/mean lymphocyte density between pre- and post-treatment samples. This ΔTILs information was either directly reported in the papers or was calculated manually with relevant data extracted by the two researchers.

In the pooled analysis of pre- and post- TILs, we observed a consistent decrease in TILs post-treatment across all breast cancer subtypes. However, it's worth noting that the number of studies including the Luminal subtype was not sufficient for analysis. Among the twenty-one studies that reported tumor-infiltrating lymphocytes (TILs) as a continuous variable, positive standardized mean difference (SMD) values were noted in the HER2-positive, triple-negative breast cancer (TNBC), and unspecified subgroups. However, no pooled analysis was conducted for the Luminal subtype due to the limited availability of data, with only two studies providing relevant information.

The definitions of disease-free survival (DFS) or recurrence-free survival (RFS) vary among the studies included. Specifically, two studies defined RFS as the time from the date of primary surgery until the date of disease recurrence. In another study, RFS was defined as the time from diagnosis to the occurrence of locoregional recurrence, distant metastasis, or death from any cause. However, one study did not provide a clear definition of RFS. We considered these definitions to be similar and treated them as a single combined endpoint. In the pooled analysis, an increased ΔTILs was considered positive indicator of DFS/RFS (HR=0.59, 95% CI=0.37, 0.95, p = 0.03). Similar analyses in other subtypes were not feasible due to the limited number of studies available.

Our study stands as a comprehensive summary on the current evidence assessing TILs dynamics in matched paired BC patient samples before and after NAT. It offers valuable insights into how TILs could potentially be leveraged to optimize neoadjuvant treatment, particularly for patients with TNBC and HER2+ disease.

The main finding of the meta-analysis is the obvious trend towards decreased TILs after NAT observed across breast cancer subtypes. Even though this trend is modest and often not statistically significant in the conducted analyses, it remains a consistent observation and might stand as a true effect. The explanation for this finding can be either that TILs decrease concurrent with the treatment effects of cytotoxic chemotherapy, as this type of therapy is generally acknowledged to be immunosuppressive, or may simply be due to analytical issues, as surgical tissue samples are typically larger than biopsy samples, potentially diluting the concentration of TILs observed. Either way, the TILs dynamics under chemotherapy treatment worth further exploration. As we have entered in the immunotherapy era, the best

combination and sequence pattern of ICIs with chemotherapeutics remains to be elucidated. Studies have suggested that different chemotherapeutic agents might affect immune cell surface marker differently and induce a stronger immunogenicity[115]. Reversely, other studies have suggested immunotherapy might induce TILs relocation from stroma to tumor nests[116].

Regarding the potential prognostic role of dynamic TILs, a potential effect of increased TILs during NACT with better prognosis was seen specifically in TNBC patients. The dynamic status of TILs could potentially serve as an early biomarker for prognostic purposes or for guiding treatment optimization strategies in patients with TNBC.

Our study has several notable limitations. Firstly, all included studies were retrospective in nature, which may introduce inherent biases. Additionally, the sample sizes in these studies were limited, potentially affecting the robustness of our findings. Furthermore, our analysis relied on published study-level results rather than individual patient data, which may have influenced the precision of our estimates. Furthermore, it's important to acknowledge the lack of current methodological standards for post-neoadjuvant chemotherapy TILs enumeration in residual cancers. Moreover, discrepancies in the methods used to assess TILs post-NACT among studies pose another challenge. While some studies counted TILs only in residual disease, others included TILs in the stroma from patients with pCR. Additionally, the use of tissue microarrays (TMAs) in some studies, which represent only a small portion of tissue, may introduce bias in heterogeneous tumors. The diversity of therapeutic regimens used as NACT among eligible studies also adds to the heterogeneity observed. Given the high between-study heterogeneity, we used random-effects models to mitigate the impact of heterogeneity. These limitations underscore the complexity of our analysis and highlight the need for careful interpretation of our findings.

## 4.2 PAPER II

In paper II, our investigation focused on exploring the potential predictive and prognostic value of baseline and serial levels of serum TK1 in patients with HER2-positive early breast cancer enrolled in the PREDIX HER2 trial.

In sequential samples, serum TK1 (sTK1) activity was generally lower at baseline for most patients. It showed a significant increase after one cycle of treatment, remained relatively consistent during the neoadjuvant treatment, and decreased at the end of adjuvant treatment. Following this, the median sTK1 activity level decreased to a level similar to baseline during the 1-year follow-up period. The fluctuation of median sTK1 activity from baseline to visit 2 was observed to be higher in the DHP arm compared to the T-DM1 arm. In the DHP arm, sTK1 activity remained consistently high, above 1000 DuA, throughout the treatment period, with a decrease observed at the end of treatment (EoT). Conversely, in the T-DM1 arm, sTK1 remained at an intermediate high level of around 1000 DuA during treatment.

sTK1 activity values were categorized into three groups: undetectable (<45 DuA), low (45 DuA ⩽ value ⩽ median), and high (> median), based on the median value at baseline. At following timepoints, patients were subsequently classified into three groups based on the median value of sTK1 at each time point: low (< median), high (median ⩽ value ⩽ 3081 DuA), and out of range (> 3081 DuA).

There was a notably higher prevalence of out-of-range (saturated) sTK1 activity observed in patients at visits 2 and 4 in the DHP arm compared to the T-DM1 arm. Conversely, a greater proportion of patients in the DHP arm exhibited low sTK1 activity at visit EoT, while no significant difference was noted at visit FU1. The median (IQR) sTK1 levels over time exhibits different patterns for pCR and non-pCR group, however no significant effect was seen with sTK1 activity level with pCR status in adjusted logistic regression model.

Regarding long-term prognostic implications, sTK1 levels at baseline and visit 2 were not found to be associated with EFS. To explore the prognostic value of sTK1 at the end of adjuvant treatment (visit EoT) with DFS, sTK1 activity levels at visit EoT were categorized into high and low groups using both the median value and an established cutoff of 250 DuA. Patients exhibiting elevated sTK1 levels showed enhanced disease-free survival (DFS); nonetheless, this disparity did not achieve statistical significance in multivariable models adjusted for variables including pathological complete response (pCR), Ki67, treatment arm, tumor grade, tumor size, estrogen receptor (ER) status, and node status. Finally, within a subset of non-pCR patients, we examined the relationship between sTK1 levels at visit EoT and survival outcomes. Although a trend was observed, it was not statistically significant.

The current study is the first to assess dynamic changes of sTK1 during different phases of HER2-positive breast cancer in a large cohort of patients recruited in a prospective clinical trial. Baseline sTK1 activity was not correlated with any patient characteristics. Our main finding is the large increase of sTK1 after two cycles of treatment, which is consistent with our previous findings in HER2-negative tumors treated with chemotherapy but contrasting with data reported on neoadjuvant endocrine-treated BC.

This could be attributed to several factors: effective targeted treatment inducing cancer cell death and subsequent release of cytosolic sTK1 into the bloodstream, or effective chemotherapy inhibiting the de novo dTMP synthesis pathway and activating the salvage pathway, resulting in increased sTK1 uptake and detection of exocytosis/exosome TK1 in the blood. Hence, sTK1 may serve as a metabolic marker, as evidenced by previous clinical and preclinical studies. Larger metabolic change might be indicated in DHP arm.

Similarly, despite previous findings demonstrating a notable early increase in serum sTK1 during neoadjuvant therapy for HER2-negative breast cancer, particularly in highly proliferative tumors, and its correlation with improved survival outcomes, our current study did not reveal any association between sTK1 levels at any timepoint during neoadjuvant therapy or sTK1 kinetics with long-term survival in HER2-positive breast cancer. The reasons behind this discrepancy in prognostic value for HER2-positive breast cancer remain

unclear. Further investigation is warranted to elucidate whether this lack of prognostic value in HER2-positive breast cancer is attributable to factors such as the small sample size with few events, limitations in the detection range of the assay, or specific biological characteristics of the disease.

The potential prognostic value of sTK1 for patients with residual invasive HER2-positive breast cancer is an intriguing finding of our study. Currently, trastuzumab emtansine is recommended as post-neoadjuvant salvage therapy for such patients. However, it's worth noting that 80% of patients treated with trastuzumab in the KATHERINE trial were disease-free at the 7-year follow-up[117]. This suggests that some patients may be overtreated with trastuzumab emtansine, leading to higher toxicity and increased costs.

We illustrate for the first time the sTK1 activity levels following surgery in HER2-positive BC with a RD and showing post-surgery sTK1 monitoring might identify distinct patients with different survival outcome. We hypothesize that combining the Residual Cancer Burden index with sTK1 could enhance prognostic value, potentially sparing patients with an excellent prognosis from unnecessary salvage treatment.

However, it is crucial to acknowledge that our findings should be regarded as hypothesis-generating given their exploratory nature and the limited number of post-surgery relapses. Nonetheless, the unmet clinical need to better stratify patients with residual invasive disease highlights the importance of validating our findings in a larger cohort in the future.

### 4.3 PAPER III

In paper III, we further validated the predictive and prognostic value of the PAM50 HER2-E subtype in HER2-positive disease Additionally, we described the subtype changes occurring during treatment.

After quality control for both sample and bioinformatics, PAM50 results were available for nearly all patients from pretreatment samples. At baseline, 55% of patients were classified as HER2-E, followed by 18% each for both luminal A and luminal B, with Basal-like being 8%. As expected, there were more Luminal-like tumors observed in hormone receptor-positive patients compared to HR-negative patients.

Patients with HER2-E tumors exhibited a higher histological grade and less frequent lymph node involvement at diagnosis compared to those with non-HER2-E tumors. Median sTILs enumeration was higher in HER2-E patients compared to non-HER2-E patients. Among non-HER2-E samples, the highest levels of sTILs were observed in the Basal-like subtype.

HER2-E tumors exhibited higher rates of pathological complete response (pCR) compared to non-HER2-E tumors. Specifically, the overall pCR rate was 76% among all patients, with 73% observed in the DHP arm and 79% in the TDM-1 arm. Basal-like (BL) tumors displayed the lowest pCR rate, with 4.6% in the total patient population, 2.3% in the DHP arm, and 7.0% in the TDM-1 arm. Baseline HER2-E subtype was significantly associated with pCR

(adjusted odds ratio [ORadj]=4.05; 95% confidence interval [CI], 2.01-8.36; $p < 0.001$) and event-free survival (adjusted hazard ratio [HRadj]=0.26; 95% CI, 0.09-0.73; $p = 0.011$). This trend remained consistent across both treatment arms.

Longitudinal PAM50 molecular subtype changes were explored by comparing baseline, visit 2 and surgery timepoints in the trial. Most patients with available results were identified as normal-breast-like (NBL) type, around 17% and 16% remained HER2-E and Luminal-like, while very few were identified as BL. Similar results were observed at the time of surgery. The dynamics in paired patients were illustrated by Sankey diagram in the figures (see Paper III). These findings are exploratory due to the limited number of samples available post-baseline, and it's notable that patients who experience an RD and have adequate tissue material tended to have biomarker results at cycle 2 (94[90%] patients tissue availability for non-pCR subgroup vs 67[77%] for pCR subgroup) and at surgery (70[67%] of tissue availability for non-pCR subgroup vs 53[60%] for pCR subgroup).

At visit 2, most patients with available biopsies (104/161, 65%) were identified as normal-breast-like (NBL) type. The remaining were classified as HER2E (28, 17%), Luminal-like (25, 16%), and BL (2, 1%). Similar results were observed at the time of surgery, with most patients with available gene expression data regardless of pCR or not (89/123, 73%) classified as NBL. Additionally, there were 13 (11%) classified as HER2E, 19 (15%) as HER2, and 2 (1%) as BL (supplementary figure 4). Of note, among the HR+ patients with a HER2E type at baseline, 6 individuals transitioned into a Luminal subtype, whereas no HR- patients with HER2E changed into a Luminal subtype. Eighty-one percent of patients experienced a change in subtypes from baseline to visit 2, with the majority of HER2-E subtype patients switching to other subtypes, representing 76% in the DHP arm and 84% in the TDM1 arm. However, an intrinsic subtype conversion from baseline to visit 2 was not associated with early treatment response.

The subset of patients with residual disease (RD) deserves particular attention due to potential therapeutic and prognostic implications. However, even among the patients with RD, the majority either lacked samples or exhibited a NBL phenotype at the surgical timepoint. Additionally, a HER2-E subtype at RD was not found to be prognostic for recurrence-free survival (RFS) in multivariable Cox regression analysis. However, per-treatment arm analysis was not conducted due to the limited number of patients available for assessment.

We also investigated the impact of intertumoral tissue heterogeneity on PAM50 molecular subtype assessment. At baseline, cycle 2, and surgery, there were 13, 35, and 23 patients, respectively, with two core biopsies available. A nearly 50% agreement of PAM50 subtypes was observed within the two replicates at baseline. High concordance rates were found between the two replicated tissues at visit 2 and surgery.

As traditional PAM50 subtyping models relies on normalization to quantify gene expression relative to a reference, like other multigene expression models, bioinformatics approaches using single sample predictors rules such as AIMS has emerged to overcoming the

shortcomings. In the current study, we apply an established RNA-seq-based SSP algorism to estimate PAM50 molecular subtypes in clinical HER2-positive breast cancer patients recruited in a prospective randomized clinical trial.

As expected, around 60% clinical HER2-positive patients are HER2-E, and align with previous trials observations, the HER2-E intrinsic molecular subtype exhibits greater anti-HER2-sensitivity in contrast to other molecular subtypes. Longitudinal data have further validated the plasticity of PAM50 molecular subtypes under neoadjuvant HER2-targeted chemotherapy treatment, with most prevalent on-treatment subtype was Normal-like. Whether the changes in tumour phenotype is a substantial biological converson or normal breast tissues contamination remains to be unclear. The tumor purity estimated by the ESTIMATE algorithm indicates high normal contamination at the following time points.

A previous study has shown that HER2-E tumors failing to achieve a pCR are associated with unfavorable survival outcomes [118], and the most frequent post-treatment subtype alteration from baseline to surgical is the luminal A subtype in the CALGB 40601 study[119]. In our study, all four subtypes can be observed at surgery, with a considerable portion still retaining the HER2-E subtype. In the KATHERINE trial, worse invasive disease-free survival (iDFS) is observed in HER2-negative residual disease patients in the trastuzumab arm, but not in the T-DM1 arm which we did not oberseve here. This suggests that biomarkers assessed in the surgical sample have an impact on outcomes and wanrants future investigation[120].

Our exploratory biomarker analysis is subject to several limitations, such as a small sample size after the baseline, a scarcity of observed events, and presence of normal tissue contamination across all timepoints.

To conclude, our study presents a prospective trial that longitudinally evaluates intrinsic molecular subtypes in HER2-positive breast cancer. We reaffirmed the prognostic significance of HER2-E subtype and observed dynamic changes in subtype change over time. These results underscore the inherent complexity of molecular diversity within HER2-positive breast cancer. Further research is warranted to capitalize on molecular subtype alterations for tailoring patient treatment strategies.

## 4.4 CONCLUSIONS

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

- TILs assessed on hematoxylin eosin (H&E) stained tissue sections is a simple-to-use and widely available immune biomarker in breast cancer research. Dynamically evaluating TILs over time can provide valuable insights into the host response to tumors and may yield clinically useful biomarkers.
- A trend towards decreased TILs after neoadjuvant therapy (NAT) was observed in all pooled analyses, regardless of breast cancer subtype. Interestingly, the magnitude of decreased TILs appeared to be numerically larger in HER2-positive breast cancer.
- The dynamic activity of serum thymidine kinase 1 during different phases of HER2-positive disease was described. An increase in sTK1 was observed even after short exposure to neoadjuvant treatment.
- There is a potential prognostic value of serum thymidine kinase 1 for patients with residual invasive HER2-positive breast cancer.
- PAM50 molecular subtypes are prognostic for response to neoadjuvant HER2-targeted treatment and for long-term outcomes.
- PAM50 molecular subtypes exhibit significant intratumoral and temporal heterogeneity.

Given that the identification of reliable prognostic and predictive biomarkersis crucial for management and therapy decision in patients with HER2-positive breast cancer, our findings could provide potentially impactful insights and warrant further validation.

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长风破浪会有时，直挂云帆济沧海。——李白(唐)

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