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The perioperative setting as a platform for breast cancer biomarker development

Thesis presented by

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DECLARATION

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Maeve Hennessy

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ABSTRACT

Breast cancer is the most common cancer in women, with 2.3 million new cases globally in 2022 (1). 'Neoadjuvant' systemic therapy prior to surgery is the standard of care for stage II-III human epidermal growth factor receptor-2 (HER2) positive breast cancer. The identification of *predictive biomarkers of response* to systemic therapy is of great research interest, as this may allow treatment to be tailored to the individual. Pathologic complete response (pCR), the absence of residual invasive carcinoma in the breast or axillary lymph nodes following completion of neoadjuvant systemic therapy, is an accepted surrogate marker for survival outcomes in clinical trials (2). An advantage of the neoadjuvant setting is that tumour response to treatment can be assessed through tissue-based, biochemical, or radiologic changes throughout administration of therapy; allowing valuable information to be obtained in a short time period. Ideally, these potential biomarkers would be used early in the treatment paradigm allowing 'individualisation' of treatment decision-making. Certain patients could avoid receiving futile therapies, while others could be directed towards a more intensive approach to maximise their outcome.

The studies in this Thesis were undertaken to evaluate imaging and tissue-based biomarkers for oestrogen receptor (ER)-negative, HER2-positive early-stage breast cancer in the Translational Breast Cancer Research Consortium (TBCRC) 026 trial (NCT01937117). TBCRC026 was a phase II, multicentre clinical trial incorporating serial positron emission tomography–computed tomography (PET-CT) imaging [baseline and 15 days after trastuzumab/pertuzumab (HP) initiation (C1D15)] and tumour biopsy collection (3). The primary hypothesis was that early measurements of tumour maximum

standardised uptake value corrected for lean body mass (SULmax) on PET/CT would predict pCR to HP. Results found that participants not obtaining a 40% reduction in SULmax by C1D15 were unlikely to obtain pCR (3). Thus, the use of PET/CT as an imaging biomarker may provide a more individualised approach to treatment in early-stage HER2-positive breast cancer.

This Thesis presents secondary analyses of the TBCRC026 clinical trial. Chapter 2 builds on the primary study analysis and evaluates the *correlation between early metabolic changes on PET/CT and survival outcomes* in this cohort. A potential association between SULmax parameters on PET/CT and recurrence free survival (RFS) and overall survival (OS) outcomes was identified. This imaging-based biomarker has not previously been assessed in patients receiving anti-HER2 therapy alone and therefore is novel and potentially practice changing if validated in future trials.

Chapter 3 explores the *relationship between HER2 and immune tissue-based biomarkers and pCR* in this dataset, through analysis of baseline archived tumour samples. NanoString codesets BC360 and IO360 were used to compare gene expression in baseline tumours that underwent pCR versus no pCR. NanoString GeoMx digital spatial profiling (DSP) assessed immune protein abundance in intraepithelial and stromal segments. There was a significant association between intraepithelial HER2 protein abundance and pCR. Overall, the results demonstrated the unique molecular and immunological features of ER-negative, HER2-positive breast cancer, which warrant further exploration as potential predictive biomarkers.

Chapter 4 investigates *improving on the prediction power of the PET/CT imaging biomarker by combining it with a HER2-tissue based biomarker*. Promising HER2-tissue biomarkers available from our analysis included high HER2 expression by immunohistochemistry (IHC), HER2-enriched (HER2-E) intrinsic subtype and high levels of HER2 protein abundance. While the composite HER2/PET biomarker comprising D15 SULmax, % reduction in SULmax and tumour HER2 protein abundance had high prediction power for pCR, it was not significantly superior to the prediction power of PET/CT parameters alone.

The combined data from the above analyses suggest clinical trials informed by early PET/CT biomarkers and promising HER2-tissue based biomarkers should be further evaluated to personalise breast cancer care. If these potential biomarkers can be validated, results will be used to design clinical utility studies, facilitating early individualisation of therapy for patients.

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ABBREVIATIONS

Abbreviation	Definition
ASCO/CAP	American Society of Clinical Oncology/College of American Pathologists
AURKA/B	Aurora kinase A/B
ER	Oestrogen Receptor
FISH	Fluorescence In Situ Hybridisation
iDFS	Invasive Disease-Free Survival
PD-L1	Programmed Cell Death Ligand 1
C1D1	Date of first course of treatment
C1D15	15 days after treatment initiation
CI	Confidence Intervals
DCIS	Ductal Carcinoma In Situ
DFS	Disease Free Survival
DNA	Deoxyribonucleic acid
DSP	Digital Spatial Profiling
EFS	Event Free Survival
EGFR	Epidermal growth factor receptor
Erbb	Epidermal growth factor receptor
FC	Fold Change
FDA	Food and Drug Administration
FDG-PET/CT	Fluorodeoxyglucose-Positron Emission Tomography with Computed Tomography
FFPE	Formalin-fixed, paraffin-embedded

GO	Gene Ontology
H&E	Haematoxylin-Eosin
HER2	Human Epidermal Growth Factor Receptor 2
HER2-E	HER2-Enriched
HLA-DR	Human leukocyte antigen-DR isotype
HP	Trastuzumab/Pertuzumab
HR	Hazard Ratio
HR	Hormone Receptor
ICI	Immune Checkpoint Inhibitors
iGESs	Immune-related Gene Expression Signatures
IgG	Immunoglobulin G
IHC	Immunohistochemistry
MAPK	Mitogen-activated protein kinase
MHC	Major Histocompatibility Complex
MMG	Mammography
MRI	Magnetic Resonance Imaging
NCCTG	North Central Cancer Treatment Group
niFDG-PET/CT	Neoadjuvant interim FDG-PET/CT
NPV	Negative Predictive Value
OR	Odds Ratio
OS	Overall Survival
pCR	Pathologic Complete Response

PFS	Progression Free Survival
PI3K	Phosphoinositide-3-kinase
PPV	Positive Predictive Value
RFS	Recurrence Free Survival
RNA	Ribonucleic acid
ROC	Receiver Operating Curve
ROI	Regions of Interest
SNR	Signal to Noise Ratio
sTILS	Stromal Tumour Infiltrating Lymphocytes
SULmax	Tumour maximum standardised uptake value corrected for lean body mass
SUV	Standardised Uptake Value
T-DM1	Trastuzumab Emtansine
T-DXd	Trastuzumab Deruxtecan
TBCRC	Translational Breast Cancer Research Consortium
THP	Docetaxel/Trastuzumab/Pertuzumab
TNF	Tumour necrosis factor
US	Ultrasound
USA	United States of America
UV	Ultraviolet

CHAPTER 1:

INTRODUCTION

HER2 POSITIVE BREAST CANCER

Human epidermal growth factor receptor 2 (HER2) is a member of the epidermal growth factor receptor (EGFR or ErbB) family of receptor tyrosine kinases and is fundamental in controlling epithelial cell growth and differentiation. Amplification and/or overexpression of the *HER2* gene is present in approximately 15-20% of breast cancers and has traditionally been associated with a more aggressive disease trajectory and a poor prognosis (4). As such, HER2 is an important therapeutic target for the treatment of breast cancer and indeed other solid tumour malignancies (4-6).

There are four transmembrane tyrosine kinase receptors present in the HER2 family and all play a role in cell proliferation and survival. These include epidermal growth factor receptor (EGFR/HER1/ErbB1), HER2 (HER2/neu/ErbB2), HER3 (ErbB3), and HER4 (ErbB4). The structure of each receptor consists of three domains: an extracellular domain, an alpha helical transmembrane domain, and an intracellular protein kinase domain. An additional four domains make up the extracellular domain comprising of domain I (ligand-binding site), domain II (dimerisation site) and domain IV (site for binding of trastuzumab) (7).

Ligand-binding results in either homo- or hetero-dimerisation of HER proteins, initiating a downstream phosphorylation signalling cascade, which stimulates cell growth, proliferation, and differentiation (8). HER2 has no known specific ligand (9), and

therefore in order to enable downstream signalling it must form either homodimers (HER2/HER2) or heterodimers (HER2/HER3) when ligands engage other ErbB protein family surface receptors. The humanised monoclonal antibody trastuzumab binds to the extracellular domain IV of the HER2 receptor and inhibits homodimerisation, which prevents HER2-mediated signalling. Additionally it is thought to stimulate antibody-dependent cytotoxicity, resulting in the death of HER2-expressing cells (7). Increasing evidence suggests that trastuzumab may also harness other immune responses against HER2 (10).

HER2-TARGETED THERAPIES

Trastuzumab was the first HER2-targeted therapy developed for the treatment of HER2-positive breast cancer and was first investigated in the metastatic setting. A phase III trial led by Slamon et al. randomised patients with HER2-positive metastatic breast cancer to first line chemotherapy alone versus chemotherapy in combination with trastuzumab. Results showed that the addition of trastuzumab improved progression free survival (PFS), (median, 7.4 versus. 4.6 months; $P<0.001$), objective response (50% versus 32%, $P<0.001$), overall survival (OS), (median survival, 25.1 versus 20.3 months; $P=0.046$), and trastuzumab was approved on this basis in the United States of America (USA) by the Food and Drug Administration (FDA) in 1998 and then globally (11). Subsequently trastuzumab was approved in the adjuvant setting in 2005, in combination with chemotherapy, reflecting a new standard of care for patients with early stage HER2-positive breast cancer (12). The NSABP B-31/NCCTG-N9831 and HERA clinical trials demonstrated that adjuvant trastuzumab for one year in addition to chemotherapy reduced breast cancer recurrence by approximately 50% and was associated with a 35% reduction

in mortality (13, 14). The use of trastuzumab in the neoadjuvant setting has been investigated in a number of trials, described below.

Pertuzumab is another humanised, monoclonal antibody targeting HER2 that was first approved in 2012 (15). It works by binding to the extracellular domain II of the HER2 receptor and inhibits HER2 heterodimerisation with HER1, HER3 and HER4 (16) and downstream activation of the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. In particular, pertuzumab blocks the most potent signalling HER2 dimer (HER2/HER3), which is considered to play a key role in driving cell proliferation in HER2-positive cancer. The mechanisms of action of pertuzumab and trastuzumab are complementary and provide a more complete blockade of HER2-mediated signalling than either agent alone. Pertuzumab can also effectively initiate antibody dependent cytotoxicity, independent of trastuzumab (17).

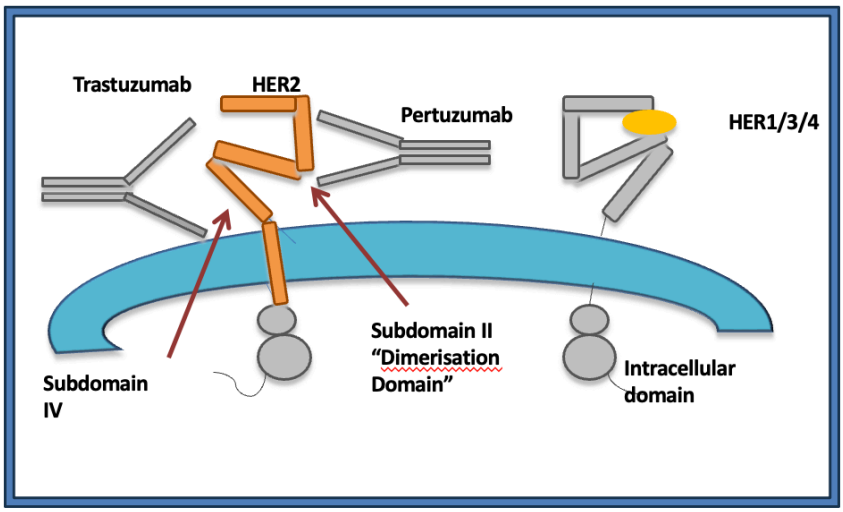


FIGURE 1: TARGETING THE HER2 SIGNALLING PATHWAY (ADAPTED FROM O'SULLIVAN, ONCOLOGY 2014, WITH PERMISSION)

The first evidence that the combination of pertuzumab and trastuzumab (without chemotherapy) was tolerable and efficacious was from a phase II trial conducted in patients with metastatic HER2-positive breast cancer, who had experienced progression on prior trastuzumab therapy (18). The overall response rate (n=66) was 24.2% and median PFS was 5.5 months. Building on this, the concept of dual HER2-targeted therapy was explored in earlier settings, such as the neoadjuvant space.

NEOADJUVANT SETTING

The neoadjuvant setting is an ideal platform for research, as new biomarkers and therapeutic strategies can be explored, with pathologic response assessed at the time of surgery. The initial aim of neoadjuvant therapy was to downstage large, inoperable tumours to allow for definitive surgery and optimise surgical outcomes. However, an advantage of this approach is that it allows the efficacy of novel therapies to be assessed and facilitates the development of prognostic and predictive biomarkers of response and

resistance to treatment. In stage II-III HER2-positive breast cancer neoadjuvant combination chemotherapy and HER2-directed therapy is now the standard of care. A pathologic complete response (pCR), is defined as the absence of residual invasive carcinoma in the breast or axillary lymph nodes following completion of neoadjuvant systemic therapy and is an accepted surrogate marker for disease-free survival (DFS) and OS in clinical trials, allowing for early drug approval contingent on confirmatory studies (2). Patients who achieve a pCR following neoadjuvant therapy tend to have improved outcomes compared to those with extensive residual disease (19). Recent trials have demonstrated the role for intensifying therapy in patients with residual disease following neoadjuvant treatment, for example with the use of adjuvant trastuzumab emtansine (T-DM1) which is an antibody drug conjugate (20). Several studies have evaluated a variety of HER2-directed approaches in the neoadjuvant setting.

The Phase II NeoSphere trial (n=417) was a four-armed study that compared neoadjuvant docetaxel (T) plus trastuzumab (H), pertuzumab (P) or both. Patients with tumours ≥2 cm were enrolled. The primary endpoint was pCR in the breast. The percentage of patients who achieved pCR and were axillary lymph node negative at surgery was higher in those who received dual anti-HER2 therapy plus chemotherapy (THP) versus those treated with single agent trastuzumab plus chemotherapy (TH) (46% versus 29%).

Another study that explored neoadjuvant dual HER2-targeted therapy was Tryphaena, in which patients with locally advanced HER2 positive breast cancer were randomised to receive either anthracycline chemotherapy concurrent with HER2-targeted therapy, anthracycline chemotherapy with sequential HER2-targeted therapy or a non-anthracycline chemotherapy arm containing dual HER2-targeted therapy. pCR was

assessed at the time of surgery and patients completed one year of adjuvant trastuzumab. The primary endpoint was cardiac safety and there was a low incidence of cardiac dysfunction noted in all three arms. Like NeoSphere, Tryphaena demonstrated high rates of pCR (64%) with the inclusion of both trastuzumab and pertuzumab, with a particular benefit noted in the oestrogen receptor (ER)-negative, HER2-positive subgroup.

Other novel HER2-targeted therapies have also been explored in the neoadjuvant setting, including the tyrosine kinase inhibitor lapatinib (21, 22) and the antibody drug conjugate T-DM1 (23, 24) with impressive pCR rates. However, based on data from the preoperative studies investigating pertuzumab in combination with chemotherapy, such as NeoSphere and Tryphaena, the FDA granted accelerated approval for neoadjuvant pertuzumab in combination with chemotherapy in 2013, with a confirmatory phase III trial required to verify clinical benefit, for full approval. *Importantly, 17% of patients in NeoSphere who achieved a pCR with HP alone i.e. without the addition of conventional chemotherapy, suggesting that certain patients may have excellent outcomes without the need for chemotherapy.*

INDIVIDUALISATION OF THERAPY FOR EARLY-STAGE HER2-POSITIVE BREAST CANCER

Based on the current evidence described above, the standard approach for stage II-III HER2-positive breast cancer is neoadjuvant combination chemotherapy plus dual HER2-directed therapy with HP. A strategy to overcome treatment resistance is to offer additional adjuvant treatment for patients that do not achieve a pCR following neoadjuvant therapy, an approach described as 'post-neoadjuvant treatment.' In early-

stage HER2-positive breast cancer, patients that achieve a pCR receive adjuvant treatment with trastuzumab, while those that do not achieve a pCR receive post-neoadjuvant T-DM1 as per the pivotal Katherine study (20). This trial investigated T-DM1 versus trastuzumab for residual HER2-positive early breast cancer following neoadjuvant treatment and reported improved PFS and OS for the T-DM1 arm (20). T-DM1 is an antibody-drug conjugate that combines the anti-HER2 mechanism of trastuzumab with the potent cytotoxic agent DM1 (25). Thus, the Katherine study provides an example of tailoring therapy to the individual, where those with residual disease and likely a higher chance of recurrence received intensified therapy in order to maximise their outcome. Several studies have explored this concept of either 'escalating' or 'de-escalating' therapy for early-stage HER2-positive breast cancer, in order to 'optimise' or 'individualise' outcomes.

The phase III DESTINY-Breast05 trial is currently underway and is an example of an 'escalation' trial, aiming to improve upon outcomes of this patient population with early-stage HER2-positive breast cancer and residual disease following neoadjuvant therapy (26). Patients are randomised to either post-neoadjuvant trastuzumab deruxtecan (T-DXd), a next generation antibody drug conjugate that has demonstrated superiority over T-DM1 in the metastatic setting (27) or T-DM1 as per the Katherine data (20). Similarly, the ongoing CompassHER2 Residual Disease trial randomises patients with high-risk HER2-positive residual disease after neoadjuvant chemotherapy plus HER2-directed treatment to either adjuvant T-DM1 and placebo, versus T-DM1 and tucatinib, a novel oral agent. The primary objective is to determine if invasive disease-free survival (iDFS) with T-DM1 plus tucatinib is superior to iDFS with T-DM1 plus placebo (28).

Other studies have attempted to 'de-escalate' therapy, one of the first examples is the APT trial led by Tolaney et al. This was a landmark adjuvant study of paclitaxel and trastuzumab and led to changes in care for patients with stage I HER2-positive breast cancer, reducing both the dose and duration of therapy, with excellent survival outcomes reported at ten years of follow up (29). This concept of therapy de-escalation has subsequently been applied to the neoadjuvant setting, for example in the ADAPT trial which explored a 'chemotherapy-free' approach. Patients with hormone-receptor (HR)-positive/HER2-positive early-stage breast cancer were randomised to four cycles of either neoadjuvant T-DM1 with or without endocrine therapy versus trastuzumab plus endocrine therapy. The omission of adjuvant chemotherapy was permitted in patients achieving a pCR. There were 41% of patients who achieved a pCR after only 4 cycles of T-DM1, highlighting impressive response rates without incorporating standard chemotherapy (30). In the Swedish PREDIX trial, which included women with HER2-positive breast cancer >2cm; pCR rates after six cycles of neoadjuvant single-agent T-DM1 without chemotherapy compared favourably to those treated with six cycles of chemotherapy plus HP (44% versus 46%) (24). Of note, *the above studies demonstrate that there is a subset of patients who obtain pCR without having received classic chemotherapy.* If these subsets can be reliably identified in future studies, this may facilitate the de-escalation of systemic anti-cancer treatment and reduce treatment associated side-effects. Improved prognostic and predictive biomarkers are thus needed to aid treatment decision-making, over and above the standard clinicopathologic features such as tumour size, grade and disease stage that are currently used in clinical practice.

PREDICTIVE AND PROGNOSTIC BIOMARKERS

The development of biomarkers to accurately select patients who may benefit from more intensive anti-HER2 therapy or those who may be able to forego chemotherapy i.e. the 'optimisation' or 'individualisation' of treatment is a key area of interest among the breast cancer community. Valuable information can be obtained using small numbers of patients in a short time frame, by measuring biochemical or radiologic changes in malignant tissue prior to, during, and following neoadjuvant therapy. Ideally, these surrogate biomarkers (tissue-based, imaging or blood-based) would be used early in the treatment paradigm to predict response to neoadjuvant chemotherapy. For example, a marker that can separate sensitive and resistant tumours to specific agent(s) early in the course of therapy could be used to determine treatment early in the course. This could avoid certain patients receiving toxic and futile therapies, and direct other patients towards an aggressive or investigational approach in an effort to maximise their breast cancer outcome.

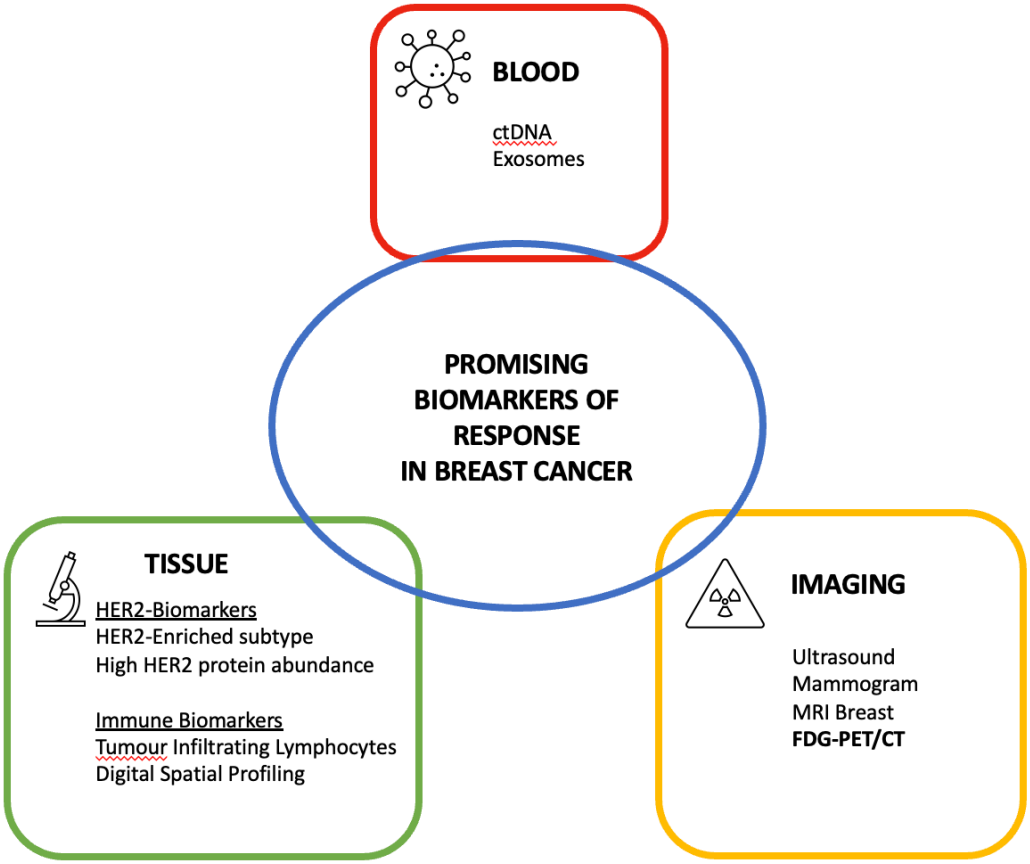


FIGURE 2: PREDICTIVE BIOMARKERS OF RESPONSE AND RESISTANCE IN BREAST CANCER

IMAGING-BASED BIOMARKERS

Imaging techniques may provide early information regarding tumour response. Traditionally, magnetic resonance imaging (MRI), mammography (MMG) and ultrasound (US) have been used to image breast cancer and assess changes during and after neoadjuvant therapy. MRI has been deemed the most accurate modality for the evaluation of residual tumour following neoadjuvant therapy, compared to MMG, US and clinical exam, yet it can still over and underestimate residual disease (31). Additionally, studies have demonstrated that the diagnostic accuracy of MRI post neoadjuvant treatment may differ depending on breast cancer subtype and treatment regimen, with a number of

clinical trials finding discordant results between MRI imaging and pathology at the time of surgery (32). The use of tri-modality imaging (MMG, US and MRI) after neoadjuvant therapy was investigated in the NRG-BR005 trial and was unsuccessful at identifying patients expected to have a pCR (33).

As contrast enhancement on MRI is based on blood flow and vascularity, this indirectly evaluates drug pathway targets and is less optimal for prediction of pCR based on early response to HER2 targeted therapy (31). The use of fluorodeoxyglucose-positron emission tomography with computed tomography (FDG-PET/CT) has been proposed as an attractive imaging biomarker of response to therapy in early breast cancer due to its ability to detect changes in tumour biology (34). An example of its successful implementation as an interim imaging biomarker is its use in lymphoma. Patients not achieving the desired response on PET/CT after two cycles of systemic therapy are recommended an 'escalated' treatment approach, as compared with those who demonstrate adequate response to treatment. As such, PET/CT has become an important imaging tool used to guide adaptation of therapy in lymphoma (35, 36). There is great interest amongst the oncology and imaging communities for expanding such a role to other cancer types such as breast cancer. Of course, the possibility of changing practice with the introduction of interim PET/CT scanning must be carefully balanced against the the risk of exposure to radiation for this cohort of patients with breast cancer. Ethics committees carefully consider radiation risk when reviewing imaging and other trials that might involve radiation exposure. This is important to consider as other imaging modalities such as MRI, which does not involve ionising radiation, are being examined in this setting (37).

Rationale for FDG-PET/CT in breast cancer

Researchers at Johns Hopkins and other institutions have been investigating the role of PET/CT in breast cancer for some time. Early work from a small serial PET/CT study conducted at Johns Hopkins in women with primary breast cancer >3cm (n=11) demonstrated a rapid and significant decrease in glucose metabolism from 8 days after the administration of one cycle of combination chemotherapy in patients who responded to the regimen (38). Those with a partial response were noted to have reduced FDG uptake at a later point in the course of their treatment, whereas there was no significant change in uptake noted in non-responders. In another study, patients with primary breast cancer (n=22) underwent FDG-PET/CT imaging before and after Cycle 1 and 2 of anthracycline-based chemotherapy. Patients with minimal residual disease had a decline in tracer uptake, while those who had gross residual disease demonstrated negligible change in tracer uptake (39). These studies suggest that FDG-PET/CT response following one cycle of chemotherapy is predictive of pCR to the therapy with high sensitivity (90-100%) and specificity (74-91%) and provided the expertise and interest in developing future studies incorporating FDG-PET/CT in breast cancer.

FDG-PET/CT in HER2-positive breast cancer

Consequently, there is an interest in the evaluation of FDG-PET/CT as a measure of early metabolic response in early HER2-positive breast cancer. One of the first reports to suggest that PET/CT may be a promising biomarker in HER2-positive early breast cancer was from the NeoALTTO PET sub-study. This trial enrolled 455 women with HER2-positive breast cancer and compared rates of pCR to neoadjuvant lapatinib,

trastuzumab, and their combination. The results (secondary endpoint) showed that changes in standardised uptake value (SUV) on PET/CT may identify patients with an increased likelihood of pCR after neoadjuvant trastuzumab, lapatinib, or their combination when given with chemotherapy (40). The PHERGain study explored a response-adapted approach in the treatment of early-stage HER2-positive breast cancer, aiming to identify patients who may be treated adequately with HER2 blockade, without the need for chemotherapy (41). Patients were randomised to neoadjuvant chemotherapy plus HP or HP alone. Early responders were identified on PET/CT, and in the HER2-blockade alone group nearly 80% of patients achieved a metabolic response on PET/CT, with an excellent 3 year IDFS of 95% (41). Updated results also demonstrated that in 37% of those who responded on PET/CT, a pCR was achieved, and thus there was a subgroup who never received chemotherapy, yet had a 3 year IDFS of 98.8% (42).

Based on the promising early studies of PET/CT in HER2-positive breast cancer such as NeoALLTO and the PET/CT experience at Johns Hopkins, in combination with the NeoSphere data suggesting some patients may have excellent outcomes with dual HER2-directed therapy (HP) alone, our team developed the translational breast cancer research consortium (TBCRC)026 trial. This was a multicentre phase II clinical trial with the primary objective to correlate baseline and early percentage change (by C1D15) in maximum standardised uptake value corrected for lean body mass (SULmax) on FDG-PET/CT with pCR to neoadjuvant dual HER2-directed therapy (HP), without chemotherapy. Eligible patients had stage II-III ER–negative, HER2–positive breast cancer. The study incorporated serial FDG-PET-CT imaging [baseline and 15 days after HP initiation (C1D15)] as well as tumour biopsy collection. (3). Eighty-eight women were

enrolled (83 evaluable), and the pCR rate after HP alone was 22%. The results found that among those obtaining a pCR, SULmax reduction ≥ 40% by C1D15 was more prevalent (83% versus 52%; P =0.03), and that participants not obtaining a 40% reduction in SULmax by C1D15 were unlikely to obtain pCR [negative predictive value (NPV) 91%] (3). These results suggest that optimised FDG-PET/CT imaging–based biomarkers may guide escalation and de-escalation of neoadjuvant therapy. Reduction in SULmax on FDG-PET/CT during the first cycle of HP had a high NPV, therefore this may best predict those patients who will not achieve pCR with HP alone and should be recommended alternative regimens.

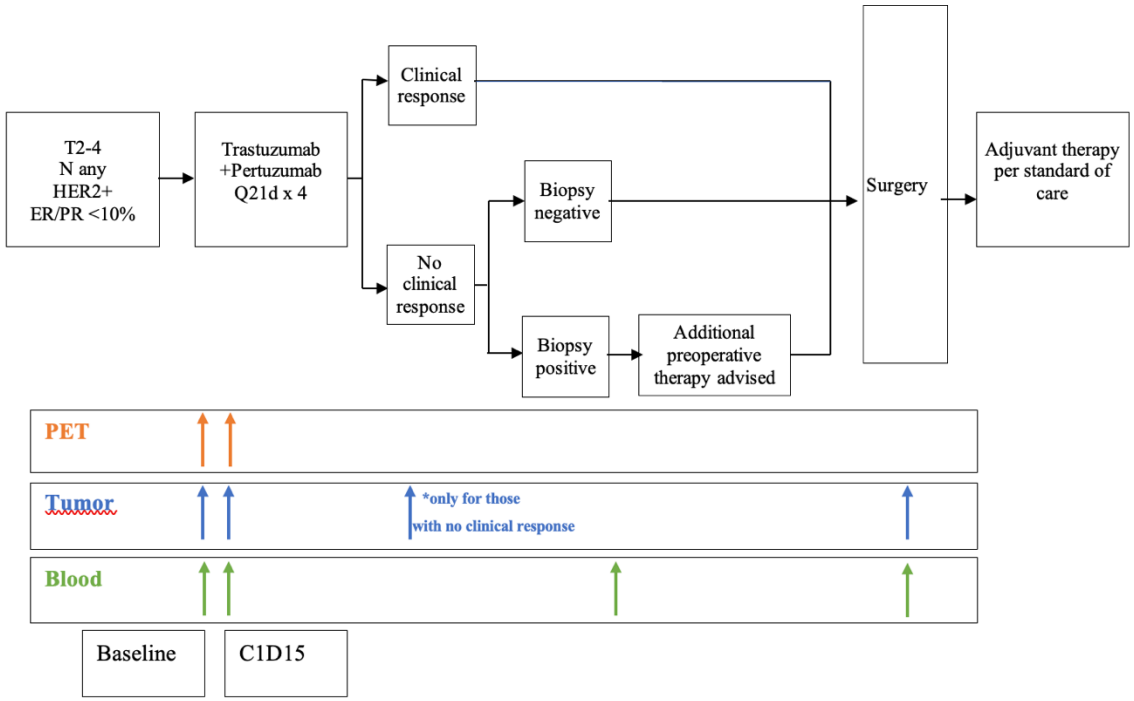


FIGURE 3:TBCRC026 STUDY SCHEMA

It is important to note that the above studies are heterogenous in design, with varying treatment regimens, hormone-receptor status, and definitions of 'metabolic response' used, thus further prospective studies are required to validate the use of PET/CT as a biomarker across several standard treatment regimens in the treatment of early stage HER2-positive breast cancer.

TISSUE-BASED BIOMARKERS

Although imaging-based biomarkers are a growing area of interest, novel tissue-based biomarkers are also under investigation. Traditionally, standard clinicopathologic factors such as receptor status, tumour grade, and proliferation index have aided in determining the choice of therapy for those with early breast cancer. However there remains a high variability in response to HER2 targeted therapy, reflective of the heterogenous nature of HER2-positive breast cancer, and to date no predictive biomarker of response to dual HER2 targeted treatment has demonstrated clinical utility. A number of biological characteristics and immune mechanisms which have relevance to the work investigated in this Thesis, have been explored and are described below.

HER2 tissue-based biomarkers

HER2 status is determined using immunohistochemistry (IHC) or fluorescence in situ hybridisation (FISH). IHC evaluates expression of the HER2 protein in cell membranes while FISH assesses *HER2* gene amplification (43). IHC is most commonly used as the primary test for HER2 status and tumours are graded based on a 0, 1+, 2+ and 3+ scoring system. The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines define HER2-positive status on the basis of IHC 3+

(uniform intense staining of >30% of invasive tumour cells) or FISH-positive (ratio of HER2 to CEP17 of >2.2 or average HER2 copy number >6 signals/nucleus) (43). Tumours graded as 0 or 1+ are considered HER2-negative, while those scored as 2+ are equivocal and require confirmation of HER2 status by ISH. While there is ongoing research into improving our existing HER2 testing methodologies, other methods of measuring HER2 are under investigation, such as analysis of HER2 protein abundance in the tumour and stroma by novel technologies such as Digital Spatial Profiling (DSP), which is discussed in further detail below and in Chapter 3.

Given the diversity among breast cancers, other efforts to subclassify breast tumours to guide prognostic information and treatment decision-making have been made. One example is PAM50, a 50-gene signature that classifies breast cancer into five molecular intrinsic subtypes: Luminal A, Luminal B, HER2-Enriched (HER2-E), Basal-like and Normal-like (44, 45). Each of these five molecular subtypes vary in terms of their biological properties and prognoses with luminal A tumours generally regarded as having the most favourable prognosis, while HER2-E and basal-like are considered more aggressive (46). Approximately 50% of HER2-amplified tumours are classified as HER2-E (47), and this subtype has been suggested to identify patients whose tumours are HER2 'addicted' and more sensitive to HER2-directed therapy as a result. Many exploratory analyses have shown a correlation between the HER2-E subtype and high pCR rates after neoadjuvant HER2-based regimens (22, 48-50). In the PAMELA trial investigating neoadjuvant dual HER2-therapy with trastuzumab/lapatinib without chemotherapy, although 89% of those achieving pCR had HER2-E tumours, reflecting increased sensitivity of this subtype to HER2 directed-therapy, more than half (59%) of the HER2-

E tumours did not undergo pCR (48). Thus intrinsic subtype while important, is perhaps insufficient for predicting benefit from therapy in its own right (48).

In terms of other intrinsic subtypes, data from studies such as the NCCTG N9831 trial of adjuvant trastuzumab in early stage HER2-positive tumours, have demonstrated that basal-like tumours are relatively unresponsive to anti-HER2 therapy (51). Similarly, in the neoadjuvant ADAPT HR-negative/HER2-positive trial, patients in the HP (chemotherapy-free) arm, with low HER2 expression and/or basal-like subtype by PAM50 did not undergo pCR (52). While the ability of intrinsic molecular subtype to serve as a potential predictive and prognostic biomarker in early HER2-positive breast cancer appears promising, further research will help to provide greater clarity.

Understanding the biological heterogeneity of HER2-positive breast cancer is crucial to identifying the prognosis of each patient and the benefit from systemic therapies targeting HER2. Another promising tool recently developed is the HER2DX assay, a novel learning algorithm designed specifically for prognostic and predictive purposes in early stage HER2-positive breast cancer. The assay incorporates clinical information (tumour size, nodal staging) and 4 gene expression signatures relating to immune infiltration, tumour cell proliferation, luminal differentiation, and the expression of the HER2 amplicon, into a single score (53). It is the first assay to integrate clinical data with genomic data encompassing tumour features, immune features, and pathologic features all in one assay. HER2DX can also be used to predict two different clinical endpoints, long-term survival outcomes (HER2DX risk score) and likelihood of achieving a pCR (HER2DX pCR). These scores can be used in combination to guide therapy. Ultimately, the HER2DX risk score aims to identify patients with early-stage, HER2-positive breast

cancer who have favourable survival outcomes with chemotherapy and trastuzumab, and could be spared additional therapies, such as pertuzumab or T-DM1. Future studies will explore the clinical utility of both HER2DX scores to help de-escalate therapy, such as the duration of trastuzumab or the amount of chemotherapy (53).

Immune-based biomarkers

The role of the host immune system in modulating response to HER2-directed therapy is an area under active investigation (54). HER2-positive breast cancer is felt to be more immunogenic than the HR-positive/HER2-negative subtype, and typically has higher levels of stromal tumour infiltrating lymphocytes (sTILs) (55). An international working group has established standardised tools for measuring TILs present in haematoxylin-eosin (H&E)–stained tumour slides (56). A number of studies have shown that high levels of TILs are associated with improved response to neoadjuvant HER2-directed therapy, and may serve as a potential predictive biomarker (57-60). In NeoALTTO, the presence of TILs at diagnosis was shown to be prognostic for pCR and event free survival (EFS) in those receiving neoadjuvant HER2 therapy and chemotherapy (57). Correspondingly, in PREDIX HER2, which compared the standard neoadjuvant regimen of docetaxel, trastuzumab and pertuzumab with T-DM1, patients with TILs ≥10%, had higher rates of pCR compared to those with TILs <10%, (51% versus 28%; p=0.003)(60). Expanding upon this, a novel biomarker called the CelTIL score was developed, which combined sTILS and tumour cellularity measured on tumour biopsies taken at D15 of HER2-directed therapy, and was found to be associated with pCR (61)

Immune activation can also be measured by gene expression and is a potential biomarker in early-stage breast cancer (62). In addition to single genes or proteins, gene-expression profiling allows for the rapid assessment of multiple genes rather than single genes using high-throughput deoxyribonucleic acid (DNA) sequencing and may be used to predict both response to therapy and clinical outcome. Several trials have evaluated multigene assays as predictors of response to therapy in the preoperative setting, and validation efforts are ongoing (63, 64). An example of a successful model is the Oncotype DX assay which has been successfully integrated into the treatment paradigm of patients with early-stage HR-positive breast cancer. This 21-gene assay predicts the likelihood of chemotherapy benefit and 10-year risk of distant recurrence and is now routinely used to inform adjuvant treatment decisions in certain patients with early-stage HR-positive/HER2-negative breast cancer (65). Patients at lowest risk of disease recurrence on the basis of the test can be spared chemotherapy, while those likely to receive most survival benefit from chemotherapy are directed towards systemic treatment (65). However, for patients with HR-negative or HER2-positive disease, the molecular tools developed thus far have not yet resulted in improved clinical decision-making and better prognostic and predictive tools for these patients are needed.

In patients with early-stage HER2-positive breast cancer treated in the neoadjuvant setting, immune-related gene expression signatures (iGESs) are associated with higher pCR rates and prolonged survival (50, 66). Specifically, the immunoglobulin G (IgG) signature (67) has previously shown strong and independent prognostic value across many studies (53, 62, 66). In a gene expression analysis encompassing the North Central Cancer Treatment Group (NCCTG) N9831 trial and the NeoALTTO trial,

enrichment of CD45 and immune-subset signatures were significantly associated with improved outcomes in N9381, and a novel 17-gene adaptive immune signature was significantly associated with improved recurrence free survival (RFS) among patients who received adjuvant trastuzumab (HR 0.66, p=0.01), although this was not observed in the chemotherapy alone group (51). This was validated in the NeoALTTO cohort, where pCR rates for trastuzumab alone were 42% versus 10% in the adaptive immune signature high versus low groups, however addition of lapatinib appeared to benefit the latter group (51). In CALGB 40601 multiple iGES were significantly associated with pCR and several immune signatures were also associated with EFS in univariable and multivariable Cox analyses (66). A combined correlative analysis of CALGB 49691 and PAMELA showed that immune activation, either by TIL analysis or through immune-related gene expression profiling, was predictive of treatment response, with the B-cell related signatures outperforming TILs for prediction of response to neoadjuvant therapy as well as prognosis (59).

These above analyses confirm the ability of HER2 tumour biology and the immune microenvironment to influence pCR in the neoadjuvant setting and illustrate the molecular heterogeneity of HER2-positive breast cancer. In general, the current evidence demonstrates that markers of an activated immune microenvironment, such as higher TILs (57) or higher expression of iGES, are associated with higher pCR rates (50) and better long-term outcomes in HER2-positive breast cancer (68).

Another novel technology that has emerged, is the use of DSP, which allows highly multiplexed spatial ribonucleic acid (RNA) and protein expression profiling in selected regions of interest (ROI), aiding characterisation of biological processes that may

be implicated in response or resistance to therapy (69). The application of DSP to a variety of cancer types is under active investigation, and will undoubtedly expand our understanding of tumour biology, with the potential for biomarker discovery (69, 70). In melanoma, Toki et al. employed DSP technology to identify protein expression that may predict response to immune checkpoint inhibitors (ICI). Programmed cell death ligand 1 (PD-L1) expression in CD68 positive cells (macrophages) and not in tumour cells, was found to be a predictive marker for PFS, OS and response (71). In breast cancer, NanoString DSP was applied to triple negative breast cancer tumours (n=10) to characterise expression of major histocompatibility complex (MHC) class II and other immune related proteins. Human leukocyte antigen-DR isotype (HLA-DR) protein expression was significantly higher in both epithelial and stromal ROIs in patients that did not experience a recurrence ($P < 0.001$, $p=0.006$), with the degree of differential HLA-DR expression between patient groups more prominent in the epithelial compartment (72). In relation to HER2-positive breast cancer, DSP technology was used to simultaneously profile forty tumour and immune markers from breast tumours sampled before, during and after neoadjuvant HER2-targeted therapy in the TRIO-US B07 clinical trial, providing an insight into biomarker dynamics on-treatment. DSP analysis revealed that changes in both tumour and immune markers on-treatment were more dramatic in tumours that went on to achieve a pCR (73). It is likely that in the future, DSP and/or other similar platforms will be used to enable a deeper understanding of cellular heterogeneity, tumour microenvironment interactions and disease mechanisms, allowing for biomarker identification.

CONCLUSION AND THESIS AIMS

Patients with early stage HER2-positive breast cancer have excellent survival outcomes, with a 5-year OS rate that varies between 98.8% for HR-positive tumours and 97.3% for HR-negative tumours [25]. Thus, for these patients, the need to overcome treatment-related toxicities is highly relevant. The challenge lies in improving identification of predictive biomarkers, to guide personalisation of therapy. Combination chemotherapy with HER2-directed therapy pre-operatively is currently the most effective treatment for women with stage II-III HER2-positive breast cancer. However, it is still difficult to precisely identify who will or will not benefit from systemic therapy. Predictive biomarkers of response and resistance to treatment may be *imaging, tissue or blood-based,* and may allow tailoring of therapy, sparing patients unwanted toxicities of chemotherapy or identifying those who need intensification of standard therapy. Several exciting advances in technology such as novel assays like the HER2DX tool and the use of DSP have been developed in an effort to facilitate biomarker discovery and are under further investigation. Collectively these results enable us to better understand how the tumour and immune microenvironment are altered during therapy and suggest new avenues to personalise cancer therapy. Nonetheless, more work is required, hence the purpose of this Thesis which aims to add to the growing literature and this rapidly evolving area.

The multicentre phase II TBCRC026 study investigated the correlation between early metabolic changes on FDG-PET/CT and pCR in patients with stage II-III ER-negative, HER2-positive breast cancer, receiving neoadjuvant HP without chemotherapy (3). The results found that among those obtaining a pCR, SULmax reduction ≥ 40% by

C1D15 was more prevalent and that participants not obtaining a 40% reduction in SULmax by C1D15 were unlikely to obtain pCR (3). In the future, pending further validation, PET/CT may be used as an interim imaging biomarker to adapt therapy and provide a more individualised approach to treatment for patients with early stage HER2-positive breast cancer. This Thesis presents secondary analyses of the TBCRC026 study. My role was to bring select predefined secondary endpoint analyses to fruition using the existing TBCRC026 dataset, and to develop a new composite biomarker analysis with the goal of refining further the predictive value of the imaging biomarker in this setting. Relevant publications and presentations to date are listed in the section linked here, Publications and Abstracts.

The work outlined in Chapter 2 aims to build on the above by evaluating *whether early changes in metabolic response on PET/CT in the TBCRC026 trial, could predict survival outcomes*, in addition to the promising role in predicting pCR. Ultimately survival is the most important endpoint for these patients who have early-stage HER2-positive breast cancer, and therefore critical to evaluate, especially in the context of administering less intensive therapy. We hypothesised that predefined PET/CT SULmax parameters would be associated with improved RFS and OS in this patient cohort. Secondary endpoints included correlation of ≥ 40% decline in SULmax between baseline and C1D15, and correlation of C1D15 SULmax value ≤3 on PET/CT with RFS and OS. Building on the available TBCRC026 dataset, my role was to formulate the statistical analysis plan with support from our biostatistics collaborator Chiung-Yu Huang, gather additional missing data required for the analyses in collaboration with the lead study coordinator at Johns Hopkins (Rita Denbow) and lead the interpretation, preparation and

presentation of results supported by my thesis supervisors (74, 75). A Data Sharing agreement between Johns Hopkins University and University College Cork was developed to enable my role in this project. This work has been published in the Journal of Nuclear Medicine (75).

In Chapter 3 we explore the relationship between various HER2 and immune-based biomarkers and response to HP, through an analysis of the TBCRC026 trial dataset. We hypothesised that immune processes and the HER2-E intrinsic subtype would be associated with pCR to HP without chemotherapy in this ER-negative, HER2-positive population. We performed a planned secondary biomarker analysis in order to test this hypothesis. Research collaboration agreements were put in place between Johns Hopkins University and the Mayo Clinic with support from the TBCRC, to enable shipping of tissue samples from study participants to the expert team at Mayo to conduct the predefined correlative analyses under supervision of the TBCRC026 study team. NanoString BC360 and IO360 compared differential gene expression in baseline tumours that underwent pCR versus those that did not respond. NanoString GeoMx DSP assessed immune protein abundance in intraepithelial and stromal segments. Secondary objectives included correlation of immune processes with pCR and correlation of breast cancer intrinsic subtype with pCR. My role in this Chapter was to align the tissue biomarker results generated by our collaborator's NanoString analysis with the clinical study results and work with the team at Mayo Clinic (including bioinformatics, pathologists) to ensure clarity and accuracy of results and figures, develop a multivariate analysis plan with support from biostatistics and prepare the manuscript, placing these findings in the context of the current literature.

Chapter 4 further expands on the results from the TBCRC026 trial by *combining promising HER2 tissue-based biomarkers with PET/CT parameters in a composite biomarker analysis*. The PET/CT imaging biomarker performed extremely well in TBCRC026, with a lack of decline in SULmax at C1D15 predictive for those unlikely to achieve pCR with HP alone. We hypothesised that a composite biomarker incorporating baseline HER2 tissue-based biomarkers in addition to PET/CT could further improve biomarker performance in patients with early-stage HER2-positive breast cancer undergoing neoadjuvant therapy with HP alone. Promising imaging biomarkers included in this analysis were ≥40% SULmax decline between baseline and C1D15, and C1D15 SULmax ≤3. Baseline tissue-based biomarkers included HER2-enriched intrinsic subtype (NanoString BC360), log2 tumour HER2 protein abundance (NanoString GeoMxTM DSP), and HER2 overexpression defined as 3+ by IHC. The associations between the exploratory composite HER2/PET-CT biomarker and pCR were analysed, with the goal of further refining predictive biomarkers of response in this setting. My role for this Chapter was to design the composite biomarker question through development of the formal statistical analysis plan with support from our biostatistics collaborator Chiung-Yu Huang, ensure that all relevant clinical, imaging and correlative data was available for the analysis, and to lead the interpretation, preparation and presentation of results (76).

Future validation and clinical utility studies will ideally be prioritised among the global breast oncology community, in order to further progress identification of predictive and prognostic biomarkers. Ultimately the goal is for the most promising biomarkers to be incorporated into daily clinical practice, facilitating individualisation of therapy for patients with early-stage HER2-positive breast cancer.

CHAPTER 2:

TBCRC026 – CORRELATION OF STANDARDISED UPTAKE VALUE ON EARLY INTERIM POSITRON EMISSION TOMOGRAPHY WITH RECURRENCE-FREE SURVIVAL AND OVERALL SURVIVAL IN PRIMARY OPERABLE HER2-POSITIVE BREAST CANCER

INTRODUCTION

Based on the data presented in Chapter 1, a rationale exists for investigating a more individualised approach to care for stage II-III HER2-positive breast cancer. The current standard of care for this patient cohort is neoadjuvant chemotherapy in combination with HER2-directed therapy. This approach has resulted in improved surgical outcomes and high rates of pCR, an accepted surrogate endpoint for survival outcomes (30, 77-79). Additionally this strategy offers the opportunity to adapt post-operative treatment based on the response to the therapy (20, 80, 81). Excellent progress has undoubtedly been made in the treatment of this disease, however the neoadjuvant approach is not without potential adverse effects. It is recognised that there may be subgroups of patients who need this aggressive standard approach, and others who may be cured with less intensive regimens with lesser short and long-term toxicities. Thus,

predictive biomarkers of response to therapy are urgently needed to help tailor treatment recommendations.

Ongoing efforts are investigating a more individualised approach to care. The use of early FDG PET/CT to predict breast cancer treatment response has been of increasing interest (38, 82). The TBCRC026 study examined dual HER2 therapy with HP without chemotherapy in primary operable ER-negative, HER2-positive breast cancer, and reported pCR rates of 22%. Early changes in SULmax on FDG PET/CT predicted pCR to neoadjuvant HP alone, suggesting that this may serve as a potential imaging biomarker of response to therapy (3). Indeed, the preoperative period has been recognised as an ideal setting for evaluating surrogate biomarkers for the prediction of treatment response, and there is a growing body of evidence to support imaging biomarkers in the HER2-positive disease (24, 40, 41). Few studies, however, have examined the relationship between metabolic response on FDG PET/CT following neoadjuvant dual HER2 therapy and long-term patient outcomes.

We thus hypothesised that predefined FDG PET/CT SULmax parameters would be associated with improved RFS and OS in patients with primary operable ER-negative, HER2-positive breast cancer receiving neoadjuvant HER2-directed therapy. To test this hypothesis, we performed a secondary planned biomarker analysis of the TBCRC026 clinical trial dataset.

METHODS

Study Design

TBCRC026 was a single arm multicentre trial investigating biomarkers of response to preoperative HER2-directed therapy without chemotherapy (Figure 3) (3). The primary objective was to correlate baseline and early percentage change (by C1D15) in SULmax on FDG PET/CT of the primary breast cancer with pCR after 4 cycles of neoadjuvant HP, without chemotherapy. The institutional review board approved this study, and all patients signed a written informed consent.

Pertuzumab (840 mg loading dose, then 420 mg) and trastuzumab (8 mg/kg loading dose, then 6 mg/kg) were administered intravenously every 3 weeks. PET/CT was performed at baseline and C1D15. Further neoadjuvant non-study therapy (chemotherapy and/or HER2-directed therapy) was administered as per physician discretion, if incomplete response or disease progression on initial therapy (3). Tumour biopsy was undertaken to histologically confirm residual disease, prior to additional treatment. Any patient who received additional systemic therapy by definition was classified as not achieving pCR following HP alone, per study protocol. Postoperative systemic therapy and radiation per standard of care were recommended. All patients regardless of pCR status were recommended to undergo adjuvant chemotherapy if this was not received preoperatively, as per standard of care.

Eligibility

Eligibility criteria included women 18 years or older with untreated, histologically confirmed infiltrating carcinoma of the breast and clinical stage T2-4(a-c), any N, M0 disease, with tumours ER ≤10% and HER2-positive, by local pathology review (43, 83). Participants agreed to study-specific procedures including two serial FDG-PET/CTs.

The women signed a written informed consent approved by the Institutional Review Boards of participating institutions. The study was registered at clinicaltrials.gov.

^{18}F-FDG PET/CT

PET/CT was performed at baseline and C1D15, with a 3 day window permitted, with detailed imaging manual published previously (3). Administration of intravenous FDG injection was followed by a 60-minute uptake phase, with subsequent PET/CT imaging from mid-skull to mid-femur. All procedures were conducted in conformance with the Uniform Protocols for Imaging in Clinical Trials ^{18}F-FDG PET/CT and Radiological Society of North America Quantitative Imaging Biomarkers Alliance profiles (84, 85). Images were assessed centrally, with reviewers blinded to clinical information.

SULmax, rather than SUV was recorded, as it is less weight dependent than SUV and has been shown to be more consistent than SUV in normal tissues among individuals. The primary breast cancer lesions were measured through the placement of a spherical volume over the target area, with avoidance of surrounding normal tissue, and recording SULmax.

Statistical Considerations

Secondary pre-planned endpoints reported here include the correlation of i) ≥ 40% decline in SULmax between baseline and C1D15, and ii) C1D15 SULmax value ≤3 on PET/CT with RFS and OS. RFS was defined as the interval from the date of first course of treatment (C1D1) to ipsilateral invasive breast tumour recurrence, loco-regional recurrence, distant recurrence, or death of any cause, whichever occurred first. OS was defined as the interval from C1D1 to death (86). Both RFS and OS were censored at the last study contact if no events were observed.

To be evaluable for this analysis, it was required that both baseline and C1D15 PET/CT were performed, SULmax and RFS/OS data collected, and pCR status after HP (without chemotherapy) were evaluated. pCR was determined in the surgical specimen and defined as no viable invasive cancer in the breast and axilla (local pathology review), following HP without chemotherapy. Participants with residual disease after 12 weeks HP or clinical progression on HP were classified as non-pCR.

RFS and OS were summarised using the Kaplan-Meier methods and compared between subgroups using log rank tests. Their associations with PET/CT (≥ 40% decline in SULmax between baseline and C1D15, or C1D15 SULmax value ≤3) and pCR were evaluated using Cox regressions, where the likelihood ratio confidence intervals [CIs] were reported due to small event numbers.

RESULTS

Patient Characteristics

Patient clinicopathologic characteristics are available in Table 1 (3). In summary, 88 women enrolled from January 2014 to August 2017; 83 were evaluable for the survival analysis. Eighty-five percent of participants completed all four cycles of neoadjuvant HP. Twenty-five patients (28%) received additional non-study therapy neoadjuvantly and were classified as not obtaining pCR (Table 1). In 22% (18/83) of patients, pCR was observed after four cycles of HP alone.

Characteristic	*n* = 83 (%)
Age (years)	
Median, Range	58 (29-82)
Race	
White	70 (84)
Black	7 (8)
Other	6 (7)
Menopausal Status	
Pre	27 (33)
Post	56 (67)
ECOG Performance Status	
0	72 (87)
1	11 (13)
Tumour Size (cm)	
Median, Range	3.9 (2-15)
Baseline Clinical Stage	
II	71 (86)
III	12 (14)
Tumour Grade	
2	20 (24)
3	63 (76)
Baseline ER Status	
<1%	72 (87)
1-10%	11 (13)
Additional Neoadjuvant Therapy	25 (30)
Taxane-based	7 (28)
Carboplatin/Taxane	15 (60)

Anthracycline-based	2 (8)
HER2-directed	1 (4)
Surgery	
Mastectomy	46 (55)
Breast Conserving Therapy	29 (35)
NA	8 (10)
pCR	
Yes	18 (22)
No	65 (78)
Adjuvant Chemotherapy	46 (55)
Taxane-based	21 (46)
Carboplatin/Taxane	17 (40)
Anthracycline-based	8 (17)
Adjuvant HER2-targeted therapy	79 (95)
Trastuzumab	56 (67)
Trastuzumab/Pertuzumab	22 (27)
TDM-1	1 (1)
Adjuvant Radiotherapy	
Yes	47 (57)
No	36 (43)
Adjuvant Endocrine Therapy	
Yes	7(8)
No	76 (92)

TABLE 1: PATIENT CHARACTERISTICS AND ADJUVANT THERAPIES RECEIVED IN THE TBCRC026 TRIAL (EVALUABLE POPULATION)

Adjuvant therapy was advised as per standard of care and a summary of treatments received is available in Figure 4, Table 1. There were 22 patients who received no (neo)adjuvant chemotherapy due to patient or physician preference. The majority of participants received adjuvant HER2-directed therapy (79/83; 95%), including trastuzumab (n=56; 67%), HP (n=22; 27%) and adjuvant T-DM1 in one patient with residual disease, which was not available for this indication in the earlier years of study conduct. Adjuvant radiation was completed by 57% (47/83), and adjuvant endocrine therapy by 8% (7/83). This was in keeping with the study eligibility criteria which permitted enrolment of patients with tumours expressing ER ≤10%.

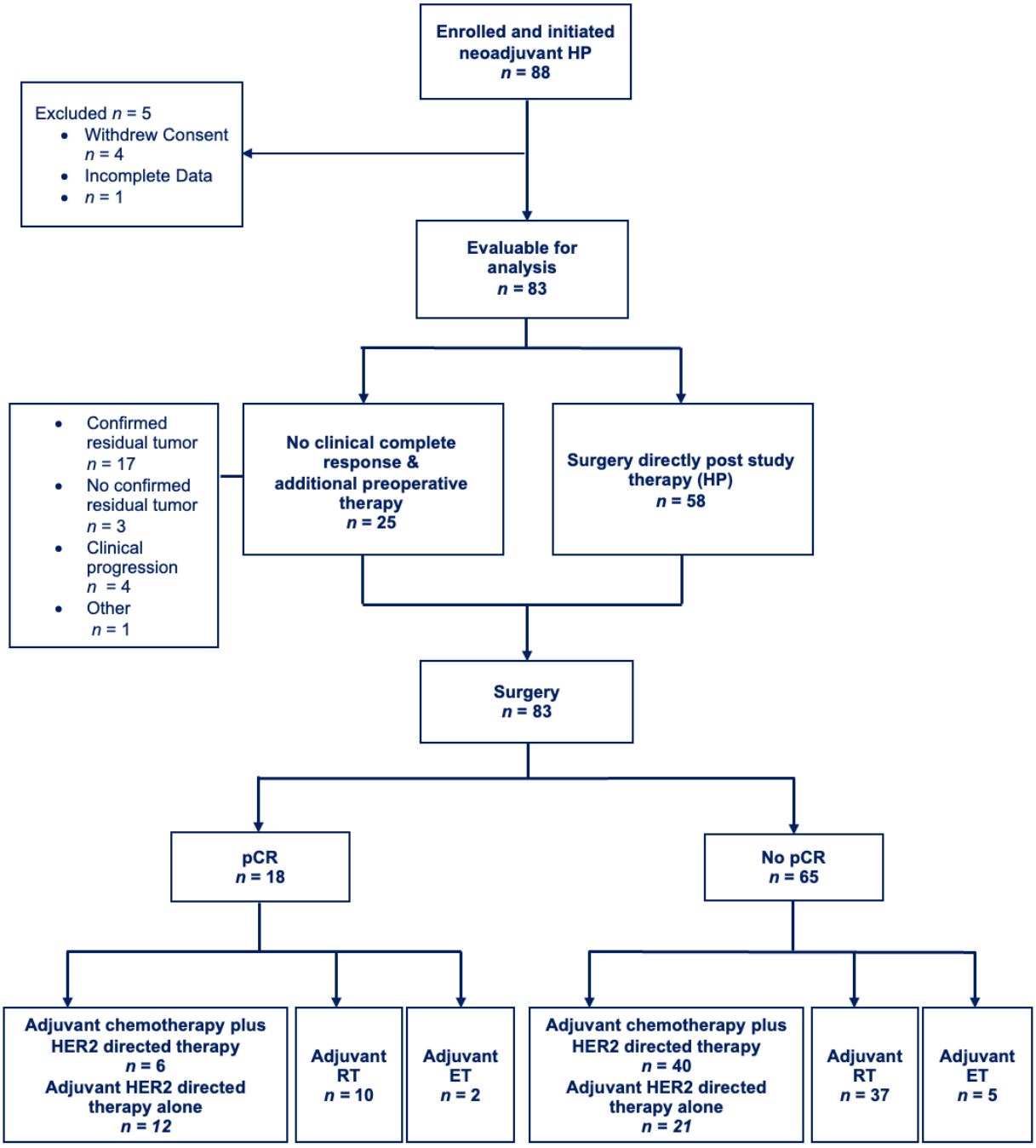


FIGURE 4: STUDY FLOW DIAGRAM INDICATING THERAPIES RECEIVED IN THE TBCRC026 TRIAL

RFS and OS Analyses

The median follow-up was 53.7 months, with 6 deaths and 14 RFS events occurring. The estimated RFS at 3 years was 84% (95% CI, 76%-92%) and the estimated OS at 3 years was 92% (95% CI, 87%-98%). RFS events included 1 loco-regional recurrence and 13 distant recurrence events. The most common site of distant relapse included lung (n=4) and liver (n=2). One patient relapsed with intracranial disease as well as liver and bone involvement and 1 patient developed bone-only disease. Of patients experiencing an RFS event, most (8/14, 57%) were lymph node-positive at baseline and all had at least T2 tumours with a median size of 3.5cm, tumour characteristics indicating higher risk disease. The majority of events were in patients who did not achieve pCR (13/14, 93%), and occurred despite the majority (10/14, 71%) receiving a complete course of (neo)adjuvant chemotherapy in addition to study therapy (HP). In terms of HER2-targeted therapy, most patients with RFS events received trastuzumab monotherapy in the adjuvant setting (8/14, 57%), with the addition of pertuzumab to trastuzumab in 3 patients and T-DM1 for 1 patient. Only one of these recurrence events, and subsequent death, occurred in a patient who had achieved a pCR after HP alone.

In keeping with the prognostic value of obtaining a pCR in published neoadjuvant breast cancer studies, pCR was associated with improved RFS (hazard ratio [HR], 0.25; 95% CI, 0.01-1.24; P=0.10) and OS (HR 0.65, 95% CI 0.03-4.06, p=0.69), although the observed trends did not reach statistical significance at this early point in the study (Figure 5).

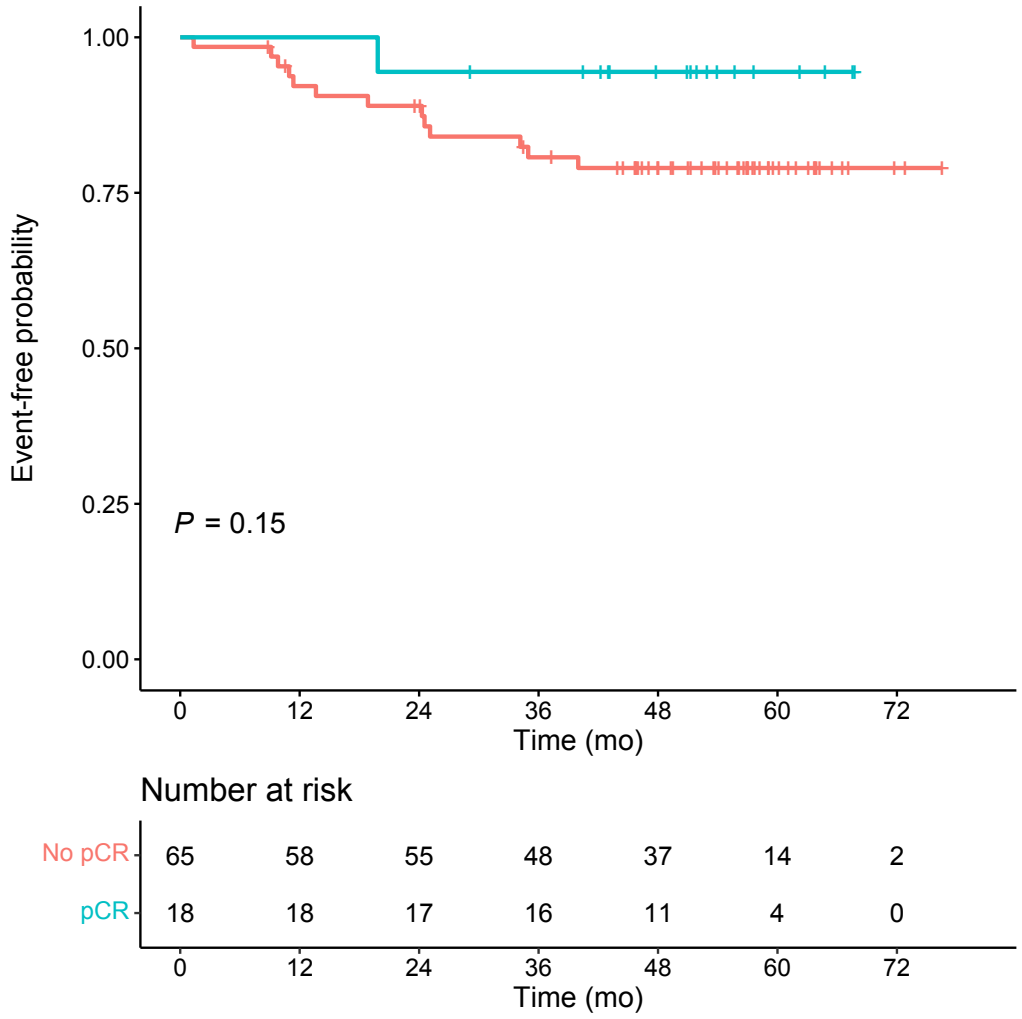


FIGURE 5: RECURRENCE FREE SURVIVAL (RFS) BY PATHOLOGIC COMPLETE RESPONSE (PCR)

Correlation of SULmax with OS and RFS

A summary of baseline and D15 SULmax values has been previously reported and is available for reference in Table 2. Regarding the association of SULmax on PET/CT and survival outcomes, achieving an SULmax value ≤3 by C1D15 after commencing study therapy was associated with an improved RFS, although not statistically significant (HR, 0.36; 95% CI, 0.11-1.05; p=0.06). The 3-year RFS probability was 91% (95% CI, 83%-100%) in those with C1D15 SULmax value ≤3 versus 74% (95% CI, 60%-90%) in

those who did not achieve an SULmax value ≤3 (Figure 6). Interestingly this biomarker parameter, achieved in 57% of patients, was associated with a statistically significant improvement in OS (HR, 0.14, 95% CI, 0.01-0.85; p=0.03). The 3-year OS was 98% (95% CI, 94%-100%) versus 85% (95% CI, 74%-98%) in those who failed to achieve an SULmax value ≤3 (Figure 7).

	No pCR (N=65)	**pCR (N=18)**	**P value**
Baseline SULmax			0.34
Mean (SD)	7.2 (3.7)	6.7 (4.6)	
Median	6.7	5.6	
Range	1.7-14.6	1.6-15.8	
D15 SULmax			<0.001
Mean (SD)	4.6 (3.9)	1.7 (0.6)	
Median	3.6	1.6	
Range	0.6-15.9	0.9-2.8	
D15 SULmax			<0.001
>3	36 (55%)	0 (0%)	
≤3	29 (45%)	18 (100%)	
% Reduction in SULmax			0.03
<40%	31 (48%)	3 (17%)	
≥40%	34 (52%)	15 (83%)	

TABLE 2: SUMMARY OF SULMAX VALUES AND ASSOCIATION WITH PCR IN THE TBCRC026 TRIAL

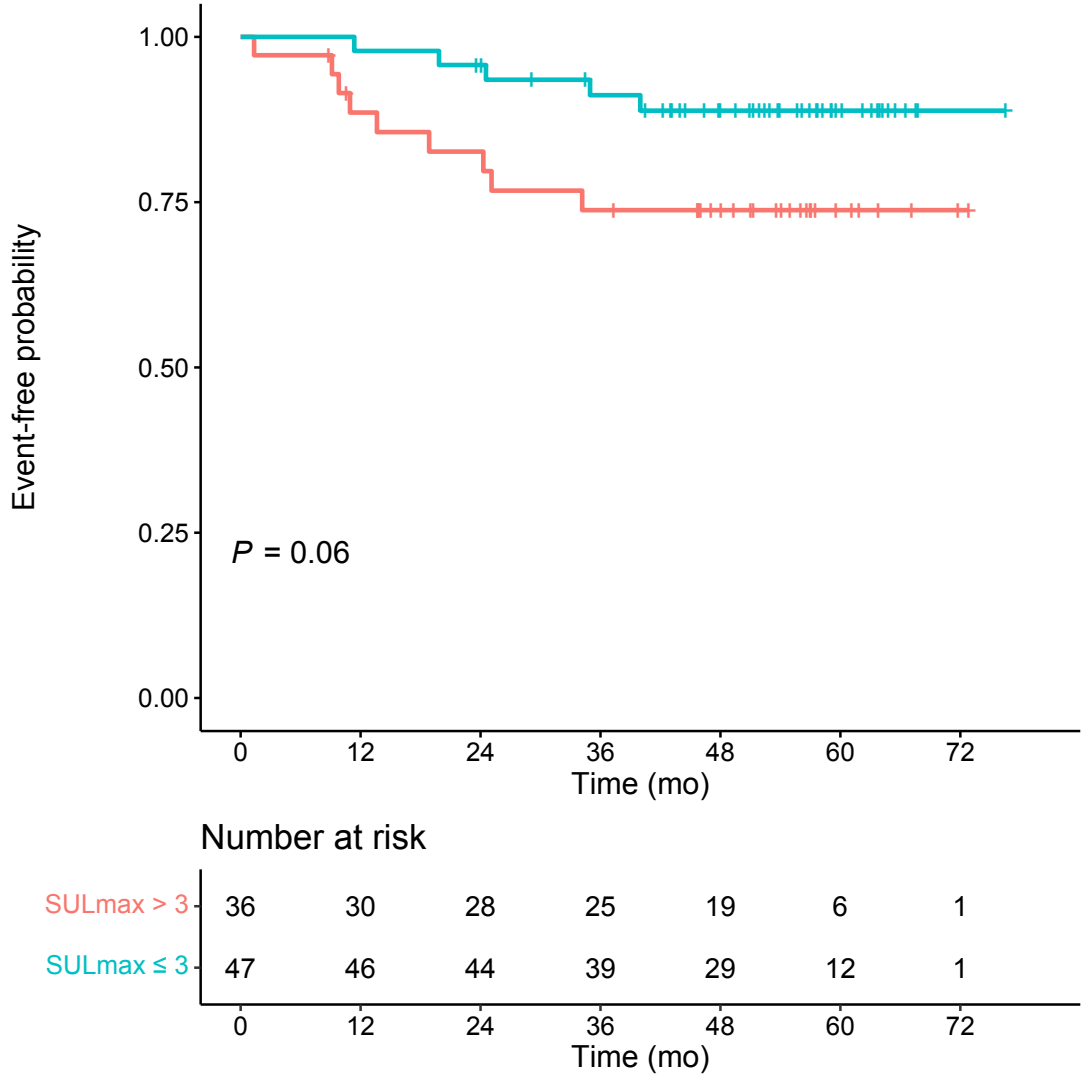


FIGURE 6: RECURRENCE FREE SURVIVAL (RFS) BY D15 SULMAX (≤3 VS >3)

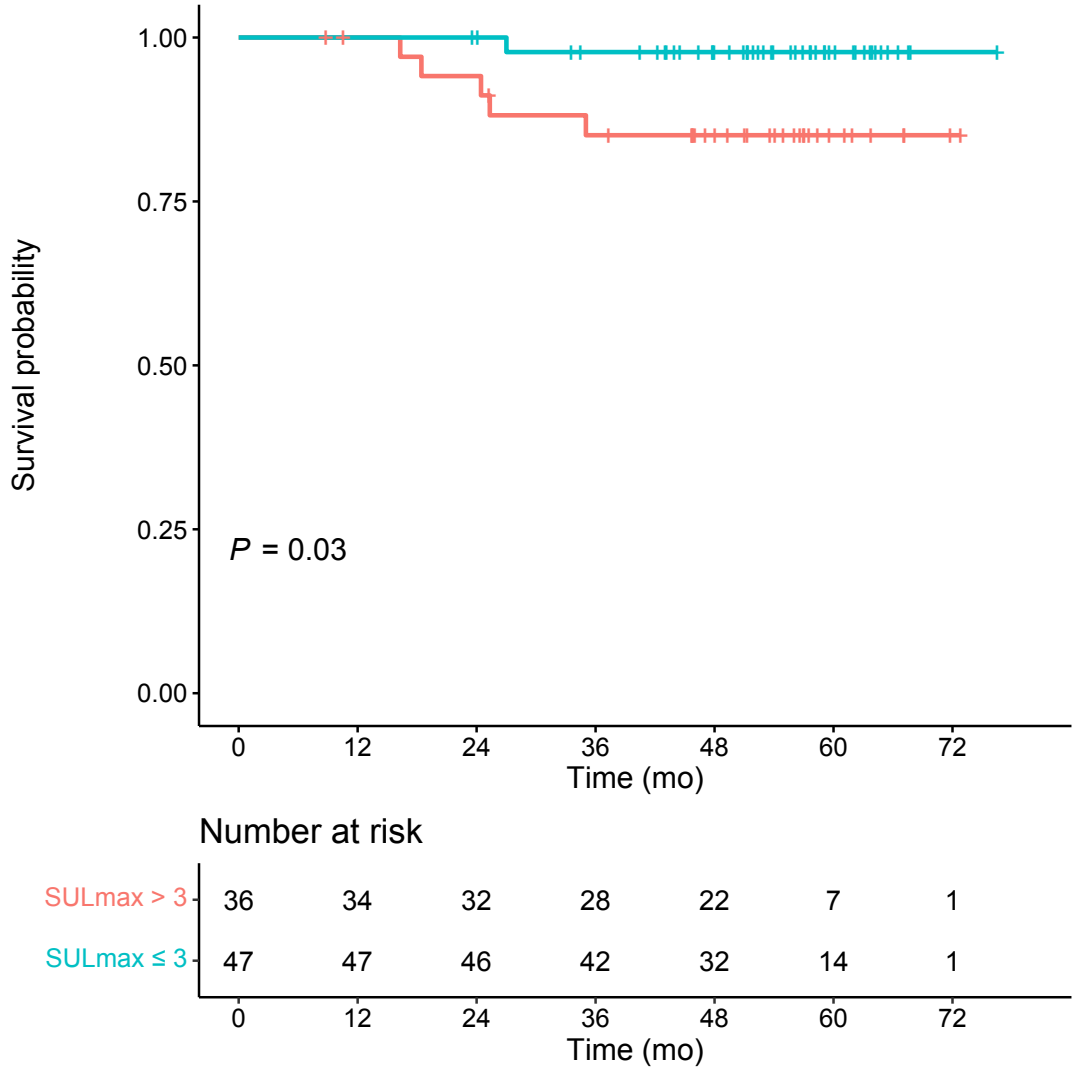


FIGURE 7: OVERALL SURVIVAL (OS) BY D15 SULMAX (≤3 VS >3)

A similar proportion of patients (59%) achieved an SULmax decline ≥ 40% between baseline and C1D15 after starting therapy. The association between SULmax decline ≥ 40% with RFS (HR, 0.45; 95% CI 0.15- 1.28; p=0.13) and OS (HR, 0.62; 95% CI 0.12-3.37; p=0.56) did not reach statistical significance (Figure 8, Figure 9). Finally, the adjusted effect of D15 SULmax ≤3 on RFS did not reach statistical significance in

the multivariate Cox regression that included the clinical variables of age, tumour grade and tumour size (Table 3).

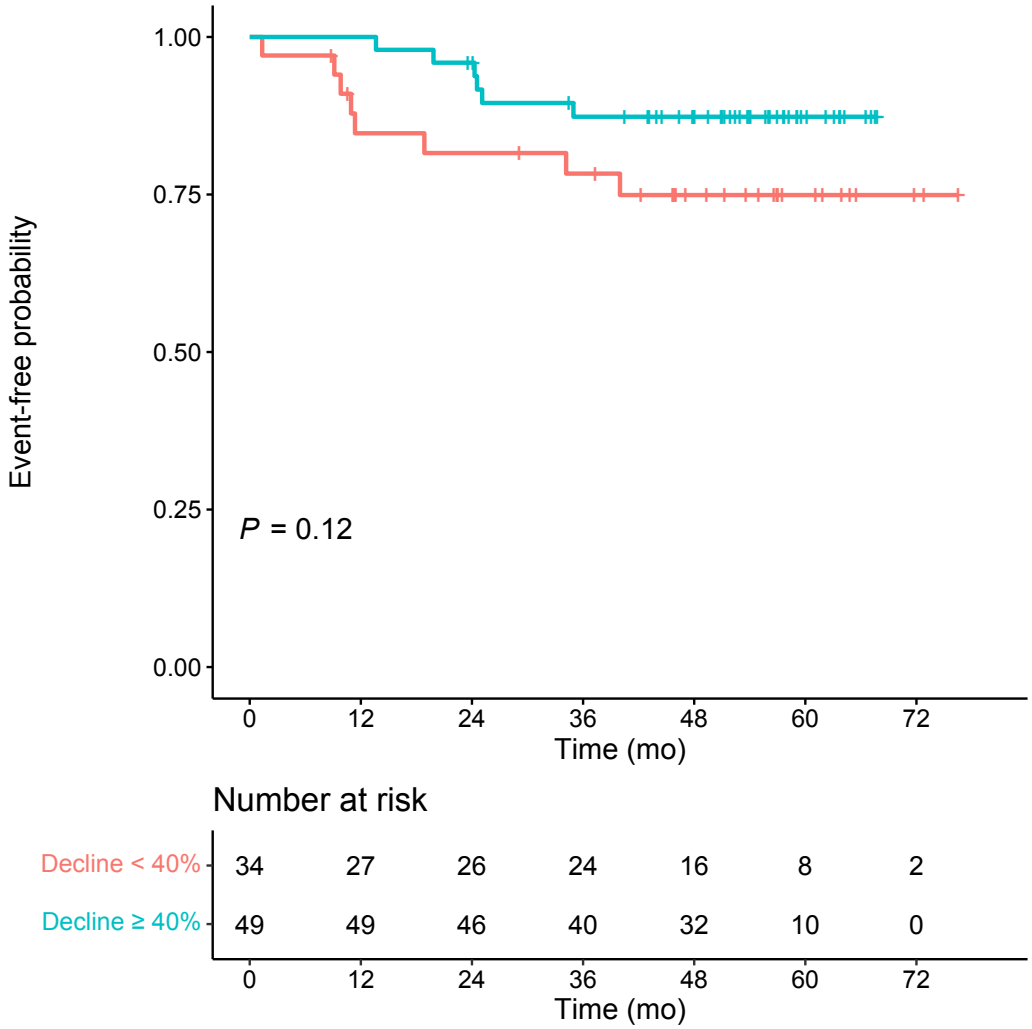


FIGURE 8: RECURRENCE FREE SURVIVAL (RFS) BY SULMAX DECLINE (≥40% VS <40%)

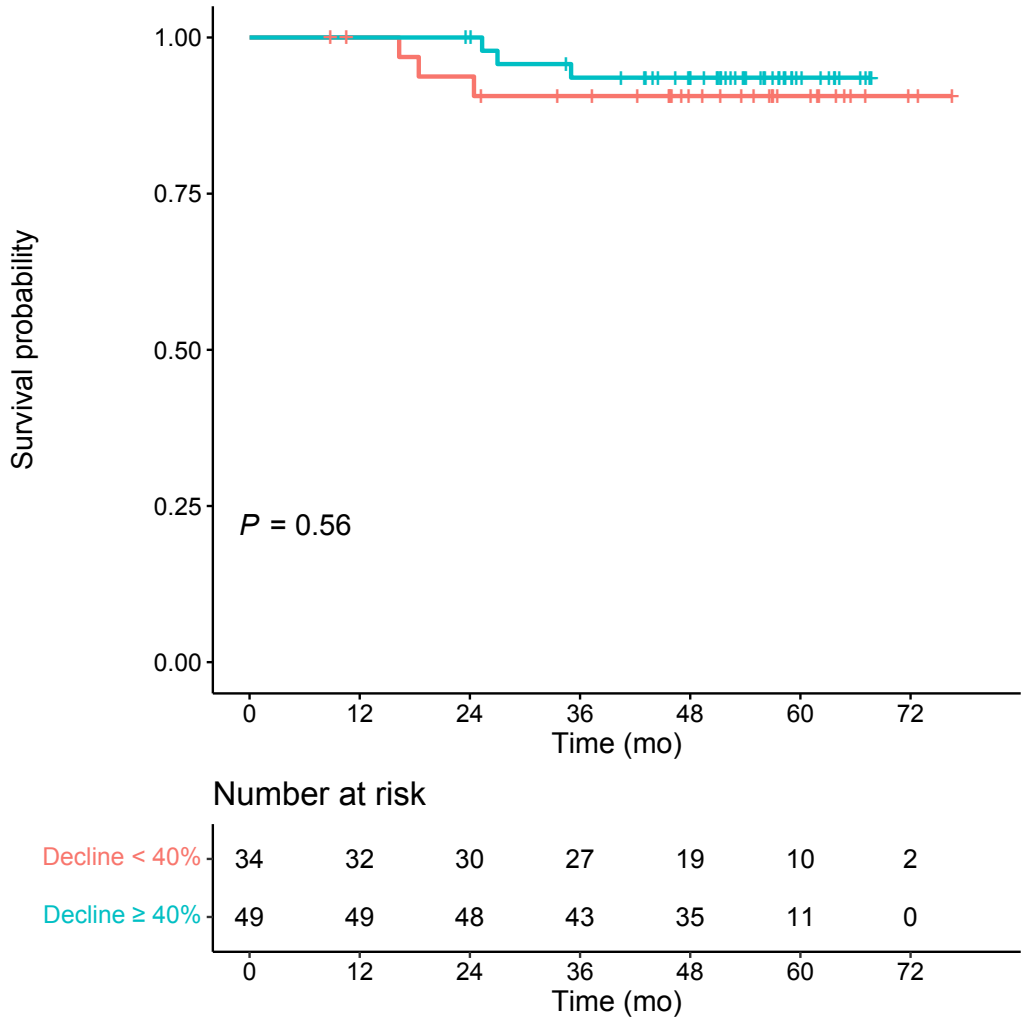


FIGURE 9: OVERALL SURVIVAL (OS) BY SULMAX DECLINE (≥40% VS <40%)

VARIABLE	UNIVARIABLE ANALYSIS		MULTIVARIABLE ANALYSIS	
	HR (95% CI)	**P**	**HR (95% CI)**	**P**
D15 SULMAX ≤3	**0.36 (0.11-1.05)**	**0.06**	**0.41 (0.12-1.22)**	**0.1**
AGE	**1.02 (0.98-1.07)**	**0.3**	**1.04 (0.99-1.1)**	**0.16**
GRADE III	**0.81 (0.27-2.94)**	**0.7**	**0.68 (0.22-2.58)**	**0.55**
BASELINE TUMOUR SIZE (CM)	**1.21 (0.97-1.45)**	**0.09**	**1.23 (0.96-1.5)**	**0.09**

TABLE 3: UNIVARIABLE AND MULTIVARIABLE ANALYSIS OF ASSOCIATION OF DAY 15 SULMAX ≤3 WITH RECURRENCE FREE SURVIVAL;

ABBREVIATIONS: D15, DAY 15; HR = HAZARD RATIO; SULMAX, STANDARDIZED UPTAKE BY LEAN BODY MASS

DISCUSSION

We demonstrated an association between C1D15 SULmax ≤3 on FDG-PET/CT and improved survival outcomes in patients with ER-negative, HER2-positive breast cancer receiving neoadjuvant HER2-directed therapy. Patients receiving HP alone, who achieved an SULmax value ≤3 on their C1D15 FDG-PET/CT, experienced improved RFS (HR, 0.36; 95% CI, 0.11-1.05; p=0.06) as well as statistically significantly improved OS (HR, 0.14, 95% CI, 0.01-0.85; p=0.03), when compared to those who did not achieve this SULmax value. This C1D15 SULmax value was achieved in almost 60% of patients, suggesting that it may be a clinically useful biomarker.

One of the earliest reports suggesting that FDG-PET/CT may be a promising biomarker in HER2-positive early breast cancer was from the NeoALTTO PET sub-study which prospectively evaluated whether changes in SUV could predict response to neoadjuvant anti-HER2 therapy comprising trastuzumab and lapatinib (40). Metabolic responders (> 15% reduction in SUV as determined by the study protocol) had higher pCR rates compared to non-responders (42% versus 21% at week 2; 44% versus 19% at week 6) (40). More recently, studies have focused on neoadjuvant regimens incorporating more modern HER2-directed regimens including HP and the antibody-drug conjugate T-DM1. The PHERGain phase II trial (n=356) randomised patients to neoadjuvant docetaxel, carboplatin and HP (n=71, group A) or HP alone (n=285, group B). PET/CT was performed at baseline and following two cycles of treatment (41). The investigators defined metabolic response after 2 cycles as an SUV decline of ≥ 40% from baseline. Approximately 40% of patients who were designated as metabolic responders and continued dual anti-HER2 therapy achieved a pCR without the addition of chemotherapy,

while ‘non-responders’ in group B switched to neoadjuvant chemotherapy combined with HP. The study met its first co-primary endpoint (41). The phase II PREDIX HER2 trial found that neoadjuvant therapy with docetaxel and HP had similar pCR rates when compared with single agent T-DM1. PET/CT was performed initially and following cycle 2 and 6 of neoadjuvant treatment. In a secondary analysis, decrease of the SUVmax ≥ 68.7% (the median SUVmax decline from baseline to cycle 2) was used as a cut-off, and was associated with pCR in 57% of patients, versus 17% for patients with SUVmax less than the median (24).

The aforementioned studies differed from TBCRC026 in their design, with heterogenous treatment regimens administered, varying eligibility criteria in regards ER status, and SUV thresholds investigated generally ranging from 40-60% assessed at varying times post initiation of therapy. It should be noted that only the TBCRC026 trial was designed prospectively to determine the optimum FDG-PET/CT threshold for response as its primary objective (3). It is thus clear that further prospective studies are required to validate FDG-PET/CT as a biomarker across several standard treatment regimens. If confirmed, this non-invasive biomarker may be incorporated in future clinical trials aiming to determine the clinical utility of this approach in treatment decision-making. This paradigm of biomarker development has led to the successful clinical implementation of interim PET/CT scanning in lymphoma, where patients not achieving the desired PET/CT response are recommended an “escalated” treatment approach (35, 36). The EA1211/DIRECT trial (NCT05710328), led by the ECOG-ACRIN research group, will aim to prospectively validate the 40% SULmax decline threshold at 15 days post initiation of therapy as the optimum cut point across standard of care HER2-directed

neoadjuvant regimens. If validated, future trials may consider a response-guided treatment strategy, with the goal of changing practice.

Ultimately, for those with early-stage, curable HER2-positive breast cancer, long-term survival outcomes are the most important endpoint. We found that pCR following HP alone was associated with numerically improved RFS, however this did not reach statistical significance. Whether the prognostic value of pCR in HER2-positive breast cancer is equivalent if obtained with or without chemotherapy has been debated (60). In addition to our single arm study results, others have observed acceptable long-term outcomes with HER2-directed therapy alone, suggesting that achieving a pCR translates into improved outcomes irrespective of the type of neoadjuvant treatment received (60, 87). That a subset of patients can achieve a pCR and have excellent long-term outcomes without conventional chemotherapy, highlights the need for identification of robust biomarkers and careful study design in order to select out this cohort. This approach is indeed appealing, however will require further validation and clinical utility studies before it can be incorporated into routine clinical practice.

We acknowledge the limitations of this study, which include the heterogeneity in the post-operative therapy received and the possible effect of this on evaluating long-term outcomes. The C1D15 SULmax ≤ 3 was associated with significantly improved OS in univariate analysis although did not reach statistical significance in the multivariable analysis. As this was a secondary analysis the study was not adequately powered for the endpoints of RFS and OS and therefore we await confirmatory studies with larger patient numbers. We used the absolute SUL max and its change as our markers of PET/CT metabolic activity, rather than the PERCIST 1.0 criteria. This study was designed before

PERCIST was widely deployed and has identified a larger percent change for response evaluation than PERCIST 1.0 which is specific to a breast cancer population. In addition, some small and non-FDG avid tumours in TBCRC026 might not have been metabolically assessable by PERCIST 1.0 at baseline. Additional studies using PERCIST 1.0 or a modified PERCIST threshold warrant further study, as the PERCIST 1.0 responders across a wide range of tumour types appear to have superior outcomes to non-responders. It should be noted that the change in SUV or SUL appears to be a more reliable technical metric than absolute SUL which can vary by manufacturer and reconstruction methods. Thus, the absolute SUL threshold would be more difficult to apply routinely than the relative change metric. Further studies and technical standardization could help address this concern.

CONCLUSION

To our knowledge, this is the first report of a potential association between C1D15 SULmax ≤3 on FDG PET/CT after two weeks of neoadjuvant HP alone and RFS/OS outcomes. If confirmed in larger studies, early neoadjuvant interim PET/CT may become a key tool used to adapt therapy for patients with breast cancer in the coming years. Patients demonstrating an early metabolic response could potentially be spared additional chemotherapy, while non-responders could go on to receive intensification of treatment. The ultimate goal will be to facilitate PET biomarker-informed early individualisation of therapy in order to maximise efficacy and minimise toxicity for patients with early-stage HER2-positive breast cancer.

CHAPTER 3:

MULTIPLEX SPATIAL PROTEOMIC ANALYSIS OF HER2-POSITIVE BREAST TUMOURS REVEALS UNIQUE MOLECULAR AND IMMUNOLOGICAL FEATURES ASSOCIATED WITH TREATMENT RESPONSE

INTRODUCTION:

The prognosis of early HER2-positive breast cancer has dramatically improved due to the emergence of HER2 targeted therapies (77, 88, 89). Preoperative chemotherapy in combination with dual HER2 therapy (HP) is the current standard of care for stage II/III disease, however several studies have indicated that a cohort of patients respond to dual HER2 blockade alone (without chemotherapy) (52, 77). Nonetheless, individual outcomes vary, reflective of the heterogenous nature of HER2-positive breast cancer, and no new predictive biomarker of response to dual HER2 targeted treatment has demonstrated clinical utility to date. The TBCRC026 clinical trial evaluated four cycles of HP alone (without chemotherapy) and provides a platform for predictive biomarker discovery in patients with early-stage ER-negative, HER2-positive breast cancer.

The high variability in response to HER2 targeted therapy has been attributed to a variety of biological characteristics, including tumour intrinsic subtype, alterations in

signalling pathways and immune activation (90). The HER2-E intrinsic subtype, comprising approximately 50% of HER2-amplified tumours (47), has shown an association with increased pCR rates following HER2-based neoadjuvant regimens (22, 48, 91-95). Accumulating evidence suggests that the host immune system plays a pivotal role in HER2-positive breast cancer, including in the modulation of response to HER2-directed therapy (54). Further, this subtype typically has higher levels of sTILs (55), which have correlated with improved response to neoadjuvant HER2-directed therapy (57-60). Immune-related gene expression signatures have also demonstrated association with improved outcomes in patients with early HER2-positive breast cancer receiving neoadjuvant therapy (50, 51, 59, 66) and are now being included in novel multi-feature prognostic tools, such as the HER2DX assay (53). Other efforts to advance biomarker discovery include multiplex proteomic characterisation using innovative DSP technology, which provides comprehensive insights into the tumour and immune microenvironment and enables a deeper understanding of complex tumour biology (69, 73).

We hypothesised that immune processes and HER2-E intrinsic subtype would be associated with pCR to HP without chemotherapy in an ER-negative, HER2-positive population. We therefore performed a planned secondary biomarker analysis of the prospective TBCRC026 clinical trial to investigate this hypothesis.

METHODS:

Clinical Trial Design

The multicentre TBCRC026 trial investigated predictive biomarkers of response to neoadjuvant HER2-directed therapy alone in stage II/III ER-negative, HER2-positive

breast cancer. The primary objective was to assess early metabolic change in the primary breast tumour on FDG-PET/CT between baseline and C1D15 and to correlate with pCR, following four cycles of pre-operative HP. Eligible participants for TBCRC026 were patients 18 years or older, with treatment-naïve infiltrating carcinoma of the breast, clinical stage T2-4(a-c), any N, and M0 disease (American Joint Committee on Cancer 7th edition staging). Tumours were histologically confirmed as ER-negative (ER ≤ 10%) and HER2-positive by local pathology review, according to ASCO/CAP guidelines (43). All patients received intravenous trastuzumab plus pertuzumab every three weeks for four cycles as follows: pertuzumab (840mg loading dose, 420mg maintenance dose), trastuzumab (8mg/kg loading dose, 6mg/kg maintenance dose). Additional non-study chemotherapy was received in 28% of patients per physician discretion, in the setting of incomplete response or disease progression on study therapy, at the discretion of the treating clinician. Post-study systemic therapy and radiation was advised as per standard of care. Research biopsies collected at baseline (prior to initiation of therapy) were utilised for biomarker assessment. Written informed consent approved by the institutional review boards of the participating institutions was signed by all patients. Evaluable data including outcome, histopathologic and molecular data were available for 83 patients, (Table 4). A summary of the various specimens available for correlative analyses is given in Table 5.

Characteristic	*n* = 83 (%)
Age (years)	
Median, Range	58 (29-82)
Tumour Size (cm)	
Median, Range	3.9 (2-15)
Baseline Clinical Stage	
II	71 (86)
III	12 (14)
Tumour Grade	
2	20 (24)
3	63 (76)
Baseline Ki67	**N = 65**
Mean, Range	16 (0.1-90)
Stromal TILS	**N= 77**
Median, Range	20 (1-90)
<30%	50 (65%)
>/=30%	27 (35%)
HER2 IHC	**N = 77**
1+	4 (5%)
2+	9 (12%)
3+	64 (83%)
Intrinsic Subtype	**N = 64**
Basal	16 (25%)
HER2 enriched	46 (72%)
Luminal A	2 (3%)
log2 HER2 protein abundance (stroma)	**N = 71**
Median (Range)	10.2 (5.1-13.9)

log2 HER2 protein abundance (tumor)	
Median (Range)	13.5 (7.1-15.9)
log2 Ki67 protein abundance (stroma)	
Median (Range)	10.3 (8.3-11.7)
log2 Ki67 protein abundance (tumor)	
Median (Range)	11.1 (8-13.1)

TABLE 4: SUMMARY OF PATIENT CHARACTERISTICS IN THE TBCRC026 TRIAL, INCLUDING HISTOPATHOLOGIC AND MOLECULAR DATA

Analysis	**N = 83 (Total tumor biopsies)**
HER2 Central IHC	77
HER2 Central FISH	11 (of 13 IHC 1+/2+)
TILs	77
Ki67	65
Intrinsic Subtype (NS)	63
NS BC260/IO 360	63
NS DSP	71

TABLE 5: SUMMARY OF SPECIMENS AVAILABLE FOR CORRELATIVE ANALYSES

HER2, TILS and Ki67 Analysis

Baseline HER2 status for study entry was determined locally by IHC and/or FISH, and HER2 status was subsequently confirmed centrally by IHC by the study pathologist according to ASCO/CAP guidelines. HER2 FISH was performed centrally to assess for HER2 amplification on all tumours with HER2 negative (IHC 0/1+) or equivocal (IHC 2+) results on central IHC (Supplementary Table 1). The degree of TILs in baseline tumour biopsies was assessed centrally on H&E stained sections as the percentage stromal

area occupied by lymphocytes and plasma cells (% sTILs), per the International TILs Working Group scoring guidelines (56). sTILs were evaluated as a continuous measure and as binary variables (<30 versus ≥30%). IHC for Ki67 was performed centrally to determine Ki67 proliferation indices (% cells with any degree of nuclear labeling) at baseline.

Sample Processing

All available H&E slides were evaluated by the study pathologist to identify and annotate regions that contained invasive carcinoma. Regions containing normal breast tissue, necrosis or acute inflammation, biopsy site changes, or ductal carcinoma in situ (DCIS) only were excluded. Core were manually macro-dissected to include only the annotated areas. Multiple tissue cores were manually dissected from formalin-fixed, paraffin-embedded (FFPE) tissue samples, mounted on glass slides. The cores were pooled, and nucleic acids were extracted manually using the Qiagen Allprep DNA/RNA FFPE co-extraction kit (Cat# 80234). RNA quality and quantity were assessed using the Agilent 2100 Bioanalyzer System, Agilent RNA 6000 Nano Ladder kit (Cat#. 5067-1529). DNA quality and quantity was assessed using the NanoDrop 2000 UV Visible spectrophotometer.

Transcriptome Analysis

RNA (300ng) was analysed using the NanoString BC360 and IO360 codesets to evaluate 1239 unique transcripts in baseline tumours, comparing differential expression in tumours that underwent pCR versus no pCR (Supplementary Table 2A-C). Data were normalised to the geometric mean of 20 housekeeping transcripts in IO360 and 18

housekeeping transcripts in BC360, according to standard NanoString protocols (https://university.nanostring.com/gene-expression-data-analysis-guidelines/894768). Quality control protocols for both BC360 and IO360 were employed, and 61 samples passed assessment.

Digital Spatial Profiling (DSP) analysis

DSP analysis was performed using NanoString GeoMxTM to assess immune protein abundance in intraepithelial and stromal segments. Unstained slides (10-micron sections) from baseline core biopsies were stained with three immuno-fluorescent marker antibodies (Pan-cytokeratin, CD68, and CD45), a nuclear dye (SYTO13), plus 77 target antibodies, each conjugated via an UV-labile linker to a unique synthetic oligonucleotide bar code. All antibodies were purchased from NanoString. The samples were digitally scanned, and immuno-fluorescent images were used to select 6 ROI from each sample. ROI selection was guided the study pathologist to avoid normal breast tissue, necrosis or acute inflammation, biopsy site changes, or DCIS. ROIs (600-micron diameter circles) were segmented into intraepithelial (cytokeratin^{+}) and stromal (cytokeratin^{-}/SYTO13^{+}) domains using computer-generated masks. These segments were illuminated in sequence (cytokeratin^{+} first, followed by cytokeratin^{-}) by a computer-guided ultraviolet (UV) laser to release antibody-bound oligonucleotides. Segment-associated oligonucleotides were collected following UV-laser illumination and quantified using conventional nCounter technology.

Data were normalised to the geometric mean of 2 housekeeping proteins (histone H3 and ribosomal protein S6) according to NanoString best practices (PMID: 34503266).

Signal to noise ratio (SNR) was calculated for each protein in each segment using the geometric mean of 2 negative controls (RbIgG and MsIgG1) in that segment as "noise." SNR analysis was used to identify proteins that were near or below background abundance (log2SNR=0). Tumour-level protein abundance was calculated as the mean of 6 intraepithelial or stromal segments per sample.

Statistical analyses

The primary endpoint was pCR, defined as no viable invasive cancer in breast and axilla by local pathology review. All other patients, including those with histologically confirmed residual disease after 4 cycles of HP or clinical progression on HP, were classified as non-pCR. Preplanned secondary objectives reported here include correlation of immune processes with pCR and correlation of breast cancer intrinsic subtype with pCR. To be evaluable for correlative analysis, histopathologic and molecular data were required as well as pCR status after HP (without chemotherapy).

The following were correlated with pCR in univariate analysis: age, tumour size, tumour grade, baseline Ki67, baseline sTILs (as continuous and binary <30% vs. ≥30%), HER2 overexpression by IHC, intrinsic molecular subtype, HER2 protein abundance (DSP) and Ki67 protein abundance (DSP). Multivariate logistic regressions were used to test associations between variables significant in univariate analysis ($p<0.05$) and pCR.

Differential expression at the transcript level (IO360 and BC360) was assessed using a generalised linear model comparing samples that underwent pCR versus no pCR (Supplementary Table 2B, C). Multiple testing error was estimated using the Benjamini-Yekutieli model (96). Gene Ontology (GO) enrichment analysis was carried out using the

GO enrichment tool (http://geneontology.org/) to identify biological processes that were associated with pCR. GO enrichment data were filtered for FDR<0.05 and ≤100 genes/GO terms (Supplementary Table 3A, B).

The linear mixed model was used to calculate differential expression of protein abundance in intraepithelial and stromal segments from DSP analysis. This model was used to estimate Fold Change (FC) and p-value for intraepithelial segments as a function of pCR (Supplementary Table 4A, B). Differential expression of both RNA and protein was calculated using R Statistical Software (version 4.2.2). Univariate descriptive statistics were calculated using Stats Direct. The association between baseline sTILS, Ki67, immune cell marker proteins (DSP) and immune processes (NS) with HER2 protein abundance was also examined.

RESULTS:

Patient Characteristics

Clinicopathologic characteristics and demographics of patients with ER ≤10% and HER2-positive breast cancer from TBCRC026 are summarised in Table 4, with 83 evaluable for biomarker assessment. The majority (85%) of patients completed all four cycles of neoadjuvant HP, and a subset (28%) of patients received additional non-study pre-operative therapy. Eighteen (22%) patients achieved a pCR after neoadjuvant therapy with HP alone.

TILs, HER2, and Ki67 Analysis

Baseline mean and median sTILs in evaluable tumours were 28% and 20%, respectively, and did not correlate with pCR (p=0.83). There was no significant difference in pCR rates in samples with < 30% vs ≥ 30% sTILs (p=0.55) (Figure 10).

Endpoint	Total Samples with sTILs	sTILS ≥30% pCR	sTILs ≥30% No pCR	sTILs<30% pCR	sTILs<30% No pCR	Fisher's Exact p-value
pCR	77	4	23	11	39	0.55

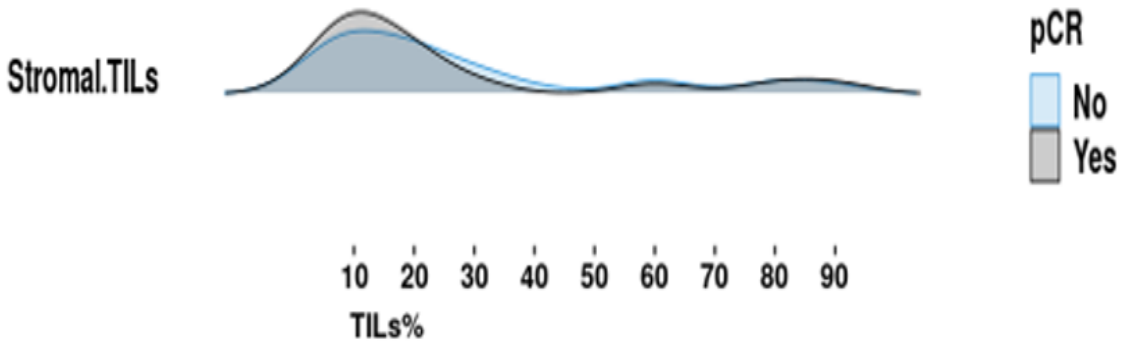


FIGURE 10: ASSOCIATION BETWEEN STILS AND PCR

Central assessment of HER2 status confirmed the majority (83%) of evaluable tumours (77/83) had HER2 overexpression (score 3+) by IHC (Table 4 and Supplementary Table 1). Thirteen tumours were negative (IHC 1+) or equivocal (IHC 2+) for HER2 overexpression, and subsequent FISH confirmed HER2 amplification in all evaluable tumours, Supplementary Table 1. Almost all cases of pCR (93%, 14/15) in this cohort occurred in tumours with HER2 overexpression (IHC 3+) versus those without (7%, 1/15) ($p<0.001$). Baseline Ki67 proliferation indices were significantly higher in cases with residual disease (no pCR) compared to those with pCR ($p=0.04$, Figure 11).

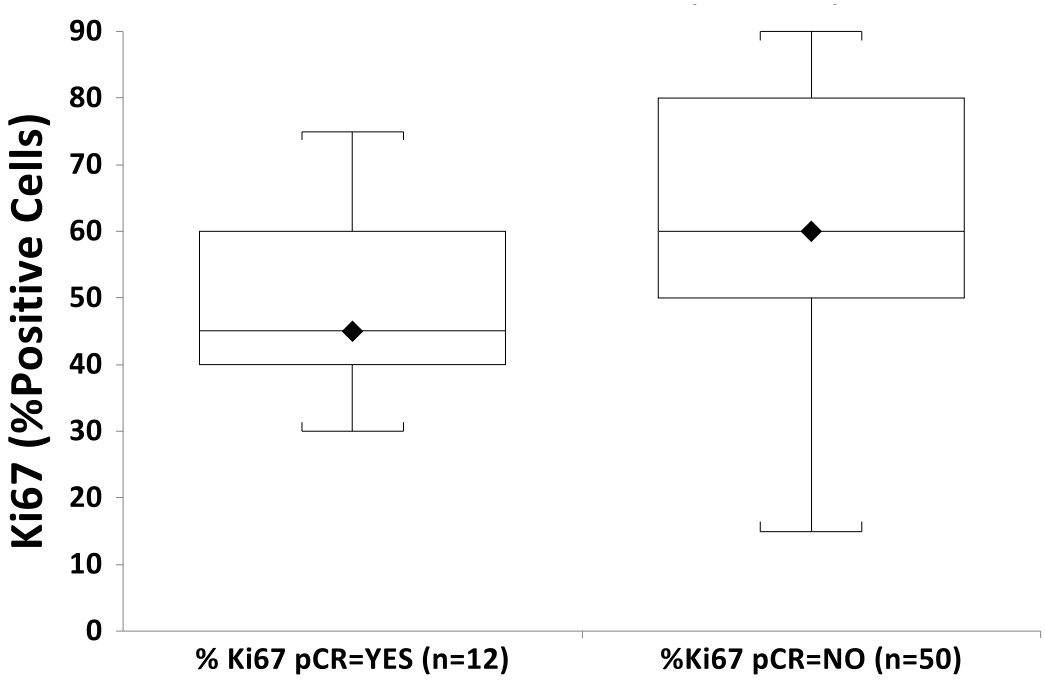


FIGURE 11: ASSOCIATION BETWEEN BASELINE KI67 AND PCR STATUS

Breast Cancer Intrinsic Subtype

NanoString BC360-based analysis of intrinsic subtype was evaluable in 64 tumours. The majority (72%, 46/64) of tumours were classified as HER2-E, with a subset classified as basal-like (25%, 16/64) or luminal A (3%, 2/64). pCR was achieved in 26% (12/46) HER2-E tumours and 50% (1/2) luminal A tumours, but in only 6% (1/16) of basal-like tumours. The association between intrinsic subtype and pCR did not reach statistical significance ($p=0.158$).

Digital Spatial Profiling of Protein Abundance

NanoString GeoMxTM DSP data were obtained for 77 proteins (including 3 negative controls and 3 housekeeping proteins) in intraepithelial and stromal segments from 62 patients' tumours (12 pCR, 50 no-pCR). Differential protein expression as a function of pCR (expressed as SNR) revealed that the majority of the immune proteins were expressed at similar abundance in both intraepithelial (Figure 12) and stromal (Figure 13) segments, regardless of pCR status. Analysis of immune cell type markers revealed no significant difference in intraepithelial or stromal markers of T cells, B cells, macrophage/myeloid cells, or antigen presenting cells (Table 6).

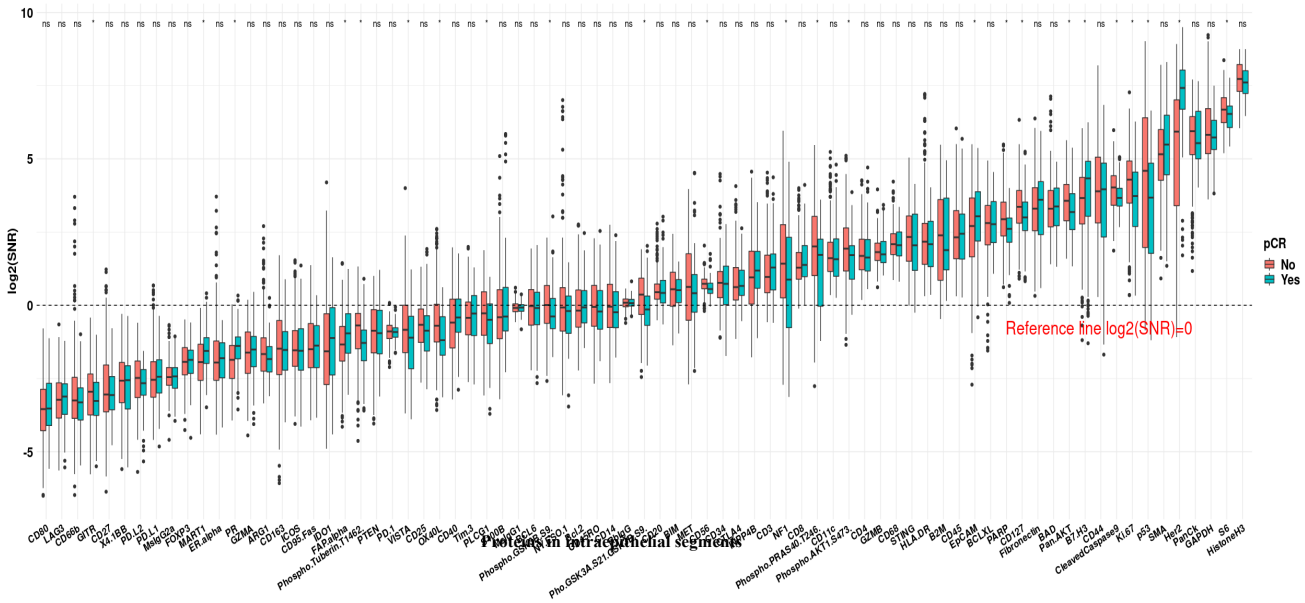


FIGURE 12: DIFFERENTIAL PROTEIN EXPRESSION IN INTRAEPITHELIAL SEGMENTS AS A FUNCTION OF PCR BY DSP

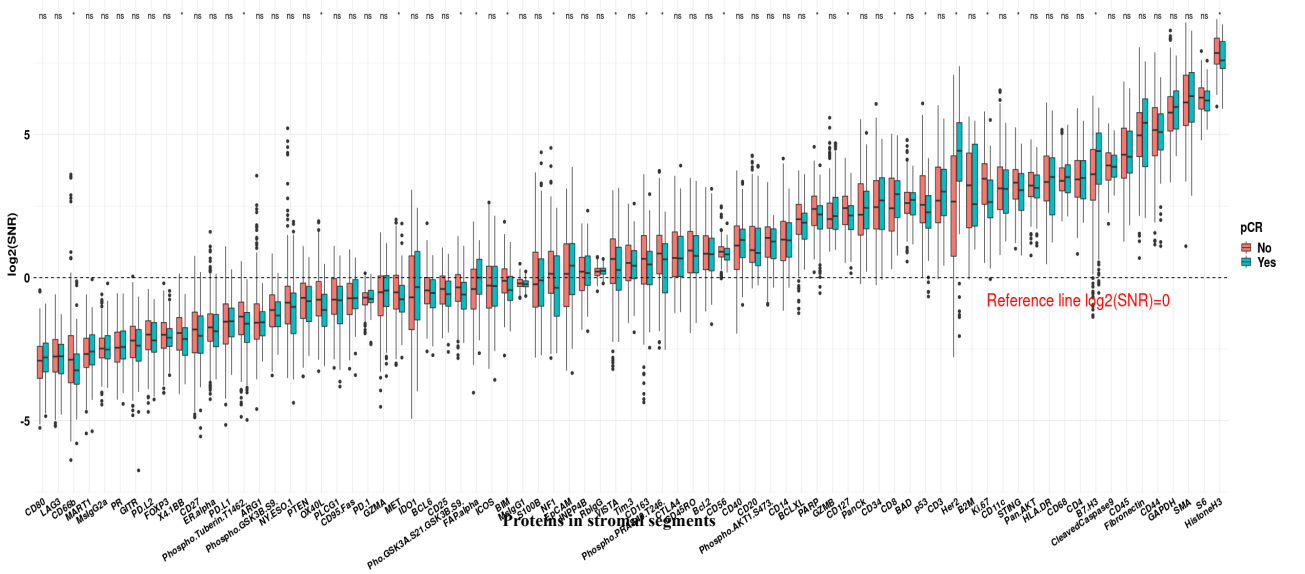


FIGURE 13: DIFFERENTIAL PROTEIN EXPRESSION IN STROMAL SEGMENTS AS A FUNCTION OF PCR BY DSP

Protein	Log2 Mean Intraepithelial pCR	Log2 Mean Intraepithelial No pCR	p-value	Log2 Mean Stroma pCR	Log2 Mean Stroma No pCR	p-value
T cells						
CD127	10.1	10.3	0.317	9.3	9.4	0.316
CD25	6.2	6.2	0.990	6.6	6.6	0.873
CD27	4.1	4.1	0.914	5.2	5.2	0.989
CD3	8.3	8.1	0.289	10.1	9.9	0.399
CD4	8.9	8.7	0.318	10.7	10.5	0.336
CD45RO	7.0	6.9	0.950	8.0	7.9	0.632
CD8	8.6	8.4	0.202	10.0	9.6	0.057
CTLA4	7.9	7.8	0.640	8.0	7.9	0.745
FOXP3	5.1	5.0	0.350	5.1	5.0	0.385
GITR	3.8	4.0	0.431	4.8	4.8	0.860
LAG3	3.9	3.7	0.250	4.3	4.3	0.700
B cells						
CD20	7.6	7.5	0.4	8.4	8.3	0.713

Macrophage/Myeloid Cells						
ARG1	5.3	5.4	0.510	5.6	5.6	0.884
CD11c	8.8	8.7	0.791	10.3	10.2	0.640
CD14	7.0	7.0	0.990	8.5	8.4	0.644
CD163	5.5	5.4	0.733	7.6	7.6	0.776
CD40	6.7	6.4	0.159	8.4	8.1	0.132
CD66b	3.7	3.9	0.491	4.0	4.4	0.267
CD68	9.1	9.1	0.610	10.7	10.5	0.301
CD80	3.6	3.3	0.126	4.4	4.1	0.097
IDO1	5.9	5.6	0.419	6.9	6.5	0.288
VISTA	5.9	6.1	0.282	7.5	7.5	0.829
Antigen Presenting Cells						
HLA.DR	9.2	9.3	0.864	10.5	10.5	0.920
B2M	9.5	9.3	0.551	10.3	10.1	0.489

TABLE 6: ASSOCIATION BETWEEN INTRAEPITHELIAL AND STROMAL IMMUNE CELL TYPE MARKERS AND PCR

Five proteins were identified with differential (fold change) intraepithelial expression levels achieving $p<0.05$ (Supplementary Table 4A), specifically PR, MART1, FAPα, phospho-GSK3A, and HER2. Intraepithelial HER2 was the only protein with relatively high abundance (log2 mean 13.84) that was significantly more abundant in tumours that achieved pCR (log2FC 2.14, p=0.001, Supplementary Table 4A, Figure 12

and Figure 14). Stromal segment HER2 expression was also significantly higher in tumours that achieved pCR (log2FC 1.97, p=0.001, Supplementary Table 4B), whereas stromal Ki67 was relatively high (log2 mean 10.47 versus 11.37 in intraepithelial segments) and was more abundant in samples without pCR (log2FC -0.45, p=0.025) (Supplementary Table 4B).

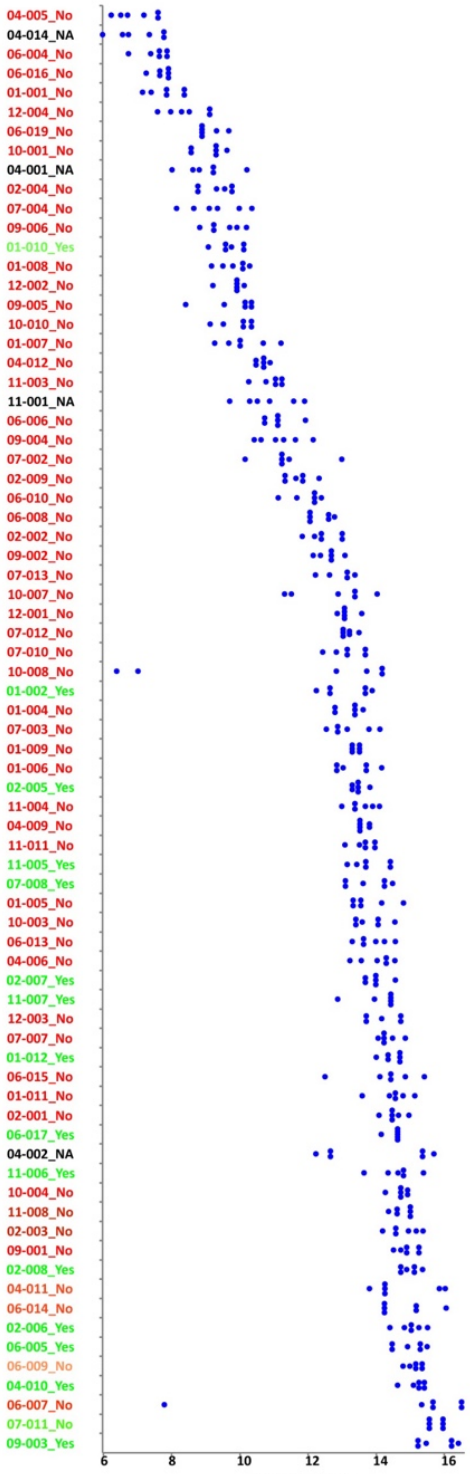


FIGURE 14: ASSOCIATION BETWEEN LOG2 HER2 INTRAEPITHELIAL ABUNDANCE AND PCR; SAMPLES WITH LOWER HER2 ABUNDANCE (HIGHLIGHTED IN RED) ALMOST NEVER UNDERGO PCR

HER2 abundance as a function of pCR

The data shown in Figure 12 and Supplementary Table 4A indicate that intraepithelial abundance of HER2 protein is strongly associated with pCR in these samples. Intraepithelial and stromal HER2 protein abundance was significantly associated with pCR after controlling for Ki67 stromal or intraepithelial abundance, respectively, and tumour size on multivariate analysis (Table 7). Figure 14 shows log2 HER2 abundance for each of six intraepithelial segments in each tumour. Three key features are apparent from these data. There was heterogeneity of HER2 abundance within a given tumour, with the majority of tumours displaying an average log2=1 (2-fold) range of expression between the 6 evaluated segments. There was extreme heterogeneity in HER2 abundance between the HER2-positive tumours, with a log2=10 (1000-fold) range from the lowest to highest segments. Finally, samples with lower HER2 abundance rarely undergo pCR (highlighted in red in Figure 14). Tumour average (mean of 6 intraepithelial segments per tumour) data indicate that the average range of HER2 abundance was about log2=9 (500-fold), (Figure 15).

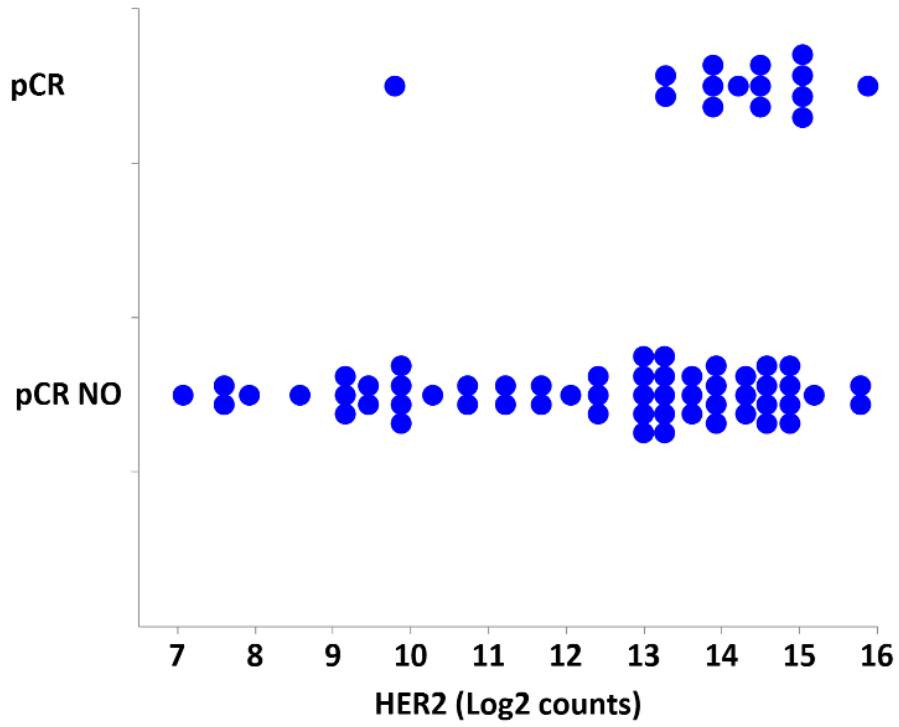


FIGURE 15: TUMOUR AVERAGE INTRAEPITHELIAL HER2 ABUNDANCE AND PCR STATUS

Variable	Univariate analysis		Multivariate analysis			
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Baseline tumour size (cm)	0.56 (0.33-0.86)	0.02	0.56 (0.29 – 0.91)	0.04	0.51 (0.25 – 0.87)	0.03
Log2 HER2 protein abundance (stroma)	1.69 (1.2 – 2.62)	0.008	1.55 (1.08 – 2.43)	0.03		
Log2 Ki67 protein abundance (stroma)	0.42 (0.18 – 0.93)	0.04	0.45 (0.26 – 1.16)	0.1		
Log2 HER2 protein abundance (intraepithelial)	1.75 (1.2 – 2.97)	0.01			1.74 (1.17 - 3.06)	0.02
Log2 Ki67 protein abundance (intraepithelial)	0.6 (0.31 – 1.1)	0.10			0.51 (0.2 – 1.15)	0.11

TABLE 7: UNIVARIABLE AND MULTIVARIATE LOGISTIC REGRESSION ANALYSIS OF TUMOUR SIZE, HER2 AND KI67 ABUNDANCE AND PCR STATUS

The majority (93%, 14/15) of tumours that achieved pCR had high intraepithelial HER2 protein abundance, whereas tumours that did not undergo pCR had either low or high HER2 abundance. The Odds Ratio (OR) for pCR comparing the upper and lower terciles of HER2 abundance was OR 4.81 (95%CI 1.82-12.72, p=0.002). Assessment of segment level distribution of HER2 abundance as a function of pCR (Figure 16) illustrates this finding in another way. While nearly all (95%, 19/20) tumours with low HER2 abundance (log2<11.5) failed to undergo pCR, tumours with high HER2 abundance (>log2 11.5) may or may not undergo pCR. Indeed, the majority (73%, 37/51) of tumours with high HER2 abundance failed to achieve pCR, while only a quarter (27%, 19/51)

achieved a pCR. The empirical breakpoint of log2(HER2 abundance) =11.5 that separates low versus high HER2 protein abundance by DSP also corresponds to the breakpoint separating tumours with no/equivocal HER2 overexpression (IHC1+/2+) versus those with HER2 overexpression (IHC3+) (Figure 17).

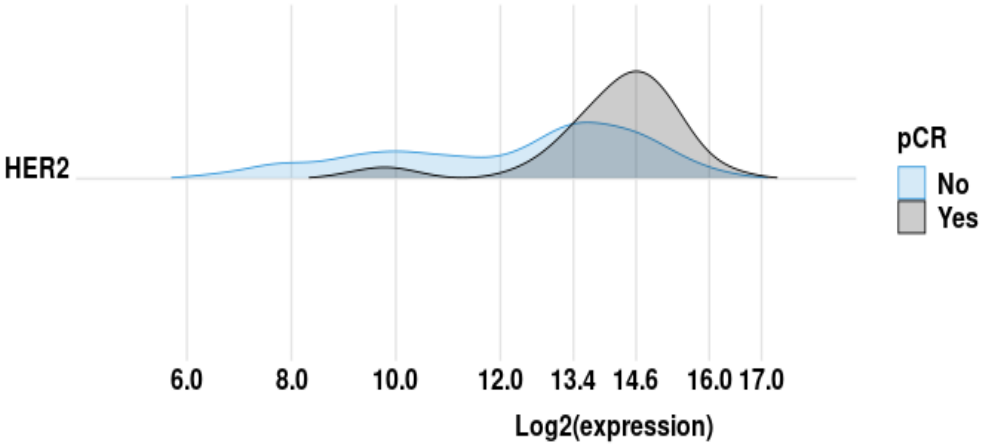


FIGURE 16: SEGMENT LEVEL DISTRIBUTION OF HER2 ABUNDANCE AS A FUNCTION OF PCR

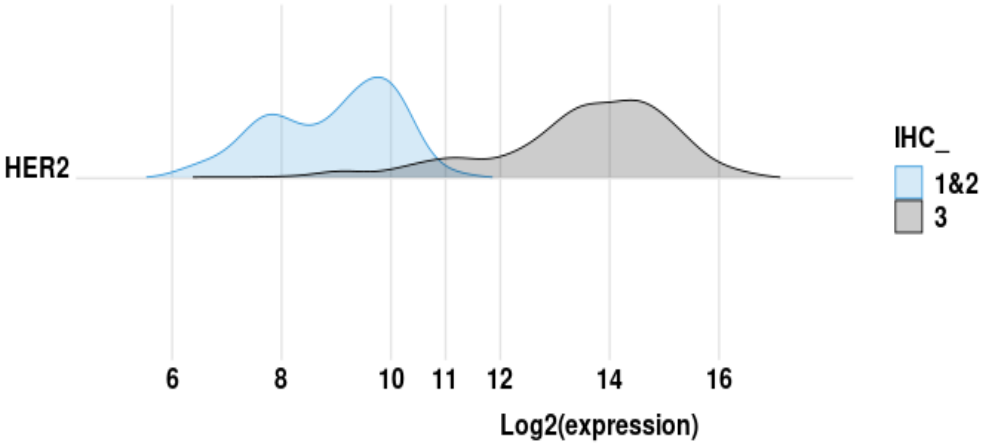


FIGURE 17: SEGMENT LEVEL DISTRIBUTION OF HER2 ABUNDANCE AS A FUNCTION OF HER2 EXPRESSION ON IHC; ILLUSTRATING THE RELATIONSHIP BETWEEN THE BREAKPOINT OF LOG2 HER2=11.5 AND IHC1/2 AND IHC3

Based on these distributions, we subsequently focused on three classes of HER2-positive tumours: low HER2 abundance/non-pCR (n=19), high HER2 abundance/non-pCR (n=37), and high HER2 abundance/pCR (n=14). We assessed histopathologic features, protein expression by DSP, and gene expression in these three categories. Half (53%, 10/19) of the low HER2 abundance/non-pCR tumours were of the basal intrinsic subtype, compared to 12% (6/51) of tumours with high HER2 abundance (Fisher's exact test, p=0.0007). There were no significant differences between baseline Ki67 or sTILs and few significant differences between immune cell marker protein expression by DSP in these three categories (Figure 18, Figure 19, Figure 20, Figure 21). However, the transcriptome landscape of the high HER2 abundance tumours was strikingly different between those that did versus did not achieve pCR.

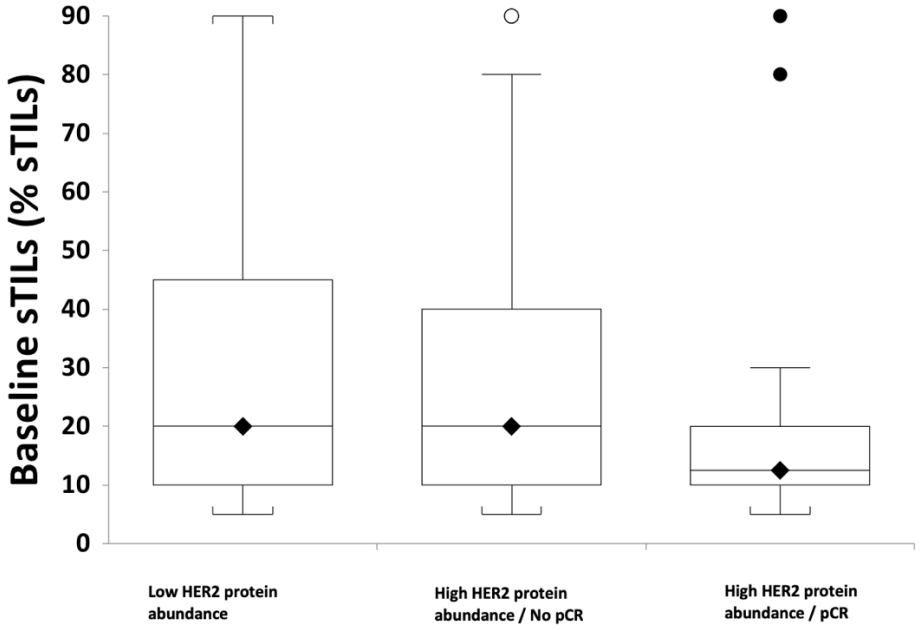


FIGURE 18: EVALUATION OF BASELINE KI67 IN THE THREE CLASSES OF HER2 POSITIVE TUMORS (BY HER2 PROTEIN ABUNDANCE [HIGH/LOW] AND PCR STATUS)

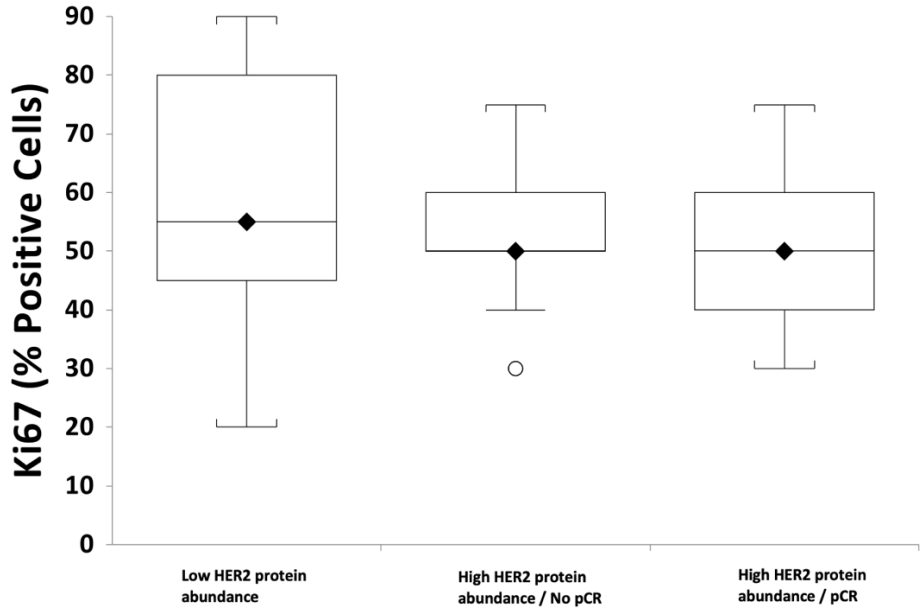


FIGURE 19: EVALUATION OF BASELINE STILS IN THE THREE CLASSES OF HER2 POSITIVE TUMORS (BY HER2 PROTEIN ABUNDANCE [HIGH/LOW] AND PCR STATUS)

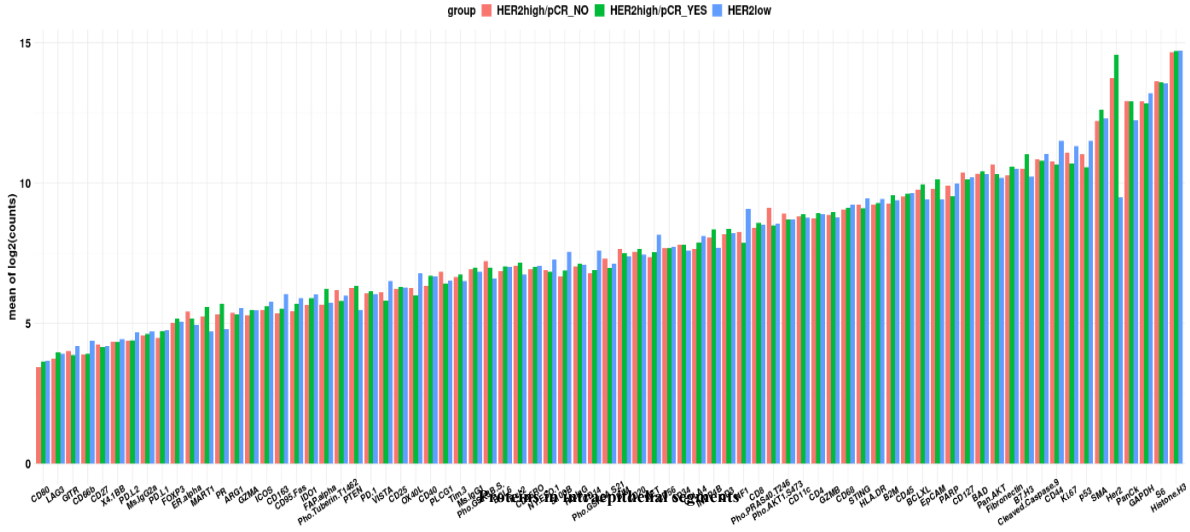


FIGURE 20: PROTEIN ABUNDANCE (DSP) BY HER2 ABUNDANCE AND PCR IN INTRAEPITHELIAL SEGMENTS

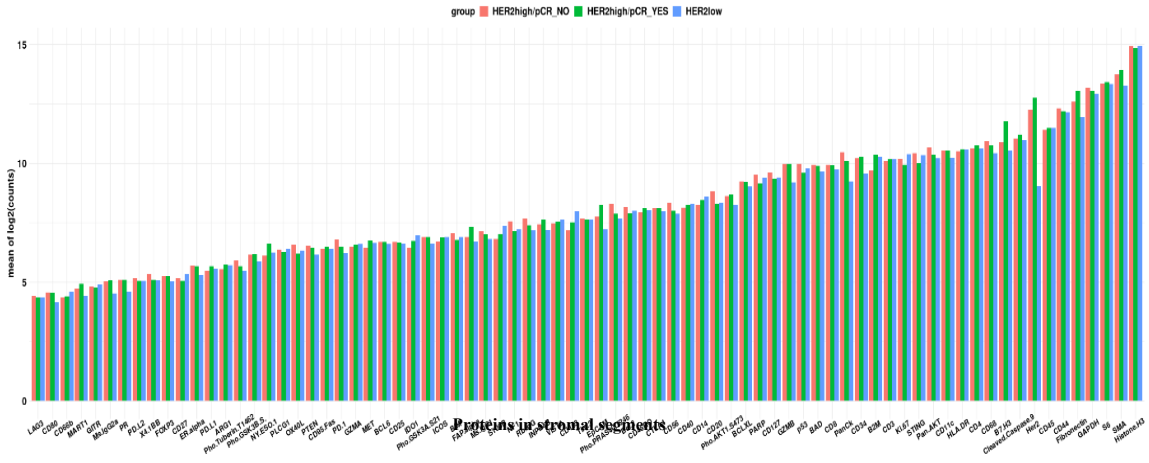


FIGURE 21: PROTEIN ABUNDANCE (DSP) BY HER2 ABUNDANCE AND PCR IN STROMAL SEGMENTS

Analysis of the transcriptome landscape of high HER2 abundance tumours

Transcript abundance data were available from high HER2 abundance/pCR (n=14) and high HER2 abundance/non-pCR (n=37) tumours. NanoString IO360 and BC360 codesets were used to interrogate 1239 unique transcripts, 246 of which were differentially expressed at nominal $p<0.05$ (Supplementary Table 2A). GO enrichment analysis was carried out to identify biological processes that were associated with pCR. Four GO terms were identified associated with Erbb/EGFR signalling. These were consolidated, and differential expression of the 10 associated transcripts is shown in Figure 22, and described below.

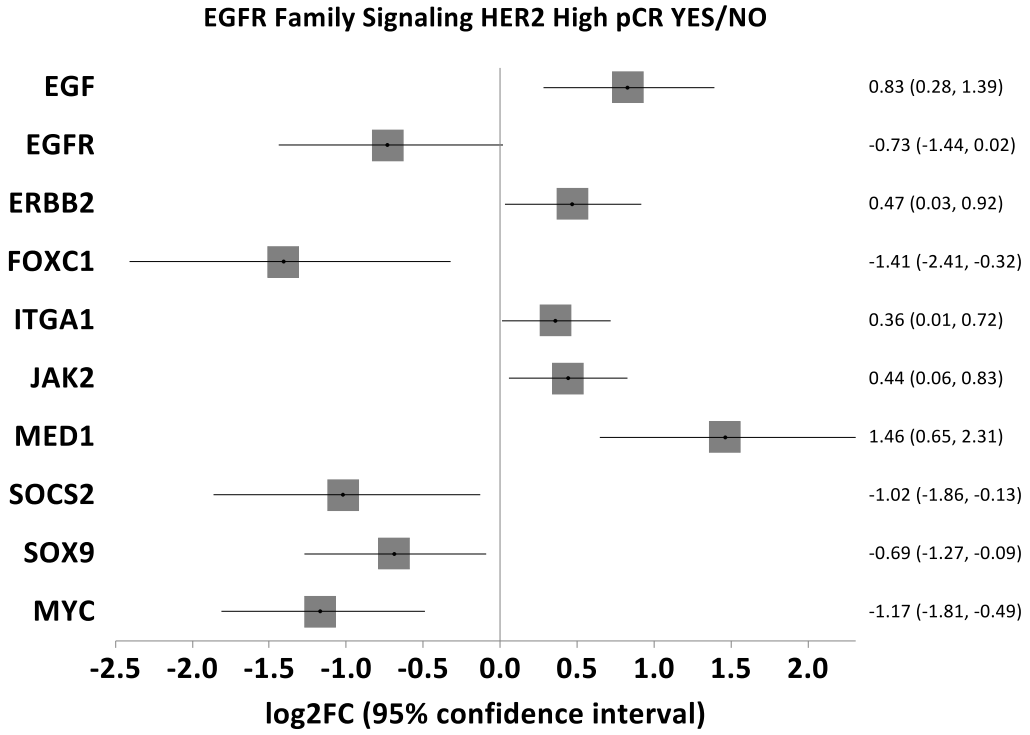


FIGURE 22: DIFFERENTIAL EXPRESSION OF TRANSCRIPTS RELATING TO EGFR SIGNALLING IN TUMOURS WITH HIGH HER2 PROTEIN ABUNDANCE AND ASSOCIATION WITH PCR; EGFR SIGNALLING IS ENRICHED IN TUMOURS WITH HIGH HER2 PROTEIN ABUNDANCE/NO PCR

High HER2 abundance/non-pCR tumours: Tumours with high HER2 abundance/non-pCR had more abundant EGFR mRNA transcripts, coupled with overexpression of the EGFR-activated transcription factor FOXC1 and the FOXC1 target gene MYC (Figure 22). GO enrichment analysis of genes overexpressed in tumours with high HER2 abundance/non-pCR further identified 230 biological processes that were significantly enriched (Supplementary Table 5A). Among these, 45 were associated with M-phase-related biological processes, demonstrating that these tumours are enriched for processes related to mitotic spindle formation, cytokinesis, and M-phase progression (Figure 23). Relevant pharmaceutical targets [e.g. Aurora kinase A/B (AURKA/B)] are

overexpressed in such tumours. Overall, this suggests that there are a subset of tumours expressing high level HER2 but driven by EGFR signalling and which manifest enrichment for targetable M-phase processes.

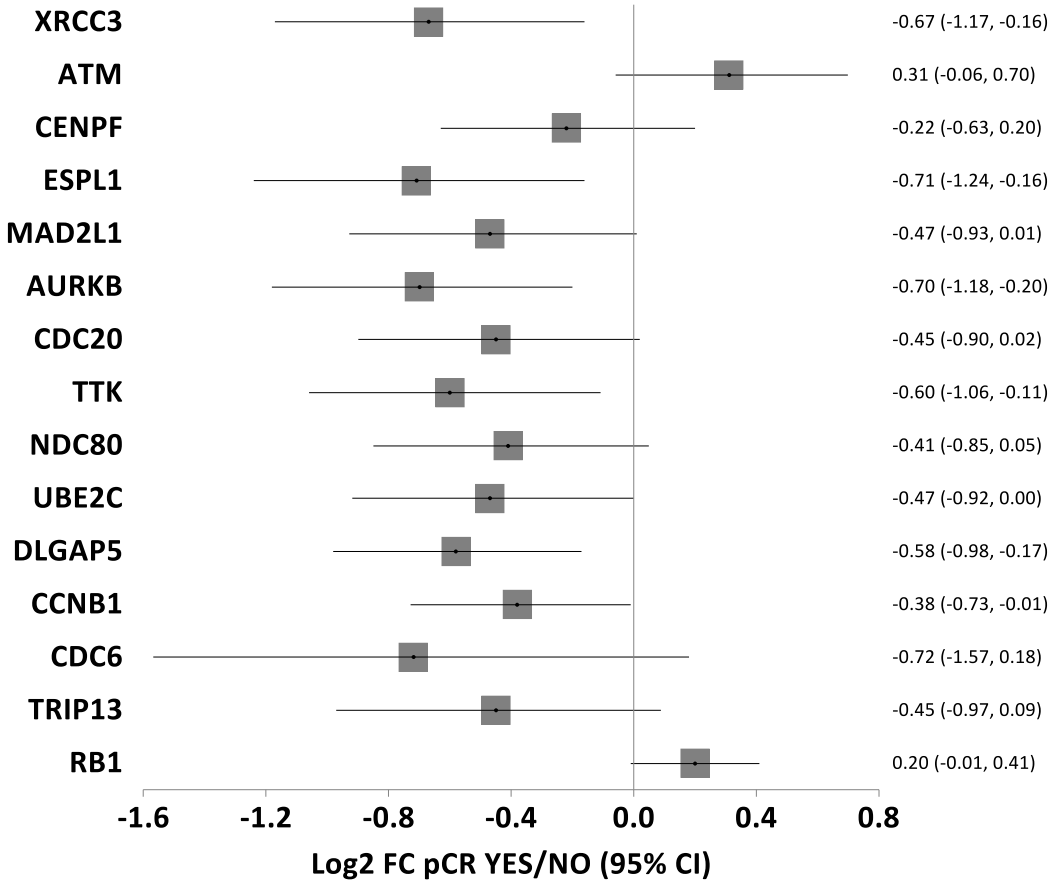


FIGURE 23: DIFFERENTIAL EXPRESSION OF TRANSCRIPTS RELATING TO M-PHASE RELATED BIOLOGICAL PROCESSES IN TUMOURS WITH HIGH HER2 PROTEIN ABUNDANCE AND ASSOCIATION WITH PCR; M-PHASE GENES ARE ENRICHED IN TUMOURS WITH HIGH HER2 PROTEIN ABUNDANCE/NO PCR

High HER2 abundance/pCR tumours: Tumours with high HER2 abundance/pCR had more abundant Erbb2 and the Erbb2 downstream target MED1 transcripts. GO enrichment analysis of genes that were overexpressed in tumours with high HER2 abundance/pCR further identified 324 biological processes that were significantly enriched (Supplementary Table 5B). Among these, almost half (46%, 150/324), were associated with immune cell signalling processes including features related to cytokine signalling, interferon gamma signalling, tumour necrosis factor (TNF) superfamily signalling and adaptive immune activation processes (Figure 24, Figure 25, Figure 26, Figure 27). Differential expression of transcripts related to GO:0001959, regulation of cytokine-mediated signalling pathway, is shown in Figure 24. These data are consistent with the hypothesis that immune cell activity is associated with response to HER2 targeted therapy in tumours with high level HER2 expression, although immune cell number by DSP or sTILs did not appear to be associated in this analysis.

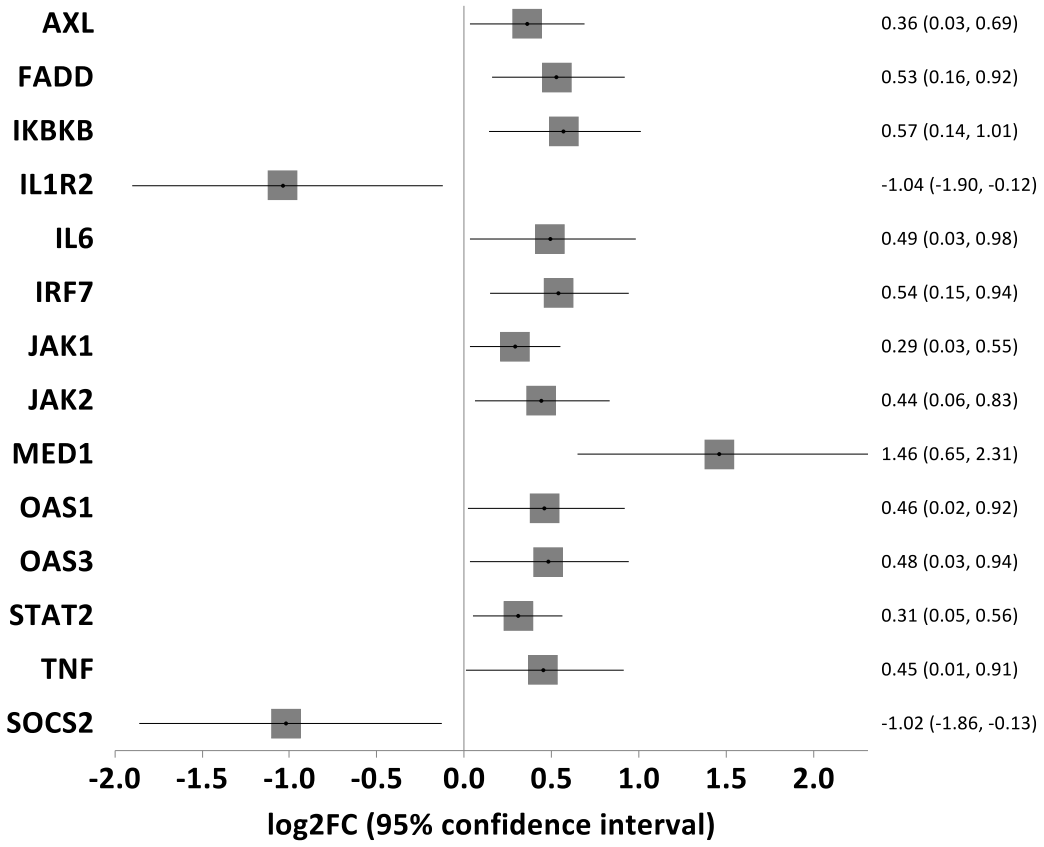


FIGURE 24: DIFFERENTIAL EXPRESSION OF TRANSCRIPTS RELATING TO CYTOKINE SIGNALLING IN TUMOURS WITH HIGH HER2 PROTEIN ABUNDANCE AND ASSOCIATION WITH PCR

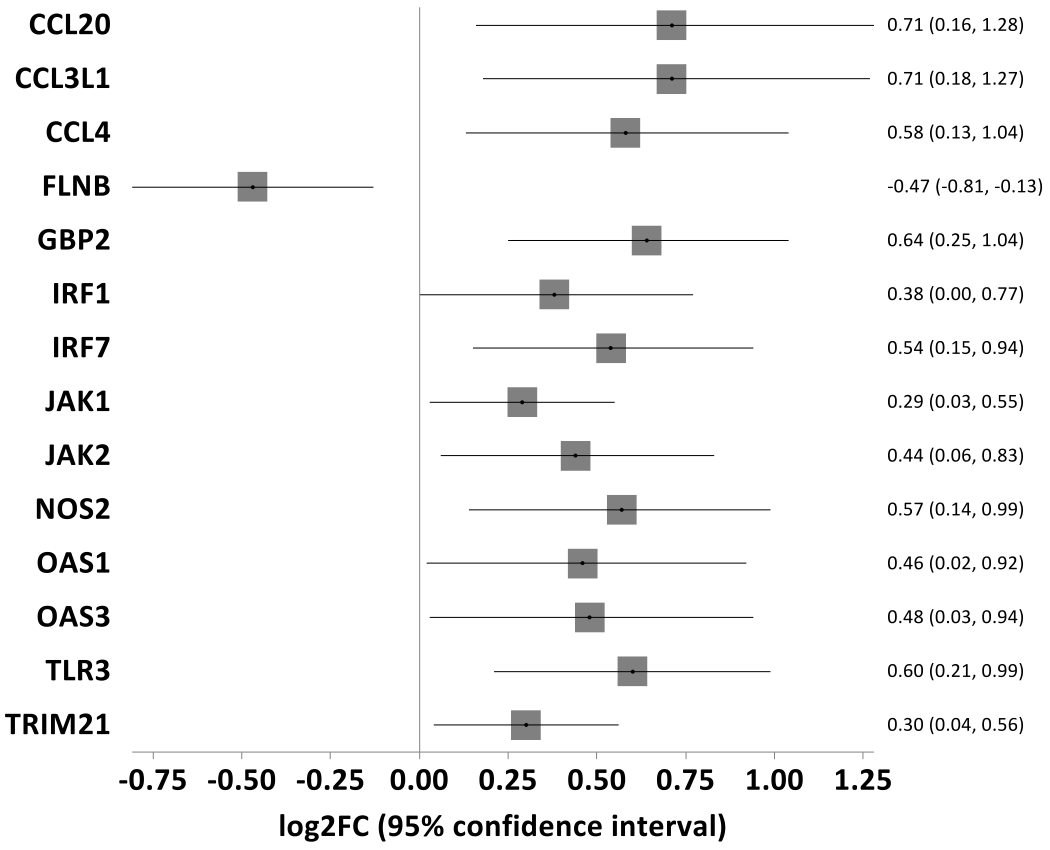


FIGURE 25: DIFFERENTIAL EXPRESSION OF TRANSCRIPTS RELATING TO INTERFERON SIGNALING IN TUMOURS WITH HIGH HER2 PROTEIN ABUNDANCE AND ASSOCIATION WITH PCR

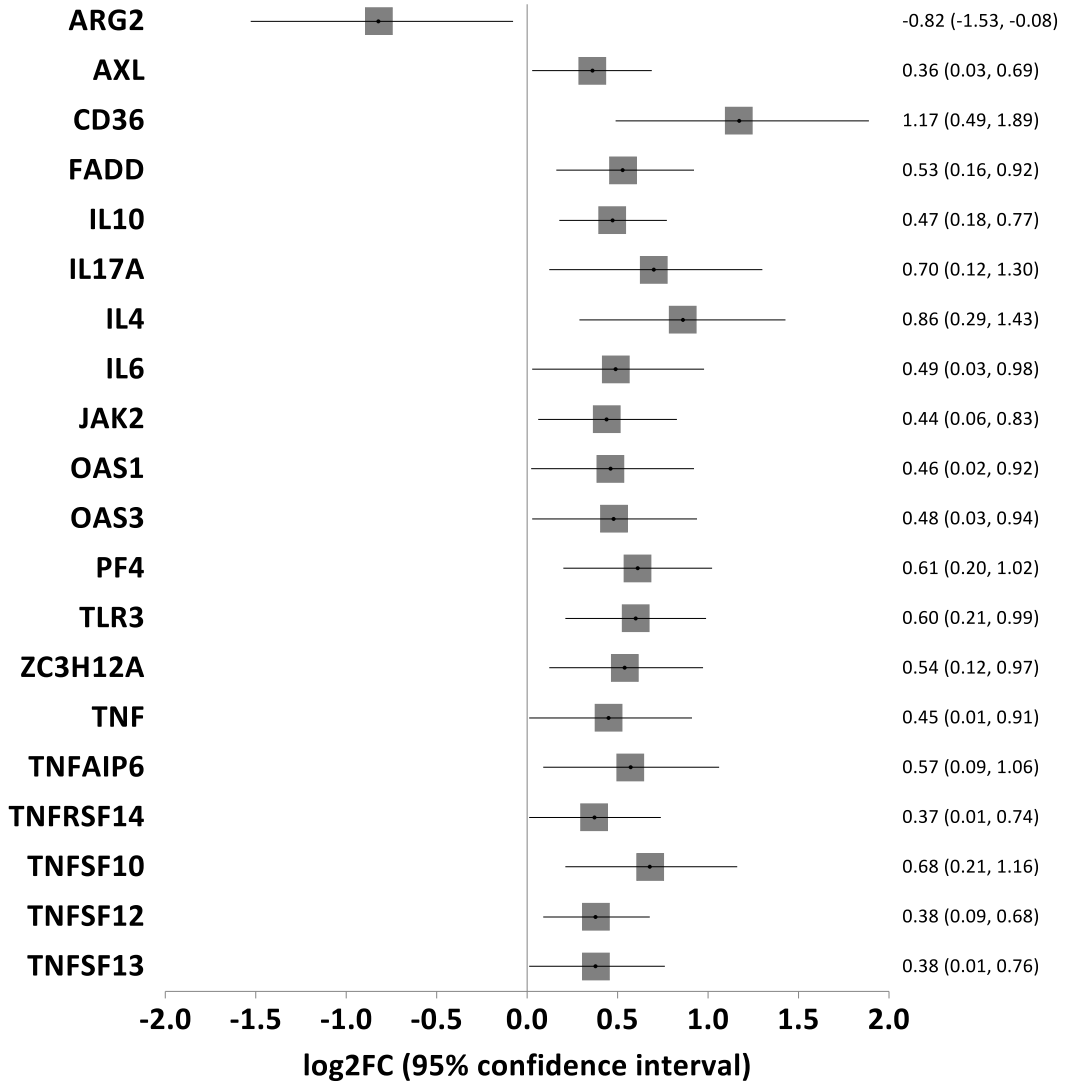


FIGURE 26: DIFFERENTIAL EXPRESSION OF TRANSCRIPTS RELATING TO TUMOUR NECROSIS FACTOR PRODUCTION IN TUMOURS WITH HIGH HER2 PROTEIN ABUNDANCE AND ASSOCIATION WITH PCR

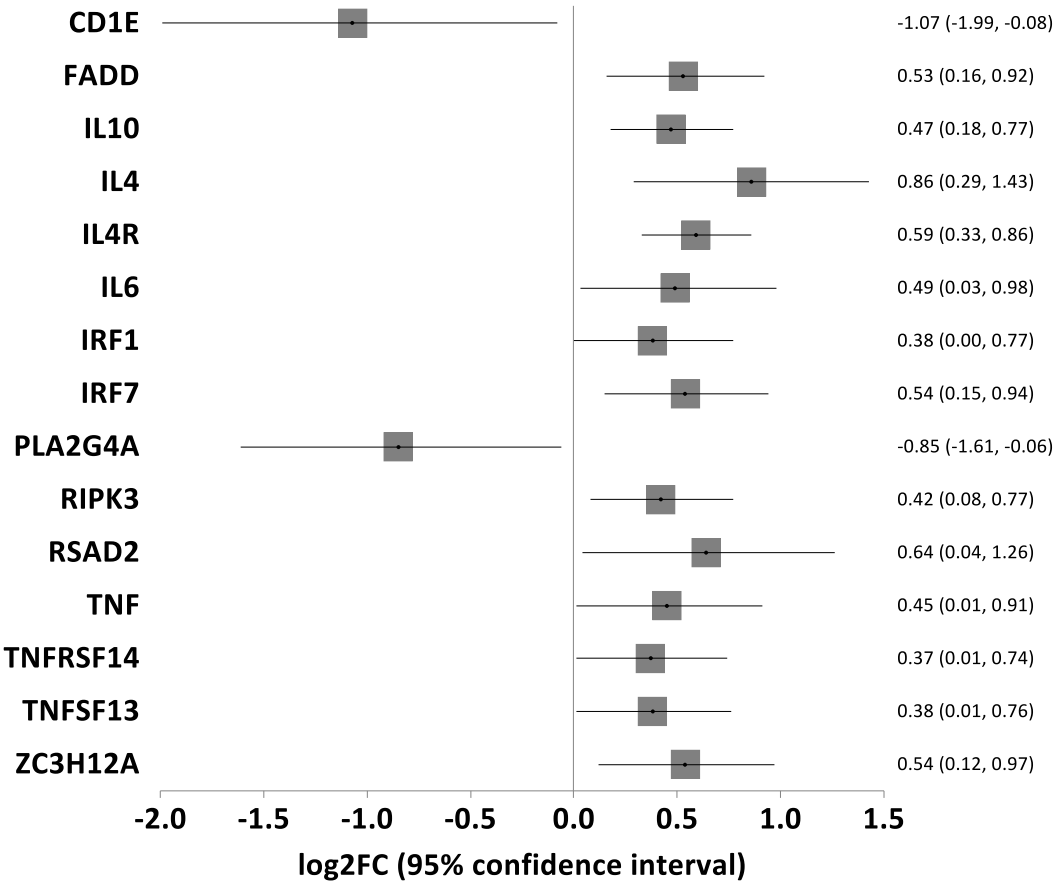


FIGURE 27: DIFFERENTIAL EXPRESSION OF TRANSCRIPTS RELATING TO REGULATION OF ADAPTIVE IMMUNE RESPONSE IN TUMOURS WITH HIGH HER2 PROTEIN ABUNDANCE AND ASSOCIATION WITH PCR

DISCUSSION

The landscape of targeted therapeutic interventions for patients with early-stage or late-stage HER2-positive breast cancer has expanded significantly in the past decade (97), and with it, there is an increased need for robust biomarkers to predict response to therapy. In the multicentre, phase II TBCRC026 clinical trial, we showed that a subset of patients with early-stage, ER-negative, HER2-positive breast cancer responded to neoadjuvant dual HER2-targeted therapy (HP) without chemotherapy (75). In this cohort, treated with HP alone, the pCR rate was 22%, in line with other similar studies (77). Identification of predictive biomarkers for pCR is critical to guide patient selection for targeted therapies, particularly in the context of omitting or including concomitant neoadjuvant chemotherapy. In this pre-planned correlative biomarker assessment of TBCRC026, we interrogated the relationship between HER2, immune processes, and breast cancer intrinsic subtype with pathologic response to neoadjuvant HP without chemotherapy.

We found that intraepithelial HER2 protein abundance by DSP was significantly associated with pCR ($p=0.001$). Two classes of tumours were identified based on HER2 protein abundance: high HER2 protein abundance and low HER2 protein abundance. Tumours with low HER2 protein abundance were primarily basal-like and were relatively unresponsive to HER2-targeted therapy, with only one achieving pCR. This is in keeping with data from other adjuvant and neoadjuvant studies (52, 98). Tumours with high HER2 protein abundance that did not achieve pCR were enriched for EGFR signalling and M-phase processes, whereas tumours with high HER2 protein abundance that underwent pCR were enriched for multiple immune processes, discussed in further detail below.

These results underscore the complexities of biologic processes regulating HER2-positive tumour response to targeted therapy.

Our analysis thus revealed marked transcriptomic differences between tumours with high HER2 protein abundance that underwent pCR compared with those that did not. Tumours with high HER2 protein abundance that failed to achieve pCR were driven by several potentially targetable biological processes including EGFR signalling and M-phase progression. The EGFR/FOS1/FOXC1/MYC pathway has also been implicated in the development of metastasis in triple-negative breast cancer (99, 100), and CDK7 inhibitors, which disrupt SOX9 and FOXC1 binding and lead to increased cell death, have been proposed as a possible targeted therapy (101). In addition, AURKA/B, involved in the regulation of mitosis, was overexpressed in the high HER2 abundance/no pCR tumours (102). Alterations in AURKA have correlated with chemoresistance and poorer outcomes in a number of tumour types, including triple negative breast cancer (103), and small molecule AURKA inhibitors have demonstrated activity in clinical trials (102, 104). A deeper understanding of alterations in these signalling networks in HER2-positive breast cancer may inform future innovative therapeutic strategies.

In contrast, tumours with high HER2 abundance and pCR contained more abundant transcripts for Erbb2 and its downstream target MED1. They were enriched for multiple immune biological processes including regulation of the cytokine-mediated signalling pathway, TNF production and interferon gamma signalling, and activation of adaptive immunity. These results are consistent with other reports suggesting an association between immune cell activity and response to HER2 directed therapy (22, 50, 105). A combined correlative analysis of early-stage HER2-positive breast cancer cohorts

in the CALGB 49691 and PAMELA trials showed that measurement of immune activation by sTIL or immune-related gene expression profiling was predictive of treatment response (59). Notably, the presence of multiple B cell-related immune gene signatures had stronger prognostic and predictive value than sTILs alone (59). Interestingly, the level of baseline sTILs in our analysis did not correlate with treatment response, in contrast to other studies (57, 60), acknowledging the difference in treatment backbone between studies. Neither were there significant differences in intraepithelial or stromal markers of T cells, B cells, macrophage/myeloid cells or antigen presenting cells between tumours that underwent pCR or not. These findings support further exploration of immune cell activation and response as a predictive biomarker in HER2-positive breast cancer.

Spatial proteomic characterisation by DSP was used in our study to evaluate baseline samples (*static* biomarker) for correlation with pCR. Others have used this tool to assess *dynamic* biomarker changes, such as in the phase II TRIO-US-B07 clinical trial in which patients with HER2-positive tumours received one cycle of neoadjuvant HER2-directed therapy (trastuzumab and/or lapatinib), followed by 6 cycles of the same targeted therapy plus chemotherapy. On-treatment proteomic changes after a single cycle of HER2-directed therapy alone were notable for reduced HER2 signalling and breast cancer-associated markers, accompanied by increased CD45 and CD8 expression consistent with immune cell infiltration (73). The on-treatment CD45 protein expression alone was highly predictive of pCR and was reproduced with CD45 IHC, suggesting a potential predictive biomarker that may guide early therapy stratification if validated (73). The TRIO-US-B07 clinical trial differs from TBCRC026 by including a more

heterogeneous cohort of patients with hormone receptor positive or negative tumours, as well as incorporates a tyrosine kinase inhibitor into its HER2-targeted therapy rather than the current approved backbone of pertuzumab. Our work thus adds to the growing body of literature investigating spatial proteomics as a biomarker in HER2-positive breast cancer, however further efforts are needed to better define its clinical applications.

Our biomarker analysis of the prospective phase II TBCRC026 study provides fresh perspectives into the complex biology of HER2-positive, early-stage breast cancer. Through the use of DSP, we interrogated differential protein expression between tumour and stromal compartments and between tumours that did or did not achieve pCR following neoadjuvant HP. We acknowledge the limitations of this study, including the small sample sizes for some subgroups, low tumour cellularity precluding evaluation of every sample across analyses, and the small number of pCR cases in this cohort treated without chemotherapy. Moreover, although DSP enables highly multiplexed spatial RNA and protein expression profiling with the potential for biomarker discovery (69, 70), DSP lacks single cell resolution and quantifies protein abundance in broad populations of cells (e.g., intraepithelial vs stromal cell niches). Use of emerging technologies with single cell resolution will be required for a more detailed understanding of the role of individual classes of immune cells in treatment response. Lastly, not all biomarkers of interest were pre-specified for analysis and as such our findings should be considered as hypothesis-generating.

CONCLUSION

ER-negative, HER2-positive breast cancer has unique molecular and immunological properties that are associated with pCR following neoadjuvant HP without chemotherapy. Our data demonstrated that response to HER2-targeted therapy is associated with high level HER2 protein abundance in a population of tumours that exhibit a high degree of immune cell activity, although not necessarily immune cell number. Tumours with high HER2 abundance that did not respond to anti-HER2 therapy were characterised by a number of biological processes including EGFR signalling and M-phase progression that may, in the future, offer novel therapeutic strategies. Future studies to validate these findings in larger cohorts treated with neoadjuvant pertuzumab-based regimens warrant consideration as the oncology community strives to personalise further therapeutic decisions for patients with early-stage HER2-positive breast cancer.

CHAPTER 4: TBCRC026 EVALUATION OF A COMPOSITE PET/CT AND HER2 TISSUE-BASED BIOMARKER TO PREDICT RESPONSE TO NEOADJUVANT HER2-DIRECTED THERAPY IN EARLY BREAST CANCER

INTRODUCTION

Strategies that optimise the treatment decision-making paradigm for individuals with HER2-positive early breast cancer are imperative, as we move further into an era of highly effective novel therapies and multi-agent regimens. Chapter 1 describes in detail how identification of predictive biomarkers of response to therapy (blood, tissue, imaging) may facilitate improved individualisation of treatment decision-making in early stage HER2-positive breast cancer. In Chapter 2, the potential for metabolic change on FDG-PET/CT (imaging biomarker) to act as a predictor of survival outcomes is described. In Chapter 3, we explore HER2 tissue-based biomarkers of response through analysis of archived baseline tumour samples from the TBCRC026 study.

While these tissue- and imaging-based biomarkers have demonstrated promise in terms of their associations with treatment response in a number of studies, further validation is required before clinical practice can be influenced. At present, we continue to rely on conventional clinicopathologic factors such as hormone receptor status, HER2 expression or amplification and pCR to guide treatment decision-making and

prognostication in daily practice. It has become clear that the heterogenous nature of HER2-positive breast cancer may render the application of a single predictive biomarker challenging, and this has led to the evaluation of composite biomarkers (60, 106, 107).

In the TBCRC026 clinical trial, in which patients with early stage HER2-positive breast cancer received neoadjuvant HP without chemotherapy; C1D15 metabolic response on PET/CT was predictive of response to therapy (3). The high NPV of 91% for the imaging biomarker suggests that a lack of decline in SULmax by 40% or greater on FDG PET/CT during the first cycle of HP may best predict those who will *not* respond to HP alone (3). We thus hypothesised that a composite biomarker incorporating baseline HER2 tissue-based biomarkers in addition to FDG PET/CT could further improve biomarker performance in this setting.

METHODS:

Clinical Trial Design

Study design, eligibility criteria and primary endpoint results have previously been reported for the neoadjuvant TBCRC026 trial (NCT01937117) (3). In summary, patients with stage II-III ER-negative/HER2-positive breast cancer received 4 cycles of neoadjuvant HER2-directed therapy alone with HP (Figure 3). FDG-PET/CT was performed at baseline, and C1D15, prior to research tumour biopsies. PET/CT facilities required approval by study team and study manuals were provided to ensure consistency in methodology, as explained in more detail in the primary study publication (3). The primary endpoint was to correlate early metabolic change on PET/CT between baseline and C1D15 with pCR, following 4 cycles of HP. Baseline research tumour biopsies were

collected prior to commencement of therapy and at C1D15, and tumour tissue was obtained from the surgical specimen at the time of surgery. Only baseline samples were evaluated for the purposes of this analysis. All patients signed a written informed consent that was approved by the institutional review boards of the participating institutions.

18F-FDG PET/CT Biomarkers

PET/CT imaging parameters included in this composite biomarker analysis were predefined based on the following primary results from TBCRC026. Results of both univariable and multivariable logistic regression revealed that median percent reduction in SULmax at C1D15 differed significantly between those with pCR and without pCR post HP (64% versus 42%; p = 0.004; Table 8). In a receiver operating characteristic (ROC) analysis, SULmax decline ≥ 40% by C1D15, (ΔSULmaxD15 ≥ 40%) had high sensitivity (83%) and NPV (91%) for predicting the lack of pCR, and this threshold was considered clinically optimal (3).

A significant difference was also observed in the median C1D15 SULmax value between those who obtained pCR and those who did not (1.6 versus 3.6; p <0.001; Table 8). An exploratory cut off of ≤ 3 versus > 3 for C1D15 SULmax was tested and a higher proportion of patients who obtained pCR had a C1D15 SULmax ≤ 3 versus those who did not (100% v 45%; P < 0.001). Compared to other cut points examined, this had a high NPV (100%) for predicting pCR (3).

Based on the above findings from the primary analysis, the following FDG PET/CT parameters were assessed in this analysis: D15 SULmax (mean, median, range),

D15 SULmax (≤ 3 versus > 3), % reduction in SULmax (mean, median, range) and % reduction in SULmax (< 40% versus ≥ 40%).

HER2 Tissue Based Biomarkers

Baseline tumour specimens were analysed for individual biomarkers based on tissue availability. For the purposes of this composite biomarker analysis, we predefined what were felt to represent the most promising baseline HER2 tissue-based biomarkers based on the current literature. This included HER2 overexpression (score 3+) assessed by central IHC according to ASCO/CAP guidelines (43); high HER2 protein abundance (tumour and stroma) evaluated using Nanostring Digital Spatial Platform (GeoMxTM DSP); and HER2-enriched intrinsic subtype, assessed using the Nanostring BC360 platform.

Statistical Analysis

The exploratory HER2/PET-CT biomarkers were compared between patients with and without pCR using Fisher's exact tests and Wilcoxon rank-sum tests, as appropriate. pCR was defined as no viable invasive cancer in the breast and axilla in the surgical specimen (local pathology review), following HP without chemotherapy. Patients who had residual disease following neoadjuvant HP or clinical progression on HP were classified as non-pCR.

The associations between the exploratory composite HER2/PET-CT biomarker and pCR were evaluated using logistic regressions, where p values were obtained using Wald-type tests. The overall discriminatory power of the predictive models was evaluated using the c-statistic, that is, the area under the ROC curve. The c-statistic measures the

concordance between model-based risk estimates and observed binary outcome status. A larger c-statistic signifies better discriminatory performance, with 0.5 indicating no discriminative ability (equivalent to random guess) and 1.0 representing perfect discrimination. Sensitivity and specificity were evaluated at the optimal cut-off point determined by maximising Youden's index. In all analyses, two-sided p values less than 0.05 were considered statistically significant.

RESULTS:

Patient Demographics

Clinicopathologic characteristics from TBCRC026 are summarised in Table 4. Enrolment of 88 women took place from January 2014-August 2017, with 83 evaluable for this composite biomarker analysis. All four cycles of neoadjuvant HP were completed by 85% of patients with additional non-study pre-operative therapy administered to 25 patients (28%), and these patients were classified as not obtaining pCR. The pCR rate was 22% (18/83) following four cycles of HP without chemotherapy.

18F-FDG PET/CT Biomarkers

To summarise the previously reported results for the PET/CT parameters, the % reduction in SULmax was significantly associated with pCR (p=0.004), with a median of 52.5% (range -34.0-91.9). ΔSULmaxD15 $\geq$ 40% was achieved in 59% (49/83) of patients and was associated with pCR (p=0.03). The median C1D15 SULmax value differed significantly between those achieving pCR and those who did not (p<0.001), and a C1D15

SULMax ≤3 was achieved in 57% (47/83) of patients and was significantly associated with pCR, p=0.001 (Table 8) (3).

	No pCR (N=65)	pCR (N=18)	Total (N=83)	P value
Intrinsic Subtype				0.32
Basal + Luminal A	16 (32%)	2 (14%)	18 (28%)	
HER2 Enriched	34 (68%)	12 (86%)	46 (72%)	
HER2 Expression (IHC)				0.44
0/1+/2+	12 (19%)	1 (7%)	13 (17%)	
3+	50 (81%)	14 (93%)	64 (83%)	
Log2 HER2 protein tumour (DSP)				0.002
Mean (SD)	12.3 (2.4)	14.2 (1.4)	12.7 (2.3)	
Median (Range)	13.2 (7.1 - 15.8)	14.5 (9.8 - 15.9)	13.5 (7.1 - 15.9)	
D15 SULmax				<0.001
Mean (SD)	4.6 (3.9)	1.7 (0.6)	3.9 (3.7)	
Median (Range)	3.6 (0.6 - 15.9)	1.6 (0.9 - 2.8)	2.4 (0.6 - 15.9)	
% Reduction in SULmax				0.004
Mean (SD)	38.3 (32.2)	62.9 (21.3)	43.6 (31.7)	
Median (Range)	41.8 (-34.9-91.9)	63.8 (25.1-91.5)	52.5 (-34.9 – 91.9)	
D15 SULmax				<0.001
>3	36 (55%)	0 (0%)	36 (43%)	
≤3	29 (45%)	18 (100%)	47 (57%)	
% Reduction in SULmax				0.03
<40%	31 (48%)	3 (17%)	34 (41%)	
≥40%	34 (52%)	15 (83%)	49 (59%)	

TABLE 8: SUMMARY STATISTICS OF 18F-FDG PET/CT AND HER2 BIOMARKERS BY PCR STATUS

HER2-Tissue Based Biomarkers

In line with emerging biomarkers of interest, baseline HER2 tissue-based biomarkers examined intrinsic subtype, HER2 expression by IHC and HER2 tumour protein abundance by DSP with a summary of results presented in Table 4. The HER2-E intrinsic subtype was present in 72% of patients (46/64 evaluable). Central evaluation of HER2 by IHC revealed high expression of HER2 (score 3+) in 83% of patients (64/77 evaluable). HER2 protein abundance by DSP was analysed in 71/83 samples, with median log 2 HER tumour protein abundance of 13.5 (range 7.1-15.9) and median log2 HER2 stroma protein abundance of 10.2 (range 5.1-13.9). HER2 tumour protein abundance differed significantly between those who obtained pCR and those who did not, p = 0.002 (Table 8).

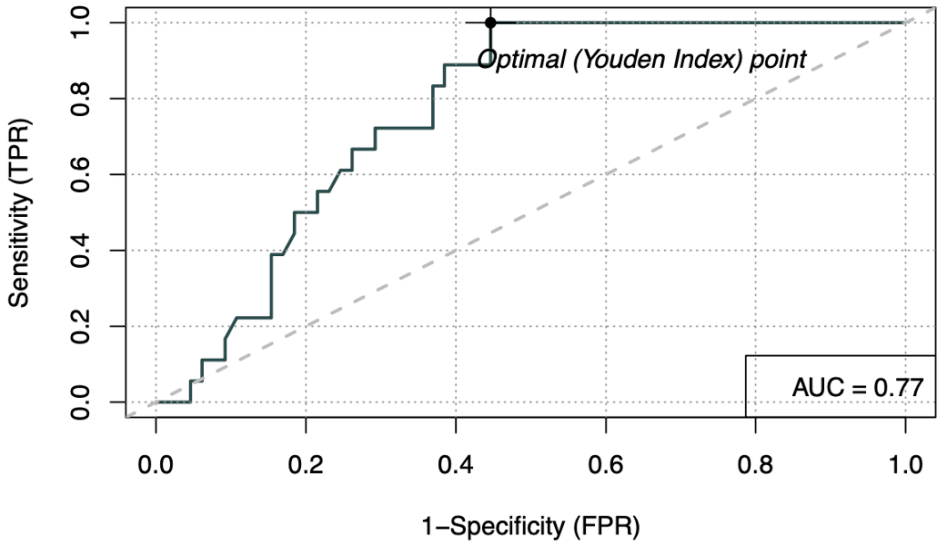
Composite Biomarker Performance

In the single variable prediction model, the PET/CT parameters most predictive for pCR included D15 SULmax (OR 0.43; p=0.007, c=0.77) and percent reduction in SULmax (OR 1.03, p=0.006, c=0.72), (Table 9). Regarding the HER2-tissue based biomarkers, HER2 tumour protein abundance was the most promising for prediction of pCR, (OR 1.75; p=0.01, c=0.76), while HER2-E and HER2 3+ expression had lower concordance (c=0.59, p=0.2 and c=0.56, p=0.3, respectively), (Table 9). The ROC analysis, with D15 SULmax as a single predictor, had a specificity of 55%, sensitivity of 100%, NPV 100% and positive predictive value (PPV) 38% and yielded a c statistic of 0.77 (

Figure 28).

	OR	CI lower	CI higher	P value	Concordance
HER2-E	2.82	0.67	19.5	0.2	0.59
HER2 3+ (IHC)	3.4	0.58	63.8	0.3	0.56
Log2 HER2 protein tumour	1.75	1.2	2.97	0.01	0.76
D15 SULmax	0.43	0.21	0.71	0.007	0.77
% Reduction in SULmax	1.03	1.01	1.06	0.006	0.72

TABLE 9: PERFORMANCE OF SINGLE PREDICTOR MODELS



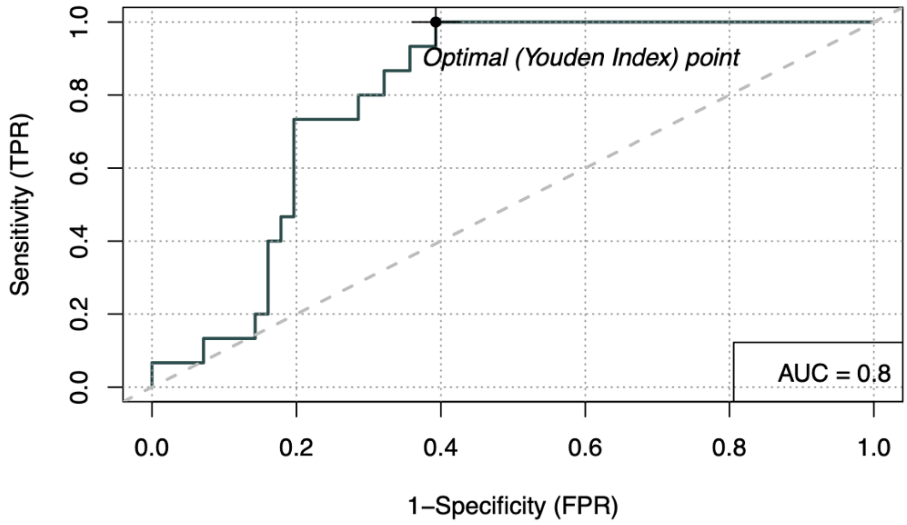
	Threshold	Specificity	Sensitivity	NPV	PPV	Youden
Threshold	0.15	0.55	1	1	0.38	1.55

FIGURE 28: D15 SULMAX SINGLE PREDICTOR MODEL

Investigating composite biomarkers that included D15 SULmax as a component did not appear to meaningfully improve prediction power (Table 10). For example, the composite biomarker of D15 SULmax and log2 HER2 protein tumour had a specificity of 61%, sensitivity 100%, NPV 100% and PPV 41% (Figure 29). Similarly, the composite of D15 SULmax and percent reduction in SULmax had a specificity of 60%, sensitivity 100%, NPV 100% and PPV 41%, c=0.78 (Figure 30).

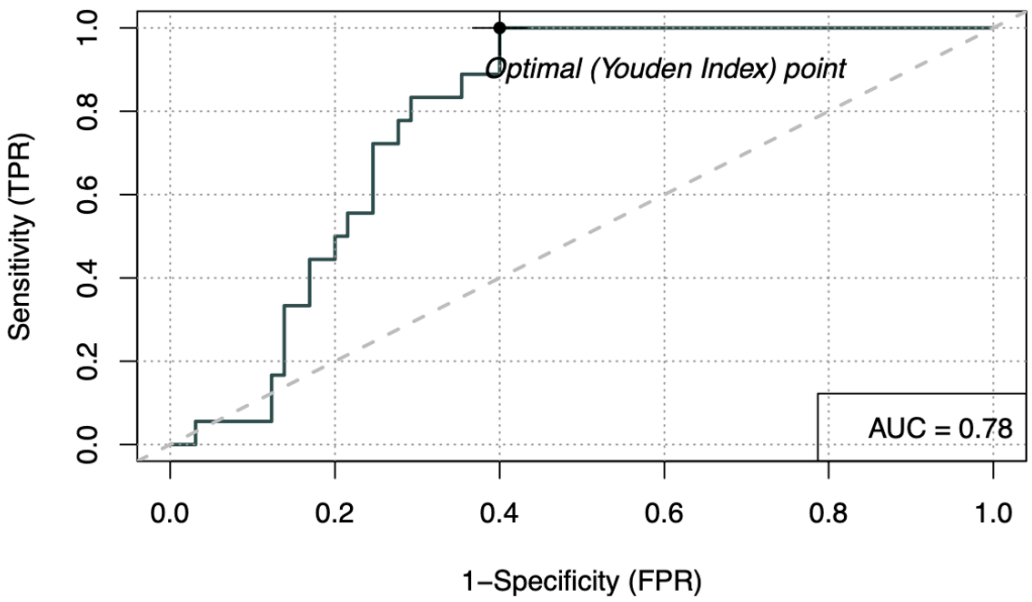
	OR	**CI lower**	**CI higher**	**P value**	**Concordance**
HER2 Enriched	1.71	0.32	13.6	0.56	0.73
D15 SULmax	0.5	0.23	0.82	0.03	
HER2 3+	0.53	0.04	13.4	0.65	0.77
D15 SULmax	0.44	0.21	0.74	0.01	
Log2 HER2 protein abundance (tumour)	1.45	0.9	2.7	0.17	0.80
D15 SULmax	0.49	0.23	0.83	0.03	
% Reduction in SULmax	1.01	0.99	1.04	0.5	0.78
D15 SULmax	0.47	0.22	0.82	0.02	

TABLE 10: COMPOSITE BIOMARKERS INCLUDING D15 SULMAX AS A COMPONENT



	Threshold	Specificity	Sensitivity	NPV	PPV	Youden
Threshold	0.15	0.61	1	1	0.41	1.61

FIGURE 29: COMPOSITE OF D15SULMAX AND LOG 2 HER2 TUMOUR PROTEIN ABUNDANCE

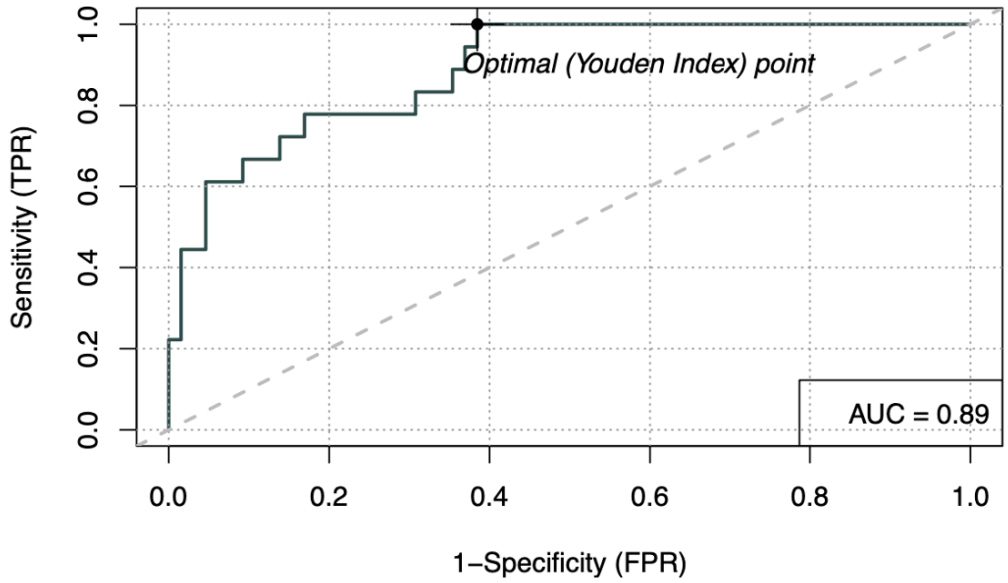


	Threshold	Specificity	Sensitivity	NPV	PPV	Youden
Threshold	0.08	0.62	1	1	0.42	1.62

FIGURE 30: COMPOSITE OF D15SULMAX AND LOG 2 HER2 TUMOUR PROTEIN ABUNDANCE

However, including the interaction term between D15 SULmax and percent reduction in SULmax in the prediction model improved the concordance probability (c=0.89 from c=0.78) (Figure 31), with high sensitivity (100%) and NPV (100%) (Table 11). The inclusion of the interaction term allows the effect of D15 SULmax on predicting pCR to vary across different levels of % reduction in SULmax (or vice versa). This composite endpoint provides a similar discrimination power when compared to the model with the three biomarkers: D15 SULmax, % reduction in SULmax, their interaction term, and tumour HER2 protein abundance (c=0.90), i.e. the addition of HER2 tumour protein

abundance, which was the most promising HER2 biomarker in the single prediction model, did not further improve prediction power (Table 12).



	Threshold	Specificity	Sensitivity	NPV	PPV	Youden
Threshold	0.08	0.62	1	1	0.42	1.62

FIGURE 31: PERFORMANCE OF COMPOSITE BIOMARKER D15 SULMAX AND % REDUCTION IN SULMAX INCLUDING INTERACTION TERM

	OR	CI lower	CI higher	P value	Concordance
				0.02	0.89
D15 SULmax	0.00	0.00	0.05	0.01	
% Reduction in SULmax	0.84	0.71	0.95	0.02	
Interaction term	1.12	1.04	1.3	0.01	

TABLE 11: PREDICTION MODEL INCLUDING D15 SULMAX, % REDUCTION IN SULMAX AND THEIR INTERACTION TERM

	OR	CI lower	CI higher	P value	Concordance
				0.186	0.90
D15 SULmax	0.00	0.00	0.05	0.02	
% Reduction in SULmax	0.81	0.65	0.94	0.02	
Interaction term	1.14	1.04	1.3	0.02	
Log2 HER2 protein tumour	1.35	0.76	2.73	0.34	

TABLE 12: PREDICTION MODEL INCLUDING D15 SULMAX, % REDUCTION IN SULMAX, HER2 PROTEIN ABUNDANCE AND THEIR INTERACTION TERM

DISCUSSION:

The TBCRC026 primary imaging trial investigated the correlation between early metabolic changes in SULmax on PET/CT and pCR to HP. Results found that a lack of decline in SULmax at C1D15 bests predicts those unlikely to achieve pCR with HP alone, with a high NPV of 91% indicating that the PET/CT biomarker performed exceptionally well. A strength of such neoadjuvant studies is the ability to collect serial biospecimens for correlative analyses that might add additional prognostic insights or help tease out mechanisms of sensitivity and resistance to anti-cancer therapy. The rationale for the use of PET/CT as an early imaging biomarker of response to HER-directed therapy, as opposed to other imaging modalities, relates to the downstream metabolism of glucose in the HER2/EGFR pathway, with studies demonstrating reduced glucose metabolism after effective HER2-pathway inhibition (34). Several studies have explored this approach and concluded that PET/CT may allow for early prediction of response, however these studies are heterogeneous in design (40, 41). The ECOG-ACRIN EA1211/DIRECT trial aims to validate PET/CT as a neoadjuvant imaging integral biomarker in patients treated with standard HER2-directed regimens and we eagerly await the results, which may ultimately facilitate a change in practice. In the interim, we considered how we might further improve biomarker performance of PET/CT through the addition of promising HER2 tissue-based biomarkers in a composite biomarker analysis.

Composite biomarkers have been previously evaluated by other investigators in the HER2-positive setting, although the incorporation of imaging and tissue-based biomarkers in the assessment of response to HER2-therapy in early breast cancer is reasonably unique. The combination biomarker of metabolic activity on FDG PET/CT

and TILs was recently explored in the PREDIX HER2 trial which compared neoadjuvant docetaxel/trastuzumab/pertuzumab with T-DM1 (60). After two cycles of treatment (C2), patients who had both C2 SUV max ≥2.60 and TILS <10% had a lower rate of pCR (8%) versus those with C2 SUV max <2.60 and TILS >10% (35-58%), p=0.002. Overall, the combination of TILs (either assessed manually or digitally) and metabolic response resulted in superior prognostic information compared with either biomarker alone, in this secondary analysis (60).

Tissue-based novel combined biomarkers that have been explored in early HER2-positive breast cancer include the CelTIL score which encompasses sTILs and tumour cellularity; evaluated by tumour biopsy after 2 weeks of HER2-directed therapy alone (61). High CelTIL scores can select tumours with high levels of immune infiltration and decreased tumour cellularity and were shown in the PAMELA and LPT109096 trials to be predictive of pCR following neoadjuvant therapy with lapatinib and trastuzumab +/- endocrine therapy (48, 108). When the association between CelTIL and long-term survival outcomes was tested in a different neoadjuvant cohort (NeoALTTO) in patients receiving trastuzumab-based chemotherapy, those that were CelTIL-high had improved 5 year EFS (76% versus 60%; HR 0.4) and OS (86% versus 74%; HR 0.43) compared to those that were CelTIL-low, supporting this as a potential early predictive biomarker (109).

Other efforts have examined combination HER2 tissue-based biomarkers and have yielded interesting and hypothesis-generating results. In an analysis of tumours from five clinical trials of early HER2-positive breast cancer treated with neoadjuvant HER2-directed therapy alone, an RNA-based assay combining ERBB2 and the HER2-E intrinsic

subtype allowed better identification of high anti-HER2 sensitivity compared to when either were used in isolation (106). A combinatorial biomarker of HER2 amplification plus markers of PI3K pathway activation and pCR was examined in an analysis of TBCRC006 trial, which investigated neoadjuvant lapatinib/trastuzumab (107, 110). A high level of HER2 amplification combined with wild-type PI3K pathway status was associated with pCR ($p = 0.0031$) and may indicate increased sensitivity to HER2-targeted therapies without chemotherapy (107). It is quite likely that in the future, prognostic models that integrate multiple variables into a single assay will be most practical in terms of guiding treatment-making decisions. One such example is the novel HER2DX assay, which measures the expression of 27-genes involved in immune infiltration, luminal differentiation, tumour proliferation and HER2 amplicon expression and combines this with clinical data to generate predictive and prognostic information (53, 111). This assay has been validated using multiple datasets and pending future clinical utility studies may be used to guide adaptation of systemic therapy for patients, particularly in the era of newer antibody-drug conjugates and tyrosine kinase inhibitors (53).

Potential limitations of our study include a relatively small patient cohort, with not all tissue samples evaluable for all analyses, perhaps limiting the ability to strengthen the imaging biomarker further. The attrition in samples is in many cases an unavoidable limitation of tissue-based biomarker analysis and renders the non-invasive PET-CT biomarker attractive. However, access to PET imaging and associated costs are important considerations, particularly in resource-limited settings. This was an exploratory analysis and as such our findings should be considered as hypothesis-generating. Note that the concordance analysis was not cross-validated, hence it may over-estimate the

performance of the prediction model. Despite these limitations, our study highlights the novel concept of combining imaging and tissue biomarkers and is one of the first studies to our knowledge to have examined this in the neoadjuvant HER2-positive setting. Going forward, the potential of combining different predictive biomarkers to identify HER2-positive patients who may benefit from HER2-targeted therapies, without the need for chemotherapy or with less toxic chemotherapy regimens, is likely to be a key area of research focus.

CONCLUSION:

The composite HER2/PET-CT biomarker comprising D15 SULmax, % reduction in SULmax and tumour HER2 protein abundance had high prediction power for pCR, however was not significantly superior to the prediction power of PET/CT parameters and their interaction term alone. These data suggest that clinical trials informed by early PET/CT biomarkers warrant further evaluation to personalise breast cancer care. Ideally, with thoughtfully designed studies and global collaboration amongst the breast oncology community we will continue to progress validation and clinical utility studies in order to bring the most promising biomarkers into the clinic, enabling optimisation of therapy.

CHAPTER 5: EPILOGUE

The work presented in this Thesis describes correlative and survival analyses from a clinical trial designed and led by Johns Hopkins investigators, Baltimore, Maryland (USA) in collaboration with the TBCRC. The TBCRC026 study was a phase II trial investigating the use of FDG-PET/CT as an imaging biomarker of response in patients with primary operable early-stage HER2-positive breast cancer, undergoing neoadjuvant dual HER2-directed therapy, without chemotherapy. This trial was multicentre in nature and involved collaborations with TBCRC academic sites nationwide, and other National Cancer Institute (NCI) supported academic collaborators. Participating sites included Johns Hopkins, Vanderbilt University, University of North Carolina, Mayo Clinic, Baylor College, University of Washington Seattle, Indiana University, MD Anderson, University of Alabama, Yale and Washington University, St Louis. Primary study results were published in the Journal of Clinical Oncology in 2021 (PMID: 33999652) and reported that early changes in SULmax predict response to HP in ER-negative/HER2-positive breast cancer (Figure 3) (3). To undertake this thesis, I was responsible for leading the secondary and exploratory endpoint analyses from this trial and bringing all the way to completion and presentation. My contribution is reflected in the authorship order in the numerous abstract and poster presentations at international oncology meetings, and the three original publications from this work; one accepted for publication in 2023 and the remaining two to be submitted in 2024 (see Publications and Abstracts section, linked here).

The secondary analysis of the TBCRC026 trial outlined in Chapter 2 demonstrates a potential association between SULmax parameters on PET/CT and RFS/OS outcomes. At a median follow up of 54 months, there were six deaths and fourteen RFS events. The estimated RFS and OS at 3 years was 84% (95% CI, 76%-92%) and 92% (95% CI, 87%-98%) respectively. Achieving a D15 SULmax ≤3 was associated with improved RFS (HR 0.36; p=0.06) and significantly improved OS (HR 0.14; p=0.03). This is the first time to our knowledge that an imaging-based biomarker has been assessed in patients receiving neoadjuvant pertuzumab-based HER2 therapy without chemotherapy and as such is innovative, with the potential to change practice pending future confirmatory studies.

Another exciting avenue of investigation relates to the use of multiplex spatial proteomic analysis as a tool for biomarker discovery. The study in Chapter 3 aimed to evaluate immunological and molecular features of baseline HER2 tumours from the TBCRC026 trial, and their association with pCR. The results showed that intraepithelial HER2 protein abundance, as assessed by DSP, was significantly associated with pCR (p=0.001). The majority of tumours with low HER2 protein abundance tumours were basal-like, and all but one failed to achieve a pCR. When the subset of tumours with high HER2 protein abundance that underwent pCR was analysed, these tumours demonstrated a high degree of immune activity. In the subset of tumours with high HER2 protein abundance tumours that failed to achieve pCR, these were enriched for M-phase processes and epidermal growth factor receptor signalling, which may represent targetable pathways for novel therapies.

The study discussed in Chapter 4 describes combining PET/CT imaging with HER2-tissue based biomarkers in order to improve on the prediction power of PET/CT

alone, through a composite biomarker analysis. The composite of D15 SULmax and % reduction in SULmax combined with their interaction term, had improved probability (c=0.89 from c=0.78), with high sensitivity (100%) and NPV (100%). However, although the composite HER2/PET biomarker comprising D15 SULmax, % reduction in SULmax and tumour HER2 protein abundance had high prediction power for pCR (c=0.90), this was not significantly superior to the prediction power of PET/CT parameters alone. These data indicate that clinical trials informed by non-invasive PET/CT biomarkers may facilitate personalisation of breast cancer care and warrant further exploration.

In summary, these three Chapters outline the promise of imaging and tissue-based predictive biomarkers of response and resistance to therapy. The work presented thus far is exciting and aims to individualise decision-making in the treatment paradigm for early-stage HER2-positive breast cancer, in order to reduce unwanted side-effects for patients. A stepwise approach is required for successful biomarker development, and validation in order to ensure accuracy, reproducibility, sensitivity and specificity is required. Confirmation of clinical utility is essential as the final step in ensuring biomarkers are clinically meaningful for patients. Challenges remain in terms of translating the latest advances in technology into widespread use in clinical practice, and these can include issues with regulatory approval, acceptability to patients and physicians, and access and cost implications. In an era where the global incidence of cancer is projected to increase by 70%, most of which will be in low and middle income countries, the affordability of treatments and potential for these studies to guide better selection of therapy and resources is crucial (112).

The use of PET/CT as an imaging biomarker of response shows promise, however several unanswered questions remain, before early interim PET/CT is incorporated into standard treatment algorithms. The optimal schedule and cut point for the use of PET/CT in breast cancer clinical trials remains to be defined. It is exciting to note that as the work in this Thesis is presented, recruitment for a large multicentre ECOG-ACRIN registration study (EA1211/DIRECT) is ongoing (NCT05710328). This trial has been developed based on the promising results observed in the phase II TBCRC026, specifically that those patients not obtaining a 40% reduction in SULmax by C1D15 following neoadjuvant HP were unlikely to obtain pCR, with NPV of 91%. The aim of EA1211/DIRECT is to validate FDG-PET/CT as a neoadjuvant interim imaging integral biomarker (niFDG-PET/CT) in patients treated with standard HER2-directed regimens. Patients with stage II/III HER2-positive breast cancer are suitable for enrolment into this multicentre, single-arm, primary imaging phase 2 study. A standard skull base-thigh FDG-PET/CT will be performed at baseline and C1D15 and patients will receive standard of care pertuzumab-based neoadjuvant therapy followed by surgery. The primary objective is to estimate the NPV of niFDG-PET/CT for pCR using the change in SULmaxD15 of the primary breast cancer, at the threshold of 40%. In addition, secondary objectives will aim to validate correlation of the imaging biomarker with survival outcomes as we have presented in Chapter 2. This trial is the first step in implementing a Response-Guided Treatment Strategy, akin to the use of FDG-PET/CT in lymphoma. If it meets its objectives, the results will be used to design clinical utility studies, and thus may change practice in the future.

CONCLUSION

The field of predictive and prognostic biomarkers is an exciting and evolving area of investigation in breast cancer research and therapeutics. Despite the approval of many novel agents for varying indications in HER2-positive breast cancer, significant challenges remain regarding the appropriate selection of patients for these therapies. The potential for early FDG-PET/CT to act as an imaging biomarker of response to neoadjuvant HER2-directed therapy certainly appears promising but requires confirmatory trials before any transition is made to everyday clinical practice. In addition, the most promising tissue based-biomarkers will likely be used in combination prognostic and predictive assays in the future, to guide individualised treatment-making decisions. In this era of personalised medicine, it is imperative that investigators continue to collaborate, in conjunction with patient advocates and the broader oncology community, to design preclinical experiments and clinical trials such that patients receive the most effective treatments for breast cancer, with the least associated toxicities.

TABLES

FIGURES

SUPPLEMENTARY TABLES

(Data available on request)

Supplementary Table 1: Summary of central HER2 IHC and FISH, *HER2: human epidermal growth factor 2, IHC: immunohistochemistry, FISH: fluorescent in situ hybridisation*

Supplementary Table 2A: Differential Expression IO360 and BC360 Codesets (NanoString), 1239 unique transcripts

Supplementary Table 2B: Differential Expression IO360 Codesets by pCR status, *pCR: pathologic complete response*

Supplementary Table 2C: Differential Expression BC360360 Codesets by pCR status

Supplementary Table 3A: GO Enrichment Data in HER2 high protein abundance tumours, *GO: Gene Ontology*

Supplementary Table 3B: GO Enrichment Data in HER2 high protein abundance tumours

Supplementary Table 4A: Differential expression in intraepithelial segments by DSP, *DSP: Digital spatial profiling*

Supplementary Table 4B: Differential expression in stromal segments by DSP

Supplementary Table 5A: GO Enrichment Analysis in HER2 high protein abundance tumours

Supplementary Table 5B: GO Enrichment Analysis in HER2 high protein abundance tumours

PUBLICATIONS AND ABSTRACTS

1. Hennessy MA, Leal J, Huang CY, Solnes L, Denbow R, Abramson V, Carey L, Liu MC, Rimawi M, Specht J, Storniolo AM, Valero V, Vaklavas C, Winer EP, Krop IE, Wolff AC, Cimino-Mathews A, Wahl RL, Stearns V, Connolly RM. 'Correlation of early change in standardized uptake value (SUV) on positron emission tomography (PET/CT) with recurrence free survival and overall survival in patients with primary operable HER2-positive breast cancer (TBCRC026)'. Abstract presented as Poster at European Society of Medical Oncology Congress (ESMO), September 2022
2. Hennessy MA, Leal J, Huang CY, Solnes L, Denbow R, Abramson V, Carey L, Liu MC, Rimawi M, Specht J, Storniolo AM, Valero V, Vaklavas C, Winer EP, Krop IE, Wolff AC, Cimino-Mathews A, Wahl RL, Stearns V, Connolly RM et al. 'Correlation of SUV on Early Interim PET with recurrence free survival and overall survival in primary operable HER2-positive breast cancer (the TBCRC026 trial)'. J Nucl Med. 2023 Aug 31:jnumed.123.265853. doi: 10.2967/jnumed.123.265853. Epub ahead of print. PMID: 37652539
3. Hennessy MA, Cimino-Mathews A, Carter J, Kachergus JM, Ma Y, Leal JP, Solnes LB, Abramson VG, Carey LA, Rimawi M, Specht J, Storniolo AM, Vaklavas C, Wolff AC, Wahl RW, Perez EA, Huang CY, Stearns V, Thompson EA, Connolly RM, 'Evaluation of a composite PET/CT and HER2 tissue-based biomarker to predict response to neoadjuvant HER2-directed therapy in early breast cancer (TBCRC026)'. Abstract presented as Poster at European Society of Medical Oncology Congress (ESMO), October 2023

4. Hennessy MA*, Fernandez-Martinez A*, Huang CY, Cimino-Mathews A, Denbow R, Abramson VG, Rimawi M, Specht J, Storniolo AM, Valero V, Vaklavas C, Winer EP, Krop IE, Wolff AC, Wahl RL, Thompson EA, Stearns V, Perou C, Carey LA, Connolly RM, 'RNA-Seq based gene expression profiling of baseline and on-treatment breast tumors to predict response to HER2-directed therapy, without chemotherapy (TBCRC026)'. *Abstract accepted for poster presentation at the American Society of Clinical Oncology (ASCO), June 2024*
5. Hennessy MA, Gatsonis C, Jacene H*, Connolly RM*, Burnette BL, Stringer-Reasor EM, Romanoff J, Taurone A, O'Sullivan CC, Le-Petross HT, Lawhn-Heath C, Stearns V, Fowler AM, Tang S, Sepulveda KA, DeMichele AM, Mankoff DA**, Wolff AC** ECOG-ACRIN EA1211: Interim FDG-PET/CT for predicting response of HER2-positive breast cancer to neoadjuvant therapy (DIRECT Trial). *Abstract accepted for poster presentation at the American Society of Clinical Oncology (ASCO), June 2024*

REFERENCES

1. Ferlay J EM, Lam F, Laversanne M, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. 2024 [Available from: https://gco.iarc.who.int/today.
2. Prowell TM, Pazdur R. Pathological complete response and accelerated drug approval in early breast cancer. N Engl J Med. 2012;366(26):2438-41.
3. Connolly RM, Leal JP, Solnes L, Huang CY, Carpenter A, Gaffney K, et al. Updated Results of TBCRC026: Phase II Trial Correlating Standardized Uptake Value With Pathological Complete Response to Pertuzumab and Trastuzumab in Breast Cancer. J Clin Oncol. 2021;39(20):2247-56.
4. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987 Jan 9;235(4785):177-82.
5. Agus DB, Akita RW, Fox WD, Lewis GD, Higgins B, Pisacane PI, et al. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell. 2002;2(2):127-37.
6. Connolly RM, Wang V, Hyman DM, Grivas P, Mitchell EP, Wright JJ, et al. Trastuzumab and Pertuzumab in Patients with Non-Breast/Gastroesophageal HER2-Amplified Tumors: Results from the NCI–MATCH ECOG–ACRIN Trial (EAY131) Subprotocol J. Clinical Cancer Research. 2024:OF1-OF8.
7. Sarup JC, Johnson RM, King KL, Fendly BM, Lipari MT, Napier MA, et al. Characterization of an anti-p185HER2 monoclonal antibody that stimulates receptor function and inhibits tumor cell growth. Growth Regul. 1991;1(2):72-82.
8. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nature Reviews Molecular Cell Biology. 2001;2(2):127-37.
9. Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, et al. Epidermal growth factor receptor (EGFR) signaling in cancer. Gene. 2006;366(1):2-16.
10. Nuti M, Bellati F, Visconti V, Napoletano C, Domenici L, Caccetta J, et al. Immune effects of trastuzumab. J Cancer.2:317-23.
11. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001; **344:** 783–92.
12. Herceptin (trastuzumab) Label- Access data FDA. www.accessdata.fda.gov/drugsatfda_docs/label/.../103792s5256lbl.pdf.
13. 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.
14. 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.

15. Blumenthal GM, Scher NS, Cortazar P, Chattopadhyay S, Tang S, Song P, et al. First FDA approval of dual anti-HER2 regimen: pertuzumab in combination with trastuzumab and docetaxel for HER2-positive metastatic breast cancer. Clin Cancer Res. 2013;19(18):4911-6.
16. Adams CW, Allison DE, Flagella K, Presta L, Clarke J, Dybdal N, et al. Humanization of a recombinant monoclonal antibody to produce a therapeutic HER dimerization inhibitor, pertuzumab. Cancer Immunol Immunother. 2006;55(6):717-27.
17. Scheuer W, Friess T, Burtscher H,et al. Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models. *Cancer Research*. 2009;69(24):9330–9336.
18. Baselga J, Gelmon KA, Verma S, Wardley A, Conte P, Miles D, et al. Phase II trial of pertuzumab and trastuzumab in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer that progressed during prior trastuzumab therapy. J Clin Oncol. 2010;28(7):1138-44.
19. Connolly R, Stearns V. A multidisciplinary approach to neoadjuvant therapy for primary operable breast cancer. Challenges and opportunities. Oncology (Williston Park).24(2):135-43, 2010.
20. von Minckwitz G, Huang C-S, Mano MS, Loibl S, Mamounas EP, Untch M, et al. Trastuzumab Emtansine for Residual Invasive HER2-Positive Breast Cancer. New England Journal of Medicine. 2018;380(7):617-28.
21. Baselga J, Bradbury I, Eidtmann H, Di Cosimo S, de Azambuja E, Aura C, et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. The Lancet. 2012;379(9816):633-40.
22. Carey LA, Berry DA, Cirrincione CT, Barry WT, Pitcher BN, Harris LN, et al. Molecular Heterogeneity and Response to Neoadjuvant Human Epidermal Growth Factor Receptor 2 Targeting in CALGB 40601, a Randomized Phase III Trial of Paclitaxel Plus Trastuzumab With or Without Lapatinib. J Clin Oncol. 2016;34(6):542-9.
23. Hurvitz SA, Martin M, Symmans WF, Jung KH, Huang CS, Thompson AM, et al. Neoadjuvant trastuzumab, pertuzumab, and chemotherapy versus trastuzumab emtansine plus pertuzumab in patients with HER2-positive breast cancer (KRISTINE): a randomised, open-label, multicentre, phase 3 trial. Lancet Oncol. 2018;19(1):115-26.
24. Bergh JCS, Andersson A, Bjohle J, Bosch A, Carlsson L, Dreifaldt AC, et al. Docetaxel, trastuzumab, pertuzumab versus trastuzumab emtansine as neoadjuvant treatment of HER2-positive breast cancer: Results from the Swedish PREDIX HER2 trial identifying a new potential de-escalation standard? Journal of Clinical Oncology. 2019;37(15_suppl):501-.
25. Pernas S, Tolaney SM. HER2-positive breast cancer: new therapeutic frontiers and overcoming resistance. Ther Adv Med Oncol. 2019;11:1758835919833519-.
26. Geyer CE, Jr, Untch M, Prat A, Rastogi P, Niikura N, Mathias E, et al. Abstract OT-03-01: Trastuzumab deruxtecan (T-DXd; DS-8201) vs trastuzumab emtansine (T-DM1) in high-risk patients with HER2-positive, residual invasive early breast cancer after neoadjuvant therapy: A randomized, phase 3 trial (DESTINY-Breast05). Cancer research. 2021;81(4_Supplement):OT-03-1-OT--1.

27. Hurvitz SA, Hegg R, Chung W-P, Im S-A, Jacot W, Ganju V, et al. Trastuzumab deruxtecan versus trastuzumab emtansine in patients with HER2-positive metastatic breast cancer: updated results from DESTINY-Breast03, a randomised, open-label, phase 3 trial. The Lancet. 2023;401(10371):105-17.
28. O'Sullivan CC, Ballman KV, McCall L, Kommalapati A, Zemla T, Weiss A, et al. Alliance A011801 (compassHER2 RD): postneoadjuvant T-DM1 + tucatinib/placebo in patients with residual HER2-positive invasive breast cancer. Future Oncol. 2021;17(34):4665-76.
29. Tolaney SM, Tarantino P, Graham N, Tayob N, Parè L, Villacampa G, et al. Adjuvant paclitaxel and trastuzumab for node-negative, HER2-positive breast cancer: final 10-year analysis of the open-label, single-arm, phase 2 APT trial. The Lancet Oncology. 2023;24(3):273-85.
30. Harbeck N, Gluz O, Christgen M, Kates RE, Braun M, Kuemmel S, et al. De-Escalation Strategies in Human Epidermal Growth Factor Receptor 2 (HER2)-Positive Early Breast Cancer (BC): Final Analysis of the West German Study Group Adjuvant Dynamic Marker-Adjusted Personalized Therapy Trial Optimizing Risk Assessment and Therapy Response Prediction in Early BC HER2- and Hormone Receptor-Positive Phase II Randomized Trial-Efficacy, Safety, and Predictive Markers for 12 Weeks of Neoadjuvant Trastuzumab Emtansine With or Without Endocrine Therapy (ET) Versus Trastuzumab Plus ET. J Clin Oncol. 2017;35(26):3046-54.
31. Hylton NM, Blume JD, Bernreuter WK, Pisano ED, Rosen MA, Morris EA, et al. Locally advanced breast cancer: MR imaging for prediction of response to neoadjuvant chemotherapy--results from ACRIN 6657/I-SPY TRIAL. Radiology. 2012;263(3):663-72.
32. Mukhtar RA, Yau C, Rosen M, Tandon VJ, Hylton N, Esserman LJ. Clinically meaningful tumor reduction rates vary by prechemotherapy MRI phenotype and tumor subtype in the I-SPY 1 TRIAL (CALGB 150007/150012; ACRIN 6657). Ann Surg Oncol. 2013;20(12):3823-30.
33. Basik M, Costantino JP, De Los Santos JF, Umphrey H, Julian TB, Mamounas EP, et al. NRG Oncology BR005: Phase II trial assessing accuracy of tumor bed biopsies (Bx) in predicting pathologic response in patients (Pts) with clinical/radiological complete response (CR) after neoadjuvant chemotherapy (NCT) in order to explore the feasibility of breast-conserving treatment (BCT) without surgery. Journal of Clinical Oncology. 2018;36(15_suppl):TPS604-TPS.
34. Lin NU, Guo H, Yap JT, Mayer IA, Falkson CI, Hobday TJ, et al. Phase II Study of Lapatinib in Combination With Trastuzumab in Patients With Human Epidermal Growth Factor Receptor 2-Positive Metastatic Breast Cancer: Clinical Outcomes and Predictive Value of Early [18F]Fluorodeoxyglucose Positron Emission Tomography Imaging (TBCRC 003). J Clin Oncol. 2015;33(24):2623-31.
35. Cheson BD, Fisher RI, Barrington SF, Cavalli F, Schwartz LH, Zucca E, Lister TA. Recommendations for initial evaluation, staging, and response assessment of Hodgkin and non-Hodgkin lymphoma: the Lugano classification. J Clin Oncol. 2014;32(27):3059-68.

36. Hoppe RT, Advani RH, Ai WZ, Ambinder RF, Armand P, Bello CM, et al. Hodgkin Lymphoma, Version 2.2020, NCCN Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw. 2020;18(6):755-81.
37. van der Voort A, Louis FM, van Ramshorst MS, Kessels R, Mandjes IA, Kemper I, et al. MRI-guided optimisation of neoadjuvant chemotherapy duration in stage II–III HER2-positive breast cancer (TRAIN-3): a multicentre, single-arm, phase 2 study. The Lancet Oncology. 2024;25(5):603-13.
38. Wahl RL, Zasadny K, Helvie M, Hutchins GD, Weber B, Cody R. Metabolic monitoring of breast cancer chemohormonotherapy using positron emission tomography: initial evaluation. J Clin Oncol. 1993;11(11):2101-11.
39. Schelling M, Avril N, Nahrig J, Kuhn W, Romer W, Sattler D, et al. Positron emission tomography using [(18)F]Fluorodeoxyglucose for monitoring primary chemotherapy in breast cancer. J Clin Oncol. 2000;18(8):1689-95.
40. Gebhart G, Gámez C, Holmes E, Robles J, Garcia C, Cortés M, et al. 18F-FDG PET/CT for Early Prediction of Response to Neoadjuvant Lapatinib, Trastuzumab, and Their Combination in HER2-Positive Breast Cancer: Results from Neo-ALTTO. Journal of Nuclear Medicine. 2013;54(11):1862.
41. Pérez-García JM, Gebhart G, Ruiz Borrego M, Stradella A, Bermejo B, Schmid P, et al. Chemotherapy de-escalation using an (18)F-FDG-PET-based pathological response-adapted strategy in patients with HER2-positive early breast cancer (PHERGain): a multicentre, randomised, open-label, non-comparative, phase 2 trial. Lancet Oncol. 2021;22(6):858-71.
42. Cortes J, Pérez-García JM, Ruiz-Borrego M, Stradella A, Bermejo B, Escrivá-de-Romaní S, et al. 3-year invasive disease-free survival (iDFS) of the strategy-based, randomized phase II PHERGain trial evaluating chemotherapy (CT) de-escalation in human epidermal growth factor receptor 2-positive (HER2[+]) early breast cancer (EBC). Journal of Clinical Oncology. 2023;41(17_suppl):LBA506-LBA.
43. 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.
44. Koboldt DC, Fulton RS, McLellan MD, Schmidt H, Kalicki-Veizer J, McMichael JF, et al. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490(7418):61-70.
45. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol. 2009;27(8):1160-7.
46. Caan BJ, Sweeney C, Habel LA, Kwan ML, Kroenke CH, Weltzien EK, et al. Intrinsic subtypes from the PAM50 gene expression assay in a population-based breast cancer survivor cohort: prognostication of short- and long-term outcomes. Cancer Epidemiol Biomarkers Prev. 2014;23(5):725-34.
47. Prat A, Carey LA, Adamo B, Vidal M, Tabernero J, Cortés J, et al. Molecular features and survival outcomes of the intrinsic subtypes within HER2-positive breast cancer. J Natl Cancer Inst. 2014;106(8).

48. Llombart-Cussac A, Cortés J, Paré L, Galván P, Bermejo B, Martínez N, et al. HER2-enriched subtype as a predictor of pathological complete response following trastuzumab and lapatinib without chemotherapy in early-stage HER2-positive breast cancer (PAMELA): an open-label, single-group, multicentre, phase 2 trial. Lancet Oncol. 2017;18(4):545-54.
49. Prat A, Bianchini G, Thomas M, Belousov A, Cheang MC, Koehler A, et al. Research-based PAM50 subtype predictor identifies higher responses and improved survival outcomes in HER2-positive breast cancer in the NOAH study. Clin Cancer Res. 2014;20(2):511-21.
50. Fumagalli D, Venet D, Ignatiadis M, Azim HA, Jr, Maetens M, Rothé F, et al. RNA Sequencing to Predict Response to Neoadjuvant Anti-HER2 Therapy: A Secondary Analysis of the NeoALTTO Randomized Clinical Trial. JAMA Oncol. 2017;3(2):227-34.
51. Chumsri S, Li Z, Serie DJ, Norton N, Mashadi-Hossein A, Tenner K, et al. Adaptive immune signature in HER2-positive breast cancer in NCCTG (Alliance) N9831 and NeoALTTO trials. npj Breast Cancer. 2022;8(1):68.
52. Nitz UA, Gluz O, Christgen M, Grischke EM, Augustin D, Kuemmel S, et al. De-escalation strategies in HER2-positive early breast cancer (EBC): final analysis of the WSG-ADAPT HER2+/HR- phase II trial: efficacy, safety, and predictive markers for 12 weeks of neoadjuvant dual blockade with trastuzumab and pertuzumab ± weekly paclitaxel. Ann Oncol. 2017;28(11):2768-72.
53. Prat A, Guarneri V, Pascual T, Brasó-Maristany F, Sanfeliu E, Paré L, et al. Development and validation of the new HER2DX assay for predicting pathological response and survival outcome in early-stage HER2-positive breast cancer. eBioMedicine. 2022;75.
54. Griguolo G, Pascual T, Dieci MV, Guarneri V, Prat A. Interaction of host immunity with HER2-targeted treatment and tumor heterogeneity in HER2-positive breast cancer. J Immunother Cancer. 2019;7(1):90.
55. Stanton SE, Adams S, Disis ML. Variation in the Incidence and Magnitude of Tumor-Infiltrating Lymphocytes in Breast Cancer Subtypes: A Systematic Review. JAMA Oncol. 2016;2(10):1354-60.
56. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol. 2015;26(2):259-71.
57. Salgado R, Denkert C, Campbell C, Savas P, Nuciforo P, Aura C, et al. Tumor-Infiltrating Lymphocytes and Associations With Pathological Complete Response and Event-Free Survival in HER2-Positive Early-Stage Breast Cancer Treated With Lapatinib and Trastuzumab: A Secondary Analysis of the NeoALTTO Trial. JAMA Oncol. 2015;1(4):448-54.
58. Denkert C, von Minckwitz G, Brase JC, Sinn BV, Gade S, Kronenwett R, et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. J Clin Oncol. 2015;33(9):983-91.
59. Fernandez-Martinez A, Pascual T, Singh B, Nuciforo P, Rashid NU, Ballman KV, et al. Prognostic and Predictive Value of Immune-Related Gene Expression Signatures vs

Tumor-Infiltrating Lymphocytes in Early-Stage ERBB2/HER2-Positive Breast Cancer: A Correlative Analysis of the CALGB 40601 and PAMELA Trials. JAMA Oncol. 2023.
60. Matikas A, Johansson H, Grybäck P, Bjöhle J, Acs B, Boyaci C, et al. Survival Outcomes, Digital TILs, and On-treatment PET/CT During Neoadjuvant Therapy for HER2-positive Breast Cancer: Results from the Randomized PREDIX HER2 Trial. Clinical Cancer Research. 2023;29(3):532-40.
61. Nuciforo P, Pascual T, Cortés J, Llombart-Cussac A, Fasani R, Paré L, et al. A predictive model of pathologic response based on tumor cellularity and tumor-infiltrating lymphocytes (CelTIL) in HER2-positive breast cancer treated with chemo-free dual HER2 blockade. Ann Oncol. 2018;29(1):170-7.
62. Iglesia MD, Vincent BG, Parker JS, Hoadley KA, Carey LA, Perou CM, Serody JS. Prognostic B-cell signatures using mRNA-seq in patients with subtype-specific breast and ovarian cancer. Clin Cancer Res. 2014;20(14):3818-29.
63. Gianni L, Zambetti M, Clark K, Baker J, Cronin M, Wu J, et al. Gene expression profiles in paraffin-embedded core biopsy tissue predict response to chemotherapy in women with locally advanced breast cancer. J Clin Oncol. 2005;23(29):7265-77.
64. Ayers M, Symmans WF, Stec J, Damokosh AI, Clark E, Hess K, et al. Gene expression profiles predict complete pathologic response to neoadjuvant paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide chemotherapy in breast cancer. J Clin Oncol. 2004;22(12):2284-93.
65. Paik S, Tang G, Shak S, Kim C, Baker J, Kim W, et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. J Clin Oncol. 2006;24(23):3726-34.
66. Fernandez-Martinez A, Krop IE, Hillman DW, Polley M-Y, Parker JS, Huebner L, et al. Survival, Pathologic Response, and Genomics in CALGB 40601 (Alliance), a Neoadjuvant Phase III Trial of Paclitaxel-Trastuzumab With or Without Lapatinib in HER2-Positive Breast Cancer. Journal of Clinical Oncology. 2020;38(35):4184-93.
67. Fan C, Prat A, Parker JS, Liu Y, Carey LA, Troester MA, Perou CM. Building prognostic models for breast cancer patients using clinical variables and hundreds of gene expression signatures. BMC Med Genomics. 2011;4:3.
68. Perez EA, Thompson EA, Ballman KV, Anderson SK, Asmann YW, Kalari KR, et al. Genomic analysis reveals that immune function genes are strongly linked to clinical outcome in the North Central Cancer Treatment Group n9831 Adjuvant Trastuzumab Trial. J Clin Oncol. 2015;33(7):701-8.
69. Merritt CR, Ong GT, Church SE, Barker K, Danaher P, Geiss G, et al. Multiplex digital spatial profiling of proteins and RNA in fixed tissue. Nature Biotechnology. 2020;38(5):586-99.
70. Moutafi M, Martinez-Morilla S, Divakar P, Vathiotis I, Gavrielatou N, Aung TN, et al. Discovery of Biomarkers of Resistance to Immune Checkpoint Blockade in NSCLC Using High-Plex Digital Spatial Profiling. Journal of thoracic oncology. 2022;17(8):991-1001.
71. Toki MI, Merritt CR, Wong PF, Smithy JW, Kluger HM, Syrigos KN, et al. High-Plex Predictive Marker Discovery for Melanoma Immunotherapy-Treated Patients Using Digital Spatial Profiling. Clin Cancer Res. 2019;25(18):5503-12.

72. Stewart RL, Matynia AP, Factor RE, Varley KE. Spatially-resolved quantification of proteins in triple negative breast cancers reveals differences in the immune microenvironment associated with prognosis. Sci Rep. 2020;10(1):6598.
73. McNamara KL, Caswell-Jin JL, Joshi R, Ma Z, Kotler E, Bean GR, et al. Spatial proteomic characterization of HER2-positive breast tumors through neoadjuvant therapy predicts response. Nature Cancer. 2021;2(4):400-13.
74. Hennessy MA, Leal J, Huang CY, Solnes L, Denbow R, Abramson V, et al. 191P Correlation of early change in standardized uptake value (SUV) on positron emission tomography (PET/CT) with recurrence-free survival (RFS) and overall survival (OS) in patients with primary operable HER2-positive breast cancer (TBCRC026). Annals of oncology. 2022;33:S623-S4.
75. Hennessy MA, Leal JP, Huang CY, Solnes LB, Denbow R, Abramson VG, et al. Correlation of SUV on Early Interim PET with Recurrence-Free Survival and Overall Survival in Primary Operable HER2-Positive Breast Cancer (the TBCRC026 Trial). J Nucl Med. 2023;64(11):1690-6.
76. Hennessy MA, Cimino-Mathews A, Carter J, Kachergus J, Ma Y, Leal J, et al. 333P Evaluation of a composite PET/CT and HER2 tissue-based biomarker to predict response to neoadjuvant HER2-directed therapy in early breast cancer (TBCRC026). Annals of oncology. 2023;34:S316.
77. 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.
78. Schneeweiss A, Chia S, Hickish T, Harvey V, Eniu A, Hegg R, et al. Pertuzumab plus trastuzumab in combination with standard neoadjuvant anthracycline-containing and anthracycline-free chemotherapy regimens in patients with HER2-positive early breast cancer: a randomized phase II cardiac safety study (TRYPHAENA). Annals of oncology. 2013;24(9):2278-84.
79. Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. The Lancet. 2014;384(9938):164-72.
80. Loibl S, Poortmans P, Morrow M, Denkert C, Curigliano G. Breast cancer. The Lancet. 2021;397(10286):1750-69.
81. Thomssen C, Balic M, Harbeck N, Gnant M. St. Gallen/Vienna 2021: A Brief Summary of the Consensus Discussion on Customizing Therapies for Women with Early Breast Cancer. Breast Care. 2021;16(2):135-43.
82. Han S, Choi JY. Prognostic value of (18)F-FDG PET and PET/CT for assessment of treatment response to neoadjuvant chemotherapy in breast cancer: a systematic review and meta-analysis. Breast Cancer Res. 2020;22(1):119.
83. 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.

84. Graham MM, Wahl RL, Hoffman JM, Yap JT, Sunderland JJ, Boellaard R, et al. Summary of the UPICT Protocol for 18F-FDG PET/CT Imaging in Oncology Clinical Trials. J Nucl Med. 2015;56(6):955-61.
85. Kinahan PE, Perlman ES, Sunderland JJ, Subramaniam R, Wollenweber SD, Turkington TG, et al. The QIBA Profile for FDG PET/CT as an Imaging Biomarker Measuring Response to Cancer Therapy. Radiology. 2020;294(3):647-57.
86. Tolaney SM, Garrett-Mayer E, White J, Blinder VS, Foster JC, Amiri-Kordestani L, et al. Updated Standardized Definitions for Efficacy End Points (STEEP) in Adjuvant Breast Cancer Clinical Trials: STEEP Version 2.0. Journal of Clinical Oncology. 2021;39(24):2720-31.
87. Nitz U, Gluz O, Graeser M, Christgen M, Kuemmel S, Grischke EM, et al. De-escalated neoadjuvant pertuzumab plus trastuzumab therapy with or without weekly paclitaxel in HER2-positive, hormone receptor-negative, early breast cancer (WSG-ADAPT-HER2+/HR-): survival outcomes from a multicentre, open-label, randomised, phase 2 trial. Lancet Oncol. 2022;23(5):625-35.
88. 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. New England Journal of Medicine. 2001;344(11):783-92.
89. 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. New England Journal of Medicine. 2005;353(16):1659-72.
90. Schettini F, Prat A. Dissecting the biological heterogeneity of HER2-positive breast cancer. Breast. 2021;59:339-50.
91. Schettini F, Pascual T, Conte B, Chic N, Brasó-Maristany F, Galván P, et al. HER2-enriched subtype and pathological complete response in HER2-positive breast cancer: A systematic review and meta-analysis. Cancer Treat Rev. 2020;84:101965.
92. Shen G, Zhao F, Huo X, Ren D, Du F, Zheng F, Zhao J. Meta-Analysis of HER2-Enriched Subtype Predicting the Pathological Complete Response Within HER2-Positive Breast Cancer in Patients Who Received Neoadjuvant Treatment. Front Oncol. 2021;11:632357.
93. Fumagalli D, Venet D, Ignatiadis M, Azim HA, Jr., Maetens M, Rothé F, et al. RNA Sequencing to Predict Response to Neoadjuvant Anti-HER2 Therapy: A Secondary Analysis of the NeoALTTO Randomized Clinical Trial. JAMA Oncol. 2017;3(2):227-34.
94. Harbeck N, von Schumann R, Kates RE, Braun M, Kuemmel S, Schumacher C, et al. Immune Markers and Tumor-Related Processes Predict Neoadjuvant Therapy Response in the WSG-ADAPT HER2-Positive/Hormone Receptor-Positive Trial in Early Breast Cancer. Cancers (Basel). 2021;13(19).
95. de Haas SL, Slamon DJ, Martin M, Press MF, Lewis GD, Lambertini C, et al. Tumor biomarkers and efficacy in patients treated with trastuzumab emtansine + pertuzumab versus standard of care in HER2-positive early breast cancer: an open-label, phase III study (KRISTINE). Breast Cancer Research. 2023;25(1):2.
96. Yoav B, Daniel Y. The control of the false discovery rate in multiple testing under dependency. The Annals of Statistics. 2001;29(4):1165-88.

97. Marra A, Chandarlapaty S, Modi S. Management of patients with advanced-stage HER2-positive breast cancer: current evidence and future perspectives. Nature Reviews Clinical Oncology. 2024.
98. Perez EA, Ballman KV, Mashadi-Hossein A, Tenner KS, Kachergus JM, Norton N, et al. Intrinsic Subtype and Therapeutic Response Among HER2-Positive Breaty st Tumors from the NCCTG (Alliance) N9831 Trial. J Natl Cancer Inst. 2017;109(2).
99. Ray T, Ryusaki T, Ray PS. Therapeutically Targeting Cancers That Overexpress FOXC1: A Transcriptional Driver of Cell Plasticity, Partial EMT, and Cancer Metastasis. Front Oncol. 2021;11:721959.
100. Tang L, Jin J, Xu K, Wang X, Tang J, Guan X. SOX9 interacts with FOXC1 to activate MYC and regulate CDK7 inhibitor sensitivity in triple-negative breast cancer. Oncogenesis. 2020;9(5):47.
101. Coombes RC, Howell S, Lord SR, Kenny L, Mansi J, Mitri Z, et al. Dose escalation and expansion cohorts in patients with advanced breast cancer in a Phase I study of the CDK7-inhibitor samuraciclib. Nat Commun. 2023;14(1):4444.
102. Du R, Huang C, Liu K, Li X, Dong Z. Targeting AURKA in Cancer: molecular mechanisms and opportunities for Cancer therapy. Molecular Cancer. 2021;20(1):15.
103. Chu MP, Hecht JR, Slamon D, Wainberg ZA, Bang Y-J, Hoff PM, et al. Association of Proton Pump Inhibitors and Capecitabine Efficacy in Advanced Gastroesophageal Cancer: Secondary Analysis of the TRIO-013/LOGiC Randomized Clinical Trial. JAMA Oncol. 2017;3(6):767-73.
104. O'Shaughnessy J, McIntyre K, Wilks S, Ma L, Block M, Andorsky D, et al. Efficacy and Safety of Weekly Paclitaxel With or Without Oral Alisertib in Patients With Metastatic Breast Cancer: A Randomized Clinical Trial. JAMA Netw Open. 2021;4(4):e214103.
105. Bianchini G, Pusztai L, Pienkowski T, Im YH, Bianchi GV, Tseng LM, et al. Immune modulation of pathologic complete response after neoadjuvant HER2-directed therapies in the NeoSphere trial. Annals of oncology. 2015;26(12):2429-36.
106. Prat A, Pascual T, De Angelis C, Gutierrez C, Llombart-Cussac A, Wang T, et al. HER2-Enriched Subtype and ERBB2 Expression in HER2-Positive Breast Cancer Treated with Dual HER2 Blockade. J Natl Cancer Inst. 2020;112(1):46-54.
107. Veeraraghavan J, De Angelis C, Mao R, Wang T, Herrera S, Pavlick AC, et al. A combinatorial biomarker predicts pathologic complete response to neoadjuvant lapatinib and trastuzumab without chemotherapy in patients with HER2+ breast cancer. Ann Oncol. 2019;30(6):927-33.
108. Holmes F, Nagarwala Y, Espina V, Liotta L, Danso M, Gallagher R, et al. Correlation of molecular effects and pathologic complete response to preoperative lapatinib and trastuzumab, separately and combined prior to neoadjuvant breast cancer chemotherapy. Journal of Clinical Oncology. 2011;29(15_suppl):506-.
109. Chic N, Luen SJ, Nuciforo P, Salgado R, Fumagalli D, Hilbers F, et al. Tumor Cellularity and Infiltrating Lymphocytes as a Survival Surrogate in HER2-Positive Breast Cancer. J Natl Cancer Inst. 2022;114(3):467-70.
110. Rimawi MF, Mayer IA, Forero A, Nanda R, Goetz MP, Rodriguez AA, et al. Multicenter phase II study of neoadjuvant lapatinib and trastuzumab with hormonal

therapy and without chemotherapy in patients with human epidermal growth factor receptor 2-overexpressing breast cancer: TBCRC 006. J Clin Oncol. 2013;31(14):1726-31.
111. Prat A, Guarneri V, Paré L, Griguolo G, Pascual T, Dieci MV, et al. A multivariable prognostic score to guide systemic therapy in early-stage HER2-positive breast cancer: a retrospective study with an external evaluation. Lancet Oncol. 2020;21(11):1455-64.
112. Cortes J, Perez-García JM, Llombart-Cussac A, Curigliano G, El Saghir NS, Cardoso F, et al. Enhancing global access to cancer medicines. CA: A Cancer Journal for Clinicians. 2020;70(2):105-24.