QuantifyHER
Quantitative Immunofluorescence and/or RT-qPCR for Measuring HER2 in HER2-low Metastatic Breast Cancer

Protocol Number	TBCRC 066
Protocol Chair	Angela DeMichele MD, MSCE Rowan Breast Center of the Abramson Cancer Center University of Pennsylvania Perelman Center, 3rd Floor 3400 Civic Center Blvd, Philadelphia PA 19104 Phone: 215-615-6820 Fax: 215-615-3349 Email: angela.demichele@pennmedicine.upenn.edu
Co-Chair	David Rimm MD, PhD Yale University School of Medicine david.rimm@yale.edu *Co-Chair*
Co-Investigators	Eleanor Taranto MD Department of Hematology & Oncology Hospital of the University of Pennsylvania Eleanor.taranto@pennmedicine.upenn.edu
Statistician	E. Paul Wyleto, PhD Perelman School of Medicine at the University of Pennsylvania
Supporters	Johns Hopkins University on behalf of the Translational Breast Cancer Research Consortium (TBCRC) Danaher/Cepheid, Inc American Society of Clinical Oncology, via Gianni Bonadonna Breast Cancer Research Fellowship
Coordinating Center	University of Pennsylvania

QuantifyHER
Quantitative Immunofluorescence and/or RT-qPCR for Measuring HER2 in HER2-low Metastatic Breast Cancer

- Protocol Revision Record -

Original Protocol: 1/19/2024

Table of Contents

1. SUMMARY / SCHEMA

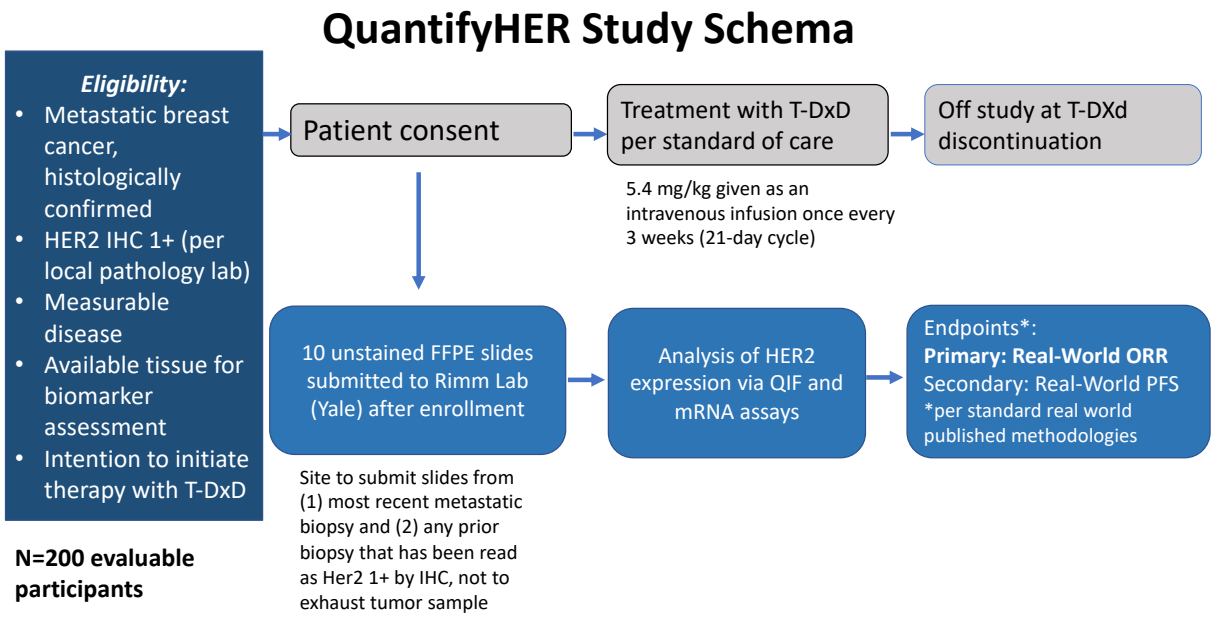
Metastatic breast cancer remains incurable despite significant advances in both understanding of the disease and treatment innovations. Balancing treatment effectiveness with quality of life is therefore integral to the care of the patient. Trastuzumab-deruxtecan (T-DXd), an antibody-drug conjugate, was recently approved for patients with metastatic breast cancer expressing low levels of HER2 by immunohistochemistry (IHC 1+ or 2+, FISH negative). However, there is significant inter-reader variability in calling HER2 IHC 1+ vs IHC 0, raising concern for inappropriate patient selection for T-DXd.

Rimm et al. have developed a quantitative immunofluorescence (QIF) HER2 assay that, in serially collected samples, has identified up to 20% of IHC 1+ samples below the level of quantification using QIF. HER2 messenger RNA (mRNA) assessment is also being explored as an alternative quantitative method.

We now propose to validate these quantitative assays by conducting a multi-site, longitudinal cohort study ("QuantifyHER") within the Translational Breast Cancer Research Consortium (TBCRC). This study will assess whether a quantitative, immunofluorescence-based HER2 assay and/or a HER2 mRNA RT-qPCR assay can accurately and reliably discriminate between responders and non-responders among patients with HER2 IHC1+ metastatic breast cancer who are receiving T-DXd.

Patients with biopsy-proven metastatic breast cancer that is HER2 1+ by IHC and who are initiating T-DXd will be enrolled prospectively and followed. Key eligibility criteria include available tissue, measurable disease, and ability to provide informed consent. Patients with prior HER2 positive disease are excluded. Patient data (demographics, clinical features, and follow up) will be recorded in case report forms and stored in a Penn-managed REDCap database. For each patient, 9 unstained FFPE sections and 1 H&E section will be sent to Dr. David Rimm at Yale for quantitative immunofluorescence analysis (a.k.a. the Troplex assay) yielding a HER2-expression value (QIF) and ERBB2 mRNA level assessment via Cepheid Xpert Breast Cancer STRAT4 (RUO) assay. Slides will be collected for these two purposes, but remaining slides will be stored and may be used for testing beyond these two planned assays (the informed consent will include the possibility of additional research to assess resistance factors or related biomarkers). Clinical endpoints (response, death) will be assessed by local investigators using real-world response categories. The primary objective is to determine if there is a relationship between QIF of HER2 protein expression and/or quantitative mRNA levels of ERBB2 and real-world objective response (rwORR). For the planned 200 patients and a one-sided type-one error of 2.5%, we will have 80% power to detect a difference in proportions of 9 to 10 percentage points for each standard deviation increase in QIF or mRNA across the range of interest, such as 29% versus 20%, or 34.5% versus 25%. This corresponds to an odds ratio of approximately 1.6. A non-binding interim review at n=70 will assess preliminary biomarker distribution and allow for necessary sample size adjustment. Secondary endpoints will include real-world progression-free survival, overall survival, threshold QIF value for response, relationship between QIF/mRNA level and estrogen-receptor status, and relationship between HER2 mRNA level and response. We will conduct an exploratory analysis of rwORR using QIF and mRNA jointly to

determine whether a combination is better than either alone at discriminating responders from non-responders.



2. OBJECTIVES

2.1 Primary Objective

2.1.1 To determine whether there is a relationship between low levels of tumor HER2 measured by quantitative immunofluorescence (QIF) and/or mRNA by RT-qPCR and real-world response (rwORR) to T-DXd among patients with metastatic breast cancer whose tumors are deemed HER2 1+ by immunohistochemistry.

Primary Endpoint: Association between quantitative HER2 expression (as a continuous variable) and real-world objective response rate (rwORR)

2.2 Secondary Objectives

2.2.1 To determine whether there is a relationship between low levels of tumor HER2 measured by QIF and/or mRNA by RT-qPCR and real-world progression-free survival (rwPFS) to T-DXd among patients with tumors deemed HER2 1+ by standard immunohistochemistry.

Endpoint: Association between quantitative HER2 expression (as a continuous variable) and rwPFS.

2.2.2 To determine the relationship between HER2 expression by QIF and mRNA and both rwPFS and rwORR, stratified by estrogen receptor (ER) expression status.

Endpoint: Association between HER2 expression by quantitative immunofluorescence and mRNA in HER2 IHC 1+ tumors and both rwORR and rwPFS, stratified by mRNA ER expression.

2.2.3 To determine whether a cut-off value exists for HER2 QIF, mRNA or the combination below which patients will not respond to T-DXd.

Endpoint: ROC thresholds for HER2 QIF and/or mRNA levels that discriminate responders from non-responders by identifying the lowest levels that perform better than a random classifier.

2.2.4 To determine, based on rwORR, whether a linear combination of HER2 QIF and mRNA is better than either alone at discriminating T-DXd responders from non-responders.

Endpoint: Association between combined mRNA + QIF and rwORR

3. BACKGROUND

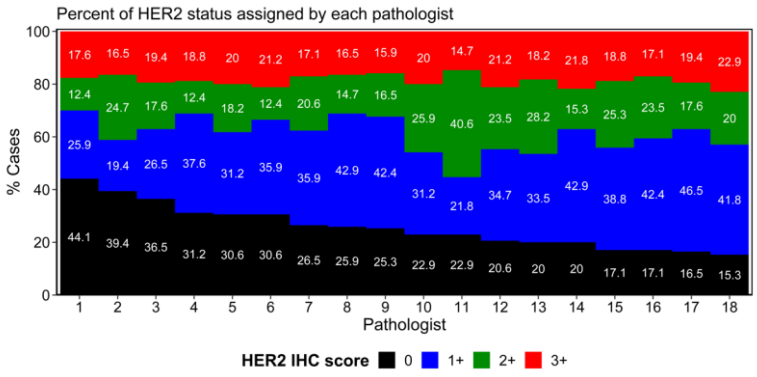
3.1 HER2-low Metastatic Breast Cancer

Metastatic breast cancer (MBC) remains incurable despite significant advances in both our understanding of the disease as well as treatment innovations. Therefore, balancing treatment effectiveness with quality of life is integral to the overall care of the patient given that therapies are palliative in nature. Although HER2-targeted therapies have historically been restricted to MBC that is defined as HER2+ by standard ASCO/CAP guidelines, recent advances in antibody-drug conjugate (ADC) design have led to striking results, as exhibited by one such ADC, trastuzumab deruxtecan (T-DXd), in patients with HER2-negative MBC that expressed low levels of HER2 (1+ or 2+ by immunohistochemistry [IHC]). T-DXd has demonstrated objective response rates (ORR) of up to 50% in patients with estrogen-receptor (ER)-positive and ER-negative MBC expressing HER2 IHC 1+/2+ and has doubled progression-free survival (PFS) and improved overall survival (OS) compared to standard chemotherapies. This has led to classification of HER2 IHC 1+ or 2+/FISH non-amplified tumors as "HER2-low."

3.2 HER2 assessment methods

The standard immunohistochemistry (IHC) assays using antibodies against HER2 were initially designed to reliably identify patient tumors with very high levels of HER2, without focus on the low end of the expression spectrum. HER2 grading by IHC has also been found to be subjective and pathologist dependent. A recent study surveying 1400 laboratories around the world suggested < 70% interrater agreement between HER2 IHC 0 and 1+ among 19% of the laboratories surveyed, and a real world sub-study of 170 tissue sections showed only a 26% concordance rate among pathologists in calling HER2 IHC 0 (see Figure 1).[1] Another study investigating HER2 mRNA levels showed similar levels between HER2 0 and HER2 IHC 1+, and significantly different compared to HER2 2+ FISH non-amplified[2], suggesting misclassification by IHC. Given the toxicity profile for T-DXd, including rates up to 12% for interstitial lung disease (ILD)/pneumonitis[3], a reliable quantitative assay with high precision for the presence of low levels of HER2 tumor expression is urgently needed to ensure optimal patient selection.

Figure 1: Percent HER2 Status By Pathologist



3.3 Quantitative HER2 Assessment Methods: Immunofluorescence

The Rimm Lab at Yale University School of Medicine has developed a quantitative immunofluorescence (QIF) assay, known as the Troplex Assay, using automated quantitative analysis (AQUA).[4] This technique has been used to look at HER2 expression in a serial collection of breast cancer cases, with work that has now defined the limits of detection (LOD), quantification (LOQ), and linearity (LOL) for the HER2 QIF assay using standard analytic methods.[4] The assay has now been validated on over 400 serially collected samples from 2011-2014 and over 250 breast core needle biopsies collected prospectively. Rimm and colleagues have found that many cases considered HER2 negative by conventional assay have quantifiable levels of HER2 protein expression (above the LOQ), and that a significant proportion of cases called IHC 1+ in fact show HER2 protein levels *below* the limit of quantification (see Table 1, Rimm et. al, Manuscript in process).

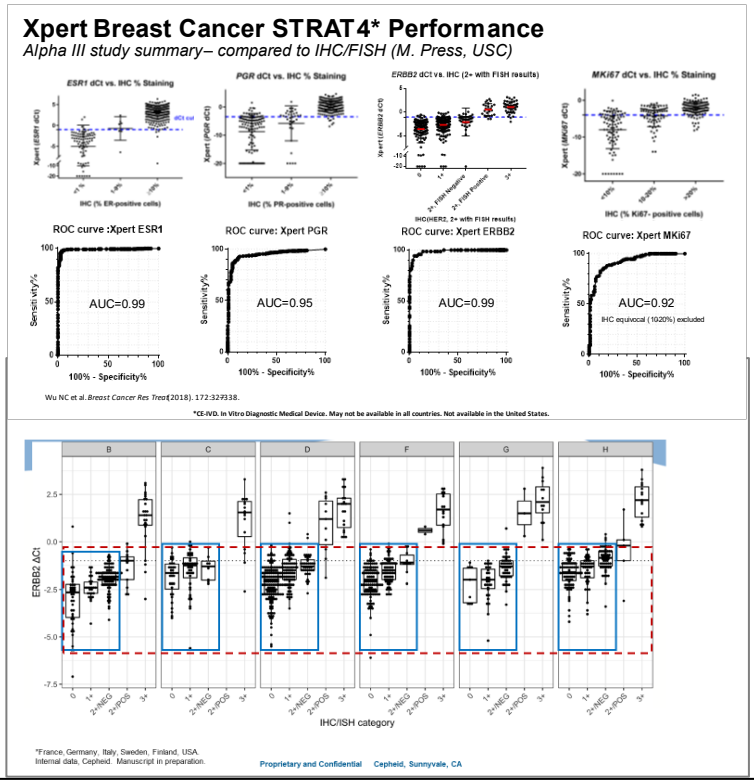
Table 1: Comparison of HER2 by IHC Versus QIF

	IHC 0	IHC 1+	IHC 2+/N	IHC 2+ & 3+	amol/mm2
below LOD	3 (3%)	1 (1%)	1 (2%)	0	5 (2%)
between LOD & LOQ	24 (21%)	15 (13%)	0	0	39 (12%)
between LOQ & LOL	59 (53%)	53 (45%)	10 (18%)	4 (13%)	126 (40%)
above LOL	26 (23%)	49 (42%)	44 (80%)	27 (87%)	146 (46%)
Total Slides	112 (35%)	118 (37%)	55 (17%)	31 (10%)	316
Total Cases	316				

3.4 Quantitative HER2 Assessment Methods: mRNA

Cepheid's Xpert® Breast Cancer STRAT4 test can assess mRNA for *ESR1, PGR, ERBB2, and MKi67* in <3 hours. The STRAT4 test, currently available for research use only (RUO) in the United States but commercially available in several other countries, produces reproducible results, demonstrating high concordance with central IHC measures.[5] In a retrospective validation study of 500 breast cancer cases, overall percent agreement between STRAT4 and centrally tested IHC/FISH was 97.8% for ESR1, 90.4% for PGR, 93.3% for ERBB2, and 78.6% for MKi67 (see Figure 2).[6] Another independent validation study of

Figure 2: Xpert Breast Cancer STRAT4 Performance



200 samples found similar rates of concordance between STRAT4 and IHC[7], and an unpublished pooled analysis representing ~1000 unique breast cancers has further confirmed the correlation. In the bottom panel of Figure 2, the cases outlined by the (red) dashed lines represent the lower end of the mRNA spectrum, highlighting significant overlap of ERBB2 mRNA expression, quantified as delta cycle threshold (delta CT, or dCt) levels across multiple IHC levels (0, 1+, 2+). In-house experiments have characterized the limits of quantification (LoQ) and detection (LoD) for ERBB2, but these proposed dCt detection cutoffs have not yet been tested or correlated with outcomes in patients with "HER2-low" breast cancer treated with HER2-ADCs.

3.5 Rationale

Given the challenges with interpreting semi-quantitative immunohistochemistry (IHC) results—especially when it comes to low levels of HER2 expression—this prospective observational study will assess two new quantitative approaches to measure HER2 protein expression, and their individual and collective abilities to predict response to treatment among those with metastatic breast cancer who are starting treatment with Trastuzumab Deruxtecan (T-DXd). We will identify a cohort of patients newly diagnosed with HER2 low (1+) metastatic breast cancer who are starting treatment with T-DXd. We will assess their biopsy samples at the time of diagnosis with metastatic disease (as well as prior HER2 low samples) for HER2 expression via the QIF Troplex and mRNA STRAT4 assays. We will then follow them prospectively throughout the course of their treatment with T-DXd.

We propose to prospectively validate the QIF assay as a way to more reliably measure low levels of HER2 surface protein, and to assess whether QIF or mRNA levels can discriminate responders from non-responders to T-DXd in the HER2 1+ population. We also plan to examine the relationship between QIF scores and other key clinical endpoints (ORR, PFS, OS) using "real world" response assessment, and to stratify results by estrogen receptor status. If cut-off points can be identified that discriminate responders from non-responders to T-DXd, this assay could not only improve patient selection for T-DXd but could be scaled and adapted for next generation ADCs for any measurable target.

Another key objective will be to investigate the utility of measuring HER2 messenger RNA (mRNA) levels as a separate predictor for response to T-DXd, and to assess whether HER2 QIF and mRNA are jointly superior to either alone for predictive modeling. By collecting real-world response data in patients with metastatic breast cancer treated with T-DXd, this approach will not only seek to validate the ability of the assays to discriminate between responders and non-responders to T-DXd but will also produce results that are immediately generalizable to a broader population and to clinical practice.

4. PARTICIPANT SELECTION

4.1 Inclusion Criteria

4.1.1 Women and men age ≥ 18 years

4.1.2 Metastatic breast cancer, histologically- confirmed. Any estrogen receptor (ER) status is allowed. ER status will be determined by local laboratory assessment utilizing ASCO/CAP guidelines.

4.1.3 Primary and/or metastatic tumor with 1+ level of expression of HER2 by immunohistochemistry as determined by local laboratory assessment utilizing ASCO/CAP guidelines.

4.1.4 Measurable disease by cross-sectional imaging at the start of treatment. Patients with measurable bone-only disease or active brain metastases are eligible.

4.1.5 Archival tissue available for biomarker assessment. One specimen should be the most recent metastatic biopsy. If HER2 1+ status was determined on a different specimen (either primary or metastatic tissue), that specimen is also required. Samples obtained from bone metastases that were processed via decalcification methods are not eligible.

4.1.6 Intention to initiate therapy with T-DXd (Enhertu) at FDA-approved dose and schedule as next line of therapy. If T-DXd was already initiated, patients must be registered within 30 days of initiation.

4.1.7 Ability to provide informed consent.

4.2 Exclusion Criteria

4.2.1 Concurrent Her2-overexpressing metastatic breast cancer (as confirmed by a metastatic biopsy with IHC 3+ or IHC 2+ with FISH amplified as per standard ASCO/CAP guidelines)

4.3 Inclusion of Underrepresented Populations

Individuals of all races and ethnic groups are eligible for this trial. There is no bias towards age or race in the clinical trial outlined. This trial is open to the accrual of both men and women.

5. REGISTRATION PROCEDURES

The University of Pennsylvania (Penn) will serve as the lead institution. Penn will serve as the central site for protocol development and amendments, data management, and study monitoring. All subjects must be registered through the study specific REDCap data portal. All subjects who sign an informed consent document will be entered into and tracked in REDCap, including for collection of demographics and outcome of eligibility. Subject eligibility must be reviewed and confirmed by the lead institution prior to formal registration. Confirmation of registration will be communicated by the lead site to the local site via email. A subject is considered registered when an "On Study" date is entered into the Registration case report form (CRF) in REDCap. This process will be managed centrally by the lead institution.

5.1 Guidelines for Registration

Key steps for site personnel to register participants and generate the study ID are summarized below. Additional details can be found in the accompanying Study Manual.

- Documentation of informed consent
- Entry into the study specific REDCap data portal which generates a unique participant study ID
- Completion of the eligibility checklist with accompanying source documentation uploaded
- Confirmation of subject eligibility by the lead site and formal registration
- Following successful registration, the site will gain access to all necessary forms (including biospecimen submission forms, clinical, imaging, and pathology CRFs)

Participants must be registered with the lead institution prior to archival tissue submission.

Participants must be registered within 30 days of the initiation of T-DXd. Participants should begin T-DXd treatment as per their treating physician. Issues that cause treatment delays should be documented in CRFs. If a participant does not receive T-DXd or if samples are not received by the Rimm Lab, the participant's registration on the study may be canceled. The lead site should be notified of events leading to cancellation as soon as possible.

Any requests for eligibility exceptions and/or deviations must be approved by the Protocol Chair or designee prior to execution.

Please contact the Penn project manager or designee via study specific email address with any questions regarding this process.

Registration will only be conducted during the business hours of 8 am – 6 pm (EST) Monday through Friday.

6. STUDY CALENDAR

Procedure	Baseline[1]	Every 6 weeks (2 cycles)	Every Scan Visit (approximately every 3 months)	End of SOC treatment with T-DXd
CLINICAL ASSESSMENT:				
Informed consent	X			
Medical History	X			
Performance Status	X			
DEMOGRAPHICS	X			
IMAGING:				
CT/MRI/Bone scan/PET	X		X	X
SAMPLES:				
Archival tumor sample[2]	X			
OTHER ASSESSMENTS:				
Follow-up/Survival[3,4]		X	X	X

Note: Additional tests may be performed at the discretion of the treating physician as clinically indicated. The schedules outlined above are based on an ideal subject, and all visits will occur as per the treating physician and the standard of care. The schedule may be modified due to problems such as scheduling delays or conflicts (e.g., clinic closure, poor weather conditions, vacations, etc.).

1. Information will be collected about the breast cancer diagnosis, pathology, imaging/radiology, treatment(s) received. This should occur within 4 weeks prior to starting treatment, unless otherwise noted.
2. Samples collection is outlined in Section 9.
3. Subjects will be followed approximately every 6-12 weeks per local clinical practice for disease status and survival until discontinuation of T-DxD, the study has ended or is cancelled, or the subject asks to discontinue participation. The exact frequency of these visits will be determined by the treating physician.
4. At each follow-up visit (i.e. each infusion visit, each scan review visit, and any visits that lead to treatment modification or discontinuation), the plan for treatment continuation or discontinuation will be recorded in CRFs, with input from the treating physician as needed. The reason for discontinuation will be recorded, including whether for disease progression, drug toxicity, or other reasons.

7. STUDY PLAN

7.1 Recruitment

Patients will be recruited through the breast cancer clinics at each of the participating centers. Patients will be automatically assigned a sequential participant Study ID when their enrollment information is initially submitted to REDCap. The instructions for subject registration are noted above, with further details supplied in the study manual. Patients must enroll at a participating site, but may receive treatment with T-DXd locally, so long as biopsy samples and source data—including records of visits with clinical status, imaging reports, treatment administration, and treatment discontinuation (with reason for discontinuation)—are available to the study team. Patients must be registered within 30 days of T-DXd initiation.

7.2 Intervention Plan

This is a prospective, observational study of patients receiving T-DXd per standard of care. The patient and treating physician will make clinical decisions involving treatment without knowledge of the findings of any investigational laboratory studies conducted using collected specimens or generated research data. The protocol does not dictate T-DXd dose reductions; any other drug-related decisions will be made per normal clinical practice and the standard of care. Information regarding decisions to discontinue therapy, whether due to toxicity, progression, or other reasons, will be recorded in visit-based case report forms (CRFs) that will be submitted to the central coordinating center via electronic database (REDCAP).

7.3 Safety Assessments

This is a prospective observational study of a standard-of-care medical intervention. It involves only the collection of data at the time of standard clinical assessments. Because there is no investigational therapy, collection and documentation of individual participant adverse events per CTCAE are not warranted. The study will collect information regarding drug toxicity only as it pertains to T-DXd dose reductions and discontinuation of therapy. This information will be recorded in visit-based case report forms (CRFs) that will be submitted to the central coordinating center via electronic database.

7.4 Duration of Follow-Up

Participants will be followed until T-DxD discontinuation, death, or written withdrawal from study participation, whichever occurs first.

In the event that a subject does not continue care at the treating institution, every attempt will be made to collect this information by direct contact or through communication with the outside physician(s).

7.5 Withdrawal from Study

Participants may choose to no longer participate in this project at any time. Specifically, withdrawal of consent to participate in the study must be requested in writing to the lead site. Participants may also ask that the previously collected samples be destroyed; however, data on any research done until the date of the request will be retained. The decision to withdraw will not affect any research already conducted and/or results generated. All reasons for study discontinuation should be documented clearly in the medical record and in the REDCAP survey.

The reasons for withdrawal from the study include:

- Subject withdraws consent for follow-up.
- Subject is lost to follow-up.
- The study is terminated for any reason.

The decision to no longer take part in this project will not affect a participant's care at the enrolling institution.

7.6 Return of Results

During the consent process, patients will be able to opt in to return-of-results. The results they will receive will be the QIF assay results, which are performed in a CLIA Laboratory. Results will be provided to patients who consent for return of results at the completion of their participation in the trial. Patients will be given the option to have their results shared with them and their treating physician, along with a patient education packet about the results. This report will go initially to the participating site where the patient was enrolled, and then the site will contact patients to disclose and share results. Patients will *not* be given the results of the mRNA/RT-qPCR testing as this test is not yet clinically validated.

7.7 Additional Information

Patients will not be paid for their participation in this observational study. Remuneration for participants in appreciation for their time (e.g., parking stickers) is not currently provided by this trial, however may be given per institutional standards.

At the conclusion of the study and upon publication of any manuscript(s) or posting of data on public websites (e.g., Clinicaltrials.gov per federal guidelines), consideration will be made to providing participants with a notification of available results. Any such notification will be approved by the respective IRB at each site prior to dissemination to participants, as needed.

8. SAFETY ASSESSMENTS

This is a prospective, observational study of standard treatment in the clinical setting. There is no risk to patient safety specifically from participating in the study. This study involves the collection of data likely to occur at the time of planned clinical assessments and sample collection for routine care. Therefore, collection of toxicity or other events related to the standard treatment and procedures being received will be collected only as it pertains to discontinuation of T-DXd.

9. CORRELATIVE STUDIES

Patients enrolled on this study are required to have tissue samples available at the time of enrollment.

NOTE: A separate laboratory worksheet/manual will be provided to outline the specific specimen identification/collection parameters and shipping instructions for all research samples.

9.1 Tissues Samples

Tumor tissue samples from a primary tumor and/or metastatic biopsy which demonstrate HER2-low (1+) disease by immunohistochemistry at the local site, must be available. Samples will be shipped directly to Dr. David Rimm's lab at Yale University as per the laboratory manual. If the HER2-low (1+) disease was established from a prior biopsy, samples from both the biopsy at the time of metastatic disease and the biopsy demonstrating HER2 1+ status must be shipped to the Rimm Lab.

9.2 Collection

Participants will be asked permission in the consent form to access tissue collected as part of routine care at the time of initial diagnosis/diagnostic core biopsy and again at surgical resection for correlative studies. Either sections/slides or blocks may be provided at the discretion of the pathologist.

The specifics of slide requirements: Formalin Fixed Paraffin Embedded (FFPE) unstained sections on charged glass slides, including an H&E stained section, must be collected from the metastatic biopsy that demonstrated HER2 1+ status by immunohistochemistry. 9 unstained FFPE sections (5-micron thick) and 1 H&E section will be collected. Site collection should avoid exhausting available tumor for a given patient.

9.3 Processing and storage

All tissue samples will be sent to and stored at Yale University until analysis. A separate laboratory worksheet/manual will be provided to outline the specific collection parameters and shipping instructions for all research samples. Slides will be stored at -80 until processing for HS-HER2 or RT-qPCR. Remaining slides will be stored at -80 until further usage.

The study specimens will be recorded, de-identified, handled, processed, and shipped according to the protocol standard operating procedures detailed in the REDCap study manual and the study laboratory manual.

9.4 Laboratory Analysis Methods

Correlative samples will be analyzed with collaboration from the University of Pennsylvania, Yale University, and Cepheid, Inc.

9.4.1 HER2 Quantitative Immunofluorescence (QIF) Assessment

Measurement of HER2 QIF via Troplex Assay is based on quantitative immunofluorescence (QIF) as described in Moutafi et al.[4] This approach uses a series of cell lines with known levels of HER2 protein as previously determined by mass spectrometry. These lines are used in a cell line microarray (CMA) used as a standard in every assay. For every stainer batch (up to 9 cases), one CMA slide is included and used to create a stainer run-specific standard curve to calculate the HER2 level (in attomole/mm^2) for each case. One section is stained with H&E stain and is used to define the region of the tissue section that is measured. A pathologist views each case and, using a pseudo-IHC image generated by the Cytefinder II scan, software conversion and the scanned H&E if necessary, chooses the area of the slide to be measured that represents the diagnostic metastatic tumor. The measurement is provided in amol/mm^2 as an average across the region of interest selected by the pathologist.

9.4.2 HER2, ER/PR mRNA Assessment

Messenger RNA levels will be measured quantitatively using the Cepheid STRAT4 assay. This assay, run on the Cepheid GeneXpert platform, measures the cycle threshold in a sensitive closed system quantitative RT-qPCR approach normalized by an internal "housekeeping" gene mRNA (CYFIP1) as described previously[6,8]. In this study, two unstained slides will be placed on top of a tumor-annotated H&E and the tumor region will be scraped and processed per STRAT4 protocol. A cycle threshold is determined for each case and normalized by the CYFIP1 gene to return a deltaCT (dCT) value.

9.5 Specimen Submission Requirements

The above specimens are to be submitted to the indicated lab. Refer to the separate laboratory manual for additional processing and shipping instructions. Sample FFPE lysis kits and STRAT4 carts will be provided to the testing lab by Cepheid.

9.6 Additional Information

The laboratory technicians will document information with the collection conditions, processing and storage information for correlative samples.

The laboratory and radiology investigators will be blinded to subject identifiers and clinical data while generating the research data. Additionally, the reported results will not disclose any unique patient identifiers.

A separate laboratory worksheet/manual will be provided to outline the specific collection parameters and shipping instructions for all research samples.

10. SPECIMEN BANKING

Any leftover study tissue samples may be stored for future research studies. The subjects will consent to the future use of samples in the consent form for the study. Any samples will only be released for use in future studies after approval by the Protocol Chair and other regulatory bodies, as appropriate.

The study Protocol Chair and collaborators have approval by the TBCRC to use all research biospecimens collected during the conduct of this trial to address the research questions described in the protocol document. All future use of leftover/residual or repository specimens collected in this trial for purposes not prospectively defined will require review and approval by the TBCRC according to its established policies, whether the specimens are stored in a central site or at a local institution in a virtual repository as described in TBCRC Protocol ADM-002. Secondary use of biospecimens for new endpoints must be submitted to the TBCRC Central Office for possible review by the TBCRC Correlative Science Review Committee.

Once the trial has closed to accrual and primary study objectives have been reported, all biospecimens and data will "roll over" (be virtually stored) under TBCRC Protocol ADM-002: *Translational Breast Cancer Research Consortium Biospecimen and Outcomes Repository,* to allow TBCRC Investigators accessibility for future research. Research participants that have opted in under this protocol will not be approached for additional consent at the time of roll over.

11. MEASUREMENT OF EFFECT

For the purposes of this study, patients should be re-evaluated for response as per the standard of care, with a case report form (CRF) completed in REDCap at all visits, including routine treatment, imaging, and follow-up visits and as otherwise dictated by the treating physician, roughly as per the study calendar. This CRF will document treatment response and disease status, any changes to treatment, and the basis for these changes—whether progression, toxicity, or otherwise.

Since this is a real-world study without the use of RECIST for measuring tumor response, we will employ real-world response methodology[9-11] to derive real-world (rw) measures of efficacy, including response and progression, (rwORR, rwPFS) based on interpretation of treating clinicians' assessments (in office notes), radiology reports, pathology reports, and laboratory studies.

Real-world response categories, as defined by Bartlett et al.[10] are listed in Appendix A. These real-world methodologies have been validated against matched clinical trial cohorts and have been shown to be feasible, reliable and highly correlated with a Spearman's $\rho = 0.99$.[11] Clinical endpoints will be correlated with HER2 QIF and ERBB2 mRNA dCT by STRAT4.

Study personnel at each site will be trained to extract this data from electronic medical records and complete electronic CRFs for data collection. It will be expected that the site research personnel will complete these CRFs using the accompanying study manual along with input from the treating

physician after each infusion visit, each scan review visit, and any visits that lead to treatment modification or discontinuation.

After site startup, data monitoring by the lead site will occur for the first several patients at each site to confirm that real world response assessment is consistent across the different sites and matches real-world response categories as defined by Bartlett et al.[11]

11.1 Evaluable for Objective Response

All patients who initiate treatment with T-DXd will be evaluable for objective response based on real-world response categories. These patients will have their responses classified according to the definitions in Appendix A. (Note: Patients who exhibit objective disease progression prior to the end of cycle 1 will also be considered evaluable.)

11.2 Response Criteria

11.2.1 Real world Response Rate (rwORR)

At every visit, with timing determined as per the treating physician/standard of care, clinical and imaging reports will be used to determine the extent of disease response to therapy, and whether there will be a change in treatment.

The dates of therapy initiation, confirmed disease progression or death, and treatment changes (including the reason for treatment changes) will be collected, as well as response based on imaging and clinical status at each visit. After each scan review visit, information will be collected regarding overall response assessment and the number and size of changing lesions to document best response. For every visit the patient has as per the treating physician, information about treatment continuation and discontinuation—and the reasons for discontinuation, including imaging progression, biochemical progression, symptomatic deterioration, drug toxicity, or other—will be recorded. This information will be documented in CRFs by the site coordinator after each visit, with input as needed from the treating physician about overall response.

Real world objective response rate (rwORR) will be calculated based on the number of those with partial or complete response as determined by the criteria in Appendix A over the absolute number of patients.

11.2.2 Progression due to Symptomatic Deterioration

Subjects with global deterioration of health status requiring discontinuation of treatment without objective imaging evidence of disease progression at that time, and not either related to study treatment or other medical conditions, should be reported as PD due to “symptomatic deterioration.” Every effort should be made to document objective progression even after discontinuation of treatment due to symptomatic deterioration.

11.2.3 Real-world Progression-Free Survival (rwPFS)

Real-world Progression-free survival (rwPFS) is defined as the time from the date of initiation of the study treatment until the date of objective disease progression, death, or discontinuation of T-DXd for any reason in the absence of progression.

11.3 Response Review

There is no independent or central review of the radiology assessments planned for this trial. Response determination will be performed by the local site radiologist in conjunction with the treating physician, as per the standard of care. The Protocol Chair (or designee) may choose to review select cases including imaging reports, pathology reports, and reports about clinical history from the electronic medical record.

12. DATA AND SAFETY MONITORING

12.1 Data Management and Reporting

All information will be collected on study-specific case report forms (CRFs) in REDCAp by the study staff at each institution. The necessary forms will be provided to each site by the Coordinating Center. The following procedures will apply to the REDCap electronic database:

- The database will be password protected; only authorized staff may enter and view study data.
- Passwords and system IDs will not be shared.
- Physical security of the workstations/files will be maintained.
- Staff is trained on the data entry system and importance of security procedures.
- Workstations with the database open will not be left unattended.

All data will be forwarded via secure REDCap to the Coordinating Center for central review and inclusion in the overall study dataset, with relevant source documentation as outlined in the case report forms.

Study data will be reviewed for completeness and accuracy by the Protocol Chair or designees as needed, with initial monitoring of data collection by clinical trial monitors at the lead site to ensure accuracy of data collection at each participating site. The Principal Investigator (or her/his designee) at each respective institution is responsible for review, and ensuring the completeness and accuracy, of the data generated by her/his institution.

12.2 Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents, and data records include: hospital records, clinical and office charts, laboratory notes, memoranda, evaluation checklists, pharmacy dispensing records,

recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, x-rays, and patient files.

12.3 Case Report Forms

The study case report form (CRF) is the primary data collection instrument for the study. All data requested on the CRF must be recorded. All entries will be entered into an electronic data capture system (EDC) via REDCap.

12.4 Meetings

The Coordinating Center will schedule regular teleconferences to take place which will include the Protocol Chair and site investigators and/or study personnel. The following study team members involved with the conduct of the trial will be included as appropriate: study site coordinators, data managers, research nurses, sub-investigators, patient advocates, collaborators (if applicable), and statistician.

During these meetings matters related to the following will be discussed: enrollment rate relative to expectation, characteristics of participants, retention of participants, adherence to protocol (potential or real protocol violations), validity and integrity of the data, safety data, analysis of samples, and progress of data for objectives.

12.5 Monitoring

This study does not include an investigational drug, agent, or device. No Data Safety Monitoring Board applies to this study; the Protocol Chair/Coordinating Center will monitor data and study conduct as above.

12.6 Confidentiality

Information about study patients will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act (HIPAA). Those regulations require a signed patient authorization informing the patient of the following:

- What protected health information (PHI) will be collected from patients in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research patient to revoke their authorization for use of their PHI.

In the event that a patient revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of patient authorization. For patients that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (i.e. that the patient is alive) at the end of their scheduled study period.

13. REGULATORY CONSIDERATIONS

13.1 Protocol Review and Amendments

Unless otherwise specified, each participating institution must obtain its own IRB approval. It is expected that the IRB will have the proper representation and function in accordance with federally mandated regulations. The IRB should approve the consent form and protocol.

Information regarding study conduct and process will be reported to the Institutional Review Board (IRB) per the current institutional standards of each participating center. Any changes to the protocol will be made in the form of an amendment and must be approved by the IRB of each institution prior to implementation.

The Protocol Chair (or her designee) is responsible for the coordination and development of protocol amendments, and will disseminate this information to the participating centers for local submission.

13.2 Informed Consent

The investigator (or her/his designee) will explain to each subject the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits involved and any discomfort it may entail. Each subject will be informed that participation in the study is voluntary, that s/he may withdraw from the study at any time, and that withdrawal of consent will not affect her subsequent medical treatment or relationship with the treating physician(s) or institution. The informed consent will be given by means of a standard written statement, written in non-technical language, which will be IRB approved. The subject should read and consider the statement before signing and dating it, and will be given a copy of the document. No subject will enter the study before his/her informed consent has been obtained.

In accordance with the Health Information Portability and Accountability Act (HIPAA), the written informed consent document (or a separate document to be given in conjunction with the consent document) will include a subject authorization to release medical information to the study sponsor and supporting agencies and/or allow these bodies, a regulatory authority, or Institutional Review Board access to subjects' medical information that includes all hospital records relevant to the study, including subjects' medical history.

13.3 Ethics and Good Clinical Practice

This study will be carried out in compliance with the protocol and Good Clinical Practice, as described in:

1. ICH Harmonized Tripartite Guidelines for Good Clinical Practice 1996.
2. US 21 Code of Federal Regulations dealing with clinical studies (including parts 50 and 56 concerning informed consent and IRB regulations).

3. Declaration of Helsinki, concerning medical research in humans (Recommendations Guiding Physicians in Biomedical Research Involving Human Subjects, Helsinki 1964, amended Tokyo 1975, Venice 1983, Hong Kong 1989, Somerset West 1996).

The investigator agrees to adhere to the instructions and procedures described in it and thereby to adhere to the principles of Good Clinical Practice that it conforms to.

13.4 Compliance with Trial Registration and Results Posting Requirements

Under the terms of the Food and Drug Administration Modernization Act (FDAMA) and the Food and Drug Administration Amendments Act (FDAAA), the sponsor-investigator of the trial is solely responsible for determining whether the trial and its results are subject to the requirements for submission to the Clinical Trials Data Bank, http://www.clinicaltrials.gov. Information posted will allow subjects to identify potentially appropriate trials for their disease conditions and pursue participation by calling a central contact number for further information on appropriate trial locations and study site contact information.

14. MULTI-CENTER GUIDELINES

14.1 Study Documentation

Each participating site is responsible for submitting copies of all relevant regulatory documentation to the Coordinating Center/Lead Site. The required documents include, but are not limited to the following: local IRB approvals (i.e., protocol, consent form, amendments, patient brochures and recruitment material, etc.), IRB membership rosters, summary of unanticipated problems or protocol deviations, and documentation of expertise of the investigators. The Coordinating Center will provide each participating site with a comprehensive list of the necessary documents. It is the responsibility of the participating sites to maintain copies of all documentation submitted to the Coordinating Center.

14.2 Records Retention

Following closure of the study, each participating center will maintain a copy of all site study records in a safe and secure location. The Coordinating Center will inform the investigator at each site at such time that the records may be destroyed.

14.3 Publication

It is understood that any manuscript or releases resulting from the collaborative research must be approved by the Protocol Chair and will be circulated to applicable participating sites/investigators prior to submission for publication or presentation.

Additionally, any publication of study data and results must conform to the publications policy as stated the Translational Breast Cancer Research Consortium's (TBCRC) "Policies and Procedures".

15. STATISTICAL CONSIDERATIONS

15.1 Study Design

15.1.1 Study Overview

QuantifyHER is a prospective, longitudinal cohort study of patients with metastatic breast cancer whose tumors express HER2 identified as IHC 1+ by pathologists' local assessment as per ASCO/CAP guidelines, and who are initiating therapy with T-DXd. See further eligibility criteria in Section 4. If a subject is included for the screen and then ultimately excluded, this subject will not be included in the N=200 sample size below.

15.1.2 Clinical Data Collection

A REDCap database will be constructed to maintain participant information. Clinical data will be abstracted from patient charts at participating sites by designated site personnel into electronic case report forms (CRFs), and will include baseline demographics, clinical and pathologic characteristics, treatment history, imaging report review, toxicity assessment as it relates to drug discontinuation, and real-world data for endpoint assessment.

15.1.3 Measurement of Effect

Since this is a real-world study without the use of RECIST for measuring tumor response, we will employ real-world response methodology[9-11] to derive real-world (rw) measures of efficacy, including response and progression, (rwORR, rwPFS) based on interpretation of treating clinicians' assessments (in office notes), radiology reports, pathology reports, and laboratory studies. Real-world response categories, as defined by Bartlett et al.[10] are listed in **Appendix A.**

These real-world methodologies have been validated against matched clinical trial cohorts and have been shown to be feasible, reliable and highly correlated with a Spearman's $\rho = 0.99$.[11] Clinical endpoints will be correlated with HER2 QIF and mRNA. Study personnel at each site will be trained to extract this data from electronic medical records and complete electronic CRFs for data collection. Please see sections 9 and 11 for further information.

15.2 Study Endpoints

15.2.1 Primary Endpoint

Association between quantitative HER2 and/or ERBB2 expression (as a continuous variable) and real-world objective response rate (rwORR)

15.2.2 Secondary Endpoint(s)

- Association between quantitative HER2 and/or ERBB2 expression (as a continuous variable) and rwPFS.
- Association between HER2 expression by quantitative immunofluorescence in HER2 IHC 1+ tumors and both rwORR and rwPFS, stratified by ER expression.
- ROC-based cut-points for HER2 QIF and/or mRNA levels that discriminate responders from non-responders by identifying the lowest levels that perform better than a random classifier.
- Association between combined mRNA + QIF and rwORR

15.3 Sample Size/ Accrual Rate

15.3.1 Sample Size: 200 evaluable patients.

With our full sample of 200 patients and a one-sided type-one error of 2.5%, we will have 80% power to detect a difference in proportions of 9 to 10 percentage points for each standard deviation increase in QIF or mRNA across the range of interest, such as 29% versus 20%, or 34.5% versus 25%. This corresponds to an odds ratio of approximately 1.6. Because there are no prior studies evaluating quantitative HER2 expression and response rates, we will structure this study as a two-stage trial, with a full sample size of N=200, and a non-binding interim review at N=70. The interim review will give us a preliminary look at biomarker distribution through descriptive statistics. We will also calculate conditional power to achieve our target odds ratio (OR=1.6). Power considerations are identical for the mRNA measure.

15.3.2 Length of Enrollment

We estimate that it will take approximately 2 years to enroll 200 evaluable patients.

15.3.3 Accrual rate

Planned 1-2 / month / institution.

15.4 Analysis of Primary Endpoint

For our primary objective (real world response rate, rwORR), we will test the hypothesis (H1) that there is a relationship between QIF measures of HER2 protein expression and/or ERBB2 mRNA levels as measured by dCt values in STRAT4 and rwORR, against the null hypothesis (H0) of no-relationship. We will analyze the data using logistic regression, with the primary outcome variable (rwORR) being binary, and with two separate continuous predictor variables (HER2 QIF value, and/or ERBB2 dCt). The hypothesis will be tested using the z-score corresponding to the odds-ratio, using a one-sided type-one error of 2.5%. Proportions such as rwORR

will be analyzed using logistic regression and tested using the z-value corresponding to the odds ratio.

15.5 Analysis of Secondary Endpoints

Time to event measures, to assess real-world PFS (rwPFS), will be analyzed using Kaplan-Meier methods and summarized as survival curves and time percentiles with 95% CI.

For assessment of the interplay between ER status and HER protein or ERBB2 mRNA quantitative assessment, we will summarize any association between HER2 QIF or ERBB2 dCt and ER expression as a Pearson Correlation with 95% CI.

If we find a relationship between QIF or ERBB2 by STRAT4 and rwORR, we will use ROC curve methodology to determine whether there is predictive potential in the model comparing AUC to a value of 50% (random prediction), and in turn identify the lowest level (threshold) QIF and ERBB2 dCT values that do better than random prediction in differentiating responders from non-responders. We will ultimately identify (estimate) the QIF values corresponding to multiple rwORR values (10%, 20%, 30% response) using the logistic model, with standard errors calculated using the delta method.

Because the logistic regression analysis presumes a linear response of the logit to our continuous biomarkers, we will explore any relationship between QIF or ERBB2 by STRAT4 and rwORR for evidence of non-linearity by examining binned Pearson residuals, and the Box-Tidwell transformation.

Lastly, we will conduct an analysis of rwORR using QIF and mRNA jointly, to determine whether a linear combination of the two values is better than either alone at discriminating responders from non-responders. We will use ROC in conjunction with cross-validation methods to assess the predictive performance of the model.

15.6 Reporting and Exclusions

15.6.1 Interim Review

Because this is the first study comparing HER2 QIF or ERBB2 mRNA levels against standard IHC for response to T-DXd, we will perform a non-binding interim review after enrolling the first 70 patients to assess biomarker distribution and test performance, allowing us to modify the sample size and inclusion criteria as needed. This interim review will give us a preliminary look at biomarker distribution through descriptive statistics. We will also calculate conditional power to achieve our target odds ratio (OR = 1.6), which would be the basis for any sample size re-estimation.

REFERENCES

1. Fernandez AI, Liu M, Bellizzi A, et al. Examination of Low ERBB2 Protein Expression in Breast Cancer Tissue. JAMA Oncol. 2022;8(4):607-610. doi:10.1001/JAMAONCOL.2021.7239

2. Shu L, Tong Y, Li Z, Chen X, Shen K. Can HER2 1+ Breast Cancer Be Considered as HER2-Low Tumor? A Comparison of Clinicopathological Features, Quantitative HER2 mRNA Levels, and Prognosis among HER2-Negative Breast Cancer. Cancers (Basel). 2022;14(17). doi:10.3390/CANCERS14174250

3. Modi S, Jacot W, Yamashita T, et al. Trastuzumab Deruxtecan in Previously Treated HER2-Low Advanced Breast Cancer. N Engl J Med. 2022;387(1):9-20. doi:10.1056/NEJMOA2203690/SUPPL_FILE/NEJMOA2203690_DATA-SHARING.PDF

4. Moutafi M, Robbins CJ, Yaghoobi V, et al. Quantitative measurement of HER2 expression to subclassify ERBB2 unamplified breast cancer. Lab Invest. 2022;102(10):1101-1108. doi:10.1038/S41374-022-00804-9

5. Erber R, Hartmann A, Fasching PA, Ruebner M, Stöhr R, Beckmann MW, Zentgraf M, Popp V, Weidler J, Simon I, Becker S, Huebner H, Fischer J, Guerini Rocco E, Viale G, Cayre A, Penault-Llorca F, Caniego Casas T, Pérez-Miés B, Palacios J, Jank P, Denkert C, Khoury L, Mairinger T, Ferrazzi F. Reproducibility of mRNA-Based Testing of ESR1, PGR, ERBB2, and MKI67 Expression in Invasive Breast Cancer: A Europe-Wide External Quality Assessment. Cancers (Basel). 2021 Sep 21;13(18):4718. doi: 10.3390/cancers13184718. PMID: 34572945

6. Wu NC, Wong W, Ho KE, et al. Comparison of central laboratory assessments of ER, PR, HER2, and Ki67 by IHC/FISH and the corresponding mRNAs (ESR1, PGR, ERBB2, and MKi67) by RT-qPCR on an automated, broadly deployed diagnostic platform. Breast Cancer Res Treat. 2018;172(2):327-338. doi:10.1007/S10549-018-4889-5

7. Wu NC, Wong W, Acca B, et al. A multicenter clinical study of Xpert® breast cancer STRAT4 demonstrates high concordance with central lab ER, PgR, HER2, and Ki67 IHC and HER2 FISH tests in FFPE breast tumor tissues. Presented at San Antonio Breast Cancer Symposium; Poster P2-03-03. Abstract available at https://www.sabcs.org/Portals/SABCS2016/Documents/SABCS-2017-Abstracts.pdf

8. Gupta S, Mani NR, Carvajal-Hausdorf DE, et al. Macrodissection prior to closed system RT-qPCR is not necessary for estrogen receptor and HER2 concordance

with IHC/FISH in breast cancer. Lab Invest. 2018;98(8):1076-1083. doi:10.1038/S41374-018-0064-1

9. DeMichele A, Cristofanilli M, Brufsky A, et al. Comparative effectiveness of first-line palbociclib plus letrozole versus letrozole alone for HR+/HER2− metastatic breast cancer in US real-world clinical practice. Breast Cancer Res. 2021;23(1):1-10. doi:10.1186/S13058-021-01409-8/TABLES/2

10. Bartlett CH, Mardekian J, Cotter MJ, et al. Concordance of real-world versus conventional progression-free survival from a phase 3 trial of endocrine therapy as first-line treatment for metastatic breast cancer. PLoS One. 2020;15(4):e0227256. doi:10.1371/JOURNAL.PONE.0227256

11. Ma X, Bellomo L, Magee K, et al. Characterization of a Real-World Response Variable and Comparison with RECIST-Based Response Rates from Clinical Trials in Advanced NSCLC. Adv Ther. 2021;38(4):1843-1859. doi:10.1007/S12325-021-01659-0/FIGURES/3

APPENDICES

APPENDIX A: Real-World Response Categories

Response Category	Definition
Complete response	Complete resolution of all visible disease
Partial response	Partial reduction in size of visible disease in some or all areas without any areas of increase in visible disease
Stable Disease	No change in overall size of visible disease (includes cases where some lesions increased, and some lesions decreased in size)
Progressive Disease	Increase in visible disease or presence of new lesions
Indeterminate/equivocal	Clinician specifically indicates that response is “indeterminate” or “uncertain” or if clinician’s interpretation of the scan(s) cannot be mapped to 1 of the above categories