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Unveiling Predictive Biomarkers for Antibody-Drug Conjugates for the Treatment of Breast Cancer

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1. ABSTRACT

Background: Trastuzumab deruxtecan (T-DXd) and trastuzumab emtansine (T-DM1) are anti-HER2 antibody-drug conjugates (ADCs) which have shown robust efficacy for treating breast cancer. For both ADCs, however, we lack effective biomarkers to predict outcomes from pre-treatment samples.

Methods: To unveil predictive biomarkers for ADCs, we first evaluated 5-year outcomes in a prospective trial (ATEMPT) testing adjuvant T-DM1 in patients with stage I HER2+ breast cancer, and reviewed real-world outcomes with T-DXd for patients with metatatic breast cancer at two academic institutions. Moreover, we conducted a multi-omic assessment of HER2 and extensive translational analyses to predict outcomes with T-DM1 in ATEMPT and T-DXd in the real world.

Results: Among 383 patients with stage I HER2+ breast cancer receiving adjuvant T-DM1, the 5-year iDFS was 97.0% (95% CI, 95.2 to 98.7). For those patients with sufficient tissue for HER2DX testing (n = 187), 5-year outcomes differed according to HER2DX risk score, with significantly better RFI and iDFS among patients with HER2DX low-risk versus high-risk tumors. We also evaluated real-world outcomes among 191 patients with metastatic breast cancer. Herein, we demonstrate that T-DXd is associated with relevant real-world activity, with a time-to-next treatment of 9.1 months (range: 7.6 – 10.4) and an overall survival of 22.2 months (range: 17.1 – 25.2). The quantitative proteomic (High Sensitivity-HER2, Reverse Phase Protein Array), transcriptomic (HER2DX) and circulating tumor DNA (DNADX) assessment of HER2 on pre-T-DXd samples enabled a refined prediction of T-DXd efficacy, including in subgroup analyses of HER2-positive and HER2-negative breast cancer.

Conclusion: a multi-omic characterization of HER2 expression in pre-treatment tumor samples showed promise in predicting the efficacy of T-DM1 in an adjuvant trial and T-DXd in the real world. Further validation of the biomarkers included in this study is planned within three ongoing phase 2 clinical trials, and may lead in the future to a refined use of anti-HER2 ADCs in clinical practice.

2. INTRODUCTION

2.1 Antibody-Drug Conjugates for treating Breast Cancer

Breast cancer is the most common invasive cancer among women in the United States, with more than 310,000 cases estimated in 2024 [1]. Approximately 20% of breast cancers are HER2-positive; the remaining 80% are HER2-negative, categorized as either hormone receptor (HR)-positive/HER2-negative or triple-negative breast cancer (TNBC) [2]. Once metastatic, breast cancer is considered incurable, with resistance to systemic treatments representing a major clinical challenge [2].

Antibody-drug conjugates (ADCs) are one of the most effective classes of therapies for the treatment of breast cancer[3]. ADCs consist of a cytotoxic payload bound via a molecular linker to a monoclonal antibody specific to a tumor-associated surface antigen.[3] The structure of ADCs enables enhanced delivery to tumor cells of potent cytotoxic molecules while retaining the antitumor effects of the antibody moiety derived from the engagement of its target. In this framework, the therapeutic effect of ADCs is linked both to the ability of the antibody to bind to its target on the cancer cell surface, as well as the ability of the cytotoxic payload to engage its intracellular target and inhibit cancer proliferation; molecular alterations that affect either of these interactions can reduce the efficacy of ADCs [3].

Within the last decade, three ADCs have been approved for treating breast cancer: trastuzumab emtansine (T-DM1), which carries a microtubule inhibitor payload (thus having microtubules as *payload target)*, and two ADCs carrying cytotoxic molecules that inhibit topoisomerase I (TOPO1; *payload target*); all three ADCs have demonstrated survival improvement in patients with MBC, and T-DM1 has also shown ability to prevent recurrence in the early-stage setting. T-DM1 targets HER2 (*antibody target*) and showed to improve outcomes in HER2-positive metastatic breast cancer in the phase 3 EMILIA trial[4] and in HER2-positive early breast cancer with residual disease after neoadjuvant

treatment in the phase 3 KATHERINE trial[5].

Sacituzumab govitecan (SG) targets Trop2 (*antibody target*) and achieved positive results in the phase 3 ASCENT [6] and TROPiCS-02 trials [7], which enrolled patients with TNBC and HR-positive/HER2-negative MBC, respectively. Trastuzumab deruxtecan (T-DXd) targets HER2 (*antibody target*) and achieved positive results in the phase 3 DESTINY-Breast03 [8] and DESTINY-Breast04 [9] trials, which enrolled patients with HER2-positive and HER2-low MBC, respectively. Additionally, the anti-Trop2 ADC datopotamab deruxtecan (Dato-DXd) may also soon join the treatment arsenal for HR-positive MBC after meeting the primary endpoint of improved progression-free (PFS) compared with chemotherapy in the randomized phase 3 TROPION-Breast01 trial [10]. More than one hundred additional ADCs are currently in early- or late-stage development, representing one of the areas of fastest growth in breast oncology.

Importantly, the choice of the ADC to use in patients with HER2-negative metastatic breast cancer remains challenging, since T-DXd and SG have never been compared head-to-head, and that no validated biomarker is available to guide treatment decisions. Similarly, no validated biomarker exists for patient selection for T-DM1 apart from HER2-positivity, leading to uncertainty on its use in certain clinical settings, such as in the post-T-DXd setting or in patients with heterogenous HER2 expression.

2.2Novel quantitative assays to predict the activity of ADCs

Given the mechanism of action of ADCs, attempts to predict their clinical activity have mostly focused on measuring the expression of the antibody target. However, the immunohistochemical (IHC) expression of Trop2 or HER2 failed to identify responders in phase 3 trials[8,9,11-13], possibly due to limitations of IHC, a semi-quantitative, subjective assay, dependent on poorly controlled variables[14]. Of note, the development of novel assays able to investigate the biology of breast cancers and quantify

target expression harbors the promise of adequately predicting response to ADCs.

Importantly, no actionable biomarker of intrinsic or acquired resistance to ADCs has emerged from any of the registrational trials, although small translational efforts have suggested multifactorial mechanisms of resistance to TOPO1 ADCs. Resistance to T-DM1 has been suggested to be linked to dysfunctional intracellular metabolism of the construct and subversion of DM1-mediated cell killing.[15] Similarly, mechanisms of resistance to T-DXd and SG appear to be multifactorial. In the DAISY phase 2 trial, most patients had a decrease in HER2 expression after treatment with T-DXd[16], suggesting a role of the ADC target in generation of resistance. However, such a decrease in HER2 may also be an epiphenomenon, not associated with resistance to T-DXd, since this agent is also active in HER2-low/ultralow tumors; moreover a preclinical study found that cancer cells exposed to T-DXd became resistant to other TOPO1 inhibitors, suggesting payload-related mechanisms of resistance to the same agent [17]. Lastly, genomic analyses from tumor lesions of a patient experiencing progression on SG revealed the emergence of mutations in both the *TOP1* gene (TOPO1; *payload target*) and the *TACSTD2* gene (Trop2; *antibody target*)[18], and emergence of *TOP1* mutations have been also observed on circulating tumor DNA collected after progression on TOPO1 ADCs[19]. Together, the abovementioned results reinforce the multifactorial nature of mechanisms of resistance to ADCs, warranting multi-omic analyses to be uncovered.

2.3 Uncovering response and resistance biomarkers for ADCs: correlative analyses of ATEMPT and analysis of samples from real-world patients with metastatic breast cancer receiving ADCs

A key opportunity to uncover biomarkers of response and resistance to ADCs was offered by the conduction of the ATEMPT randomized phase 2 trial at the Dana-Farber Cancer Institute (DFCI). This was a prospective, randomized clinical trial aimed at demonstrating that the administration of adjuvant T-DM1 for one year may be associated with favorable outcomes and a better profile of tolerability compared to traditional treatment with paclitaxel and trastuzumab (TH) among patients with surgically resected, stage I, HER2-positive breast cancer. I had the opportunity to contribute to the study and to leverage the tumor samples collected from the patients participating in the studies conducting extensive correlative analyses aimed at investigating biomarkers of efficacy and resistance to T-DM1.

Concomitantly, to develop biomarkers for TOPO1 ADCs (T-DXd and SG), I worked on the translational analysis of tissue samples collected from patients with metastatic breast cancer treated at DFCI within the last 5 years, with extensive molecular profiling that was enabled by multiple translational collaborations with DFCI investigators and external institutions.

3. METHODS

3.1 Prediction of T-DM1 efficacy: 5-year update of the ATEMPT trial and multiomic translational analyses

ATEMPT Randomized Trial: Study Design

The phase II ATEMPT trial was designed with two main objectives: to test the activity of one year of adjuvant T-DM1 and to compare its toxicity to the TH regimen in patients with stage I centrally confirmed HER2-positive breast tumors.

The study was conducted through the Translational Breast Cancer Research Consortium (TBCRC), a collaborative group founded in 2005 to conduct innovative and high-impact clinical trials for breast cancer, with a strong translational perspective. Twenty-four institutions across the United States were involved and accrued patients to the trial. Patient trial eligibility included age ≥ 18 years, a performance status ≥ 1 according to the Eastern Cooperative Oncology Group (ECOG) criteria, a left ventricular ejection fraction (LVEF) ≥ 50%, no history of prior breast cancer, and be within 90 days of their most recent breast surgery. Tumor requirements included a diagnosis of pathologic stage I, HER2-positive (per ASCO/College of American Pathologists 2013 guidelines) invasive breast cancer that was node-negative (N0) or had ≤ 1 lymph node with micrometastasis (N1mi). Patients were stratified by age (< 55 vs ≥ 55 years old), planned use of radiation therapy (yes or no), and planned use of endocrine therapy (yes or no) and were randomly assigned in a 3:1 ratio to receive T-DM1 or TH, respectively (**Figure 1**).

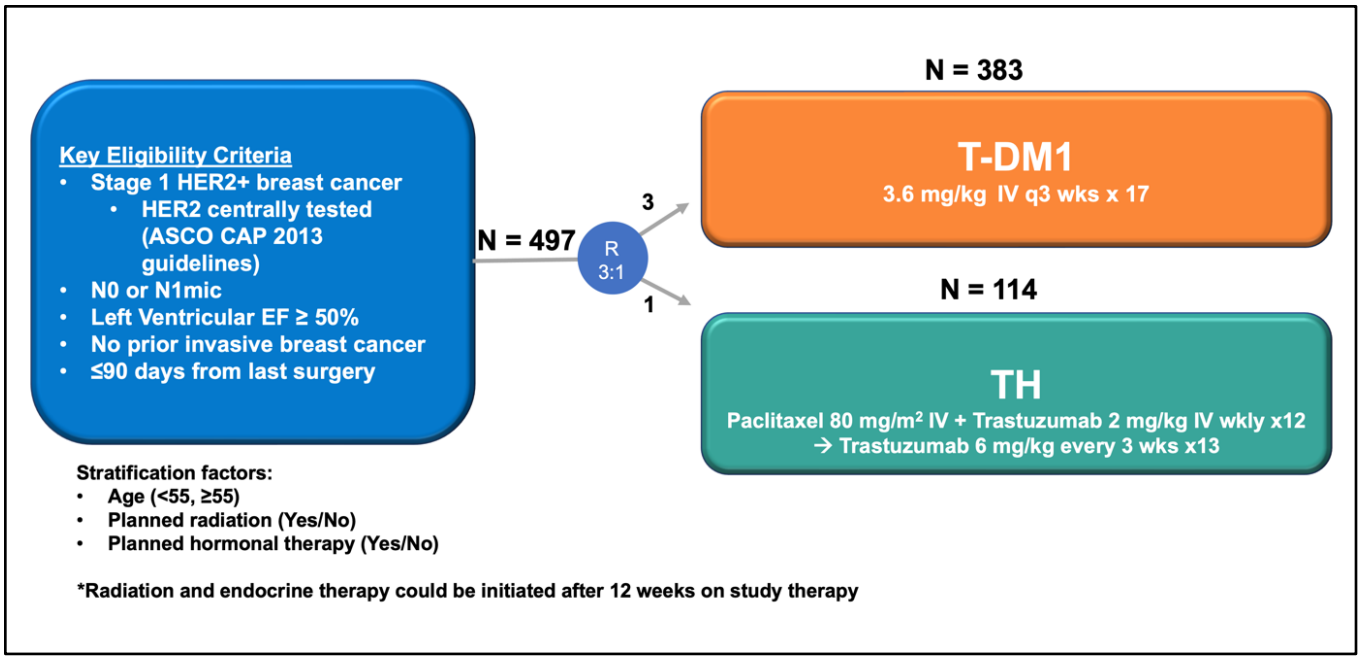

Figure 1 – Design of the randomized phase 2 ATEMPT trial.

Patients enrolled in the T-DM1 arm were administered intravenous T-DM1 at standard dose (3.6 mg/kg) on day 1 of each 21-day cycle for a total of 17 cycles or 1 year of duration. Patients enrolled in the TH arm received intravenous paclitaxel 80 mg/m2 weekly for 12 weeks, combined with concurrent intravenous trastuzumab, administered at a loading dose of 4 mg/kg followed by 2 mg/kg. Once the 12 weeks of paclitaxel and trastuzumab was completed, patients received intravenous trastuzumab at a dose of 6 mg/kg every 21 days to complete one year (13 cycles). After completing the assigned study treatment, patients enrolled in the study were followed for recurrence, and those discontinuing treatment for toxicities remained on study and were monitored for resolution of toxicity and recurrence. Tissue samples for correlative analyses were collected at baseline (i.e. primary breast tumor, before initiation of T-DM1 treatment) and at disease recurrence, if feasible and safe.

HER2DX testing

The standardized HER2DX genomic test[20] was performed at Hospital Clinic of Barcelona (Spain) on 225 primary tumor samples from patients enrolled in ATEMPT, and was successful for 187 samples with enough invasive component (T-DM1 arm: 147, TH arm: 40).

The pre-established HER2DX risk score cut-off was used to create HER2DX risk groups (low [0-50]) and high [50-100]).[20] The association between HER2DX risk groups and survival outcomes was assessed among all patients treated in ATEMPT. Genomic analyses were performed blinded from clinical outcome data.

Machine-learning analysis of HER2 expression

PathAI's ML-based HER2 algorithms were deployed on whole slide images of the HER2-stained slides from the ATEMPT clinical trial. These ML algorithms are developed using annotations from PathAI's network of expert pathologists to train models capable of extracting human-interpretable features (HIFs).[21] The HER2 Cell model detects cancer cells and classifies the HER2 stain intensity of each cell. Slide-level HIFs, such as cell counts and tissue area proportion, are derived from the model output across a slide. We calculated the proportion of invasive cancer cells with each stain intensity and the circumferential completeness based on the ASCO/CAP 2018 guidelines.[22]

The distribution of each stain intensity proportion feature across cases was compared for multiple dataset splits of interest using the Mann-Whitney U test. The following data splits were tested for significant differences in the distribution across patients: (1) RFI event vs. no RFI event (RFI event n=11, no RFI event n=431); (2) Estrogen receptor (ER)-positive vs. ER-negative (ER-positive n=318, ER-negative n=124); (3) Progesterone receptor (PR)-positive vs. PR-negative (PR-positive n=239, PR-negative n=203).

The heterogeneity in stain intensity was quantified by calculating the entropy of the distribution

across stain intensities across one slide[23] and compared between the 3 dataset splits listed above using the Mann-Whitney U test. All reported p-values were corrected using the Benjamini-Hochberg method for false discovery rate (FDR) correction.

HER2 genetic heterogeneity

Central pathology evaluation of HER2 heterogeneity was performed as described previously[24] at the European Institute of Oncology (Milan, Italy) on 13 baseline samples from patients experiencing RFI events (7 ipsilateral recurrences, 5 distant recurrences, 1 breast-cancer related death) (cases) and 39 controls matched on study arm, tumor size and hormone receptor (HR) status. The study included 24 patients from the T-DM1 arm and 28 patients in the TH arm.

HER2 gene status was assessed by FISH using the HER2 IQFISH pharmDx assay. FISH-stained slides were evaluated at a Zeiss Axio Imager Z2 microscope, equipped with a Metafer 4 Metacyte software for automating microscopy. Each slide was evaluated visually and independently by two trained readers blinded to outcomes, counting at least 60 invasive tumor cells, and subsequently by the Metafer software counting an average number of approximately 300 invasive tumor cells. HER2 heterogeneity was defined as the existence of a population of tumor cells with HER2 amplification (i.e., HER2/CEP 17 ratio >2.0 or a gene copy number [GCN]>6) representing more than 5% but less than 50% of invasive tumor cells, following recommendations from dedicated guidelines.[25,26]

NanoString GeoMX Digital Spatial Profiling

FFPE tumor samples from 13 patients who experienced local ipsilateral (n=10) or contralateral (n=3) events with either T-DM1 (n=6) or TH (n=7) (cases) and 21 controls matched on study arm, tumor size and HR status were analyzed using the NanoString GeoMX Digital Spatial Profiler (DSP) as

described previously[27]. One hematoxylin and eosin-stained slide from each tumor sample was used to identify regions of interest. Each slide was stained with a multiplexed panel of protein antibodies (62 distinct proteins, Supplementary Methods). Differential expression analyses were performed using linear mixed effect models with relevant groups of interest (e.g., treatment, time point) included as the fixed effect and patient ID included as a random intercept to account for non-independence of patient-specific AOIs. Proteins were considered differentially expressed with a P-value<0.05 and absolute log2 fold change >0.5. Differential expression results were visualized in volcano plots. P-values were corrected for multiple testing using the Benjamini-Hochberg method for false discovery rate (FDR) correction. Interquartile range (IQR) of log_2 normalized HER2 expression was used to classify patients as HER2 heterogeneous (0.85 quartile).

3.2 Prediction of T-DXd efficacy: real-world effectiveness and multiomic translational analyses

Novel quantitative assays to evaluate ADC target expression

The availability of novel, quantitative assays to evaluate the expression of ADC targets provides the opportunity to improve the prediction of response and resistance to ADCs. These include the HS-HER2 assay developed by the Rimm Lab[14,28], the HER2DX assay from Reveal Genomics[29] and the Reverse-Phase Protein Array (RPPA) assay by Ignite Proteomics, among others[30].

Furthermore, evaluating circulating tumor DNA (ctDNA) from plasma provides a means of non-invasively characterizing the genomic landscape of tumors before treatment, on treatment, and after treatment with an ADC, unveiling potential genomic alterations that arise during treatment with ADCs [31].

The opportunity to utilize novel assays to unveil biomarkers of response and resistance to TOPO1 ADCs derives from a rapidly expanding cohort of patients treated with SG and T-DXd in clinical practice. All patients with MBC seen at DFCI are invited at their first visit to join the EMBRACE program[32]. As a component of EMBRACE, patients are asked to participate in the collection of archival tissue, clinical data, and blood samples for future research. Since its inception in 2009, the EMBRACE research cohort study (DFCI protocol #09-204) has enrolled 3,456 patients with MBC, and over 9,079 research blood samples have been collected. Additionally, as part of the EMBRACE program, 1,061 patients also have been enrolled on a dedicated protocol (DFCI protocol #05-246) allowing for the collection of fresh biopsies for research purposes.

Real-world T-DXd population

We retrospectively analyzed data for patients with MBC who received T-DXd at Dana-Farber Cancer Institute (DFCI) (Boston, MA) between July 1, 2017 and January 20, 2023 and Duke Cancer Institute (Durham, NC) between March 31, 2020 and April 30, 2022. The following clinical data were collected from electronic medical records: age at time of metastatic diagnosis, sex, race, stage at initial diagnosis, tumor histology, estrogen receptor (ER), progesterone receptor (PR), and HER2 status at primary diagnosis, initial metastatic diagnosis, and subsequent biopsy closest to the T-DXd initiation, number/type of disease sites at time of initial metastatic diagnosis, receipt of prior neoadjuvant and/or adjuvant therapy, and receipt/type of prior lines of therapy in the metastatic setting. The start and end date of T-DXd; reduction in dose of T-DXd; reason of discontinuation for T-DXd; toxicities with T-DXd and subsequent lines of therapy after T-DXd, if relevant, with start date, end date, and associated reason of discontinuation were also collected. Vital status along with date of last contact or death were documented.

HER2, ER, and PR expression levels were abstracted from pathology records. Patients were categorized as having HER2-positive disease if they had any biopsy testing HER2 IHC 3+ or ISH amplified at any timepoint. Patients classified as HER2-negative were further divided into HER2-low or HER2-0 based on the most recent biopsy prior to the start of T-DXd. Patients with a HER2 IHC score of 1+ or 2+ with negative ISH were classified as HER2-low, whereas patients with a HER2 IHC score of 0 were classified as HER2-0. Tumors were considered HR-positive if at least 1% of invasive tumor cells exhibited immunostaining for either ER or PR on the last biopsy before starting T-DXd.

We determined TTNT, OS, toxicities, TTNT based on changes in HER2 status, and TTNT with post-T-DXd regimens. OS was defined as time from the initiation of T-DXd treatment to death or censored at the last known vital status date. TTNT was defined as the time interval between the initiation of treatment with T-DXd and the initiation of a subsequent regimen or death or censored at the last known vital status date. Detailed review of the medical records was performed to determine the incidence, grade, management and outcomes of key toxicities with T-DXd.

High-Sensitivity HER2 (HS-HER2)

To determine quantitative HER2 protein, we leveraged an analytic assay based on protein concentrations in cell line standard, determine by mass spectrometry. This method is described previously.[14] In brief, cell line microarrays (CMA) composed of 5 cell lines (including JURKAT #HTB-152, BT20 #HTB-19, T47D #HTB-133, ZR-75-1 #CRL-1500 and BT483 #HTB-121 created by Array Science LLC) and patient tissue slides were offline baked at 60^{o}C for at least 1 hour prior to autostaining. One CMA is processed with every 9 patient tissue slides in each BOND slide tray. The Leica Bond Rx autostainer protocol is as follows; deparaffinization with BOND dewax solution (AR9222), antigen retrieval with BOND HIER epitope retrieval solution 2 (AR9640) at 97^{o}C for 20

minutes, blocking with ReadyProbes Endogenous HRP & AP blocking solution (R37629, Invitrogen) for 10 minutes and with BSA for 30 minutes, 1 hour- incubation with primary rabbit monoclonal HER2 antibody (clone 29D8, #2165, IgG, Cell Signaling) at optimal concentration of 1ug/ml mixed together with 1:100 concentration of pan-CK (Clones AE1/AE3, REF#M3515, Dako), amplification with Rabbit Envision+ System – HRP labelled polymer anti-Rabbit (K400311-2, Dako) mixed together with 1:100 dilution of a green-fluorescent Alexa Fluor 546 Goat-anti-Mouse IgG (H+L)Cross-Adsorbed Secondary Antibody (REF#A11003) for 1 hour, staining with 1:50 dilution of a red-fluorescent Tyramide Signal Amplification (TSA) Cyanin 5 (SAT705A001EA, Akoya Biosciences) for 10 minutes and nuclear staining with 1:500 dilution of a blue-fluorescent 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes. Subsequently, the excess DI water on each slide were wiped off carefully and coverslipped with ProLong Gold Antifade mounting reagent (P36930, Invitrogen).

The HS-HER2 assay was performed on 74 pre-T-DXd treatment samples which were 5 um sections from formalin-fixed, paraffin embedded (FFPE) tumors including 32 surgical biopsies and 42 resections. The control CMAs and pre-T-DXd samples were scanned at 20X magnification on the Rarecyte CyteFinder HT II multiplexed fluorescent imaging platform (RareCyte, serial number: HT-0452201). After the patient tissue slide is scanned, a pseudo-IHC image is generated and regions of interest (ROIs) of each biopsy are selected by a board-certified pathologist. Three cases were omitted from further analysis due to their insufficient or damaged tumor cells. Then, using the CMA standard (calibrated by liquid chromatography-tandem mass spectrometry (LC-MS/MS) of each cell line), the signal intensity in the ROI is converted to amol/mm^2.

We evaluated the association of HS-HER2 in the closest samples collected prior to T-DXd with time to next treatment (TTNT) and overall survival (OS) in continuous terms and by quartiles of HS-HER2. Cox proportional hazards models were utilized to estimate hazard ratios, and log-rank test p-

values were reported. On top of that, Kaplan-Meier method was used to calculate median estimates. Additionally, Weighted Cohen's kappa statistic was calculated to assess the agreement in HS-HER2 score between primary and metastatic samples for patients that had both samples.

Reverse Phase Protein Array (RPPA)

In total, 47 formalin-fixed, paraffin embedded (FFPE) tumor surgical biopsy specimens were available for RPPA analysis, three of which did not contain a sufficient quantity of tumor cells and were removed from further analysis. Specimens were serially sectioned at 8 μm and stored at 4°C for up to 292 days prior to microdissection.

Sections were stained with hematoxylin, followed by tumor epithelial cell enrichment (~5-10 um^2) via laser capture microdissection using an ArcturusXT laser capture microdissection system (Molecular Devices, LLC, San Jose, CA, USA). Samples were stored at -80°C prior to lysis. Samples were lysed by heating a solution of 225mM tris hydrochloride (Rockland Immunochemicals, Pottstown, PA, USA), 50 mM tris(2-carboxyethyl)phosphine (TCEP) (ThermoFisher, Waltham, MA, USA), 10% glycerol (FisherScientific, Hampton, NH, USA), and 4% SDS (FisherScientific) to 95°C for 20 min, followed by a 2 hour water bath incubation at 80°C, and then centrifuged for 15 min.

RPPA analysis was performed as previously described[33,34] in a Clinical Laboratory Improvement Amendments (CLIA)-certified and College of American Pathologists (CAP)- accredited laboratory. In short, lysates were diluted to a concentration of 250 ug/mL and stored at -80°C before printing. Samples were printed in triplicate at 9nL per spot onto nitrocellulose backed slides (Grace Biolabs, Bend, OR, USA) using a Quanterix 2470 arrayer (Quanterix, Billerica, MA, USA). Positive and negative control cell lysates, bovine serum album (BSA) standards, and analyte-specific calibrator curves were printed alongside samples for quality control purposes.

Prior to staining, nitrocellulose slides were pre-treated with ReBlot (MillliporeSigma, Rockville, MD, USA) and blocked with I-Block (Applied Biosystems, Waltham, MA, USA). Nitrocellulose slides were probed with the respective primary antibodies (EGFR T654, Abcam ab75986; EGFR Y1068, Cell Signaling Technology (CST) 3777; EGFR Y1173, CST 4407; HER2, Thermo Fisher Scientific MA5-14509; HER2 Y1248, Abcam ab201013; HER3 Y1289, CST 4791; SLFN11, CST 34858; TOPO1, Abcam ab109374; TROP2, CST 47866) for 30 minutes, followed by secondary antibody incubation using biotinylated goat anti-rabbit IgG (H+L) (BA1000, Vector Laboratories, Newark, CA, USA). Signal amplification and staining was performed using tyramide based avidin/biotin amplification, followed by a streptavidin-conjugated IRDye 800 CW (LI-COR, Lincoln, NE, USA). Negative control slides were stained in the absence of the primary antibody. Total protein deposition was measured using Fast Green FCF (FisherScientific). Slides were scanned using an InnoScan 710IR scanner (Innopsys, Carbonne, France) and spot intensities were measured using Mapix software (Innopsys).

Total protein content per sample was calculated using a BSA standard curve printed on the same slide. Background and negative subtracted intensity values were fit to an analyte-specific calibrator and total protein normalized. Antibodies used for immunostaining were validated for major band with appropriate molecular weight using western blotting as previously described.[33,35] We evaluated the association of different biomarkers (HER2, phosphoHER2, TOPO1, SLFN11, Trop2, HER3, and EGFR) with TTNT and OS in continuous terms and categorized by quartiles of each biomarkers. Cox proportional hazards models were utilized to estimate hazard ratios, and log-rank test p-values were reported. On top of that, Kaplan-Meier method was used to calculate median estimates.

HER2DX

RNA samples were extracted from 41 FFPE tumor tissue samples using the ReliaPrep FFPE Total RNA Miniprep System (Promega) following manufacturer's protocol. From FFPE RNA, the HER2DX standardized assay was centrally performed in Reveal Genomics (Barcelona, Spain) using the nCounter platform (NanoString Technologies, Seattle, WA, USA) as previously described.[29]

The HER2DX assay is based on 4 different gene comprising 27 genes, including the 14-gene immunoglobulin (IGG) module (i.e., *CD27, CD79A, HLA-C, IGJ, IGKC, IGL, IGLV3-25, IL2RG, CXCL8, LAX1, NTN3, PIM2, POU2AF1, TNFRSF17*), a 4-gene tumor cell proliferation signature (*EXO1, ASPM, NEK2, KIF23*), a 5-gene luminal differentiation signature (*BCL2, DNAJC12, AGR3, AFF3, ESR1*), and the 4-gene HER2 amplicon signature (*ERBB2, GRB7, STARD3, TCAP*). For each signature, the normalized gene expression was calculated for each patient. The HER2DX ERBB2 score was calculated based on the *ERBB2* mRNA levels. Pre-established cutoffs were used for each signature/variable. Missing data was not imputed. We evaluated the association of different biomarkers (HER2 amplicon, ERBB2 gene, IGG, luminal, and proliferation) with TTNT and OS in continuous terms and categorized by tertiles of each biomarker. Cox proportional hazards models were utilized to estimate hazard ratios, and log-rank test p-values were reported. On top of that, Kaplan-Meier method was used to calculate median estimates.

OncoPanel

OncoPanel is a targeted NGS assay that detects genomic alterations in tissue samples, including insertions, deletions, single-nucleotide variants, copy number alterations, and structural variants across a panel of 447 genes with evidence as drivers of cancer biology.[36,37] OncoPanel data was retrieved for patients that received clinical NGS testing during the course of their disease. Analyses were restricted to

patients with HER2-negative disease. We compared TTNT and OS among patients with presence vs. absence of *ERBB2* hemizygous deletions.

DNADX

A total of 81 pre-treatment and 59 post-treatment plasma samples collected from patients with MBC treated with T-DXd were shared with Reveal Genomics (Barcelona, Spain) for the purpose of DNADX analyses[38]. Of the pre-treatment samples, 66 (81.5%) were collected within 6 months before starting T-DXd and 15 (18.5%) were collected more than 6 months before T-DXd initiation.

cfDNA was obtained from 3 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (QIAGEN Inc.) according to the manufacturer's instructions and quantified with a Qubit dsDNA high-sensitivity assay kit and the Qubit 4.0 fluorometer (Life Technologies, Carlsbad, CA, USA). cfDNA was concentrated using SpeedVac to fulfil the requirements for library preparation. Library preparation was performed by ligating unique dual indexes (UDI) custom adapters to a minimum of 10 ng of the isolated cfDNA (10-50 ng dsDNA). More specifically, the fragment ends of cfDNA were blunted and 5' phosphorylated and, after that, 3' ends were A-tailed to favor adapter ligation. Adapters were 10 bp – UDI as recommended to mitigate errors introduced by index-hopping or switching in Illumina instruments with patterned flow cells, such as the NovaSeq 6000. Indexed libraries were quantified by qPCR using the KAPA Library Quantification Kit (Roche Sequencing Solutions), pooled, and sequenced in a NovaSeq 6000 Illumina at 0.5x mean coverage with read length of 2 x 150 bp. ShWGS was analyzed with hmmcopy_utils (https://github.com/shahcompbio/hmmcopy_utils) and ichorCNA v0.2.0 (https://github.com/broadinstitute/ichorCNA), with a bin size of 500kb and default parameters.[39]

A total of 150 DNA-based phenotypic signatures including the DNADX HER2 signature, and 5 DNADX subtypes (Clusters-0 [TF=0], -1 [copy number-low], -2 [luminal-like], -3 [basal-like] and -4

[proliferative]), were identified as previously described.[38,40] Briefly, DNA-sequencing segmentation files from CNVkit output (for tumor DNA) were first mapped to gene-level features. The signal of 519 DNA segments was calculated using the mean copy number score across genes within each segment. The coefficients of DNA segments for predicting gene signatures were obtained from Xia et al. DNA-based signature scores were calculated as the weighted average of DNA segment values for each sample: a final signature score was obtained by adding all values (i.e., coefficient of segment A x signal of segment A plus coefficient of segment B x signal of segment B)[40]. The 5 DNA-based subtypes or clusters were identified using a previously reported DNA-based subtype predictor, which is based on unsupervised analysis of tumor samples and the 150 DNA-based signatures.[38] For all samples with the 150 DNA-based signatures available, we calculated the Euclidean distances to the 4 centroids and assigned a cluster class to each sample based on the nearest centroid.

DNADX mutation calling

ctDNA was processed for library preparation using a custom hybridization-based capture panel targeting 124 genes with reported somatic mutations performed with Agilent SureSelectXT Low Input Target Enrichment System (Agilent Technologies, Inc). Indexed libraries were quantified by qPCR using the KAPA Library Quantification Kit (Roche Sequencing Solutions), pooled and sequenced in a HiSeq 2500 Illumina (2 x 100bp) at an average coverage of 500x. Frequent single nucleotide polymorphisms (SNPs) in the population were removed based on the gnomAD database (allele frequency ≤ 0.0001). Data was manually curated, and classification of identified variants was performed using publicly available databases (COSMIC, cBioPortal, ClinVar, VarSome, OncoKB).

4. RESULTS

4.1 Five-year outcomes and correlative analyses from ATEMPT

The ATEMPT randomized study enrolled 512 patients with stage I, HER2-positive breast cancer between May 17, 2013, and December 13, 2016. Overall, 384 patients were randomized to T-DM1, of which 383 received protocol treatment and one withdrew consent and did not receive the intervention. Baseline patient and tumor characteristics did not differ significantly between study arms. When comparing the HER2DX and no-HER2DX populations, no significant difference was observed in terms of age, sex, race, ethnicity, nodal status, grade and HR status, whereas significant differences were observed in terms of tumor size and HER2 immunohistochemical (IHC) distribution. (**Table 1**)

	Arm 1: T-DM1				**Arm 2: TH**			
	ITT (n = 383)	**No HER2DX (n=236)**	**HER2DX (n=147)**	**p-value**	**ITT (n = 114)**	**No HER2DX (n=74)**	**HER2DX (n=40)**	**p-value**
Age, Years								
Median (range)	56 (32-85)	55 (33-79)	57 (32-85)	0.14	55.5 (23-82)	56 (29-82)	53 (23-82)	0.64
Sex								
Female	378 (99%)	232 (98%)	146 (99%)	0.65	113 (99%)	74 (100%)	39 (98%)	0.35
Male	5 (1%)	4 (2%)	1 (1%)		1 (1%)	0 (0%)	1 (2%)	
Race								
White	327 (85%)	198 (84%)	129 (88%)	0.82	93 (82%)	61 (82%)	32 (80%)	0.29
Black or AA	21 (5%)	14 (6%)	7 (5%)		7 (6%)	6 (8%)	1 (2%)	
Asian	22 (6%)	14 (6%)	8 (5%)		4 (4%)	3 (4%)	1 (2%)	
More than one race	2 (1%)	2 (1%)	0 (0%)		1 (1%)	0 (0%)	1 (2%)	
Other	11 (3%)	8 (3%)	3 (2%)		9 (8%)	4 (5%)	5 (12%)	
Ethnicity								
Hispanic or Latino	11 (3%)	8 (3%)	3 (%)	0.60	1 (1%)	1 (1%)	0 (0%)	0.86
Non-Hispanic	352 (92%)	214 (91%)	138 (%)		97 (85%)	62 (84%)	35 (%)	
Unknown	20 (5%)	14 (6%)	6 (%)		16 (14%)	11 (15%)	5 (%)	
Tumor Size								
≤ 0.1 cm	4 (1%)	4 (2%)	0 (%)	0.03	7 (6%)	7 (9%)	0 (0%)	0.05
> 0.1-0.5	57 (15%)	40 (17%)	17 (12%)		13 (11%)	11 (15%)	2 (5%)	
> 0.5-1.0	127 (33%)	80 (34%)	47 (32%)		40 (35%)	26 (35%)	14 (35%)	
> 1.0-1.5	124 (32%)	64 (27%)	60 (41%)		29 (25%)	18 (24%)	11 (28%)	
> 1.5-2.0	71 (19%)	48 (20%)	23 (16%)		25 (22%)	12 (16%)	13 (32%)	
Nodal micrometastasis								
Yes	18 (5%)	13 (6%)	5 (3%)	0.46	1 (1%)	1 (1%)	0 (0%)	1

No	365 (95%)	223 (94%)	142 (97%)		113 (99%)	73 (99%)	40 (100%)	
Histologic Grade								
Well differentiated Moderately diff. Poorly differentiated Unknown	11 (3%) 148 (39%) 219 (57%) 5 (1%)	6 (3%) 92 (39%) 135 (57%) 3 (1%)	5 (3%) 56 (38%) 84 (57%) 2 (1%)	0.96	4 (4%) 46 (40%) 62 (54%) 2 (2%)	2 (3%) 34 (46%) 36 (49%) 2 (3%)	2 (5%) 12 (30%) 26 (65%) 0 (0%)	0.20
HER2 by IHC (by central testing)								
(0, 1+) (2+) (3+) Not done/unknown	5 (1%) 92 (24%) 277 (72%) 9 (2%)	5 (3%) 86 (36%) 137 (58%) 8 (2%)	0 (0%) 6 (4%) 140 (95%) 1 (1%)	<0.001	1 (1%) 25 (22%) 87 (76%) 1 (1%)	1 (1%) 25 (34%) 47 (64%) 1 (1%)	0 (0%) 0 (0%) 40 (100%) 0 (0%)	<0.001
Hormone receptor Status								
Positive Negative	289 (75%) 94 (25%)	186 (79%) 50 (21%)	103 (70%) 44 (30%)	0.07	84 (74%) 30 (26%)	52 (70%) 22 (30%)	32 (80%) 8 (20%)	0.37

Table 1. Patient and tumor characteristics by arm and receipt of HER2DX testing. No significant difference was observed between the HER2DX and non-HER2DX populations in terms of age, sex, race, ethnicity, nodal status, grade and HR status, whereas significant differences were observed in terms of tumor size and HER2 immunohistochemical (IHC) distribution. AA=African American.

After a median follow-up of 5.8 years, there were a total of 11 iDFS events in the T-DM1 arm, consistent with a 5-year iDFS of 97.0% (95% CI: 95.2-98.7%). The 5-year RFI observed with adjuvant T-DM1 was 98.3% (95% CI: 97.0-99.7%), the 5-year OS was 97.8% (95% CI: 96.3-99.3%) and the 5-year Breast-Cancer Specific Survival (BCSS) was 99.4% (95% CI: 98.6-100%). (**Figure 2**)

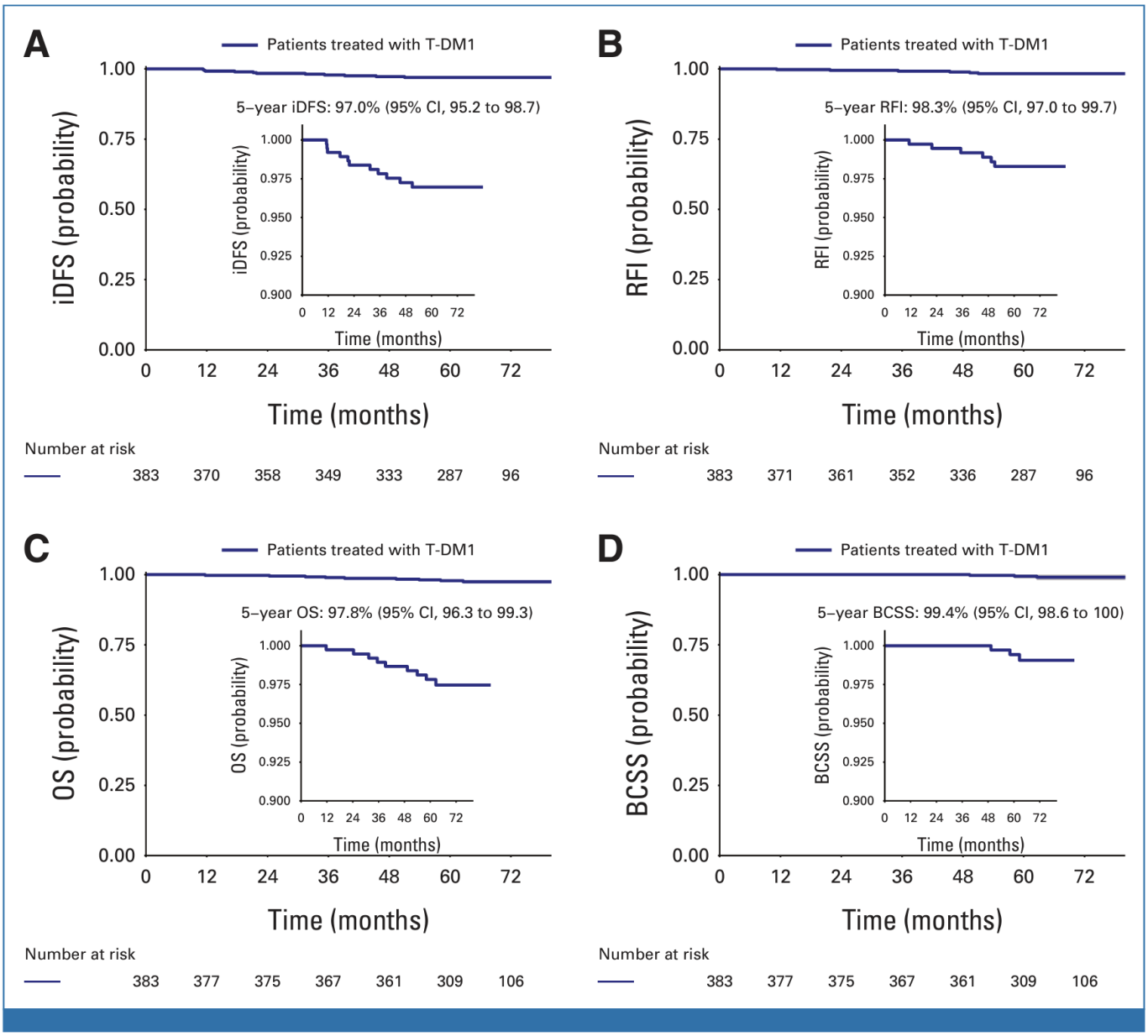

Figure 2. Survival outcomes in the T-DM1 arm of ATEMPT in terms of (A) iDFS, (B) RFI, (C) OS, and (D) BCSS.

Survival outcomes in the T-DM1 arm were comparable, regardless of the tumor size, hormone receptor (HR) status of the tumor and receipt of more or less than 6 months of T-DM1 (**Figure 3**). The 5-year iDFS was 98.7% (95% CI: 97.0-100%) for patients having tumors <1 cm, and 95.6% (95% CI: 92.9-98.5%) tumors ≥1 cm. Similarly, 5-year iDFS was 96.8% (95% CI: 94.7-98.7%) for patients with

HR-positive tumors, and 97.6% (95% CI: 94.4-100%) for patients with HR-negative tumors. (**Figure 3**).

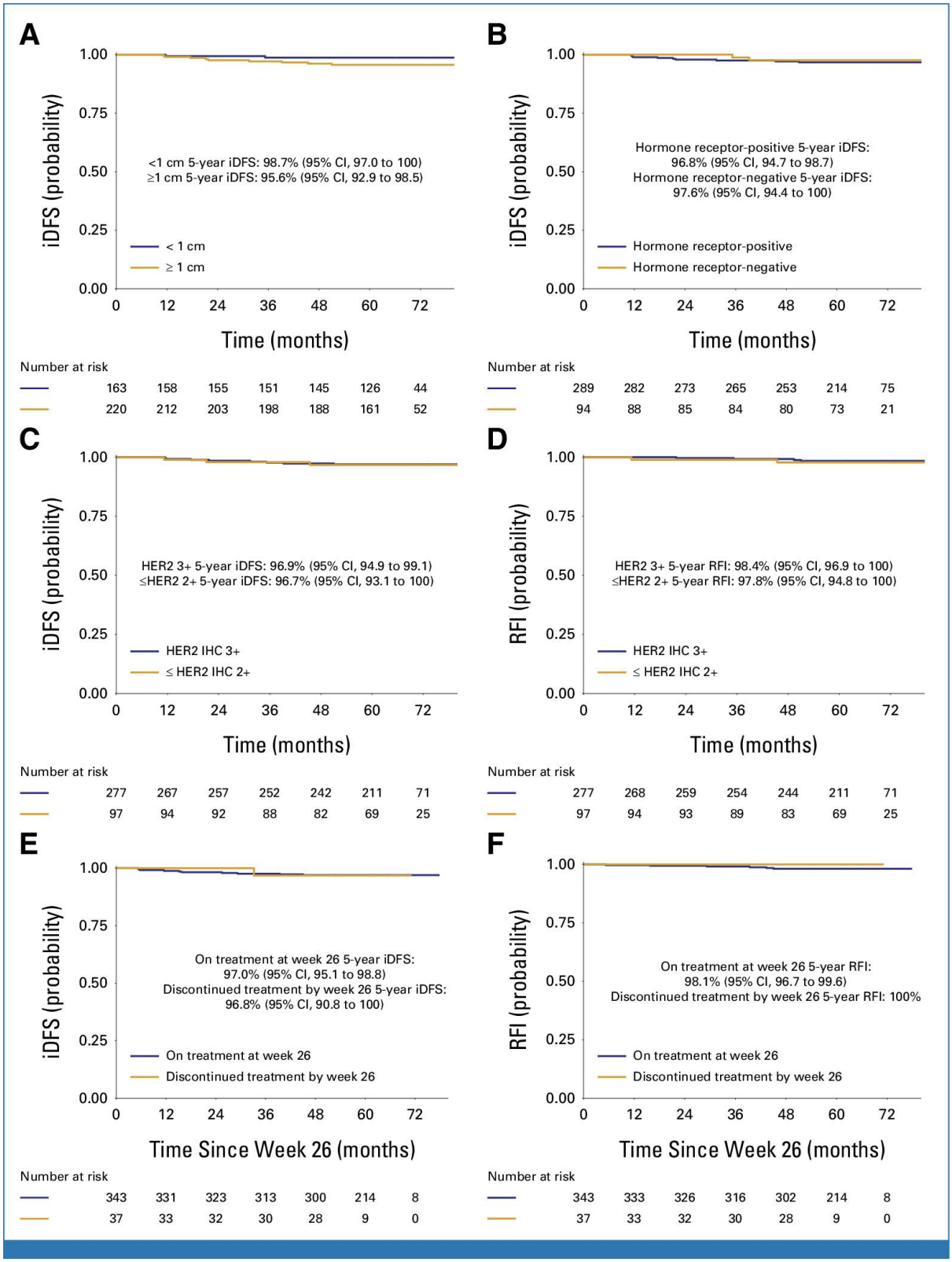

Figure 3. Outcomes in the T-DM1 arm by tumor size ((A) iDFS), hormone receptor status ((B) iDFS), HER2 IHC ((C) iDFS, (D) RFI), and duration of exposure to T-DM1 ((E) iDFS, (F)RFI).

Among the iDFS events observed with T-DM1, only three were distant recurrences, with the remaining events being two ipsilateral recurrences (one of which HER2-negative), three new contralateral primary breast cancers (all HER2-negative) and three non-breast-cancer related deaths. (**Table 2**). Although the study was not powered to evaluate the efficacy of adjuvant TH, the 5-year iDFS in this arm was 91.3% (95% CI: 86.0-96.9%) with 9 iDFS events observed, including two distant events. The 5-year RFI was 93.2% (95% CI: 88.5-98.2%), the 5-year OS was 97.9% (95% CI: 95.2-100%) and the 5-year BCSS was 99.0% (97.2-100%). (**Table 2**; **Figure 4**).

	T-DM1 arm (n = 383)		**TH arm (n = 114)**	
iDFS Events	**n (%)**	**Time to Event (months)**	**n (%)**	**Time to iDFS Event (months)**
Any recurrence or any death	11		9	
Local/Regional Recurrence				
Ipsilateral breast (HER2+)	1	35	5	18, 31, 32, 33, 47
Ipsilateral breast (HER2-)	1	11	0	
New Contralateral Primary Breast Cancer				
HER2+	0		0	
HER2-	3	11, 18, 21	1	23
Distant Recurrence	3	22, 45, 51	2	12, 30
Death				
Non-breast cancer related*	3	12, 31, 39	1	59

Table 2. Five-year survival outcomes and types of iDFS events in each study arm. *Deaths due to: diabetic coma, stroke, Creutzfeldt-Jakob disease, coronary artery disease/diabetes

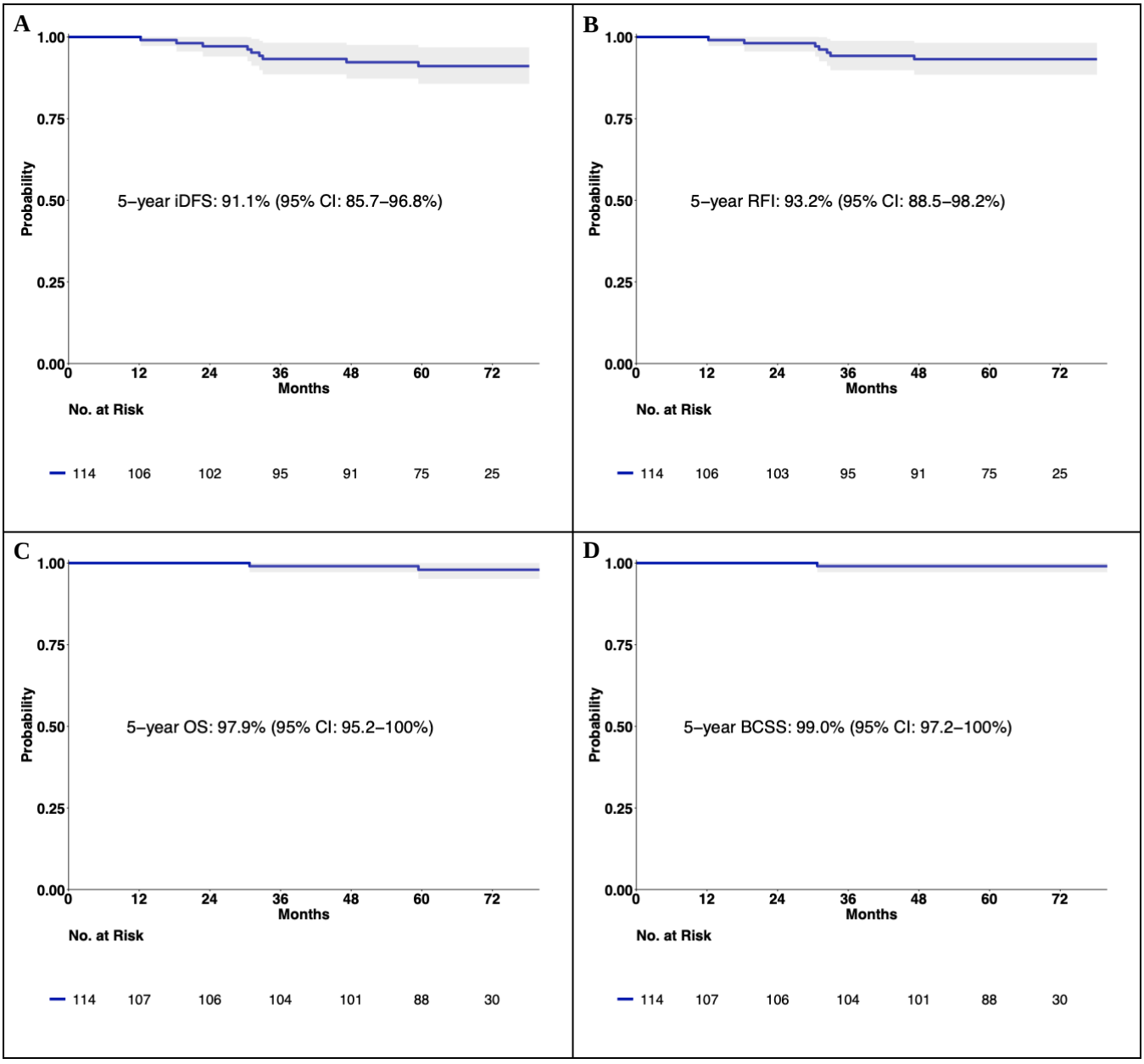

Figure 4. Five-year iDFS (A), RFI (B), OS (C) and BCSS (D) among patients with stage I HER2-positive breast cancer receiving adjuvant paclitaxel and trastuzumab in ATEMPT.

4.2 Results of HER2DX testing and association with five-year outcomes in ATEMPT

The standardized HER2DX genomic test was obtained for 187 (37.6%) patients enrolled in ATEMPT with enough available tissue (147 receiving T-DM1, 40 receiving TH). Baseline characteristics for the patients included in this study were comparable to those of the overall study cohort (**Table 1**). The 5-year iDFS and 5-year RFI in the cohort of patients with available HER2DX testing were 95.4% (92.3-98.6%) and 97.0% (94.5-99.6%), respectively.

The proportion of patients with high expression of luminal, proliferation and IgG signatures was 34.8%, 57.8% and 48.1%, respectively, and 133 patients (71.1%) had high HER2 expression (**Figure 5A**). The proportion of patients with HER2DX low-risk disease was 93.6% (n=175), while 6.4% (n=12) had HER2DX high-risk disease.

Patients with HER2DX low-risk disease were found to have significantly more favorable RFI compared with patients having HER2DX high-risk disease (5-year 98.1% vs 81.8%, hazard ratio 0.10 [0.02-0.57], p=0.01, **Figure 5D**) as well as better iDFS (5-year 96.3% vs 81.8%, hazard ratio 0.20 [0.04-0.98], p=0.047) (**Figure 5B-C**).

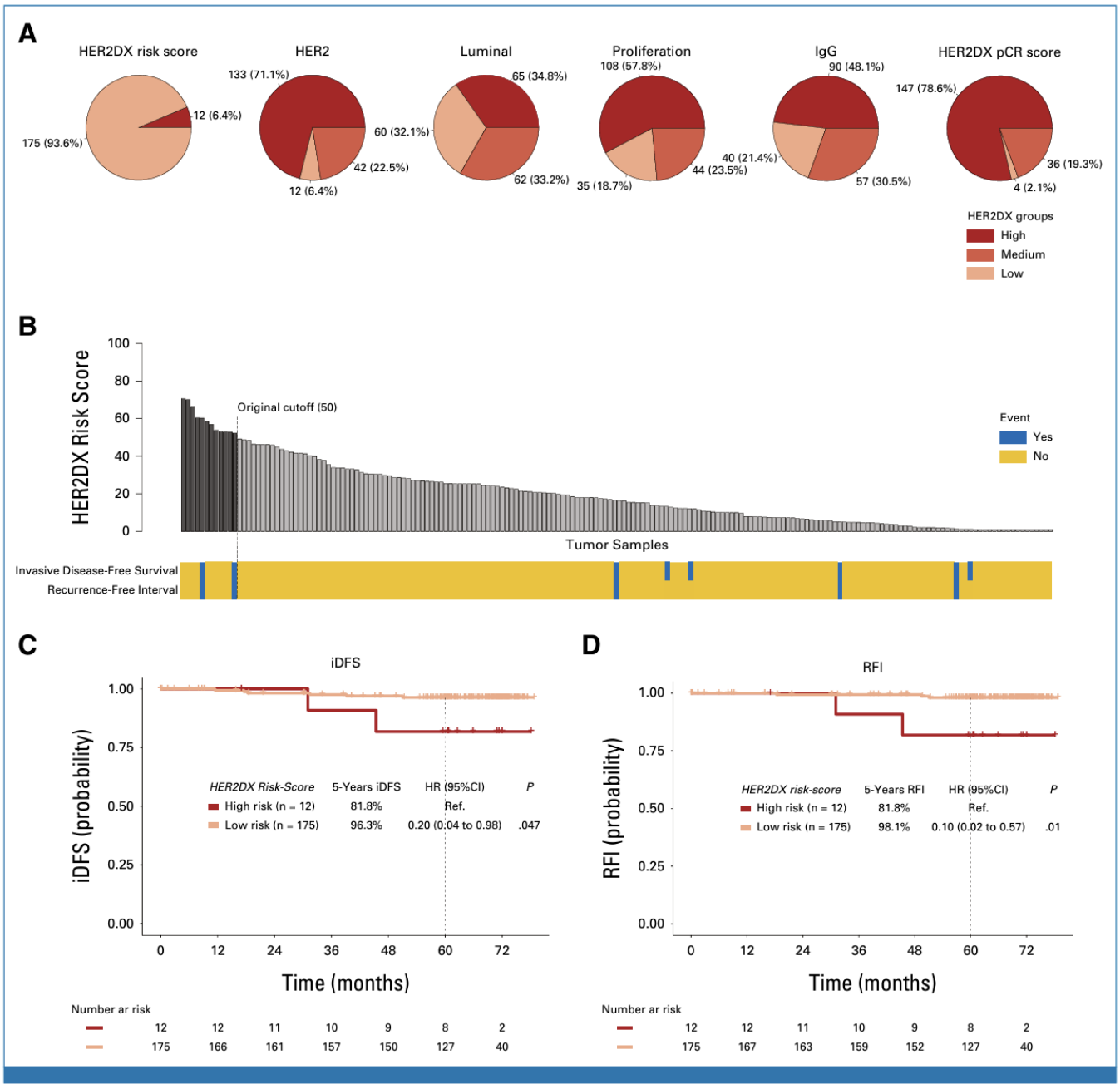

Figure 5. A. Distribution of the HER2DX risk score, pCR score and main biological features captured by HER2DX, including the expression of HER2 mRNA, the luminal signature, the proliferation signature, and the IgG signature. B. HER2DX risk score ranking and association with invasive disease-free survival events and recurrence-free interval events. C-D. 5-year invasive disease-free survival (C) and 5-year recurrence free survival (D), stratified by HER2DX risk group.

Multiomic evaluation of HER2 heterogeneity in ATEMPT

A wide range of variability in HER2 staining intensity was observed at a cancer cell level within and across the tumors (**Figure 6A**), with variability in both ER-positive and negative cases (**Figure 6B**).

HR-positive tumors were found associated with a higher proportion of tumor cells expressing weak-to-moderate and incomplete membrane staining (ER: $p=0.002$; PR: $p<0.001$), whereas HR-negative tumors were associated with a higher proportion of tumor cells with intense and complete circumferential staining for HER2 (ER: $p<0.001$; PR: $p<0.001$, **Figure 6C-D**). However, none of the HER2 stain intensity proportion features were significantly associated with the risk of recurrence among patients enrolled in ATEMPT (**Figure 6E**), although this analysis had limited power.

The entropy of HER2 stain intensity distributions across each slide was evaluated as a measure of HER2 heterogeneity. Examples of tumors exhibiting high entropy (entropy=1.55, i.e., high HER2 heterogeneity) and low entropy (entropy=0.39, i.e., low HER2 heterogeneity) are shown in **Figure 6F**. Numerically higher entropy in HER2 staining distribution was observed among ER-positive vs. ER-negative cases, as well as PR-positive vs. PR-negative cases, though neither effect was significant (ER: $U=21972$, $p=0.062$; PR: $U=26866$, $p=0.051$; **Figure 6G-H**). No significant difference in entropy distribution was seen between patients experiencing or not experiencing recurrence, either in the overall study cohort ($U=2649$, $p=0.221$) (**Figure 6I**), or when evaluating each arm separately (T-DM1 arm: $U=1209$, $p=0.086$; TH arm: $U=247$, $p=0.982$) (**Figure 6L**).

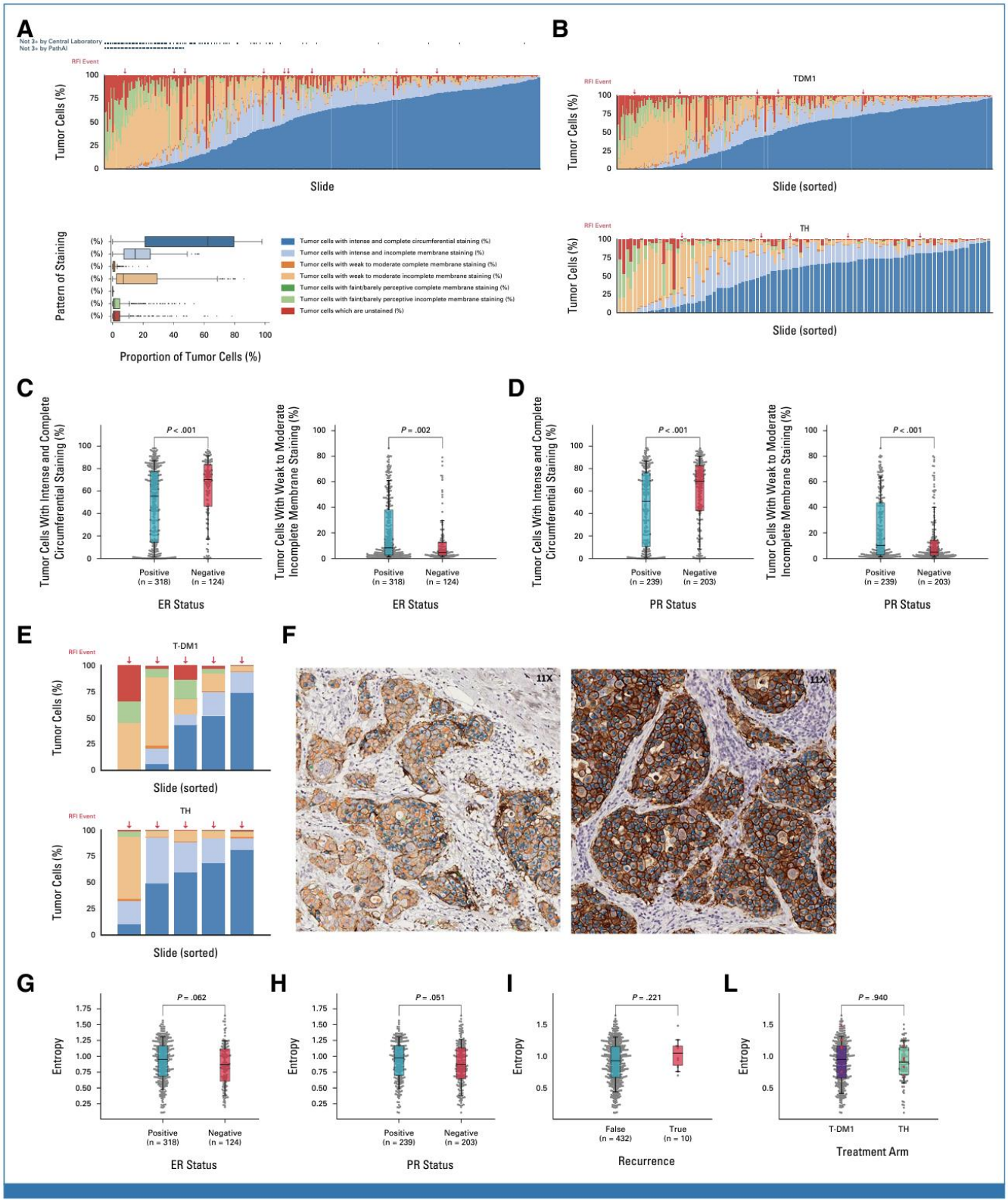

Figure 6. Machine learning assisted analysis of HER2 expression patterns. A-B. Machine learning-derived HER2 stain intensity variability in the overall population (A) and by ER status (B). C-D.

Percentage of tumor cells exhibiting intense and complete HER2 staining or weak and incomplete HER2 staining depending on ER expression (C) and PR expression (D). E. Distribution of tumor cells with different HER2 staining intensities among patients experiencing RFI events in each study arm. F. Examples of tumors exhibiting high entropy (left panel, entropy=1.55, i.e., high HER2 heterogeneity) and low entropy (right panel, entropy=0.39, i.e., low HER2 heterogeneity). G-L. Entropy distribution (i.e., level of HER2 heterogeneity) according to the ER expression (G) and PR expression of the tumor (H), according to the recurrence status overall (I) and depending on the study arm (L).

A case-control study was conducted to characterize HER2 heterogeneity via single-cell FISH on primary tumors of 52 patients with evaluable tissue enrolled in ATEMPT, including 13 patients experiencing RFI events and 39 matched controls.

Fifty-one of the 52 samples showed HER2 gene amplification according to the ASCO/CAP guidelines.[22] Visual and Metafer-assisted evaluations were highly concordant (Kendall's Tau: 0.93 for both HER2/CEP17 ratio and GCN) with mean ratio values of 5.44 (min 1.66, max 9.93) and 5.37 (1.64-9.79) respectively, and a mean GCN of 11.11 and 10.82. Intratumoral genetic heterogeneity was encountered in 4 of the 51 amplified cases (**Figure 7A-D**); HER2 IHC was 2+ or 3+ in all but 8 of the 51 amplified cases (7 were 1+, 1 non-evaluable).

No significant association was observed between HER2 genetic heterogeneity and recurrence (7.7% of patients had HER2 genetic heterogeneity among both those with and without recurrence, p=1) (**Figure 7A**). Among patients receiving T-DM1, 16.7% of those that experienced recurrence and 5.6% without recurrence had HER2 genetic heterogeneity (p=0.45) (**Figure 7B**).

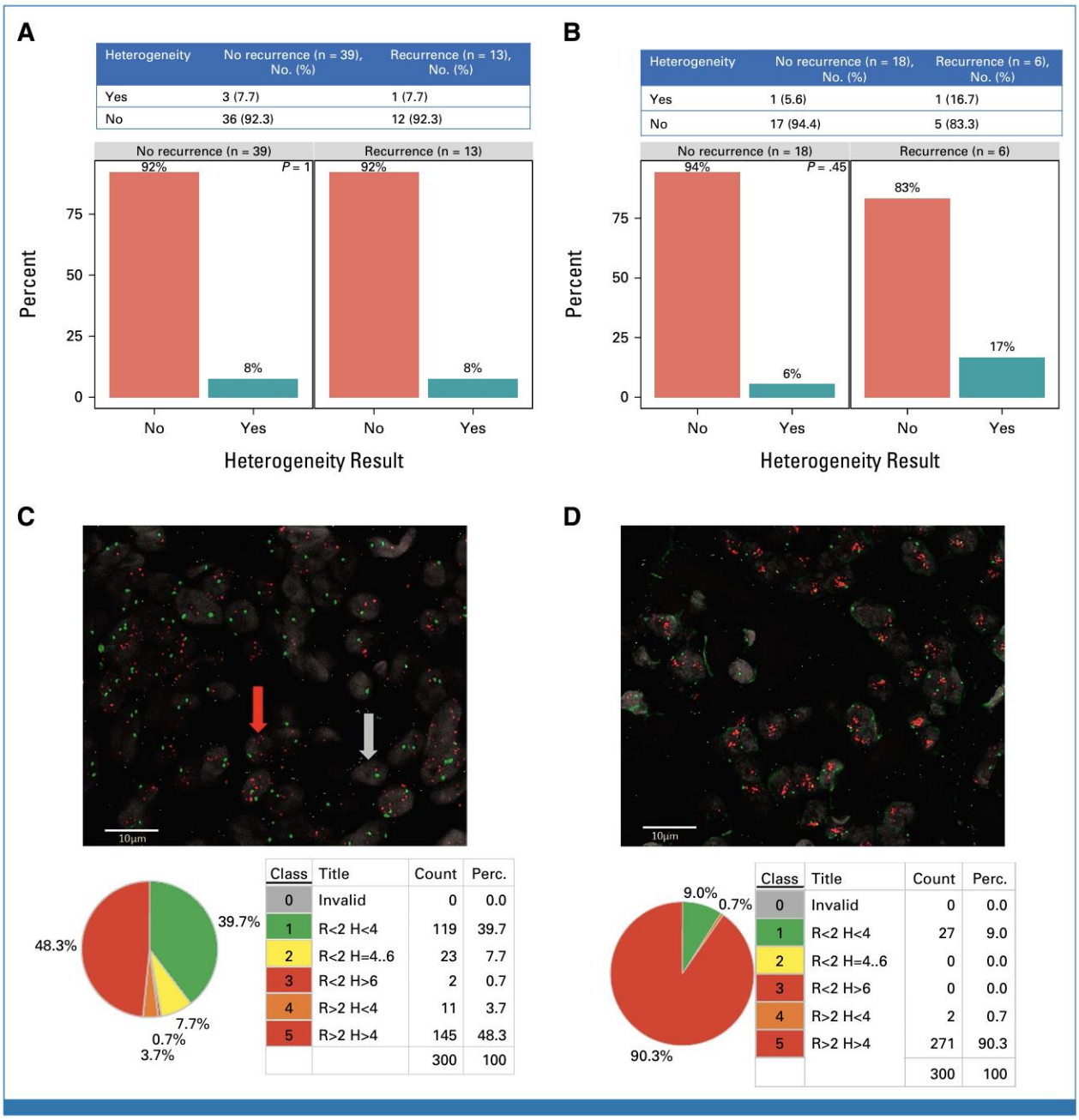

Figure 7. Characterization of HER2 genetic heterogeneity through single-cell FISH. A-B. Rates of recurrence by presence of absence of HER2 genetic heterogeneity in the overall case-control study population (A) and only among patients receiving adjuvant T-DM1 (B). C. Example of HER2 heterogenous case with dissection in populations of cells (in terms of absolute numbers and percentage) having different HER2 ratios and copy numbers; D. Example of HER2 non-heterogenous case with dissection in populations of cells (in terms of absolute numbers and percentage) having different HER2 ratios and copy numbers.

To investigate prognostic biomarkers among the patients enrolled in ATEMPT, spatial proteomic analyses were conducted on tumor samples of 13 patients experiencing ipsilateral or contralateral events (cases) in the trial and 21 matched controls.

Differential expression analyses between primary tumors of cases and controls revealed four up-regulated and nine down-regulated proteins ($P<0.05$, $|\log2 \text{ fold change}| >0.5$). Proteins found significantly upregulated among cases included NF1 ($\log_2FC=3.2$, $p=3.2\times10^{-9}$, p-value adjusted for FDR [q]$=1\times10^{-7}$), CTLA4 ($\log_2FC=2.5$, $P=1.3\times10^{-7}$, $q=2.7\times10^{-6}$) and CD20 ($\log_2FC=0.6$, $P=2\times10^{-6}$, $q=3\times10^{-5}$), whereas Cleaved Caspase 9 ($\log_2FC=-2.5$, $P=8.5\times10^{-14}$, $q=5.3\times10^{-12}$), CD25 ($\log_2FC=-1.0$, $P=0.00033$, $q=0.004$), ICOS ($\log_2FC=-.91$, $P=0.0026$, $q=0.023$), GITR ($\log_2FC=-.79$, $P=0.0025$, $q=0.023$) and GZMB ($\log_2FC=-0.8$, $P=0.004$, $q=0.031$) were found significantly upregulated in controls. (**Figure 8A**).

Further comparing primary tumor samples from cases to recurrence samples revealed an upregulation of NF1 ($\log_2FC=-2.0$, $P=0.00089$, $q=0.014$), CTLA4 ($\log_2FC=-2.2$, $P=0.00029$, $q=0.0084$) and CD20 ($\log_2FC=-.65$, $P=0.0004$, $q=0.0084$) among the primary tumors, whereas an upregulation of Cleaved Caspase 9 ($\log_2FC=2.5$, $P=0.0000066$, $q=0.00041$) was observed in the recurrences (**Figure 8B**).

Of the 6 patients classified as HER2 heterogeneous by spatial proteomic analysis, only one was from a primary tumor of a patient experiencing recurrence, whereas the remaining five were from controls (**Figure 8C**). Differential expression analyses between HER2 heterogenous (n=6, 17.1%) and non-heterogenous tumors (n=29, 82.9%) revealed slight down-regulation of NY-ESO-1 ($\log_2FC=-0.73$, $P=0.029$, $q=0.89$) and Fibronectin ($\log_2FC=-0.81$, $P=0.04$, $q=0.89$) in HER2-heterogeneous cases, though neither remained significant after FDR correction (**Figure 8D**).

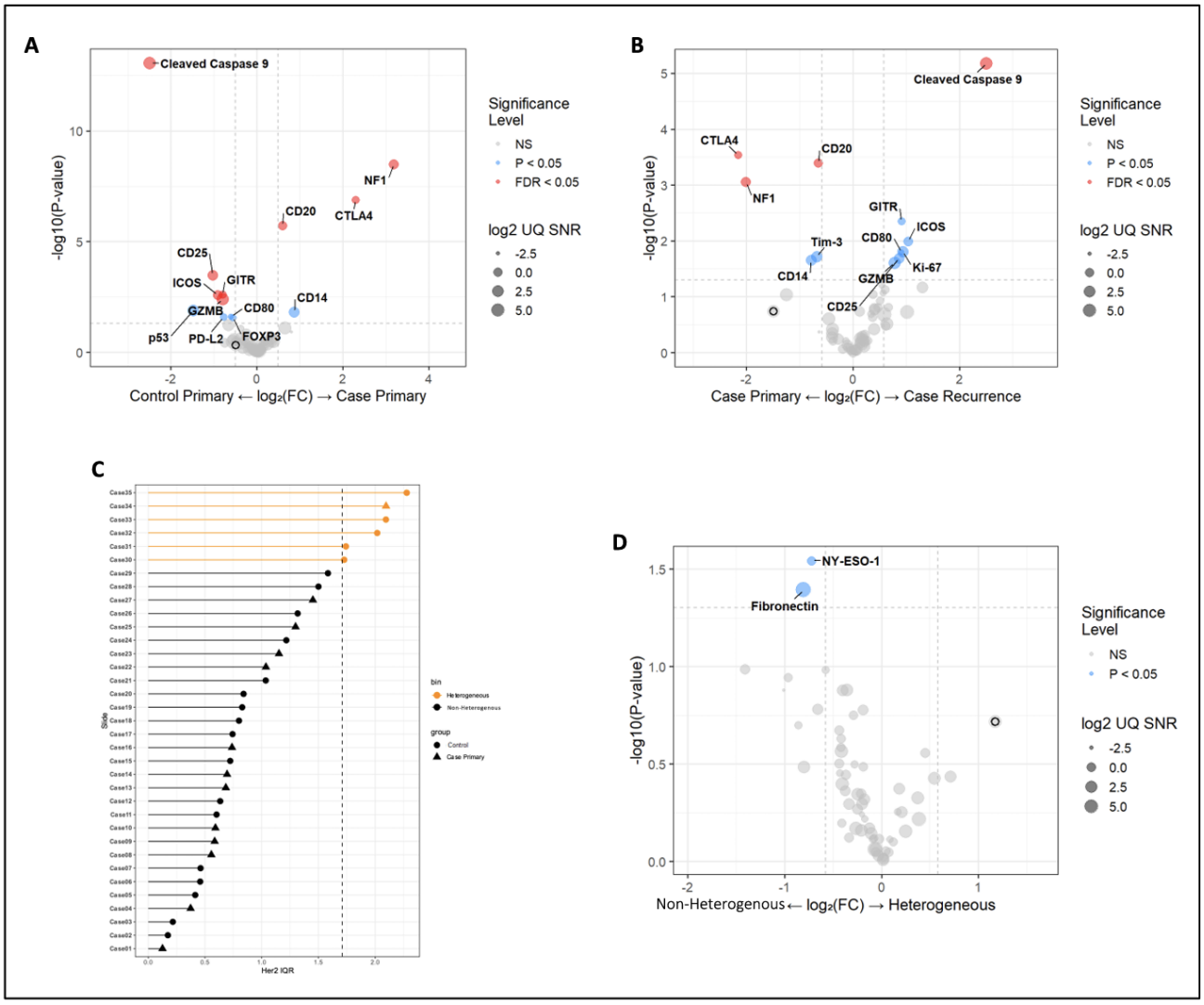

Figure 8. Spatial proteomic analyses in ATEMPT. A-B. Differential proteomic expression between primary tumors of cases and controls (A) or primary tumors of cases and matched local recurrences or contralateral tumors (B). C. Distribution of HER2 IQR values for primary tumors of cases and controls. Patients with HER2 IQR in the 0.85 quantile were considered heterogeneous (orange line color). The point shape distinguishes controls (circles) from cases (triangles). D. Differential proteomic expression between HER2 heterogenous and non-heterogenous samples

4.3 Real-world analysis of outcomes with T-DXd

As previously mentioned, T-DXd is a novel generation ADC that has demonstrated improved outcomes compared with T-DM1 in a phase 3 trial[41], leading to its second-line implementation in HER2-positive metastatic breast cancer, which has been followed by implementation in a much wider population of patients with HER2-low breast cancer, based on the results of the DESTINY-Breast04 trial.[9] To unveil predictive biomarkers of T-DXd efficacy, we conducted a real-world study and multiple translational analyses leveraging samples collected from patients receiving treatment with T-DXd for metastatic breast cancer.

A total of 191 patients were included in the real-world analysis of outcomes with T-DXd. Demographic and clinicopathologic characteristics for the study population are described in **Table 3**. The median age at metastatic diagnosis was 50.9 years (range: 21.4 – 78.0). Most patients were White (84%), and roughly one quarter (26%) were diagnosed with *de novo* stage IV breast cancer, while the remainder experienced metastatic recurrence after an initial diagnosis of early-stage disease

At the initiation of T-DXd therapy, 126 patients (66.0%) had HER2-positive disease, 44 (23.0%) had HER2-low disease, and 21 (11.0%) had HER2-0 disease. In addition, 57.1% of patients had hormone receptor-positive (HR-positive) disease and 42.4% had HR-negative disease. Patients had received a median of four prior lines of therapy for MBC, including a median of two lines of chemotherapy.

	HER2-zero (n = 21)	**HER2-low** (n = 44)	**HER2+** (n = 126)	**Total** (n = 191)
Median age at metastatic diagnosis, years (range)	57.5 (25.4 – 76.5)	52.6 (27.6 – 70.1)	48.3 (21.4 – 78.0)	50.9 (21.4 – 78.0)
Sex				
Female	21 (100%)	44 (100%)	124 (98.4%)	189 (99.0%)
Male	0 (0%)	0 (0%)	2 (1.6%)	2 (1.0%)
Time from metastatic diagnosis to initiation of T-DXd, months (range)	40 (11 – 110)	45 (2 – 185)	37.9 (12 – 256)	40 (12 – 256)
Median number of metastatic sites at diagnosis (range)	4 (1 – 7)	4 (1 – 7)	3 (0 – 9)	3 (0 – 9)
Hormone receptor status at initiation of T-DXd				
HR+	12 (57.1%)	30 (68.2%)	67 (53.2%)	109 (57.1%)
HR-	9 (42.9%)	14 (31.8%)	58 (46.0%)	81 (42.4%)
Not Done	0 (0%)	0 (0%)	1 (0.8%)	1 (0.5%)
HER2 status at diagnosis of metastatic disease				
HER2-positive	0 (0%)	0 (0%)	108 (85.7%)	108 (56.5%)
HER2-low	5 (23.8%)	25 (56.8%)	9 (7.1%)	39 (20.4%)
HER2-zero	16 (76.2%)	19 (43.2%)	7 (5.6%)	42 (22.0%)
Not Done	0 (0%)	0 (0%)	2 (1.6%)	2 (1.0%)
Prior lines of treatment in the advanced setting, median (range)	4 (3 – 8)	4.5 (0 – 12)	4 (0 – 16)	4 (0 – 16)
Prior lines of chemotherapy, median (range)	2 (1 – 4)	2 (0 – 6)	2 (0 – 9)	2 (0 – 9)
Prior lines of endocrine therapy, median (range)	1 (0 – 4)	2 (0 – 5)	0 (0 – 5)	0 (0 – 5)

Table 3. Description of the real-world T-DXd patient population by HER2 status at the time of initiation of T-DXd treatment.

Real-world Treatment Outcomes with T-DXd by static and dynamic HER2 status

The median follow-up was 16.6 months from the initiation of T-DXd treatment (interquartile range [IQR]: 8.8 – 29.5). At last follow-up, 55 patients remained on T-DXd (28.8%), 108 stopped T-DXd due to disease progression (56.5%), and 28 stopped T-DXd due to toxicity (14.7%).

The TTNT among the overall cohort was 9.1 months (range: 7.6 – 10.4), and the overall survival (OS) was 22.2 months (range: 17.1 – 25.2). The TTNT varied significantly by HER2 status of the disease, ranging from 10.4 months (95% confidence interval [CI]: 8.9 – 13.2) in patients with HER2-

positive disease; to 7.6 months (95% confidence interval [CI]: 5.3 – 10.2) in patients with HER2-low disease and 3.7 months (95% CI: 3.7 – 5.8) in patients with HER2-0 disease (**Figure 9A**; $p < 0.0001$). Similar results were observed based on breast cancer clinical subtypes at time of T-DXd initiation (**Figure 9B**; $p < 0.0001$).

Next, we evaluated TTNT by change in HER2-low status between the primary breast tumor and the latest metastatic biopsy performed prior to T-DXd. The TTNT was 3.0 months (95% CI: 1.7 – 5.6) for patients who switched from HER2-low to HER2-0, 5.6 months (95% CI: 4.4 – 15.8) among patients who switched from HER2-0 to HER2-low, and 9.4 months (95% CI: 4.4 – 13.5) for patients who maintained stable HER2-low disease between the two timepoints (**Figure 9C**; $p=0.006$). Conversely, TTNT did not vary based on change in HER2 status between the first and the latest biopsy of metastatic disease performed prior to T-DXd (**Figure 9D**; $p = 0.4119$).

OS after starting T-DXd differed significantly depending on the HER2 status of the disease. Patients with HER2-positive disease had a median OS of 23.1 months (95% CI: 18.6 – 27.2), followed by 14.2 months for HER2-low (95% CI: 9.4 – 25.5) and 9.5 months for HER2-0 MBC (95% CI: 3.7 – 28.2) (**Figure 9E**; $p = 0.0039$). When patients were classified by breast cancer subtype, the longest OS were observed among those with HR-positive/HER2-negative and HER2-positive disease, while patients with TNBC experienced the shortest OS (**Figure 9F**; $p = 0.0032$).

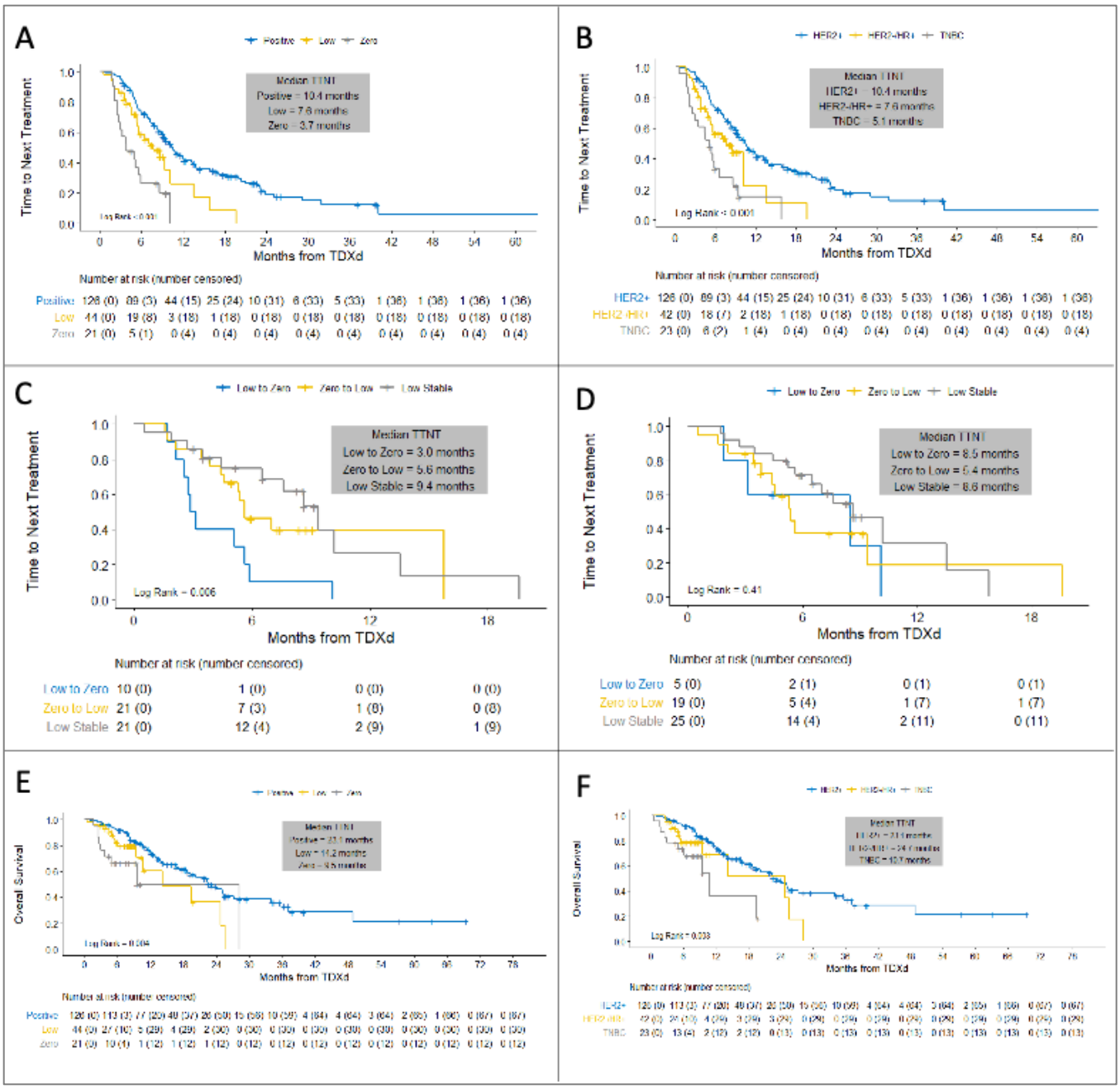

Figure 9. Real-world activity of T-DXd in patients with metastatic breast cancer (n=191). A. TTNT with T-DXd by HER2 status of the disease; B. TTNT with T-DXd by clinical subtype of the disease; C. TTNT with T-DXd depending on changes in HER2 status (low vs. 0) between the primary tumor and last metastatic biopsy; D. TTNT with T-DXd depending on changes in HER2 status (low vs. 0) between the first and last metastatic biopsy; E. OS with T-DXd by HER2 status of the disease; F. TTNT of treatments administered immediately after T-DXd.

Outcomes with treatments administered immediately after T-DXd

When evaluating the TTNT of treatments administered immediately after T-DXd, results did not vary significantly by HER2 status or breast cancer subtype. Median TTNT with post-TDXd regimens was 4.0 months for patients with HER2-positive disease, 3.1 months for patients with HER2-low disease and 4.3 months for patients with HER2-0 disease (p=0.63).

Real-world safety of T-DXd

A total of 61 patients (31.9%) required T-DXd dose reductions during treatment, most commonly for fatigue (n=28, 14.7%), nausea/vomiting (n=19, 9.9%), or hematologic toxicity (n=12, 6.3%). Two thirds of the patients required one level of dose reduction, whereas one third required 2 levels of dose reduction (**Table 4**)

Interstitial lung disease (ILD) was diagnosed in 22 patients (11.5%) during treatment with T-DXd, among whom 6.2% had grade 1 (G1), 3.1% had G2, 1.6% had G3, and 0.5% had G4. There were no cases of fatal (G5) ILD. ILD occurred after a median of 8 months from initiation of T-DXd treatment and resolved in 63.6% of cases, with a median time to resolution of 4 months (2 months for G1 events) (**Table 4**)

Five patients (2.6%) developed cardiotoxicity, defined as a decrease in left ventricular ejection fraction (LVEF) ≥ 10% from baseline to a value < 50%. Four out of five patients that developed cardiotoxicity with T-DXd had received prior treatment with anthracyclines. (**Table 4**)

	Total *n (%)*
Starting Dose of T-DXd	
3.2 mg/kg	10 (5.2%)
4.4 mg/kg	12 (6.3%)

	Total *n (%)*
5.4 mg/kg	150 (78.5%)
6.4 mg/kg	9 (4.7%)
Unknown	10 (5.2%)
Required Dose Reduction during the course of treatment	
Yes	61 (31.9%)
No	117 (61.3%)
Unknown	13 (6.8%)
Levels of dose reduction required	
1 level	41 (67.2%)
2 levels	19 (31.1%)
Unknown	1 (1.6%)
Reason for Dose Reduction	
Fatigue	28 (45.9%)
Hematalogical Toxicity	12 (19.7%)
Nausea/Vomiting	19 (31.1%)
Neuropathy	3 (4.9%)
Pulmonary Toxicity	6 (9.8%)
Cardiac Toxicity	1 (1.6%)
abnormal LFTs	1 (1.6%)
colitis , diarrhea	1 (1.6%)
diarrhea, hepatic failure, acute kidney	1 (1.6%)
mucositis	1 (1.6%)
stomatitis, diarrhea	1 (1.6%)
weakness, renal insufficiency	1 (1.6%)
Other	14 (23.0%)
Cardiotoxicity Occurrence	
Yes	5 (2.6%)
No	178 (93.2%)
Unknown, outside records not available	8 (4.2%)
INCIDENCE OF CHARACTERIZATION OF ILD	
ILD Occurrence	
Yes	22 (11.5%)
No	160 (83.8%)
Unknown	9 (4.7%)
Received steroid treatment for ILD	
Yes	18 (81.8%)
No	4 (18.2%)

	Total *n (%)*
Time from T-DXd Start to ILD onset (in Months) [Median (Min, Max)]	8 (1, 51)
Distribution of ILD Grade	
Grade 1	12 (54.5%)
Grade 2	6 (27.3%)
Grade 3	3 (13.6%)
Grade 4	1 (4.5%)
Type of ILD Diagnosis	
Clinical and radiological	12 (54.5%)
Radiological only	10 (45.5%)
Treatment received for ILD	
Steroids	19 (86.4%)
Antibiotics	7 (31.8%)
Oxygen Therapy	3 (13.6%)
ILD Resolved	
Yes	14 (63.6%)
No	6 (27.3%)
Unknown	2 (9.1%)
Time to ILD Resolution (in Months) [Median (Min, Max)]	4 (1, 16)
Prior History of pulmonary comorbidities among patients with ILD	
Pneumonia	1 (4.5%)
Prior Asthma	1 (4.5%)
RSV Pneumonia	1 (4.5%)
diagnosed with COPD on pulmonary consult	1 (4.5%)
Smoking Status of patients with ILD	
Current/Former Smoker	7 (31.8%)
Not Smoker	13 (59.1%)
Unknown	2 (9.1%)
Pulmonary Consultation	
Yes (multiple)	10 (45.5%)
Yes (once)	6 (27.3%)
No	4 (18.2%)
Unknown	2 (9.1%)

Table 4. Dosing, dose reductions and toxicities experienced by patients receiving T-DXd

4.4 Prediction of T-DXd activity with novel multiomic biomarkers

Quantitative evaluation of HER2 with HS-HER2

The QuPathQymia method of quantitative immunofluorescence was used to determine HS-HER2 on tissue samples collected before the start of T-DXd (**Figure 10A**). Samples from 51 patients (32 with HER2-positive, 19 with HER2-negative MBC at time of T-DXd) were analyzed with HS-HER2. We analyzed the primary tumor in 20 patients (39.2%), a metastatic biopsy in 13 (25.5%), both in 18 patients (35.3%). (**Table 5**).

	HER2 Positive (N=32)	**HER2 Negative (N=19)**	**Total (N=51)**
Age at MetDx			
Median	49.2	53.1	51.4
Range	30.3 - 70.8	25.4 - 67.4	25.4 - 70.8
Time from MetDx to TDXD (Months)			
Median	42.0	32.0	34.0
Range	1.0 - 137.0	2.0 - 155.0	1.0 - 155.0
Count of Sites Involved at TDXD			
Median	3.0	3.0	3.0
Range	0.0 - 6.0	1.0 - 5.0	0.0 - 6.0
HR Status at TDXD			
Positive	14 (43.8%)	9 (47.4%)	23 (45.1%)
Negative	18 (56.2%)	10 (52.6%)	28 (54.9%)
HER2 status at diagnosis of metastatic disease			
HER2 Positive	27 (84.4%)	0 (0.0%)	27 (52.9%)
HER2 Low	3 (9.4%)	7 (36.8%)	10 (19.6%)
HER2 Negative	2 (6.2%)	12 (63.2%)	14 (27.5%)
HER2 status of the primary tumor			
HER2 Positive	24 (75.0%)	0 (0.0%)	24 (47.1%)
HER2 Low	1 (3.1%)	10 (52.6%)	11 (21.6%)
HER2 Negative	4 (12.5%)	8 (42.1%)	12 (23.5%)
Unknown	3 (9.4%)	1 (5.3%)	4 (7.8%)
Prior lines of treatment in the advanced setting			

	HER2 Positive (N=32)	HER2 Negative (N=19)	Total (N=51)
Median	5.0	4.0	5.0
Range	1.0 - 16.0	0.0 - 10.0	0.0 - 16.0
Prior lines of chemotherapy			
Median	2.5	2.0	2.0
Range	0.0 - 9.0	0.0 - 6.0	0.0 - 9.0
Prior lines of endocrine therapy			
Median	0.0	1.0	0.0
Range	0.0 - 3.0	0.0 - 3.0	0.0 - 3.0
Receipt of Sacituzumab Govitecan			
Yes, before T-DXd	6 (18.8%)	12 (63.2%)	18 (35.3%)
Yes, after T-DXd	3 (9.4%)	3 (15.8%)	6 (11.8%)
No	23 (71.9%)	4 (21.1%)	27 (52.9%)
HS-HER2 tumor type			
Both	10 (31.2%)	8 (42.1%)	18 (35.3%)
Primary only	13 (40.6%)	7 (36.8%)	20 (39.2%)
Metastasis only	9 (28.1%)	4 (21.1%)	13 (25.5%)
Time from Procedure Date to Start of TDXd (In Months)			
Median	33.6	23.6	31.3
Range	6.6 - 184.6	2.5 - 204.0	2.5 - 204.0

Table 5. Clinicopathologic characteristics of patients included in the HS-HER2 analysis

HS-HER2 was found to be continuously associated with TTNT (hazard ratio [HzR] per 5-unit increment 0.78 [95% CI: 0.69 – 0.88], p<0.001) and OS (HzR per 5-unit increment 0.79 [95% CI: 0.69 – 0.92], p=0.002) (**Table 6**). Additionally, there was a significant association between TTNT and HS-HER2 when divided into quartiles, with a trend of increasing median TTNT (**Figure 10B**, p<0.001) and OS (**Figure 10C**, p=0.011) with increasing HS-HER2 quartiles. Consistent findings were observed in sub-analyses of patients with HER2-positive MBC (**Figure 10D**, TTNT HzR per 5-unit increment 0.82 [95% CI: 0.71 – 0.94], p=0.005) and patients with HER2-negative MBC (TTNT HzR per 5-unit

increment 0.70 [95% CI: 0.53 – 0.94], p=0.017).

A total of 17 patients had both primary tumor and metastatic samples undergoing HS-HER2 testing. The weighted Cohen's k of HS-HER2 score agreement between primary and metastatic samples was 0.32 (confidence boundary 0.031 – 0.60), denoting fair agreement.

Among the 51 patients included in the HS-HER2 analysis, the standard HER2 IHC subtypes (HER2-positive, HER2-low, HER2-0) sub-optimally predicted outcomes, with the numerically longest median TTNT observed for patients with HER2-low disease, followed by HER2-positive and HER2-0 (**Figure 10E**).

	Group	**Median TTNT (month)**	**95% CI**	**HR**	**95% CI**	**p-value**
Continuous (amol/mm2)	1 unit increase	-	-	0.95	0.93-0.97	<0.001
	5 unit increase	-	-	0.78	0.69-0.88	<0.001
Median	< median (ref)	4.67	2.87-5.37			
	>= median	7.68	5.23-15.77	0.31	0.16-0.59	<0.001
Quartile						(0.002)
	<=25% (ref)	3.70	2.73-NA			
	>25%-50%	5.07	2.1-NA	0.61	0.27-1.37	0.23
	>50%-75%	6.98	4.37-NA	0.28	0.11-0.68	0.005
	>75%	11.67	5.83-NA	0.17	0.07-0.44	<0.001

Table 6. Association between HS-HER2 (as continuous variable, median or quartiles) and TTNT with T-DXd

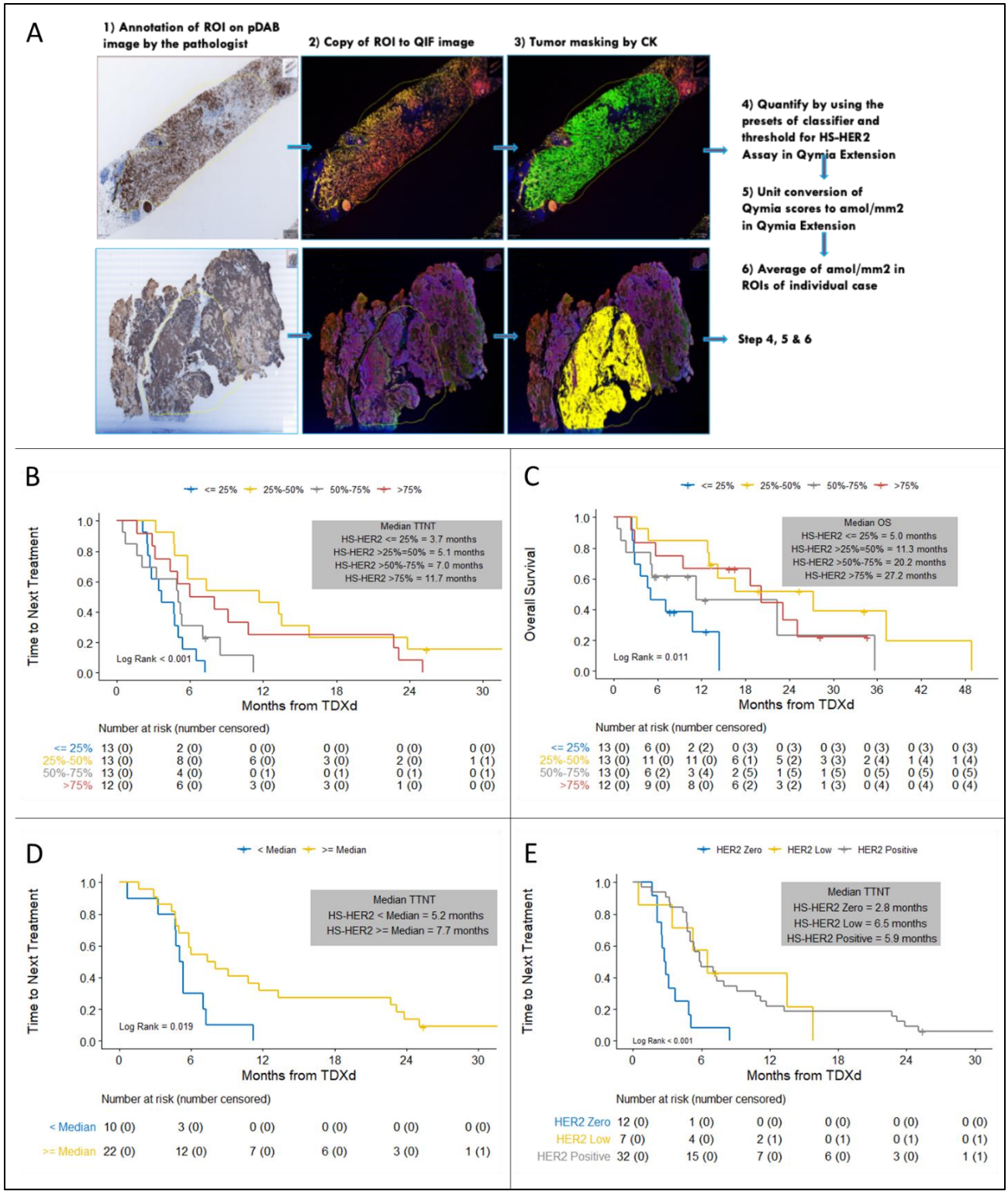

Figure 10. Outcomes with T-DXd according to pre-treatment HS-HER2 status. A. Workflow for the quantification of HS-HER2; B. TTNT with T-DXd according to HS-HER2 quartiles; C. OS with T-DXd according to HS-HER2 quartiles; D. TTNT with T-DXd according to HS-HER2

median (HER2-positive disease only); E. TTNT with T-DXd according to the traditional HER2 IHC classification of HER2-positive, HER2-low-HER2-0 in the HS-HER2 population.
Abbreviations: T-DXd, trastuzumab deruxtecan; TTNT, time to next treatment; HS-HER2, High Sensitivity-HER2; IHC, immunohistochemistry.

Quantitative evaluation of HER2 with RPPA

Pre-T-DXd tissue samples from 38 patients (24 with HER2-positive, 14 with HER2-negative disease at time of T-DXd) were analyzed with RPPA (**Figure 11A**, **Table 7**). A total of 7 different analytes were evaluated and associated with real-world outcomes with T-DXd, including two markers associated with T-DXd antibody target (HER2, phosphoHER2), two putative markers associated with payload sensitivity (TOPO1, SLFN11) and three additional ADC targets (Trop2, HER3 and EGFR).

	HER2 Positive (N=24)	**HER2 Negative (N=14)**	**Total (N=38)**
Age at metastatic diagnosis			
Median	51.9	54.3	53.1
Range	30.3 - 70.8	33.1 - 65.3	30.3 - 70.8
Gender			
Female	24 (100.0%)	14 (100.0%)	38 (100.0%)
Time from metastatic diagnosis to initiation of T-DXd (Months)			
Median	59.0	35.5	37.0
Range	1.0 - 125.0	2.0 - 110.0	1.0 - 125.0
Number of metastatic sites at start of T-DXd			
Median	3.0	3.0	3.0
Range	1.0 - 6.0	1.0 - 5.0	1.0 - 6.0
HR Status for Primary Tumor			
Positive	12 (57.1%)	12 (92.3%)	24 (70.6%)
Negative	9 (42.9%)	1 (7.7%)	10 (29.4%)
Unknown	3	1	4
HR Status for Metastatic Tumor			

	HER2 Positive (N=24)	HER2 Negative (N=14)	Total (N=38)
Positive	6 (54.5%)	5 (100.0%)	11 (68.8%)
Negative	5 (45.5%)	0 (0.0%)	5 (31.2%)
N/A (no metastatic tumor)	13	9	22
HR Status at T-DXd			
Positive	9 (37.5%)	8 (57.1%)	17 (44.7%)
Negative	15 (62.5%)	6 (42.9%)	21 (55.3%)
HER2 Status for Metastatic Tumor			
Positive	6 (54.5%)	5 (100.0%)	11 (68.8%)
Negative	5 (45.5%)	0 (0.0%)	5 (31.2%)
N/A (no metastatic tumor)	13	9	22
HER2 status of the primary tumor			
HER2 Positive	17 (81.0%)	0 (0.0%)	17 (50.0%)
HER2 Negative	4 (19.0%)	13 (100.0%)	17 (50.0%)
Unknown	3	1	4
Prior lines of treatment in the advanced setting			
Median	5.0	4.0	5.0
Range	1.0 - 16.0	0.0 - 9.0	0.0 - 16.0
Prior lines of chemotherapy			
Median	3.0	2.0	2.0
Range	0.0 - 9.0	0.0 - 5.0	0.0 - 9.0
Prior lines of endocrine therapy			
Median	0.0	1.0	0.5
Range	0.0 - 2.0	0.0 - 4.0	0.0 - 4.0
N/A (Always HR-)	11	1	12
Receipt of sacituzumab govitecan			
Yes, after T-DXd	2 (8.3%)	2 (14.3%)	4 (10.5%)
Yes, before T-DXd	4 (16.7%)	8 (57.1%)	12 (31.6%)
No	18 (75.0%)	4 (28.6%)	22 (57.9%)
HS-HER2 performed on			
Primary only	13 (54.2%)	9 (64.3%)	22 (57.9%)
Metastasis only	8 (33.3%)	2 (14.3%)	10 (26.3%)
Both	3 (12.5%)	3 (21.4%)	6 (15.8%)

Table 7. Clinicopathologic characteristics of patients included in the RPPA analysis

Across all patients, the RPPA HER2 expression was found to be directionally associated with TTNT (HzR per 10-unit increment 0.95 [95% CI: 0.90 – 1.01], p=0.08) and significantly associated with OS (HzR per 10-unit increment 0.89 [95% CI: 0.82 – 0.98], p=0.015) (**Table 8**). Additionally, there was a significant association between outcomes with T-DXd and HER2 RPPA when divided into quartiles, with a trend of increasing median TTNT and OS with increasing RPPA HER2 quartiles (**Figure 11B-C**). A significant difference in OS was seen when RPPA HER2 expression was dichotomized at the median, in patients with HER2-positive disease (p<0.001).

	Group	Median TTNT (month)	95% CI	HR	95% CI	p-value
ITT (n=38)						
Continuous	1 unit increase	-	-	1.00	0.99-1.00	0.083
	10 unit increase	-	-	0.95	0.90-1.01	
Median	< median (ref)	4.37	2.87-8.33			
	≥ median	8.00	5.97-23.13	0.37	0.18-0.74	(0.005)
						(0.03)
Quartile	≤ 25% (ref)	4.03	2.87-NA			
	>25%-50%	5.83	2.13-NA	0.64	0.25-1.61	0.3399
	>50%-75%	8.00	5.33-NA	0.28	0.10-0.74	0.0109
	>75%	9.07	5.83-NA	0.30	0.12-0.75	0.0104
HER2+ at T-DXd (n=24)						
Continuous	1 unit increase	-	-	1.00	0.99-1.00	0.28
	10 unit increase	-	-	0.97	0.91-1.03	
Median	< median (ref)	5.02	4.37-NA			
	≥ median	9.38	5.83-23.83	0.30	0.12-0.78	(0.018)
HER2- at T-DXd (n=14)						
Continuous	1 unit increase	-	-	0.99	0.94-1.03	0.54
	10 unit increase	-	-	0.86	0.54-1.38	
Median	< median (ref)	3.13	2.53-NA			
	≥ median	6.50	4.90-NA	0.74	0.20-2.76	(0.64)

Table 8. Association between RPPA HER2 (as continuous variable, median or quartiles) and TTNT with T-DXd, across all patients and separately in HER2-positive and HER2-negative MBC

PhosphoHER2 expression was also found to be directionally associated with TTNT (HzR per 10-unit increment 0.89 [95% CI: 0.78 – 1.02], p=0.087) and significantly associated with OS (HzR per 10-unit increment 0.80 [95% CI: 0.66 – 0.96], p=0.017), with a trend of increasing median OS with increasing RPPA HER2 quartiles, whereas the trend was less clear for TTNT (**Figure 11D-E**). When analyzed by median of RPPA phosphoHER2 expression, a significant difference in OS was seen in the ITT population, a significant result which remained consistent only in the HER2-positive disease subgroup (both p=0.002).

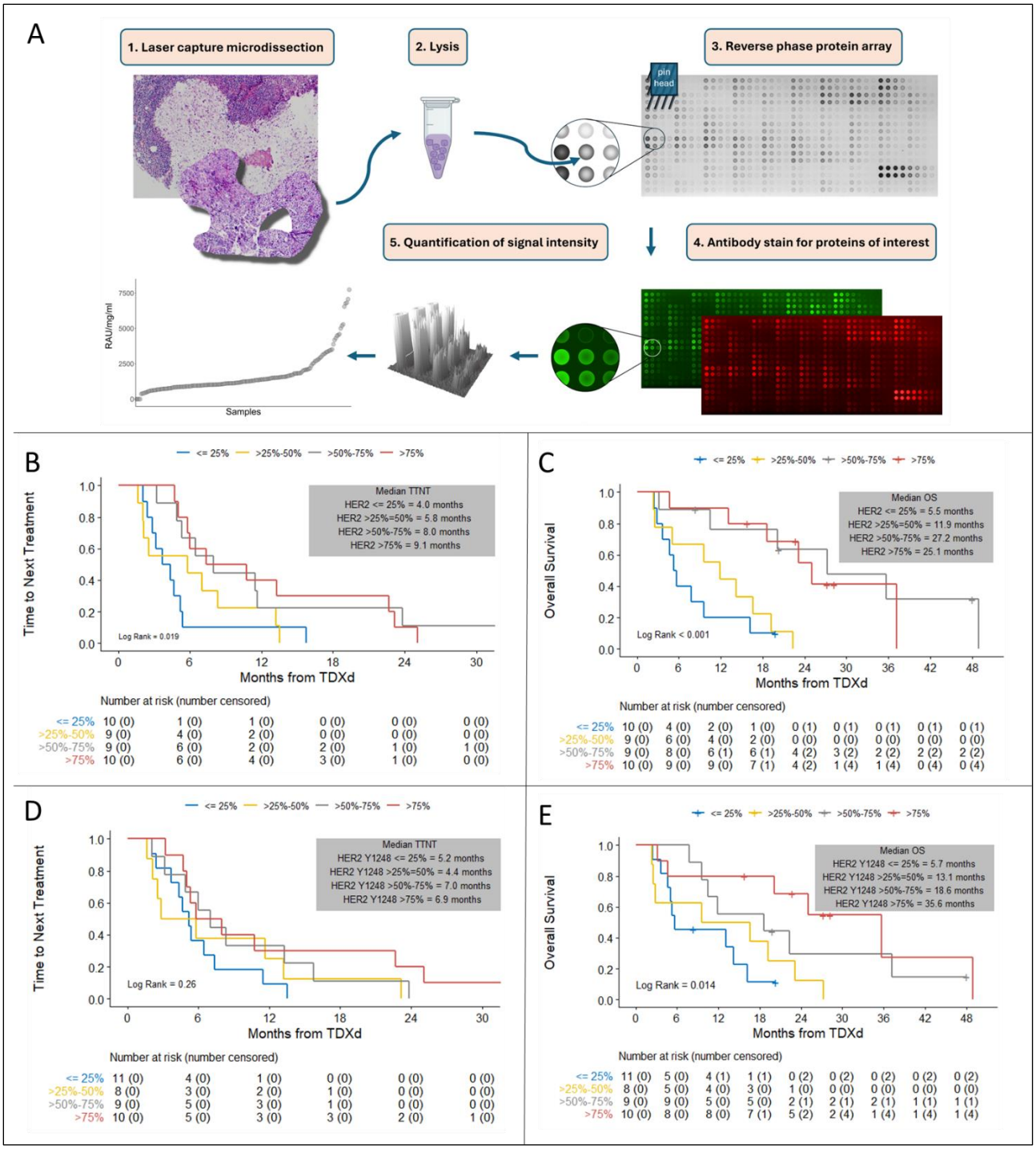

Figure 11. Outcomes with T-DXd according to pre-treatment RPPA HER2 status. A. Workflow for the RPPA analysis; B. TTNT with T-DXd according to RPPA HER2 quartiles; C. OS with T-DXd according to RPPA HER2 quartiles; D. TTNT with T-DXd according to RPPA phosphoHER2 Y1248 quartiles; E. OS with T-DXd according to RPPA phosphoHER2 Y1248 quartiles

Neither TOPO1 nor SLFN11 were found significantly associated with TTNT in the overall cohort. However, TOPO1 expression was found associated with outcomes among patients with HER2-negative MBC, with higher expression of TOPO1 (when divided by median) found significantly associated with worse TTNT and OS with T-DXd (**Figure 12A-B**).

No clear association was observed between outcomes with T-DXd and the RPPA expression of Trop2, EGFR and HER3 (**Figure 12C-E**), although higher Trop2 expression was found associated with shorter TTNT with T-DXd in patients with HER2-negative disease. Very weak correlation was observed between HER2 and Trop2 RPPA expression (r=0.06, p=0.73), whereas a weak correlation was observed between HER2 and SLFN11 (r=0.39, p=0.016) and a moderate correlation between HER2 and TOPO1 (r=0.47, p=0.003).

Among the 38 patients included in the RPPA analysis, the standard HER2 IHC subtypes (HER2-positive, HER2-low, HER2-0) sub-optimally predicted outcomes, with the numerically longest TTNT observed for patients with HER2-low disease, followed by HER2-positive and HER2-0 (**Figure 12F**).

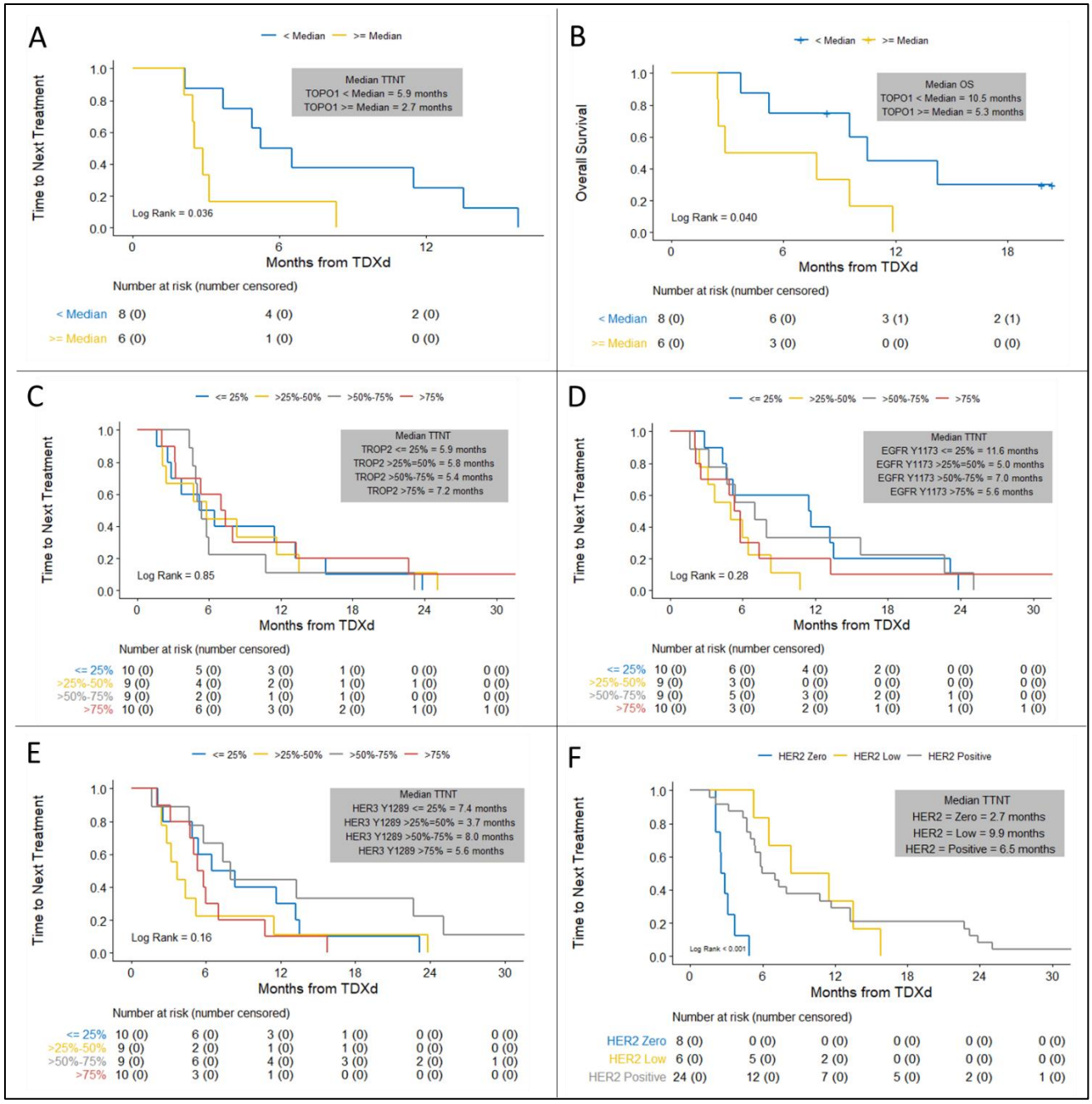

Figure 12. Outcomes with T-DXd according to pre-treatment RPPA status of markers beyond HER2. A. TTNT with T-DXd according to RPPA TOPO1 median (HER2-negative only); B. OS with T-DXd according to RPPA TOPO1 median (HER2-negative only); C. TTNT with T-DXd according to RPPA Trop2 quartiles; D. TTNT with T-DXd according to RPPA EGFR quartiles; E. TTNT with T-DXd according to RPPA HER3 quartiles; F. TTNT with T-DXd according to the traditional HER2 IHC classification of HER2-positive, HER2-low-HER2-0 in the RPPA population

Transcriptomic evaluation of HER2 with HER2DX

Pre-T-DXd tissue samples from 41 patients (25 with HER2-positive, 16 with HER2-negative disease at time of T-DXd, **Table 9**) were analyzed with the standardized HER2DX assay, with analysis of different transcriptomic modules (HER2 amplicon, 14-gene immunoglobulin (IGG), luminal, proliferation and ERBB2 gene, **Figure 13A**).

	HER2 Positive (N=25)	**HER2 Negative (N=16)**	**Total (N=41)**
Age at metastatic diagnosis			
Median	50.6	54.3	53.1
Range	30.3 - 70.8	33.9 - 65.3	30.3 - 70.8
Gender			
Female	25 (100.0%)	16 (100.0%)	41 (100.0%)
Time from metastatic diagnosis to initiation of T-DXd (Months)			
Median	50.0	27.5	34.0
Range	1.0 - 119.0	2.0 - 110.0	1.0 - 119.0
Number of metastatic sites at start of T-DXd			
Median	3.0	3.0	3.0
Range	1.0 - 6.0	1.0 - 5.0	1.0 - 6.0
HR Status for Primary Tumor			
Positive	11 (50.0%)	13 (92.9%)	24 (66.7%)
Negative	11 (50.0%)	1 (7.1%)	12 (33.3%)
HR Status for Metastatic Tumor			
Positive	4 (36.4%)	8 (100.0%)	12 (63.2%)
Negative	7 (63.6%)	0 (0.0%)	7 (36.8%)
N/A (no metastatic tumor)	14	8	22
HR Status at T-DXd			
Positive	10 (40.0%)	9 (56.2%)	19 (46.3%)
Negative	15 (60.0%)	7 (43.8%)	22 (53.7%)
HER2 status at initial metastatic tumor diagnosis			
HER2 Positive	4 (36.4%)	8 (100.0%)	12 (63.2%)
HER2 Negative	7 (63.6%)	0 (0.0%)	7 (36.8%)
N/A (no metastatic tumor)	14	8	22
HER2 status of the primary tumor			

	HER2 Positive (N=25)	HER2 Negative (N=16)	Total (N=41)
HER2 Positive	18 (72.0%)	0 (0.0%)	18 (43.9%)
HER2 Negative	4 (16.0%)	14 (87.5%)	18 (43.9%)
Unknown	3 (12.0%)	2 (12.5%)	5 (12.2%)
Prior lines of treatment in the advanced setting			
Median	5.0	4.0	5.0
Range	1.0 - 16.0	0.0 - 9.0	0.0 - 16.0
Prior lines of chemotherapy			
Median	2.0	1.5	2.0
Range	0.0 - 9.0	0.0 - 5.0	0.0 - 9.0
Prior lines of endocrine therapy			
Median	0.0	1.0	1.0
Range	0.0 - 2.0	0.0 - 4.0	0.0 - 4.0
N/A (Always HR-)	13	1	14
Receipt of sacituzumab govitecan			
Yes, after T-DXd	3 (12.0%)	4 (25.0%)	7 (17.1%)
Yes, before T-DXd	5 (20.0%)	8 (50.0%)	13 (31.7%)
No	17 (68.0%)	4 (25.0%)	21 (51.2%)
HS-HER2 performed on			
Primary only	14 (56.0%)	8 (50.0%)	22 (53.7%)
Metastasis only	7 (28.0%)	4 (25.0%)	11 (26.8%)
Both	4 (16.0%)	4 (25.0%)	8 (19.5%)

Table 9. Clinicopathologic characteristics of patients included in the HER2DX analysis

The HER2 amplicon signature was found to be significantly associated with TTNT (HzR per 1-unit increment 0.70 [95% CI: 0.56 – 0.87], p=0.001) and OS (HzR per 1-unit increment 0.65 [95% CI: 0.5 – 0.84], p=0.001) with T-DXd, including in subgroup analyses of TTNT in HER2-positive and HER2-negative MBC (**Table 10**). Additionally, there was a significant association between TTNT and the HER2DX HER2 amplicon module when divided into tertiles, with a trend of increasing median TTNT (p=0.019, **Figure 13B**) and OS (p=0.009, **Figure 13C**) with increasing HER2 amplicon tertiles, including when restricting to patients with HER2-positive disease (**Figure 13 D-E**).

A significant continuous association was also observed between TTNT and the HER2DX ERBB2 score (p=0.002), whereas no significant association was observed with the IGG module (p=0.29), the proliferation module (p=0.86) and the luminal module (p=0.32) when evaluated in continuous terms, although a trend towards better TTNT was observed with higher expression of luminal genes among patients with HER2-negative disease (**Figure 13F**). Similar findings were observed with OS.

Among the 41 patients included in the HER2DX analysis, the standard HER2 IHC subtypes (HER2-positive, HER2-low, HER2-0) sub-optimally predicted outcomes, with the longest TTNT observed for patients with HER2-low disease, followed by HER2-positive and HER2-0 (**Figure 13G**).

	Group	Median TTNT (month)	95% CI	HR	95% CI	p-value
ITT (n=41)						
Continuous	1 unit increase	-	-	0.7	0.56-0.87	0.001
Group						(0.019)
	Low (ref)	4.52	3.13-7.23			
	Medium	5.33	4.7-NA	0.71	0.33-1.55	0.39
	High	12.02	7.37-NA	0.23	0.08-0.69	0.009
HER2+ at T-DXd (n=25)						
Continuous	1 unit increase	-	-	0.63	0.47-0.86	0.004
Group						(0.012)
	Low (ref)	4.70	3.27-NA			
	Medium	5.33	4.7-NA	0.57	0.22-1.5	0.25
	High	12.02	7.37-NA	0.17	0.05-0.59	0.006
HER2- at T-DXd (n=16)						
Continuous	1 unit increase	-	-	0.45	0.21-0.94	0.033
Group						(-)
	Low (ref)	3.57	2.73-11.5			
	Medium			-	-	-
	High			-	-	-

Table 10. Association between HER2DX HER2 amplicon score (continuous, median or quartiles) and TTNT with T-DXd, across all patients and separately in HER2-positive and -negative MBC.

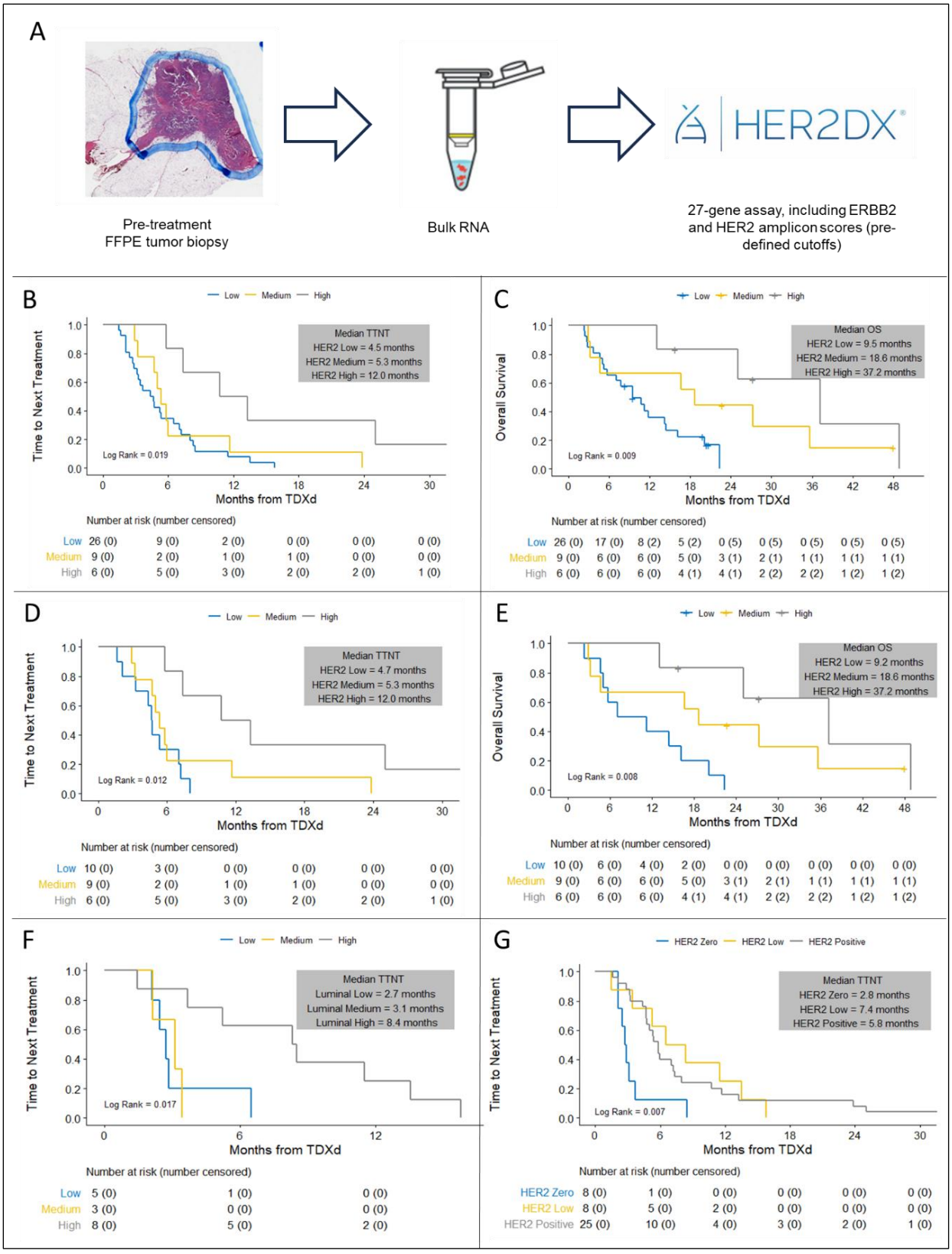

Figure 13. Outcomes with T-DXd according to pre-treatment HER2DX modules. A. Description of

HER2DX gene expression modules; B-C. association between the HER2 amplicon module with TTNT (B) and OS in all patients (C); D-E. association between the HER2 amplicon module with TTNT (D) and OS in HER2-positive MBC (E); F. association between TTNT and the luminal module in patients with HER2-negative disease; G. TTNT with T-DXd according to the traditional HER2 IHC classification of HER2-positive, HER2-low-HER2-0 in the HER2DX population

NGS testing of *ERBB2* hemizygous deletions

NGS data (OncoPanel assay) was retrieved for 53 patients with HER2-negative MBC at T-DXd start. This subgroup included 36 (67.9%) patients with HR-positive/HER2-negative and 17 (32.1%) patients with HR-negative/HER2-negative (i.e., triple-negative) disease. A total of 8 patients (15.1%) exhibited *ERBB2* hemizygous deletions (6 with HR-positive disease, 2 with HR-negative disease). A trend towards shorter TTNT (4.9 months vs. 7.6 months, p=0.60, **Figure 14**) and OS (8.0 vs. 11.4 months, p=0.55, **Figure 14B**) was observed among patients harboring *ERBB2* hemizygous deletions, although neither difference was found to be statistically significant.

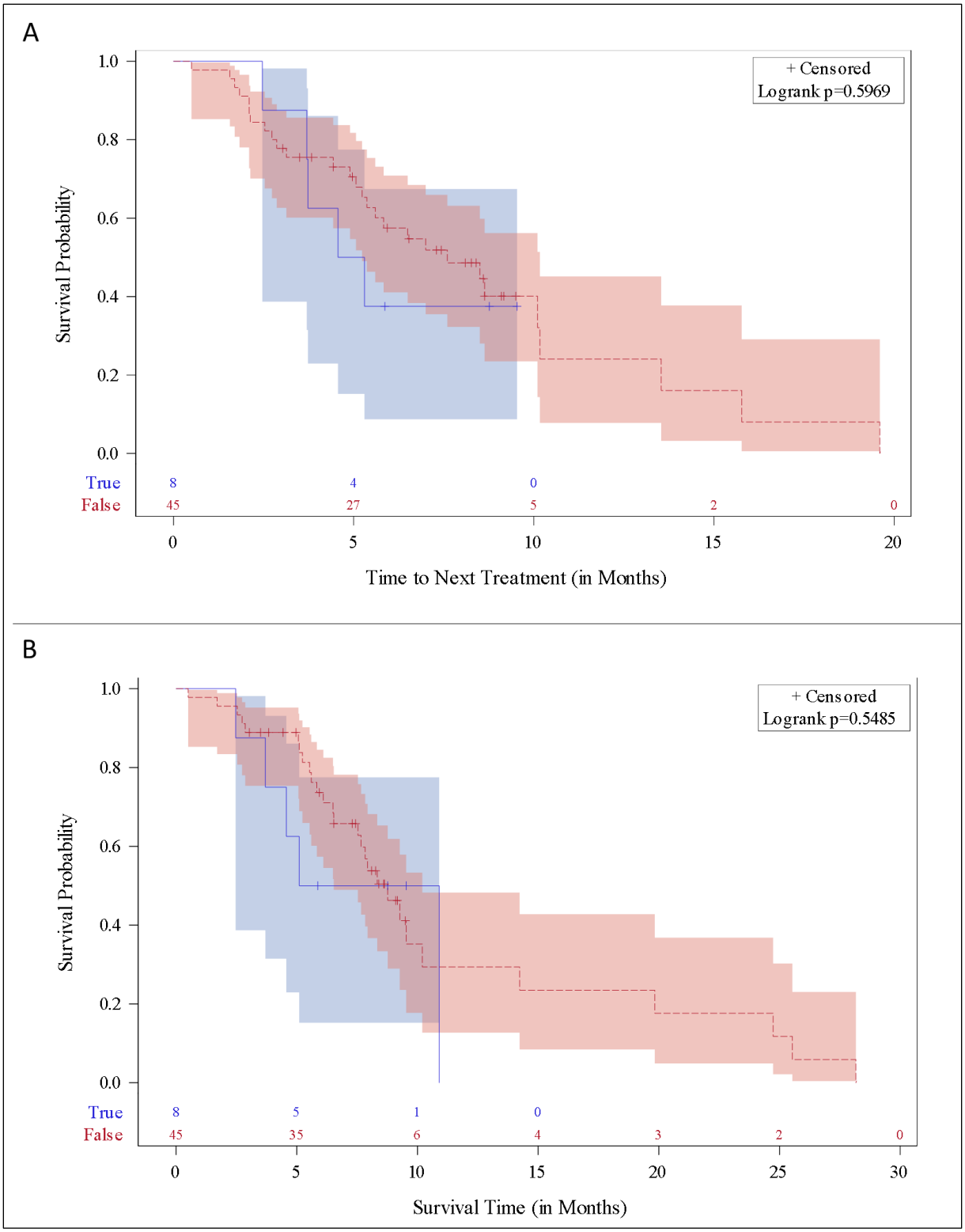

Figure 14. TTNT (A) and OS (B) by presence of absence of ERBB2 hemizygous deletions among patients with HER2-negative metastatic breast cancer that received T-DXd and had available clinical NGS testing.

DNADX signature

DNADX was performed on a total of 140 plasma samples from 98 patients, including 81 samples taken at baseline and 59 taken after progression on T-DXd. The population of patients included in the DNADX analyses included 40 (49.4%) with HER2-positive and 41 (50.6%) with HER2-negative disease, with the latter further divided into HER2-low (n=27, 33.3%) and HER2-0 (n=14, 17.3%). A total of 51 (63%) patients had HR-positive disease and 30 (37%) had HER2-negative disease; patients had received a median of 5 prior lines of treatment (range 1 to 14). The median follow-up for DNADX analyses was 18.6 months (range 0.5 – 63 months).

Among pre-T-DXd plasma samples, the median tumor fraction (TF) was 9% (range: 0% - 63%), with 30 patients (37%) having a TF of 0%, and 51 (63%) having a TF≥1%. DNADX classified the baseline ctDNA samples into 5 cluster subtypes: Cluster-0 (TF=0%, n=30, 37%); Cluster-1 (copy number-low, 0%); Cluster-2 (luminal-like, n=29, 36%); Cluster-3 (basal-like, n=8, 10%); and Cluster-4, (proliferative, n=14, 17%). There was no significant association between DNADX 5-class cluster subtype and HER2 IHC status, whereas the 5-class cluster subtype was significantly associated with HR IHC status ($p<0.0001$).

A significant association was detected between DNADX TF and outcomes with T-DXd (**Figure 15A**; $p =0.0489$), with patients having undetectable TF experiencing better outcomes comparing to patients with detectable TF. Additionally, the DNADX cluster subtype distribution was found significantly associated with TTNT (**Figure 15B**; $p = 0.004$)

The DNADX HER2 signature was calculated on baseline samples from 51 patients with TF>1% (8.6% HER2-0, 24.7% HER2-low, 29.6% HER2-positive). There was a significant association of the HER2 signature with TTNT ($p = 0.0446$), particularly when comparing patient with DNADX HER2-low vs. HER2-high disease (**Figure 15C**; HR=2.64; $p=0.0152$), whereas IHC was less predictive of benefit

in this population, with longer TTNT in HER2-low tumors compared with HER2-positive or HER2-0 tumors (**Figure 15D**).

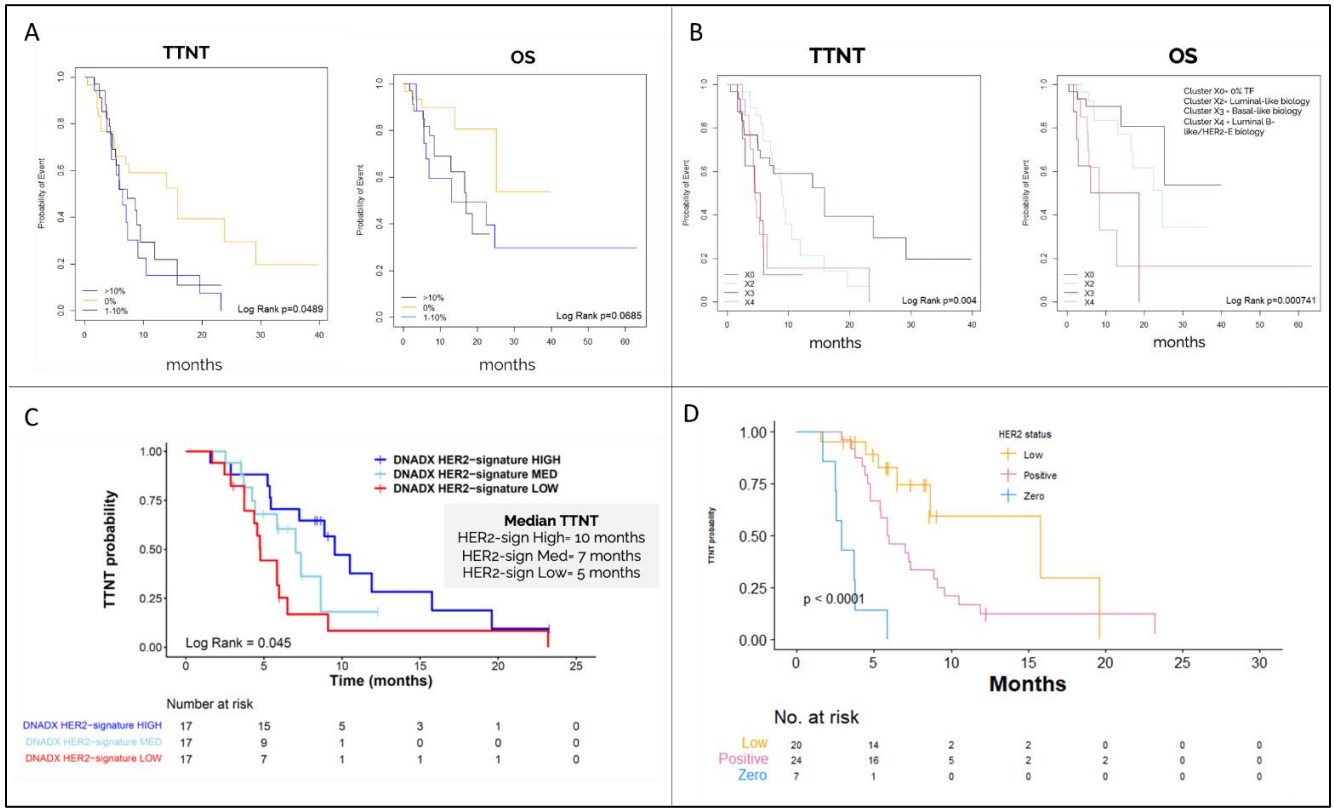

Figure 15. Outcomes with T-DXd for metastatic breast cancer according to DNADX-detected tumor fraction (A), DNADX subtype (B), DNADX HER2 signature (C) and outcomes depending on HER2 IHC status in the cohort of patients with detectable tumor fraction.
Abbreviations: T-DXd, trastuzumab deruxtecan.

A mutational panel covering 124 genes was assessed on a total of 127 plasma samples from 91 patients, including 73 samples taken at baseline and 54 taken after progression on T-DXd.

The most frequent mutations observed in pre-treatment samples were in *TP53* (26%), *MYC* (17%), *KMT2C* (17%), *PIK3CA* (17%), *GATA3* (14%), *ATM* (12%), *ERBB2* (11%), and *ESR1* (11%). Baseline mutations in *TP53* were significantly associated with worse TTNT with T-DXd in univariable

(HR=3.4, p<0.001) and multivariable analysis after adjusting by DNADX subtype and DNADX HER2 signature (HR=4.02; p<0.001).

The most frequent mutations observed in post-treatment samples were in *TP53* (26%), *PIK3CA* (26%), *TERT* (20%), *ARID1B* (20%), *ATM* (19%), *CDH1* (17%), and *ESR1* (15%). *ARID1B* mutations were significantly enriched in post-treatment samples (odds ratio=5.88; p=0.009). Mutations in *NFE2L2, HCFC2, GNA11, FGFR1,* and *USP9X* were only detected in post-treatment samples**.**

5. CONFIRMATION OF THE TRANSLATIONAL FINDINGS IN CLINICAL TRIALS OF NOVEL ADCS

The long-term plan for this project involves the clinical testing of novel ADCs in key areas of unmet need, with prospective confirmation of the biomarker findings emerging from the retrospective analyses. During the last three years, I designed and developed the protocol for three investigator-initiated trials testing ADCs, all of which opened to accrual in 2023 and are currently ongoing: (1) the SATEEN phase 2 trial tests SG and trastuzumab for patients with HER2-positive MBC that have progressed on T-DXd (NCT06100874); (2) the TRUDI phase 2 trial tests neoadjuvant T-DXd and durvalumab for patients with HER2-expressing inflammatory breast cancer (IBC) (NCT05795101); (3) the DATO-BASE phase 2 trial tests Dato-DXd for MBC patients with brain metastases (NCT06176261).

SATEEN phase 2 trial

SATEEN (NCT06100874) is an open label, multi-center, phase 2 trial that tests the activity of SG plus trastuzumab in patients with HER2-positive MBC that have previously received treatment with T-DXd and experienced progression on discontinued treatment for toxicity (**Figure 16).**

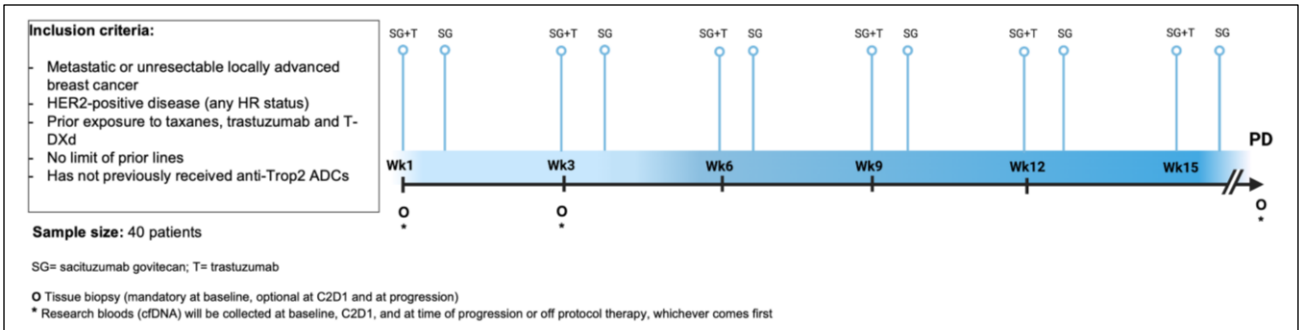

Figure 16. Schema of the SATEEN phase 2 trial

This represents the first study to test SG in HER2-positive MBC. Tumor biopsies and blood samples are collected at baseline, during treatment, and at progression, with plans to conduct in-depth analyses of these samples. I wrote the concept and protocol for this trial; the study opened to accrual in November 2023 and has currently accrued 15 patients (out of 40 planned). We anticipate to complete accrual by Q1-2 2026. The primary endpoint of the study is objective response rate (ORR), which will be used to validate the Trop2 quantitative thresholds identified within the retrospective analyses.

TRUDI phase 2 trial

TRUDI (NCT05795101) is an open label, multi-center, phase 2 trial that tests neoadjuvant T-DXd plus durvalumab for patients with HER2-expressing IBC, tackling a major unmet need in breast oncology **(Figure 17)**.

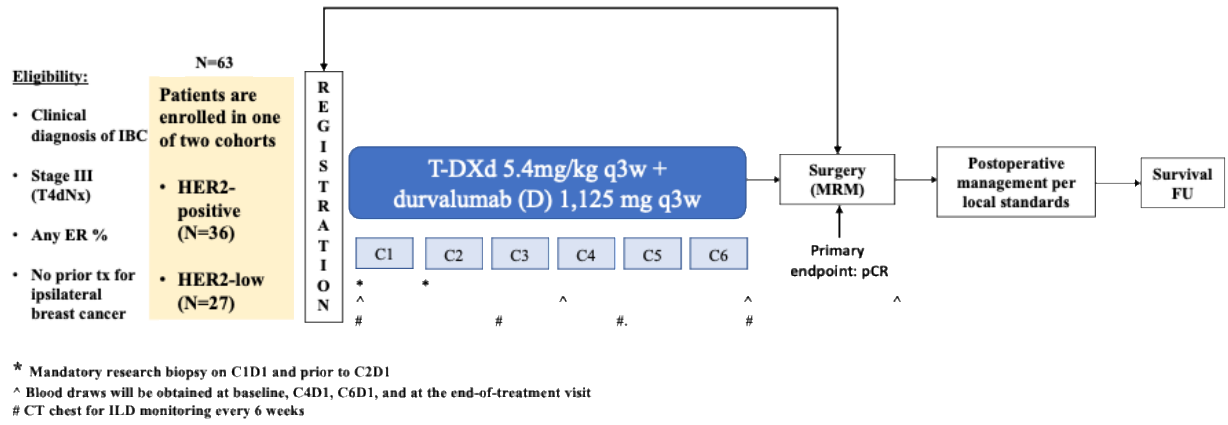

Figure 17. Schema of the TRUDI phase 2 trial

I wrote the protocol for this trial. The study activated on May 4th, 2023, and enrolled the first patient on July 17th, 2023. To date, it has accrued 23 patients (out of a total of 63 planned). The primary endpoint is pathologic complete response (pCR), which will be used to validate the HER2 quantitative thresholds identified within the retrospective analyses.

DATO-Base phase 2 trial

DATO-BASE (NCT06176261) is an open label, multi-center, phase 2 trial that tests the activity of Dato-DXd among patients with HER2-negative active brain metastases or leptomeningeal disease, populations for which very few active treatment options are currently available (**Figure 18**).

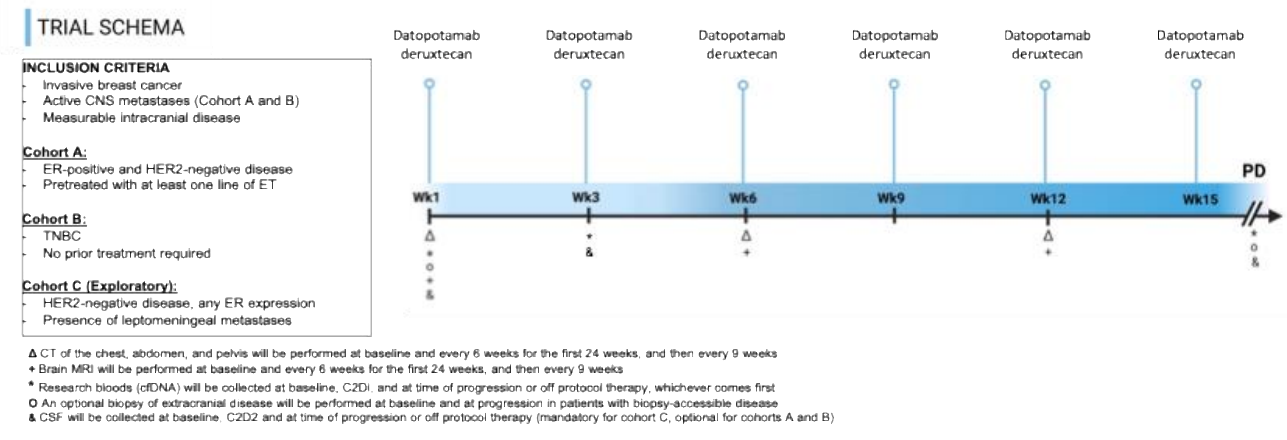

Figure 18. Schema of the DATO-Base phase 2 trial

I designed the concept and wrote the protocol for this trial. The study opened to accrual at DFCI on December 26th, 2023, and has accrued 16 patients (out of a total of 58 planned). The primary endpoint is the intracranial ORR with Dato-DXd, which will be used to validate the Trop2 quantitative thresholds identified within the retrospective analyses.

6. DISCUSSION

As few treatment classes, ADCs are currently reshaping the way we treat metastatic breast cancer, among other cancer types. After the first approval of an ADC for breast cancer in 2013 (T-DM1), two further ADCs have been recently approved for this disease (T-DXd and SG), and T-DXd has been granted the first ever agnostic approval for an ADC, with its use that is now recommended for any patient with a HER2 overexpressing advanced solid tumor. Despite the striking improvements in efficacy observed with novel ADCs over traditional chemotherapy, however, this class of drugs remains associated with relevant side effects, warranting precision in their clinical use. Given that no biomarker (besides classical HER2 IHC) is currently available for guiding treatment choices in clinical practice, we leveraged tissue from patients treated with T-DM1 and T-DXd to unveil potential tissue and plasma-based biomarkers that may refine the use of these conjugates. Moreover, we conducted extensive multi-omic analyses to unveil the molecular correlates of response and resistance with ADCs, which may inform the development of rational combinations and of the next generation of ADCs.

From our analyses of tissue samples collected before initiation of adjuvant T-DM1 for early-stage, HER2-positive breast cancer, we derived several observations. First, single-cell FISH analysis confirmed that HER2 heterogenous tumors account for a minority of HER2-positive tumors,

representing approximately 8% in ATEMPT. However, this feature had no significant impact on survival outcomes with T-DM1, which is compatible with the lack of significant differences in survival by HER2 heterogeneity observed in a trial of neoadjuvant T-DM1,[42] despite the same trial showing major differences in pCR rates.[43] These results highlight the complex relationship between pCR and survival in HER2-positive tumors, given that certain features (e.g., luminal genes expression) can be concomitantly associated with decreased likelihood of pCR but also with better long-term survival.[20] Even when assessed through ML-based algorithms and with spatial proteomic analyses, HER2 heterogeneity did not show a clear relationship with survival. It should be noted that the number of samples included in each of these translational analyses were small, thus the power to detect any statistically significant difference was limited.

Of note, a comprehensive assessment of the tumor biology with the HER2DX tool showed that a pre-established risk score threshold was able to discriminate a small proportion of patients (6.4%) with significantly higher risk of recurrence in ATEMPT. This finding further reinforces what was previously observed in the APT trial, where a similar percentage of patients (4.9%) were found to have HER2DX high-risk disease, which was associated with increased risk of recurrence.[44] Although based on a small number of recurrence events, the results from both trials underscore the promise of HER2DX in tailoring treatments for patients with early-stage HER2-positive tumors, warranting prospective validation.

In addition to looking at HER2 heterogeneity and HER2DX, we utilized spatial proteomic analyses to identify biomarkers associated with a higher risk of recurrence. NF1 and CTLA4 were found to be overexpressed in primary tumors of patients experiencing ipsilateral or contralateral events, whereas Cleaved Caspase 9, CD25, ICOS, GITR and GZMB were found upregulated in matched controls. NF1 alterations have been previously found enriched in advanced breast cancers compared with matched primary tumors,[45-47] as well as potentially associated with increased sensitivity to T-

DM1.[48,49] CTLA4 is an immune checkpoint molecule that negatively regulates T cell activation,[50] often upregulated by tumors as a strategy to achieve immune evasion.[51] Our findings provide a potential rationale for therapeutic CTLA4-blockade in HER2-positive breast cancer, although the current availability of highly effective anti-HER2 agents may raise challenges in the development of immunotherapy strategies in HER2-positive tumors. Conversely, most proteins found upregulated in controls are either associated with the activation of the apoptotic pathway (Cleaved Caspase 9) or with immune activation (CD25, ICOS, GITR and GZMB), suggesting that an active antitumor immune response, inducing a higher degree of apoptosis among cancer cells, may signal a better prognosis.

Overall, the most actionable finding from our translational analyses from ATEMPT proved to be the HER2DX analysis, based on the statistical results, the concordance with similar prior analyses and the ongoing efforts to prospectively validate this biomarker, which may enable for a refined way to treat early-stage HER2-positive breast cancer in the future.

Besides improving treatment tailoring for early-stage breast cancer, a major unmet need is currently represented by treatment tailoring for the metastatic disease, where multiple treatments are rapidly becoming available, and where treatment choices are becoming increasingly complex due to the lack of biomarkers and head-to-head comparisons between novel treatments. One key challenge is represented by the choice of which ADC to use in HER2-negative metastatic breast cancer, where both T-DXd and SG are available, both delivering a similar chemotherapy payload, and where several additional ADCs are in phase 3 testing and may join the treatment arsenal soon. We and others have previously demonstrated that the utilization of TOPO1 ADCs in sequence is associated with some degree of cross-resistance[52-55], highlighting the importance of understanding the mechanisms of resistance of these agents and developing predictive biomarkers to inform treatment decisions.

To address the unmet need of predicting the activity of T-DXd, we conducted a multiplicity of translational analyses with samples collected at DFCI from patients receiving T-DXd. A summary of the results from these analyses is provided in **Table 11**.

ANALYSIS	RESULTS
HS-HER2	Pre-TDXd tissue samples from 51 patients were analyzed with HS-HER2. HS-HER2 was found to be continuously associated with TTNT and OS with T-DXd. Consistent findings were observed in sub-analyses of patients with HER2-positive and HER2-negative MBC.
RPPA	Pre-TDXd tissue samples from 38 patients were analyzed with RPPA. The RPPA HER2 expression and PhosphoHER2 expression were found to be directionally associated with TTNT and significantly associated with OS. TOPO1 expression was found associated with outcomes among patients with HER2-negative MBC, with higher expression of TOPO1 found significantly associated with worse TTNT and OS with T-DXd. No clear association was observed between outcomes with T-DXd and the RPPA expression of Trop2, EGFR and HER3
HER2DX	Pre-T-DXd tissue samples from 41 patients were analyzed with the standardized HER2DX assay. The HER2 amplicon signature was found to be significantly associated with TTNT and OS with T-DXd, including in subgroup analyses of TTNT in HER2-positive and HER2-negative MBC. A significant continuous association was also observed between TTNT and the HER2DX ERBB2 score, whereas no significant association was observed with the IGG module, the proliferation module and the luminal module.
Oncopanel	NGS data was retrieved for 53 patients with HER2-negative MBC at T-DXd start.

	A total of 8 patients (15.1%) exhibited ERBB2 hemizygous deletions. A trend towards shorter TTNT and OS was observed among patients harboring ERBB2 hemizygous deletions.
DNADX	The DNADX HER2 signature was calculated on baseline samples from 51 patients with TF>1%. There was a significant association of the HER2 signature with TTNT, particularly when comparing patient with DNADX HER2-low vs. HER2-high disease.
DNADX mutation calling	The most frequent mutations observed in pre-treatment samples were in TP53, MYC, KMT2C, PIK3CA, GATA3, ATM, ERBB2, and ESR1. Baseline mutations in TP53 were significantly associated with worse TTNT with T-DXd. The most frequent mutations observed in post-treatment samples were in TP53, PIK3CA, TERT, ARID1B, ATM, CDH1, and ESR1. ARID1B mutations were significantly enriched in post-treatment samples. Mutations in NFE2L2, HCFC2, GNA11, FGFR1, and USP9X were only detected in post-treatment samples.

Table 11. Summary of the results of the multiomic predictive analyses for T-DXd

Herein, we demonstrate that multiple strategies of pre-T-DXd HER2 quantitation initiation can continuously predict the efficacy of T-DXd, each with distinctive features and with improvement compared with traditional HER2 IHC subtypes. The quantitative immunofluorescence-based HS-HER2 was found continuously associated with outcomes with T-DXd, with a 22% increase in TTNT for each 5-unit increment in HS-HER2. Notably, the assay proved to be predictive both in HER2-positive and HER2-negative MBC, and its dynamics did not seem to impact its predictive value. Similarly, the RPPA quantitation of HER2 and phosphoHER2 showed promise in predicting outcomes with T-DXd, with a significant association of both biomarkers with OS after starting T-DXd. Noteworthy, the multiplex

capabilities of RPPA testing enabled to concomitantly quantitate markers associated with payload sensitivity: higher TOPO1 expression was found to be associated with shorter TTNT and OS with T-DXd used for HER2-negative MBC, suggesting that additional aspects of ADCs activity beyond the antibody target may further refine prediction of activity of T-DXd. The fact that TOPO1 appeared prognostic only in HER2-negative disease may signal that, in the presence of high overexpression of the antibody target (e.g., HER2-positive disease), this feature dominates the prediction of ADCs activity, whereas multiple factors may predict the performance of ADCs in tumors expressing low/intermediate levels of the antibody target (e.g., HER2-negative/low). The quantification of the HER2 mRNA via HER2DX was also found continuously associated with the activity of T-DXd, with the strongest association with TTNT/OS observed with the HER2 amplicon module of HER2DX. Further confirming the strength of this analysis, no significant association was observed with other HER2DX modules (IGG, proliferation), although better outcomes were observed among patients with HER2-negative MBC with higher luminal expression, further signaling that the prediction of ADC efficacy in this subgroup may be more complex than in the HER2-positive disease. Lastly, the machine learning assisted analysis of pre-T-DXd plasma samples with DNADX enabled to evaluate a HER2 signature among patients with detectable tumor fraction, which showed a strong association with T-DXd efficacy. Confirmation of this finding may provide a non-invasive method for the prediction of T-DXd efficacy in MBC. Importantly, for each of the biomarker subsets of patients, the traditional HER2 IHC subtypes (HER2-positive, HER2-low, HER2-0) seemed to provide less predictive information, with the longest TTNT observed for patients with HER2-low disease rather than for those with HER2-positive disease.

We further analyzed the genomic landscape of MBC before and after treatment with T-DXd to unveil resistance mechanisms and genomic correlates of T-DXd activity. We show that *ERBB2* hemizygous deletions are relatively common events in HER2-negative MBC (15.1% of the patients in

our study) and are associated with numerically worse outcomes compared with patients having wild-type *ERBB2*. This is in line with a prior report from DAISY, where most patients harboring *ERBB2* hemizygous deletions (n=4/6) did not respond to T-DXd.[16] Given the small sample size of both analyses, these trends should be considered hypothesis generating. In addition, we describe a detrimental effect of *TP53* mutations on outcomes with T-DXd, and describe the enrichment of several mutations after treatment with T-DXd, including *NFE2L2, HCFC2, GNA11, FGFR1, USP9X* and *ARID1B*. Confirmation of these mutations in further datasets may provide insights on resistance mechanisms to T-DXd and other novel ADCs.

Key limitations these translational studies are represented by their retrospective nature, the limited sample size and the absence of prospectively collected metrics of T-DXd efficacy, such as PFS. However, the meaningful prognostic value observed with HER2 testing via multiple different assays, as opposed to the lack of prognostic value for other ADC markers (e.g., Trop2, HER3, EGFR) or unrelated markers (IGG and proliferation modules of HER2DX), highlights the key relevance of a quantitative HER2 assessment in the assessment of treatment benefit with T-DXd.

To validate the findings from our translational analyses, our long-term plan involves the analysis of samples from three ongoing, prospective phase 2 trials of novel ADC, which we anticipate completing enrollment by the end of 2026, namely TRUDI, SATEEN and DATO-Base. Each of these trials will provide detailed efficacy data with anti-topoisomerase1 ADCs in key settings of unmet need, which we will plan to associate with quantitative measures of target expression explored in this pilot study, building on its preliminary findings. The ultimate goal is to bring helpful biomarkers to the clinic, through the prospective validation in clinical trials designed and powered to demonstrate the clinical utility of biomarkers in tailoring treatment with ADCs.

7. CONCLUSION

After decades dedicated to the discovery of new anticancer drugs, we are now finding ourselves in an era of rapidly expanding treatment options for breast cancer. This is particularly true for ADCs, with over 200 compounds currently in clinical development for oncologic indications. Nonetheless, the paucity of biomarkers available to guide treatment with ADCs challenges their practical use, which is in most cases blinded to the tumor molecular characteristics.

Our research effort tried to address this unmet need, unveiling novel quantitative proteomic and transcriptomic assays which appear to granularly predict the benefit from T-DM1 and T-DXd in our pilot studies. This effort represents only the first of many steps needed to validate and bring a molecular biomarker to the clinic. We believe that only similar efforts in biomarker discovery may lead us to a future era of precision ADC treatment, allowing each of our patients to receive the right treatment, within the right sequence, at the right time and with the right prediction of its outcome.

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