Observational Retrospective-Prospective Study Protocol	
Study title	RADIomics to predict HER2 status And T-DXd efficacy in metastatic breast cancer: the RADIOSPHER2 study.
Sponsor	Fondazione IRCCS Istituto Nazionale dei Tumori, via G. Venezian, 1 – 20133 Milano
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Background and Rationale	***HER2-low and BC***. Breast cancer (BC) is the first cancer in terms of incidence worldwide, with a critical impact on public health due to its global disease burden.[1] Historically, BC is classified in three major histopathological categories based on the expression of Hormone Receptors (HR) and Human Epidermal growth factor Receptor 2 (HER2) expression. While HER2-positive subtype is characterized by expression defined as 3+ by immunohistochemistry (IHC) or 2+ with in situ hybridization (ISH) amplification, the other two subtypes, defined as HR+/HER2- and triple-negative BC (TNBC), are characterized by a low (1+ or 2+, not ISH amplified) or null HER2 expression on cells surface, and so classified as "HER2-low" or "HER2-0", respectively. As neither HER2-low nor HER2-0 BC subgroups demonstrated any survival benefit from the addition of HER2-directed monoclonal antibodies to chemotherapy in historical trials, these patients kept to be treated accordingly to HR status, without HER2-directed therapies.[2,3] ***Trastuzumab-Deruxtecan in mBC***. Recently, the development of antibody drug conjugates (ADCs) revolutionized the landscape of treatment for BC pts with both HER2-positive and previously defined "HER-2 negative" disease. In particular, Trastuzumab Deruxtecan (T-DXd), an ADC composed of an anti-HER2 antibody conjugated with a potent topoisomerase I inhibitor as cytotoxic payload, demonstrated impressive activity in pre-treated HER2-positive mBC, with a median Progression-Free Survival (mPFS) of 28.8 months as second-line therapy.[4-6] Based on the intrinsic characteristics of T-DXd, in particular the potent chemotherapy (ChT) payload and the demonstrated bystander effect, the randomized phase III trial Destiny-Breast 04 was designed to investigate the efficacy of T-DXd on HER2-low metastatic BC (mBC), who received 1-2 prior lines of ChT for metastatic disease.[7] T-DXd demonstrated superior efficacy compared to the control arm, reaching a mPFS of 9.9 months and OS of 23.4

months, thus becoming the preferred treatment option for most pts with HR+/HER2- aBC and HER2-low status progressing on first-line cycline-dependent kinase 4/6 inhibitors (CDK4/6i) in combination with endocrine therapy (ET) and at least one line of ChT. The ongoing Destiny-Breast 06 trial will provide answers about the use of T-DXd as second line treatment after ET+CDK4/6i for HR+ mBC. The recently published DAISY trial was a prospective, phase 2, clinical trial assessing T-DXd efficacy in pts with mBC and different HER2 expression.[8] Notably, differently from pivotal studies, a tumor specimen collected within 3 months before inclusion was mandatory. Among the three cohort, i.e. HER2 overexpressed (N = 72), HER2 low (N = 74) and HER2 IHC 0 (N = 40), the ORR, which was the primary endpoint of the trial, was 70.6%, 37.5% and 29.7%, respectively. Interestingly, also the mPFS seemed to be significantly proportional to the level of HER2 expression (11.1 months, 6.7 months and 4.2 months in the three subgroups, respectively). This observation suggests that the HER2 expression level, if assessed at treatment baseline, can predict differential benefit from T-DXd. Finally, the activity of T-DXd in the HER2 0+ population could reflect the limitation of HER2 assessment, perhaps due to the insufficient sensitivity of IHC to low ranges of HER2 expression, the low capacity of pathologist to detect HER2 ultra-low expression, the spatial intratumor heterogeneity, or a combination of these factors.

Biology of HER2-low mBC. From a biological point of view, it remains unclear if HER2-low BC is characterized by a peculiar biology compared to HER2-0 BC, reflecting in different clinical behaviour and impact on prognosis.[9-10] In general, compared to HER2-0, HER2-low BC has been observed to have higher prevalence of *PIK3CA* mutation, lower prevalence of *TP53* mutation, higher *ERBB2* mRNA expression, less responsivity to neoadjuvant ChT and slightly better survival compared to HER2-0. However, a strong association between HER2-low expression and HR positivity has been observed, with HER2-low BC characterized by a higher HR expression rate, likely as a consequence of the HR-HER2 molecular crosstalk. Therefore, after adjusting to HR status, the abovementioned differences among the groups seem to be lost.[11] However, some prognostic effect conferred by HER2 low status seems to be evident in some subgroups, such as HR+/HER2- BC treated with first-line CDK4/6i with ET, where a potential detrimental effect of low HER2 expression on survival was observed.[12]

HER2 heterogeneity. HER2 expression in mBC is characterized by a strong heterogeneity both among different metastases and even at intratumor

level.[13] In particular, intratumoral heterogeneity, which refers to the co-existence of multiple tumor cell subpopulations with varying HER2 statuses within the same tumor, is not a rare phenomenon in BC, with a prevalence up to 40%. This heterogeneity, defined by different levels of protein expression or gene amplification evaluated by pathologists on tumor slides, has obvious clinical implication: firstly, a lower rate of pathologic complete response was observed in patients with documented HER2 heterogeneity underwent anti-HER2-based neoadjuvant treatment; furthermore, it may contribute to inaccurate assessment of HER2 status and lead to inappropriate treatment regimens.[14-15] HER2 heterogeneity at macroscopic level is less known, however HER2 different metabolic expression among metastatic sites was documented by HER2-positron emission tomography (PET)/computed tomography (CT) with (89) Zr-trastuzumab in patients enrolled in the prospective ZEPHIR trial.[16] HER2 is also characterized by a deep dynamicity over time (temporal heterogeneity), with up to 35% of discordance between paired primary and metastatic tumors, due to the selection of HER2-negative clones induced by treatment, genetic shift or analytic issues.

Application of AI-based methodology for BC care. Artificial Intelligence (AI) recently emerged as a new methodologic approach to medical images.[17] In particular, radiomics and pathomics are promising fields of application, identifying relevant features not accessible by raw human eye from radiological images and from pathology slides, respectively.[18] In the last two years, a plethora of works demonstrated the feasibility and ability of radiomics in predicting patient outcomes, tumor response to therapies and in identifying molecular subtypes of human malignancies that are commonly identified through the use of specific omics analysis performed in tumor specimens, such as genomics, transcriptomics and proteomics.[19-21] However, none of these tools are currently used in clinical practice. Two studies already demonstrated the feasibility of the radiomics use on predicting the benefit of mBC patients from novel therapeutics. In particular, Khorrami M et al built a CT scan-based radiomic model able to predict survival benefit in mBC with liver metastases treated with CDK4/6i in combination with endocrine therapy, reaching a notable accuracy (AUC = 0.77).[19] Indeed, Zhao J et al conducted a retrospective radiomic analysis on a multicentre cohort of 240 mBC treated with immunotherapy to predict Disease Control Rate, with excellent results (AUC = 0.92 in the validation set) and a discriminatory capacity superior to the clinical model.[20]

	Radiogenomics aims to predict, through highly complex analysis of radiological images, those molecular alterations that could otherwise be reached only by high-costly analyses on tumor samples. This use of AI comes with several advantages: a) it allows the monitoring of tumor evolution over time and its temporal heterogeneity, avoiding repeated and potentially risky biopsy procedures (which are not always feasible); b) it allows the assessment of the inter-lesion tumor heterogeneity through the evaluation of all metastatic lesions at the same time, which could not be individually biopsied; c) it is a ready-to-use and easy-to-access tool, which could reduce the economic burden and save time required for extensive molecular analyses. ***Rationale.*** HER2 status may be heterogeneous across tumor lesions of the same pt (inter-lesion heterogeneity) and can also evolve during disease history (temporal heterogeneity).[22-26] Therefore, to understand if a patient could benefit from HER2-directed therapies, including T-DXd, it is important to obtain HER2 status assessment close to the treatment start and possibly from different disease sites. However, re-biopsy is not always feasible and, even when feasible, not all disease sites can be approached. In addition, HER2 status by pathological assessment is characterized by several limitations (analytic issues, spatial heterogeneity) and re-biopsy is often not feasible in clinical practice (or associated with risky procedures, e.g. bone metastases).[27] Therefore, there is an urgent need to find a non-invasive, reliable and low-cost method capable of providing quantitative HER2 status assessment and, consequently, predictive information of potential benefit from HER2-directed therapies, in mBC patients. Based on these data, we hypothesized that innovative Machine Learning and Deep Learning-based radiogenomics methodologies can allow an updated and comprehensive evaluation of HER2 status in pts with mBC, breaking down the barrier of spatial and temporal heterogeneity. To test this hypothesis, we aim at identifying a radiomics score as surrogate of pathological-assessed HER2 status and, finally, at improving the performance of actual HER2 assessment in predicting pts benefit form T-DXd treatment.
Study design	RADIOSPHER2 study is a monocentric, retrospective, observational study aiming at identifying a radiomics signature able to predict HER2 expression (0 vs low vs overexpression) and T-DXd efficacy in mBC patients. The study also encompasses translational analyses and inter-modal correlations in order to provide novel insights about HER2 spatial and temporal heterogeneity, at the macroscopic and microscopic levels.

	In particular, to pursue the study aims it will be conducted different analyses, which are structured in the subsequent sections. Figure 1 summarize the whole study design. **Section 1: Identification of the HER2 radiomic signature («radiobiopsy»).** We hypothesize that several features extracted from radiological images can predict the HER2 status (positive vs low vs null) on different specific metastatic sites of mBC patients. Accordingly to the study rationale, it will chosen to analyse images from those metastatic sites usually difficulty to approach with biopsy, such as lung, liver, pleural and bone lesions. The population of this first part of the study will consists with a cohort of patients with mBC underwent a liver, lung, pleura or bone biopsy in the metastatic setting at "Fondazione IRCCS Istituto Nazionale Tumori" in Milan (INT) with available imaging (~500 patients expected). This first analysis will consist of extracting and selecting features that will be included in the «radiobiopsy» model for HER2 status prediction. **Section 2: T-DXd treatment efficacy prediction with «radiobiopsy» score.** As previously underlined, a positive and significant association was seen between T-DXd clinical benefit (defined as mPFS) and HER2 status, if evaluated on tissue collected right before treatment start. The «radiobiopsy» model will be applied to retrospectively calculate HER2 status from the baseline scan of HER2+ or HER2-low mBC pts underwent any-line T-DXd treatment for metastatic disease. The T-DXd PFS of patients assigned to the three cohorts (HER2-0, HER2-low or HER2-overexpressed) based on «radiobiopsy» model will be then calculated and differences among groups will be tested with Cox regression analysis. Clinical characteristics (e.g., number of therapy line for metastatic disease, HR status, HER2 IHC expression) of the same cohort will be extracted from clinical health records, in order to build a multivariate Cox regression model. In case of the HER2 status calculated with the «radiobiopsy» model would demonstrate an insufficient accuracy in predicting T-DXd survival benefit, a new multimodal model will be build taking in account radiological baseline scan and clinical features. To this proposal, the same modality used for building the «radiobiopsy» model will be applied, adopting T-DXd survival outcomes (12-month survival status) as clinical outcome for classification.

	Section 3: HER2 heterogeneity prediction. HER2 heterogeneity is frequent in BC and is associated with worse prognosis.[28] To assess the HER2 spatial heterogeneity at intra-tumor microscopic level, pathology slides derived from biopsies of patients enrolled to build the «radiobiopsy» model will be also reviewed from pathologists for HER2 heterogeneity evaluation and digitalized for pathomics analyses.[29] The HER2 heterogeneity will be evaluated by pathologist based on IHC expression and distribution. Both Hematoxilin-Eosin (HE) and HER2 stained slides will be analysed for this purpose. After splitting the entire cohort in training (80%) and testing (20%), through the DL-based pathomic workflow described in the "methodology" section, the training cohort will be assigned to a heterogeneity class and pathomic model trained for heterogeneity classification. The validation cohort will be used to assess the performance of the model. In order to evaluate if radiomic features can predict HER2 heterogeneity at intra-tumor macroscopic level, a *different* radiomic model will be developed for this purpose, using the HER2 heterogeneity score assigned by pathologist in the previous task as outcome. In addition, in order to confirm and quantify inter-lesion HER2 spatial heterogeneity, the features included in the discovered «radiobiopsy» signature will be extracted and the HER2 status calculated for the other metastatic lesions. To reach this purpose, different lesions from the same CT scan exam included in Task 1 and 2 will be segmented using the following algorithm: (1) at least one lesion from the same organ different to the one used for model development, if available; (2) at least one lesion for each of the different metastatic organs involved, if present. A minimum of 3 lesions and a maximum of 5 lesions will be considered for this task. A correlation analysis between HER2 status assignment from different lesions of the same patients will be performed to quantify the prevalence of HER2 variability among lesions in the cohort. In order to clarify if HER2 heterogeneity at microscopic level is associated with inter-lesion heterogeneity, a single-patient correlations between the heterogeneity pathomic score and the inter-lesion radiomic heterogeneity will be performed. Finally, to test if the intratumoral and inter-lesion heterogeneity can be predictable of T-DXd efficacy, both intratumor heterogeneity and inter-lesion heterogeneity score will be correlated with survival outcomes of patients treated with T-DXd enrolled for Section 2 proposal. A Cox regression analysis

	will be used to test the ability of these scores to predict different outcomes of patients treated with T-DXd. Section 3 proposal is summarized at Figure 2. **Section 4: Explanation of agnostic features with multi-omic correlations.** To have insight into the features included into the «radiobiopsy» model, transcriptomics analysis will be conducted through bulk-RNA sequencing from available tumor FFPE specimens of lung, pleural and liver biopsies (~100 samples from INT). The differential expression of several genes and transcriptomic signatures with known prognostic/predictive relevance, such as immune and metabolic genes and signatures, will be assessed among samples with differently expressed features included in the model. This will allow to dissect tumor biology by finding the biological counterpart of agnostic radiomics features that would remain otherwise unexplained. In addition, to test if the «radiobiopsy» model is able to predict the HER2 status defined by transcriptomic profile, correlation between radiomics classes and single-gene ERBB2 expression, HER2-pathway associated signature and PAM50 classification will be also performed.
Study objectives	The primary objectives of the study are: • To identify a radiomic signature («radiobiopsy») able to predict the HER2 status on a specific metastasic site of mBC patients based on radiologic images (CT scan, PET-FdG) • To test the accuracy of «radiobiopsy» in predicting PFS in a cohort of mBC patients treated with T-DXd. • To identify a pathomic-based model able to predict intratumoral HER2 heterogeneity on pathology slides of HER2-positive and HER2-low mBC patients. • To test the concordance between intratumoral and inter-lesional HER2 heterogeneity in HER2-positive and HER2-low mBC patients. • To test the effect of inter-lesional heterogeneity on PFS mBC patients treated with T-DXd. • To correlate the features included in the radiomic model with the transcriptomics data obtain from bulk-RNAseq from biopsies, in order to explain agnostic features discovered with radiomics. Secondary objectives include:

	• To identify a radiomic-based model able to predict intratumoral HER2 heterogeneity on baseline radiological scan of HER2-positive and HER2-low mBC patients. • To characterize the prevalence of inter-lesional HER2 heterogeneity in pluri-metastatic BC patients. • To identify a new radiomic signature able to predict efficacy of T-DXd in metastatic HER2-positive metastatic BC patients. • To integrate radiomics with clinical information to finally create a tool able to predict HER2 status based on available pts characteristics (e.g. HER2 status on primary biopsy, ER status) and actual radiological images. • To test the accuracy of HER2 status predicted by «radiobiopsy» model to predict the efficacy of subsequent anti-HER2 therapy (Section 1 cohort).
Study population	**<u>Section 1:</u>** The population of this first part of the study will consists with a cohort of patients with mBC underwent a liver, lung, pleural or bone biopsy in the metastatic setting at INT with available imaging (CT scan and/or PET-FdG scan), performed from 01Jan2005 to 01Jan2024. Patients will be excluded in the case of: unknown HER2 status; matched available imaging carried out more than three months before the biopsy procedure or before the last previous treatment interruption; node, soft tissue or other visceral as biopsy site. Patients underwent tumor biopsy in the context of surgery will be included (e.g. orthopaedic surgery to bone stabilization). **<u>Section 2:</u>** The study population for section 2 will include HER2+ or HER2-low mBC patients underwent any-line T-DXd treatment for metastatic disease. HER2 status will be defined from the last tissue biopsy, but patients with only surgical specimen or diagnostic biopsy can be included as well. Patients without available baseline CT or PET scan or patients with a follow up less than 6 months will be excluded. **<u>Section 3:</u>** For the proposal of this section, the same inclusion and exclusion criteria for Section 1 and 2 will be applied. For inter-lesional HER2 heterogeneity

	evaluation task, patients with less than three metastatic sites will be excluded from the analysis. **Section 4:** Patients included in this section must reflect the same criteria adopted for Section 1, with these additional exclusion criteria: patients underwent bone biopsy; patients underwent biopsy before 2010.
Methodologies	*Radiomics Methodology:* A team of four radiologists with experience on CT scan/PET images segmentation (G.G., R.V., M.R., G.C.) will identify the Volume Of Interest (VOI) through a semi-automated 3D segmentation process performed with Syngo.via software. The VOI will be selected as the same lesion underwent diagnostic biopsy. Peritumoral area will be obtained through automated augmentation techniques (+5 mm, +10 and +15 mm); if the peritumoral area obtained crosses blood vessels, gas, bone, bile duct, or exceeded the organ edge, the contour will be manually modified to exclude this area. After image pre-processing through different techniques (e.g., gray discretization, intensity normalization, and voxel resampling), radiomic features will be extracted from VOIs and peritumoral areas using the PyRadiomics library.[30] In order to prevent signature overfitting, dimensionality of features is reduced before signature construction, firstly excluding features with high intraclass correlation coefficient and significantly different between the two outcome groups as assessed by one-way analysis of variance (ANOVA). Least absolute shrinkage and selection operator (LASSO) regression and/or Maximum Relevance Minimum Redundancy (MRMR) will be then used for the selection of feature that will be included in the final model with 5-fold cross-validation.[31-32] The INT cohort will be splitted in a training (80%) and testing cohort (20%). Different standard machine learning classifiers, such as Random Forest, Multilayer perceptron, Logistic Regression, Support Vector Machine, CatBoost, AdaBoost, XGBoost, will be trained and evaluated for this task. AUC of training cross-validation and testing sets will be used to evaluate the diagnostic performance of radiomics signature in distinguishing HER2 classes (high vs. low vs. 0) and PFS classes during T-DXd therapy (<12 vs ≥12 months). *Pathomics Methodology:* Pathology slides analyzed for HER2 review (Task 1) will be digitalized with Leica Scanscope AT2 and stored as ".svs" files to obtain Whole Slide Images (WSIs). In addition to the HER2-stained slides, Hematoxilin and Eosin (HE) slides will be also digitalized for pathomics purposes. WSIs will be analysed through Slideflow, a Python package that provides a unified

	Application Programming Interface for building and testing deep learning models for histopathology.[33] After color normalization procedures, quantitative pathomics feature will be extracted using a DL approach. Model creation and performance evaluation will follow the same methodology discussed in the "Radiomics" workflow. *Transcriptomics analysis methodology:* After cellular RNA extraction, RNA quality check and preparation of libraries, RNA will be sequenced with the NovaSeq 6000 System. Counts will be analyzed to dissect differential gene expression among radiomics-defined HER2 classes both at single gene level – through differential gene expression analysis (with the Deseq2 method) – and at pathway level – through Gene Set Enrichment Analysis (GSEA), Over Representation Analyses (ORA) and Gene Set Variation Analysis (GSVA).[34-35] The pathway-level analysis will be carried out using the Bioconductor packages fgsea, gsva, and clusterprofiler, taking advantage of the HALLMARK, KEGG, and REACTOME gene sets available from the GSEA Molecular Signatures Database (http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp). Intrinsic subtypes will be assessed through the Absolute Intrinsic Molecular Subtyping (AIMS) algorithm.[36] *Biological evaluation on pathology specimen:* To guarantee the univocal class attribution, a pathologist with experience on BC diagnosis (A.V.) will reassess the HER2 status of all the enrolled pts in a blinded manner, both from training and validation cohort (Task 1). Accordingly to the study proposal, the pathological HER2 status will be intended as categorical, in particular will be considered three classes: HER2 0 (HER2-0), HER2 low (HER2-L) and HER2 overexpressed (HER2-E). The HER2 assessment will be performed according to the updated 2023 ASCO-CAP recommendations for HER2 testing.[37] HER2 heterogeneity will be assessed from the same pathologist in both quantitative and qualitative way. For quantitative evaluation, a measure of dispersion of the distribution between HER2 IHC classes will be calculated; for qualitative evaluation, an heterogeneity pattern between "clustered", "mosaic" and "scattered" will be attributed to the sample.[38] *Statistical considerations:* Kaplan-Meier curves will be compared using the Cox proportional hazards model. AUC values of the models will be calculated using the Caret and pROC R package and compared using Delong's test. The association between two continuous variables will be assessed using the Pearson's correlation test. The continuous variables will be compared using Student's t-test or one-way analysis of variance (ANOVA) followed by post-hoc

	Tukey's test, as appropriate. The optimal cutoff values will be determined to maximize the sum of sensitivity and specificity of each model. For multiple comparisons, P-values will be adjusted using Holm's method. All statistical analyses will be performed using R software version 4.1.0.
Informed consent	Patients who are alive at the time of study conduction, will be asked to sign the informed consent for the treatment of personal data for research purposes. For patients included in the Section 4 cohort will be asked to provide consent for the use of available archival tumor material for research purposes. For patients who are not alive at the moment of study initiation and data collection, we will be unable to ask an informed consent for the use of personal data. In this case, our study will adhere to the rules for the protection of personal data ("Autorizzazione Generale al trattamento dei dati personali effettuata per scopi di ricerca scientifica – 1 marzo 2012", published on "Gazzetta Ufficiale n. 72 del 26 marzo 2012").
Study timelines	The study will start on 1st Mar 2024. Primary results on the first radiomics score are expected by 1st Jan 2025. Secondary analysis on external validation cohort are expected by 1st Jul 2025. Final results including intermodal correlations are expected by 1st Jan 2027.
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