**Supplemental Material**

**A biologic signature for breast ductal carcinoma *in situ* to predict radiation therapy (RT) benefit and assess recurrence risk**

July 20, 2018

**Elements of Supplement**

**Text Sections**

Multi-Site Study

Biologic Signature Factor Selection

Retrospective Data Collection

Sample Preperation and Assay Methodology

Population Definitions

Biologic Signature Development

Construction of Linear Panel for Comparison to Biologic Signature

**Supplementary** **Tables**

Table S1. Biomarker Assay Methodology

Table S2. Multivariate Cox Proportional Hazard Analysis of DS, RT and Study Site by Risk Groups

Table S3. Clinicopathologic Features by Treatment and Risk Group

Table S4. Interaction Analysis of RT by DS

Table S5. DS Group Classification within Select Clinicopathologic Factors

Table S6. Analysis of Linear Model at Select Thresholds

**Supplementary** **Figures**

Figure S1. Study Population Diagram

Figure S2. Biologic Signature Interrogates Comprehensive Set of Pathways.

Figure S3. Baseline Risks as a Function of Year of Diagnosis

Figure S4. Multivariate Analysis of Clinicopathologic and Molecular Factors Excluding Patients with Positive Margins for 10 Year IBE Risk

**SUPPLEMENT**

**Multi-Site Study**

DCIS cohorts that included annotated tissue sections with long-term follow-up are rare and precious resources. The strategy to include multiple sites was chosen to increase patient diversity and numbers, as high quality DCIS studies with annotated tissue samples and 10-year outcome are rare. A multiple cross-validation approach was used to further improve model generalization to the real-world population. To conserve such resources both whole tissue sections and tissue microarrays were included in the development and cross-validation. When utilized, TMAs were constructed of two 1mm cores per patient which were assayed and then scored jointly.

**Biologic Signature Factor Selection**

Biomarker candidates were selected based on a literature review of biomarker studies conducted between 1990 and 2012. Additional candidates of interest were added based on private discussions with researchers at top research institutions. Selection criteria was based on several factors; (1) univariate marker prognostic utility for identifying ipsilateral breast events (IBE) being either ductal carcinoma in situ (DCIS) or invasive breast cancer (IBC); (2) availability of antibodies; (3) freedom to operate based on review of intellectual property and licensing negotiations, **(**1**,**2**,**3**,**4**,**5**)** Clinicopathologic factors were also screened for candidate selection. Considerations for selection included prognostic utility, reproducibility and availability of data.

**Retrospective Data Collection**

This was a retrospective study on archived tissue samples. Patient outcome and clinicopathologic data was collected for all patients, regardless of their treatment or whether tissue was available. Consistent with “Reporting recommendations for tumor Marker prognostic studies” (REMARK) guidelines, the numbers of patients deemed ineligible and the specific reasons for their ineligibility will be reported. The study was conducted with the appropriate institutional approvals. Uppsala University Regional Ethical Review Board: 1995/170, 1999/422 and 2005:118 and UMass Medical School Tissue and Tumor Bank Institutional Review Board, Institutional Biosafety Committee.

**Sample Preparation and Assay Methodology**

*University of Massachusetts Cohort*

FFPE tissue specimens were selected according to a standardized protocol by a centralized pathology review of H&E sections by UMass. Generally, the tissue block containing the largest amount of high grade DCIS was selected. Histotechnologists cut sections and mounted them on positively charged glass microscope slides. At least 10 sections (10 slides) were prepared for each patient. Slides were labeled with anonymized study IDs to link tissue specimens to outcome data in the study database. If insufficient tissue was available to prepare 10 slides, an alternate block was selected and sectioned. Specimens were shipped in batches from UMass to PreludeDx. Immunohistochemistry (IHC) assays were conducted according to established protocols on a Leica BOND MAX (Leica). Positive and Isotype controls were included in all staining runs. HER2 was assessed by FISH and IHC. FISH was used as the primary data source when available. Markers were scored according to standard protocols and reported as percentage, intensity, positive or negative or Allred score, depending on the marker, by PreludeDx. HER2 was also independently assessed by Phenopath.

*Uppsala University Hospital Cohort*

UUH conducted a central pathology review of H&E sections to select FFPE tissue blocks. Tissue microarrays (TMAs) were constructed using the selected tissue blocks. Histotechnologists cut sections from the constructed TMAs and whole blocks, when available. IHC was conducted according to established protocols(2). HER2 by silver in situ hybridization (SISH) was also performed by UUH on an automated instrument, Ventana Benchmark (Ventana Medical Systems, Tucson, AZ) per the manufacturer's protocols for the INFORM HER2 DNA probe and chromosome 17 probes. Positive and Isotype controls were included in all staining runs. Whole sections and dual core TMAs were both scored when both were available, although TMAs were the predominate source. Markers were scored according to standard protocols and reported as percentage, intensity, positive or negative or Allred score, depending on the marker, by UUH and PreludeDx. Consensus scores were used when results from multiple sources existed. In the TMAs, HER2 was assessed by IHC and SISH. SISH was used as the primary data source when available (2). The concordance of IHC staining between original whole section slides and TMA-slides (including between biopsies from the same DCIS lesion) was previously evaluated and demonstrated good concordance(6).

**Population Definitions**

### *Study Population*

All subjects who meet the eligibility criteria, as listed herein .

Inclusion Criteria

* Histologically confirmed DCIS in a single breast in original Path report.
* Age >26 years at diagnosis with known age
* Diagnosed and underwent BCS in specified year range in specified region
* Known Adjuvant RT Therapy

Exclusion Criteria

* Sex ≠ Female
* Any prior invasive cancer
* Carcinoma or suspicious mammogram findings in other breast
* Prior in situ or invasive breast cancer
* Mastectomy or unknown surgery
* No known previous biologic therapy, chemotherapy, endocrine therapy, or chest RT
* Suspicious post-operative mammogram within 6 months, unless re-excised
* Less than 6 month follow-up
* RT Status Unknown

Tissue and Data Availability:

* FFPE tissue block was available
* HER2 and PR biomarker results available
* Not more than 3 biomarkers missing

**Biologic Signature Development**

A series of literature reviews of publications and patents were conducted to identify molecular markers and clinicopathologic factors associated with DCIS recurrence or progression to invasive breast cancer from 1990 to 2012. Subsequently, candidate features were selected from this review as well as from prior unpublished research **(**1**,**5**,**7**,**8**,**9**,**10**,**11**).** The utility of constructed features (single factors or interactions between and within markers and clinicopathologic factors) was explored usingmachine learning techniques such as forward-backward feature selection, false discovery rate analysis, and cross-validated modeling in patients treated with breast conserving surgery. In order to account for interdependencies known to be present in the oncogenic pathways, a non-linear modeling process was used. This allows for each composite factor value to depend on two biomarker values whereas previous development efforts focused on prognostic or predictive tests for breast cancer have used linear weighting for each biomarker, which is unable to account for these complex interactions. The non-linear modelling techniques employed herein do not use a single threshold to interpret an assay signal as a positive or negative result. Instead, the techniques define a monotonic mapping from the assay signal to a DS input value for each factor that ranges from 0 to 1. For each individual factor, if there is an “equivocal” range where the DS input value varies between 0 and 1. Above and below this range, the DS input value is 0 or 1, respectively. This approach eliminates the commonly encountered issues with single threshold systems. Final selection of the candidate molecular markers and clinicopathologic factors was based on overall ability to accurately identify recurrences (e.g. prognostic utility).

The staining and scoring of biomarkers was conducted according to standardized protocols, which are described in supplementary Table S1. Invasive breast cancer (IBC) events included all first invasive breast cancer events that were ipsilateral (local, regional) or distant metastatic disease. A contralateral invasive breast event prior to a distant metastatic event, censored the IBC event. Total ipsilateral breast events (IBE) included all ipsilateral DCIS events or IBC after the primary DCIS. Analyses were based on time from primary DCIS diagnosis to recurrence. If a patient did not have any subsequent event, censoring occurred at death or last follow-up.

Within the study population, the non-linear biologic signature was developed using machine learning techniques with the goal of identifying clinically relevant low and elevated risk groups with differential RT benefit (12,13,14,15). The biologic signature was parameterized and tested using multiple cross-validation and produced a continuous score. A risk threshold was selected using the training datasets in the cross-validated development with the goal of identifying an average 10-year IBE risk of ten percent or less and an average 10-year IBC risk of six percent or less. Patients with a score greater than the threshold belonged to the Elevated Risk Group. A cost function balanced the objectives of prevalence in the Low Risk Group with the goal of maximizing the hazard ratio (HR) between the Low and Elevated Risk Groups.

During cross-validation, the data was mapped into an effect space so that each factor was pre-normalized to have a maximum of unity and a minimum of zero. Training and validation addressed incomplete biomarker data for each subject. Incomplete biomarker data was assigned a 0.5 effect value for use during parameter weighting. Parameterization was performed with the training folds and evaluated using the validation folds. The final set of markers included COX-2 (1,16), FOXA1(1,17,18), HER2 (1,19), Ki-67(1,20), p16/INK4A(1,21), PR (1,22) and SIAH2 (23,24) and four clinicopathologic features (age, size, margin status and palpability)(1), see supplementary Figure S2. A continuous score ranging from zero to ten, termed the Decision Score (DS), was reported for each patient as the median of the multiple cross-validated results. The threshold between the Low and Elevated Risk groups was scaled to three (3) with the Low Risk Group including patients with DS ≤3, and the Elevated Risk Group including patients with DS >3.

**Construction of Linear Panel for Comparison to Biologic Signature**

Individual factors from the biologic signature with the addition of grade were used to construct a linear panel for comparison to the biologic signature. The risk panel was constructed by linearly weighting each of these factors. A multiple cross-validation approach was used to further improve the weighted panel’s generalization and serve as a realistic comparator such that parameterization was performed with the training folds and evaluated using the validation folds. The consensus result from the simple linear panel was normalized from 0 to 10. The ability of the linear model to identify a lower and higher risk subset of patients was assessed with survival analysis for 10-year risk of IBC and IBE using thresholds from 2 to 7.

**SUPPLEMENTAL REFERENCES**:

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**Supplementary Tables**

**TITLE:** Table S1. Biomarker Assay Methodology

**CAPTION:** None

**TITLE:** Table S2. Multivariate Cox Proportional Hazards Analysis of DS, RT and Study Site by Risk Groups

**CAPTION:** A multivariate Cox proportional hazards analysis was performed with DS, RT as a function of DS and study site, and Year of Diagnosis, excluding margin positive patients (n=474). Abbreviations: 95%CI = 95% Confidence Interval; DS = Decision Score; RT = Radiation therapy; HR = Hazard Ratio; UMass = University of Massachusetts; UUH = Uppsala University Hospital.

**TITLE:** Table S3. Clinicopathologic Features by Treatment & Risk Group (margin negative)

**CAPTION:** None

**TITLE:** Table S4. Interaction Analysis of RT by DS

**CAPTION:** Interaction analysis of RT and DS, with covariates DS, RT, and (RT & DS>X) in the subset of patients with clear margins. IBC and IBE risk was dependent on DS (prognostic), and RT benefit was dependent on DS for both IBC and IBE risk (predictive). For DS above 2.1 the RT benefit for IBC was substantial and statistically different from the baseline RT benefit, which had a non-significant HR near unity. For DS above 2.7 the RT benefit for IBE was substantial and statistically different from the baseline RT benefit which had a non-significant HR near

unity. Abbreviations: 95%CI = 95% Confidence Interval; DS = Decision Score; RT = Radiation therapy; HR = Hazard Ratio.

†For IBC, X=2.1; for IBE, X=2.7

**TITLE:** Table S5. DS Group Classification within Select Clinicopathologic Factors

**CAPTION:** DS Groups were used to stratify patients within clinicopathologic factors to show the number and percentage of patients at low and elevated risks, excluding margin positive patients (n=474). Abbreviations: Grade = Nuclear Grade; DS = Decision Score

**TITLE:** Table S6. Analysis of Linear Model at Selected Thresholds

**CAPTION:** A Cox proportional hazards analysis was performed using a linear model that caclulates a score on a scale from 0 to 10. The score correlates to a 10 year IBE risk for patients treated with BCS, excluding patients with Positive Margins (n=196). Abbreviations: IBE = Ipsilateral Breast Event

**Supplementary** **Figures**

**TITLE:** Figure S1. Study Population Diagram

**CAPTION:** None

**TITLE:** Figure S2. Biologic Signature Interrogates Comprehensive Set of Pathways

**CAPTION:** The Decision Score on a scale from zero to ten is derived from protein expression of a comprehensive biological and clinico-pathologic profile.

**TITLE:** Figure S3. Baseline Risks as a Function of Year of Diagnosis

**CAPTION:** The risk of IBC (panel A) and IBE (panel B) were determined from Kaplan Meier analysis as a function of year of diagnsis (+/- 2 year window) for the study population. For all patients in each window (+/- 2 years) centered around the year of diagnosis, the 10-year IBC or IBE risk and the 95% confidence interval was calculated. The mean 10-year risks are points, and the 95%CI are lines. The transition period after 1995 is observable in both the IBC and IBE 10-year risks. Abbreviations: IBC = invasive breast cancer; IBE = ipsilateral breast event

**TITLE:** Figure S4. Multivariate Analysis of Clinicopathologic and Molecular Factors Excluding Patients with Positive Margins for 10 Year IBE Risk

**CAPTION:** A multivariate Cox proportional hazards analysis was performed with clinicopathologic and molecular factors as well as RT as covariates, excluding patients positive margins (n=474) for 10 year IBE risk. Significant hazard ratios are indicated by larger filled dots and non-significant hazard ratios are indicated with hollow dots. Abbreviations: IBE = Ipsilateral Breast Event; RT = Radiation Therapy.