Supplementary Online Content

Datta J, Fracol M, McMillan MT, et al. Association of depressed anti-HER2 $CD4^+$ T-helper type 1 response with recurrence in patients with completely treated HER2-positive breast cancer: role for immune monitoring? *JAMA Oncol*. Published online December 30, 2015. doi:10.1001/jamaoncol.2015.5482

This supplementary material has been provided by the authors to give readers additional information about their work.

eMethods

Scheduling and dosing of trastuzumab and chemotherapy regimens

T+C-treated patients received one of the following regimens, either preoperatively or post-operatively: (1) AC/TH: Adriamycin/Cytoxan (every 2 weeks for 4 cycles) followed by Taxol and concurrent trastuzumab (weekly for 12 weeks); (2) TCH: Taxotere/carboplatin with concurrent trastuzumab (every 3 weeks for 6 cycles), or (3) TC-H: Taxotere/Cytoxan with concurrent trastuzumab (every 3 weeks for 4 cycles). All patients received additional trastuzumab alone (every 3 weeks) to complete a full year of therapy.

PBMC processing and handling

Between 25 and 30 mL of whole blood was collected from each study participant in 5 heparinized collection tubes (BD Bioscience). Shortly after phlebotomy (<4-6 hours), PBMCs were isolated using density gradient centrifugation (i.e., Ficoll-Paque method; Fisher Scientific) according to manufacturer's instructions, and cryopreserved at $10x10^6$ cells/mL in 10% DMSO in human serum at -80°C; all PBMCs were utilized within 4 weeks of cryopreservation. Viability upon thaw was between 60 and 80%. Briefly, PVDF membrane plates (Mabtech Inc.) were coated overnight with anti-IFN-γ capture antibody. Cryopreserved PBMCs were thawed into pre-warmed DMEM+5% human serum, and prepared at $1x10^6$ PBMCs/mL in said medium. After plates were washed and blocked, PBMCs were plated in triplicate ($2x10^5$ cells/well), and incubated at 37°C for 24-36 hours with either HER2 peptide(s), medium alone, or positive control (refer to **Methods** section).

Flow cytometry

PBMC were suspended in FACS buffer (PBS+1% FCS+0.01% azide) and PE/FITC/Cy5 -conjugated mouse anti-human CD3, CD4, CD25, T-bet, GATA-3, IFN-γ, or subclass-matched controls (BD Bioscience) were used to determine relative PBMC immunophenotype. Intracellular staining with anti-FoxP3 using FoxP3 fixation/permeabilization kit (Biolegend) was performed according to manufacturer's instructions. Analysis was performed using BD LSR-II cytometer, and datasets analyzed using CellQuest Pro software.

Functional contribution of Th1 vs. Th2 subtypes

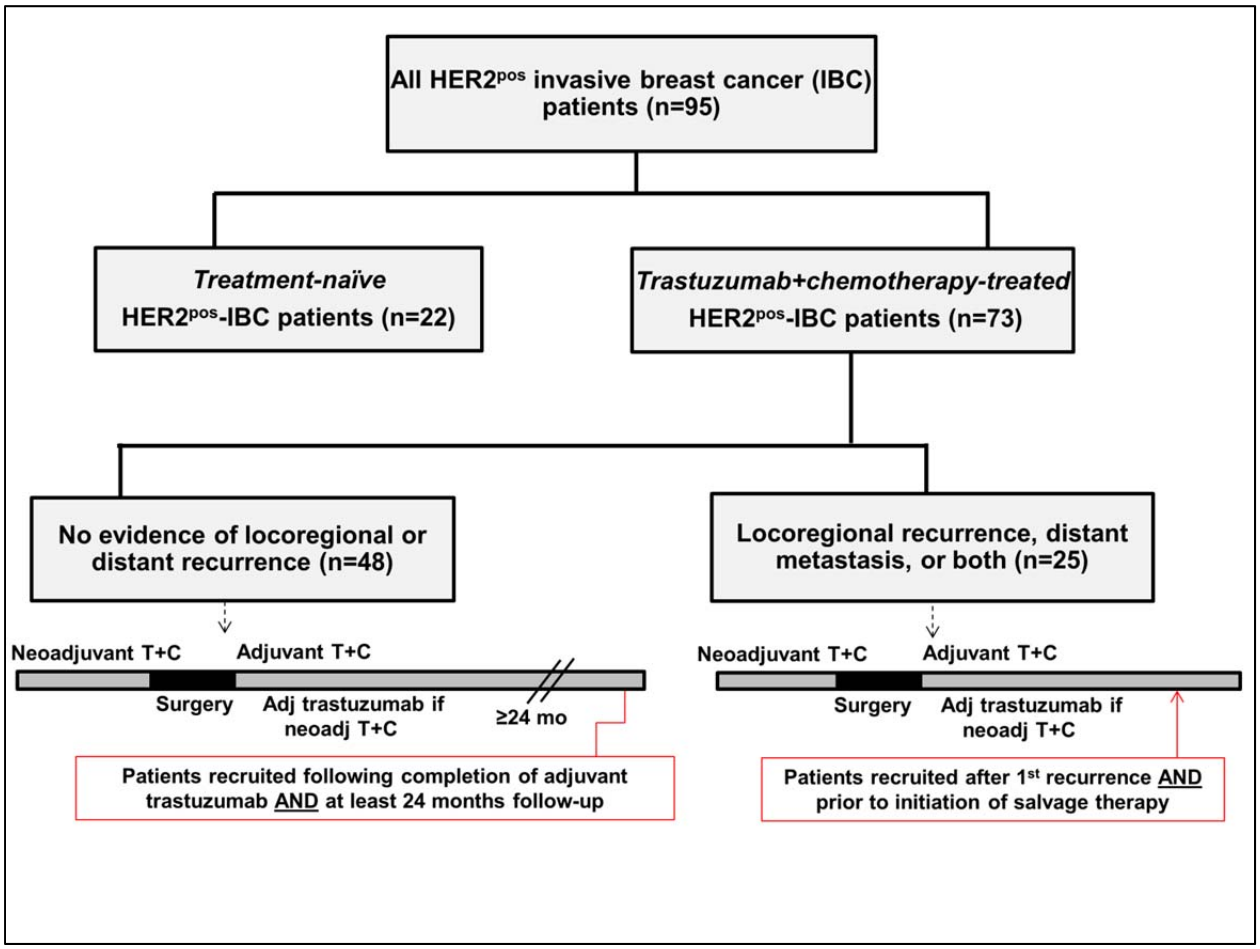
PBMCs were resuspended at $1.2x10^6$ cells/mL in DMEM + 5% human serum in 24-well plates, and pulsed with HER2-class II peptide mix (24 μg/mL). Unstimulated and anti-CD3/CD28 antibody-pulsed PBMCs from each donor served as negative and positive control, respectively. Following incubation for 6 hours at 37°C, protein transport inhibitor Brefeldin-A (Sigma Aldrich; 10 μg/ml) was added to each sample, and incubated overnight. Following washing, cells were stained with anti-human CD4 for 30 min at room temperature. Cells were subsequently washed twice, fixed and permeabilized using Foxp3 fixation/permeabilization kit (Biolegend) according to manufacturer's instructions, and stained with anti-T-bet, anti-GATA-3 and anti-IFN-γ (Biolegend) for 30 min. After incubation, cells were washed and analyzed on a BD LSR-II cytometer.

Statistical analysis

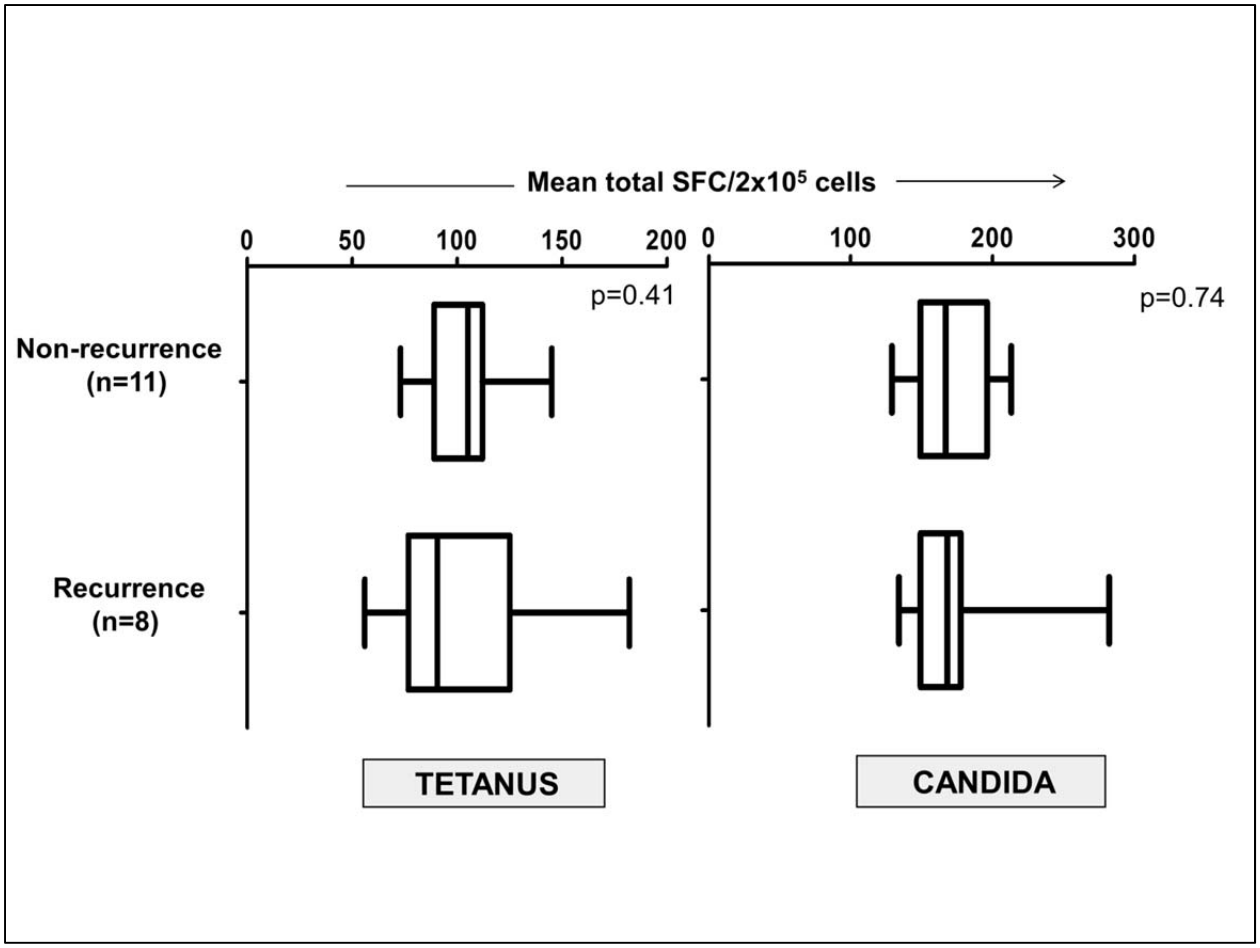
Descriptive statistics summarized distributions of patient characteristics and immune response variables. Comparisons between non-recurrent and recurrent $HER2^{pos}$-IBC cohorts were performed as indicated: (a) 2-group/univariate testing — unpaired Student's t-test (parametric continuous), Wilcoxon rank-sum test (non-parametric continuous), and $\chi 2$ tests (categorical); (b) >2-group testing — one-way

ANOVA with post-hoc Bonferroni testing. Variables with $p<0.1$ on univariate testing were entered into a forward, stepwise multivariable logistic regression model ($p<0.05$ for entry) to determine independent correlates to recurrence (binary outcome yes/no). Missing data for post-neoadjuvant pathologic response status (n=36) and LVI (n=12) were imputed using the Markov chain Monte Carlo method. Five imputation data sets were created and five sets of analyses were combined per Rubin's formula.

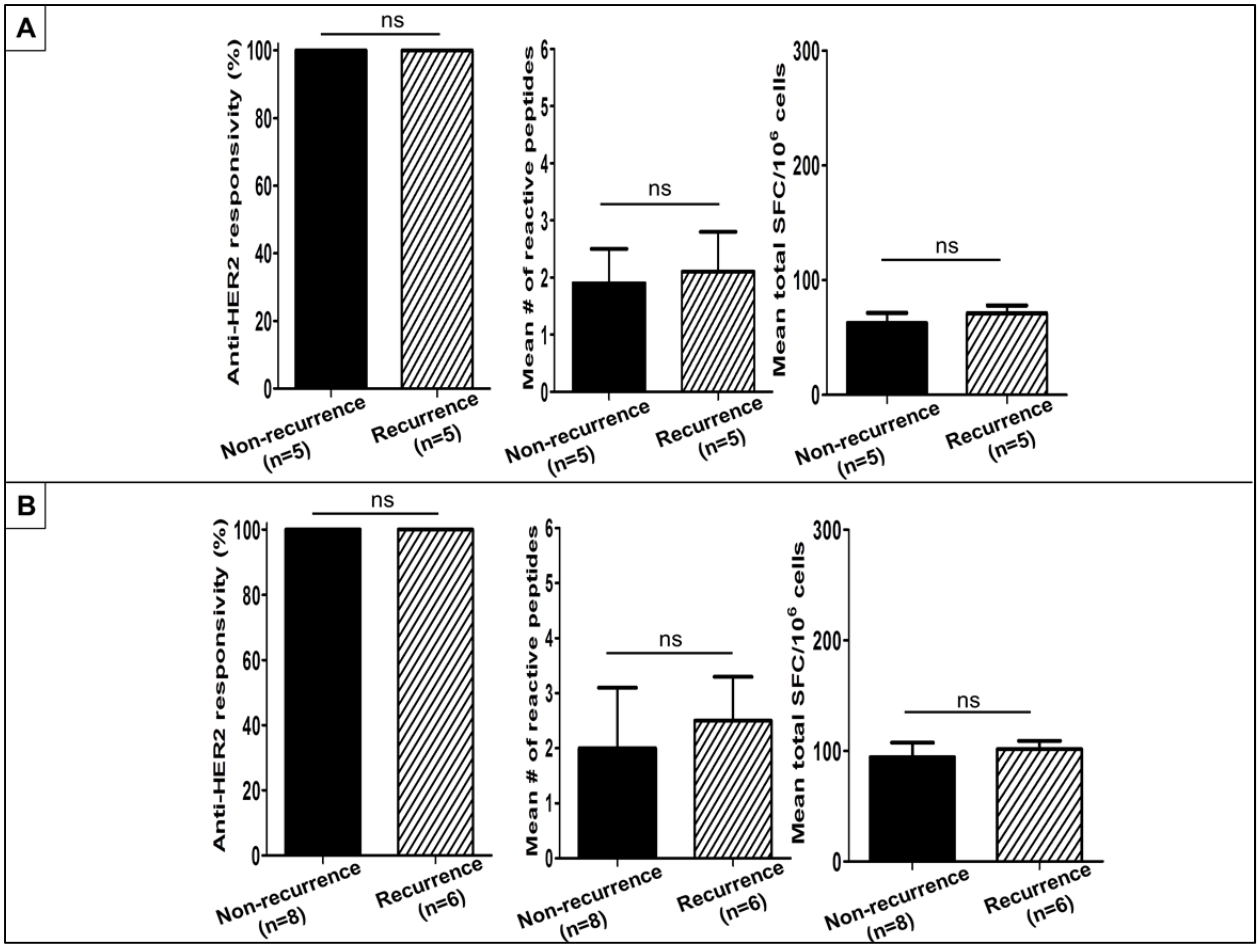
Univariate disease-free survival (DFS) estimates were examined by Kaplan-Meier methodology, stratifying by anti-HER2 Th1 responsivity and other covariates. Observations of non-recurrent patients (minimum 24 month follow-up) were censored at last known follow-up. To analyze the instantaneous hazard of all variables and control for varied follow-up, Cox proportional hazards modeling was performed. The assumptions of the Cox model were assessed, including interactions and proportionality of hazards over time. $P<0.05$ was considered statistically significant. All tests were two-sided. Analyses were performed using SPSS version 22 (IBM Corp).



eFigure 1. **Flow diagram of study populations**. In this study, 95 HER2^{pos} breast cancer patients were enrolled; all tumors were histologically confirmed invasive breast cancer with HER2 overexpression (3+ or 2+/FISH-positive). Time-points at which blood was drawn are indicated in red callout boxes. Median follow-up in the trastuzumab and chemotherapy (T+C)-treated cohort was 44 (IQR 31) months.



eFigure 2. PBMCs from non-recurrence and recurrence cohorts did not differ significantly in IFN-γ production to recall stimuli tetanus toxoid (*left*) and *Candida albicans* (*right*) by ELISPOT. Results presented as median ± interquartile range (IQR) IFN-γ SFC/2x10^5 cells.



eFigure 3. Circulating HER2-specific Th2 and T_{reg} functional contributions. (A) Circulating HER2-specific IL-4 production — a surrogate for Th2 function — does not vary between non-recurrence and recurrence cohorts, when assessed by anti-HER2 Th1 responsivity, repertoire, and cumulative response. Results expressed as proportion or mean ± SEM. **(B)** HER2-specific IL-10 production — a surrogate for T_{reg} function — is similar between non-recurrence and recurrence cohorts across all three Th1 metrics. Results expressed as proportion or mean ± SEM.

eTable 1. Demographic and tumor-related characteristics of study populations.
Age, race, AJCC pathologic stage, hormone receptor status, and time from completion of trastuzumab (when applicable) for study populations.

Characteristic	*Treatment-naïve* HER2^{pos}-IBC (n=22)		*T+C-treated* HER2^{pos}-IBC (n=73)			
			Non-recurrence (n=48)		Recurrence (n=25)	
Age						
Mean ± SE	54.7 ± 3.1		49.6 ± 1.8		47.2 ± 2.2	
Range	24 – 88		25 – 85		24 – 80	
	#	%	#	%	#	%
Race/Ethnicity						
Caucasian	17	77.3	39	87.5	18	72.0
African-American/Asian/Hispanic	5	22.7	9	7.5	7	28.0
AJCC stage at diagnosis*						
Stage 1	14	63.6	5	10.4	2	8.0
Stage 2	6	27.3	23	47.9	12	48.0
Stage 3	2	9.1	20	41.7	11	44.0
Hormone receptor status						
ER/PR^{pos}	12	54.5	26	54.2	17	68.0
ER/PR^{neg}	10	45.5	22	45.8	8	32.0
Time from completion of trastuzumab to study enrollment						
<6 months			23	47.9	11	44.0
≥6 months			25	52.1	14	56.0

*clinical stage indicated for all cohorts

Abbreviations: HER2^{pos}: Human epidermal growth factor receptor overexpressing; IBC: invasive breast cancer; T+C: trastuzumab and chemotherapy, AJCC: American Joint Committee on Cancer; ER: estrogen receptor; PR: progesterone receptor

eTable 2. Demographic, tumor-related and treatment characteristics of HER2-overexpressing breast cancer patients incurring recurrence following trastuzumab and chemotherapy (T+C).

Pt no.	Age (yrs)	Stage at initial diagnosis	Timing of T+C receipt	ER/ PR	Initial operation	Initial HER2 agent	Type of recurrence	Location, if distant	Time to recurrence (months)	Salvage therapy following recurrence
1	48	3	Neoadj	+	L MRM, R simple	Trast	Distant	Bone	22	Chemo, Lapatinib
2	51	2	Adj	+	L BCS	Trast	Locoregional	--	16	Trastuzumab, AI
3	24	2	Neoadj	-	R BCS	Trast	Locoregional	--	49	Chemo, Trastuzumab
4	52	2	Neoadj	-	L BCS	Trast	Locoregional, Distant	Lung	16	Chemo, Trastuzumab
5	35	3	Neoadj	-	R MRM	Trast	Distant	Lung, liver, bone	27	Chemo, Trastuzumab, Pertuzumab
6	58	2	Neoadj	+	L MRM	Trast	Distant	Lung, Bone	43	Fulvestrant, Chemo, Trastuzumab, Pertuzumab
7	80	2	Neoadj	+	Bilateral BCS	Trast	Distant	Bone	48	Trastuzumab, Pertuzumab
8	39	2	Neoadj	+	L BCS + ALND	Trast	Locoregional, Distant	Lung	42	Chemo, Trastuzumab, Pertuzumab
9	47	3	Adj	-	R BCS	Trast	Locoregional	--	64	Chemo, Trastuzumab
10	41	3	Adj	+	L BCS + ALND	Trast	Locoregional	--	113	Chemo, Trastuzumab
11	52	1	Adj	+	R BCS + ALND	Trast	Locoregional	--	82	Chemo, AI, Trastuzumab
12	30	2	Adj	+	L BCS	Trast	Locoregional	--	43	HER2-DC1 vaccine
13	50	3	Adj	-	R BCS + ALND	Trast	Locoregional	--	42	Resection alone
14	55	2	Adj	+	R MRM	Trast	Locoregional	--	26	Chemo, Trastuzumab, Pertuzumab, Lapatinib
15	49	3	Adj	-	L MRM, R simple	Trast, Lap	Locoregional	--	13	Chemo, Lapatinib, TDM-1
16	54	3	Adj	+	R MRM	Trast, TDM-1	Locoregional	--	19	Resection, HER2-DC1 vaccine
17	42	2	Adj	+	R MRM, L simple	Trast, TDM-1	Distant	Lung	51	Chemo, Pertuzumab, HER2-DC1 vaccine
18	45	3	Adj	-	L MRM	Trast, Lap	Distant	Lung, Brain	60	AI, Trastuzumab, Pertuzumab, HER2-DC1 vaccine
19	34	1	Adj	+	L BCS	Trast	Locoregional	--	47	Resection + HER2-DC1 vaccine

20	41	2	Adj	-	L MRM	Trast, Lap	Distant	Liver	16	Chemo, Trastuzumab, Lapatinib, Kadcycla, CDK 4/6 inhibitor
21	42	3	Adj	+	L MRM	Trast	Locoregional, Distant	Bone, Brain	23	AI, Trastuzumab, TDM-1
22	51	3	Adj	+	L MRM, R BCS	Trast	Distant	Lung, Bone	58	Chemo, AI, Trastuzumab, Lapatinib, Pertuzumab
23	54	3	Adj	+	L BCS + ALND	Trast	Distant	Bone	53	Chemo, Trastuzumab, Pertuzumab
24	44	2	Adj	+	R MRM	Trast	Locoregional, Distant	Lung	10	Chemo, Trastuzumab, Pertuzumab, TDM-1
25	62	2	Adj	+	R BCS	Trast	Distant	Liver, Lung	46	Chemo, Trastuzumab, Pertuzumab

Abbreviations: T+C: trastuzumab and chemotherapy; ER: estrogen receptor; PR: progesterone receptor; Neoadj: Neoadjuvant; Adj: Adjuvant; MRM: Modified radical mastectomy; Simple: Simple mastectomy; BCS: Breast conservation surgery; ALND: Axillary Lymph Node Dissection; Trast: Trastuzumab; AI: Aromatase Inhibitor; DC1: Type 1-polarized dendritic cell vaccine.

eTable 3. Univariate comparison of demographic and tumor-related characteristics between $HER2^{pos}$-IBC patients with or without disease recurrence. Variables with p<0.10 were entered into a multivariable logistic regression to identify independent correlates to disease recurrence

Characteristic	**Non-recurrence** *n (%) or median (IQR)*	**Recurrence** *n (%) or median (IQR)*	**Univariate p-value**	**OR (95% CI)**	**Multivariable p-value**
Overall population	48 (65.8)	25 (34.2)	--	--	--
Age Median (years)	49.5 (39-57)	48.0 (41-53)	0.518		
BMI Median (kg/m^2)	27 (25-32)	27.3 (24.2-31.0)	0.506		
Race White Black/Asian/Hispanic	39 (81.3) 9 (18.8)	18 (72.0) 7 (28.0)	0.365		
Charlson comorbidity index 0 ≥1	27 (56.3) 21 (43.7)	12 (48.0) 13 (52.0)	0.532		
Menopausal status Pre-menopausal Post-menopausal	24 (50.0) 24 (50.0)	13 (52.0) 12 (48.0)	0.871		
AJCC clinical stage I II III	5 (10.4) 23 (47.9) 20 (41.7)	2 (8.0) 12 (48.0) 11 (44.0)	0.941		
ER/PR status Negative Positive	22 (45.8) 26 (54.2)	8 (32.0) 17 (68.0)	0.254		
Lymphovascular invasion* Absent Present	14 (34.1) 27 (65.9)	11 (55.0) 9 (45.0)	0.120		
Nuclear grade Low/intermediate High	15 (31.3) 33 (68.8)	11 (44.0) 14 (56.0)	0.280		
Chemotherapy sequence‡ Neoadjuvant T+C Adjuvant T+C	29 (60.4) 19 (39.6)	8 (32.0) 17 (68.0)	**0.021**	3.28 (0.67-15.99)	0.142
Pathologic response (if neoadjuvant) *‡ Incomplete response Complete response	13 (44.8) 16 (55.2)	8 (100.0) 0 (0.0)	**0.006**	0.63 (0.03-15.03)	0.764
Operative approach BCS + XRT Mastectomy ± XRT	23 (47.9) 25 (52.1)	11 (44.0) 14 (56.0)	0.750		

Anti-HER2 Th1 responsivity‡ Non-responsive Responsive	8 (16.7) 40 (83.3)	23 (92.0) 2 (8.0)	**<0.0001**	0.015 (0.003-0.083)	**<0.0001**

*****The statistics of imputed variables are pooled over *m*=5 multiple imputations.

‡ Included in multivariable logistic regression analysis to determine independent correlates to recurrence

Abbreviations: BMI: Body mass index; AJCC: American Joint Committee on Cancer; ER: estrogen receptor; PR: progesterone receptor; IQR: interquartile range; AC/TH: Adriamycin/Cytoxan/Taxol/Herceptin; BCS: Breast-conserving surgery; XRT: radiotherapy