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## Journal
PLOS ONE, 16(5)

## ISSN
1932-6203

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## Publication Date
2021

## DOI
10.1371/journal.pone.0251163

Peer reviewed

PLOS ONE

RESEARCH ARTICLE

# Clinical evaluation of BCL-2/$X_L$ levels pre- and post- HER2-targeted therapy

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**Citation:** Zoeller JJ, Press MF, Selfors LM, Dering J, Slamon DJ, Hurvitz SA, et al. (2021) Clinical evaluation of BCL-2/$X_L$ levels pre- and post- HER2-targeted therapy. PLoS ONE 16(5): e0251163. https://doi.org/10.1371/journal.pone.0251163

**Editor:** Elda Tagliabue, Fondazione IRCCS Istituto Nazionale dei Tumori, ITALY

**Received:** January 19, 2021

**Accepted:** April 16, 2021

**Published:** May 5, 2021



**Data Availability Statement:** The TRIO-B-07 data files are publicly available from the GEO database (GSE130788).

**Funding:** Our work was supported by a Department of Defense Breast Cancer Research Fellowship Award [https://cdmrp.army.mil/bcrp/], Award Number W81XWH-11-1-0572 (to J.J. Zoeller), a Breast Cancer Research Foundation Grant [https://www.bcrf.org] (to M.F. Press) and a Stand Up To Cancer-American Association for Cancer Research Dream Team Translational Cancer Research Grant [https://standuptocancer.

## Abstract

Our previous pre-clinical work defined BCL-2 induction as a critical component of the adaptive response to lapatinib-mediated inhibition of HER2. To determine whether a similar BCL-2 upregulation occurs in lapatinib-treated patients, we evaluated gene expression within tumor biopsies, collected before and after lapatinib or trastuzumab treatment, from the TRIO-B-07 clinical trial (NCT#00769470). We detected BCL2 mRNA upregulation in both HER2+/ER- as well as HER2+/ER+ patient tumors treated with lapatinib or trastuzumab. To address whether mRNA expression correlated with protein expression, we evaluated pre- and post-treatment tumors for BCL-2 via immunohistochemistry. Despite BCL2 mRNA upregulation within HER2+/ER- tumors, BCL-2 protein levels were undetectable in most of the lapatinib- or trastuzumab-treated HER2+/ER- tumors. BCL-2 upregulation was evident within the majority of lapatinib-treated HER2+/ER+ tumors and was often coupled with increased ER expression and decreased proliferation. Comparable BCL-2 upregulation was not observed within the trastuzumab-treated HER2+/ER+ tumors. Together, these results provide clinical validation of the BCL-2 induction associated with the adaptive response to lapatinib and support evaluation of BCL-2 inhibitors within the context of lapatinib and other HER2-targeted receptor tyrosine kinase inhibitors.

## Introduction

Adaptive responses to targeted-therapies compromise treatment effectiveness and confer drug resistant phenotypes [1]. Treatment-associated adaptive reprogramming often includes parallel upregulation of alternative receptors and/or activation of alternative pathways, which compensate for target inhibition and maintain tumor growth and survival [2–8]. Our previous work identified the upregulation of an anti-apoptotic program as an adaptive response to lapatinib-mediated inhibition of HER2 in vivo [9] and BEZ235-mediated inhibition of PI3K/mTOR in vitro [10]. BCL-2 induction, mediated via FOXO-dependent transcription and cap-independent translation, was a critical component of this pro-survival response. To determine

org], Grant Number SU2C-AACR-DT0409 (to J.S. Brugge and D.J. Slamon). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** I have read the journal's policy and the authors of this manuscript have the following competing interests: S.A. Hurvitz Contracted Research: Ambrx Amgen Astra Zeneca Arvinas Bayer Daiichi-Sankyo Genentech/Roche Gilead GSK Immunomedics Lilly Macrogenics Novartis Pfizer OBI Pharma Pieris PUMA Radius Sanofi Seattle Genetics Dignitana Zymeworks Phoenix Molecular Designs, Ltd. Stock Options: NK Max Travel: Lilly M.F. Press Consulting or Advisory Role: AstraZeneca Biocartis SA CEPHEID Clinical Care Options, LLC CME Outfitters, LLC Lilly USA, LLC Merck & Co. Puma Biotechnology Zymeworks Inc. Eli Lilly & Company Private Equity Role: TORL BIOTHERAPEUTICS LLC All other authors have declared that no competing interests exist. We have confirmed that the declared competing interests do not alter adherence to all PLOS ONE policies on sharing data and materials.

**Abbreviations:** B×, biopsy; CI, confidence interval; DUA, Data Use Agreement; DF, Dana-Farber; GSE, Gene Series Expression; HCC, Harvard Cancer Center; HER2, human epidermal growth factor receptor-2; ER, estrogen receptor; FOXO, members of the class O of forkhead box transcription factors; H&E, hematoxylin-and-eosin; H-score, histo-score; IDC, invasive ductal carcinoma; IHC, immunohistochemistry; ILC, invasive lobular carcinoma; IRB, institutional review board; L, lapatinib; LN, lymph node; mTOR, mammalian target of rapamycin; N, unavailable; NCT, National Clinical Trial; p, p-value; PI3K, phosphoinositide 3-kinase; r, Spearman correlation; RNA, ribonucleic acid; SAM, Significance Analysis of Microarrays; T, trastuzumab; T×, treatment; UCLA, University of California Los Angeles; Y, available.

whether BCL-2 upregulation could be identified in patients treated with HER2-targeted therapies, we utilized tumor samples that were previously collected during the TRIO-B-07 clinical trial (NCT#00769470). TRIO-B-07 was designed to include patients with HER2+/ER- or HER2+/ER+ breast cancers and biopsies before and after 2–3 weeks of either lapatinib and/or trastuzumab treatment (Fig 1). Prior to definitive surgery, patients were treated with additional neo-adjuvant DNA-damaging and anti-mitotic chemotherapies. To specifically address HER2-associated events, we focused on the pre- (baseline) and post- treatment (run-in) biopsies prior to chemotherapy-treatment. To specifically compare the effects of lapatinib versus trastuzumab, we excluded tumors treated with both agents and focused on tumors treated with single agents. As previously described [11], these tumor biopsies were profiled via microarrays (GSE130788) and prepared as FFPE slides.

## Materials and methods

### Biospecimens

Tumor biopsies were collected from patients enrolled in the TRIO-B-07 clinical trial (NCT#00769470) as previously described [11]. TRIO-B-07 was reviewed and approved by multiple institutional review boards (UCLA; Olive View; Western), and all participants signed an IRB-approved informed consent form. The Institutional Review Board (IRB) of the Harvard University Faculty of Medicine reviewed and determined that our study is not human subjects research. The UCLA IRB also reviewed and approved our study. Our UCLA Data Use Agreement (DUA) confirmed that all samples were de-identified and that access to identifiers (or links to identities) of individuals from whom the samples were collected would not be granted under any circumstances. Tables 1–4 summarize patient-relevant characteristics corresponding to the subsets of samples analyzed.

### RNA isolation and microarrays

RNA isolation and gene expression profiling (GSE130788) was performed as previously described [11]. Pre-treatment and post-treatment samples from the lapatinib and trastuzumab-treatment arms of GSE130788 were subjected to Significance Analysis of Microarrays (SAM) analyses using the siggenes (v1.62.0) package in R (v4.0.1). The log10 ratios from GEO for all probes of human BCL-2 homologous and BH3-containing proteins from the BCL-2 Database [12] were tested using the single class (for post-treatment comparisons) or 2-class (for ER+/ER- pre-treatment) d.stat method. For post-treatment, delta was 0.10, corresponding to an FDR of 3.8%. For pre-treatment, delta was 1.0, corresponding to an FDR of 2.6%. Heatmaps were generated with ComplexHeatmap (v2.4.3). For genes with multiple significant probes, the probe with highest variance is presented.

### Tumor histology & IHC assays

Tissue was processed for paraffin embedding, sectioning and hematoxylin-and-eosin (H&E) staining at UCLA. Unstained sections were analyzed by IHC analysis according to previously described procedures [9]. Marker specifics are as follows: BCL-2 (DAKO M0887), BCL-$X_L$ (CST 2764), ER (DAKO M6364), Ki67 (DAKO M7240) and HER2 (Epitomics 42011 or DAKO A0485). BCL-2, BCL-$X_L$, ER and Ki67 results were scored blindly by a board-certified pathologist using H-scores as previously described [13]. For Ki67, the proportion positive reflects the total percentage of tumor cells qualitatively scored with intensities as strong (3+), moderate (2+) and weak (1+).

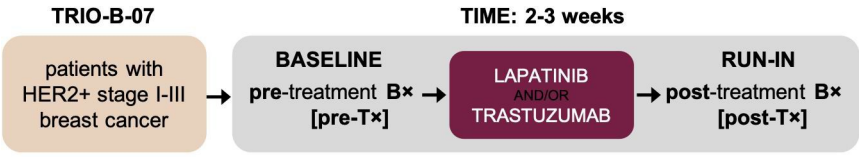


**Fig 1. TRIO-B-07 neo-adjuvant HER2-targeted treatment course.** Patients with HER2+ breast cancer underwent biopsies (B×) before (baseline; pre-treatment; pre-T×) and after (run-in; post-treatment; post-T×) two to three weeks of HER2-targeted therapies (lapatinib; L and/or trastuzumab; T). The number of paired clinical samples available for RNA or protein analysis are described within Tables 1–4.

https://doi.org/10.1371/journal.pone.0251163.g001

## Microscopy

IHC images were digitized at the DF/HCC Tissue Microarray Core or the UCLA Translational Pathology Core on the Aperio Digital Pathology Slide Scanner (Leica Biosystems). The Aperio ImageScope software (Leica Biosystems) was used for image visualization and acquisition.

## Statistics

Statistical analyses were performed using GraphPad Prism version 8 for MAC. For statistical tests, Wilcoxon matched-pairs signed rank test or Mann-Whitney test was applied. For correlation analyses, Spearman correlation was applied.

# Results

Gene expression profiles corresponding to twenty-five lapatinib-treated and twenty-six trastuzumab-treated cases were available for analysis (Tables 1–4). We sub-divided the HER2+/ER-

**Table 1. Lapatinib-treated HER2+/ER- tumors[a].**

| ID | AGE | Histological Type | Histological Grade | Tumor Status | LN Status | Tumor Staging | RNA | PROTEIN |
|---|---|---|---|---|---|---|---|---|
| **30** | 51 | IDC | G2 | T2 | N1 | IIB | Y | N |
| **38** | 51 | IDC | G3 | T2 | N1 | IIB | Y | N |
| **43** | 37 | IDC | G3 | T2 | N0 | IIA | Y | Y |
| **47** | 37 | IDC | G3 | T3 | N0 | IIA | Y | Y |
| **51** | 48 | IDC | G2 | T2 | N1 | IIB | Y | Y |
| **53** | 35 | IDC | G3 | T2 | N1 | IIB | Y | Y |
| **62** | 52 | IDC | G2 | T3 | N1 | IIIA | Y | Y |
| **67** | 54 | IDC | G3 | T4 | N0 | IIIB | Y | Y |
| **74** | 44 | IDC | G3 | T2 | N0 | IIA | Y | Y |
| **85** | 48 | IDC | G2 | T2 | N0 | IIA | Y | Y |
| **90** | 56 | IDC | G1 | T2 | N0 | IIA | Y | Y |
| **91** | 47 | OTHER[b] | G3 | T3 | N0 | IIB | Y | N |
| **97** | 52 | IDC | G3 | T2 | N0 | IIA | Y | Y |
| **109** | 47 | IDC | G1 | T3 | N0 | IIA | N | Y |
| **116** | 50 | IDC | GX | T3 | N0 | IIB | Y | Y |
| **119** | 45 | IDC | G3 | T3 | N3 | IIIC | Y | N |
| **125** | 53 | OTHER[c] | NA[d] | TX | N3 | IIIB | Y | N |

[a]ER H-score = 0.

[b]multi-focal Invasive Carcinoma.

[c]LN.

[d]unknown.

https://doi.org/10.1371/journal.pone.0251163.t001

**Table 2. Trastuzumab-treated HER2+/ER- tumors[e].**

| ID | AGE | Histological Type | Histological Grade | Tumor Status | LN Status | Tumor Staging | RNA | PROTEIN |
|---|---|---|---|---|---|---|---|---|
| **24** | 56 | IDC | G3 | T1 | N0 | I | Y | Y |
| **26** | 62 | IDC | GX | T2 | N1 | IIB | Y | N |
| **27** | 56 | IDC | G2 | T2 | N1 | IIB | Y | N |
| **37** | 51 | ILC | G3 | T3 | N1 | IIIA | Y | N |
| **41** | 52 | IDC | G3 | T3 | N3 | IIIC | Y | Y |
| **60** | 51 | IDC | G2 | T2 | N0 | IIA | Y | N |
| **61** | 46 | IDC | G3 | T2 | N0 | IIA | Y | Y |
| **69** | 59 | IDC | G3 | T4 | N0 | IIIB | Y | Y |
| **72** | 65 | IDC | G3 | T2 | N0 | IIA | N | Y |
| **79** | 44 | IDC | G2 | T1 | N1 | IIA | Y | N |
| **84** | 53 | IDC | G3 | T3 | N1 | IIIA | Y | Y |
| **86** | 35 | IDC | G3 | T1 | N0 | I | N | Y |
| **101** | 28 | IDC | G3 | T2 | N0 | IIA | Y | Y |
| **104** | 76 | IDC | G3 | T2 | N1 | IIB | Y | N |
| **111** | 32 | IDC | G3 | T2 | N0 | IIA | Y | Y |
| **124** | 40 | IDC | NA[f] | T2 | N1 | IIB | Y | Y |

[e]ER H-score = 0.

[f]unknown.

https://doi.org/10.1371/journal.pone.0251163.t002

(pre-treatment ER H = 0) and HER2+/ER+ (pre-treatment ER H > 0) tumors, compared pre-treatment BCL2 mRNA levels within both sub-types and determined that HER2+/ER- tumors were associated with significantly lower BCL2 expression levels (S1 Fig).

We evaluated 2-color Agilent array data comparing the tumor biopsies collected after 2–3 weeks of treatment with either lapatinib or trastuzumab to the pre-treatment tumor biopsies from the same patient and specifically investigated the mRNA expression of BCL2 and other apoptotic regulatory genes from the BCL-2 Database [12]. While there was significant alteration of several apoptotic genes within post-treatment samples, BCL2 mRNA was upregulated

**Table 3. Lapatinib-treated HER2+/ER+ tumors[g].**

| ID | AGE | Histological Type | Histological Grade | Tumor Status | LN Status | Tumor Staging | RNA | PROTEIN |
|---|---|---|---|---|---|---|---|---|
| **35** | 51 | IDC | G2 | T2 | N1 | IIB | Y | Y |
| **54** | 63 | IDC | G1 | T2 | N1 | IIB | N | Y |
| **56** | 66 | IDC | G2 | T2 | N1 | IIB | N | Y |
| **68** | 51 | IDC | G3 | T2 | N0 | IIA | Y | N |
| **71** | 32 | IDC | G3 | T2 | N1 | IIA | Y | Y |
| **78** | 57 | IDC | G3 | T2 | N1 | IIB | Y | N |
| **81** | 54 | IDC | G2 | T1 | N0 | I | Y | Y |
| **100** | 37 | IDC | G3 | T3 | N1 | IIIA | Y | Y |
| **105** | 51 | IDC | GX | T3 | N3 | IIIA | Y | Y |
| **120** | 53 | IDC | G3 | T2 | N1 | IIB | Y | Y |
| **126** | 54 | IDC | G2 | T2 | N1 | IIB | N | Y |
| **128** | 40 | IDC | G2 | T2 | N0 | IIA | Y | Y |
| **129** | 44 | IDC | GX | T2 | N1 | IIB | N | Y |

[g]ER H-score > 0.

https://doi.org/10.1371/journal.pone.0251163.t003

**Table 4. Trastuzumab-treated HER2+/ER+ tumors[h].**

| ID | AGE | Histological Type | Histological Grade | Tumor Status | LN Status | Tumor Staging | RNA | PROTEIN |
|---|---|---|---|---|---|---|---|---|
| **21** | 46 | IDC | G2 | T2 | N0 | IIA | Y | N |
| **22** | 63 | IDC | G3 | T3 | N1 | IIIA | Y | Y |
| **40** | 45 | IDC | G3 | T1 | N1 | IIA | Y | Y |
| **49** | 46 | IDC | G2 | T3 | N1 | IIIA | N | Y |
| **52** | 41 | IDC | G2 | T2 | N0 | IIA | Y | Y |
| **58** | 49 | IDC | G3 | T4 | N2 | IIIB | Y | Y |
| **63** | 49 | IDC | G3 | T4 | N0 | IIIA | N | Y |
| **73** | 37 | IDC | GX | T2 | N1 | IIB | Y | Y |
| **77** | 46 | IDC | G3 | T3 | N1 | IIIA | Y | N |
| **80** | 35 | IDC | GX | T2 | N0 | IIA | Y | N |
| **83** | 46 | IDC | G2 | T3 | N1 | IIIA | Y | N |
| **106** | 45 | IDC | G3 | T3 | N0 | IIB | Y | Y |
| **107** | 44 | IDC | G1 | T3 | N1 | IIIA | Y | Y |
| **122** | 70 | IDC | G3 | T3 | N0 | IIB | Y | Y |

[h]ER H-score > 0.

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in HER2+/ER- (10 out of 16) as well as HER2+/ER+ (6 out of 9) lapatinib-treated tumors (Fig 2). BCL2 mRNA upregulation was also detected in both HER2+/ER- (10 out of 14) and HER2+/ER+ (10 out of 12) trastuzumab-treated tumors (Fig 2). These results indicate that BCL2 mRNA upregulation occurred in both HER2+/ER- and HER2+/ER+ patient tumors treated with lapatinib or trastuzumab.

To address whether BCL2 mRNA expression correlated with BCL-2 protein expression, pre- and post-treatment FFPE tumors were evaluated for BCL-2 by IHC. We evaluated BCL-2 by intensity and proportion scores to calculate an H-score [13] on a case-by-case basis. Twenty-three lapatinib-treated and twenty trastuzumab-treated cases were evaluated by blinded pathological assessment (Tables 1–4). In contrast to HER2+/ER+ tumors, pre-

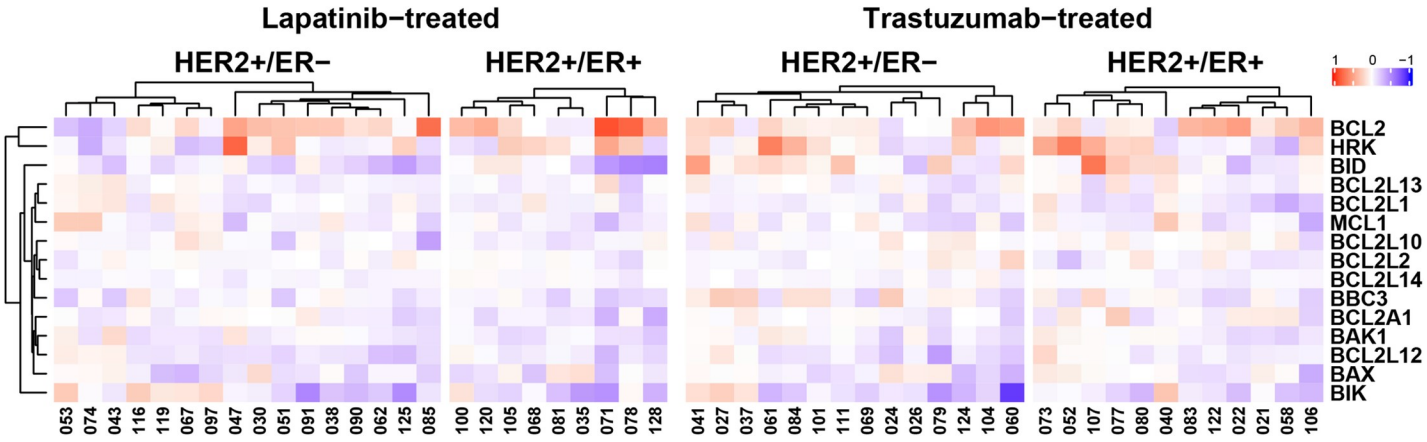


**Fig 2. RNA-based analysis of BCL2 and other apoptotic regulatory genes.** Heatmaps represent lapatinib-treated HER2+/ER- (n = 16) and HER2+/ER+ (n = 9) tumors, and trastuzumab-treated HER2+/ER- (n = 14) and HER2+/ER+ (n = 12) tumors. Each numerical identifier represents a clinical sample pair. Data are the log10 ratio of run-in (post-treatment) sample compared to paired baseline (pre-treatment) sample. Heatmaps include genes with statistically significant differences post-treatment. Heatmaps were generated with ComplexHeatmap (v2.4.3). Note, genes (proteins): BCL2 (BCL-2); HRK (HRK); BID (BID); BCL2L13 (BCL-rambo, MIL1); BCL2L1 (BCL-$X_L$); MCL1 (MCL-1); BCL2L10 (NRH, BCL-B); BCL2L2 (BCL-w); BCL2L14 (BCL-G); BBC3 (PUMA); BCL2A1 (A1); BAK1 (BAK); BCL2L12 (BPR); BAX (BAX); BIK (BIK, NBK).

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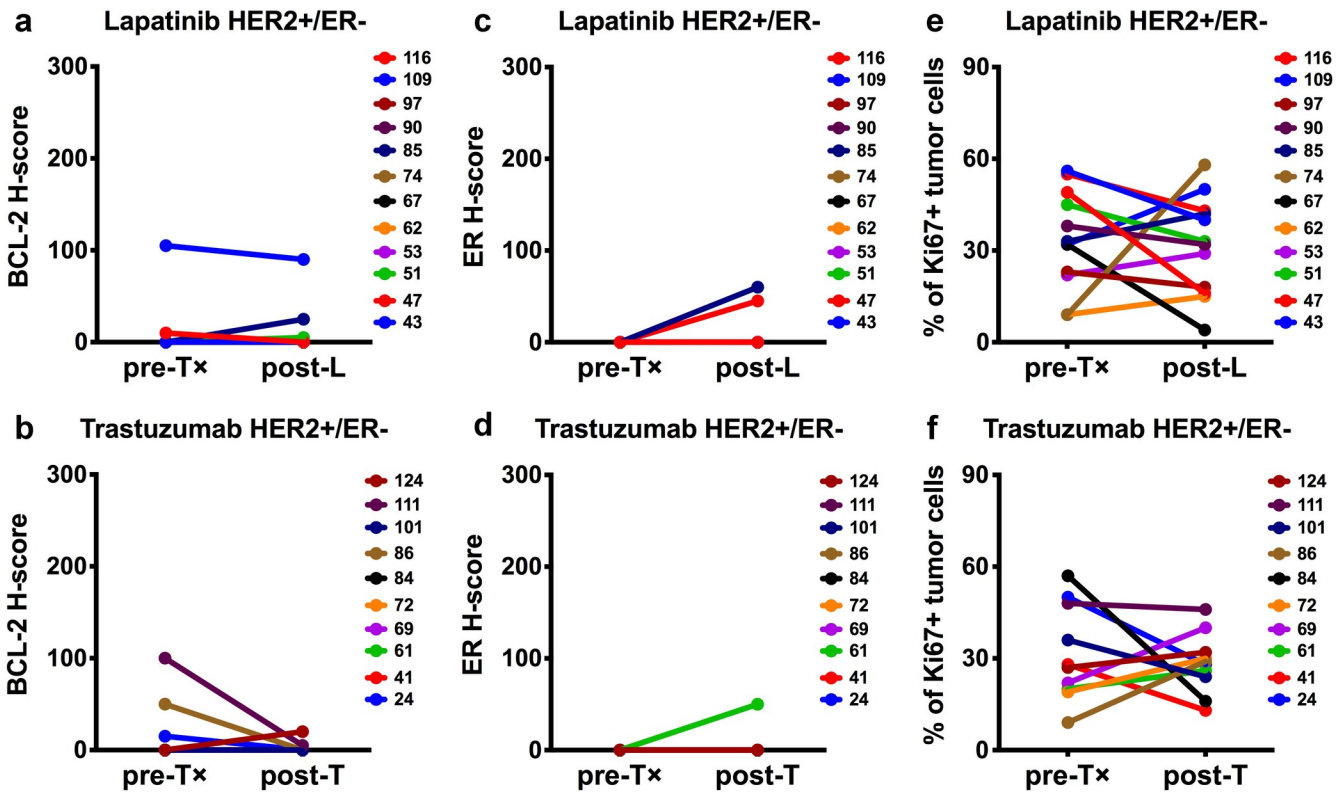


**Fig 3. [HER2+/ER-] comparison of BCL-2, ER and Ki67 protein levels pre- and post-lapatinib [L] or post-trastuzumab [T] treatment.** Twelve HER2+/ER- lapatinib-treated and ten HER2+/ER- trastuzumab-treated sample pairs were evaluated for BCL-2 (**a** and **b**), ER (**c** and **d**) and Ki67 (**e** and **f**). BCL-2 and ER were semi-quantitated via H-score assessment. Ki67 values represent the estimated percentage of Ki67+ tumor cells. Each numerical identifier represents a clinical sample pair. Each plot compares baseline (pre-treatment) and run-in (post-treatment) paired samples. Statistical comparisons utilized Wilcoxon matched-pairs signed rank two-tailed test (p-values > 0.05). p-values = [**a**] >0.9999; [**b**] 0.3750; [**c**] 0.5000; [**d**] >0.9999; [**e**] 0.6353; [**f**] 0.7695.

https://doi.org/10.1371/journal.pone.0251163.g003

treatment BCL-2 protein levels were undetectable in most of the HER2+/ER- tumors (S1 Fig). Despite BCL2 mRNA upregulation within HER2+/ER- tumors, only 2 out of 12 lapatinib-treated cases (Fig 3A) and 1 out of 10 trastuzumab-treated cases (Fig 3B) displayed a minor increase in BCL-2 protein levels following treatment. Eleven matched lapatinib-treated cases (Fig 4A) and ten matched trastuzumab-treated cases (Fig 4B) were HER2+/ER+; all of these cases were BCL-2-positive before treatment (H-scores = 15–218). Post-lapatinib, significant BCL-2 upregulation was evident in 9 out of 11 HER2+/ER+ cases (Fig 4A). Examples of BCL-2 and HER2 immunostaining of pre- and or post-treatment tumors from three lapatinib-treated cases [#35; #54; #71] are presented in Fig 5. Post-trastuzumab, BCL-2 upregulation was observed in 6 out of 10 HER2+/ER+ cases; however, these pairwise comparisons were statistically insignificant (Fig 4B). To determine whether treatment altered the expression of BCL-$X_L$, another pro-survival protein, we performed BCL-$X_L$ IHC on the lapatinib-treated and trastuzumab-treated HER2+/ER+ cases. BCL-$X_L$ was upregulated in 4 out of the 9 cases where BCL-2 was upregulated in response to lapatinib (Fig 4C). Interestingly, upregulation in the absence of BCL-2 upregulation was detected within the remaining 2 out of 11 lapatinib-treated HER2+/ER+ tumors. S2 Fig includes BCL-$X_L$ and BCL-2 correlation analysis for the 11 lapatinib-treated HER2+/ER+ tumors. Changes in BCL-2 and BCL-$X_L$ expression were anti-correlated (r = -0.424; 95% CI [-0.8231, 0.2548]); however, the correlation was not significant (p = 0.192). For the trastuzumab-treated HER2+/ER+ cases, BCL-$X_L$ was upregulated in only 1 out of the 6 cases where BCL-2 was upregulated (Fig 4D) and within two additional cases; however, one of

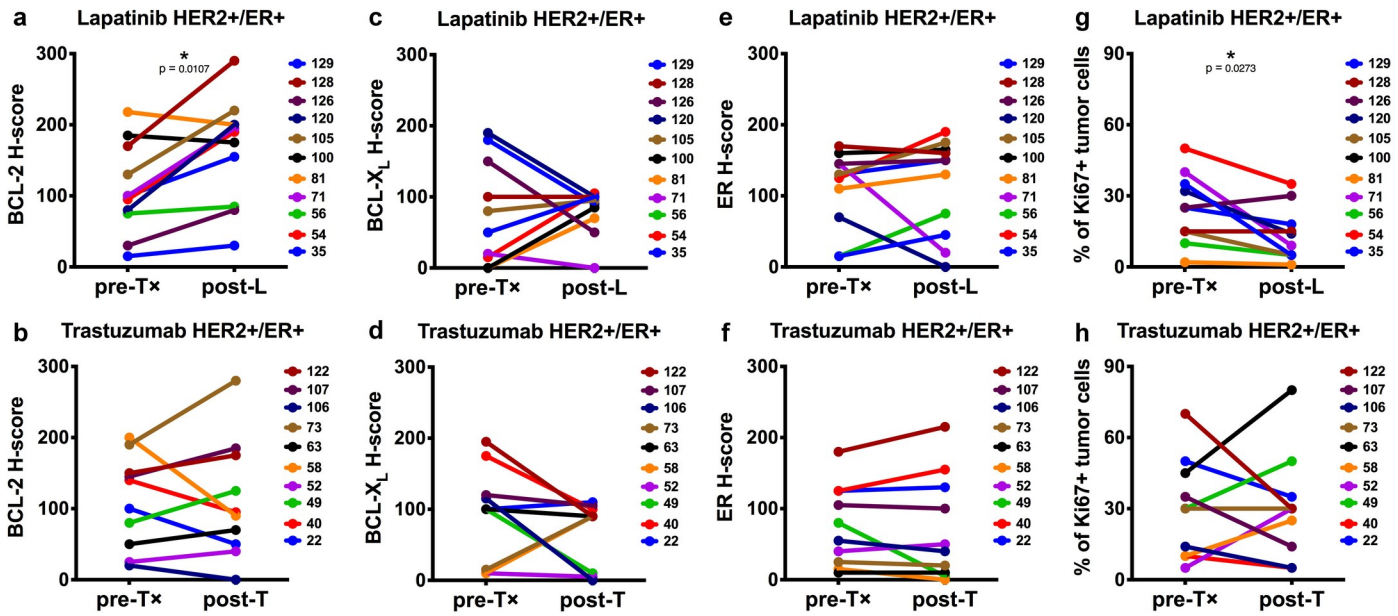


**Fig 4. [HER2+/ER+] comparison of BCL-2, BCL-$X_L$, ER and Ki67 protein levels pre- and post-lapatinib [L] or post-trastuzumab [T] treatment.** Eleven HER2+/ER+ lapatinib-treated and ten HER2+/ER+ trastuzumab-treated sample pairs were evaluated for BCL-2 (**a** and **b**), BCL-$X_L$ (**c** and **d**), ER (**e** and **f**) and Ki67 (**g** and **h**). BCL-2, BCL-$X_L$, ER and Ki67 were evaluated as described in Fig 3. Each numerical identifier represents a clinical sample pair. Each plot compares baseline (pre-treatment) and run-in (post-treatment) paired samples. Statistical comparisons utilized Wilcoxon matched-pairs signed rank two-tailed test and p-values $< 0.05$ are indicated (*). p-values = [**a**] 0.0107; [**b**] 0.9922; [**c**] 0.9727; [**d**] 0.2207; [**e**] 0.4521; [**f**] 0.8945; [**g**] 0.0273; [**h**] 0.9297.

https://doi.org/10.1371/journal.pone.0251163.g004

these cases displayed only a minor increase in BCL-$X_L$ protein levels post-treatment. These results indicate that BCL-2 is upregulated within the majority of lapatinib-treated HER2+/ER+ tumors and that BCL-2 protein is undetectable pre- and post-treatment in most HER2+/ER- tumors.

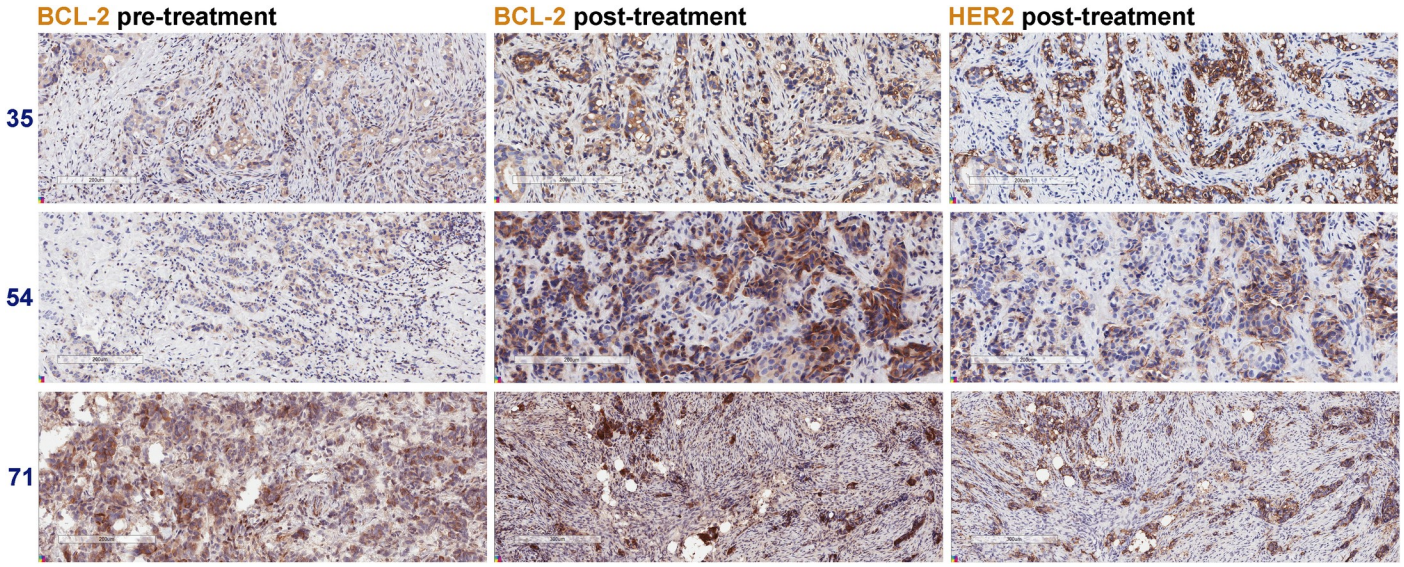


**Fig 5. Lapatinib-induced BCL-2 upregulation post-treatment.** Comparison of BCL-2 levels pre- and post-treatment for cases #35, #54 and #71. HER2 serial section IHC identified the tumor cells post-lapatinib. Scale bars, 200–300 μm.

https://doi.org/10.1371/journal.pone.0251163.g005

Since BCL2 is a direct transcriptional target of ER [14], we performed ER IHC and examined whether lapatinib-associated BCL-2 induction paralleled ER upregulation. ER was upregulated in 6 out of the 9 HER2+/ER+ cases where BCL-2 was upregulated in response to lapatinib (Fig 4E and S2 Fig) and in 2 out of the 6 HER2+/ER+ cases where BCL-2 was upregulated in response to trastuzumab (Fig 4F). By comparison, ER upregulation was detected within 2 out of 12 HER2+/ER- lapatinib-treated cases (Fig 3C) and 1 out of 10 HER2+/ER- trastuzumab-treated cases (Fig 3D); however, only one of these cases displayed a minor increase in BCL-2. These results indicate that BCL-2 upregulation in lapatinib-treated HER2+/ER+ tumors is often, but not always, coupled with increased ER expression.

To determine whether BCL-2 upregulation correlated with the inhibition of tumor proliferation, we performed Ki67 IHC. Pair-wise comparisons revealed non-significant Ki67 alterations within the HER2+/ER+ tumors post-trastuzumab (Fig 4H) and the HER2+/ER- tumors treated with either lapatinib (Fig 3E) or trastuzumab (Fig 3F). For HER2+/ER+ tumors, we detected an overall reduction in Ki67+ tumor cells post-lapatinib (Fig 4G). The percentage of Ki67+ tumor cells was reduced in the majority of cases where BCL-2 was upregulated in response to lapatinib. Correlation analysis (S2 Fig) supported these observations and indicated BCL-2 and Ki67 were anti-correlated ($r = -0.421$; 95% CI [-0.8218, 0.2585]); however, the correlation was not significant ($p = 0.196$). These results indicate that BCL-2 upregulation within lapatinib-treated HER2+/ER+ tumors is frequently associated with lapatinib-induced proliferative blockade.

## Discussion

Here, we provide clinical validation of the BCL-2 induction associated with the adaptive response to lapatinib-mediated HER2 blockade. The upregulation of BCL2 mRNA occurred in both HER2+/ER- as well as HER2+/ER+ patient tumors treated with lapatinib or trastuzumab; however, BCL-2 protein levels pre- and post-treatment were undetectable in most HER2+/ER- tumors. The upregulation of BCL-2 protein was evident within the majority of lapatinib-treated HER2+/ER+ tumors and was often coupled with increased ER expression and decreased proliferation. Comparable BCL-2 upregulation was not observed within the trastuzumab-treated HER2+/ER+ tumors. Together, these results support clinical evaluation of BCL-2 inhibitors within the context of lapatinib and the treatment of HER2+/ER+ breast cancers.

Our results are consistent with in vitro studies of ER-mediated lapatinib resistance mechanisms [15, 16], which provided evidence for ER and BCL-2 induction within lapatinib-resistant models. Our results are further supported by similar lapatinib-focused analyses of ER and BCL-2 co-expression [17], which demonstrated parallel upregulation of ER and BCL-2 within two pre-clinical models of treatment resistant HER2+ breast cancer as well as a subset of lapatinib-treated HER2+ clinical samples. The relationship between lapatinib-mediated HER2 blockade and ER induction is consistent with evidence that pathways activated (MAPK/ERK or PI3K/AKT) downstream of HER2-signaling negatively regulate ER through both epigenetic and transcriptional mechanisms [15, 18–24]; whereas, the correlation between BCL-2 upregulation and ER upregulation is consistent with evidence that BCL-2 is a direct target of ER transcriptional regulation [14]. Since BCL-2 upregulation was also observed in a few tumors without ER upregulation, our results also suggest that there can be ER-independent regulation of BCL-2. These results are consistent with multiple previous reports demonstrating that anti-estrogen treatment only decreased BCL-2 expression in a subset of tumors analyzed [25–28].

The correlation between BCL-2 upregulation and decreased proliferation is consistent with evidence that HER2 inhibition confers adaptive reprogramming. Since lapatinib and

trastuzumab have differential mechanisms of action [29] and variable impacts on HER2--signaling blockade [30], the lapatinib-associated BCL-2 responses could be directly related to superior HER2 inhibition achieved with lapatinib versus trastuzumab.

Endpoint analysis of pathological responses in the TRIO-B-07 clinical trial indicated that lapatinib-treated HER2+/ER+ tumors had the lowest pathological complete response rates [11]. Since these tumors were subsequently treated with additional neo-adjuvant DNA-damaging and anti-mitotic chemotherapies, we are unable to address the extent to which BCL-2 induction clinically impacted the lapatinib-associated pathological responses observed within HER2+/ER+ tumors. Nonetheless, we would predict that tumor populations distinguished by elevated BCL-2 could contribute to residual and recurrent disease. These predictions are based upon our own pre-clinical in vivo data demonstrating BCL-2 induction in lapatinib-treated HER2+ residual tumors [9] as well as additional in vitro and in vivo studies demonstrating BCL-2 induction within lapatinib-resistant models [15–17].

BCL-2 dependencies have been described with the context of HER2-/ER+ breast cancers, which are enriched for BCL-2 expression [31–33]. Based upon these observations, pre-clinical studies have determined that selective targeting of BCL-2 via ABT-199/venetoclax [34] enhanced the effectiveness of ER-targeted treatment [35] and have motivated clinical investigations of venetoclax combined treatment strategies within HER2-/ER+ tumors [36]. Since our results demonstrated similar BCL-2 enrichment within the context of HER2+/ER+ breast cancers, we would predict that HER2-targeted and ER-targeted treatments of these tumor types could be enhanced via venetoclax.

The pro-survival functions of BCL-2, BCL-$X_L$ and MCL-1 have been described within various contexts of HER2-targeted therapies. Using cell-derived and patient-derived xenograft models of HER2+/ER- breast tumors, our previous pre-clinical work provides evidence that dual targeting of BCL-2 and BCL-$X_L$, via ABT-737 [37] or ABT-263/navitoclax [38], enhanced the in vivo effectiveness of lapatinib [9] or T-DM1 [39]. Another report identified an increased BCL-2:BAX ratio within a trastuzumab-resistant in vitro model and demonstrated that dual targeting of BCL-2 and BCL-$X_L$ enhanced the effectiveness of trastuzumab [40]. Multiple pre-clinical studies have identified MCL-1 as a critical component of lapatinib-resistant phenotypes and demonstrated that blockade of MCL-1, via obatoclax [41] or S63845 [42], enhanced treatment effectiveness [43–47]. Although several studies have demonstrated trastuzumab-associated downregulation of MCL-1 [48] or BCL-2 [49], these studies also reported that further disruption or modulation of the pro-survival proteins enhanced treatment effectiveness. Together with our TRIO-B-07 clinical results, these studies support the evaluation of BCL-targeted agents within the context of HER2-targeted treatments.

## Supporting information

**S1 Fig. Pre-treatment expression levels of BCL2 mRNA or BCL-2 protein.** Each plot compares baseline (pre-treatment) HER2+/ER- (pre-treatment ER H = 0) and HER2+/ER+ (pre-treatment ER H $>$ 0) tumors. Statistical comparisons of BCL2 mRNA utilized Significance Analysis of Microarrays (SAM) analyses, FDR-p = 0.00319 (**). Statistical comparisons of BCL-2 protein utilized Mann-Whitney two-tailed test, $p < 0.0001$ (****). Each line represents the median.
(JPG)

**S2 Fig. Correlation analyses for lapatinib-treated HER2+/ER+ tumors.** Each graph compares BCL-2 and BCL-$X_L$, ER or Ki67 alterations ($\Delta$ = Fig 4 post-value–Fig 4 pre-value). The Spearman correlation [r] and associated p-value [p] is indicated for each comparison. 95% CI:

BCL-2 and BCL-$X_L$ [-0.8231, 0.2548], ER [-0.7980, 0.3219] or Ki67 [-0.8218, 0.2585].
(JPG)

## Acknowledgments

We would like to thank Enrique Guandique (UCLA), Lillian Ramos (UCLA) and Aleksandr Vagodny (Harvard Medical School) for IHC-related technical assistance. We would like to acknowledge the Dana-Farber Harvard Cancer Center Tissue Microarray Imaging Core and UCLA Translational Pathology Core for access to and usage of the Aperio slide scanner.

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