



original reports

# Survival, Pathologic Response, and Genomics CALGB 40601 (Alliance), a Neoadjuvant Phase III Trial of Paclitaxel-Trastuzumab With or Without Lapatinib in HER2-Positive Breast Cancer

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abstract

**PURPOSE** CALGB 40601 assessed whether dual versus single human epidermal growth factor receptor 2 (HER2) –targeting drugs added to neoadjuvant chemotherapy increased pathologic complete response (pCR). Here, we report relapse-free survival (RFS), overall survival (OS), and gene expression signatures that predict pCR and survival.

**PATIENTS AND METHODS** Three hundred five women with untreated stage II and III HER2-positive breast cancer were randomly assigned to receive weekly paclitaxel combined with trastuzumab plus lapatinib (THL), trastuzumab (TH), or lapatinib (TL). The primary end point was pCR, and secondary end points included RFS, OS, and gene expression analyses. mRNA sequencing was performed on 264 pretreatment samples.

**RESULTS** One hundred eighteen patients were randomly allocated to THL, 120 to TH, and 67 to TL. At more than 7 years of follow-up, THL had significantly better RFS and OS than did TH (RFS hazard ratio, 0.32; 95% CI, 0.14 to 0.71;  $P = .005$ ; OS hazard ratio, 0.34; 95% CI, 0.12 to 0.94;  $P = .037$ ), with no difference between TH and TL. Of 688 previously described gene expression signatures, significant associations were found in 215 with pCR, 45 with RFS, and only 22 with both pCR and RFS (3.2%). Specifically, eight immune signatures were significantly correlated with a higher pCR rate and better RFS. Among patients with residual disease, the immunoglobulin G signature was an independent, good prognostic factor, whereas the HER2-enriched signature, which was associated with a higher pCR rate, showed a significantly shorter RFS.

**CONCLUSION** In CALGB 40601, dual HER2-targeting resulted in significant RFS and OS benefits. Integration of intrinsic subtype and immune signatures allowed for the prediction of pCR and RFS, both overall and within the residual disease group. These approaches may provide means for rational escalation and de-escalation treatment strategies in HER2-positive breast cancer.

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## INTRODUCTION

Human epidermal growth factor receptor 2 (HER2) is amplified and overexpressed in 15% to 20% of all breast cancers and, when untreated, is the most aggressive breast cancer phenotype. Over the past 20 years, the prognosis of HER2-positive breast cancer has been markedly improved with the implementation of anti-HER2 targeted therapies. In neoadjuvant randomized trials, dual HER2 blockade is associated with a higher pathologic complete response (pCR) rate compared with that of single agents.<sup>1-5</sup> In the absence of chemotherapy, dual HER2 blockade alone is associated with pCR rates of approximately 25%.<sup>6,7</sup>

however, large adjuvant trials have demonstrated only marginal survival benefit of dual HER2 blockade compared with standard trastuzumab added to chemotherapy.<sup>8,9</sup>

Both the intrinsic complexity of HER2-positive tumors and the properties of its tumor microenvironment are responsible for the differences in response to anti-HER2 targeted therapies. HER2-positive disease is highly heterogeneous. All the intrinsic subtypes—Luminal A, Luminal B, HER2 Enriched, Basal like, and Normal like—can be identified within HER2-positive tumors by gene expression. Among them, the HER2-Enriched subtype has been systematically associated

## ASSOCIATED CONTENT

### Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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## CONTEXT

### Key Objective

Here, we report CALGB 40601 prespecified secondary end points of relapse-free survival (RFS) and overall survival with a median follow-up of 7 years and a comprehensive exploratory analysis testing the ability of hundreds of genomic biomarkers to predict not only pathologic complete response (pCR), but also RFS.

### Knowledge Generated

In CALGB 40601, there was a significant improvement in RFS and overall survival at 7 years with dual (lapatinib and trastuzumab) versus single anti-HER2 therapy. Biomarker analysis showed that the HER2-enriched subtype, a key independent predictor of pCR, was also an inverse predictor of RFS, whereas immune gene expression signatures were significantly correlated with higher pCR rates and better RFS.

### Relevance

For future escalation and de-escalation strategies in HER2-positive breast cancer, we may need to integrate the information provided by clinical parameters, intrinsic subtype, and immune signatures to best predict response and survival.

with higher pCR rates to anti-HER2 targeted therapies across multiple neoadjuvant clinical trials.<sup>3,6,10-17</sup> At a DNA level, *PIK3CA* mutations have also been associated with lower treatment response.<sup>18,19</sup> Apart from intrinsic tumor characteristics, different immune system features have been correlated with a higher pCR rate in HER2-positive breast cancer, including the presence of tumor-infiltrating lymphocytes (TILs),<sup>14,20-24</sup> programmed death ligand-1 protein expression,<sup>23</sup> T-cell receptor diversity metrics,<sup>25</sup> and immune gene-expression signatures.<sup>3,12,26</sup> TILs in the NeoALTTO trial<sup>22</sup> showed a significant association with survival.

Patients with clinical stage II and III HER2-positive breast cancer are typically treated with neoadjuvant chemotherapy plus HER2 targeting because of the effect of neoadjuvant treatment on surgical end points (smaller surgeries and less need for axillary lymph node dissection) and the tailoring of therapy by pCR status now that patients with residual disease (RD) receive ado-trastuzumab emtansine.<sup>27</sup> Thus, pCR is no longer merely an intermediate biomarker of outcome, rather both pCR and survival are clinically meaningful end points.

We have previously reported the pCR end points for CALGB 40601 (now part of the Alliance for Clinical Trials in Oncology), including finding a significant contribution to pCR of intrinsic subtype and immune gene signatures.<sup>3</sup> Here, we report CALGB 40601 prespecified secondary end points of relapse-free survival (RFS) and overall survival (OS), with a median follow-up of 7 years, and the association between pCR and RFS/OS. Using the transcriptome analyses from pretreatment biopsies, we performed an exploratory analysis to test the ability of clinical and genomic biomarkers to predict pCR and RFS at 7 years. Understanding which biomarkers are going to have an influence on both pCR and survival outcomes will help build prognostic tools to design future

escalation and de-escalation trials in HER2-positive breast cancer.

## PATIENTS AND METHODS

### CALGB 40601 Study Design and Patients

The CALGB 40601 study design and pCR results have been previously published.<sup>3</sup> A total of 305 women with stage II and III HER2-positive breast cancer were randomly assigned to receive paclitaxel (T) at 80 mg/m<sup>2</sup> once per week, with the addition of trastuzumab (H; 4 mg/kg loading dose followed by 2 mg/kg), lapatinib (L; 1,500 mg/d), or both (L at 1,000 mg/d plus the same dose of H) for 16 weeks. On the basis of reports of inferiority and higher toxicity, the TL arm was closed early; patients who were randomly assigned to that arm completed treatment. Protocol-defined therapy ended at surgery. It was recommended that all patients receive dose-dense doxorubicin and cyclophosphamide (AC) and complete 1 year of trastuzumab adjuvantly.

The primary end point was pCR, defined as no invasive tumor in the breast at surgery. Secondary end points included RFS and OS. RFS was defined as the interval from surgery to ipsilateral invasive breast tumor recurrence, regional recurrence, distant recurrence, or death of any cause, whichever occurred first. Patients without an event were censored at the date of the last clinical assessment. OS was defined as the interval from random assignment to death or last follow-up. Clinical data collection and statistical analyses were conducted by the Alliance Statistics and Data Center.

### Sample Acquisition and Biospecimen Processing

Participants underwent four pretreatment 16-gauge core biopsies for research. The CONSORT diagram (Data Supplement) shows the flow of participants from the intention-to-treat (ITT) population to the gene expression

cohort. Patients with inadequate RNA quality were excluded from the final gene expression cohort, which consisted of 264 patients. All 264 patients signed an institutional review board–approved, protocol-specific informed consent document following federal and institutional guidelines. This document also included consent for biomarker research.

### Tumor Gene Expression Analyses

Gene expression profiles from pretreatment core biopsies were generated by RNA sequencing using an Illumina HiSeqEquation 2000 (Data Supplement).<sup>28</sup> Intrinsic subtype biomarkers were obtained by the PAM50 predictor<sup>29</sup> after applying a new HER2/estrogen receptor subgroup-specific gene normalization method (Data Supplement).<sup>30</sup> Gene expression signatures and single genes representing multiple biologic pathways and cell types (Data Supplement) were also assessed following different specifications (Data Supplement).

### Data Analysis and Interpretation

ANOVA and Fisher exact test were used to compare clinicopathologic variables between different treatment and biomarker groups. Proportions and 95% CIs were provided. We estimated 7-year RFS and OS rates for each treatment arm and pCR group using the Kaplan-Meier method. The relationship between clinical and genomic biomarkers with pCR and/or RFS was assessed using univariable and multivariable logistic and Cox proportional hazards regression models, respectively. Odds ratios, hazard ratios (HRs) and 95% CIs were calculated for each response variable. The significance level was set to a two-sided  $\alpha$  of .05, and *P* values were unadjusted for multiplicity. The Akaike Information Criterion and Bayesian Information Criterion were used to analyze the goodness of fit of two competing statistical models. Collinearity between gene expression continuous variables was evaluated using Pearson correlation. High-dimensional modeling for pCR and RFS was done by Elastic Net (R package glmnet<sup>31</sup>). A detailed description can be found in the Data Supplement. Analyses were based on the clinical study database frozen on April 3, 2019, and all statistical analyses were performed using R 3.5.2 and Python 3.6.

## RESULTS

### Clinicopathologic Characteristics

From December 2008 to February 2012, 305 patients were randomly assigned to one of three treatment groups: 118 to trastuzumab plus lapatinib (THL), 120 to TH, and 67 to TL. Of the 305 patients, 299 began protocol treatment; pCR was evaluable in 295 patients. RNA sequencing was performed on 264 pretreatment tumor samples (Data Supplement). Patient characteristics were balanced by treatment arm in both the RNA sequencing and the ITT populations (Table 1). pCR rates in the ITT subpopulation were 57% (95% CI, 47% to 66%) in the THL arm,

45% (95% CI, 36% to 54%) in the TH arm, and 30% (95% CI, 19% to 42%) in the TL arm, slightly different from the original pCR rates<sup>3</sup> that were calculated using the pCR-evaluable cohort (*n* = 295). After surgery, as recommended by the protocol, 51% received AC and 73% completed 1 year of trastuzumab. There was no imbalance by treatment arm in either the RNA sequencing and the ITT cohorts (Table 1).

### RFS and OS Analyses

With a median follow-up of 83 months from random assignment (interquartile range, 71 to 90 months), RFS events were recorded in 16% of participants: 18 (26.9%) in the TL arm, 24 (20%) in the TH arm, and eight (6.8%) in the THL arm, with corresponding 7-year RFS rates of 69% (95% CI, 58% to 82%; TL), 79% (95% CI, 71% to 87%; TH), and 93% (95% CI, 88% to 98%; THL). The RFS difference between the THL and control TH arms was highly statistically significant (HR, 0.32; 95% CI, 0.14 to 0.71; *P* = .005; Fig 1A). Nine deaths (13.4%) occurred in the TL arm, 14 (11.7%) in the TH group, and four (3.4%) in the THL group, with corresponding 7-year OS rates of 84% (TL), 88% (TH), and 96% (THL). OS was significantly higher in the THL compared with the TH arm (HR, 0.34; 95% CI, 0.12 to 0.94; *P* = .037; Fig 1B). Neither receipt of adjuvant AC, nor whether the full year of adjuvant trastuzumab was completed, altered these relationships (Data Supplement).

When all treatment groups were combined, a significant association between pCR and RFS was found. Of the 141 patients who achieved pCR, 14 (9.9%) had an RFS event compared with 35 (23%) of 154 patients with RD (HR, 0.42; 95% CI, 0.23 to 0.78; *P* = .006; Fig 1C). Patients who achieved pCR also had improved OS benefit compared with patients with RD (HR, 0.3; 95% CI, 0.12 to 0.74; *P* = .009; Fig 1D). pCR and treatment effect in RFS and OS were preserved also in the RNA sequencing subpopulation (Data Supplement).

### Intrinsic Subtype Association With Response and RFS

Using a new and improved PAM50 normalization method (Data Supplement), the majority of tumors were HER2 Enriched (146 of 264; 55%), followed by Luminal B (13%), Normal like (12%), Luminal A (11%), and Basal like (8%; Data Supplement). Although subtype distribution significantly differed by hormone receptor status (*P* < .001), the HER2-Enriched subtype was the most frequent subtype identified in both hormone receptor–positive and –negative groups (44% and 72%, respectively; Data Supplement).

pCR in the breast was achieved in 89 (61%) of 146 HER2-Enriched patients compared with 29 (25%) of 118 non-HER2-Enriched patients (odds ratio, 3.8; 95% CI, 2.23 to 6.72; *P* < .001; Data Supplement). Significant RFS differences were found among the different subtypes. Luminal A tumors, with the lowest pCR rate (14.3%), carried the best RFS outcome, with no events recorded

**TABLE 1.** Baseline Characteristics of the Intention-to-Treat and Biomarker Populations by Treatment Arm

Characteristic	ITT Subpopulation (n = 305), No. (%)			P	RNA Sequencing Subpopulation (n = 264), No. (%)			P
	THL Arm (n = 118)	TH Arm (n = 120)	TL Arm (n = 67)		THL Arm (n = 103)	TH Arm (n = 104)	TL Arm (n = 57)	
Age, years				.396				.602
Median	48	50	48		48	49	51	
Range	24-70	30-75	25-74		24-70	30-75	25-74	
Menopausal status				.348				.293
Pre	72 (61.0)	63 (52.5)	39 (58.2)		65 (63.1)	59 (56.7)	30 (52.6)	
Post	42 (35.6)	54 (45.0)	27 (40.3)		34 (33.0)	42 (40.4)	26 (45.6)	
Missing	4 (3.4)	3 (2.5)	1 (1.5)		4 (3.9)	3 (2.9)	1 (1.8)	
Racial or ethnic group				.23				.293
Black	10 (8.5)	10 (8.3)	3 (4.5)		9 (8.7)	9 (8.7)	3 (5.3)	
White	90 (73.6)	99 (82.5)	60 (89.3)		79 (76.7)	85 (81.7)	51 (89.4)	
Other	18 (15.3)	11 (9.2)	4 (6.0)		15 (14.6)	10 (9.6)	3 (5.3)	
ECOG PS				.296				.426
0	110 (93.2)	107 (89.2)	63 (94)		95 (92.2)	93 (89.4)	54 (94.7)	
1	5 (4.2)	11 (9.2)	3 (4.5)		5 (4.9)	9 (8.7)	2 (3.5)	
Missing	3 (2.5)	2 (1.7)	1 (1.5)		3 (2.9)	2 (1.9)	1 (1.8)	
HR status				.988				.91
Positive	70 (59.3)	70 (58.3)	39 (58.2)		60 (58.3)	62 (59.6)	32 (56.1)	
Negative	48 (40.7)	50 (41.7)	28 (41.8)		43 (41.7)	42 (40.4)	25 (43.9)	
Clinical stage				.79				.556
II	81 (68.6)	80 (66.7)	48 (71.6)		69(67)	68 (65.4)	42 (73.3)	
III	37 (31.4)	40 (33.3)	19 (28.4)		34(33)	36 (34.6)	15 (26.3)	
Adjuvant AC				.863				.503
No	59 (50.0)	60 (50.0)	31 (46.3)		68 (66.0)	71 (68.3)	41 (71.9)	
Yes	59 (50.0)	60 (50.0)	36 (57.7)		35 (34.0)	33 (31.7)	16 (28.1)	
1-year adjuvant trastuzumab				.204				.624
No	30 (25.4)	29 (24.2)	24 (35.8)		24 (23.3)	25 (24.0)	17 (29.8)	
Yes	88 (74.6)	91 (75.8)	43 (64.2)		79 (76.7)	79 (76.0)	40 (70.1)	

NOTE. The *P* value for the distribution differences between treatment arms in the ITT and RNA sequencing cohorts were assessed by a one-way ANOVA test (median age) and Fisher exact test (menopausal status, racial or ethnic group, ECOG PS, HR status, clinical stage, adjuvant AC, 1 year of adjuvant trastuzumab).

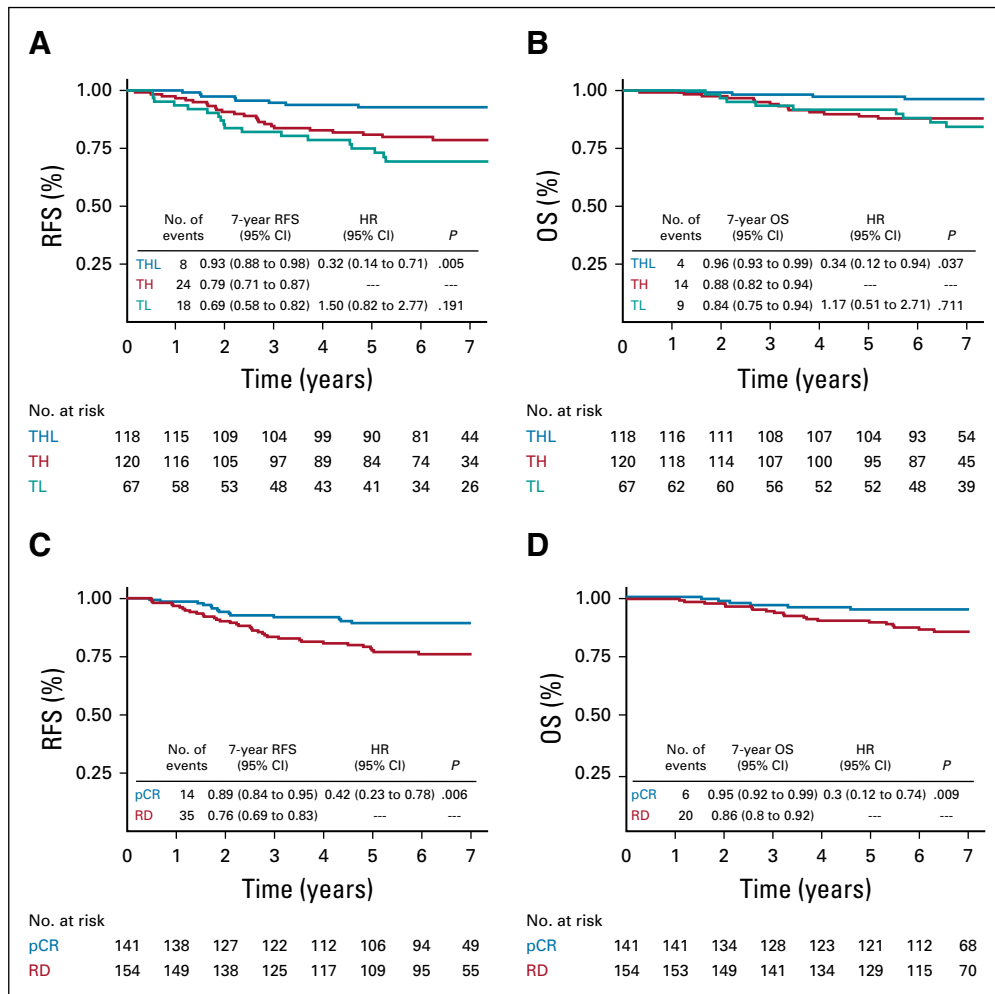
Abbreviations: AC, doxorubicin and cyclophosphamide; ECOG PS, Eastern Cooperative Oncology Group; HR, hormone receptor; ITT, intention to treat; pCR, pathologic complete response; TH, paclitaxel plus trastuzumab; THL, paclitaxel, trastuzumab and lapatinib; TL, paclitaxel and lapatinib.

after 7 years of follow-up. In contrast, HER2-Enriched patients, with the highest pCR rate, carried a significantly worse RFS outcome, with 30 (20.5%) of 146 RFS events recorded (Data Supplement). Of interest, only 10 (11%) of 89 HER2-Enriched pCR patients had an RFS event compared with 20 (35%) of 57 HER2-Enriched RD patients.

### Genomic Modeling for pCR and RFS

In our previous analyses,<sup>3,26</sup> prespecified gene expression signatures—*intrinsic* subtype, immune activation—significantly and independently contributed to pCR. Building

on this, we sought to create a unified predictive model for both pCR and RFS. OS data, with a low number of events (*n* = 28), were not mature enough for high-quality predictive modeling. We first built 688 logistic regression models to test the ability of each genomic variable to predict pCR in the presence of clinical information, including treatment arm, clinical stage, and hormone receptor status. A total of 215 genomic variables (31.25%) were significantly associated with pCR (Data Supplement). In concordance with our previous results,<sup>3,26</sup> subtype-related biomarkers, like the HER2-Enriched signature, the



**FIG 1.** Kaplan-Meier curves for relapse-free survival (RFS) and overall survival (OS) in the intention-to-treat (ITT) population. (A) Kaplan-Meier estimates of RFS at 7 years by treatment arm in the ITT population, showing a significant benefit of THL (paclitaxel, trastuzumab, and lapatinib) versus TH (paclitaxel plus trastuzumab) treatment arms. (B) Kaplan-Meier estimates of OS at 7 years by treatment arm in the ITT population, showing a significant benefit of THL versus TH treatment arms. (C) Kaplan-Meier estimates of RFS at 7 years by pathologic complete response (pCR) status in the ITT population, showing a significant improvement in outcome in pCR versus residual disease (RD). (D) Kaplan-Meier estimates of OS at 7 years by pCR status in the ITT population, showing a significant improvement in outcome in pCR versus RD. HR, hazard ratio; TL, paclitaxel and lapatinib.

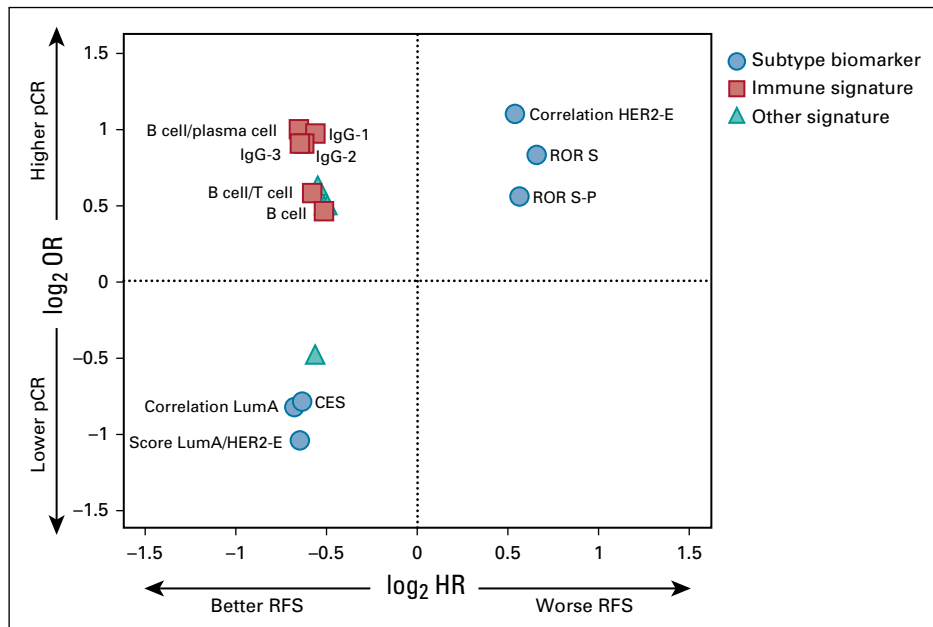
PAM50 risk of recurrence, *ERBB2* gene expression, and B cell/immunoglobulin G (IgG) immune signatures were found to powerfully and positively predict pCR. In contrast, luminal parameters, like *ESR1* gene expression, the Luminal A signature, the Luminal Tumor Score,<sup>32</sup> the chemodendocrine score (CES),<sup>33</sup> and the LumA-HER2-E scores were negative predictors of response.

Similarly, we created Cox proportional hazards regression models to predict RFS at 7 years. Only 45 (6.54%) of the 688 biomarkers tested were significantly associated with RFS, and neither receipt of adjuvant AC, nor completion of adjuvant trastuzumab altered this association (Data Supplement). Twenty-two (3.2%) were significantly associated with both pCR and RFS (Fig 2 and Data Supplement),

including seven intrinsic subtype-related biomarkers and eight immune signatures. Of interest, all the immune signatures, which were strongly correlated with higher pCR rate, were also associated with longer RFS. In contrast, all the tumor-related biomarkers worked in opposite directions for the prediction of pCR and RFS. Akaike Information Criterion and Bayesian Information Criterion tests showed a better fit of the univariable LumA-HER2-E score pCR and RFS models compared with the HER2-Enriched and Luminal A models (Data Supplement).

A high Pearson correlation among the different genomic biomarkers was observed (Data Supplement). To handle this collinearity, we used Elastic Net to build two high-dimension models for predicting pCR and RFS at 7 years





**FIG 2.** Association between odds ratio (OR) and hazard ratio (HR) in 16 significant biomarkers of response and survival. OR and HR values have been log transformed. Whereas all the immune-related biomarkers predicted a higher response and a longer relapse-free survival (RFS), subtype-related biomarkers showed the opposite direction for the prediction of response and survival. For subtype-related biomarkers, the correlation to each PAM50 centroid was used. CES, chemoendocrine score; HER2-E, HER2-enriched; IgG, immunoglobulin G; LumA, Luminal A; P, proliferation; pCR, pathologic complete response; ROR, risk of recurrence; S, subtype.

using as outcome time to an event. The best pCR model included 55 (7.9%) of 696 (eight clinical and 688 genomic) variables and an area under the curve value of 0.82 in the train set and 0.75 in the test set, in the line of the expected values from cross-validation (Data Supplement). This model included clinical features, such as treatment arm, as well as several subtype and immune B-cell and T-follicular helper signatures, *ESR1* and *ERBB2* gene expression, and some supervised signatures, such as the CES<sup>33</sup> and LumA-HER2-E scores. The optimal RFS model included 46 (6.6%) of 697 (nine clinical and 688 genomic) variables, with a C-index of 0.88 in the train set and 0.63 when applied to the test set, in line with the expected values from cross-validation (Data Supplement). In this model, clinical parameters, such as pCR status and treatment arm, carried the greatest weights. Subtype-related biomarkers and multiple B-cell and T-cell signatures were also present. From all 688 genomic variables tested, only nine (1.3%) were present in both models (Data Supplement). In particular, five immune signatures were included within the significant positive features for response and RFS prediction: two IgG signatures, one B-cell/plasma cell signature, one T-helper signature, and a T-cell/B-cell cooperation signature.<sup>34</sup> In contrast, all subtype-related biomarkers worked in opposite directions for the prediction of pCR and RFS. Of interest, the LumA-HER2-E score was selected together with the HER2-Enriched and the Luminal A signatures for both models. The CES<sup>33</sup> score

and the *ESR1* and *ERBB2* gene expression, present in the pCR model, were not selected by Elastic Net as RFS predictors.

The predictive value of HER2-Enriched and the IgG signatures, or whether these two biomarkers can predict the benefit of dual over single HER2 blockade was also explored (Data Supplement). HER2-Enriched and IgG-high patients treated with THL had a significant RFS benefit compared with TH (HR, 0.28; 95% CI, 0.1 to 0.77;  $P = .014$ ; and HR, 0.09; 95% CI, 0.01 to 0.72;  $P = .023$ ) with no significant differences in non-HER2-Enriched and IgG-low subgroups; however, both interaction tests were nonsignificant. Consequently, we could not establish the predictive value of these two biomarkers.

#### Genomic Signatures for RFS Within Patients With RD

The RD subset was of particular interest given the therapeutic and prognostic implications, as well as the discordance of the pCR and RFS relationship in key signatures. In two univariable Cox proportional hazards regression models in patients with RD, the HER2-Enriched signature was significantly correlated with shorter RFS (HR, 1.77; 95% CI, 1.19 to 2.62;  $P = .005$ ), whereas the IgG signature was correlated with longer RFS (HR, 0.65; 95% CI, 0.46 to 0.93;  $P = .019$ ). Moreover, HER2-Enriched or IgG-low RD patients had significantly shorter RFS interval (Data Supplement). In multivariable Cox analysis, the IgG signature remained an independent good prognostic factor (HR,

0.60; 95% CI, 0.41 to 0.86;  $P = .005$ ), whereas patients with RD with higher HER2-Enriched signature had significantly worse RFS (HR, 2.12; 95% CI, 1.35 to 3.35;  $P = .001$ ; Fig 3). Of interest, hormone receptor status was not significantly associated with RFS in this group.

## DISCUSSION

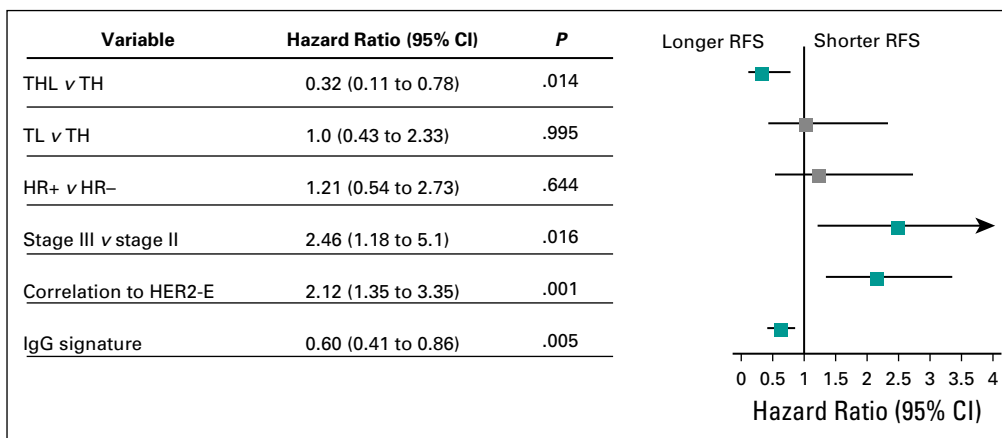
In CALGB 40601, women whose tumors had pCR to neoadjuvant chemotherapy plus HER2 targeting had significantly better RFS and OS than did women with RD, a finding consistent with many other neoadjuvant trials. Of interest, there was a significant improvement in RFS and OS at 7 years with dual therapy in this trial, a surprising finding given that a large adjuvant trial, ALTT0, which included a lower clinical risk but otherwise similar patient population, demonstrated only a modest and statistically nonsignificant effect (disease-free survival HR, 0.84) of adding lapatinib administered for a longer duration.<sup>8</sup> A trial similar in population and intervention to CALGB 40601, NeoALTT0, found numerically higher but nonsignificant EFS differences with dual therapy (84% v 76%)<sup>35</sup>; as in CALGB 40601, survival outcome was a secondary end point. For these reasons, our statistically significant effect of dual therapy on relapse and survival should be considered in the context of its secondary analytic nature and the results of other trials suggesting a far more limited impact of dual therapy.

Accumulating evidence supports the clinical validity of two prognostic biomarkers in HER2-positive breast cancer: intrinsic subtype and immune cell features. The HER2-Enriched subtype, a key and independent predictor of pCR, was also found to be an inverse predictor of RFS. Specifically, HER2-Enriched patients, with the highest pCR rate of the tumor intrinsic subtypes, had significantly worse RFS

than did patients with Luminal A tumors, even in the presence of HER2-targeted drugs. This finding, which fails to conform to the known association of pCR with outcome, is consistent with emerging data regarding the complexity of the relationship of pCR with RFS and the effect of confounding but unmeasured variables when the focus is entirely on pCR. This is an example of Simpson's paradox,<sup>36</sup> in which the confounding variable is the substantially worse RFS outcome among HER2-Enriched tumors with RD. Genomically, the HER2-Enriched subtype is the most HER2 oncogene addicted of the subtypes, which likely explains the high sensitivity to anti-HER2 therapies and may also explain why an HER2-Enriched tumor resistant to anti-HER2 therapies administered neoadjuvantly carries a particularly poor prognosis. Luminal tumors, which comprise the majority of non-HER2-Enriched among clinically HER2-positive tumors, are genomically driven by hormone receptor-related pathways and are usually hormone receptor positive, likely benefiting from 5 to 10 years of adjuvant endocrine therapy.

Conversely, evidence of immune activation using multiple RNA-based signatures was significantly and independently directly associated with a higher likelihood of pCR and better RFS. Whether a pathology-based approach, such as TILs, will perform similarly as well as multigene expression profiling is unknown. In NeoALTT0, TILs and immune signatures seemed to predict higher pCR,<sup>12,22</sup> whereas only TILs pretended statistically significant better event-free survival.<sup>22</sup>

The combination of HER2-Enriched and IgG signatures provided more prognostic information than either alone; to build a useful prognostic tool for patients with HER2-positive breast cancer, both biomarkers should be taken into account. We also found that these signatures may provide augmented clinical value in patients with RD.



**FIG 3.** Forest plot representing a multivariable Cox proportional hazards regression analysis within the residual disease group of patients. HER2-enriched (HER2-E) subtype was correlated with shorter relapse-free survival (RFS), whereas immunoglobulin G (IgG) signature was an independent good prognosis factor. The correlation to the HER2-E centroid and the IgG gene expression signature, both as continuous variables, were used for the multivariable Cox proportional hazards regression analysis. HR, hormone receptor; TH, paclitaxel plus trastuzumab; THL, paclitaxel, trastuzumab, and lapatinib; TL, paclitaxel and lapatinib.

Our study has several strengths. First, the clinical trial has mature RFS and OS estimates. Second, we were able to perform a comprehensive genomic analysis on a significant proportion (86.6%) of the pretreatment specimens. Third, we performed an integrated analysis of clinical parameters and 688 genomic biomarkers, testing their ability to predict not only response but also survival. Although the gene expression correlative analysis of the NOAH and the NeoALTTO trials previously identified an association between a small number of signatures with pCR and event-free survival,<sup>10,12</sup> the current study is the most comprehensive effort to date to correlate pCR and RFS predictors in HER2-positive breast cancer. Efforts of this type are key to identifying mechanisms to more precisely select patients who are appropriate for de-escalation strategies, as was done in the APT trial using only low-risk clinical features<sup>37</sup> or escalation strategies, such as the use of adjuvant ad-trastuzumab emtansine.

In contrast, our study also has several limitations. First, CALGB 40601 was not powered to detect a survival benefit, and it was not designed to be prescriptive regarding adjuvant therapy other than the recommendation of

completion of AC chemotherapy and of 1 year of adjuvant trastuzumab. For this reason, RFS biomarker analysis should be also interpreted with caution. Second, the anti-HER2 approaches used in the trastuzumab-containing arms are consistent with the current standard of care, but lapatinib is not used in the early breast cancer setting because of the negative results of the ALTTO trial.<sup>8</sup> Finally, TILs assessment in the pretreatment specimens has not been performed yet and will be reported in a future analysis.

To conclude, in CALGB 40601, dual HER2 blockade with lapatinib added to trastuzumab and chemotherapy demonstrated a significant effect on RFS compared with trastuzumab plus chemotherapy alone, and patients who achieved pCR had significantly better outcomes than patients with RD. However, most patients with RD did not experience relapse, and some pCR patients did experience relapse. Our genomic data suggest that future escalation and de-escalation strategies may benefit from integrating the information provided by clinical parameters, intrinsic subtype, and immune signatures to predict not only response, but also survival.

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## CLINICAL TRIAL INFORMATION

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## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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**AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST****Survival, Pathologic Response, and Genomics in CALGB 40601 (Alliance), a Neoadjuvant Phase III Trial of Paclitaxel-Trastuzumab With or Without Lapatinib in HER2-Positive Breast Cancer**

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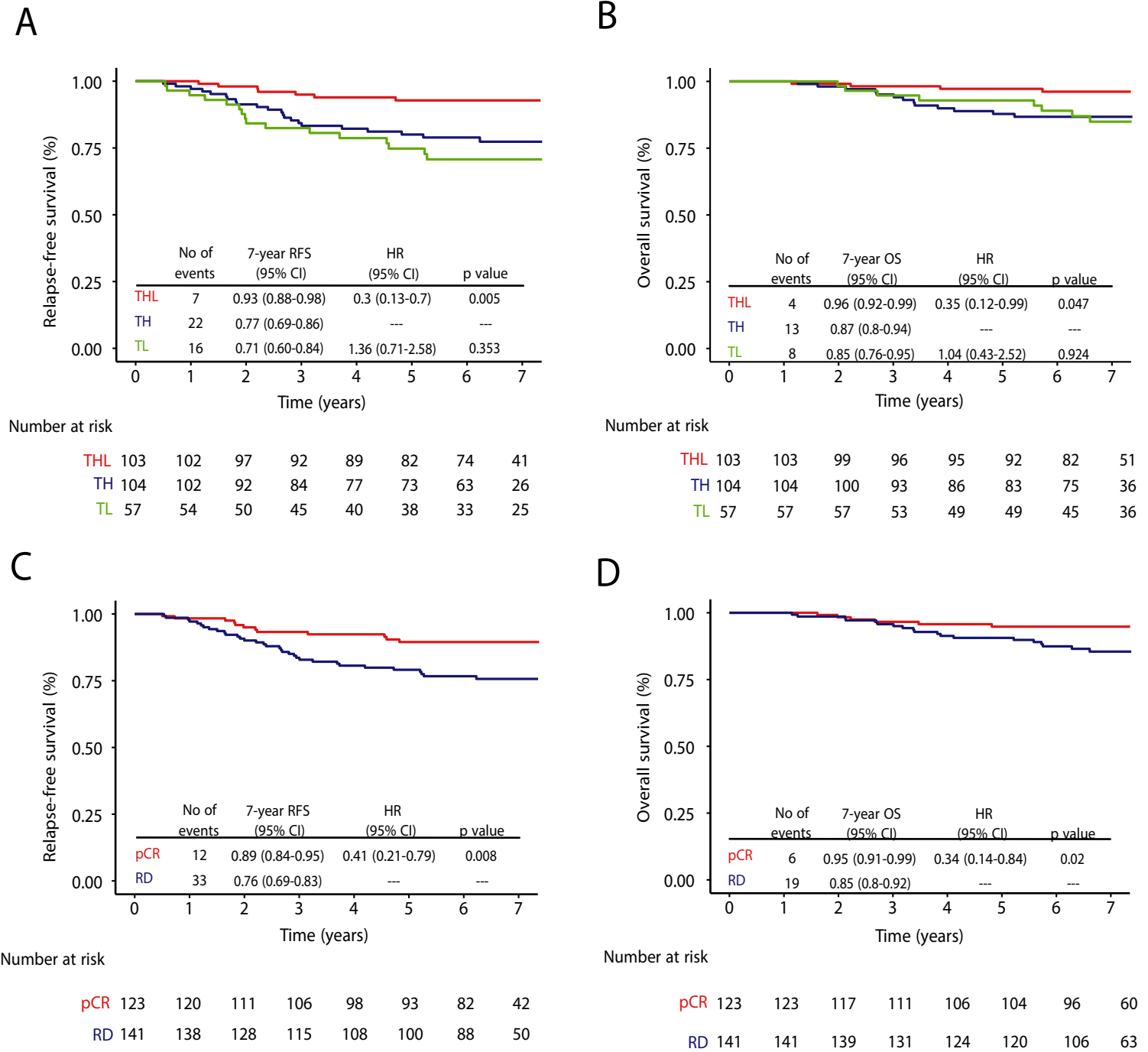
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Supplemental Figure 1. Kaplan-Meier curves for relapse-free survival (RFS) and overall survival (OS) in the gene-expression cohort of patients.



1A. Kaplan-Meier estimates of RFS at 7 years by treatment arm in the gene-expression population, showing a significant RFS benefit of the THL vs. TH treatment arm.

1B. Kaplan-Meier estimates of OS at 7 years by treatment arm in the gene-expression population, showing a significant OS benefit of the THL vs. the TH treatment arm.

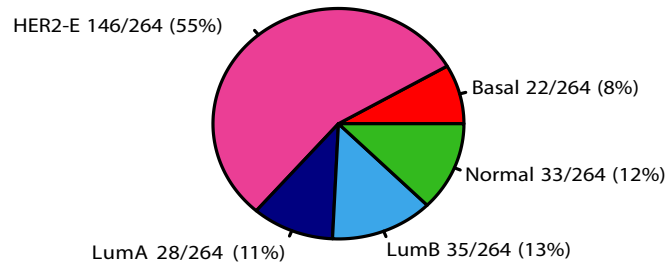
1C. Kaplan-Meier estimates RFS at 7 years by pCR status in gene-expression population, showing a RFS benefit of pCR vs. RD patients.

1D. Kaplan-Meier estimates of OS at 7 years by pCR in the gene-expression population, showing a OS benefit of pCR vs. RD patients.

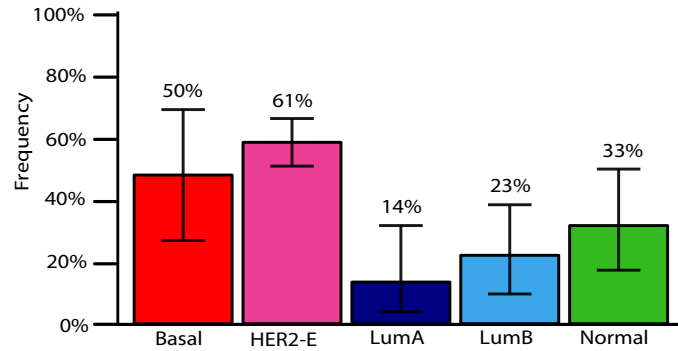
ITT, intention-to-treat; pCR, pathologic complete response; THL, paclitaxel, trastuzumab and lapatinib; TH, paclitaxel plus trastuzumab; TL, paclitaxel and lapatinib; RD, residual disease; HR Hazard ratio; RFS, relapse-free survival; CI, confident interval.

Supplemental Figure 2. Intrinsic subtype distribution, pCR, and relapse-free survival implications. The absolute tumor intrinsic subtype as a discrete variable was used for these figures.

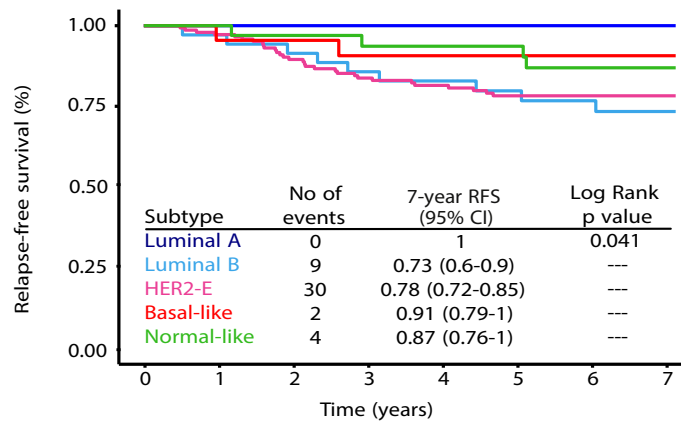
A



B



C



Number at risk

Subtype	0	1	2	3	4	5	6	7
Luminal A	28	28	28	27	27	25	22	11
Luminal B	35	34	33	30	29	26	24	14
HER2-E	146	141	126	116	104	97	85	48
Basal-like	22	22	20	19	18	17	14	4
Normal-like	30	33	32	29	28	28	26	16

2A. Intrinsic subtype distribution among the study population of clinically HER2-positive tumors, demonstrating that all the intrinsic subtypes can be found inside HER2-positive disease.

2B. Pathologic complete response (pCR) rates demonstrating significant variation across different subtypes. Error bars represent 95% confidence limits.

2C. Kaplan-Meier estimates of relapse-free survival at 7 years, demonstrating the prognostic differences by tumor intrinsic subtype.

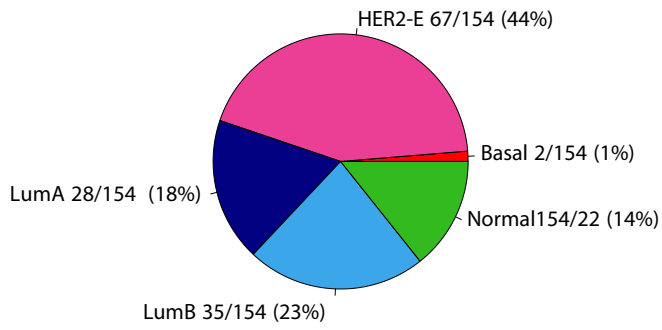
HER2-E, HER2-enriched; LumA, Luminal A; LumB, Luminal B; RFS, relapse-free survival; CI, confident interval.



Supplemental Figure 3. Intrinsic subtype distribution differed between hormone receptor (HR) positive (3A) and negative (3B) tumors (Fisher's exact test p-value < .001).

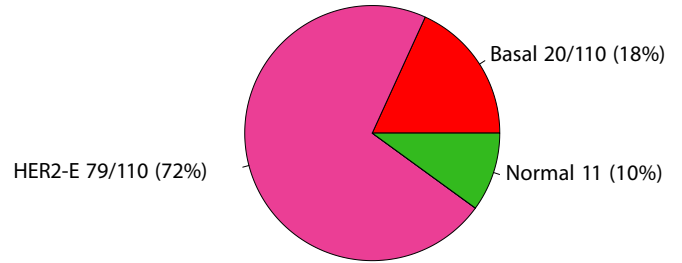
A

HR positive



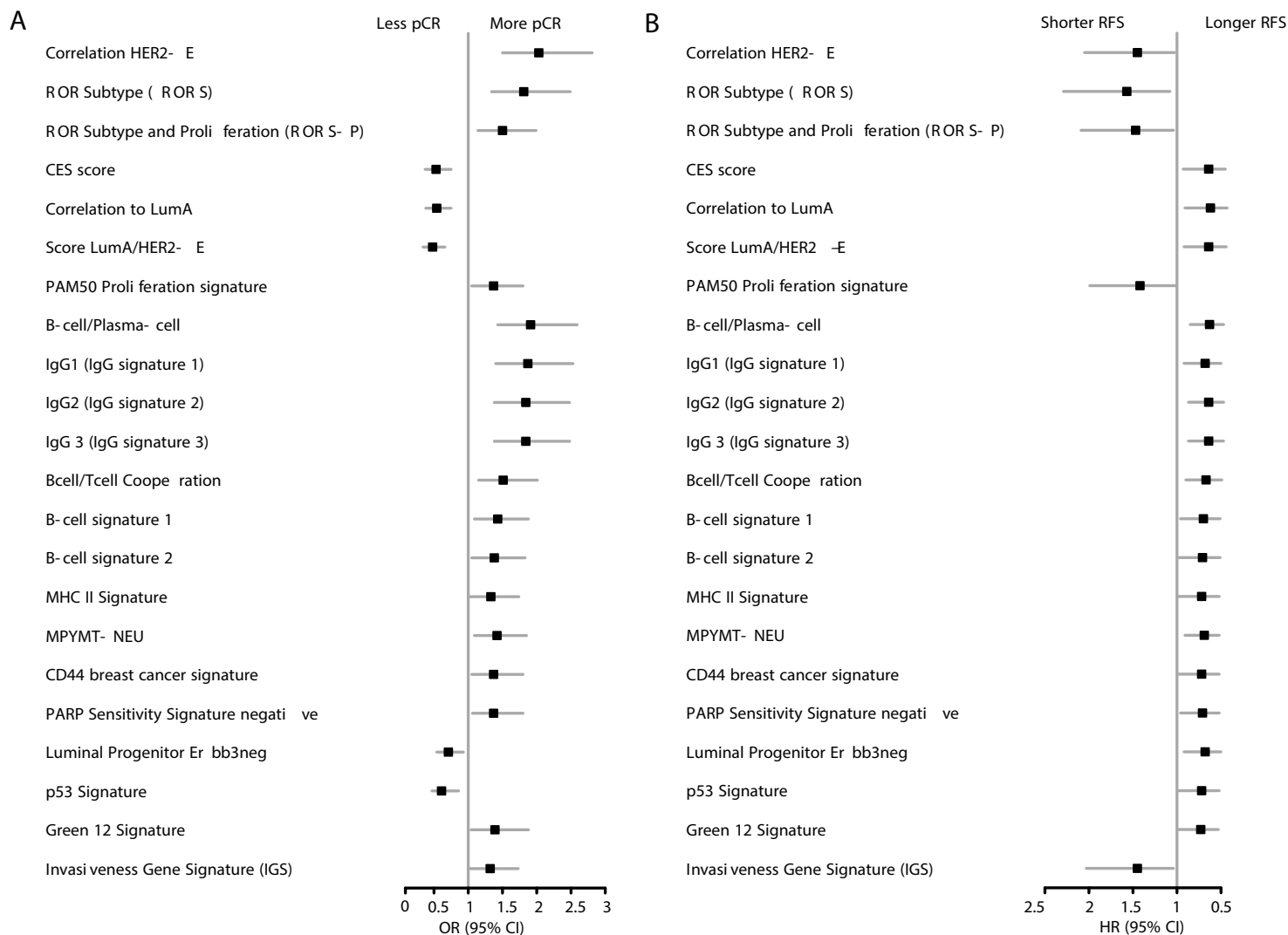
B

HR negative



HR, hormone receptor; HER2-E, HER2-Enriched; LumA, Luminal A; LumB, Luminal B.

Supplemental Figure 4. Twenty-two genomic biomarkers significantly associated with pathologic complete response (pCR) and relapse-free survival (RFS). For subtype-related biomarkers, the correlation to each PAM50 centroid was used.

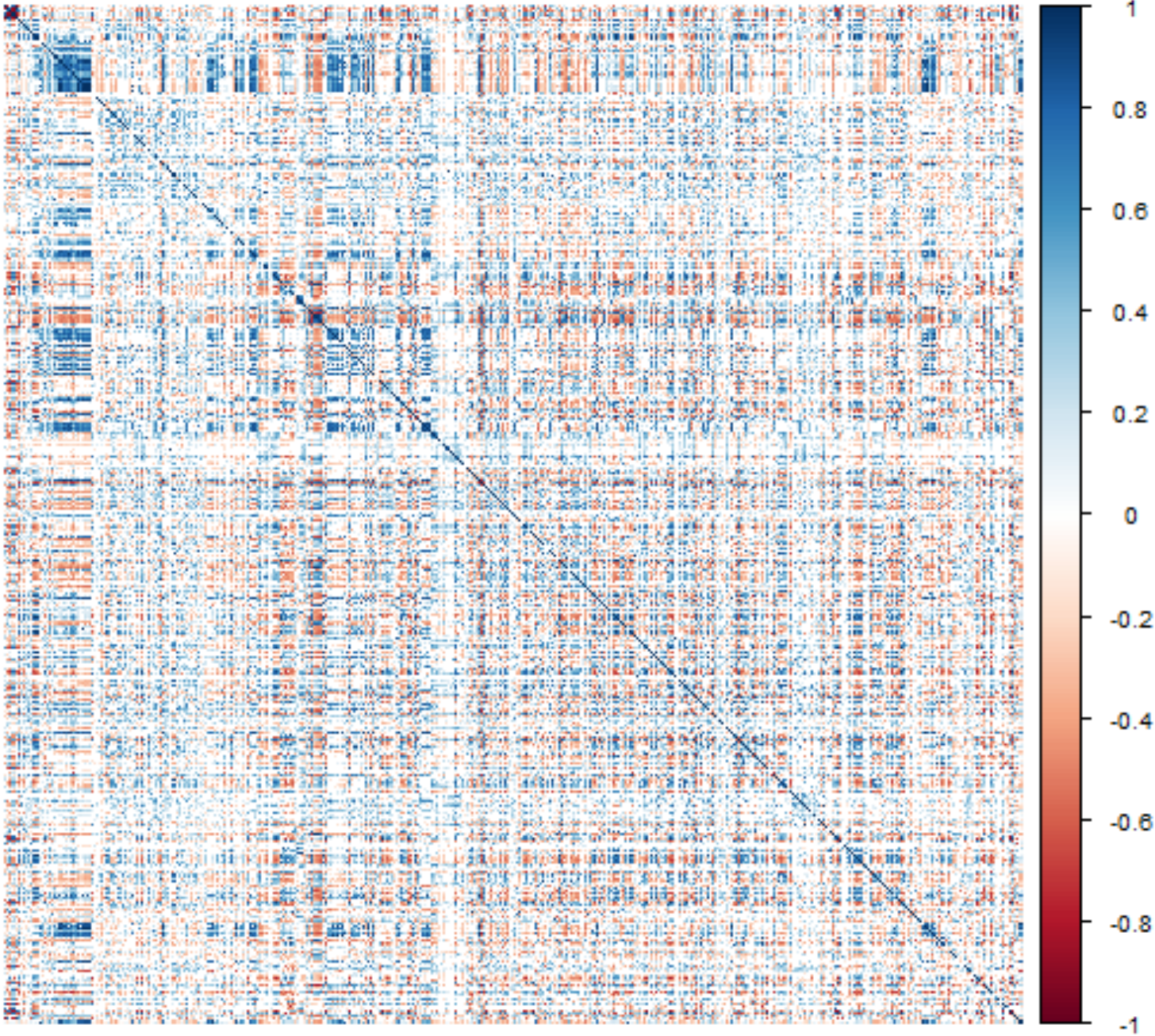


4A. Forest plot representing 22 logistic-regression models significantly associated with pCR.

4B. Forest plot representing 22 Cox-regression models significantly associated with RFS.

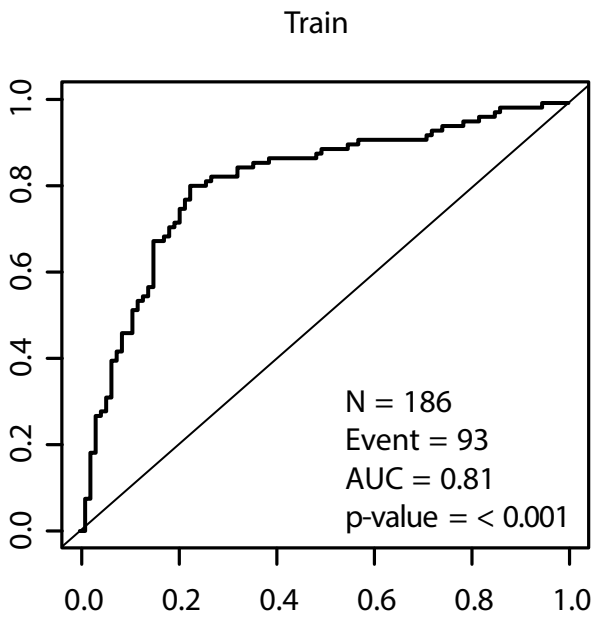
pCR, pathologic complete response; RFS, relapse-free survival; HER2-E, HER2-Enriched; ROR, risk of recurrence; S, subtype; S-P, subtype and proliferation; CES, chemo-endocrine score; LumA, luminal A, IgG, immunoglobulin G; MHC, major histocompatibility complex.

Supplemental Figure 5. Pearson's correlation matrix of 688 genomic biomarkers demonstrating the high collinearity between them. Positive significant Pearson correlations are displayed in blue, negative significant correlations in red, and non-significant correlations are displayed in white color. Color intensity is proportional to the correlation coefficient. For subtype-related biomarkers, the correlation to each PAM50 centroid was used.

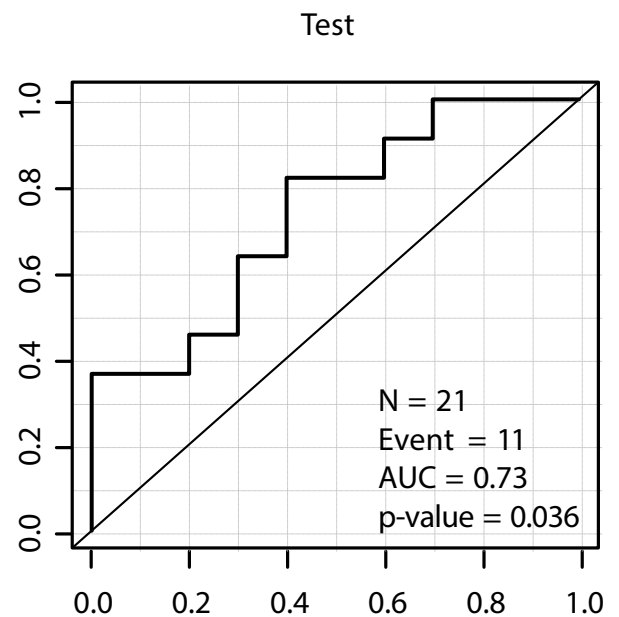


Supplemental Figure 6. Optimum Elastic-Net models for the prediction of response and survival. This figure represents the the final model fit in the train and the test sets.

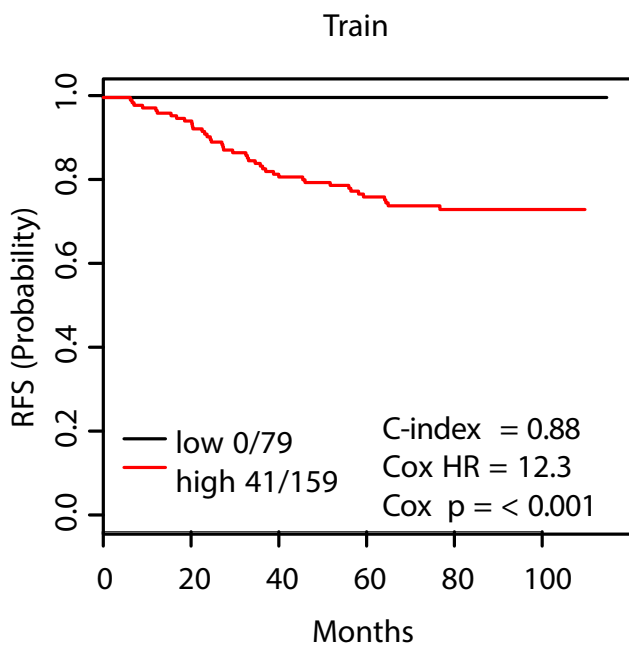
A



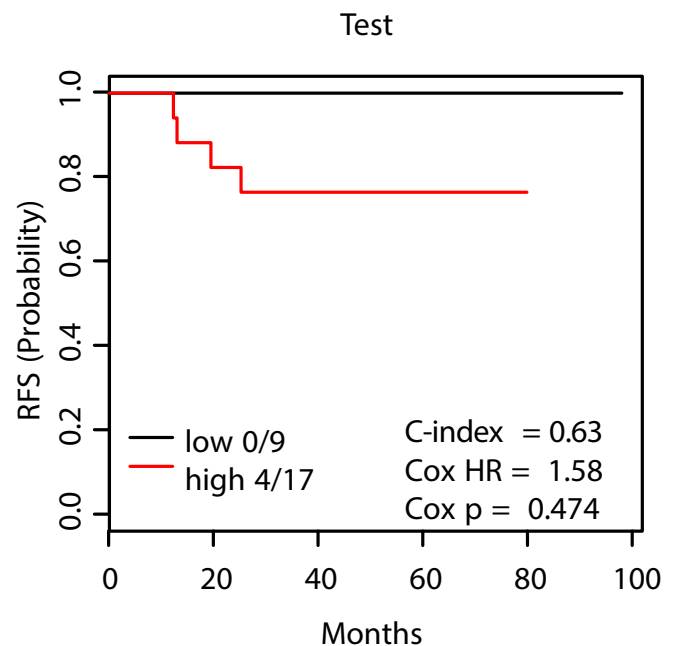
B



C



D

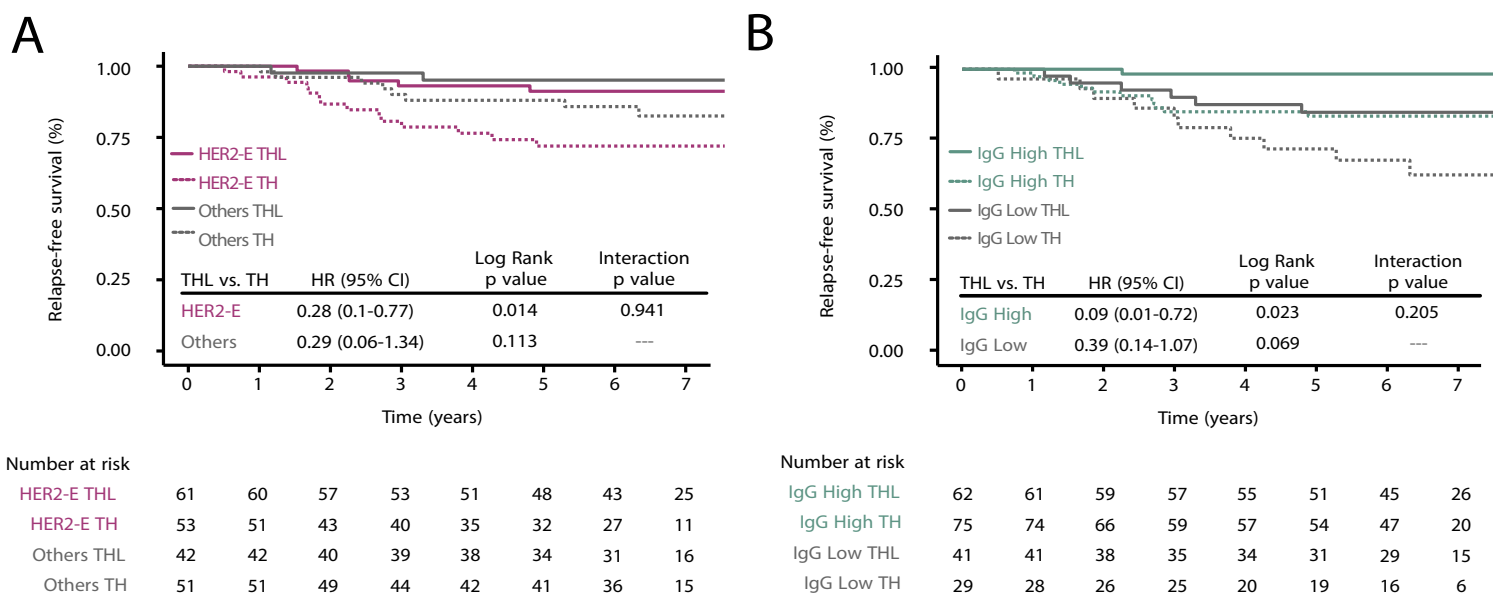


6A and 6B. Area under the curve (AUC) from the Receiver operating characteristic (ROC) curves were estimated for the optimum Elastic net model for pCR prediction in the train ( 7A) and the test ( 7B) sets. Clinical parameters and 688 gene expression signatures were tested as predictors. All RNAseq cohort was used to create this model (n = 264).

6C and 6D. Kaplan Meier curves and c-index were estimated for an Elastic Net model for RFS prediction at 7 years in the train ( 7C) and the test ( 7D) sets. Clinical parameters and 688 gene expression signatures were tested as predictors. All RNAseq cohort was used to create this model (n = 264).

AUC, area under the curve; THL, paclitaxel, trastuzumab and lapatinib; TH, paclitaxel plus trastuzumab; TL, paclitaxel and lapatinib; RFS, relapse-free survival; RNAseq, RNA sequencing.

Supplemental Figure 7. Evaluation of the predictive value of HER2-Enriched (HER2-E) subtype and Immunoglobulin G (IgG) signature. There was a significant benefit of dual (trastuzumab-lapatinib) vs. single (trastuzumab) treatment in the HER2-E patients and IgG-high patients, with no significant differences in other subtypes or IgG-Low patients. However, the interaction tests were non-significant.



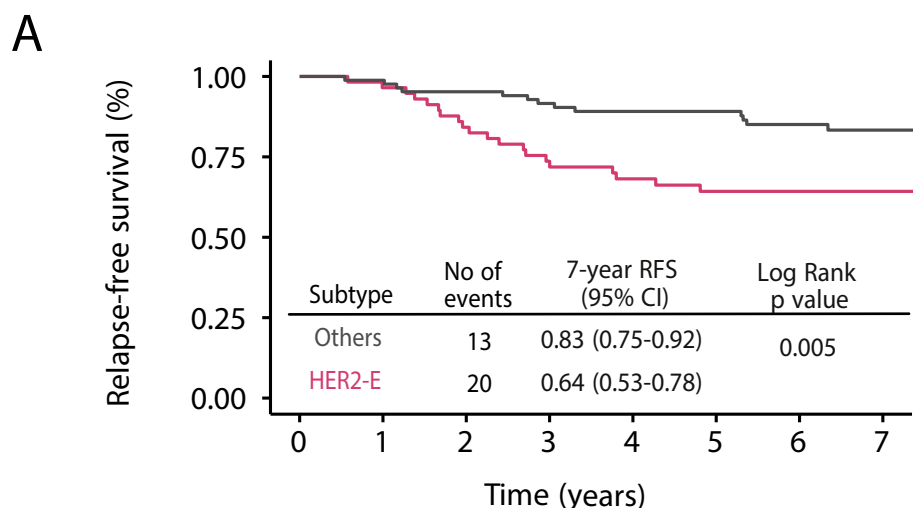
7A. Evaluation of the predictive and prognostic value of HER2-Enriched (HER2-E) biomarker in EFS at 7 years according to treatment arm. The absolute tumor intrinsic subtype HER2-Enriched vs. others (Luminal A, Luminal B, Normal-like and Basal-like) was used.

7B. Evaluation of the predictive and prognostic value of IgG signature biomarker in EFS at 7 years according to treatment arm. Patients were labelled as IgG-high (two upper tertiles) vs. IgG-low (lower tertile).

THL, paclitaxel, trastuzumab and lapatinib; TH, paclitaxel plus trastuzumab; HR Hazard ratio; RFS, relapse-free survival; CI, confident interval; HER2-E, HER2-Enriched, IgG immunoglobulin G.

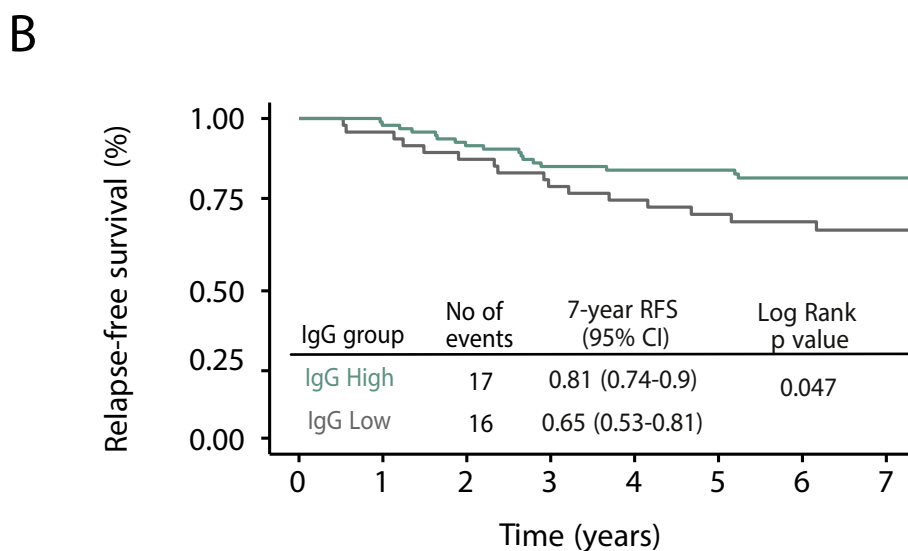


Supplemental Figure 8. Prognostic value of HER2-Enriched subtype and IgG status within the residual disease (RD) cohort of patients.



Number at risk

Others	84	83	80	74	72	67	60	32
HER2-E	57	55	48	41	36	33	28	18



Number at risk

IgG High	94	93	87	76	73	69	60	36
IgG Low	47	45	41	39	35	31	28	14

8A. Kaplan Meier curves illustrating a significant shorter relapse-free survival (RFS) time in HER2-Enriched patients vs. others (Luminal A, luminal B, Normal-like and Basal-like).

8B. Kaplan Meier curves illustrating a significant longer relapse-free survival (RFS) time in IgG-high patients (two upper tertiles) vs. IgG low patients (lower tertile).

RFS, relapse-free survival; CI, confident interval; HER2-E, HER2-enriched, IgG immunoglobulin G.

**Survival, pathologic complete response, and genomics correlates from CALGB 40601 (Alliance), a neoadjuvant phase III trial of paclitaxel-trastuzumab with or without lapatinib in HER2+ breast cancer.**

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**SUPPLEMENTAL METHODS.**

*RNA sequencing and expression quantification*

Whole transcriptome analyses by RNA sequencing (RNAseq) and gene expression analyses were performed in the Genomics Core High Throughput Sequencing Facility and analyzed by the University of North Carolina Lineberger Comprehensive Cancer Center Bioinformatics Core. Briefly, RNAseq libraries were made from total RNA using the Illumina TruSeq mRNA sample preparation kit and sequenced on an Illumina HiSeq 2000 using a 2x50bp configuration. Purity-filtered reads were aligned to the human reference GRCh38/hg38 genome using Spliced Transcripts Aligned to a Reference (STAR) version 2.4.2a<sup>1</sup>. Transcript (GENCODE v22) abundance estimates were generated by Salmon version 0.6.0<sup>2</sup> in ‘-quant’ mode, based on the STAR alignments. Raw read counts for all RNAseq samples were normalized to a fixed upper quartile<sup>3</sup>. RNAseq normalized gene counts were then log<sub>2</sub> transformed (zeros were unchanged), and genes were filtered for those expressed in 70% of samples. FASTQ files from RNAseq data are available via the NCBI dbGAP repository under accession number phs001570.v2.p1. The star-salmon upper quartile normalized gene expression matrix is available in GEO under the accession number GSE116335.

*Intrinsic subtype prediction and HER2/ER subgroup-specific gene centering method.*

One of the two goals of normalization before applying the PAM50 predictor was to correct the technical bias between the gene expression of two different platforms: the mRNAseq platform used in our study samples and the Agilent Human Microarrays utilized to create the original PAM50 UNC232 training set<sup>4</sup>. The second goal of this normalization method was to correct the differences in the biological composition between our study samples, all of them HER2-positive, and the original UNC232 training samples, with only 13% of HER2-positive tumors. The standard median centering that is routinely done before molecular subtyping prediction can produce inaccurate classifications when there is a difference in the distribution of clinicopathological characteristics between the study and the train set. To solve this problem, we applied a HER2/ER subgroup-specific gene centering method based on a previous publication<sup>5</sup>. We first collected the ER, PR, and HER2 IHC status from the original UNC232 training set samples, partially published<sup>4</sup>. 30% of the training set samples (n = 63) did not have any HER2 IHC information. For these samples, the HER2 IHC status was calculated from gene expression data as follows: we first extracted the *ERBB2* gene expression values from a collection of 345 samples with HER2 IHC information analyzed with the same Agilent Human Microarray platform than the original PAM50 UNC232 training set<sup>4</sup>; 265 samples were HER2-negative, and 80 samples were HER2 positive (IHC 3+ or IHC 2+ with a positive FISH). Then, we evaluated the ability of the *ERBB2* gene expression to predict the HER2 IHC status as a binary variable. The area under the ROC curve was 0.86. With a sensitivity of 70% and a specificity of 93.2%, the Youden index, corresponding to an *ERBB2* gene expression value of 1.265, was applied as a cutoff to the 63 training set samples without HER2 information. In total, 18/232 (7.76%) samples were considered HER2-positive/ER-positive, and 12/232 (5.17%) samples were labeled as HER2-positive/ER-negative within the

UNC232 training set. To select the best sample combination possible, a bioinformatic algorithm was designed and applied to an external RNAseq gene-expression dataset of 142 HER2-positive samples from the PAMELA clinical trial (SOLTI, NCT01973660), to obtain the higher concordance between the RNAseq derived subtypes and Prosigna® (NanoString Technologies, Seattle, WA) subtypes. With a kappa index of agreement of 0.9, 13/18 HER2-positive/ER-positive, and 10/12 HER2-positive/ER-negative samples from the UNC232 training set were selected to create the final HER2/ER subgroup-specific gene centering columns<sup>5</sup>. The gene expression values of the PAM50 genes in the CALGB 40601 samples were then normalized by this method. After the gene normalization, the PAM50 predictor<sup>4</sup> was applied. For each sample, we calculated the correlation coefficient to the PAM50 centroids (Basal-like, HER2-Enriched, Luminal A, Luminal B, and Normal-like signatures, respectively), the PAM50 proliferation signature and two PAM50 risk of recurrence models (ROR-subtype and ROR-subtype-proliferation). Correlation to each PAM50 centroid as continuous variables were used for pCR and RFS logistic regression, Cox and Elastic Net modeling.

### *Single genes and gene expression signatures*

Previous analysis of the CALGB 40601 and NeoALTTO trials showed a significant correlation of the single gene expression of ERBB2 and ESR1 with higher and lower pCR rates, respectively<sup>6,7</sup>. Also, in a correlative analysis of the APT trial, the protein expression of programmed death-ligand 1 (PD-L1) by IHC was highly correlated with TILs infiltration and other immune gene-expression signatures<sup>8</sup>. Based on these results, the single gene expression of *ERBB2*, *ESR1*, PD-1 (*PDCD1*), and PD-L1 (*CD274*) genes was extracted for each sample after median-centering and standardizing the gene expression matrix.

We next applied a collection of 676 known gene signatures, representing multiple biological pathways and cell types, to the median-centered and standardized gene expression matrix. The list of signatures is summarized in **Supplemental Table 1** and includes 48 signatures extracted from the Molecular Signature Database<sup>9</sup> and 628 signatures from 105 publications partially summarized before<sup>10-13</sup>. The signature scores for each sample were calculated in different ways. For example, 637 were computed by calculating the median expression of all the genes inside a signature, and 39 supervised models were obtained using different predefined algorithms<sup>14-35</sup>. These supervised models include two PAM50-derived biomarkers, the chemo-endocrine (CES) score<sup>15</sup>, and a new LumA-HER2-E score. This last signature is a new finding building upon some of our previous findings. We have previously showed<sup>6</sup> how correlation to HER2-Enriched centroid was highly correlated with higher pCR rate while the correlation to Luminal A centroid was highly correlated with lower pCR rate. Based on this result, we selected the correlation to Luminal A, and then we subtracted the correlation to HER2-Enriched subtype by sample, creating the new LumA-HER2-E score. The performance of this biomarker in a univariate logistic regression analysis on a CALGB 40601 train and test sets and the external validation in a chemotherapy and trastuzumab treated cohort of the NOHA<sup>36</sup> (ISRCTN86043495) and CHERLOB<sup>37</sup> (NCT00429299) trials (ISRCTN86043495) is summarized in **Supplementary Table 6**. Multiple immune-related biomarkers were also included in this gene-signatures collection, most of them initially extracted by comparing the gene expression pattern of different immune cell sub-populations<sup>38,39</sup>. Other immune signatures, like our IgG/B cell signatures, were obtained by an unsupervised cluster of different breast cancer samples as previously described<sup>10,40</sup>.



### *High dimensional modeling for pCR and RFS*

Elastic Net (R package glmnet<sup>41</sup>) is a regularized regression method that linearly combines the L1 and L2 penalties of the Ridge Regression and Least Absolute Shrinkage and Selection Operator<sup>42</sup>. This model addresses the collinearity of many biomarker variables (e.g., intrinsic subtype covaries with *ESR1* gene expression). Our data was divided (90% train, 10% test) using a stratified sampling<sup>43</sup> incorporating HR status, clinical stage, treatment arm, and, in the case of the RFS model, also pCR. Models were built to predict pCR or RFS in the training set, selecting lambda values over a grid of alpha values from 0.1 to 0.8 by 0.1 increments and lambdas recommended by glmnet, via 10-fold cross-validation<sup>41</sup>. Then, we calculated the accuracy of the different models over the training set, and we identified the optimal lambda and alpha combination with the minimum cross-validation error. Finally, we applied the final models to the test set. To evaluate the model fitting to the train/test sets, we evaluated the area under the receiver operating characteristic (ROC) curve for pCR prediction and the C-index for RFS. The primary purpose of using Elastic Net was to analyze which clinical and genomic features were most often included in both pCR and RFS optimized models.

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