Integrated evaluation of PAM50 subtypes and immune modulation of pCR in HER2-positive breast cancer patients treated with chemotherapy and HER2-targeted agents in the CherLOB trial

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Background: The aim of this work was to evaluate the impact of (and relative contribution of) tumor-related and immune-related diversity of HER2-positive disease on the response to neoadjuvant chemotherapy plus anti-HER2 agents.

Patients and methods: The CherLOB phase II study randomized 121 HER2-positive breast cancer patients to neoadjuvant chemotherapy plus trastuzumab, lapatinib or both. Tumor samples from diagnostic core biopsy were centralized. Tumor-infiltrating lymphocytes (TILs) were evaluated on H&E slides. Intrinsic subtyping was carried out using the research-based 50-gene prediction analysis of a microarray (PAM50) subtype predictor. Immune-related gene signatures were also evaluated.

Results: Continuous Str-TILs and It-TILs were significantly associated with pCR [OR 1.03, 95% CI 1.02–1.05 ($P < 0.001$) and OR 1.09, 95% CI 1.04–1.15 ($P < 0.001$) for Str-TILs and It-TILs, respectively]. According to PAM50, the subtype distribution was as follows: HER2-enriched 26.7%, Luminal A 25.6%, Luminal B 16.3%, Basal-like 14% and Normal-like 17.4%. The highest rate of pCR was observed for the HER2-enriched subtype (50%), followed by Basal-like, Luminal B and Luminal A (χ^2 test, $P = 0.026$). Immune gene signatures significantly associated with pCR in univariate analyses were identified: most of them maintained a significant association with pCR in multivariate analyses corrected for PAM50 subtypes, whereas TILs did not.

Conclusions: In this study, both tumor-related and immune-related features contribute to the modulation of pCR after neoadjuvant chemotherapy plus anti-HER2 agents. Immune signatures rather than TILs added significant prediction of pCR beyond PAM50 intrinsic subtypes.

Key words: breast cancer, HER2, immune signatures, neoadjuvant, PAM50, tumor-infiltrating lymphocytes

introduction

The heterogeneity of HER2-positive breast cancer (BC) constitutes an obstacle to the success of anti-HER2 treatments.

HER2-positive BCs display a different clinical behavior depending on hormone receptor (HR) status [1–5]. Moreover, within clinically defined HER2-overexpressing BC, all the five main intrinsic molecular subtypes (Luminal A, Luminal B, HER2-enriched, Basal-like, Normal-like) can be identified [6, 7].

Tumor–microenvironment interactions and immune aspects add another layer of complexity. In HER2-positive disease, higher levels of tumor-infiltrating lymphocytes (TILs) and the activation of immune pathways in the primary tumor have been suggested to predict for trastuzumab benefit in the adjuvant setting; moreover, TILs correlate with a higher chance of pathological complete response (pCR) after neoadjuvant chemotherapy plus trastuzumab [8–11].

The aim of this work was to evaluate the relative contribution of tumor-related and immune-related diversity of HER2-positive

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disease on the response to neoadjuvant chemotherapy plus anti-HER2 agents. We carried out an exploratory translational evaluation of PAM50 molecular intrinsic subtypes, TILs and immune gene signatures in the context of the neoadjuvant prospective CherLOB trial.

methods

clinical platform

The phase II CherLOB study randomized 121 patients with stage II–IIIA, HER2-positive BC to preoperative chemotherapy with weekly paclitaxel followed by FEC (fluorouracil, epirubicin and cyclophosphamide) plus trastuzumab (Arm A), lapatinib (Arm B) or both (Arm C). Study details and clinical results are described elsewhere [5]. Briefly, the CherLOB study showed that dual HER2 blockade (trastuzumab + lapatinib) combined with chemotherapy resulted in a significantly increased pCR rate compared with single HER2 blockade plus chemotherapy. Ethical Committees of all participating sites approved the study, and an informed consent was obtained from all patients before study entry.

Formalin-fixed paraffin-embedded (FFPE) tumor blocks from diagnostic core biopsies and surgical specimens were centralized and reviewed for quality and tumor content. Fresh-frozen samples from the diagnostic core biopsy were centralized.

TIL evaluation

Hematoxylin and eosin-stained (HES) slides from the diagnostic core biopsy were evaluated for Intratumoral (It) and Stromal (Str) TILs (%), according to predefined criteria [12].

Cases were defined as lymphocyte-predominant BC (LPBC) if presenting either It-TILs or Str-TILs (or both) ≥60%, and as non-LPBC if presenting both It-TILs and Str-TILs <60% [12]. TILs were also assessed after neoadjuvant treatment for those patients with residual invasive disease in the breast and availability of surgical sample.

Other pathology methods have already been reported [5, 13].

gene expression analysis

Methods for RNA extraction, quantification, quality assessment and gene expression microarray analysis are reported elsewhere [13]. Gene expression data have been deposited (GEO database, GSE66399). Principal components 1 and 2 loading plots using all genes revealed two outlier samples (77 and 78), which were removed from analyses.

intrinsic subtyping

The research-based, 50-gene prediction analysis of microarray (PAM50) subtype predictor was applied as previously described [14–16] to classify into one of the following groups: Luminal A, Luminal B, HER2-enriched, Basal-like and Normal-like [14].

immune signatures

Fifteen immune-related gene signatures were evaluated (supplementary Table S1, available at *Annals of Oncology* online). Ten gene lists associated with different immune cell types were identified from the literature [17–19]. Another signature composed of 28 immune-related genes, which we called RibasImmune, has been reported previously as predictive of anti-PD1 therapy in patients with melanoma [20]. Moreover, we carried out an unsupervised clustering analysis of the 4909 most variable genes across the 86 patients with gene expression data. We handpicked the only three gene clusters with immune-related genes (i.e. Immune 1, Immune 2, Immune 3) that met the following criteria: at least 20 genes and a coefficient correlation among them >0.80 (supplementary Figure S1 and Table S1, available at *Annals of Oncology* online). The SigClust method was applied to assess the statistical (https://cran.r-project.org/web/packages/sigclust/index.html) significance of our three clusters. To make sure the three gene clusters were stable, we evaluated the expression of each signature/cluster (i.e. each gene list) in the latest TCGA RNAseq-based dataset ($n = 1218$), where all subtypes are represented. The genes of each signature were found highly correlated among them similar to our observations in our Affymetrix-based dataset (supplementary data, available at *Annals of Oncology* online).

Finally, we checked for genes whose expression correlated with stromal TILs % (12 normal-like samples excluded) using a SAM quantitative analysis. Thus, both variables (i.e. the signature and stromal TILs) were evaluated as a continuous variable. A total of 82 genes showed a false discovery rate (FDR) below 10% (seven genes with an FDR below 5%). This gene list was found significantly enriched for the following biological processes: immunoglobulin, immune response, cytokine activity, lymphocyte activation. We called this signature Stromal-TIL SAM signature (supplementary Table S2, available at *Annals of Oncology* online).

The same analysis in correlation with It-TILs was conducted, but only three genes showed an FDR below 10% (*NPFR*, *CXCL13* and *C8Orf83*) and no signature was derived.

statistical analysis

Statistical analysis was carried out using IBM SPSS Version 22. Association between variables was evaluated by Student's *t*-test, Pearson's χ^2 test or Fisher's exact test. pCR was defined as the absence of residual invasive cancer in breast and axillary nodes following neoadjuvant therapy (ypT0/is ypN0). To calculate the score of each signature, the median expression of all genes within a signature was calculated. Odds ratios (ORs) with 95% confidence interval (95% CI) were calculated by logistic regression analysis. All statistical tests were two-sided and considered significant when $P \leq 0.05$. Event-free survival (EFS) was defined from the time of surgery to relapse, death or last follow-up. Hazard ratios (HRs) and 95% CI were calculated by Cox regression analysis.

results

patients' characteristics

Table 1 reports the characteristics of the patient population (supplementary Figure S2, available at *Annals of Oncology* online). None of the features listed in Table 1 significantly correlated with pCR (supplementary Table S3, available at *Annals of Oncology* online).

tumor-infiltrating lymphocytes

Pretreatment TILs assessment was available for 105 patients. Median It-TILs and Str-TILs% were 5% and 17% (interquartile ranges 0%–15% and 9%–40%), respectively. Seventeen patients (16.2%) had an LPBC and 88 (83.8%) presented a non-LBPC phenotype. The presence of higher levels of TILs was significantly associated with estrogen receptor (ER) negative status and high Ki67 (supplementary Table S4, available at *Annals of Oncology* online).

Str-TILs and It-TILs were significantly associated with pCR [OR 1.03, 95% CI 1.02–1.05 ($P < 0.001$) and OR 1.09, 95% CI 1.04–1.15 ($P < 0.001$) for each 1% increase in Str-TILs and It-TILs, respectively]. LPBC patients were more likely to achieve pCR compared with non-LPBC patients (Figure 1). Rates of

Table 1. Clinicopathological characteristics of patients included in the TIL and gene expression analyses compared with the overall CherLOB population

	Overall CherLOB population			TIL analysis population				Gene expression analysis population			
	N (%)	Mean (range)	IQ (Q1;Q3)	*N* (%)	Mean (range)	IQ (Q1;Q3)	*P**	*N* (%)	Mean (range)	IQ (Q1;Q3)	*P**
Age											
Available	121 (100)	49 (26–68)	12 (44;56)	105 (100)	50 (25–68)	12(45;57)	ns	86 (100)	49 (27–68)	13 (43;56)	ns
Missing	0 (0)			0							
Clinical stage											
IIA	38 (31.4)			37 (35.2)			ns	28 (32.5)			ns
IIB	61 (50.4)			51 (48.6)				41 (47.7)			
IIIA	22 (18.2)			17 (16.2)				17 (19.8)			
Missing	0			0				0			
Histotype											
Ductal	115 (95)			101 (96.2)			ns	83 (96.5)			ns
Lobular	6 (5)			4 (3.8)				3 (3.5)			
NA	0			0				0			
Grade											
1–2	23 (19)			20 (19)			ns	17 (19.8)			ns
3	77 (63.6)			68 (64.8)				55 (63.9)			
Missing	21 (17.4%)			17 (16.2)				14 (16.3)			
ER											
ER−	50 (41.3)			40 (38.1)			ns	36 (41.9)			ns
ER+	71 (58.7)			65 (61.9)				50 (58.1)			
Missing	0			0				0			
Ki67											
Available	113 (93.4)	29.6 (4–90)	15 (20;35)	101 (96.2)	28.3 (4–70)	15 (20;35)	ns	82 (95.3)	29.4 (4–90)	22 (18;40)	ns
Missing	8 (6.6)			4 (3.8)				4 (4.7)			
Arm											
A (CT + T)	36 (29.8)			32 (30.5)			ns	22 (26.6)			ns
B (CT + L)	39 (32.2)			34 (32.4)				30 (34.9)			
C (CT + T+L)	46 (38)			39 (37.1)				34 (39.5)			
Missing	0			0				0			
Evaluable for path response											
Yes	118			105				84			
No	3			0				2			

N, number; IQ, interquartile; Q, quartile; *P*, *P*-value; ER, estrogen receptor; CT, chemotherapy; T, trastuzumab; L, lapatinib; path, pathological; ns, non-significant.

**P*-value for χ^2 test (categorical variables) and Student's *t*-test (continuous variables) for the comparison versus the overall CherLOB population.

pCR were 64.7% for LPBC and 25% for non-LPBC patients, respectively ($P < 0.001$).

The association between 1% TILs increment and pCR was observed in both the ER-positive [OR 1.03, 95% CI 1.00–1.06 ($P = 0.035$) and OR 1.08, 95% CI 1.00–1.15 ($P = 0.049$) for Str-TILs and It-TILs, respectively] and ER-negative groups [OR 1.04, 95% CI 1.01–1.06 ($P = 0.009$) and OR 1.01, 95% CI 1.02–1.18 ($P = 0.012$) for Str-TILs and It-TILs, respectively]. According to treatment, continuous TILs significantly predicted pCR only for patients included in arm B [OR 1.05, 95% CI 1.02–1.09 ($P = 0.005$) and OR 1.15, 95% CI 1.04–1.28 ($P = 0.009$) for Str-TILs and It-TILs, respectively] and C [OR 1.03, 95% CI 1.00–1.06 ($P = 0.041$) and OR 1.11, 95% CI 1.01–1.22 ($P = 0.025$) for Str-TILs and It-TILs, respectively], whereas no effect was seen in arm A [OR 1.03, 95% CI 0.99–1.06 ($P = 0.132$) and OR 1.08, 95% CI 0.97–1.21 ($P = 0.178$) for Str-TILs and It-TILs, respectively]. Figure 1 reports the association between LPBC and pCR according to ER status and treatment, including interaction tests. However, due to the small sample size, these findings should be interpreted with caution.

No significant change in It-TIL and Str-TIL before and after treatment was observed in the 57 assessable patients with residual invasive disease in the breast (supplementary Table S5A, available at *Annals of Oncology* online). Ten patients showed a 10% increase from pre- to post-treatment Str-TILs: 7 of them were ER negative and all of them received lapatinib as part of the neoadjuvant treatment (supplementary Table S5B, available at *Annals of Oncology* online).

intrinsic subtypes

The majority of tumors were not identified as belonging to the HER2-enriched subtype by PAM50 (supplementary Figure S3, available at *Annals of Oncology* online). The subtype distribution was as follows: HER2-enriched 26.7%, Luminal A 25.6%, Luminal B 16.3%, Basal-like 14% and Normal-like 17.4%. When

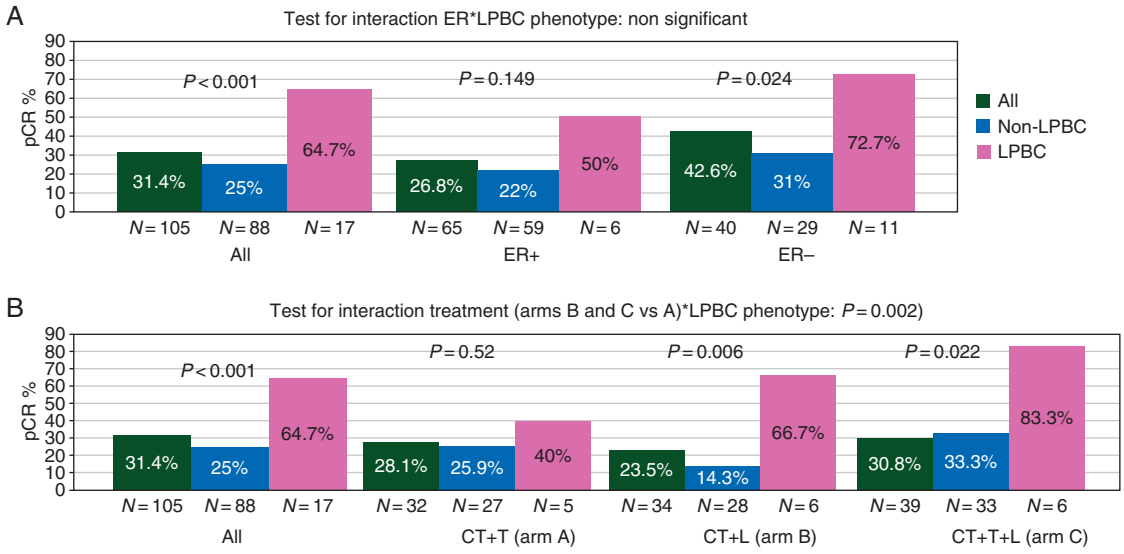

Figure 1. pCR rates in LPBC and non-LPBC tumors. Overall, in the ER-positive and ER-negative groups (A) and according to treatment (B). LPBC, lymphocyte-predominant breast cancer; ER, estrogen receptor; pCR, pathologic complete response.

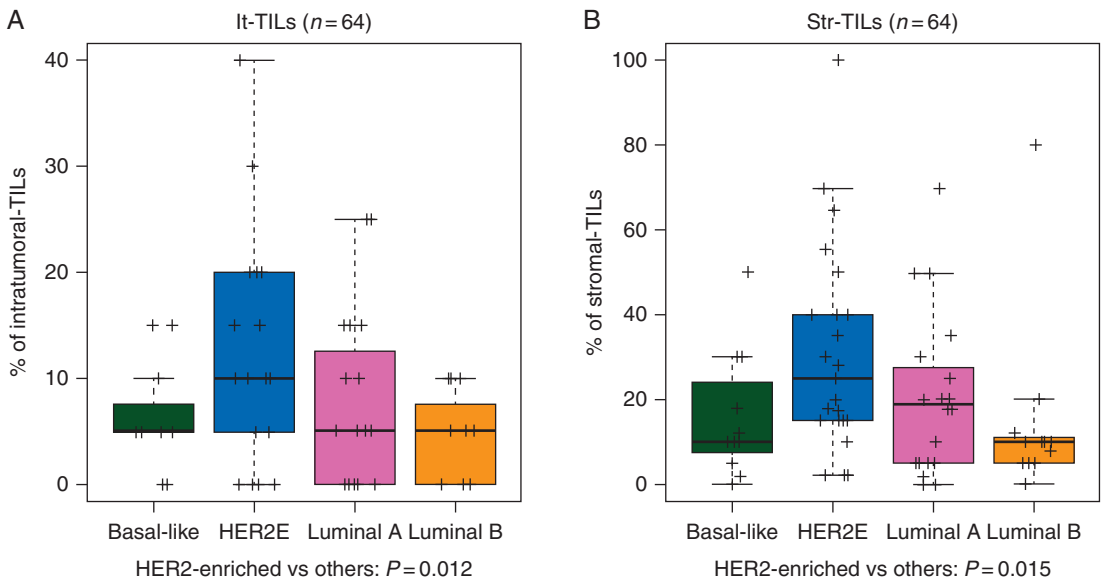

Figure 2. TIL levels across the intrinsic subtypes. (A) It-TILs; (B) Str-TILs. TILs, tumor-infiltrating lymphocytes.

considering ER-negative cases, more than a half were HER2-enriched and 11% were Luminal A or B. To the opposite, ER-positive BCs mostly displayed a Luminal profile (Luminal A 38% and Luminal B 26%), with only 8% of the samples being classified as HER2 enriched.

The highest levels of TILs were observed in HER2-enriched BCs (Figure 2): median It-TILs and Str-TILs were, respectively, 10% and 27.5% in HER2-enriched compared with 5% and 10% in non-HER2-enriched samples (*t*-test, $P = 0.012$ and $P = 0.015$, respectively, Normal-like excluded). The number of LPBC patients across PAM50 subtypes was as follows: LumA ($n = 1$), LumB ($n = 1$), HER2-enriched ($n = 3$), Basal-like ($n = 0$), Normal-like ($n = 2$).

The pCR rates according to PAM50 subtypes for the 69 patients who were evaluable for pCR (Normal-like cases excluded) are reported in Table 2. The highest rate of pCR was observed for the HER2-enriched subtype (50%), followed by Basal-like, Luminal B and Luminal A (pCR rates 25%, 21% and 10%, respectively; χ^2 test, $P = 0.026$).

immune signatures

We investigated whether immune signatures add information beyond PAM50 and/or TILs. For each sample, we identified a gene score for the 15 identified immune signatures (Stromal-TIL SAM, Immune1, Immune2, Immune3, immune-related gene signatures tracking T cells, CD8 cells, activated dendritic cells -aDC-, T-helper cells, immature dendritic cells -iDC-, B cells, TH1 cells, TH2 cells, Natural Killer cells, regulatory T-cells -FOXP3- and for the RibasImmune signature) [17–20]. The majority of immune

gene signatures were positively correlated with each other (supplementary Figure S4, available at *Annals of Oncology* online). The correlation coefficient among the various immune-related gene signatures, except those tracking Treg-FOXP3 and NK cells, was high (Pearson correlation coefficient = 0.816). When the two types of TILs (It-TILs and Str-TILs) were included, the correlation coefficient among all variables (signatures and TILs) was moderate (Pearson correlation coefficient = 0.572).

Poor correlation was found between PAM50 subtype or proliferation signatures with immune signatures or TILs. No immunohistochemical or flow cytometry data supporting the immune variables were available.

We also investigated the association of previously described biomarkers [13] with TILs and immune signatures (supplementary Table S6, available at *Annals of Oncology* online). A positive correlation between PTEN expression and T-cell signatures and a negative correlation between phospho-akt expression and T_{helper} Cell signature were noted.

The association of each gene signature with pCR was evaluated in the 84 patients with gene expression data who were also evaluable for response. High expressions of Immune 2, Immune 3, CD8-cell and T-cell signatures were statistically significantly correlated with a higher probability of achieving a pCR (Figure 3A).

Table 2. pCR rates according to PAM50 intrinsic subtypes (normal-like excluded)

PAM50 intrinsic subtype	*N* tot	pCR; *n* (%)	χ^2 *P*
Her2-enriched	22	11 (50)	0.026
Basal-like	12	3 (25)	
Luminal B	14	3 (21.4)	
Luminal A	21	2 (9.5)	
Total	69	19 (27.5)	

N, *n*, number; tot, total; pCR, pathological complete response; *P*, *P*-value.

We then evaluated whether those immune-related parameters that were significant predictors of pCR in univariate analysis (four immune gene signatures, It-TIL and Str-TIL) added independent information to PAM50 (Normal-like cases excluded) in multivariate models (Figure 3B). Three of the four immune signatures that correlated with pCR in univariate analyses maintained their predictive value in a multivariate model corrected for PAM50. The association between It-TILs and Str-TILs with pCR did not maintain statistical significance beyond PAM50. The results were similar for multivariate analyses of each immune-related parameter after adjusting for ERBB2 and ESR1 expression instead of PAM50 (supplementary Figure S5, available at *Annals of Oncology* online).

survival analyses

Increasing It-TILs levels (per 1%) were associated with a better EFS in univariate analyses (HR 0.93, 95% CI 0.87–1.00, $P = 0.047$). Full exploratory survival analyses are reported in supplementary Table S7, available at *Annals of Oncology* online; however, due to the low number of events, the data must be interpreted with caution.

discussion

In this work, both tumor molecular heterogeneity and the immune microenvironment contribute to modulating the response of clinically defined HER2-positive tumors to neoadjuvant chemotherapy and anti-HER2 agents. Intrinsic molecular subtypes and immune gene signatures provide distinct biological information independently affecting treatment sensitivity.

Our results are in line with previous reports showing that HER2-positive BCs encompass a spectrum of distinct molecular entities. In our population of HER2-positive patients, all the main intrinsic molecular subtypes were identified [6, 7, 21]. Moreover, the majority of cases were not classified as HER2 enriched. The different distribution of the PAM50 subtypes

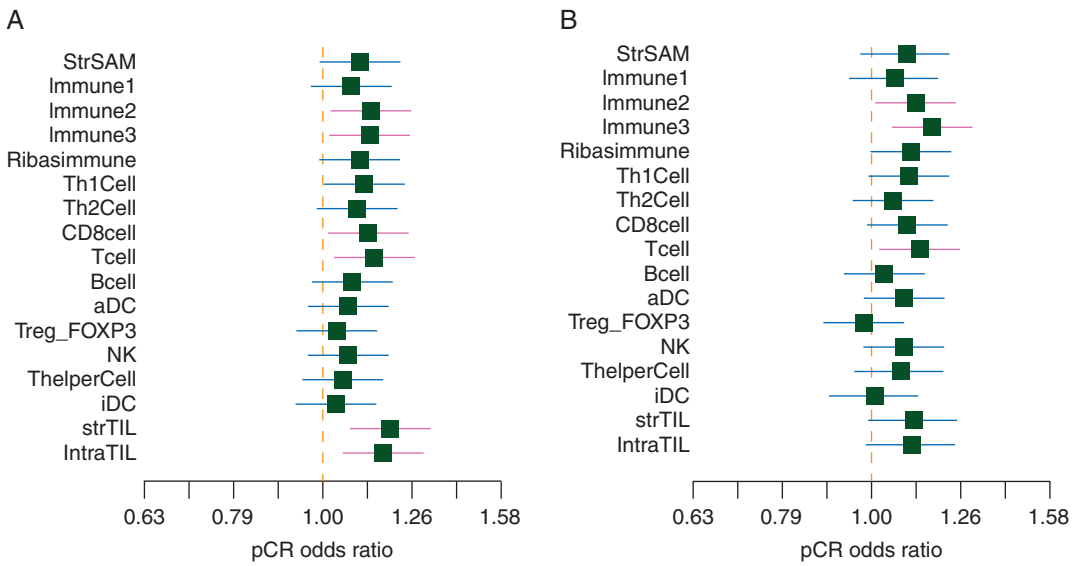

Figure 3. Odds ratio (ORs) for pathologic complete response (pCR) of each immune signature or TIL (for unit increase) in (A) univariate analysis and (B) bivariate analysis of each signature when adjusted by intrinsic subtype. Each row represents an individual analysis. TIL, tumor-infiltrating lymphocyte.

according to ER status is similar to the data by Carey *et al.* [21], highlighting that the drivers of HER2-positive/ER-positive and HER2-positive/ER-negative cancers are different. The significant prediction of pCR by PAM50 subtypes that we observed indicate that capturing the molecular diversity of HER2-positive BC may be clinically relevant [6, 21]. Nevertheless, this consideration should be limited to the neoadjuvant setting, since so far, PAM50 subtyping has not demonstrated to predict benefit from trastuzumab in the adjuvant setting [22].

Cancer is a disease that does not exclusively involve tumor cells. The immune tumor microenvironment seems particularly relevant in HER2-positive disease for two main reasons. First, oncogene-addicted tumors are able to mediate an immunosuppressive microenvironment that favors tumor growth [23]; second, both chemotherapy and trastuzumab are able to elicit an antitumor immune response [24, 25]. The evaluation of TILs represents the simplest method to provide immune-related information [12]. In our study, the levels of TILs significantly correlated with the likelihood of achieving a pCR after chemotherapy and anti-HER2 treatments, in line with data from other groups [10, 26] but in contrast with the results from the NeoALTTO and NeoSphere trials [27, 28]. Differences between these trials, including the neoadjuvant chemotherapy backbone (anthracyclines + taxanes or taxanes only), may have accounted for discrepancies. Another point could be that TILs probably provide only raw information on the complexity of the tumor-immune microenvironment. Data from other studies have suggested that immune gene signatures or metagenes can predict response to and benefit from HER2-targeted therapies [11, 21, 28]. The fact that TILs evaluation is relatively easy and cheap represents an advantage; however, in our study, TILs failed to provide an independent prediction of pCR beyond molecular subtypes and were outperformed by immune-related gene signatures in this sense.

In conclusion, the complexity of HER2-positive BC relies on both tumor cell-related features and the stroma, both contributing to a significant extent to the modulation of treatment sensitivity. In the era of the rapidly increasing knowledge on molecular cancer classification and oncoimmunology, the search for prognostic and predictive biomarkers should move from focusing on one single piece of the puzzle to a more comprehensive and integrated view.

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disclosure

The authors have declared no conflicts of interest.

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