Immune modulation of pathologic complete response after neoadjuvant HER2-directed therapies in the NeoSphere trial

G. Bianchini1, L. Pusztai2, T. Pienkowski3, Y.-H. Im4, G. V. Bianchi5, L.-M. Tseng6, M.-C. Liu7, A. Lluch8, E. Galeota1, D. Magazzù9, J. de la Haba-Rodríguez10, D.-Y. Oh11, B. Poirier12, J. L. Pedrini13, V. Semiglazov14, P. Valagussa9 & L. Gianni1*

1Department of Medical Oncology, IRCCS Ospedale San Raffaele, Milan, Italy; 2Medical Oncology, Yale Cancer Center, Yale School of Medicine, New Haven, USA; 3Centrum Onkologii, Warsaw, Poland; 4Department of Medicine, Samsung Medical Center, Seoul, Republic of Korea; 5Oncologia Medica, Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy; 6Taipei-Veterans General Hospital, National Yang-Ming University, Taipei; 7Koo Foundation Sun Yat-Sen Cancer Center, Taipei, Taiwan; 8Hospital Clínico Universitario, INCLIVA Biomedical Research Institute, Valencia, Spain; 9Fondazione Michelangelo, Milan, Italy; 10Hospital Reina Sofia, Córdoba, Spain; 11Division of Medical Oncology, Seoul National University Hospital Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea; 12Centre des maladies du sein, Hôpital du Saint-Sacrement, CHU de Québec, Québec, Canada; 13Hospital Ernesto Dornelles, Porto Alegre, Brazil; 14NN Petrov Research Institute of Oncology, St Petersburg, Russia

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Background: To investigate in the NeoSphere trial the contribution of the immune system to pathologic complete response in the breast (pCRB) after neoadjuvant docetaxel with trastuzumab (TH), pertuzumab (TP), or both (THP), or monoclonal antibodies alone (HP).

Patients and methods: Immune gene mRNA expression (n = 350, 83.8%), lymphocyte infiltration (TIL, n = 243, 58.3%), and PDL1 by immunohistochemistry (n = 305, 73.1%) were correlated with pCRB. We studied five selected genes (IFNG, PD1, PDL1, PDL2, CTLA4) and six immune metagenes corresponding to plasma cells (IGG), T cells (CD8A), antigen-presenting cells (MHC2), and to MHC1 genes (MHC1), STAT1 co-expressed genes (STAT1), and interferon-inducible genes (IF-I). Gene expression data from the NOAH trial were used for validation.

Results: TIL as continuous variable and PDL1 protein expression were not significantly associated with pCRB. Expression of immune genes/metagenes had different association with pCRB after THP than after other therapies. With THP, higher expression of PD1 and STAT1, or any among PDL1, CTLA4, MHC1, and IF-I was linked with lower pCRB. In the combined TH/TP/HP treatment group, in multivariate analysis, higher expression of PD1, MHC2, and STAT1 were linked with pCRB, and higher PDL1, MHC1, or IF-I to lower pCRB. In the NOAH, a similar association of higher STAT1 with higher pCRB, and higher MHC1 and IF-I with lower pCRB was found for trastuzumab/chemotherapy but not for chemotherapy treatment only.

Conclusions: The immune system modulates response to therapies containing trastuzumab and pertuzumab. Greatest benefit from THP is observed for low expression of some immune markers (i.e. MHC1, CTLA4). The involvement of PDL1 in resistance supports testing combinations of HER2-directed antibodies and immune-checkpoint inhibitors.

Key words: breast cancer, pertuzumab, immune system, predictive marker, gene expression, trastuzumab

introduction

In the randomized NeoSphere study, we showed that a 12-week-long neoadjuvant course with docetaxel and the HER2-directed monoclonal antibodies trastuzumab and pertuzumab (THP regimen) significantly increased the rate of pathologic complete response in the breast (pCRB) over that with conventional docetaxel and trastuzumab (TH) or docetaxel and pertuzumab (TP) [1]. Treatment with the two monoclonal antibodies without chemotherapy (HP) led to 17% pCRB [1].

In the trial, we collected tumors biopsies before therapy from almost all patients. There is increasing evidence that the presence and composition of immune cells in the tumor microenvironment can modulate tumor response to chemotherapy and HER2-targeted antibody therapies [2, 3]. Preclinical experiments and correlative science observations suggest that...
the anti-cancer activity of trastuzumab is partially mediated by the immune system independent of its effects on HER2 signaling [3].

Whether similar or distinct immune mechanisms contribute to chemotherapy and antibody-therapy induced tumor responses is currently unknown. The goal of the current study was to examine association between pCRB and several distinct mRNA expression-based immunoparameters as well as PD-L1 protein expression and tumor-infiltrating lymphocyte (TIL) count in pretreatment biopsies from the NeoSphere trial. The four arms of the trial, including one non-chemotherapy arm, provided a unique opportunity to test if different immune parameters are predictive of responses in different arms. We also assessed the generalizability of our observations in the previously published gene expression data from the NOAH trial.

materials and methods

patients

The results of the NeoSphere trial were previously published [1]. All patients were centrally confirmed HER2-positive operable or locally advanced breast cancer (LABC) and were randomized into one of four different neoadjuvant therapies: trastuzumab (H) plus docetaxel (T) (TH); pertuzumab (P) and trastuzumab plus docetaxel (THP); pertuzumab and trastuzumab without chemotherapy (HP); or pertuzumab plus docetaxel (TP). Four cycles q 3 weeks were administered before surgery. The primary end point of the study was pathologic complete response in the breast (pCRB) (supplementary Methods, available at Annals of Oncology online). Ninety-eight percent of the 417 patients accrued to the trial had pretreatment baseline breast tumor biopsy collected. All patients provided written informed consent. Approvals for the study protocol (and any modifications thereof) were obtained from independent ethics committees.

gene expression

RNA was extracted from baseline (pretreatment) formalin-fixed paraffin-embedded core biopsies (n = 408, 98.1% of the trial population) and gene expression profiling (GEP) was carried out with Affymetrix U133 Plus 2.0 gene chips as previously described (also supplementary Methods, available at Annals of Oncology online) [4]. Three-hundred and fifty biopsies (84%) yielded high-quality GEP results (supplementary Figure S1, available at Annals of Oncology online). The patient characteristics of the GEP biomarker analysis group was similar to the entire NeoSphere trial population (Table 1). Thirteen patients were not assessable for pCR.

The generalizability of the associations between immune parameter and pCRB observed in NeoSphere were assessed on an independent publically available gene expression dataset (GSE50948) from pretreatment biopsies of HER2-positive patients treated with neoadjuvant chemotherapy with or without trastuzumab in the NOAH trial [4].

TIL count and PD-L1 immunohistochemistry

Intratumoral (TumTIL) and stromal (StrTIL) TILs were scored in hematoxylin and eosin-stained full-face slides from baseline core biopsies as previously described [5, 6]. Three groups were defined: lymphocyte predominant BC (LPBC) as ≥50% of either stromal or intratumoral lymphocytic infiltration [6]; low lymphocyte infiltration (LowTIL) defined as <5% of TumTIL and StrTIL, and an intermediate group (IntTIL) including all other cases.

Paraffin-embedded tissue sections of 4 µm thickness were assessed for the PD-L1 protein by immunohistochemistry (IHC) using a rabbit monoclonal antibody (clone SP142, Ventana); staining was carried out on an automated stainer with EDTA-based antigen retrieval (Discovery Ultra, Ventana). Any PD-L1 staining was considered positive. PD-L1 expression was assessed on tumor and tumor-infiltrating immune cells by estimating percentage of tumor and immune cells stained regardless of intensity. An expert pathologist scored all the samples.

immune genes and immune metagenes

We carried out a pre-planned analysis for five genes and six additional metagenes. Five immune genes (IFNγ, PD1, PDL1, PDL2, CTLA4) were a priori selected for analysis because of their key roles in regulating local immune response and for their direct therapeutic relevance. The probe sets that represent these genes are listed in supplementary Table S1, available at Annals of Oncology online. The six immune metagenes used in this analysis represent the average expression of a set of highly co-expressed genes that were defined from a collection of publicly available GEP datasets (n = 1186) (supplementary Method, Table S1 and Figure S2, available at Annals of Oncology online) [7–9]. The metagenes capture semi-quantitative signals from distinct cell types, including plasma cells (IgG metagene), T-cells

<table>
<thead>
<tr>
<th>Table 1. NeoSphere patients characteristics</th>
<th>Overall study population (n = 417)</th>
<th>Biomarker population (GEP available) (n = 350)</th>
<th>P-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (years, range)</td>
<td>50 (22–80)</td>
<td>50 (22–80)</td>
<td>0.969</td>
</tr>
<tr>
<td>Type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Operable</td>
<td>254 (60.9%)</td>
<td>213 (60.9%)</td>
<td>0.965</td>
</tr>
<tr>
<td>Locally advanced</td>
<td>134 (32.1%)</td>
<td>111 (31.7%)</td>
<td></td>
</tr>
<tr>
<td>Inflammatory</td>
<td>29 (7.0%)</td>
<td>26 (7.4%)</td>
<td></td>
</tr>
<tr>
<td>Lymph node statusb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>123 (29.6%)</td>
<td>99 (28.4%)</td>
<td>0.907</td>
</tr>
<tr>
<td>N1</td>
<td>188 (45.2%)</td>
<td>163 (46.7%)</td>
<td></td>
</tr>
<tr>
<td>N2/N3</td>
<td>105 (25.2%)</td>
<td>87 (24.9%)</td>
<td></td>
</tr>
<tr>
<td>ER status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>186 (44.7%)</td>
<td>161 (46.0%)</td>
<td>0.721</td>
</tr>
<tr>
<td>Negative</td>
<td>230 (55.3%)</td>
<td>189 (54.0%)</td>
<td></td>
</tr>
<tr>
<td>PGR status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>138 (33.2%)</td>
<td>116 (33.1%)</td>
<td>0.993</td>
</tr>
<tr>
<td>Negative</td>
<td>278 (66.8%)</td>
<td>234 (66.9%)</td>
<td></td>
</tr>
<tr>
<td>Arm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TH (Arm A)</td>
<td>107</td>
<td>87</td>
<td>0.994</td>
</tr>
<tr>
<td>THP (Arm B)</td>
<td>107</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>HP (Arm C)</td>
<td>107</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>TP (Arm D)</td>
<td>96</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>pCRBreast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>121 (29.0%)</td>
<td>104 (29.7%)</td>
<td>0.976</td>
</tr>
<tr>
<td>Noc</td>
<td>280 (67.2%)</td>
<td>233 (66.6%)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>16 (3.8%)</td>
<td>13 (3.7%)</td>
<td></td>
</tr>
</tbody>
</table>

Data are number (%) unless otherwise specified. *P-value for the comparison between the overall population and the biomarker group by the t-test or χ² test as appropriate.

Data missing for one patient. The group includes also nine patients with progressive disease who were not assessable for pCR.

T, docetaxel; H, trastuzumab; P, pertuzumab.

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Results

Age, tumor size, LABC or IBC status, and clinical nodal status were not associated with expression of immune markers. PD1 mRNA and IGG metagene expression were higher in ER-negative (P < 0.01), while the IF-I metagene expression was higher in ER-positive cancers (P < 0.01) (supplementary Figure S3, available at Annals of Oncology online).

Tumor-infiltrating lymphocyte

Intratumor and stromal TIL results were available in 243 patients; 17% were classified as lymphocyte predominant (LPBC) and 14% as lowTIL (<5% TumTIL and StrTIL) (Table 2). Neither tumor nor stromal TIL counts as continuous variables were significantly associated with pCRB (supplementary Table S2, available at Annals of Oncology online). Because a non-linear association between TILs and pCR was recently reported in the NeoALTTO study [10], we also evaluated TILs as categorical variable (Table 2). The association with pCR was not statistically significant, but we also observed a non-linear pattern. Of note, the pCRB rate in THP compared with other arms was numerically higher in low and intermediate but not in the LPBC groups. As exploratory analysis, we combined the three more similar arms (TH, HP, and TP), in which the group with lowTIL had the lowest rate of pCRB (4.3%), compared with intermediate TIL and LPBC (26.9% and 26.7%, respectively) (P = 0.018 for lowTIL versus intermediate TIL/LPBC group by the χ² test) (Table 2). All immune markers, except for PD1 mRNA expression, were comparable between TH, HP, and TP arms, while PD1 mRNA expression was significantly lower in THP compared to other arms (TH and HP arms).

Figure 1. Rate of pathologic complete response in the breast (%) with each regimen by tertile of expression of MHC1 (A) and CTLA4 (B). The rate of pathologic complete response in the breast with THP was compared with those after the other treatments within each tertile. Significant differences are tagged (*P < 0.05; **P < 0.01; ***P < 0.001). (C) Forest plot of logistic regression analysis results for each immune marker adjusted for ER status (bivariate models), in docetaxel (T), trastuzumab (H), and pertuzumab (P) (THP) arm and in the group combining cases treated with TH, HP, and TP. Regression coefficients larger than 0 indicate higher likelihood of pathologic complete response in the breast.
associated with TIL count to various degrees ($P < 0.01$), but there was substantial heterogeneity within each TIL group (supplementary Figures S4 and S5, available at Annals of Oncology online). The immune parameters, except PD1 mRNA expression, also showed positive correlation with one another. The $R^2$ values ranged from 0.14 to 0.77 ($P < 0.01$, supplementary Figure S6, available at Annals of Oncology online).

### immune genes and metagenes: univariate analysis

We next examined the associations between the 11 immune parameters and pCRB in the four separate treatment arms. Logistic regression analysis for each immune covariate adjusted for ER status (bivariate models) identified 10 significant associations at $P < 0.05$, corresponding to a false discovery rate (FDR) <20% (supplementary Table S3, available at Annals of Oncology online). Interestingly, the associations were qualitatively different in the THP arm compared with the other three arms. There was a consistent trend that higher expression of all 11 immune parameters was associated with lower probability of pCRB in the THP arm. Such a trend reached statistical significance for PD1, PD1, and CTLA4 genes and the MHC1 metagene. In the other three treatment arms, only six markers were significant and they were different in different arms. High expression of IFNG and IGG were significantly associated with higher probability of pCRB in the TH arm; CTLA4 and MHC2 in the HP arm; and STAT1 and IF-I in the TP arm (supplementary Table S3, available at Annals of Oncology online). However, in these three arms, a similar common trend opposite to that observed in arm THP was observed for several immune markers (supplementary Figure S7, available at Annals of Oncology online). Figure 1 describes the pCRB rate for tertiles of expression of MHC1 and CTLA4 (Figure 1A and B) plotted for each treatment arm, as a graphical representation of the treatment–biomarker interaction. These markers were selected for illustrative purpose, but other interactions were present. For instance, in the highest tertile group of MHC1, all treatments led to similar rates of pCRB (37.5% TH; 29.6% HP; 22.2% TP; 40% TP), while in the lowest tertile group, the rate of pCRB with THP was significantly higher (58.6%) compared with any of the other treatments (25% TH; 4.3% HP; 19.3% TP) (all difference $P < 0.05$). This effect was qualitatively similar in ER-positive and -negative tumors and suggests that the greatest synergy between combined HER2-targeting antibodies and chemotherapy (THP arm) occurs in tumors with low expression of immune markers related with immune-regulation (i.e. PD1, CTLA4, MHC1, and STAT1) (Figure 1C, supplementary Figure S8, available at Annals of Oncology online).

To formally test if the associations of different immune parameters with pCRB were different among the different regimens used in the study, we carried out pairwise treatment-by-biomarker interaction tests. We observed a significant marker–treatment interaction with THP compared with the other three regimens for PD1, CTLA4, IFNG, MHC2, MHC1, STAT1, and IF-I (at $P < 0.05$, corresponding to an FDR < 10%) (supplementary Table S4, available at Annals of Oncology online). No significant interactions were observed for the other three treatment arms (except IF-I in the TH versus TP comparison). Therefore, for all subsequent analysis, we combined the TH, HP, and TP treatment arms to improve the power of the effect size estimates. In the combined group (TH, HP, and TP), high expression of CTLA4, PD1, IFNG, IGG, CD8, MHC2, and STAT1 were all associated with higher pCRB in bivariate analysis adjusted for ER status ($P < 0.05$) (Figure 1C and supplementary Table S5, available at Annals of Oncology online).

### immune genes and metagenes: multivariate analysis

We next carried out multivariate analyses adjusted for significant clinico-pathological variables. In the group pooling TH, HP, and TP, high expression of PD1 ($P = 0.0001$), MHC2 ($P = 0.003$), and STAT1 ($P = 0.0007$) were each independently associated with higher pCRB, while high expression of PD1 (0 = 0.004) and MHC1 (0 = 0.0476) were associated with a lower rate (Table 3). A similar effect was observed including the IF-I metagene instead of MHC1 in the model (data not shown). Notably, all the markers were either less or non-significant in univariate analysis. Among clinical variables, ER-positive tumors, older age, and TP treatment were also significantly

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**Table 2.** Frequency distribution of tumor-infiltrating lymphocytes as categorical variable and its association with pCRB by treatment arm, and as exploratory analysis in the combined group of TH, HP, and TP arms

<table>
<thead>
<tr>
<th>No. (%)</th>
<th>Arm TH ($n = 57$)</th>
<th>Arm THP ($n = 63$)</th>
<th>Arm HP ($n = 57$)</th>
<th>Arm TP ($n = 54$)</th>
<th>Combined arms (TH, HP, TP) ($n = 168$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCRB</td>
<td>P-value$^a$</td>
<td>PCRB</td>
<td>P-value$^a$</td>
<td>PCRB</td>
<td>P-value$^a$</td>
</tr>
<tr>
<td>Overall TIL$^b$</td>
<td>31.6%</td>
<td>42.8%</td>
<td>15.8%</td>
<td>24.1%</td>
<td>23.8%</td>
</tr>
<tr>
<td>LowTIL</td>
<td>33 (13.6%)</td>
<td>0.0%</td>
<td>0.157</td>
<td>28.6%</td>
<td>0.240</td>
</tr>
<tr>
<td>IntTIL</td>
<td>169 (69.5%)</td>
<td>36.4%</td>
<td>48.9%</td>
<td>17.9%</td>
<td>25.0%</td>
</tr>
<tr>
<td>LPBC</td>
<td>41 (16.9%)</td>
<td>33.3%</td>
<td>22.2%</td>
<td>20.0%</td>
<td>28.6%</td>
</tr>
</tbody>
</table>

$^a$2 test.

$^b$LowTIL = StrTIL and TumTIL <5%; LPBC = StrTIL and/or TumTIL ≥50%; IntTIL = all other samples.

pCRB, pathologic complete response in the breast; TILs, tumor-infiltrating lymphocytes; IntTIL, intermediate TIL; LPBC, lymphocyte predominant breast cancer.
associated with lower pCRB. The multivariate model carried out similarly in ER-positive and -negative tumors (supplementary Figure S9, available at Annals of Oncology online).

In the THP arm, high expression of PD1 ($P = 0.004$), STAT1 ($P = 0.0057$), and ER-positivity ($P = 0.0004$) were all independently associated with lower pCRB rate, and it represented the best performing model (Table 3). However, alternative multivariate models in which STAT1 was replaced by either PDL1, CTLA4, MHC1, or IF-I were also significant providing similar, even if inferior goodness of fit compared with the main model (supplementary Table S6, available at Annals of Oncology online). All these markers are positively correlated and seem to have redundant predictive value. In the THP group, the predictive functions of INFG, CTLA4, and MHC2 expression showed

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**Figure 2.** Graphical display by forest plot of bivariate (immune marker adjusted for ER status) and multivariate logistic regression results in chemotherapy only and chemotherapy/trastuzumab arms of the NOAH trial (A). A regression coefficient $>0$ means a higher likelihood of achieving a pathologic complete response in the breast. Representation of predicted probability of pathologic complete response in the breast estimated in each arm using the multivariate models defined in the chemotherapy/trastuzumab arm (B). CT, chemotherapy.

**Table 3.** Odds ratio (OR) for the likelihood of having a pCRB based on univariate and multivariate logistic regression analysis in combined arms TH, HP, and TP and arm THP, separately

<table>
<thead>
<tr>
<th></th>
<th>Combined arms (TH, HP, and TP)</th>
<th>Arm THP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(249 pts, 63 pCRB)</td>
<td>(88 pts, 41 pCRB)</td>
</tr>
<tr>
<td></td>
<td>Univariate $^a$</td>
<td>Multivariate $^b$</td>
</tr>
<tr>
<td></td>
<td>OR (CI 95%)</td>
<td>P-value</td>
</tr>
<tr>
<td>PDL1</td>
<td>1.05 (0.80–1.39)</td>
<td>0.7189</td>
</tr>
<tr>
<td>PD1</td>
<td>1.55 (1.12–2.15)</td>
<td>0.0088</td>
</tr>
<tr>
<td>MHC2</td>
<td>1.50 (1.10–2.05)</td>
<td>0.0109</td>
</tr>
<tr>
<td>MHC1</td>
<td>1.27 (0.95–1.71)</td>
<td>0.1080</td>
</tr>
<tr>
<td>STAT1</td>
<td>1.44 (1.07–1.95)</td>
<td>0.0167</td>
</tr>
<tr>
<td>ERpos (versus ERneg)</td>
<td>0.23 (0.12–0.45)</td>
<td>0.00001</td>
</tr>
<tr>
<td>Age $^c$</td>
<td>0.78 (0.58–1.04)</td>
<td>0.091</td>
</tr>
<tr>
<td>Treatment arm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arm HP (versus TH)</td>
<td>0.41 (0.20–0.85)</td>
<td>0.0173</td>
</tr>
<tr>
<td>Arm TP (versus TH)</td>
<td>0.81 (0.42–1.60)</td>
<td>0.5503</td>
</tr>
</tbody>
</table>

OR $>1$ or OR $<1$ are, respectively, associated with higher or lower likelihood of achieving a pCRB. Statistical results with $P < 0.05$ are in bold.

$^a$Univariate analysis is carried out without any adjustment and reported only for variables included in the multivariate model.

$^b$Multivariate models by backward elimination of not significant variable (by likelihood ratio test, $P < 0.05$). Clinico-pathological variables evaluated in the starting model: ER_IHC; tumor and stromal lymphocyte infiltration, age, treatment arm, type (operable, LABC, IBC), clinical node (N0, N1, N2–3).

$^c$Age as continuous variable (by 10-year increase).
an interaction with ER status ($P < 0.05$ for the test of interaction), justifying separate multivariate analysis in ER-negative and -positive cases. In ER-negative cancers, high expression of INFG was associated with higher pCRB ($P = 0.003$), whereas high expression of CTLA4 ($P = 0.003$), PD1 ($P = 0.026$), and STAT1 ($P = 0.037$) were associated with lower pCRB. In ER-positive tumors, no variable was significantly associated with response (supplementary Table S7, available at *Annals of Oncology* online).

**independent assessment in the NOAH trial**

To test the generalizability of the associations observed in the NeoSphere trial, we used GEP data from the NOAH trial in which neoadjuvant chemotherapy with or without trastuzumab was given [11]. The overall quality of GEPs from the NOAH was significantly lower than in NeoSphere (supplementary Figure S10, available at *Annals of Oncology* online), precluding the analysis of the five individual immune genes but allowing to test the metagenes (MHC2, MHC1, STAT1, and IF-I) that emerged as significant from multivariate analyses for the treatment group TH/TP/HP. Three of these four markers were significantly associated with pCRB in multivariate analysis in patients who received chemotherapy plus trastuzumab in the NOAH trial (Figure 2). Similar to the NeoSphere results, higher expression of STAT1 was associated with higher pCRB ($P = 0.0028$), while higher expression of MHC1 ($P = 0.009$) and IF-I ($P = 0.015$) were associated with lower pCRB (supplementary Table S8, available at *Annals of Oncology* online). None of the immune markers were associated with pCRB in the chemotherapy only arm.

**PD1 by immunohistochemistry**

We assessed PD1 protein expression by IHC ($n = 305$, 73.1%). Positive staining was observed in 99 cases (32.4%), in 9 cases (2.9%) in tumor cells only, in 79 cases (25.9%) in stromal cells only, and 11 cases (3.6%) had both types of staining. PD1 mRNA expression correlated strongly with protein expression in tumor and stromal cells ($P = 2.6E−07$ and $P = 7.9E−10$, respectively) (supplementary Figures S11 and S12, available at *Annals of Oncology* online). Among the PD1 IHC-negative cases, we observed large variations in PD1 mRNA expression. The PD1 IHC positivity was significantly associated with high TIL ($P = 0.00002$) (supplementary Table S9, available at *Annals of Oncology* online), and also with higher expression of 9 out of 11 immune mRNA markers ($P < 0.05$). However, PD1 protein expression by IHC was not associated with pCRB in any of the treatment arms (univariate and multivariate analyses; data not shown).

**discussion**

The antibody nature of the HER2-directed therapies used in NeoSphere and the known contribution of innate and adaptive immunity to their activity [2, 3, 6, 7, 12] led us to focus the analysis of gene expression from biopsies before therapy on few individual genes and gene families associated with specific immune cells and functions. The results establish the contribution of the immune system to the probability of response to the different therapies in study. In particular, we show that expression of the immune check point PD1 is relevant to resistance to all regimens used in the trial, and that resistance is also influenced by expression of MHC1-related genes, which inhibit antibody-dependent cell-cytotoxicity (ADCC) [13]. The findings have relevant clinical implications.

Differently from some previous studies, in which immune biomarkers were not purposely investigated or investigated one at the time, we simultaneously tested diverse immune functions and markers within the context of a randomized trial, in which high-quality gene expression profiles were obtained in 84% of patients. Such features lend robustness to the findings.

We considered immune-checkpoint molecules (PD1, PD2, PD1, CTLA4) that are now direct targets of therapy, and interferon-$\gamma$ because of its key role in innate and adaptive immune response [12, 13]. We also used metagenes as surrogates of immune cell sub-populations and functions.

In logistic regression analyses, higher probability of pCRB in the combined TH, HP, and TP group was linked to higher expression of the PD1, CTLA4, IFNG genes, and the IGG, CD8, MHC2, and STAT1 metagenes. The results are consistent with reports that high lymphocyte infiltration and high expression of IGG, PD1, CTLA4, and IFNG are associated with higher benefit from trastuzumab and chemotherapy [12, 14, 15]. The scenario was different in patients treated with THP, in whom a tendency for lower pCRB was observed for tumors with higher expression of all the immune metagenes, and reached the significance for the negative immune regulators PD1, PD1, CTLA4, and the MHC1 metagene.

The different association between immune markers and pCRB after THP, in which the immune system seemed to be associated with resistance instead of activity of monoclonal antibodies, was confirmed by significant treatment-by-biomarker interactions. In tumors with high expression of immune markers, the activity of all NeoSphere treatments was of similar order of magnitude, including the chemotherapy-free arm (HP). Such immune markers-rich group of tumors is also the one with the highest reported benefit from anthracycline-containing chemotherapy or trastuzumab-chemotherapy combinations [7, 12, 14–16]. However, the likelihood of pCRB with THP was 2- to 20-fold higher than with the other therapies in trial for tumors with low expression of PD1, CTLA4, and MHC1. Thus, it seemed to provide the largest benefit to the very group of tumors, in which chemotherapy alone or with trastuzumab performs the least. MHC1-related genes inhibit antigen-dependent cellular cytotoxicity, and loss of MHC1 is involved in metastatic progression [13, 17–20]. The data should therefore be taken into account when considering the excellent clinical benefit derived from THP in the metastatic setting [21].

The presence of multiple positive correlations between the expression of immune markers is coherent with the complex regulation of the immune system, which rely on mutual interactions, but potentially masked the prognostic/predictive contribution of each function [22–26]. The common paradigm in biomarker studies is to select variables in univariate analysis to be included in multivariate models [27]. However, to unravel the contribution of each immune marker, we conducted multivariate analyses including variables that were not significant in
univariate analysis. This approach unmasked the ‘foe-and-friend’ role of different components and functions of the immune system, which was overlooked in univariate analysis. In multivariate, high expression of PDL1, MHC1, and IF-1 metagenes were associated with resistance (lower pCRB) to TH, HP, and TP, whereas high expression of PD1, STAT1, and MHC2 were associated with higher pCRB. These last markers were also associated with pCRB in univariate analysis, but this association appeared to be even stronger in the multivariate model. The strength of the multivariate approach is also supported by the independent confirmation obtained in patients treated with chemotherapy and trastuzumab in the NOAH trial [11]. Similarly to NeoSphere, in the NOAH, high expression of MHC1 and IF-1 metagenes were associated with lower pCR, while high STAT1 was linked with higher pCR rate. Such associations were significant in multivariate analysis only. Interestingly, none of the associations was present in patients of the NOAH study who received chemotherapy without trastuzumab, suggesting the key role of monoclonal antibodies for the ADCC and the engagement of the adaptive immune system [2, 3]. Multivariate analysis also showed that high expression of PD1 and PDL1 were independently associated with lower pCR rate with THP, strengthening the contribution of immune escape mechanisms to resistance to this otherwise very effective combination.

TILs have been attributed a relevant role in the prediction of response to breast cancer therapies in the neoadjuvant and adjuvant setting [5, 6, 14]. In our study, TILs were significantly associated with immune markers confirming the technical quality of the assessment, but they were not associated as continuous variable with pCRB. Lack of association between stromal TILs and pCR has been also recently reported in the NeoALLTO trial [10]. This lack of association may be partially due to a non-linear association between TILs and pCRB, indeed lowTIL group had a significantly lower pCRB both in our study and in NeoALLTO [10], or because TILs are only a dim mirror of the complex interplay between the immune system and tumor [28].

Currently, PDL1 protein expression is being investigated as predictive marker to use PD1/PDL1-directed drugs in several indications in oncology. In NeoSphere, PDL1 measurement by immunohistochemistry was not predictive of pCRB in spite of the statistically significant association between IHC staining and mRNA expression. This may depend from a different performance of the two methods, or to the different biological information captured by mRNA and protein expression [29, 30]. Of interest, in our series, only a minority of samples showed PDL1 expression on tumor cells. This observation and the strong association between PDL1 expression and TILs is in agreement with the concept that immunosuppressive pathways may be more strongly induced as negative feedback mechanisms by the immune system than directly orchestrated by cancer cells [26].

It has been recently shown that the clinical benefit of immune-checkpoint inhibitors was significantly linked to high mutational burden [31] and in particular to the corresponding number of tumor neo-antigens [32] and the specific TCR repertoires identified at baseline and after treatment [33]. Such information would have provided a more comprehensive interpretation of our biological findings, especially to link benefit from HER2-targeted agents and immune-checkpoint inhibitors, but it is not available in NeoSphere.

This study has limitations like the small sample size of treatment arms, and the lack of a validation series to test the associations between immune markers with pCRB in the THP arm. The lack of correlation between PD1 and others immune markers could simply be due to poor performance of the corresponding probe set. Moreover, unavailability of outcome information at this time does not allow us to assess the role of the immune system in determining PFS and DFS, including the relevant analysis by HR status.

In summary, our data show the involvement of the immune system in modulating the response to HER2-directed therapies based on the monoclonal antibodies trastuzumab and pertuzumab, and are in agreement with preclinical evidence of synergy between drugs targeting immune checkpoints and monoclonal antibodies [15, 34, 35]. The data provide a strong rationale for testing such combinations, in particular with THP, in HER2-positive breast cancers.

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