NK cell infiltrates and HLA class I expression in primary HER2+ breast cancer predict and uncouple pathological response and disease-free survival

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Conflict of interest statement

J. Albanell, A. Lluch and M. Martínez-García report being advisory board members from Roche. J. Albanell, A. Lluch, M. Martínez-Garcia, A. Muntasell report receiving other honoraria from Roche as speaker’s bureau or travel grants. Other authors individually declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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Translational Relevance:

Predictive biomarkers are needed for personalized breast cancer treatment. This study identifies tumor-infiltrating NK cells and tumor HLA class I as complementary biomarkers with predictive and prognostic value in primary HER2-positive breast cancer patients treated with...
neoadjuvant anti-HER2 mAbs. Our observations provide clues for future patient stratification and treatment adaptation as well as for rationally designing NK- and T cell-targeted immunotherapy in a personalized manner. In a general context, our study reveals the importance of defining TIL subpopulations while integrating HLA class I tumor expression for optimizing the development of strategies for cancer treatment.
Abstract

Purpose: We investigated the value of tumor-infiltrating NK cells (TI-NK) and HLA class I tumor expression as biomarkers of response to neoadjuvant anti-HER2 antibody-based treatment in breast cancer.

Methods:

TI-NK cells and HLA-I were determined by immunohistochemistry in pre-treatment tumor biopsies from two cohorts of HER2-positive breast cancer patients [discovery cohort (n=42) and validation cohort (n=71)]. TIL were scored according to international guidelines. Biomarker association with pathological complete response (pCR) and disease-free survival (DFS) was adjusted for prognostic factors. Gene set variation analysis was used for determining immune-cell populations concomitant to NK cell enrichment in HER2-positive tumors from the TCGA (n=190).

Results:

TI-NK cells were significantly associated with pCR in the discovery cohort as well as in the validation cohort (p<0.0001), independently of clinicopathological factors. A ≥3 TI-NK cells/50xHPF cut off predicted pCR in the discovery and validation cohort [OR 188 (11-3154); OR 19.5 (5.3-71.8)]. Presence of TI-NK cells associated with prolonged DFS in both patient cohorts [HR 0.07 (0.01-0.6), p=0.01; HR 0.3 (0.08-1.3), p=0.1]. NK-, activated dendritic- and CD8 T-cell gene expression signatures positively correlated in HER2-positive tumors, supporting the value of NK cells as surrogates of effective anti-tumor immunity. Stratification of patients by tumor HLA-I expression identified patients with low and high relapse risk independently of pCR.

Conclusions: This study identifies baseline TI-NK cells as an independent biomarker with great predictive value for pCR to anti-HER2 antibody-based treatment and points to the complementary value of tumor HLA-I status for defining patient prognosis independently of pCR.
**Introduction**

HER2 overexpression and/or HER2 gene amplification occur in approximately 15-20% of breast tumors and are associated with aggressive disease (1). Combination of chemotherapy and anti-HER2 monoclonal antibodies (mAb), trastuzumab as standalone or trastuzumab plus pertuzumab, is the prevailing neoadjuvant approach for patients with primary HER2-positive breast cancer (2, 3). Achievement of pathological complete response (pCR) to neoadjuvant treatment has been associated with improved disease-free and overall survival; nonetheless, 35-50% of patients do not achieve pCR and/or eventually relapse. On the other hand, a subgroup of patients presents excellent clinical outcomes to anti-HER2 mAbs in the absence of concomitant chemotherapy (2, 3). This heterogeneity in clinical efficacy highlights: i) the importance of further understanding the mechanisms of action underlying anti-HER2 mAb-based treatment efficacy in different patients, and ii) the need for biomarkers aiding in patient stratification for tailoring treatment escalation or de-escalation.

Approved anti-HER2 mAbs are immunoglobulins of the G1 subclass (IgG1) that specifically block HER2-mediated oncogenic signaling and can trigger anti-tumor immunity by engaging Fcγ receptors (FcγR) expressed by immune cells (4, 5). In experimental models, tumor antigen-specific mAbs rapidly kill tumor targets via FcγR-mediated cytotoxicity (ADCC) (6-8). This short-term process also induces a vaccine effect, linking ADCC with the development of long-term anti-tumor adaptive immunity (9). Natural Killer (NK) cells are innate lymphocytes capable of recognizing antibody-coated targets through the activating Fcγ receptor CD16A (FcγRIIA). CD16-mediated NK cell activation triggers the release of cytotoxic mediators, pro-inflammatory cytokines and immune cell-recruiting chemokines (10-12). The contribution of NK cells, together with CD8 T cells, to anti-HER2 mAb activity (13) and their role as commanders of anti-tumor adaptive responses has been recently established in *in vivo* models (14, 15). In breast cancer patients, the presence of tumor-infiltrating lymphocytes (TILs) has been associated with response to neoadjuvant treatments including anti-HER2 mAbs (16, 17) and increased tumor-infiltration by NK cells after trastuzumab-docetaxel and the antibody-drug conjugate T-DM1 therapies has been reported (18-20). Despite these evidences, clinically relevant predictive biomarkers are lacking.

Expression of HLA class I molecules is an additional parameter determining the susceptibility of cancer cells to NK and CD8 T cell recognition. Indeed, tumor-antigen (neoantigen) presentation by HLA class I is required for triggering tumor-specific CD8 T cell cytotoxicity, a pathway commonly hijacked in different tumor types, including breast cancer (21-23). HLA-I downregulation facilitates NK cell-mediated tumor cell recognition whilst high HLA-I expression may repress NK cell activation against tumors through the interaction with inhibitory receptors of the KIR, CD94/NKG2 and LILRB1 families (10-12). Previous studies have reported high HLA-I expression as well as total HLA class I loss as good prognosis indicators in breast carcinomas (23-25).

We hypothesized that tumor-infiltrating NK cells and HLA class I expression in breast carcinomas could influence the efficacy of anti-HER2 mAbs-based neoadjuvant treatment.
Materials and Methods

Clinical samples

This study includes pre-treatment biopsy samples from two cohorts of primary HER2-positive breast cancer patients. The discovery cohort included patients prospectively recruited along 2014-2016 at Hospital del Mar, Barcelona (n=42). A retrospective cohort including patients recruited along 2008-2013 at Hospital del Mar, Barcelona and Hospital Fundación Jiménez Díaz, Madrid was used as validation cohort (n=71; Cohort diagram in Supplementary Figure 1). HER2-positive subtype classification was defined following 2013 ASCO/CAP guidelines (26). All patients received a neoadjuvant combination treatment of standard chemotherapy and anti-HER2 mAbs. The primary efficacy endpoint was pCR defined as ypT0ypN0 based on histopathological analysis of the resection specimen (27); DFS as the time from surgery until any breast cancer relapse or death by any cause and DMFS as the time from surgery to distant metastasis occurrence were secondary efficacy endpoints.

The study was conducted following Declaration of Helsinki guidelines. All patients gave written informed consent for the analysis of tumor biopsies for biomarker assessment. This study was approved by the Hospital del Mar Ethics Committee (2013/5307) and is reported according to the REMARK guidelines.

Stromal TILs and NK cell quantification

Stromal tumor-infiltrating lymphocytes were quantified on H&E sections of pre-treatment tumor biopsies following the guidelines of the international TIL Working group (17). NK cells were identified as CD56+CD3- cells in tumor stroma immune infiltrates by double immunohistochemical staining. Briefly, heat antigen retrieval was done in pH 9 citrate-based buffered solution and endogenous peroxidase was quenched. Mouse monoclonal anti-CD56 (clone 123-3, Dako-Agilent) and rabbit monoclonal anti-CD3 (2GV6, Ventana-Roche) antibodies were used, followed by incubation with a polymer coupled with peroxidase (UltraView, Ventana-Roche). Sections were visualized with 3,3’diaminobenzidine and alkaline phosphatase, and counterstained with Hematoxylin. All incubations were performed at Ultra platform (Ventana-Roche). CD56 and CD3 were evaluated by a computerized measurement using a DM2000 Leica microscope equipped with the Nuance FX Multispectral Imaging System (PerkinElmer). A computer-aided analysis yielded quantitative data of CD56 and CD3 on pseudo-fluorescence images. CD56 positive CD3-negative cells were counted in 50 adjacent x400 microscopic fields considering tumor stroma and expressed in absolute numbers. TILs and NK cell scoring was performed by an expert pathologist (F.R.) blinded to clinical data.

HLA class I immunohistochemistry and quantification

HLA-I expression was evaluated in baseline biopsy sections using the mouse monoclonal antibody HC10, kindly provided by Prof. Dr. Hidde Ploegh (Whitehead Institute for Biomedical Research, Massachusetts, USA). HC10 mostly reacts with HLA-B and HLA-C heavy chains and some HLA-A (HLA-A10, -A28, -A29, -A30, -A31, -A32, and –A33) (28). Staining of stromal cells served as internal positive control for HLA-I antibody reactivity. A semi quantitative histoscore
was calculated for HLA-I by estimation of the percentage of cells positively stained with low, medium, or high staining intensity. The final score was determined after applying a weighting factor to each estimate. The following formula was used: histoscore = (low%)X1 + (medium%)X2 + (high%)X3; the results ranged from 0 to 300. HLA-I staining H score was separately quantified in tumor and stromal cells as well as in normal epithelium, when available. Four tumor groups were defined based on HLA-I H score quartiles in transformed cells: i) HLA-I negative (HLA-I\(^{\text{neg}}\)) group: tumors with HLA-I H Scores \( \leq 3 \) (n=28); ii) HLA-I low (HLA-I\(^{\text{low}}\)) group: HLA-I H Scores >3 and \( \leq 60 \) (n=25); iii) HLA-I normal (HLA-I \(^{\text{norm}}\)) group: H Scores >60 and \( \leq 180 \) (n=32) and iv) HLA high (HLA-I\(^{\text{high}}\)) group: H Score >180(n=20).

TCGA data analysis for immune populations

RNAseq and clinical data of the HER2-positive breast cancer cohort (n=190) generated by the Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/abouttcga) was downloaded from the Firebrowse latest available release (2016/01) (http://firebrowse.org). Relative infiltration levels of sixteen immune populations were computed by gene set variation analysis (GSVA) through the gsva R package using non-overlapping gene sets specifically overexpressed in each cell type as described (29). The GSVA produced normalized enrichment scores ranging from -1 to 1, which represent the abundance of the immune cell population in the sample relative to the remaining analysed cohort. Hierarchical clustering of resulting immune infiltration patterns was done using the Ward’s linkage method (through Scipy Python library). Correlations between the relative abundance of cell populations across tumors were computed through a linear regression, using the Scipy Python library.

Statistical analysis

Bivariate analyses by Mann-Whitney U test were used to compare continuous variables in patients achieving or not pCR. pCR odds ratio for TILs and TI-NK cell numbers calculated by binary logistic regression are reported for 10% increments or individual units, respectively. Multivariate analysis was conducted with binary logistic regression in which patient baseline clinical and tumor characteristics: age (as a continuous variable), tumor size (T1 and T2 versus T3 and T4), lymph node stage (LN+ versus LN=0), estrogen receptor status (ER+ versus ER-), tumor grading (G1 and G2 versus G3) and Ki67 status (Ki67 <20% versus Ki67 ≥20%) were added in the model separately. Thresholds for stromal TILs and TI-NK cells that best discriminated pCR were determined using receiver operating characteristic (ROC) curve analysis in the discovery cohort. Optimal cut off points for TIL score and TI-NK cell numbers were determined by seeking the maximum Youden’s index (\( J = \text{sensitivity} + \text{specificity} - 1 \)). Predictive effects over pCR of TILs and TI-NK as categorical variables were calculated by Fisher’s exact test. Cox proportional hazards regression was used to estimate the HRs of TILs and TI-NK biomarkers in the analysis of DFS. Kaplan-Meier curves for DFS and DMFS were used to compare time to event in patients categorized according to pCR, TILs or TI-NK cells analysed by the log-rank test. All p values were two-sided; p values lower than 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad software, La Jolla, CA) and STATA version 15 (STATA Corp., Texas, USA).
Results

Baseline tumor-infiltrating NK cell numbers predict pathological complete response in HER2-positive breast cancer patients receiving anti-HER2 mAb-based neoadjuvant therapy

We hypothesized that tumor-infiltrating NK cell numbers could serve as predictive biomarkers of response to anti-HER2 mAb-based therapy. For that purpose, TILs and tumor-infiltrating (TI)-NK cells were analysed in diagnostic core biopsies from two HER2-positive breast cancer patient cohorts presented as discovery and validation datasets (Cohort characteristics in Table 1). NK cells, identified as CD56+CD3- lymphocytes by double immunohistochemistry, were mainly located in tumor stroma areas immersed within T cell (CD3+) infiltrates (Figure 1A). Eventually, intra-tumor NK cells in close proximity to transformed epithelia could also be detected, yet enumeration of TI-NK cells was restricted to stroma-associated NK cells.

In the discovery cohort, TIL and TI-NK cells were detected in 100% and 65% of core-biopsies. Median TIL score and TI-NK cell numbers were 20% (IQR 10-40) and 1.5 cells/50xHPF (IQR 0-15), respectively. In the validation cohort, TIL and TI-NK cells were detected in 93% and 72% of core-biopsies with a median TIL score of 20% (IQR 10-30) and 2 TI-NK cells/50xHPF (IQR 0-12). Higher TI-NK cell densities were detected in ER-negative as compared to ER-positive tumors in the discovery dataset whereas TIL scores were comparable. TI-NK cell numbers were not consistently related to any other classical pathological factor (Supplementary Table I).

Pathological complete response rate was 48% and 42% in the discovery and validation cohort, respectively. Global pCR rate upon combining both cohorts was 43%. ER was the only traditional clinicopathological factor associated with pCR in both cohorts. Baseline TIL score positively associated with pCR in the discovery, in the validation and in the global analysis combining both patient cohorts (Figure 1B). Baseline TI-NK cell numbers showed a remarkable association with pCR in all analysed datasets (Figure 1C). TIL score, and TI-NK cell numbers remained significantly associated with pCR when adjusted by ER status in multivariate analysis [TILs discovery OR 1.05 (1.00-1.10), p<0.005; TILs validation OR 1.04 (1.01-1.09), p=0.006; TILs Total OR 1.04 (1.00-1.07), p=0.001; TI-NK discovery OR 6.9 (4.0-10), p=0.03; TI-NK validation OR 1.45 (1.2-1.7), p<0.0001; TI-NK Total OR 1.49 (1.2-1.79), p<0.0001]. Remarkably, the association between TIL score and pCR vanished upon adjusting by TI-NK cell numbers in multivariate logistic regression [Discovery: TILs OR 0.9 (0.76-1.08), p=0.3; Validation: TILs OR 1.0 (0.95-1.05), p=0.8; Total: TILs OR 0.9 (0.93-1.03), p=0.4], indicating that pCR was selectively associated to NK cell-enriched TILs.

ROC curves and cut-off values providing best discrimination between pCR and non pCR were calculated for TI-NK cells and TILs in the discovery cohort (Supplementary Figure 2) and used for patient stratification in subsequent analysis (Supplementary Figure 2). Odd ratios for pCR were higher in the group of patients with ≥50% TILs and remarkably high in tumors with ≥3 TI-NK cells /50xHPF upon adjusting by ER status in both patient cohorts (Supplementary Figure 2). Tumors with ≥3 NK cells /50xHPF showed pCR rates of 100% and 77% in the discovery and validation cohorts, respectively. Remarkably, ≥3 TI-NK cell/50xHPF cut off performed as a biomarker with great sensitivity, specificity, positive and negative predictive value, superior to TILs cut off, in both patient cohorts (Supplementary Figure 2).
We also assessed the relationship between baseline TILs and TI-NK cells with DFS (Figure 2). Median clinical follow-up was of 31 and 49 months in the discovery and validation cohort, respectively (Table I). Despite the limited follow-up, TI-NK cell presence (≥1/50xHPF) associated to improved DFS in the discovery, in the validation as well as in the combined analysis of both patient cohorts (Figure 2C, F, I). TI-NK cell association with DFS was comparable to that of pCR in both patient datasets (Figure 2A, D, G). On the contrary, TIL score was not significantly associated to DFS in any of the datasets (Figure 2B, E, H). In an exploratory analysis of the association between TI-NK, TILs and pCR with distant metastasis free survival (DMFS; Supplementary Figure 3), TI-NK maintained its statistical significance (p=0.03) but not pCR (p=0.06), perhaps due to the reduced number of events in DMFS as compared to DFS. Overall, TI-NK cells predicted pCR and DFS better than TILs in primary HER2-positive breast cancer patients treated with neoadjuvant anti-HER2 mAbs and chemotherapy.

**NK cell-related gene set in breast carcinomas overexpressing HER2 correlate with activated dendritic cell and CD8 T cell gene signatures and are associated with increased overall survival**

In order to get an insight into the immune context associated with TI-NK cells, we estimated by gene set variation analysis (GSVA) the relative abundance of 16 immune cell populations [cytotoxic NK cells (NK\textsuperscript{dim}), B cells, eosinophils, macrophages, mast cells, CD56\textsuperscript{bright} NK cells (NK\textsuperscript{bright}), neutrophils, T helper cells (Th), central memory T cells (Tcm), effector memory T cells (Tem), follicular helper T cells (Tfh), activated dendritic cells (aDC), immature dendritic cells (iDC), activated CD8 T cell (CD8), gamma delta T cells (Tgd) and regulatory T cells (Treg)] across HER2-positive breast tumors in the public TCGA dataset (n=190). Figure 3A shows the distribution of the relative enrichment of these cell populations across the 190 tumors in the cohort. In agreement with immunohistochemistry data, the relative enrichment in NK\textsuperscript{dim} gene signature varied in distinct tumors and tended to be higher in ER-negative as compared to ER-positive tumors (Mann Whitney U p-value 0.052; Figure 3B), yet not significant association was found with other clinical parameters such as tumor size or lymph node status (data not shown). NK\textsuperscript{dim} gene set enrichment showed a positive correlation with gene signatures identifying activated dendritic and CD8 T cells, hallmarks of productive anti-tumor immunity, as well as B and regulatory T lymphocytes (Figure 3A). Of note, tumor stratification according to the top quartile NK\textsuperscript{dim} gene set enrichment identified patients showing good prognosis (p=0.008) (Figure 3C) whereas regulatory T cell (p=0.07) or activated CD8 T cell (p=0.34) gene expression signatures showed no significant correlation with overall survival in the same dataset. Hence, NK\textsuperscript{dim} cell associated gene expression signature may also perform as surrogate biomarker of effective anti-tumor immunity in primary HER2 breast carcinomas.

**High tumor HLA class I expression identifies patients with prolonged DFS, independently of pCR**

Since expression of HLA-I molecules regulates the susceptibility of cancer cells to NK and CD8 T cell recognition, we next evaluated whether HLA-I tumor expression could further modulate the efficacy of anti-HER2 mAbs. A total of 107 biopsies from both patient cohorts were evaluated for HLA-I expression by immunohistochemistry using the HC10 antibody and
combined in the analysis of HLA-I association with clinical outcomes. The H score corresponding to tumor, stroma cells and normal epithelia was separately annotated. The majority of transformed cells within a biopsy presented an homogeneous HLA-I labeling, though occasionally, tumor areas with distinct intensities were observed, likely reflecting tumor clone variants for HLA-I expression. HLA-I expression was variable in different tumors, ranging from complete absence to high intensity; independently of HLA-I levels in stroma or normal epithelial cells (Figure 4A-B). As continuous variable, tumor HLA-I H score did not associate with pCR or with any tested clinicopathological factor (Figure 4C and Supplementary Table II). Of note, HLA-I expression was higher in stroma from tumors achieving pCR in comparison to those not achieving it (Figure 4C), correlating with higher TIL content (r=0.27; p=0.004). For the analysis of extreme HLA-I tumor phenotypes, patients were stratified in four quartiles as defined in methods. pCR rate in HLA-I<sup>neg</sup> tumors was 57% and progressively decreased with increasing HLA-I tumor expression, being 40% in HLA-I<sup>low</sup> and 26% in HLA-I<sup>norm</sup> tumors; suggesting a relationship between pCR and susceptibility to NK cell recognition. However, tumors in the HLA-I<sup>high</sup> group showed the highest pCR rate (67%), perhaps reflecting tumor contexts in which tumor-specific CD8 T cells contributed to the efficacy of anti-HER2 mAb-based treatment (Figure 4D). Interestingly, the group of patients with HLA-I<sup>high</sup> tumors remained disease-free along the follow-up, regardless of prior pCR achievement whereas patients with HLA-I<sup>neg</sup> tumors displayed a trend for delayed relapses as compared to patients with HLA-I<sup>low</sup> and HLA-I<sup>normal</sup> tumors, despite showing high pCR rates (Figure 4E).

**Patient stratification by baseline HLA-I tumor expression enhances the predictive performance of TI-NK cells on pCR and DFS**

We next analysed whether TILs and TI-NK cell numbers performed equally well as pCR predictors in tumors expressing different HLA-I levels. Average TIL scores and TI-NK cell numbers were comparable among tumors of all HLA-I expression groups and TI-NK cell numbers were significantly associated with pCR in all HLA-I subgroups (Supplementary Figure 4). For subsequent analysis, the ≥3 Ti-NK cell/50xHPF cut off was applied to the overall patient population.

Stratification of patients by tumor HLA-I quartiles generated 3 contexts with distinct clinical behavior (Figure 5): i) Patients with HLA-I<sup>high</sup> tumors: association between TI-NK cells and pCR was complete in this group of tumors with all 13 cases with baseline ≥3 Ti-NK cells developing pCR post-treatment (5A). Of note, all 20 patients remained relapse-free along the study, regardless of baseline TI-NK cell density and of pCR achievement (5B); ii) Patients with HLA-I<sup>interm</sup> (low+norm) tumors: TI-NK cells predicted pCR in 83% (15/18) of cases; (5C) and performed better than pCR in the prediction of DFS (5D and 5E); tumors with HLA-I low and normal expression showed similar clinical behavior (Supplementary Figure 6); and iii) Patients with HLA-I<sup>neg</sup> tumors: TI-NK cells predictive performance on pCR and DFS was lower in this group of patients as compared to other HLA-I groups (5F, 5G and 5H).

**Discussion**

Despite many efforts, clinically useful predictive biomarkers of response to anti-HER2 mAb-based treatments in breast cancer remain elusive. Data of the herein reported study
A highly significant and independent association between tumor-infiltrating NK cell numbers in the diagnostic core biopsy and the probability of achieving complete pathological response to anti-HER2 mAb-based neoadjuvant treatment. Moreover, stratification of patients according to HLA-I tumor expression levels enhanced the prediction of long-term clinical outcomes, identifying patients with either decreased or increased relapse risk independently of pCR. From the clinical standpoint, our study identifies two independent and complementary biomarkers which might support clinical decision making. Mechanistically, our data supports the combined action of NK and CD8 T cells as major contributors to anti-HER2 mAb-based treatment efficacy in primary breast cancer and points to HLA-I expression as a tumor-intrinsic factor further influencing response to treatment.

A relationship between baseline TILs and anti-HER2 mAb efficacy has been described in primary HER2-positive breast cancer. However, the magnitude of the association and its clinical usefulness remains controversial. Some studies, including a recent analysis of six patient cohorts from Gepar trials (16), have reported a positive association between TILs as continuous variable and likelihood of pCR achievement (30, 31) or DFS (17, 32) upon anti-HER2 mAb-based treatment, whilst in others, the association with pCR was more evident in lymphocyte predominant breast tumors, defined by variable TILs cut-offs ranging from 30-60% in different studies (33-35). Hence, a consensus TILs cut-off associated to pCR or DFS prediction across different HER2-positive breast cancer patient cohorts is still lacking. In our study, baseline TIL score was associated to higher pCR rates as continuous variable. Nonetheless, the optimal cut-off value determined in the discovery cohort performed with low sensitivity in the prediction of pCR and was not associated to DFS in any studied cohorts. It is recognized that TILs comprise diverse immune cell populations that might differentially influence tumor development and treatment outcome. Our study discloses TI-NK cells as an excellent biomarker labeling TIL contexts with anti-tumor potential in response to anti-HER2 mAb-based treatment. In fact, TI-NK cells not only associated to pCR but also underlie the association between TILs and pCR in both analysed patient cohorts, as indicated by multivariate analysis. In addition, the quantitative approach for TI-NK cell determination allowed the identification of a precise cut-off value in the discovery cohort with a very significant predictive potential for pCR in the validation cohort, as well as in the global analysis including both patient cohorts. TI-NK cell numbers also associated with DFS in both patient cohorts. However, the follow up of the patients was limited, precluding definite conclusions regarding the durable clinical outcome, particularly in the ER positive subgroup. Nonetheless, the association between overall survival and NK cell-rich tumors could also be detected by the analysis of NK cell gene expression signature in HER2 tumors from the TCGA.

Beyond the biological difference between TILs and NK cells which can justify their different prognostic and predictive value, the fact that prognostic and predictive differences between TILs and NK cells could be also due to the precision on their semi-quantitative versus quantitative enumeration cannot be ruled out. However, TI-NK cell numbers ranged from 0 to 30 cells in 50 high power fields (which include roughly a total of 3000 cells) precluding their semi-quantitative assessment.
In a complementary perspective, analysis of baseline HLA class I expression on tumor cells identified patients with distinct long-term outcomes, in some cases discordant for pCR (patients in tumor HLA-I high and negative quartiles). Stratification of intermediate HLA-I expressing tumors enhanced TI-NK cell performance for pCR and DFS prediction. However, due to the very small numbers of patients in each subgroup, results should be considered hypotheses-generating and no clinical conclusions should be drawn from that. Nonetheless, our results point to HLA-I as a putatively tumor-intrinsic factor likely influencing on the clinical efficacy of anti-HER2 mAbs in breast cancer control.

From a mechanistic perspective, our data supports the complementary action of NK and CD8 T cell responses along tumor control by anti-HER2 mAb-based treatment. Baseline TI-NK cell presence may indicate tumor permissiveness to NK cell infiltration/proliferation/survival. Indeed, an increase in NK cell infiltration in tumors treated with anti-HER2 mAbs has been previously reported (18, 20, 36). Enhanced NK cell infiltration could directly contribute to anti-HER2 mAb-triggered ADCC as well as facilitate the development of tumor-specific T cell immunity, as recently described in preclinical models (9, 14, 15). In this regard, the positive correlation between gene expression sets identifying NK cells, activated dendritic cells and CD8 T cells would support a role of TI-NK cells as orchestrators of effective anti-tumor immunity in HER2+ breast tumors, as recently proposed in melanoma patients (14). In our study, the association between pCR and Ti-NK cells in HLA-I negative tumors indicates the dispensable action of CD8 T cells along tumor control in some neoadjuvant contexts. In contrast, the elevated pCR rates together with the absence of relapses in patients with high HLA-I expressing tumors, supported the decisive contribution of CD8 T cell memory both for treatment-dependent tumor control as well as for metastasis surveillance in HER2 breast cancer along anti-HER2 mAb therapies. Hence, patients with HLA-I negative or low tumors at diagnosis may benefit from treatments enhancing NK cell rather than T cell anti-tumor immunity whereas in patients with high HLA-I-expressing tumors substitution of chemotherapy treatment by PD1/PD-1L-checkpoint blockade, enhancing tumor T cell immunity, could be a reasonable option.

Limitations of this study include a modest cohort size, the heterogeneity in the anti-HER2 mAb regimen as well as the lack of standardized methodology for TI-NK and HLA-I assessment. In contrast, its hypothesis-driven prospective-retrospective design and the strength of the associations reported enhance its intrinsic value. For HLA-I staining by immunohistochemistry, the use of HC10 mAb, provided a general yet partial view biased towards HLA-B and HLA-C expression assessment, including β2 microglobulin-free HLA-I heavy chains (37). Despite the fact that further studies addressing the molecular mechanisms underlying the specific HLA class I alterations in distinct tumors (21, 22, 38) would be of interest, the here implemented analysis might facilitate a meaningful stratification of patients with distinct clinical behaviors for treatment adaptation. The predictive value of both biomarkers deserve validation for their potential clinical utility in HER2-positive breast cancer, likely extending to general biomarkers for patients’ stratification and selection for immunotherapy.

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**Author contributions**


**References**


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**FIGURE LEGENDS**

**Figure 1:** Baseline tumor-infiltrating NK cell numbers predict complete response in HER2-positive breast cancer patients receiving anti-HER2 mAb-based neoadjuvant therapy

Staining of pre-treatment tumor biopsy paraffin-embedded sections using anti-CD3 (green), anti-CD56 (red) and nuclei counterstaining (blue). A) Merged image and composition indicating tumor and stromal areas are shown. NK cells were identified as CD56+CD3- cells. Each image row corresponds to an independent biopsy representative of TIL areas either lacking or presenting low or high NK cell numbers. B-C) TIL score and tumor-infiltrating NK cell numbers in baseline biopsies of patients stratified by their clinical response to neoadjuvant treatment in the discovery, validation and total cohort. Mann-Whitney U test was used to compare medians between groups. D) Odds Ratios (OR) and 95% confidence intervals for pCR prediction of clinicopathological factors, TIL score and TI-NK cells as categorical variables in the discovery, validation and total cohort total cohort. Values adjusted by ER status.

**Figure 2:** Baseline TI-NK cell number association with DFS in HER2-positive breast cancer patients receiving anti-HER2 mAb-based neoadjuvant therapy

Kaplan-Meier analysis for disease free survival of patients in the discovery cohort stratified by (A) pCR, (B) baseline TILs (≥ 25% cut off) and (C) baseline TI-NK cells (≥ 1 NK cells/50 HPF cut off); validation cohort stratified by (D) pCR, (E) baseline TILs or (F) baseline TI-NK cells; total cohort stratified by (G) pCR, (H) baseline TILs or (I) baseline TI-NK cells. HR: Hazard Ratios.
Hierarchically clustered heatmap of the relative abundance of sixteen immune populations across 190 HER2-positive breast carcinomas computed using Gene Set Variation Analysis (GSVA). Each column is a tumor sample and each row illustrates the relative enrichment for specific immune cell populations. GSVA scores range from -1 (blue) to +1 (red). Coefficient of the correlation between NKdim GSVA scores and those corresponding to other immune subsets are indicated in the right column and colored from white ($R^2=0$) to dark magenta ($R^2=1$).

**Figure 4:** High HLA class I tumor expression in diagnostic biopsies identifies patients with good prognosis, independently of prior pCR.

HLA class I expression was analysed by immunohistochemistry in baseline tumor biopsies using the HC10 mAb. HLA-I H score was separately analysed in tumor, stroma cells and normal epithelia (n=107). A) Representative examples of tumors with high (case A), intermediate (Case B) and negative (Case C) HLA-I staining in tumors. B) Distribution of HLA-I H scores in tumor, stroma and normal epithelia. C) Tumor and stroma HLA-I H scores in patients achieving or not pCR to neoadjuvant treatment. D) pCR rate in tumors stratified by HLA-I H score quartiles. Dashed line indicates 50% pCR rate. E) Kaplan Meier for DFS in patients stratified according to tumor HLA-I H score quartiles.

**Figure 5:** Patient stratification by baseline HLA-I tumor expression enhances the predictive performance of Ti-NK cells on pCR and DFS

The predictive and prognostic value of Ti-NK cell cut offs were analysed in tumors stratified according to HLA-I high, HLA-I interm (low+normal) and HLA-I neg expression. A, C, F) pCR rates of tumors with Ti-NK≥3 versus those with Ti-NK<3 in the three HLA-I groups. Insets indicate the number of patients in each group. B, D, G) Kaplan Meier for DFS in patients stratified by Ti-NK cell≥1 cut off in the three HLA-I groups. E, H) Kaplan Meier for DFS in patients stratified by pCR in HLA-I intermediate and negative groups. OR: odd ratios; 95% CI: confidence interval. HR: hazard ratios; 95% CI: confidence interval.
<table>
<thead>
<tr>
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<th>Discovery</th>
<th>Validation</th>
<th>p</th>
<th>Overall</th>
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<tbody>
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<td>Retrospective</td>
<td>Prosp+Retrosp</td>
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<td>Neoadjuvant</td>
<td>Neoadjuvant</td>
<td>Neoadjuvant</td>
<td></td>
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<tr>
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<td>71</td>
<td></td>
<td>113</td>
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<td>54 (36-87)</td>
<td>0.01a</td>
<td>57 (36-88)</td>
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<td>T1-T2</td>
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<td>37 (52%)</td>
<td>0.01b</td>
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<tr>
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<td>31 (43%)</td>
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<td>25 (59%)</td>
<td>39 (55%)</td>
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<td>6 (8%)</td>
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<td>CDI</td>
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<td><strong>Histological Grading (n/%)</strong></td>
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<td>G1-G2</td>
<td>17 (40%)</td>
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<td>26 (37%)</td>
<td></td>
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<tr>
<td>PR-</td>
<td>21 (50%)</td>
<td>40 (56%)</td>
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<td>62 (54%)</td>
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<td><strong>Ki67 index (n/%)</strong></td>
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<tr>
<td>&lt;20%</td>
<td>7 (17%)</td>
<td>7 (10%)</td>
<td>0.38b</td>
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<td>61 (86%)</td>
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<td>3 (4%)</td>
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<td>3 (3%)</td>
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<td></td>
<td>Discovery cohort</td>
<td>Validation cohort</td>
<td>p-value</td>
<td>Ac+T cohort</td>
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<td>--------------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>-----------</td>
<td>----------------</td>
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<tr>
<td>Anthracyclines + Taxanes</td>
<td>30 (71%)</td>
<td>30 (42%)</td>
<td>0.001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60 (53%)</td>
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<td>Taxanes</td>
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<td>Hormonal</td>
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<td>--</td>
<td></td>
<td>3 (3%)</td>
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<tr>
<td>Others</td>
<td>--</td>
<td>3 (4%)</td>
<td></td>
<td>3 (3%)</td>
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<tr>
<td>pathologically Clinical Response</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>pCR</td>
<td>20 (48%)</td>
<td>30 (42%)</td>
<td>0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50 (43%)</td>
</tr>
<tr>
<td>no pCR</td>
<td>21 (50%)</td>
<td>40 (56%)</td>
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<td>61 (54%)</td>
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<td>1 (2%)</td>
<td>1 (1%)</td>
<td></td>
<td>2 (2%)</td>
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<tr>
<td>Anti-HER2</td>
<td></td>
<td></td>
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<tr>
<td>Trastuzumab</td>
<td>27 (64%)</td>
<td>71 (100%)</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98 (87%)</td>
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<tr>
<td>Trastuzumab and Pertuzumab</td>
<td>15 (36%)</td>
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<td>15 (13%)</td>
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<td>Follow-up (median, IQR; months)</td>
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<tr>
<td>Clinical follow-up</td>
<td>31 (22-38)</td>
<td>49 (24-71)</td>
<td>0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34 (24-55)</td>
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<tr>
<td>DFS at 3 years (value, 95%CI)</td>
<td>0.90 (0.73-0.96)</td>
<td>0.91 (0.8-0.96)</td>
<td>0.583</td>
<td>0.91 (0.82-0.95)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mann Whitney discovery versus validation cohort  
<sup>b</sup> Fisher's test discovery versus validation cohort  
<sup>c</sup> Ac+T versus T in discovery and validation cohort
Figure 2

A. Discovery cohort

- pCR: n=0/20
- non pCR: n=4/21

DFS

HR 0.1 (0.1-1.5); Log-rank p=0.18

B. Discovery cohort

- TILs >25%: n=1/17
- TILs <25%: n=3/24

DFS

HR 0.4 (0.06-3); Log-rank p=0.7

C. Discovery cohort

- TIL-NK ≥1: n=0/26
- TIL-NK <1: n=4/14

DFS

HR 0.07 (0.01-0.6); Log-rank p=0.01

D. Validation cohort

- pCR: n=2/30
- non pCR: n=8/40

DFS

HR 0.4 (0.1-1.5); Log-rank p=0.18

E. Validation cohort

- TILs >25%: n=5/32
- TILs <25%: n=5/39

DFS

HR 1.9 (0.5-7.1); Log-rank p=0.32

F. Validation cohort

- TIL-NK ≥1: n=5/60
- TIL-NK <1: n=5/19

DFS

HR 0.3 (0.08-1.3); Log-rank p=0.11

G. Total cohort

- pCR: n=2/60
- non pCR: n=12/61

DFS

HR 0.3 (0.1-0.9); Log-rank p=0.03

H. Total cohort

- TILs >25%: n=6/49
- TILs <25%: n=8/63

DFS

HR 1.2 (0.4-3.8); Log-rank p=0.6

I. Total cohort

- TIL-NK ≥1: n=5/76
- TIL-NK <1: n=9/93

DFS

HR 0.19 (0.06-0.6); Log-rank p=0.006
Figure 5

A. HLA-I High

<table>
<thead>
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<th>PCR rate</th>
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<td>NK&lt;3</td>
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<tr>
<td>NK&gt;3</td>
<td>n=7</td>
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Fishers' p<0.0001

B. DFS

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Ti-NK≥1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ti-NK&lt;1</td>
<td>0.4</td>
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C. HLA-I Low+Norm

<table>
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<tbody>
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<td>NK&gt;3</td>
<td>n=3</td>
</tr>
<tr>
<td></td>
<td>n=34</td>
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</table>

OR 57 (10-314); p<0.0001

D. DFS

<table>
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<tbody>
<tr>
<td>Ti-NK≥1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ti-NK&lt;1</td>
<td>0.4</td>
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HR 0.16 (0.04-0.6); Log rank p=0.006

E. DFS

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>pCR</td>
<td>1.0</td>
</tr>
<tr>
<td>non pCR</td>
<td>0.8</td>
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</table>

HR 0.36 (0.09-1.4); Log rank p=0.13

F. HLA-I H&g

<table>
<thead>
<tr>
<th>PCR rate</th>
<th>PCR rate</th>
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<tbody>
<tr>
<td>NK&lt;3</td>
<td>n=12</td>
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<tr>
<td>NK&gt;3</td>
<td>n=2</td>
</tr>
<tr>
<td></td>
<td>n=7</td>
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OR 5.6 (1-31); p=0.05

G. DFS

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Ti-NK≥1</td>
<td>1.0</td>
</tr>
<tr>
<td>Ti-NK&lt;1</td>
<td>0.4</td>
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HR 1.2 (0.1-10.7); Log rank p=0.8

H. DFS

<table>
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</thead>
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<tr>
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<td>1.0</td>
</tr>
<tr>
<td>non pCR</td>
<td>0.8</td>
</tr>
</tbody>
</table>

HR 0.3 (0.03-2); Log rank p=0.2
Clinical Cancer Research

NK cell infiltrates and HLA class I expression in primary HER2+ breast cancer predict and uncouple pathological response and disease-free survival

Aura Muntasell, Federico Rojo, Sonia Servitja, et al.

Clin Cancer Res Published OnlineFirst December 6, 2018.

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