Improved Survival of HER2\textsuperscript{+} Breast Cancer Patients Treated with Trastuzumab and Chemotherapy Is Associated with Host Antibody Immunity against the HER2 Intracellular Domain

Keith L. Knutson\textsuperscript{1}, Raphael Clynes\textsuperscript{2}, Barath Shreeder\textsuperscript{1}, Patrick Yeramian\textsuperscript{3}, Kathleen P. Kemp\textsuperscript{3}, Karla Ballman\textsuperscript{4}, Kathleen S. Tenner\textsuperscript{4}, Courtney L. Erskine\textsuperscript{5}, Nadine Norton\textsuperscript{6}, Donald Northfelt\textsuperscript{7}, Winston Tan\textsuperscript{8}, Carmen Calfa\textsuperscript{9}, Mark Pegram\textsuperscript{10}, Elizabeth A. Mittendorf\textsuperscript{11}, and Edith A. Perez\textsuperscript{12}

Abstract

The addition of trastuzumab to chemotherapy extends survival among patients with HER2\textsuperscript{+} breast cancer. Prior work showed that trastuzumab and chemotherapy augments HER2 extracellular domain (ECD)-specific antibodies. The current study investigated whether combination therapy induced immune responses beyond HER2-ECD and, importantly, whether those immune responses were associated with survival. Prettreatment and post-treatment sera were obtained from 48 women with metastatic HER2\textsuperscript{+} breast cancer on NCCTG (now Alliance for Clinical Trials in Oncology) studies, N0337 and N983252. IgG to HER2 intracellular domain (ICD), HER2-ECD, p53, IGFBP2, CEA, and tetanus toxoid were examined. Sera from 25 age-matched controls and 26 surgically resected HER2\textsuperscript{+} patients were also examined. Prior to therapy, some patients with metastatic disease had elevated antibodies to IGFBP2, p53, HER2-ICD, HER2-ECD, and CEA, but not to tetanus toxin, relative to controls and surgically resected patients. Treatment augmented antibody responses to HER2-ICD in 69% of metastatic patients, which was highly associated with improved progression-free survival (PFS; HR = 0.5, \(P = 0.0042\)) and overall survival (OS; HR = 0.7, \(P = 0.038\)). Augmented antibody responses to HER2-ICD also correlated (\(P = 0.03\)) with increased antibody responses to CEA, IGFBP2, and p53, indicating that treatment induces epitope spreading. Paradoxically, patients who already had high preexisting immunity to HER2-ICD did not respond to therapy with increased antibodies to HER2-ICD and demonstrated poorer PFS (HR = 1.6, \(P < 0.0001\)) and OS (HR = 1.4, \(P = 0.0006\)). Overall, the findings further demonstrate the importance of the adaptive immune system in the efficacy of trastuzumab-containing regimens. Cancer Res; 76(13): 3702--10. ©2016 AACR.

Introduction

Increased understanding of the molecular pathogenesis of breast cancer has led to the development of targeted therapies including the humanized mAb trastuzumab, which specifically recognizes the HER2 protein (1, 2). HER2 is part of a family of transmembrane receptors and it is overexpressed in about 20% of invasive breast cancers (3). HER2 has a well-established role in breast cancer pathogenesis, and trastuzumab in combination with chemotherapy has become the standard treatment for patients with HER2\textsuperscript{+} breast cancer (4). The single-agent effectiveness of trastuzumab is limited in patients with HER2\textsuperscript{+} metastatic breast cancer as only a fraction of patients initially respond and among the responders, and a high proportion will develop resistance to therapy within a year (5). The clinical efficacy of the combination of trastuzumab and chemotherapy likely involves several pathways, which currently remain incompletely understood (4). Increasing our understanding of these pathways would yield several clinically useful advances. First, identifying markers involved in antitumor response could lead to the discovery and implementation of enhancements that improve clinical efficacy. Second, the identification of additional mechanisms of action could provide insights into the biology of acquired resistance. Finally, predictive biomarkers of activity could identify patients most likely to benefit from trastuzumab and chemotherapy.

We published a small study that suggests an adaptive immune response begins following initiation of combination chemo- and trastuzumab therapy (6). Specifically, we examined...
anti-HER2 antibody and T-cell responses in 27 patients with HER2+ breast cancer and found that anti-HER2 antibodies were detectable in approximately one-third of patients before combination chemo- and trastuzumab therapy that increased to over half after initiation of treatment. Of the 22 individuals treated for metastatic disease, patients with objective clinical responses more frequently exhibited anti-HER2 antibody responses than those with progressive or stable disease. In addition, about one half of the patients developed concordant HER2-specific CD4 T cells early in the course of treatment, which is generally thought to be responsible for coordinated immunity and IgM to IgG class switching (7). This preliminary study established the hypothesis that adaptive immunity may be associated with improved survival. In the current study, we determined whether combination therapy induced adaptive immune responses to HER2 and other tumor antigens and whether immune responses were associated with improved progression-free survival (PFS) and overall survival (OS).

**Patients and Methods**

**Patient populations**

For this study, sera samples were acquired from 25 healthy donors, 54 patients with HER2+ metastatic breast cancer who participated in two North Central Cancer Treatment Group (now the Alliance for Clinical Trials in Oncology) trials, N0337 and N983252, and 26 patients who participated in Mayo Clinic Comprehensive Cancer Center Protocol MC1135 (Adjuvant treatment; Supplementary Table S1).

N0337 Metastatic breast cancer trial. This single-arm prospective multicenter phase II study enrolled 45 evaluable patients from March 2005 to June 2008. Patients received capecitabine (days 1–14), vinorelbine on days 1 and 8 every 3 weeks and trastuzumab on day 1, week 1, and then every 3 weeks as first- or second-line therapy for confirmed HER2+ invasive breast cancer with clinical evidence of metastases. Duration of treatment (mean and median times) with trastuzumab was 7.4 and 5.6 months, respectively, (range 1.4–10.2 months, N = 16; ref. 8).

N983252 Metastatic breast cancer trial. This two-arm prospective multicenter randomized phase II study enrolled 91 evaluable patients from September 1999 to July 2003. In Arm A, patients (N = 43) received every-3-week therapy consisting of paclitaxel, carboplatin, and trastuzumab administered every 21 days for 8 cycles. In Arm B, patients (N = 48) received weekly therapy consisting of paclitaxel, carboplatin for 3 of 4 weeks, with trastuzumab administered every 4 weeks for 6 cycles. Duration of treatment (mean and median times) with trastuzumab was 7.4 and 5.6 months, respectively, (range 2.1–15.9 months) in N983252 Arm A (N = 17), and 12.0 and 10.2 months, respectively, (range 3.0–37 months) in N983252 Arm B (N = 21). Mean and median time to metastatic disease for all patients, from initial diagnosis, was 3.2 and 1.9 months, respectively (range 0–15.8 months; ref. 9).

Patients enrolled in N0337 and N983252 had not previously received trastuzumab in any setting prior to enrollment and serum samples were available from 54 patients in these two clinical trials and 48 patients had paired pretreatment and posttreatment samples. All posttreatment samples for both metastatic trials were taken at the end of treatment at the time of disease progression, death, or study closure (with the exception of one patient, for which serum was taken at the time of evaluation where a response was noted).

**Adjunct patient samples from Mayo Clinic Study MC1135.** Sera samples were collected from 26 nonmetastatic HER2+ breast cancer patients treated in the adjuvant setting. A pretreatment sample was collected following surgery and AC (doxorubicin and cyclophosphamide) chemotherapy but prior to treatment with paclitaxel and trastuzumab. The posttreatment sample was collected at 4 months after initiation of adjuvant therapy. Mean and median duration of treatment with trastuzumab was 10.5 and 11.8 months, respectively (range 3.3–14.1 months).

**Normal healthy control samples.** Twenty-five serum samples were obtained from Bioreclamation and were similar to the metastatic patients based on age, race, and gender. Informed consent was obtained from all patients for obtaining biospecimens, and this study was approved by the Mayo Clinic Institutional Review Board.

**Direct ELISAs**

Research-level ELISAs were conducted in the laboratory of K.L. Knutson. Flat-bottom polystyrene 96-well microplates (Fisher Scientific) were coated overnight at 4°C with 100 μL/well of 0.05 mol/L carbonate-bicarbonate buffer containing 20 ng/mL of HER2-intracellular domain (ICD; aa 676–1255, Cell Sciences), HER2-extracellular domain fragment (ECD Frag; aa 22–122, Novus Biologicals), p53 (Abcam), insulin growth factor binding proteins 2 (IGFBP2; R&D Systems), or carcinoembryonic antigen (CEA; Abcam). HER2-ECD Frag was used because it lacks the trastuzumab-binding site that would interfere with detection of endogenous antibody responses. Tetanus toxoid (TT, Calbiochem) was coated at 100 ng/mL. Human IgG (Sigma) was used as a protein standard that was added at a concentration range of 25 to 0.195 ng/well. All wash steps were carried out with PBS containing 0.05% Tween 20 using a programmable automatic plate washer (Key Scientific Inc.). PBS containing 1% BSA was used as blocking and assay buffer for the ELISA. The wells were blocked with 200 μL/well of the blocking/assay buffer and were incubated for 1 hour at room temperature on a rocking platform. After washing again, human serum was added to the plate at a 1:100 dilution and plates were incubated for 2 hours at room temperature. After washing, 100 μL/well of goat anti-human IgG HRP (Santa Cruz Biotechnology) was diluted 1:2,000 and incubated for 1 hour at room temperature. After a final wash, each well was incubated with 100 μL TMB substrate (BD Biosciences). Color development was stopped by the addition of 50 μL/well of diluted HCl. Absorbance was read at 450 nm on a plate reader, which was subsequently converted into an antibody concentration using the IgG standard curve.

**Statistical analysis**

Categorical variables were compared using a χ2 test; antibody levels were compared with a paired t test or a two-sample t test, whichever was appropriate. Comparisons among three groups were made with an ANOVA test. For continuous variables with considerably skewed values, we used a sign-rank test for paired data, a Wilcoxon rank sum test for unpaired data, and Kruskal–Wallis for comparisons among three
groups. Kaplan–Meier plots were used to summarize patient survival and curves for two or more groups were compared using a log-rank test. Cox proportional hazards models were also used to assess the relationship between antibody levels and survival.

Results
Patients with HER2+ metastatic breast cancer demonstrate elevated preexisting antibody immunity to multiple tumor-associated antigens

A previous study reported that only a small fraction (7%) of patients with advanced (stages III and IV) HER2+ breast cancer had elevated antibody immunity against HER2-ECD (10). In the current study, we evaluated immunity toward HER2-ICD as well as other tumor antigens (IGFBP2, P53, CEA) in patients with either HER2+ metastatic or surgically resected breast cancers (see demographics in Supplementary Table S1). Relative to the levels of antibodies observed in normal healthy controls, patients with HER2+ metastatic breast cancer demonstrated elevated levels (> than the mean ± 2 SDs of the normal control levels) of circulating antibodies to all of the antigens except TT (Fig. 1A–F; Supplementary Table S2). Antibodies to TT were robust in patients with metastatic disease as they were in controls. In surgically resected patients, treated in the adjuvant setting, mean concentrations of antibodies to tumor antigens did not differ significantly from healthy controls, and were significantly lower than levels observed in patients with metastatic disease with the exception of antibodies to HER2-ECD Frag.

A comparison of the proportion of patients who had elevated levels of antibodies was also performed. The approximate percentage of patients with HER2+ metastatic disease with preexisting antibody levels were 32% for IGFBP2, 50% for P53, 100% for HER2 ICD, 20% for CEA, and 73% for HER2 ECD Frag (Fig. 1G). None of the metastatic patients demonstrated elevated immunity to TT relative to controls. It can also be observed from Fig. 1G that the percentage of adjuvant HER2+ patients with preexisting antibody levels were significantly lower compared with the metastatic patients. These results demonstrate that HER2+ metastatic breast cancer is naturally immunogenic.

Associations of the preexisting levels of antibodies to each of the tumor antigens and TT with progression-free (PFS) and overall survival (OS) were evaluated in the patients with metastatic disease using multivariable Cox proportional hazards analysis, adjusting for time from initial diagnosis to the development of metastatic disease (mean = 3.2 years; range 0–15.8 years). In contrast to expectations, preexisting antibody immunity to HER2 (both the ECD and ICD) was associated with a poorer outcomes relative to those patients without preexisting immunity (Table 1). Elevated antibodies to HER2-ICD and HER2-ECD Frag were associated with PFS HRs of 1.64 (P < 0.0001) and 1.50 (P = 0.009), respectively. Preexisting immunity IGFBP2, CEA, P53, and TT was not associated with PFS. A statistically significant association of preexisting antibodies with overall survival (OS) was observed only for elevated levels of HER2-ICD antibodies (HR = 1.36, P = 0.0006). Elevated levels of antibodies to the other antigens were not found to be statistically associated with OS despite preexisting antibodies to those proteins. However, there appeared to be a trend for antibodies to both HER2-ECD (P = 0.07) and P53 (P = 0.06) to be associated with worse OS.

Antibody levels to multiple tumor-associated antigens increase following treatment with trastuzumab and chemotherapy in patients with HER2+ metastatic breast cancer

We previously observed that treatment of HER2+ metastatic breast cancer patients with trastuzumab in combination with chemotherapy increased the mean levels of lambda subtype IgG antibodies specific to the ECD of HER2 (6). In the current study, we compared pretreatment and posttreatment total IgG (lambda and kappa) antibody responses in the metastatic patients to HER2-ECD as well other tumor antigens of interest including HER2-ICD (Fig. 2). As predicted from our earlier work, treatment led to an increase in the mean levels of antibodies to HER2-ECD. Notably, we also found that patients generated immunity to the HER2 ICD, IGFBP2, and p53 (See also Supplementary Table S3). Treatment did not appear to increase mean levels of antibodies against CEA or TT (Supplementary Table S3).

We found that the induction of HER2-ICD–specific antibodies (i.e., >25% increase in antibody levels) after treatment was highly correlated with induction of antibody responses to the HER2-ECD Frag as well as epitope spreading to other tumor antigens, (CEA, IGFBP2, and p53; Table 2). In contrast, epitope spreading did not correlate with the generation of antibodies to HER2-ECD (data not shown). Finally, as multiple chemotherapy regimens were used, we examined correlations with chemotherapies, finding that the only significant correlation was that a higher fraction of patients in trial 983252 demonstrated induction of immunity to HER2-ICD (Supplementary Table S4). This could suggest that the immune system may be more responsive in the presence of carboplatin and paclitaxel as opposed to capecitabine and vinorelbine combinations. Overall, our results demonstrate that treatment with combination trastuzumab and chemotherapy resulted in the induction of HER2-ICD immunity as well as epitope spreading to other common tumor antigens.

The generation of HER2-specific immunity is associated with improved PFS during treatment with trastuzumab and chemotherapy

Using a posttreatment antibody level of 125% of baseline as the cutoff for a positive response, we observed that the generation of HER2-ICD or HER2-ECD Frag antibody responses (Table 2; Fig. 3A and B) were significantly associated with improved 1-year PFS (log-rank, P = 0.004 and P < 0.001, respectively). When adjusted for the time from pretreatment to posttreatment blood draws (median = 9 months; range: 3–30 months), the association with improved PFS remained significant (Table 3). Antibody responses to other tumor antigens (i.e., epitope spreading) were not significantly associated with PFS (Supplementary Table S5).

None of the antibody responses were significantly associated with OS by univariate analysis, although an association of HER2-specific immunity was close to statistical significance (P = 0.06; Fig. 3C and D; Supplementary Table S6). However, when the model was adjusted for posttreatment collection time, which accounts for the number of total cycles of trastuzumab, antibody responses for HER2-ICD and HER2-ECD Frag were significantly associated with improved OS (P = 0.038 and P = 0.026), respectively (Table 3). Similar to PFS, antibody responses to other tumor antigens were not significantly associated with OS (Supplementary Table S6). Thus, as opposed to the preexisting immunity, therapy-induced immunity was associated with improved survival.
Optimal induction of antibodies in response to trastuzumab and chemotherapy is observed in patients with normal preexisting levels of tumor antigen–specific antibodies. The ability of a patient to augment antibodies to tumor antigen was dependent on the preexisting immune response (Fig. 4). In this analysis, the change in antibody response for each patient was compared with their preexisting immune response in scatter plots using linear regression (Fig. 4). For all antigens, there was a statistically significant negative correlation ($P < 0.01$) between preexisting immunity and the change in the immune response. Notably, patients with the highest preexisting immunity tended to have declines in the levels of immune responses during treatment.
Mean antibody levels to tumor-associated antigens do not increase after treatment with trastuzumab and chemotherapy in patients with HER2+ adjuvant breast cancer

To determine whether combination trastuzumab and chemotherapy also resulted in increased antibodies in patients treated in the adjuvant setting, we compared pretreatment and posttreatment levels (4 months after initiation of treatment). In contrast to what was observed for patients treated in the metastatic setting, we did not observe any statistically significant increases in mean antibody levels (Supplementary Fig. S1; Supplementary Table S7).

The fractions of patients in the metastatic and adjuvant groups that responded with elevated (defined as >25% increase) antibodies after treatment was compared as shown in Supplementary Fig. S2. A higher fraction of patients treated in the metastatic setting responded to IGFBP2 (54% vs. 8%; \( P < 0.0001 \)), to p53 (44% vs. 15%; \( P = 0.02 \)), and to HER2-ICD (69% vs. 24%; \( P = 0.0002 \)). The difference in HER2-ECD Frag responses did not significantly differ between the metastatic patients and the adjuvant patients (63% vs. 47%; \( P = 0.22 \), a finding that is consistent with our previous report that surgically resected patients elicit immunity to the ECD following treatment with trastuzumab in combination with chemotherapy (6). The difference in the percentage of metastatic or adjuvant patients that demonstrated elevated immunity to TT following treatment did not statistically differ. Thus, these results show that while surgically resected patients treated in the adjuvant setting with trastuzumab in combination with chemotherapy generate HER2-specific immunity, immunity is limited predominantly to the HER2 ECD with minimal epitope spreading to HER2-ICD and other local tumor antigens.

**Discussion**

These results demonstrate that patients with HER2+ metastatic breast cancer have elevated levels of antibody immunity against HER2 and other well-established tumor antigens prior to initiation of combination trastuzumab and chemotherapy compared with patients with earlier stage HER2+ breast cancer. Preexisting immunity to HER2 appears to be deleterious associating with poorer PFS and OS. Despite that, the combination of trastuzumab and chemotherapy not only further augments immunity against HER2 but also may change the immune response from pathogenic to protective. While we also provide evidence of preexistent or augmented immunity to other breast cancer antigens, we were unable to see a similar relationship to survival as was seen with immunity to HER2. A final notable observation made is that

**Table 1. Preexisting immunity to HER2 is associated with poorer PFS and OS**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Cox Multivariate HR (95% CI)</th>
<th>Cox score, ( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP2</td>
<td>1.06 (0.57–1.99)</td>
<td>0.90</td>
</tr>
<tr>
<td>P53</td>
<td>1.07 (0.84–1.37)</td>
<td>0.77</td>
</tr>
<tr>
<td>HER2-ICD</td>
<td>1.64 (1.35–2.00)</td>
<td>-0.0001</td>
</tr>
<tr>
<td>HER2-ECD Frag</td>
<td>1.50 (1.13–1.99)</td>
<td>0.009</td>
</tr>
<tr>
<td>CEA</td>
<td>1.29 (0.53–3.14)</td>
<td>0.79</td>
</tr>
<tr>
<td>TT</td>
<td>1.05 (0.98–1.12)</td>
<td>0.41</td>
</tr>
<tr>
<td>OS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP2</td>
<td>1.03 (0.56–1.89)</td>
<td>0.28</td>
</tr>
<tr>
<td>P53</td>
<td>1.24 (0.97–1.59)</td>
<td>0.06</td>
</tr>
<tr>
<td>HER2-ICD</td>
<td>1.36 (1.14–1.63)</td>
<td>0.0006</td>
</tr>
<tr>
<td>HER2-ECD Frag</td>
<td>1.26 (0.96–1.66)</td>
<td>0.07</td>
</tr>
<tr>
<td>CEA</td>
<td>1.10 (0.42–2.85)</td>
<td>0.27</td>
</tr>
<tr>
<td>TT</td>
<td>0.99 (0.91–1.08)</td>
<td>0.26</td>
</tr>
</tbody>
</table>

**NOTE:** Data adjusted for time from initial diagnosis to metastatic breast cancer. All \( P \) values for interaction between antibody levels and years to metastasis are not significant.
surgically resected patients treated in the adjuvant setting had little evidence of preexisting immunity, which was not augmented during therapy with trastuzumab-based regimens. Because the trials from which we obtained specimens did not have monotherapy arms, the current study was not designed to detect the component of the therapy, either the chemotherapy or trastuzumab, or both, that induces adaptive immunity. One could postulate that the primary driver of adaptive immunity is 

trastuzumab mediated through activating antigen-presenting cells that take up antibody-complexed HER2 in the tumor microenvironment (11). Alternatively, Fc- mediated ADCC may lead to apoptosis releasing antigens to which the immune system is not tolerant to (e.g., p53). Regardless, that trastuzumab is the primary mediator of activating the adaptive immune system is supported by several lines of evidence. For example, we recently published, in the adjuvant setting following surgical resection in the N9831 phase III clinical trial that patients on chemotherapy alone do not generate anti-HER2 antibody responses, whereas the majority of patients treated sequentially with paclitaxel followed by trastuzumab do (12). In further support, we also found in our previous smaller study that administration of trastuzumab as monotherapy resulted in induction of anti-HER2 antibody in 2 of 3 metastatic patients (6). Finally, in animal modeling of the effects of trastuzumab, Park and colleagues recently found that monotherapy of mice bearing HER2- tumors induced tumor-specific CD8+ T-cell responses that played a crucial role (13). Together, these results collectively demonstrate that anti-HER2 mAb therapy is necessary and sufficient for the activation of the adaptive immune response.

Table 2. Epitope spreading correlates with development of a HER2-ICD antibody response

<table>
<thead>
<tr>
<th>Epitope spreading</th>
<th>HER2-ICD Response</th>
<th>Total (N = 54)</th>
<th>Exact P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response</td>
<td>No (N = 15)</td>
<td>Yes (N = 33)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>10 (67%)</td>
<td>8 (24%)</td>
<td>18 (38%)</td>
</tr>
<tr>
<td>Yes</td>
<td>5 (33%)</td>
<td>25 (76%)</td>
<td>30 (63%)</td>
</tr>
<tr>
<td>Epitope spreading</td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>7 (47%)</td>
<td>5 (15%)</td>
<td>12 (25%)</td>
</tr>
<tr>
<td>Yes</td>
<td>8 (53%)</td>
<td>28 (85%)</td>
<td>36 (75%)</td>
</tr>
</tbody>
</table>

*Percentage of patients.
*Epitope spreading is response to at least one of the three tumor antigens, CEA, IGFBP2, and P53.

![Figure 3](https://example.com/figure3.png)

The generation of HER2-specific immunity is associated with improved survival during treatment with trastuzumab and chemotherapy. Kaplan-Meier curves comparing PFS in those patients who demonstrated a HER2-ICD (A) or HER2-ECD (B) antibody response (yes) to those patients who did not (no) are shown. C and D, Kaplan-Meier curves for overall survival (OS). Within each plot are the numbers of patients at each time point who were free of disease progression or who had not died within each group.
One of the key findings in our study is the generation of immunity to antigens other than HER2 following treatment, which is reminiscent of epitope spreading induced by vaccination. Epitope spreading is a phenomenon first described in the case of autoimmune disease (14). Theoretically, epitope spreading represents endogenous processing of antigen at sites of immune activation initiated by a specific T-cell response. Indeed, although we were unable to study T-cell responses in the current study, we previously reported that trastuzumab and chemotherapy induce activation of HER2-specific T-cell responses (6). Epitope spreading has been identified after immunization with cancer vaccines. For example, we found that immunization with HER2 T-helper peptide-based vaccines resulted in epitope spreading within the HER2 protein and to other tumor-associated antigens (15, 16). Epitope spreading has been linked with the progression and tissue destruction in several autoimmune disorders such as arthritis, systemic lupus erythematosus, insulin-dependent diabetes mellitus, and multiple sclerosis (17). The phenomenon of epitope spreading has been linked with survival benefit after immunotherapy in patients with cancer (18–20). In contrast, in our study, we did not see any benefit of the generation of immunity to other tumor antigens beside HER2.

Preexisting immunity has been observed in prior studies in patients with advanced HER2+ breast cancer. Our group previously reported that patients with HER2+ breast or ovarian cancers had preexisting T-cell and antibody immunity to HER2 (10). In that sampling of advanced stage (IlI and IV) breast or ovarian cancer patients, HER2- ECD-specific antibodies were detected in only three of the 45 patients examined. In stark contrast, in the current study, we found a markedly higher percentage of metastatic patients demonstrating preexisting immunity, specifically 100% demonstrated preexisting immunity to the HER2-ICD and 73% to the HER2-ECD fragment. The reasons for the striking differences in the percentage of patients demonstrating preexistent immunity is unclear but could be due to several factors. First, in the prior study, the patient population consisted of both stage III (n = 17) and IV (n = 28) patients and it may be possible that the generation of immune responses is proportional to the antigen load, which is likely to be higher in patients with more advanced stage IV (metastatic) disease who tend to have more tumor volume than those with localized earlier stage disease. Indeed, this would be consistent with previous studies of patients with NY-ESO-1 antibody who tend to have advanced stage higher burden disease (21, 22). Second, we also examined unique recombinant HER2 regions, HER2- ECD Frag and HER2- ICD; in our prior studies, whole recombinant HER2-ECD was used, which may have had differential exposure of antibody-binding regions due to different folding or glycosylation patterns inherent in the synthetic preparation (23).

The magnitude of the preexisting HER2 antibody response in patients with metastatic disease was an important factor and was strongly associated with poor survival following treatment with combination trastuzumab and chemotherapy. This observation is somewhat paradoxical given that increased antibodies are associated with better survival in other studies (24, 25) and that we observed significantly improved PFS and OS in those patients who demonstrated an increase in HER2-specific antibody responses while on treatment. One possible explanation is that patients with preexisting HER2-specific immunity may harbor more aggressive immunemediated metastatic lesions that demonstrate lower or no levels of HER2 expression, which could be addressed to extent by evaluating both the extent of pre- and posttreatment of HER2 expression as well as levels of infiltration. However, because the patients were all metastatic patients, samples of tumor were not available. The possibility of immunoeediting is underscored by recent studies that show that HER2 expression in distant metastasis is not always concordant with the primary lesions. In one study by Lower and colleagues, for example, there were 90 of 382 cases in which the primary lesion was HER2+ while the metastatic lesions were HER2− (26). Also, recent work in early-stage HER2+ breast cancer shows that induction of immune responses leads to outgrowth of HER2-loss variants (27, 28).

Alternatively, the poor survival in the patients with elevated preexisting immunity could be the result of the inability to maintain or augment preexisting immunity during combination therapy. The reasons for this remain unclear, but the preexisting immune response may have been eliminated or suppressed by the chemotherapy, which might have preferentially targeted an active immune response that was occurring at the time of initiation of chemotherapy, rendering a void in the numbers of antigen-specific B cells available for eradication of the tumor over the course of therapy. In contrast, those patients with lower level or normal preexistent immune reactivity may not have been generating an active immune response prior to therapy and may have escaped chemotherapy-induced immune suppression. Indeed, several chemotherapeutic regimens are known to suppress antibody responses during vaccination (29, 30). We considered the possibility that the different chemotherapy regimens used in the metastatic patients (capcitabine and vinorelbine in N0337, paclitaxel and carboplatin in N983252) could differentially influence induction of immunity and address the role of chemotherapy. We found that the ability to generate immune responses did not differ significantly between patients in N983252 and N0337 for most antigens, specifically CEA, P53, IGFBP2, TT, and HER2-ECD antigens. However, we did observe that a higher percentage.

### Table 3. Generation of antibody responses to HER2 is associated with improved PFS and OS

<table>
<thead>
<tr>
<th>Marker or collection time</th>
<th>Cox Univariate</th>
<th>Score</th>
<th>P</th>
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<tr>
<td></td>
<td>PFS</td>
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<tr>
<td>HER2-ICD</td>
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<tr>
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<td>Collection time</td>
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<tr>
<td>Test for interaction</td>
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<tr>
<td>HER2-ECDFrag</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No</td>
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<tr>
<td>Yes</td>
<td>0.038</td>
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<tr>
<td>Collection time</td>
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<td>Test for interaction</td>
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<td>No</td>
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<td>Yes</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collection time</td>
<td>0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for interaction</td>
<td></td>
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</tr>
</tbody>
</table>

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of patients in N983252 who generated an immune response to HER2-ICD (78%) compared with N0337 (50%), which could be related to the pronounced inhibitory effects of nucleoside analogues on humoral immune responses to vaccination (29).

These results suggest that combination treatment results in induction of adaptive immunity to antigens released by tumor and that the adaptive immune response specifically to HER2 may have a significant correlation with disease outcome. These findings may pave the way to the development of biomarkers predictive of therapeutic outcome and new therapeutic strategies with mAbs.

Disclosure of Potential Conflicts of Interest

P. Yeramian is a chief medical officer at Tapimmune. M.D. Pegram is a consultant/advisory board member for Roche/Genentech. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: K.L. Knutson, R. Clynes, K. Ballman, C. Calfa, E.A. Perez

Development of methodology: K.L. Knutson, E.A. Perez

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.L. Knutson, K. Kemp, C.L. Enkine, D.W. Northfelt, W. Tan, E.A. Perez


Writing, review, and/or revision of the manuscript: K.L. Knutson, R. Clynes, P. Yeramian, K. Ballman, N. Norton, D.W. Northfelt, W. Tan, C. Calfa, M.D. Pegram, E.A. Mittendorf, E.A. Perez

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Yeramian, K.S. Tenner, N. Norton

Study supervision: K.L. Knutson, D.W. Northfelt, W. Tan, E.A. Perez

Other (optimized protocols and performed experiments on patient samples and calculated antibody levels from the results that was sent to the senior author for analysis): B. Shreeder

Other (Optimized protocols, performed assays on patient samples and calculated antibody levels): K. Kemp

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Keith L. Knutson, Raphael Clynes, Barath Shreeder, et al.


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