The HER2 peptide nelipepimut-S (E75) vaccine (NeuVax™) in breast cancer patients at risk for recurrence: correlation of immunologic data with clinical response

Nelipepimut-S (formerly known as E75) is an immunogenic peptide from the HER2 protein that is highly expressed in breast cancer. The NeuVax™ (Galena, OR, USA) vaccine, nelipepimut-S plus granulocyte–macrophage colony-stimulating factor, is designed for the prevention of clinical recurrences in high risk, disease-free breast cancer patients. Although cancer vaccines such as NeuVax represent promising approaches to cancer immunotherapy, much remains to be elucidated regarding their mechanisms of action: particularly given that multiple cancer vaccine trials have failed to demonstrate a correlation between immunologic data and clinical outcome. Here, we briefly discuss our clinical trial experience with NeuVax focusing on immunologic response data and its implication on how the immune system may be affected by this peptide vaccine. Most importantly, we demonstrate the potential capability of certain immunologic assays to predict clinical benefit in our trials.

Keywords: breast cancer • E75 • HER2 peptide vaccine • immunotherapy • nelipepimut-S

Background
The HER2/neu proto-oncogene, a member of the EGF receptor family, is expressed in a variety of malignancies including breast cancer. Historically, HER2 overexpression (immunohistochemistry [IHC] 3+ and/or FISH >2.2) has been associated with a poor prognosis. Given that the overexpression of HER2 is associated with more aggressive subtypes of breast cancer and other adenocarcinomas, HER2 is an attractive immunologic target for both cell-mediated immunity, including cytotoxic T cells, and antibody-mediated immunotherapy approaches [1,2]. Currently, in addition to traditional therapies of surgery, chemotherapy, radiation and endocrine therapy, a small percentage of breast cancer patients benefit from trastuzumab and other HER2-targeted therapies including pertuzumab [3,4] and trastuzumab emtansine [5]. Trastuzumab, a monoclonal antibody targeting the highest level of HER2-overexpressing tumor cells, provides a proven disease-free survival (DFS) benefit to high-risk node-negative and node-positive breast cancer patients decreasing recurrence rates by approximately 50% [6–8].

Recent data show that patients with a HER2-overexpressing tumor treated with trastuzumab have a much better prognosis than HER2 low-to-intermediate (IHC 1–2+) patients not treated with trastuzumab [9]. However, trastuzumab is limited in that it has only been shown to be efficacious in the 20–30% of patients with tumors of the highest level of HER2 expression (IHC 3+) [10]. HER2 low-to-intermediate patients represent over 50% of breast cancer patients [10,11]. Thus, there exists a need to develop other adjuvant approaches to treat this HER2 low-to-intermediate-expressing population while avoiding the toxicities associated with trastuzumab and other HER2-targeted therapies.

An ideal immunologic therapy would induce immunologic memory, making clinical response long-acting and sustainable. Trastuzumab and other monoclonal antibodies are limited by passive mechanism of actions such as ADCC. Without full activation of the immune system, there is little to no immunologic...
memory. Cancer vaccines are a form of active immunotherapy where the body’s own immune system is utilized to target and kill tumor cells. The appeal of this active immunotherapeutic strategy includes the ability to generate a sustained T-cell response and immunologic memory with minimal toxicity. More specifically, vaccines target tumor-associated antigens (TAAs) that are recognized by human cytotoxic T lymphocytes (CTLs), which are critical for the removal of tumor cells in vivo.

**HER2-expressing targets.**

Table 1. Selected preclinical studies of the ability for nelipepimut-S to stimulate cytotoxic T lymphocytes to lyse HER2-expressing targets.

<table>
<thead>
<tr>
<th>Study (year)</th>
<th>Study design</th>
<th>Immunologic response/study conclusion</th>
<th>Ref.</th>
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</table>
| Fisk et al. (1995)    | Fresh cell lines from ovarian cancer pts | • First described nelipepimut-S (KIFGSLAFL, HER2/neu, p369-377)  
• Identified common immunogenic epitopes of HER2 that were recognized by isolated CD3+CD4+CD8+ ovarian-specific CTL lines  
• Nelipepimut-S epitope from tumor-associated lymphocytes from HLA-A2+ tumor-associated lymphocytes dominantly recognized by all CTL lines tested | [13] |
| Lustgarten et al. (1997) | *In vitro*/*in vivo* animal model | • Nelipepimut-S capable of stimulating potent CTL response that lysed tumor cells *in vivo*  
• Nelipepimut-S dose-dependent HER2 tumor-killing effect by nelipepimut-S-specific CTL in this mouse model | [28] |
| Disis et al. (1998)   | *In vitro*/*in vivo* animal model; nelipepimut-S-containing HER2-derived peptides plus GM-CSF or IFA | • No dose-limiting toxicity on necropsy  
• No evidence of autoimmunity  
• Significant T-cell response detected | [29] |
| Anderson et al. (2000) | Ten healthy human donors presented on autologous DCS elicited in 50% of healthy donors | • Nelipepimut-S-specific cytolytic activity in healthy donors when presented on autologous DCS elicited in 50% of healthy donors | [30] |

**Laboratory & clinical studies**

Nelipepimut-S has been studied in multiple laboratory and clinical studies [13,14,20–27]. The ability for nelipepimut-S to stimulate CTL to lyse HER2-expressing targets in the preclinical setting was established in both *ex vivo*/*in vitro* studies of ovarian and breast tumor cell lines [13,27] and animal models (Table 1) [28,29]. Pharmokinetic studies within animal models were not performed given the small peptide dose used and rapid peptide clearance. As such, typical pharmokinetic parameters (e.g., absorption, plasma protein binding, distribution, metabolism and excretion) were not established.

In addition to administering nelipepimut-S with GM-CSF, other vaccine formulations utilizing the peptide have included: loading the peptide onto autologous dendritic cells [20,24], inclusion into longer peptides to elicit both CTL and CD4+ helper T-cell response [21,22], and use of a single peptide with different immunoadjuvants [23,25,26]. All Phase I clinical trials utilizing GM-CSF as an immunoadjuvant revealed nelipepimut-S-specific CTL expansion [23,25,27]. However, despite the effective stimulation of a nelipepimut-S-specific T-cell response, little was known from the initial trials regarding antitumor activity secondary to the fact that these trials enrolled patients with advanced disease (Table 2) [23,25,26].

Our group has extensively studied the nelipepimut-S peptide as a vaccine but with a shift of focus from the treatment of late-stage disease to the prevention of disease recurrence in high-risk patients after standard-of-care therapy. In our Phase I/II clinical trials, we enrolled both node-positive and high-risk node-negative breast cancer patients who have been rendered clinically free of disease...
Table 2. Selected clinical studies examining the ability of nelipepimut-S to stimulate cytotoxic T lymphocytes.

<table>
<thead>
<tr>
<th>Study (year), phase, design</th>
<th>Patient sample size</th>
<th>Clinical response</th>
<th>Toxicity/immunogenic response</th>
<th>Ref.</th>
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</thead>
</table>
| Zaks and Rosenberg (1998), nelipepimut-S plus IFA | Four pts (breast, ovarian and colorectal cancer) | Not disclosed | ▪ Peptide-specific CTLs detected in post- but not pre-immunization blood  
▪ CTLs failed to react with HER2+ tumor cells | [26] |
| Disis et al. (1999) and Disis et al. (2002), subdominant HER2-derived epitope plus GM-CSF | 64 pts (breast, ovarian and NSCLC) | Not disclosed | ▪ 92% of patients develop T-cell immunity to HER2 peptide and 68% to HER2 protein domain  
▪ Epitope spreading in 84% of patients  
▪ 38% of patients maintained immunity to HER2 protein at 1 year | [21,22] |
| Brossart et al. (2000), pilot, peptide loaded onto autologous DCs | Ten pts (advanced breast and ovarian cancer) | Not disclosed | ▪ No side effects noted  
▪ Major CTL response in vivo was induced with the HER2-derived nelipepimut-S and the MUC1-derived M1.2 peptide, which lasted >6 months  
▪ Patient immunized with the HER2-derived peptides, MUC1-specific T lymphocytes were induced after seven immunizations, suggesting that antigen spreading in vivo may occur after successful immunization with a single tumor antigen | [32] |
| Knutson et al. (2002), Phase I, nelipepimut-S peptide plus GM-CSF | Six pts; stage III/IV breast (4)/ ovarian (2) cancer | Not disclosed | ▪ Minimal toxicity  
▪ Nelipepimut-S-specific precursors developed in two out of four evaluable patients and the responses were short-lived and not detectable at 5 months after the final vaccination | [23] |
| Murray et al. (2002), Phase I, nelipepimut-S plus GM-CSF | 14 pts; stage IV breast (13) and ovarian (1) cancer pts | Not disclosed | ▪ Vaccine found to be safe, no grade 3/4 toxicities  
▪ Four out of the eight patients showed CTL-mediated lytic activity | [25] |
| Kono et al. (2002), Phase I, peptide loaded onto autologous DCs | Nine pts (gastric cancer) | One out of nine pts with decreased tumor marker; one out of nine with stabilization of disease status x3 months | ▪ No serious adverse side effects  
▪ HER2 peptide-specific recognition demonstrated in six out of nine patients after immunization, whereas no HER2 peptide-specific recognition was present prior to immunization  
▪ Peptide-specific delayed-type hypersensitivity response occurred in three out of nine patients | [24] |
| Peoples et al. (2005), Phase I, nelipepimut-S plus GM-CSF | 53 pts; early-stage NP breast cancer post-standard-of-care therapy | DFS VG versus CG: 85.7 versus 59.8% at 22 months (p < 0.19) | ▪ Minor toxicity only (one grade 3–4%)  
▪ Nelipepimut-S-specific CTL expansion exhibited in all pts  
▪ DTH VG versus CG (33 vs 7 mm; p < 0.01) | [31] |
| Peoples et al. (2008), Phase I/II, nelipepimut-S plus GM-CSF | 186 pts: early-stage breast cancer post-standard-of-care therapy (NP = 95; NN = 91)  
VG = 101,  
CG = 85 | VG versus CG recurrence (5.6 vs 14.2%, p = 0.04) at 20 months;  
(8.3 vs 14.8%) at 26 months; VG with increased time to recurrence (11 vs 8 months) | ▪ Mild local/systemic toxicities  
▪ 74% positive postvaccine DTH with an average induration of VG versus CG (14.0 ± 1.4 mm vs 2.1 ± 0.5 mm, p < 0.0001)  
▪ Nelipepimut-S-specific CTL proliferation peak levels after the third or fourth dose in 65% of patients  
▪ Statistically significant increase in median percentage nelipepimut-S-CTLs pre- to post-vaccination  
▪ 43% pts maintained significant residual immunity (dimer > 0.5) 6 months postvaccination | [34] |

CG: Control group; CTL: Cytotoxic T-cell lymphocyte; DC: Dendritic cell; DFS: Disease-free survival; DTH: Delayed-type hypersensitivity; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IFA: Incomplete Freund’s adjuvant; MUC1: Mucin 1; NN: Node negative; NP: Node positive; NSCLC: Non-small-cell lung cancer; pts: Patients; VG: Vaccine group.
after surgery, chemotherapy and radiation as indicated (Table 2) [31,33,34]. In the Phase I/II trials, HLA-A2/A3+ breast cancer patients with any level of HER2 expression were enrolled into the vaccine group while HLA-A2/A3+ patients were followed prospectively as a control group. HLA-A2/3 has never been reported as an independent prognostic factor in breast cancer. Furthermore, a comparison of control patients from a separate randomized trial of adjuvant vaccines found no difference in survival between HLA-A2-positive and -negative patients [35]. The vaccine group received 4–6 monthly inoculations of NeuVax during the primary vaccine series. A voluntary booster program was initiated, and patients electing to participate were administered booster inoculations every 6 months out to 5 years post-primary vaccination series. These Phase I/II NeuVax clinical trials demonstrated that the vaccine has minimal toxicity and is capable of stimulating peptide-specific T-cell expansion, even within the context of low-level antigen expression [31,33,34,36] and after multiple booster doses [37]. Perhaps most importantly, dose-responsive clinical benefit was shown. In the combined trials of node-positive and node-negative patients, 60-month DFS was 89.7% in the vaccine group versus 80.2% in the control group (p = 0.08), although only 35% of patients were optimally dosed secondary to trial design. The 5-year DFS approached significance with 94.7% surviving disease free in the optimally dosed patients in comparison to 80.2% in the control group (p = 0.05) [38]. Interestingly, superior clinical benefit was also shown in HER2 low-to-intermediate-expressing patients versus controls (88.1 vs 77.5%, p = 0.16) [38]. Patients with low-to-intermediate HER2 expression will thus serve as the vaccination population for the ongoing Phase III clinical trial.

**Correlating immune & clinical responses**

An integral component of cancer vaccine trials is in vivo and ex vivo immune response monitoring as a means to predict reaction to vaccination and potential clinical benefit [39]. Current assays are designed to assess the type and magnitude of T-cell response over multiple time points throughout the trial. Theoretically, immune-monitoring modalities could provide insight into a vaccine’s specific immunologic mechanisms of action as well as early information regarding clinical efficacy. However, the optimal method to monitor activity of these peptide vaccines is not clear.

Multiple failed cancer vaccine trials (e.g., Theratope, Stimuvax and Canvaxin) [40–42] have supplied information regarding the endogenous immune response to malignancy. Particularly within the context of melanoma research, past cancer vaccine trials have assessed efficacy with immune surrogate end points but have rarely been able to correlate this data to clinical benefit or prevention of tumor progression [43]. In 2013, Hailemichael et al. specifically addressed clinical trials where CTL proliferation occurs without an associated beneficial tumor response [44]. Here, incomplete Freund’s adjuvant (IFA), the most commonly used peptide vaccine adjuvant to date, was assessed. IFA, a water-in-oil emulsion, acts slowly to release antigen to the immune system to enhance immunogenicity of vaccines. In a series of experiments using a gp100 peptide plus IFA vaccine in a mouse model, the authors observed a T-cell trap with sequestration of CTL at the vaccination site. The sequestered T cells underwent deletion without evidence of immunologic memory and subsequent hyporesponsiveness. This diminished response occurred even in vaccination of B cell-deficient and CD4 knockout mice, thus eliminating B cells and Tregs as causative sources [44]. Interestingly, vaccine trials utilizing IFA have failed to reveal objective clinical benefit [43], which, based on the work by Hailemichael et al., one may hypothesize is due to IFA-associated CTL sequestration, dysfunction and deletion [44].

CTL degradation within a T-cell trap provides some explanation as to the lack of observed clinical response despite the demonstration of CTL proliferation in response to vaccines. The Phase I clinical trials of nelipepimut-S plus adjuvant [23,25,26,31] revealed E75-specific CTL proliferation with associated antitumor activity in all but the trial utilizing IFA [26]. As discussed above, our Phase I/II trials [31,33,34,36] evaluating nelipepimut-S have utilized GM-CSF (as opposed to IFA) as the

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**Table 2. Selected clinical studies examining the ability of nelipepimut-S to stimulate cytotoxic T lymphocytes (cont.).**

<table>
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<tr>
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<th>Clinical response</th>
<th>Toxicity/immunogenic response</th>
<th>Ref.</th>
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<tr>
<td>Mittendorf et al. (2012), Phase I/II, nelipepimut-S plus GM-CSF</td>
<td>182 pts: 106 (VG), 76 (CG)</td>
<td>VG versus CG 24-month DFS 94.3 versus 86.8% (p = 0.08)</td>
<td>DFS subset analyses (VG vs CG):</td>
<td>[33]</td>
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<td></td>
<td></td>
<td></td>
<td>• Lymph node-positive (90.2 vs 79.1%; p = 0.13)</td>
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<td>• HER2 IHC 1–2+ (94.0 vs 79.4%; p = 0.04)</td>
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<td></td>
<td></td>
<td></td>
<td>• Grade 1 or 2 tumors (98.4 vs 86.0%; p = 0.01)</td>
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<td></td>
<td></td>
<td></td>
<td>• Optimally dosed (97.3 vs 86.8%; p = 0.08)</td>
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</table>

CG: Control group; CTL: Cytotoxic T-cell lymphocyte; DC: Dendritic cell; DFS: Disease-free survival; DTH: Delayed-type hypersensitivity; GM-CSF: Granulocyte–macrophage colony-stimulating factor; IFA: Incomplete Freund’s adjuvant; MUC1: Mucin 1; NN: Node negative; NP: Node positive; NSCLC: Non-small-cell lung cancer; pts: Patients; VG: Vaccine group.
immunoadjuvant. As will be discussed below, we have been able to correlate nelipepimut-S-specific immune response and increased DFS. Such ability to correlate immune response with clinical benefit has alluded past cancer vaccine trials set in the metastatic setting or in patients with residual tumor burden. However, our trial design differs from previous studies in that we have administered the vaccine as an adjuvant therapy to high-risk, disease-free patients. Just as vaccination site T-cell trap is attributed to long-lasting adjuvants such as IFA, T-cells may also be sequestered at the site of extensive tumor burden with its hostile microenvironment. Utilizing a vaccine in the setting of minimal to no residual disease and with a rapidly degrading adjuvant such as GM-CSF, such as in our Phase I/II trials, avoids these limitations.

**Immunogenic testing**

Our group has performed extensive immunologic studies to elucidate the specific immunologic mechanisms by which NeuVax confers clinical benefit. Again, as opposed to clinical trials intended to treat later-stage patients, our group has been able to correlate magnitude of peptide-specific immune response with clinical benefit [33]. We have previously outlined the immunologic monitoring methods used in these trials including delayed-type hypersensitivity (DTH) reaction, dimer assay, ELISPOT assay, assessment of regulatory T cells, cytokine evaluation, epitope spreading and circulating tumor cell (CTC) quantification as surrogates for immunologic response to NeuVax [35].

Phase I/II NeuVax clinical trials assessed *ex vivo* and *in vivo* response by evaluating nelipepimut-S-specific CTL expansion and DTH, respectively. Peptide-specific CTL expansion was measured by the dimer assay. Briefly, nelipepimut-S peptide-loaded dimers are mixed with the patient’s peripheral blood sample. This sample is analyzed by flow cytometry to quantify the number of CD8+ T-cells specifically bound to the peptide dimer molecules [45]. The assay is run on blood without any *ex vivo* manipulation and/or stimulation. Of note, vaccinated HER2 low-to-intermediate-expressing patients have displayed larger maximum immunologic responses compared with overexpressed patients (p = 0.04), an intriguing finding given the reduced mortality of low-expressor patients compared with controls (p = 0.08) [36]. DTH is assessed by injecting 100 μg of nelipepimut-S in 0.5 ml of normal saline (without GM-CSF) 1 month after completion of the vaccine series. Normal saline is injected as a parallel volume control. 48–72 h later, the area of erythema and/or induration that develops in response to this injection is measured in two dimensions and recorded as the orthogonal mean. In both of our node-positive and node-negative early-phase trials, a postvaccination DTH response was assessed. In the node-negative trial, a pre-vaccination DTH was measured as well and compared with the postvaccination DTH [54].

Using the Luminex® assay (Luminex Molecular Diagnostics Inc., ON, Canada) to delineate cytokine profiles in our vaccinated patients, significant differences were elucidated between vaccinated and unvaccinated patients. Specifically, monocyte chemotactic protein-1 (MCP-1), a chemokine associated with stimulating tumor-associated macrophages, angiogenesis and metastasis [46], showed the most significant elevation postvaccination (p = 0.003) [47]. However, as opposed to the metastatic setting where increased MCP-1 levels are implicated in tumor progression, high MCP-1 levels act as favorable prognostic indicators within the context of an adjuvant cancer vaccine. An association between MCP-1 and pre-existing HER2 immunity has also been demonstrated [48–50]. NeuVax vaccination correlated with the largest MCP-1 increase in higher-risk patients with lower initial serum MCP-1 levels. This suggests that the larger the MCP-1 response, the greater the vaccine-induced, peptide-specific immunity [51]. Thus, cytokine profiling may identify patients with low initial serum MCP-1 levels who are most likely to benefit from vaccination.

Despite the presence of an immune response to NeuVax, the interplay between malignancy and the immune system is a complex process where tumors are able to escape immune recognition. The fact that most TAA are also self-antigens tolerated by the immune system is a major obstacle in cancer immunotherapy. A subpopulation of regulatory CD4+ T-cells, Tregs, are implicated in the process of immune response downregulation and escape from immune recognition [52]. Identification of the regulatory CD4+CD25+ T-cell population, appreciated for prevention of autoimmunity and regulation of inflammatory reactions, as well as implicated in suppressing immune response against certain malignancies, enhanced the understanding of CD4+ T-cells’ importance in immune response regulation [53,54]. Our group analyzed Treg levels in a subset of vaccinated patients. Here, postvaccination Treg levels significantly decreased despite an overall increase in levels of circulating CD4+ T-cells [55].

Having shown that vaccination with nelipepimut-S plus GM-CSF can elicit clonal expansion of nelipepimut-S-specific CTL by dimer assay, we next sought to determine whether vaccination with this single peptide was capable of inducing epitope spreading [56]. Although the mechanism of epitope spreading is not fully understood, this phenomenon refers to the natural spread of immunity from one portion of an immunogenic protein to other areas within the same antigen (intra-antigenic) or to other antigens (interantigenic) [22]. We examined this
phenomenon in 44 patients enrolled in the early stage NeuVax clinical trials. Intra-antigenic epitope spreading to GP2, a second MHC class I epitope derived from the HER2 protein, was observed in 100% of node-positive and 85% of node-negative patients [56]. Of note, inter-antigenic spreading to E41, an epitope from the folate binding protein antigen, was also shown in 63% of tested patients. All patients with node-positive and 95% of the node-negative patients showed nelipeminut-S-specific clonal expansion, although node-negative patients showed only moderate expansion to this subdominant epitope. This high percentage of epitope spreading in clinically disease-free patients suggests the presence of occult disease [56].

Figure 1. Post-vaccination (R6) dimer levels in vaccinated patients and disease-free survival benefit of vaccine patients with a R6 dimer above the mean. (A) R6 dimer levels in vaccinated patients. Recurrences are shown in red. The mean R6 dimer in the vaccine group is 0.63 mdi ± 0.08. Of the 30 patients with an R6 dimer above the mean, only one recurred, compared with eight of the 56 below the mean (p = 0.09). (B) Disease-free survival benefit of vaccine patients with a R6 dimer above the mean (p = 0.12), a 76.9% relative risk reduction. f/u: Follow-up; mdi: Mean dimer index.
In order to further investigate the presence of occult disease, we have used the CellSearch® system (Veridex, NJ, USA) to quantify and phenotype CTCs [57]. Whereas *ex vivo* phenotypic (dimer/multimer) and functional (ELISPOT, cytokine flow cytometry) T-cell assays are limited by lower sensitivity given the potential for interfering background cytokines and inconsistent assay conditions, CTC hold potential as an extremely sensitive, clinically relevant surrogate to monitor vaccine response in clinically disease-free patients. In the metastatic setting, substantial numbers of CTC have been identified in up to 70% of patients [58]. Here, CTC burden is shown to predict treatment efficacy, progression-free and overall survival prior to

Figure 2. Mean dimer change in vaccinated patients and disease-free survival benefit of vaccine patients with a maximum dimer change above the mean. (A) Mean dimer change in vaccinated patients. The difference between baseline and max. mdi was available in 56 HER2 underexpressing, vaccine group patients. Of the 26 patients above the mean difference (1.08 mdi ± 0.17), one recurred, compared with six clinical recurrences in the 30 patients below the mean (p = 0.06). (B) Disease-free survival benefit of vaccine patients with a maximum dimer change above the mean (p = 0.07), a 80.4% relative risk reduction. f/u: Follow-up; Max.: Maximum; mdi: Mean dimer index.
and at any point after initiation of systemic therapy [59–61]. Data from our pilot study evaluating CTC revealed quantifiable levels of CTC in peripheral blood samples both pre- and post-vaccination [62]. Expanding this pilot study, data was collected in 25 patients within the NeuVax trial. CTC were present in 76% of patients (mean: 4.8 ± 1.0 CTC/20 ml of blood) with 68% of patients exhibiting at least two CTCs. CTC appeared to decrease from pre- to post-vaccination. Six months after the primary vaccination series, nine out of ten patients exhibited stable or decreasing CTC levels. Following NeuVax booster administration, there was a >50% reduction in CTC/20 ml (pre- 1.1 ± 0.5 vs post-0.4 ± 0.2, p = 0.14) in a matched cohort [63]. Although initial data is promising, correlating CTC with traits to include HLA expression levels, immunologic response and DFS remains challenging secondary to low CTC detection levels. The specific clinical significance has yet to be determined and will require larger studies to make meaningful correlations. However, recent studies suggest that even a single CTC detected by the CellSearch system in the adjuvant setting has prognostic significance [64,65].

**Biomarkers associated with NeuVax response**

As discussed above, multiple modalities are available for immunologic monitoring in cancer vaccine trials. Importantly, our group has been able to associate immune response magnitude with the clinical benefit of NeuVax™ [66]. We examined the relationship between *ex vivo* immunologic response and clinical recurrence after 5-year follow-up of the prospective, completed Phase I/II NeuVax clinical trials [34,38]. *Ex vivo* immune response was assessed for E75-specific CTL clonal expansion by the dimer assay prevaccination (R0), after the primary vaccination series (R6) and 6 months after completing the primary vaccination series (RC6). *In vivo* immune response was assessed by DTH reactions to nelipepimut-S at baseline and post-primary vaccine series. Of the 195 patients enrolled, 187 evaluable patients were available for analysis; 108 in the vaccine group and 79 in the control group. R6 dimer assays were available for 86 patients in the vaccine group. R6 dimer levels of individual patients were compared with the mean R6 dimer level for the entire vaccine group. At 60 months, 3% of the patients above the mean recurred, while 14% recurred below the mean (p = 0.09), a 76.9% relative risk reduction (Figure 1A & 1B). No clinical recurrence occurred in patients with HER2 low-to-intermediate expression with a mean difference in dimer levels ranked in the top third. Examining the mean dimer change from R0 to R6, the mean dimer level was also compared. Here, the greatest dimer change also demonstrated a potential DFS advantage and 80.4% relative risk reduction (Figure 2A & 2B). Together, these analyses suggest that patients exhibiting robust *ex vivo* immune responses have lower recurrence rates suggesting E75-specific CTL clonal expansion as a valid biomarker predicting response in patients treated with NeuVax [66].

From this initial analysis, our group advanced to a logistical regression model to determine the best immune response parameters for predicting disease recurrence after completion of the primary vaccination series [67]. Here, the immunologic data from all vaccinated patients were examined. Subgroup analysis was performed to include HER2 low-to-intermediate-expressing and nonboosted patients as well. The logistical regression model was performed with backwards elimination of *ex vivo* *in vivo* tests to predict the chance of recurrence (Table 3). The odds ratio and the area under the curve from the receiver operating characteristic curves was reported for statistical analysis (Figure 3). Results revealed that patients lacking pre-existing immunity, or a low R0 dimer level, have improved DFS after NeuVax.

Immunologic response of HER2 low-to-intermediate expressing patients is of importance based on the DFS benefit shown in the Phase I/II trials. Those patients with minimal pre-existing HER2-specific immunity who experience a robust T-cell response after vaccination are less likely to recur than those who have pre-existing immunity to HER2 or have a small T-cell response. In HER low-to-intermediate-expressing patients, a single variable, R0 dimer, tended to predict recurrence with an odds ratio of 1.9; meaning patients with an R0 level of nelipepimut-S specific CTL greater than 1.19 were nearly twice as likely to recur. Here, the initial dimer value at R0 had a negative predictive

<table>
<thead>
<tr>
<th>Sample (n)</th>
<th>Variables in the optimal model</th>
<th>R0 odds ratio (p-value)</th>
<th>Sensitivity, specificity</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine group (n = 93)</td>
<td>R0, RC6</td>
<td>2.36 (0.09)</td>
<td>75%, 73%</td>
<td>0.71</td>
</tr>
<tr>
<td>HER2 low-to-intermediate expression (IHC 1 or 2+, FISH &lt;2.2; n = 58)</td>
<td>R0</td>
<td>1.90 (0.19)</td>
<td>80%, 83% (R0 &lt; 1.19)</td>
<td>0.72</td>
</tr>
<tr>
<td>Unboosted (n = 52)</td>
<td>R0, post-PVS DTH</td>
<td>3.37 (0.12)</td>
<td>85%, 70%</td>
<td>0.76</td>
</tr>
</tbody>
</table>

AUC: Area under the curve; DTH: Delayed-type hypersensitivity; IHC: Immunohistochemistry; PVS: Primary vaccine series.
value of 97% and the sensitivity and specificity were 80 and 83%, respectively. Interestingly, the R0 dimer was the dominant variable in multiple modeling groups demonstrating that a low pre-existing immunity is the most important factor in predicting clinical benefit. This analysis may explain why NeuVax works best in HER2 low-to-intermediate-expressing patient with less HER2 antigen exposure.

Conclusion
In the context of clinical trials, the immune monitoring of a vaccine administered in the adjuvant setting differs than that in metastatic setting. In past trials conducted within the metastatic setting, CTL proliferation in response to vaccination failed to show a correlation with clinical response. An explanation may be the sequestering of CTL away from a patient’s peripheral circulation towards the hostile environment of an existing tumor burden. Within the context of adjuvant therapies, sampling peripheral blood mononuclear cells brings new significance to real-time immune monitoring. As a short-lived vaccination delivered to patients without significant disease burden, NeuVax may avoid trafficking of T cells either to an existing tumor burden or sequestration at the vaccination sites where antigen-driven T-cell dysfunction and deletion ensues.

The promise of NeuVax exists in the prevention of breast cancer recurrence by stimulation of the active immune system. Extensive immunologic monitoring of the NeuVax clinical trials has revealed a correlation of clinical outcome with the magnitude of immune response measured across multiple modalities. Specifically, R0 dimer holds potential as a predictive biomarker. Exhibition of a low R0 dimer indicates a lack of pre-existing immunity. Data suggest that patients with a low R0 dimer have improved DFS with those demonstrating a more robust response recurring less often. This suggests that induction, rather than amplification, of an anti-HER2 immune response confers a survival advantage. The clinical utility lies in the ability of an initial R0 dimer assessment to identify patients who will benefit from the vaccine prior to inoculation. This holds important implications in future trial design.

Confirmation of NeuVax’s clinical utility will require a large, multicenter, randomized, double-blind trial. Moving forward, the Phase III adjuvant trial (Clinicaltrials.gov identifier: NCT01479244) entitled ‘PRESENT: Prevention of Recurrence in Early-Stage, Node-Positive Breast Cancer with Low-to-Intermediate HER2 Expression with NeuVax Treatment’ is currently enrolling high-risk, node-positive patients with HER2 1+ and 2+.
tumors. Due to a potential relationship between HLA type and prognostic factors, the Phase III study is only enrolling HLA-A2* and HLA-A3* patients to be randomized to nelipepimut-S plus GM-CSF or GM-CSF alone as the control group. The primary end point is 36 month DFS [68]. Ultimately, combination, both active and passive, immunotherapy may portend the most significant contribution to the treatment of HER2-expressing breast cancer patients. The synergistic mechanism of action between trastuzumab and nelipepimut-S was examined in a preclinical study of trastuzumab-pretreated breast cancer cells, which were lysed more efficiently by E75-primed CTL compared with untreated breast cancer cells. This enhanced CTL-mediated lysis occurred regardless of the level of HER2 expression [69]. Subsequently, a pilot clinical study revealed lower recurrence rates in breast cancer patients receiving adjuvant trastuzumab followed by vaccination with a CD8-eliciting peptide vaccine compared with adjuvant trastuzumab alone [70]. These findings prompted the initiation of an ongoing Phase II clinical trial, Combination Immunotherapy with Herceptin and the HER2 Vaccine NeuVax (Clinicaltrials.gov identifier: NCT01570036). This trial hopes to demonstrate the clinical efficacy of combining the known benefits of proven passive immunotherapy with the promise of active specific immunotherapy.

**Executive summary**

**Background**
- Cancer vaccines are a promising immunotherapeutic strategy given minimal toxicity and the ability to generate a sustained immunologic response with immunologic memory.
- Vaccines work by targeting tumor-associated antigens that can be recognized by cytotoxic T lymphocytes (CTLs), critical for the removal of tumor cells in vivo.

**Introduction to NeuVax™ (Galena, OR, USA)**
- NeuVax™ (Galena, OR, USA) utilizes E75 or nelipepimut-S, a nine-amino-acid peptide derived from the HER2 protein (KIFGLSAFL, HER2 p369-377) as its immunologic focus.
- NeuVax is administered to disease-free patients in the adjuvant setting after completion of standard-of-care therapy with concurrent endocrine therapy as indicated.
- The vaccine administers nelipepimut-S in 6-monthly intradermal inoculations with granulocyte–macrophage colony-stimulating factor (GM-CSF) as an immunoadjuvant with booster inoculations every 6 months after the primary vaccine series.

**Laboratory & clinical studies**
- NeuVax shows promise in Phase I/II clinical trials although the mechanism of action, whether by inducing an immune response versus augmenting a pre-existing immune response, has been unknown.

**Correlating immune & clinical response**
- An integral component of clinical trials for cancer vaccines is in vivo and ex vivo immune response monitoring with multiple cancer vaccine studies failing to correlate peptide-specific CTL expansion with clinical benefit.
- T-cell trafficking at vaccination and tumor sites may account for T-cell degradation and deletion leading to absence of immunologic memory and subsequent hyporesponsiveness.
- Vaccination models utilizing rapidly degrading adjuvant (GM-CSF) within the adjuvant setting may avoid the pitfalls of CTL sequestration.

**Immunologic testing**
- NeuVax immunogenicity has been assessed by multiple immunologic parameters to include delayed-type hypersensitivity, dimer assay, ELISPOT assay, Treg population assessment, cytokine evaluation, epitope spreading and CTC quantification.

**Biomarkers associated with NeuVax response**
- Recent correlation of immunogenic variables with DFS supports nelipepimut-S-specific CTL clonal expansion as a valid biomarker for predicting clinical recurrence after NeuVax administration.
- Examination of nelipepimut-S-specific CTL clonal expansion data with clinical benefit suggests induction, rather than amplification, of an anti-HER2 immune response correlates with NeuVax's efficacy.
- Whereas a large tumor burden may sequester CTLs, in vitro immune monitoring may be more accurate within the adjuvant setting.

**Future perspective & conclusion**
- Confirmation of NeuVax's clinical utility will require a large, multicenter, randomized trial.
- The Phase III adjuvant trial (NCT01479244) titled ‘PRESENT: Prevention of Recurrence in Early-Stage, Node-Positive Breast Cancer with Low to Intermediate HER2 Expression with NeuVax Treatment’ is currently enrolling HLA-A2* and/or HLA-A3* node-positive patients with HER2 1+ and 2+ tumors. Enrollment will be complete in 2014.
- The vaccine may also be clinically synergistic in combination with Herceptin®. A Phase II trial examining the clinical efficacy of trastuzumab plus NeuVax versus trastuzumab plus GM-CSF alone is currently enrolling.
Financial & competing interests disclosure
GE Peoples has inventor rights to nelipepimut-S. This vaccine has been licensed for commercial development. He is entitled to financial proceeds associated with this license per Federal policy. GE Peoples also consults in the development of the vaccine. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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HER2 peptide nelipepimut-S (E75) vaccine (NeuVax™) in breast cancer patients at risk for recurrence


68 Efficacy and safety study of NeuVax(TM)(nelipepimut-S or E75) vaccine to prevent breast cancer recurrence. http://clinicaltrials.gov/show/NCT01479244
