

## Neuregulin Expression Modulates Clinical Response to Trastuzumab in Patients With Metastatic Breast Cancer

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### ABSTRACT

#### Purpose

Human epidermal growth factor receptor 2 (HER-2) overexpression has been associated with the genesis and progression of a subset of breast cancers. The function of HER-2 may be upregulated by overexpression or by the availability of neuregulins (NRGs), a group of transmembrane growth factors. Transmembrane NRGs strongly activated HER-2 and cell proliferation in breast cancer cells that did not overexpress HER-2, and treatment with trastuzumab prevented the proliferative action of transmembrane NRG. This raised the relevant clinical question of whether patients considered as HER-2 negative, but expressing transmembrane NRG, may benefit from treatment with trastuzumab.

#### Patients and Methods

MCF7 cells expressing transmembrane NRG (MCF7-NRG $\alpha$ 2c) were injected into mice, and their sensitivity to trastuzumab was assessed. A retrospective study of 124 patients with early-stage or metastatic breast cancer was conducted. Expression of transmembrane NRG was evaluated by immunohistochemistry. In 11 patients, Western blot for NRGs was also carried out. Statistics were performed to analyze possible correlations between NRG expression and response to trastuzumab-based therapies, event-free survival, and overall survival (OS).

#### Results

Trastuzumab inhibited tumor growth in mice injected with MCF7-NRG $\alpha$ 2c cells. Transmembrane NRG was frequently expressed in breast cancer patients. Overexpression of transmembrane NRG significantly correlated with a longer event-free survival and OS in patients with low or normal HER-2 expression who were treated with trastuzumab-based therapies but not in patients with HER-2 overexpression.

#### Conclusion

We suggest that the spectrum of patients who may benefit from trastuzumab-based therapies may be widened to include patients with metastatic breast cancer without HER-2 amplification but who express transmembrane NRGs.

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### INTRODUCTION

The human epidermal growth factor receptor (ErbB/HER) tyrosine kinases and their ligands are involved in numerous biologic and pathologic processes including cancer.<sup>1</sup> The following four ErbB receptors have been described in mammals: ErbB1 (epidermal growth factor receptor or HER-1), ErbB2 (HER-2 or neu), ErbB3 (HER-3), and ErbB4 (HER-4).<sup>2,3</sup> Activation of these receptors may occur by the following three different mechanisms<sup>4-6</sup>: specific HER ligands; overexpression; or molecular alterations such as point mutations or truncations.

ErbB2/HER-2 overexpression has been implicated in the genesis and progression of a subset of

breast and ovarian tumors.<sup>7,8</sup> This led to the development of therapies to decrease the activation of this receptor. One of these therapeutic developments is trastuzumab (Herceptin; Genentech, South San Francisco, CA), a monoclonal antibody that has demonstrated clinical benefit.<sup>9,10</sup> One important problem of trastuzumab therapy is the selection of responsive patients based on HER-2 overexpression, which is assessed using immunohistochemical techniques frequently combined with fluorescent in situ hybridization (FISH) to determine the level of amplification. Immunohistochemical detection of HER-2 was initially based on the use of different anti-HER-2 antibodies, which caused certain discrepancy in the evaluation of HER-2 positivity. More recently, availability of diagnostic kits based

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on well-established immunohistochemical staining and evaluation methods (ie, DAKO HercepTest; DAKO, Carpinteria, CA) has favored the standardization of HER-2 detection. Yet, these criteria for patient selection are insufficient because only 30% of patients respond to trastuzumab monotherapy.<sup>9</sup>

An alternative mode of HER-2 receptor activation is the presence of ligands, such as neuregulins (NRGs; also termed heregulins).<sup>11</sup> The NRGs are produced as transmembrane ligands, known as proNRGs, that can be released as soluble factors by the action of cell surface proteases<sup>12,13</sup> (Fig 1A). Increasing evidence indicates that NRGs may play a relevant role in breast cancer. Expression of NRG in the mammary gland induces adenocarcinomas in animal models<sup>14</sup> and favors metastatic spread of breast cancer cells.<sup>15</sup> Expression of transmembrane NRGs in breast cancer cells activates HER-2 and favors their proliferation *in vitro*.<sup>16-18</sup> This activation occurs in the absence of HER-2 overexpression and is highly sensitive to trastuzumab.<sup>18</sup> These findings raised the question of whether trastuzumab could be clinically useful in patients lacking HER-2 amplification but expressing transmembrane NRG.<sup>19</sup> In this report, we have analyzed NRG expression in samples from patients with breast cancer and have retrospectively studied a potential correlation between transmembrane NRG expression and clinical response. Our results show that transmembrane NRG expression is frequent in breast cancer patients and, in the absence of HER-2 amplification, is associated with objective clinical response to trastuzumab. Therefore, the presence of transmembrane NRG in patients without HER-2 amplification may indicate trastuzumab sensitivity and, thus, could open a new therapeutic option for this subset of patients.

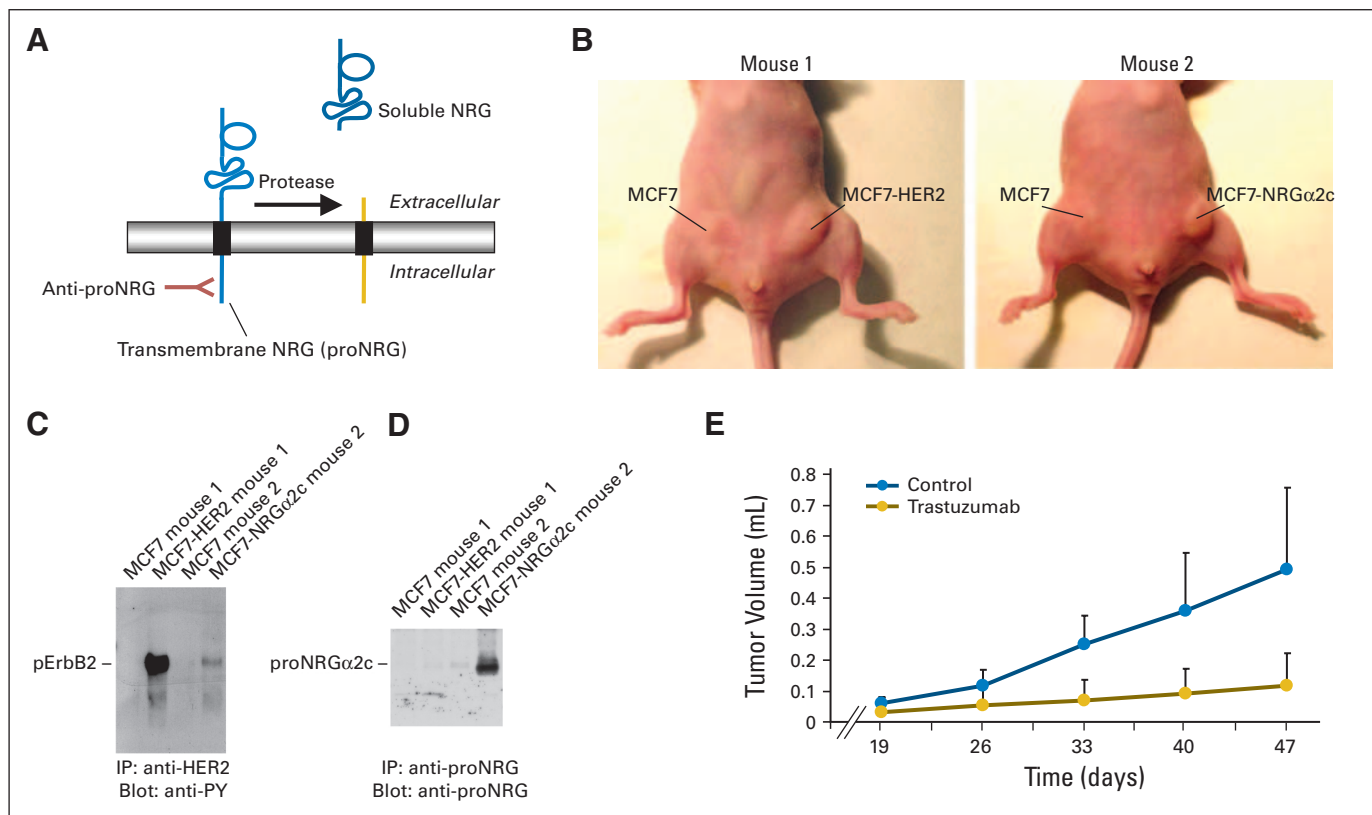
## PATIENTS AND METHODS

### Patient Selection and Statistical Analyses

One hundred fifty-one samples from 124 patients who were treated at the University Hospital of Salamanca from 2000 to 2005 were studied for NRG expression. Statistical analyses were performed in 63 patients for whom enough demographic characteristics or clinical follow-up data were available. Because some of these patients were evaluated for HER-2 expression before the HercepTest assay (DAKO), a retrospective review of their HER-2 status was performed. Data were tabulated into a Microsoft Excel worksheet (Microsoft, Redmond, WA) and exported to the SPSS 12.0 statistical suite (SPSS Inc, Chicago, IL). The Spearman's  $\chi^2$  test was used for correlation analyses, and a  $P < .05$  was used as the cutoff for decisions of statistical significance.  $\chi^2$  and Fisher's exact tests were used to assess correlation of two dichotomous variables. In the case of one ordinal variable and one dichotomous variable, comparison was performed using the Mann-Whitney  $U$  test. Kaplan-Meier survival analyses were carried out for both overall survival (OS) and time to disease progression (TDP). Log-rank statistics were also calculated. The Student's  $t$  test (two sided) was used to compare tumor sizes in mice.

### NRG Expression

For immunohistochemical staining, three tissue microarrays containing three 0.6-mm cores of the 151 breast carcinoma samples were analyzed for transmembrane NRG using an antibody (anti-proNRG, #311) raised against the intracellular domain of NRG.<sup>12</sup> Sections (3  $\mu$ m) from the tissue



**Fig 1.** (A) Representation of a transmembrane neuregulin (NRG), and the region identified by the anti-proNRG antibody. (B) Photographs of mice injected with the indicated cell lines. (C) Tyrosine phosphorylation of human epidermal growth factor receptor 2 (HER-2), and (D) expression of NRG in tumors isolated from mice. (E) Analysis of tumor growth in mice injected with MCF7-NRG $\alpha$ 2c cells and treated or not with trastuzumab. A  $P$  value of .038 was obtained upon comparing the volumes of the tumors of untreated and trastuzumab-treated animals.

microarrays were dewaxed and hydrated, washed with ethanol, and finally washed with distilled water. Three cell conditioning periods of 8 minutes at 95°C, 4 minutes at 100°C, and 36 minutes at room temperature on hot plate buffer with Tris-EDTA pH 8.0 were performed. These sections were incubated at 37°C for 1 hour with the anti-proNRG antibody. Staining was performed with the immunohistochemistry DAB MAP system (Ventana Medical Systems, Tucson, AZ). Each image was interpreted by two coauthors (E.d.A. and M.A.) for immunoreactivity using a 0 to 3 semiquantitative scoring system for both the intensity of stain and the percentage of positive cells (labeling frequency percentage). For the intensity, the grading scale ranged from no detectable signal (score of 0) to strong signal at low power (score of 3). A moderate signal seen at intermediate power was designated as a score of 2, whereas a score of 1 indicated a weak signal seen only at intermediate to high power. Labeling frequency was scored as 0 (0%), 1 (1% to 33%), 2 (34% to 66%), or 3 (67% to 100%). The multiplicative index of intensity and labeling was considered for analysis (range, 0 to 9). Maximum allowed discrepancy between both observers was 2; and in these cases, the final score was the mean value of both individual scores. Expression was also recoded into a new variable, a two-tier distribution (low or high) in which values greater than 5.5 were considered as high expression. This cutoff was chosen because the median value of NRG expression in the large data set of breast carcinoma samples used as a training set for NRG evaluation described in the previous section ( $n = 151$ ) was 5.5. Final case score was determined by obtaining the mean of three cores corresponding to each specimen.

For the detection of NRG expression by Western blot, the tumors were minced, washed with phosphate-buffered saline, and homogenized with ice cold lysis buffer<sup>12</sup> with a tight-fitting Dounce homogenizer. This homogenate was centrifuged at  $10,000 \times g$  for 20 minutes at 4°C, and the supernatants were transferred to new tubes. The samples (60  $\mu$ g) were processed for Western blotting as previously described.<sup>20</sup>

#### HER-2 and Hormonal Status Assessment

HER-2 amplification was performed by FISH (DAKO HER-2 FISH pharmDx Kit; DAKO), and HER-2 expression was evaluated by the DAKO HercepTest kit. Hormonal receptor status was performed by immunohistochemistry, and the results were scored semiquantitatively between 0 and 3.

#### In Vivo Assessment of Tumor Growth

Mice were manipulated at the animal facility following legal and institutional guidelines;  $10^7$  cells in 150  $\mu$ L of DMEM and 150  $\mu$ L of Matrigel (BD Biosciences, San Jose, CA) were injected into the mammary fat pad, and tumor growth was measured weekly for 6 to 8 weeks.

## RESULTS

### Transmembrane NRG Confers Proliferation Advantage In Vivo

Formerly, we reported that expression of transmembrane NRG in MCF7 breast cancer cells increased their in vitro proliferation.<sup>18</sup> To analyze whether such a proliferation advantage could be reproduced in vivo, MCF7 cells expressing the transmembrane form of NRG $\alpha$ 2c (MCF7-NRG $\alpha$ 2c), wild-type MCF7 cells, or MCF7 cells overexpressing HER-2 (MCF7-HER-2) were injected into the mammary fat pad of female nude mice, and the size of the masses were measured weekly. All of the mice overexpressing HER-2 developed tumors by 8 weeks after injection (Fig 1B; Table 1). However, only 31% of mice injected with wild-type MCF7 cells developed tumors. Expression of transmembrane NRG resulted in a substantial percentage of mice (87%) bearing tumor masses. Transmembrane NRG was expressed in the tumoral masses isolated from mice injected with MCF7-NRG $\alpha$ 2c cells (Fig 1D) and provoked tyrosine phosphorylation of HER-2 (Fig 1C). Tyrosine phosphorylation of HER-2 was high in tumors created by injection of MCF7-HER-2 cells. These data indicate that expression of

**Table 1.** Characteristics of Tumor Growth in Nude Mice Injected With Three Different Cell Lines

Week of Evaluation	Cell Line Injected					
	MCF7 (n = 16)		MCF7-HER-2 (n = 8)		MCF7-NRG $\alpha$ 2c (n = 8)	
	No. of Mice With Tumor Growth	%	No. of Mice With Tumor Growth	%	No. of Mice With Tumor Growth	%
2	3	18	5	62	4	50
4	4	25	6	75	6	75
6	5	31	8	100	7	87

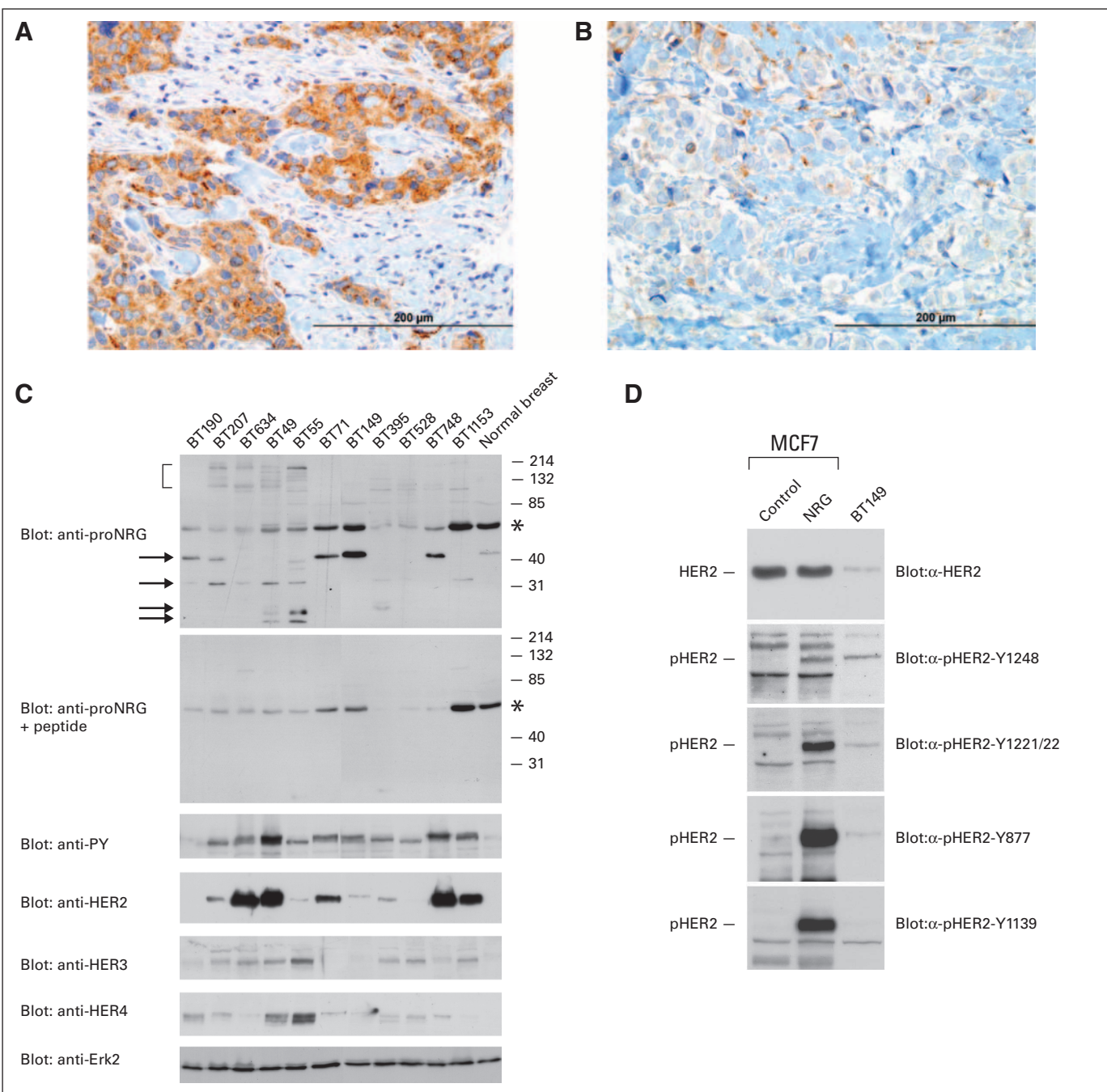
transmembrane NRG also confers a proliferation advantage in vivo. Because we formerly reported that trastuzumab prevented in vitro proliferation of MCF7-NRG $\alpha$ 2c cells,<sup>18</sup> we also explored the in vivo sensitivity to trastuzumab of tumors created by MCF7-NRG $\alpha$ 2c cells. As shown in Figure 1E, trastuzumab hampered tumor growth of MCF7-NRG $\alpha$ 2c–derived tumors.

### NRG Expression in Breast Cancer Samples

We studied the expression of transmembrane NRG in patients with breast cancer tumors by immunohistochemistry with the anti-proNRG antibody. Because we did not have previous experience of NRG expression assessment by immunohistochemistry using this antibody, a training set of 151 samples derived from 124 patients was analyzed. NRG expression was seen as a focal or diffuse staining of the tumor cell cytoplasm; some staining was seen in normal fibroblasts surrounding the infiltrative tumor nests (Fig 2A). Using a two-tier distribution (low/high) in which values greater than the median value of 5.5 were considered positive, high levels of NRG were observed in half of the patients. In the subset of patients ( $n = 32$ ) with metastatic breast cancer for whom data of response to trastuzumab were available, 24 patients (75%) had high levels of NRG, whereas only eight patients (25%) showed low levels.

We also analyzed the presence of NRG forms in 11 of the breast tumor samples by Western blotting, using tissue derived from normal breast glandular tissue as a control. This approach is complementary to the immunohistochemical analysis because it allows for the distinction between different molecular forms of transmembrane NRGs. Western blotting using the anti-proNRG antiserum revealed the presence of several reactive bands of 150 to 200, 45, 35, 25, and 21 Kd in the breast tumor samples (Fig 2C, top panel). That these bands were specifically recognized by the anti-proNRG antiserum was demonstrated by preincubation of the anti-proNRG antibodies with an excess of the peptide against which the antibody had been raised (Fig 2C, second panel). The 45-Kd band was also detected in the normal breast tissue sample.

In these samples, we also performed Western blotting analyses of HER-2, HER-3, and HER-4 receptors, together with an assessment of tyrosine phosphorylation. Most of the tumor samples contained higher levels of the HER receptors than the normal tissue. These samples also contained higher levels of tyrosine phosphorylated proteins in the 150- to 220-Kd region. To investigate whether HER-2 was tyrosine phosphorylated, we explored the level of HER-2 tyrosine phosphorylation using phosphospecific antibodies. As a representative sample, we used the BT149 tumor sample, whose level of HER-2 was low and contained high levels of the 45-Kd NRG form. Tyrosine phosphorylation of HER-2 in the BT149 sample was



**Fig 2.** (A) Immunohistochemical pattern of a sample from a patient positive for transmembrane neuregulin (NRG), compared with (B) a negative sample. (C) Western blotting of breast cancer samples incubated with the indicated antibodies. The asterisk marks a nonspecific band. The anti-Erk2 blot was used as a loading control. (D) Tyrosine phosphorylation of specific human epidermal growth factor receptor 2 (HER-2) residues from the BT149 sample, using as a control MCF7 cells treated with NRG.

detected, especially with the antibodies that recognized phosphorylation at tyrosines 1248 and 1221/1222 (Fig 2D). As a control for the activation of HER-2 in this experiment, we used MCF7 cells, whose amount of HER-2 is considered low to normal,<sup>21,22</sup> treated with soluble NRG.

**NRG Expression and Breast Cancer Prognostic Factors**

We evaluated the potential association of NRG expression with prognostic factors in patients for whom medical records were available. Prognostic factors could be studied in 63 patients (31 patients

with early-stage breast cancer treated in the adjuvant setting, and 32 patients with metastatic breast cancer). Selected prognostic factors in the adjuvant setting included tumor grade, tumor size, number of metastatic lymph nodes, and hormonal receptor status. A Spearman’s test showed no correlation between any of these factors and NRG expression (tumor grade:  $P = .819$ ; tumor size:  $P = .245$ ; number of metastatic lymph nodes:  $P = .120$ ; and hormonal receptor status: estrogen receptor  $+++++$ ,  $P = .919$ ; progesterone receptor  $+++++$ ,  $P = .296$ ). In patients with metastatic breast cancer,

the location of metastases in terms of visceral involvement was studied as a prognostic factor. Analogously, no statistically significant association was observed (Mann-Whitney *U* test,  $P = .436$ ).

### Correlation of NRG Expression With Response

We explored whether expression of transmembrane NRG predicted response to trastuzumab-based therapies. To this end, we evaluated transmembrane NRG expression in 32 patients with metastatic breast carcinoma treated with chemotherapy plus trastuzumab (Table 2). After evaluating the HER-2 status of all patients using FISH and immunohistochemistry (DAKO HercepTest), we found that 12 patients were FISH negative and HercepTest negative. These patients had previously been considered as positive using other immunohistochemical methods, before the routine use of FISH and HercepTest, and had received trastuzumab-based chemotherapy. In these HER-2-negative patients and to avoid the difference in response secondary to the distinct chemotherapy regimens and drugs, we selected only the patients who received taxanes plus trastuzumab (10 patients). Among these patients, seven patients had high levels of NRG expression, and three patients had low levels. From the group of seven patients with high levels, six patients responded to treatment compared with only one patient in the group of patients with low levels. However, this difference was not statistically significant (Fisher's exact test,  $P = .183$ ; Fig 3A). As a control, we compared response in the subgroup of

patients with HER-2 overexpression treated only with taxanes and trastuzumab. In this group, the same number of patients responded independently of the expression level of NRG (two patients in each group; Fisher's exact test,  $P = .429$ ; Fig 3B).

### Correlation of NRG Expression With Time to Progression

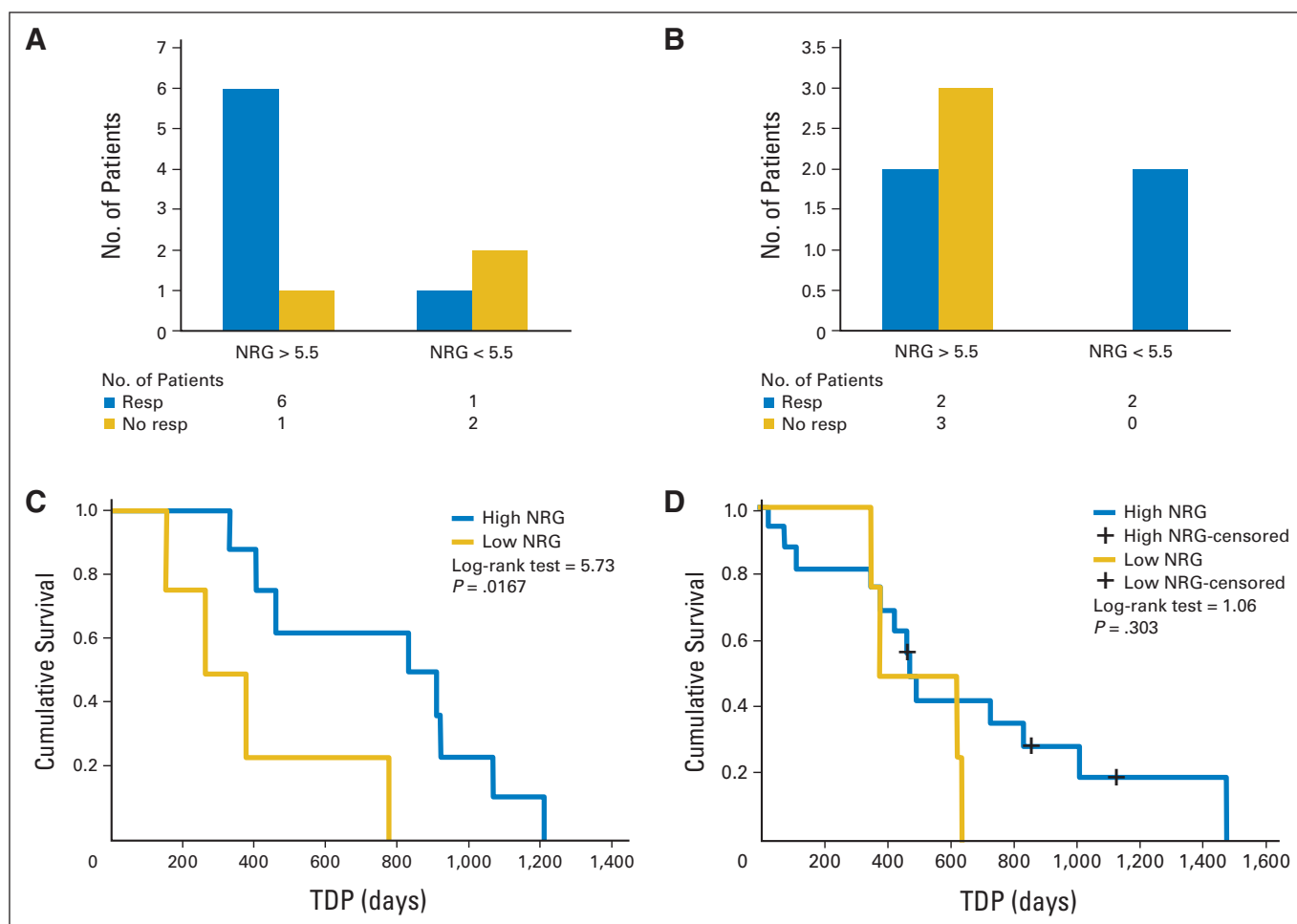
Additional parameters to evaluate the efficacy of a given treatment in metastatic breast cancer included TDP and OS. We studied TDP in the 12 HER-2-negative patients treated with trastuzumab-based therapy and observed that patients with high NRG levels had a higher TDP compared with patients with low NRG levels (log-rank test, 5.73;  $P = .0167$ ; Fig 3C). As a control group, we studied TDP in the subgroup of patients with HER-2 overexpression (Table 2). In this subgroup of patients, no differences were observed (log-rank test, 1.06;  $P = .303$ ; Fig 3D).

To further analyze whether the chemotherapy could influence these results, we studied the subgroup of 10 patients with HER-2-negative tumors (Table 2) who were treated only with taxanes and trastuzumab as first-line treatment. We observed a clear difference in TDP for patients with high levels of NRG (log-rank test, 5.54;  $P = .0186$ ; Fig 4A). When selecting the subgroup of patients with HER-2 overexpression treated only with taxanes plus trastuzumab (seven patients), there was no difference in TDP depending on NRG

**Table 2.** Characteristics of the Patients Who Received Trastuzumab-Based Therapies

Patient No.	FISH	Response	Chemotherapy	Metastases Localization	NRG	TDP (days)	Progression	OS (days)	Event
1	NA	R	P + Ht	No visceral	3	155	Yes	302	Death
2	NA	R	P + Ht	No visceral	7	835	Yes	1,761	Death
3	NA	Pr	P + Ht	No visceral	6	465	Yes	465	Death
4	NA	R	P + Ht	Visceral	6	1,211	Yes	1,226	Death
5	NA	R	P + Ht	No visceral	6	1,074	Yes	1,086	Alive
6	NA	R	D + Ht	No visceral	9	407	Yes	1,417	Alive
7	NA	R	P + Ht	No visceral	6	908	Yes	1,070	Alive
8	NA	R	P + Ht	No visceral	9	924	Yes	1,570	Death
9	NA	Pr	P + Ht	No visceral	3	778	Yes	821	Death
10	NA	Pr	P + Ht	No visceral	3	262	Yes	693	Death
11	NA	R	Ht	No visceral	4	378	Yes	744	Death
12	NA	R	Vinorelbine + Ht	Visceral	9	333	Yes	391	Death
13	A	R	P + Ht	No visceral	9	735	Yes	2,025	Death
14	A	Pr	P + Ht	No visceral	7	1,022	Yes	1,084	Death
15	A	R	Ht	No visceral	7	381	Yes	750	Death
16	A	Pr	P + Carb + Ht	Visceral	6	23	Yes	23	Death
17	A	Pr	P + Ht	No visceral	9	465	Yes	503	Death
18	A	R	LDoxorubicin + P + Ht	Visceral	6	499	Yes	1,090	Death
19	A	R	Vinorelbine + Ht	No visceral	9	866	Yes	866	Alive
20	A	Pr	P + Carb + Ht	Visceral	9	466	No	466	Alive
21	A	R	LDoxorubicin + P + Ht	Visceral	6	1,137	No	1,137	Alive
22	A	R	Vinorelbine + Ht	No visceral	6	846	Yes	1,442	Alive
23	A	R	P + Ht	Visceral	3	374	Yes	419	Death
24	A	R	Vinorelbine + Ht	No visceral	8.5	475	Yes	1,079	Alive
25	A	R	P + Carb + Ht	Visceral	7.5	74	Yes	74	Alive
26	A	R	Vinorelbine + Ht	No visceral	5.5	344	Yes	489	Death
27	A	R	P + Ht	Visceral	9	426	Yes	505	Death
28	A	Rr	P + Carb + Ht	No visceral	4	644	Yes	902	Alive
29	A	Pr	P + Ht	No visceral	7	1,490	Yes	1,774	Death
30	A	R	P + Ht	Visceral	5	629	Yes	1,102	Death
31	A	R	Vinorelbine + Ht	No visceral	9	355	Yes	1,628	Death
32	A	Pr	Cisplatin + Ht	Visceral	8	112	Yes	145	Death

Abbreviations: FISH, fluorescent in situ hybridization; NRG, neuregulin; TDP, time to disease progression; OS, overall survival; NA, no amplification; R, response; P, paclitaxel; Ht, trastuzumab; Pr, progression; D, docetaxel; A, amplification; Carb, carboplatin; LDoxorubicin, liposomal doxorubicin.



**Fig 3.** Clinical response of (A) human epidermal growth factor receptor 2 (HER-2)–negative or (B) HER-2–positive patients treated with trastuzumab plus taxanes. The number of patients responding or not responding to the therapy, with respect to their content of transmembrane neuregulin (NRG), is shown graphically at the bottom of each panel. Kaplan-Meier representation of the time to disease progression (TDP) in (C) HER-2–negative or (D) HER-2–positive patients treated with trastuzumab-based chemotherapy. *P* values are shown on top of the panels. FISH, fluorescent in situ hybridization.

levels (log-rank test, 1.74; *P* = .186; Fig 4B). However, the limited number of patients in this subgroup (only seven patients) makes the *P* value inconclusive.

### Correlation of NRG Expression and Survival

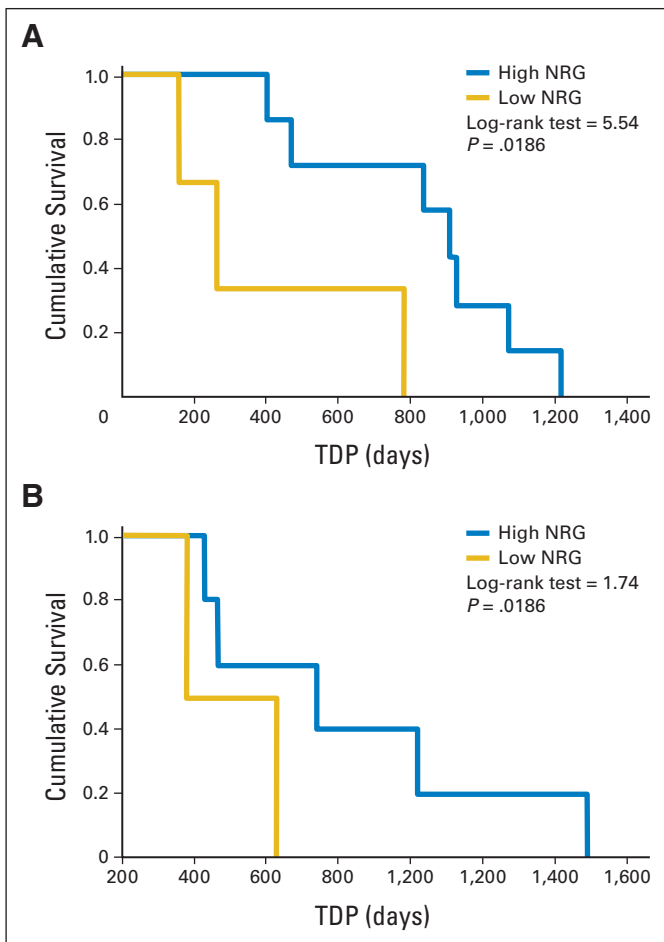
If NRG could predict the response to trastuzumab in patients without HER-2 overexpression, then patients with high levels of NRG treated with trastuzumab could have a longer OS. In the subgroup of 12 patients with HER-2–negative tumors who had been treated with a trastuzumab-based chemotherapy (Table 2), an increase in OS was observed in patients with high expression of NRG (log-rank test, 4.82; *P* = .0281; Fig 5A). In the subgroup of 10 HER-2–negative patients treated with taxanes and trastuzumab as first-line treatment, we also observed an improvement in OS in patients with high levels of NRG (log-rank test, 6.27; *P* = .0122; Fig 5B). However, patients with HER-2 overexpression and high NRG levels did not show any improvement in OS compared with patients with low levels (log-rank test, 1.08; *P* = .298; Fig 5C). Analogous analyses in the subgroup of patients with HER-2 overexpression who were only treated, as a first-line therapy, with taxanes and trastuzumab (seven patients) showed no differences in survival regardless of the NRG level (log-rank test, 0.64; *P* = .432). These results suggest that expression of transmembrane NRG can

predict response to trastuzumab-based therapies in tumors with normal or low HER-2 expression.

## DISCUSSION

This study was initiated with the purpose of evaluating whether expression of transmembrane NRG could facilitate response to trastuzumab in patients lacking overexpression of HER-2. This idea was supported by preclinical in vitro data that showed that transmembrane NRG was a potent inducer of cell proliferation in MCF7 breast cancer cells, which do not overexpress HER-2.<sup>18</sup> Furthermore, this proliferation potential was inhibited by treatment with trastuzumab.<sup>18</sup> The results we present here extend our former in vitro studies and agree with reports that indicated increased tumorigenic and metastatic potential of MCF7 cells expressing NRG.<sup>15,16</sup>

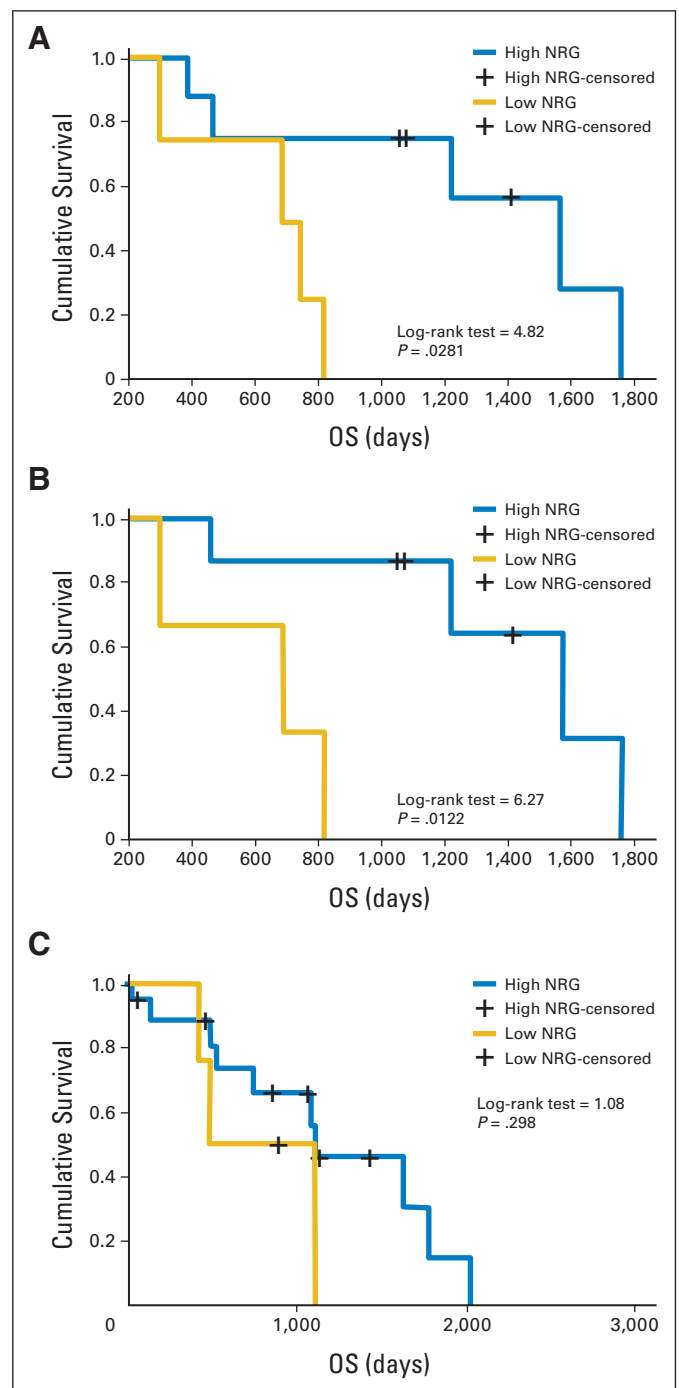
The design of the clinical part of the study merits some considerations. First, it was important to carry out a retrospective study because we already had access to the pathologic and clinical material, and more importantly for the objective of this study, we expected to find patients who received trastuzumab in the absence of HER-2



**Fig 4.** Kaplan-Meier representation of the time to disease progression (TDP) in (A) human epidermal growth factor receptor 2 (HER-2) –negative or (B) HER-2–positive patients treated with taxanes and trastuzumab as first-line therapy with respect to their transmembrane neuregulin (NRG) levels. The *P* values are shown at the top of the panels.

overexpression, as per current criteria. This was a critical aspect of the study because we wanted to be able to evaluate the potential effectiveness of trastuzumab in patients who expressed transmembrane NRG but who were HER-2 negative. Using a specific antibody, we detected transmembrane NRG in a substantial number of patient samples. Of 32 patients with metastatic breast cancer treated with trastuzumab and after re-evaluation of their HER-2 status by DAKO HercepTest and FISH, 12 patients were HER-2 negative. In this subset of patients, we analyzed a potential correlation between transmembrane NRG expression and response, TDP, and OS. A correlation between transmembrane NRG expression and TDP or OS was found in patients with low levels of HER-2 who were treated with trastuzumab, whereas no correlation was found in patients with HER-2 overexpression. When we focused on response rates, no difference was observed, probably because of the limited number of patients. However, FISH-negative patients with high NRG levels responded better than patients with low NRG levels.

These results open new avenues for future research into the clinical use of receptor-targeted therapies. One of the consequences of our work is that the spectrum of patients who may benefit from these therapies may extend to those who express transmembrane growth



**Fig 5.** Kaplan-Meier representation of overall survival (OS) in human epidermal growth factor receptor 2 (HER-2) –negative patients with respect to their transmembrane neuregulin (NRG). (A) Data obtained from the 12 patients treated with trastuzumab plus chemotherapy. (B) Kaplan-Meier curves of the 10 patients treated with trastuzumab plus taxanes. (C) Kaplan-Meier representation of OS in HER-2–positive patients treated with trastuzumab plus chemotherapy with respect to their transmembrane NRG.

factors but do not overexpress the specific receptors. Some scattered precedents on this have been reported. A study on the activity of trastuzumab in breast cancer patients indicated that 10% of HER-2–negative patients had clinical benefit from trastuzumab monotherapy.<sup>9</sup> A recent report in colon cancer patients treated with

cetuximab, a monoclonal antibody that interacts with the extracellular domain of HER-1, also demonstrated clinical effectiveness in patients who were HER-1 negative by immunohistochemistry and were treated with that antibody.<sup>23</sup>

Our work also indicates that proper molecular pathologic analysis of breast cancer patients should include the determination of HER-2 levels as well as other parameters such as ligand expression and phosphorylation status of the HER receptors. Because of the limited number of patients analyzed, our study must be considered exploratory. A larger clinicopathologic study considering these parameters may help in establishing the conditions for a better selection of patients susceptible to clinical benefit on trastuzumab treatment.

#### AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

#### REFERENCES

- Hynes NE, Lane HA: ERBB receptors and cancer: The complexity of targeted inhibitors. *Nat Rev Cancer* 5:341-354, 2005
- Yarden Y, Sliwkowski MX: Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2:127-137, 2001
- Holbro T, Civenni G, Hynes NE: The ErbB receptors and their role in cancer progression. *Exp Cell Res* 284:99-110, 2003
- Ullrich A, Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. *Cell* 61:203-212, 1990
- Cruz JJ, Ocana A, Barco ED, et al: Targeting receptor tyrosine kinases and their signal transduction routes in head and neck cancer. *Ann Oncol* 18:421-430, 2007
- Hynes NE, Schlange T: Targeting ADAMS and ERBBs in lung cancer. *Cancer Cell* 10:7-11, 2006
- Mendelsohn J, Baselga J: The EGF receptor family as targets for cancer therapy. *Oncogene* 19:6550-6565, 2000
- Hynes NE, Stern DF: The biology of *erbB-2/neu/HER2* and its role in cancer. *Biochim Biophys Acta* 1198:165-184, 1994
- Vogel CL, Cobleigh MA, Tripathy D, et al: Efficacy and safety of trastuzumab as a single agent

in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol* 20:719-726, 2002

- Slamon DJ, Leyland-Jones B, Shak S, et al: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 344:783-792, 2001
- Falls DL: Neuregulins: Functions, forms, and signaling strategies. *Exp Cell Res* 284:14-30, 2003
- Montero JC, Yuste L, Díaz-Rodríguez E, et al: Differential shedding of transmembrane neuregulin isoforms by the tumor necrosis factor- $\alpha$  converting enzyme. *Mol Cell Neurosci* 16:631-648, 2000
- Massagué J, Pandiella A: Membrane-anchored growth factors. *Annu Rev Biochem* 62:515-541, 1993
- Krane IM, Leder P: NDF/hereregulin induces persistence of terminal end buds and adenocarcinomas in the mammary glands of transgenic mice. *Oncogene* 12:1781-1788, 1996
- Atlas E, Cardillo M, Mehmi I, et al: Heregulin is sufficient for the promotion of tumorigenicity and metastasis of breast cancer cells in vivo. *Mol Cancer Res* 1:165-175, 2003
- Tsai MS, Shamon-Taylor LA, Mehmi I, et al: Blockage of heregulin expression inhibits tumorigenicity and metastasis of breast cancer. *Oncogene* 22:761-768, 2003
- Menendez JA, Mehmi I, Lupu R: Trastuzumab in combination with heregulin-activated Her-2

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(erbB-2) triggers a receptor-enhanced chemosensitivity effect in the absence of Her-2 overexpression. *J Clin Oncol* 24:3735-3746, 2006

- Yuste L, Montero JC, Esparis-Ogando A, et al: Activation of ErbB2 by overexpression or by transmembrane neuregulin results in differential signaling and sensitivity to Herceptin. *Cancer Res* 65:6801-6810, 2005
- Arteaga CL: Can trastuzumab be effective against tumors with low HER2/Neu (ErbB2) receptors? *J Clin Oncol* 24:3722-3725, 2006
- Cabrera N, Díaz-Rodríguez E, Becker E, et al: TrkA receptor ectodomain cleavage generates a tyrosine-phosphorylated cell-associated fragment. *J Cell Biol* 132:427-436, 1996
- Montero JC, Rodríguez-Barrueco R, Yuste L, et al: The extracellular linker of pro-neuregulin- $\alpha$  2c is required for efficient sorting and juxtacrine function. *Mol Biol Cell* 18:380-393, 2007
- Agus D, Akita R, Fox W, et al: Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell* 2:127-137, 2002
- Chung KY, Shia J, Kemeny NE, et al: Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol* 23:1803-1810, 2005

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