

# The NEW ENGLAND JOURNAL of MEDICINE

ESTABLISHED IN 1812

MAY 18, 2006

VOL. 354 NO. 20

## HER2 and Responsiveness of Breast Cancer to Adjuvant Chemotherapy

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### ABSTRACT

#### BACKGROUND

Amplification of the human epidermal growth factor receptor type 2 (*HER2*, also called *HER2/neu*) gene and overexpression of its product in breast-cancer cells may be associated with responsiveness to anthracycline-containing chemotherapy regimens.

#### METHODS

In the randomized, controlled Mammary5 trial, we studied 639 formalin-fixed paraffin-embedded specimens obtained from 710 premenopausal women with node-positive breast cancer who had received either cyclophosphamide, epirubicin, and fluorouracil (CEF) or cyclophosphamide, methotrexate, and fluorouracil (CMF) as adjuvant chemotherapy. *HER2* amplification or overexpression was evaluated with the use of fluorescence in situ hybridization, immunohistochemical analysis, and polymerase-chain-reaction analysis.

#### RESULTS

Amplification of *HER2* was associated with a poor prognosis regardless of the type of treatment. In patients whose tumors showed amplification of *HER2*, CEF was superior to CMF when assessed on the basis of relapse-free survival (hazard ratio, 0.52; 95 percent confidence interval, 0.34 to 0.80;  $P=0.003$ ) and overall survival (hazard ratio, 0.65; 95 percent confidence interval, 0.42 to 1.02;  $P=0.06$ ). For women whose tumors lacked amplification of *HER2*, CEF did not improve relapse-free survival (hazard ratio for relapse, 0.91; 95 percent confidence interval, 0.71 to 1.18;  $P=0.49$ ) or overall survival (hazard ratio for death, 1.06; 95 percent confidence interval, 0.83 to 1.44;  $P=0.68$ ). The adjusted hazard ratio for the interaction between treatment and *HER2* amplification was 1.96 for relapse-free survival (95 percent confidence interval, 1.15 to 3.36;  $P=0.01$ ) and 2.04 for overall survival (95 percent confidence interval, 1.14 to 3.65;  $P=0.02$ ).

#### CONCLUSIONS

Amplification of *HER2* in breast-cancer cells is associated with clinical responsiveness to anthracycline-containing chemotherapy. (cancer.gov number, NCI-V90-0027.)

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N Engl J Med 2006;354:2103-11.

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**I**N THE 1980S, TRIALS OF ADJUVANT CHEMOTHERAPY for breast cancer that compared regimens containing an anthracycline (epirubicin or doxorubicin) with a combination of cyclophosphamide, methotrexate, and fluorouracil (CMF) yielded inconsistent results.<sup>1-3</sup> More than a decade later, the safety and efficacy of an intensive regimen of cyclophosphamide, epirubicin, and fluorouracil (CEF) as adjuvant therapy for breast cancer were demonstrated.<sup>4</sup> In the National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) Mammary.5 (MA.5) randomized trial involving premenopausal women with node-positive breast cancer, CEF was shown to be superior to CMF<sup>5</sup> and remained superior in terms of relapse-free survival and overall survival after a median follow-up of 10 years.<sup>6</sup> As compared with CMF, however, CEF was associated with increased rates of alopecia, nausea, vomiting, stomatitis, and neutropenia and febrile neutropenia; a temporary reduction in the quality of life; and an increase in the risk of congestive heart failure (1.1 percent) and acute leukemia (1.4 percent).<sup>5,6</sup> Treatment with CEF is also considerably more expensive than CMF therapy.

It has been suggested that amplification of the gene for human epidermal growth factor receptor type 2 (*HER2*, also referred to as *HER2/neu*), overexpression of its product, or both in breast-cancer cells not only predicts responsiveness to trastuzumab<sup>7,8</sup> but also identifies patients whose tumors will not respond to CMF<sup>9-13</sup> and who could benefit from high-dose chemotherapy<sup>14-19</sup> or from anthracycline-containing regimens.<sup>20-29</sup> To pursue this suggestion, we studied formalin-fixed, paraffin-embedded specimens from all patients enrolled in the MA.5 trial to determine whether amplification of *HER2*, overexpression of *HER2*, or both in breast-cancer cells identifies women who could benefit from CEF, as compared with CMF.

## METHODS

### PATIENTS

We conducted a study involving premenopausal women, described previously,<sup>5,6</sup> with histologically confirmed axillary-node-positive breast cancer who had undergone a modified radical mastectomy or lumpectomy plus axillary dissection. The MA.5 trial protocol was approved by the institutional review board at each participating center,

and written informed consent was obtained from each woman before randomization.

### TREATMENT REGIMENS

As described previously,<sup>5,6</sup> the CMF regimen consisted of six cycles of oral cyclophosphamide (Cytoxan, Bristol-Myers Squibb) at a dose of 100 mg per square meter of body-surface area on days 1 through 14, 40 mg of methotrexate (Wyeth [formerly Lederle] per square meter intravenously on days 1 and 8, and 600 mg of fluorouracil (Efudex, Valeant Pharm) per square meter intravenously on days 1 and 8. The CEF regimen consisted of six cycles of oral cyclophosphamide at a dose of 75 mg per square meter on days 1 through 14, 60 mg of epirubicin (Pharmorubicin, Pfizer) per square meter intravenously on days 1 and 8, and 500 mg of fluorouracil per square meter intravenously on days 1 and 8. During CEF therapy, the women also received antibiotic prophylaxis with trimethoprim-sulfamethoxazole (Septra, Glaxo Wellcome) at a dose of two tablets orally twice daily or, for those who could not tolerate trimethoprim-sulfamethoxazole, norfloxacin (Norflox, Merck) at a dose of 400 mg orally twice daily or ciprofloxacin (Cipro, Bayer) at a dose of 500 mg orally twice daily. Endocrine therapy was not to be used after the completion of chemotherapy.

### OUTCOMES

The primary outcomes for the MA.5 study were relapse-free survival and overall survival. They have been defined previously.<sup>5,6</sup>

### SPECIMEN COLLECTION

As part of supporting documentation for the MA.5 trial, the NCIC CTG collected diagnostic pathology reports for each woman. For the current analysis, pathologists were asked to submit a representative formalin-fixed, paraffin-embedded block of tumor tissue from each woman, or if tumor blocks were unavailable, 20 4- $\mu$ m unstained sections, to the central office of the NCIC CTG. Paraffin blocks were stored at room temperature, and unstained sections were kept at 4°C. Samples were identified only by an identification number assigned to each patient at randomization. A stained section of each tumor sample was prepared from blocks or slides to confirm the diagnosis and identify representative tumor areas for microdissection and DNA ex-

traction for analysis by the polymerase chain reaction (PCR). An adjacent 10- $\mu\text{m}$  section was also obtained for PCR analysis. Further 4- $\mu\text{m}$  sections were obtained for immunohistochemical analysis and fluorescence in situ hybridization (FISH). Assay results were reported to the NCIC CTG central office, where the statistical analysis was performed.

#### MEASUREMENT OF HER2 EXPRESSION

Expression of the HER2 protein was measured by immunohistochemical analysis with the CB11 antibody (Novocastra) and the TAB 250 antibody (Zymed), as previously described.<sup>30</sup> Only complete membrane staining of invasive tumor cells was considered in the results. The proportion of cells with complete membrane staining was assessed with the use of the Allred semiquantitative scoring system.<sup>31</sup> A score of five or greater on the immunohistochemical analysis was considered to be positive for overexpression of the HER2 protein on the basis of previous technical validation of this cutoff point.<sup>30</sup>

#### FISH

Representative sections of tumor were hybridized with the use of a HER-2 DNA probe kit (Path-Vysion, Vysis), as previously described.<sup>32</sup> A staining-intensity ratio of HER2 to chromosome 17 (assessed with the use of the centromere enumeration probe cep 17) of 2 or more was considered to indicate amplification, in accordance with the manufacturer's recommendations.

#### PCR

PCR analysis for amplification of *HER2* was performed as described by O'Malley et al.<sup>30</sup> A relative increase in the number of gene copies of two or more was considered to indicate amplification.

#### STATISTICAL ANALYSIS

Kaplan–Meier estimates of survival according to the presence or absence of amplification of *HER2* or overexpression of *HER2* or according to the treatment regimen were compared with the use of a log-rank test. We use the phrase “amplification of *HER2*” throughout to refer to the results of FISH and PCR and the term “overexpression” to refer to the results of the immunohistochemical analysis. We used the Cox proportional-hazards model with

**Table 1. Results of Assays of *HER2* Amplification on FISH and PCR and *HER2* Overexpression on Immunohistochemical Analysis with the TAB 250 and CB11 Antibodies.\***

Method	TAB 250 Antibody		CB11 Antibody		PCR	
	Overexpression (N=116)	No Overexpression (N=516)	Overexpression (N=124)	No Overexpression (N=510)	Amplified (N=195)	Not Amplified (N=429)
FISH						
Amplified — no. (%)	101 (16)	59 (9)	116 (18)	46 (7)	125 (20)	32 (5)
Not amplified — no. (%)	15 (2)	445 (70)	8 (1)	452 (71)	68 (11)	394 (63)
Kappa statistic (95% CI)	0.66 (0.59–0.73)		0.76 (0.70–0.82)		0.60 (0.53–0.67)	
TAB 250 antibody						
Overexpression — no. (%)			101 (16)	15 (2)	96 (15)	19 (3)
No overexpression — no. (%)			23 (4)	493 (78)	96 (15)	406 (65)
Kappa statistic (95% CI)			0.80 (0.75–0.86)		0.51 (0.44–0.59)	
CB11 antibody						
Overexpression — no. (%)					107 (17)	14 (2)
No overexpression — no. (%)					85 (14)	411 (66)
Kappa statistic (95% CI)					0.58 (0.51–0.65)	

\* Specimens were not available for all patients in the trial. CI denotes confidence interval.

**Table 2.** Baseline Characteristics of Women for Whom FISH Data Were Available.\*

Characteristic	HER2 Amplified	HER2	P Value
	(N = 163)	Not Amplified (N = 465)	
	number (percent)		
Age			0.02
≤29 yr	6 (4)	3 (1)	
30–39 yr	44 (27)	100 (22)	
40–49 yr	88 (54)	281 (60)	
≥50 yr	25 (15)	81 (17)	
Node-positive breast cancer			0.48
1–3 nodes	93 (57)	289 (62)	
4–10 nodes	58 (36)	142 (31)	
≥10 nodes	12 (7)	34 (7)	
Estrogen-receptor levels			0.12
<10 fmol/mg	57 (35)	125 (27)	
≥10 fmol/mg	92 (56)	286 (62)	
Unknown	14 (9)	54 (12)	
Surgery			0.36
Partial mastectomy	76 (47)	236 (51)	
Mastectomy	87 (53)	229 (49)	
Tumor stage			0.59
T1 (≤2 cm)	57 (35)	188 (40)	
T2 (>2 cm to ≤5 cm)	84 (52)	226 (49)	
T3 (>5 cm)	8 (5)	23 (5)	

\* P values were calculated with the use of the chi-square test.

a single covariate to obtain the hazard ratios for relapse or death and associated 95 percent confidence intervals for the comparison between the two groups. We used the Cox model, with treatment, amplification status, and their interaction as covariates, to assess the interaction between treatment and amplification status. Multivariable analyses were performed with the use of the Cox model and were adjusted for age (≥50 years vs. <50 years), number of positive lymph nodes (<3 vs. ≥4), estrogen-receptor level (≥10 vs. <10 fmol per milligram), type of surgery (total vs. partial mastectomy), and tumor size (T1, T2, or T3, according to the tumor–node–metastasis staging system). The kappa statistic and associated 95 percent confidence intervals were used to measure agreement among the four assays of HER2 (with a value >0.80

indicating near-perfect agreement, values of 0.61 to 0.80 substantial agreement, values of 0.41 to 0.60 moderate agreement, values of 0.21 to 0.40 fair agreement, values >0 to 0.20 slight agreement, and values of 0 no agreement or a random association).<sup>33</sup>

Drs. Shepherd and Tu, of the NCIC CTG central office, were responsible for study coordination, collection and management of the data, statistical analyses, and administrative activities. Most of the study funding was provided by the NCIC, the Canadian Cancer Society, and the Canadian Breast Cancer Research Alliance. Pharmacia (now Pfizer) provided epirubicin, and Vysis provided FISH kits at a reduced price.

## RESULTS

The outcome data reported here are based on the data set from the MA.5 trial, which was updated on April 5, 2002,<sup>34</sup> and published in 2005.<sup>6</sup> All women who were alive were followed for a minimum of 9 years and a median of 10 years. Among the 710 women in the study, 363 had a recurrence of breast cancer and 284 died.

Tissue samples were available from 639 of 710 eligible women (90 percent) in the MA.5 trial. Immunohistochemical analysis with the use of the CB11 antibody was successful in 634 tumor samples (89 percent), immunohistochemical analysis with the use of the TAB 250 antibody was successful in 632 samples (89 percent), and the results of FISH were interpretable for 628 samples (89 percent). Amplification of *HER2* was assayed by PCR in 624 tumors (88 percent). On FISH, 163 of 628 tumors were found to have *HER2* amplification (26 percent). On immunohistochemical analysis, overexpression of the *HER2* protein was found in 124 of 634 tumors analyzed with the CB11 antibody (20 percent) and in 116 of 632 tumors analyzed with the TAB 250 antibody (18 percent). PCR analysis detected amplification of *HER2/neu* in 195 of 624 tumors (31 percent).

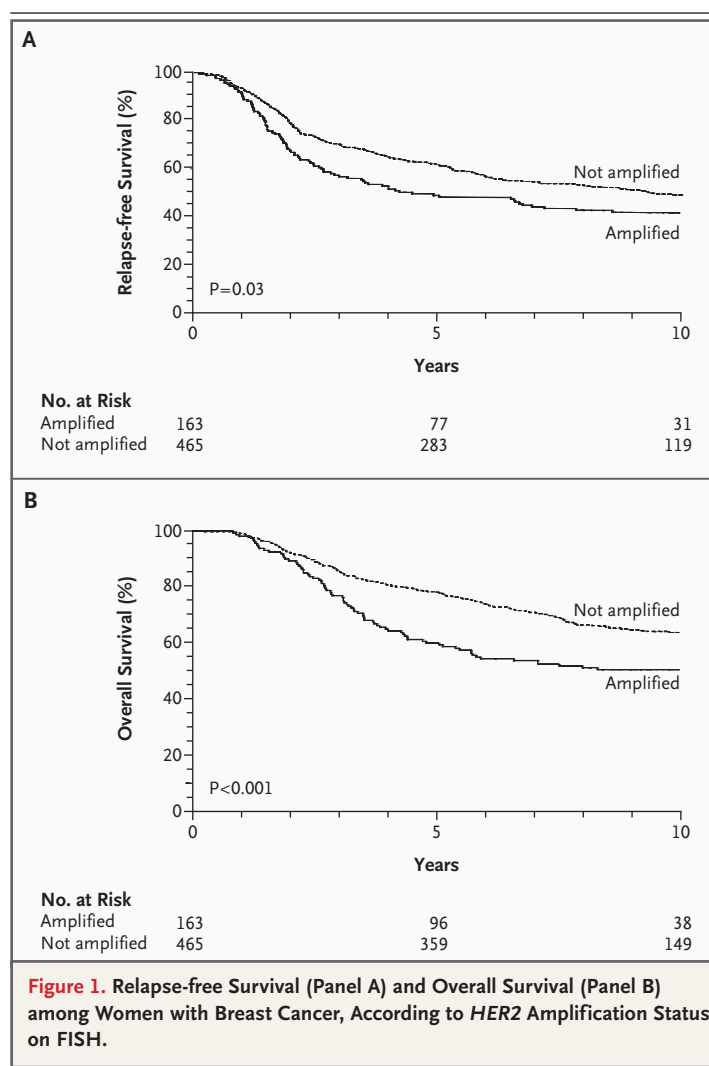
Results with the two antibodies, CB11 and TAB 250, were in substantial agreement (kappa statistic, 0.80; 95 percent confidence interval, 0.75 to 0.86) (Table 1). There was also substantial agreement between the results of FISH and those of the immunohistochemical analyses with the CB11 antibody (kappa statistic, 0.76; 95 percent

confidence interval, 0.70 to 0.82) and the TAB 250 antibody (kappa statistic, 0.66; 95 percent confidence interval, 0.59 to 0.73) and between FISH and the PCR analysis (kappa statistic, 0.60; 95 percent confidence interval, 0.53 to 0.67) (Table 1). Kappa statistics for other analyses showed moderate agreement (Table 1).

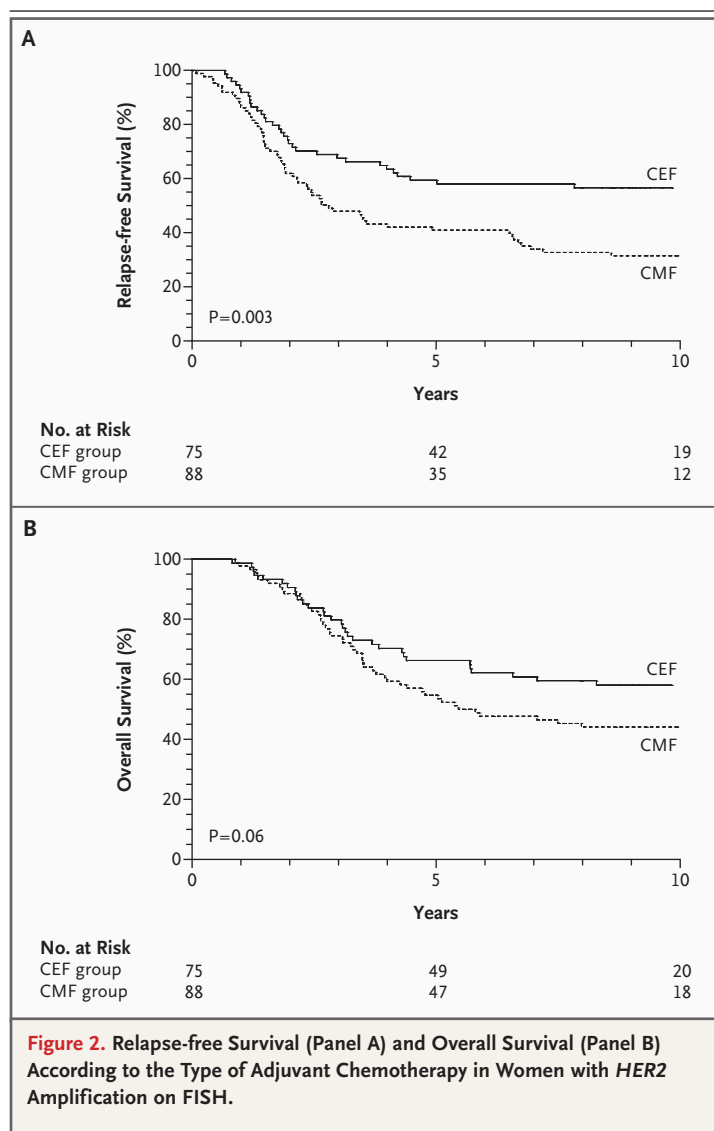
Associations between the results of analyses of amplification or overexpression of HER2 and relapse-free survival and overall survival were assessed with each of the four assay methods. The results of FISH are presented in detail, because this method is the gold standard for measurement of HER2 status.<sup>35,36</sup> Table 2 shows the baseline characteristics of the patients for whom FISH-based measurements were available. The distribution of the characteristics in this subgroup was similar to that among all 710 eligible patients who underwent randomization in the MA.5 study.<sup>6</sup> There was no significant difference between women with HER2 amplification and those without amplification, except for a shift toward younger age among those whose tumors exhibited HER2 amplification on FISH.

As compared with women without HER2 amplification, women with HER2 amplification had a decreased likelihood of both relapse-free survival (hazard ratio for relapse, 1.31; 95 percent confidence interval, 1.03 to 1.67;  $P=0.03$ ) and overall survival (hazard ratio for death, 1.62; 95 percent confidence interval, 1.24 to 2.11;  $P<0.001$ ) (Fig. 1). After adjustment for age, nodal status, estrogen-receptor status, type of surgery, and tumor size, the likelihood of relapse-free survival remained lower among women with HER2 amplification than among women without HER2 amplification (hazard ratio for relapse, 1.24; 95 percent confidence interval, 0.96 to 1.60;  $P=0.09$ ), as did the likelihood of overall survival (hazard ratio for death, 1.53; 95 percent confidence interval, 1.15 to 2.02;  $P=0.003$ ).

Among the women whose tumors showed HER2 amplification, adjuvant chemotherapy with CEF was superior to that with CMF in terms of both relapse-free survival (hazard ratio for relapse, 0.52; 95 percent confidence interval, 0.34 to 0.80;  $P=0.003$ ) and overall survival (hazard ratio for death, 0.65; 95 percent confidence interval, 0.42 to 1.02;  $P=0.06$ ) (Fig. 2). By contrast, among those whose tumors did not show amplification of



HER2, there was no significant difference between adjuvant chemotherapy with CEF and that with CMF in terms of relapse-free survival (hazard ratio for relapse, 0.91; 95 percent confidence interval, 0.71 to 1.18;  $P=0.49$ ) or overall survival (hazard ratio for death, 1.06; 95 percent confidence interval, 0.83 to 1.44;  $P=0.68$ ) (Fig. 3). Unadjusted hazard ratios for the interaction between treatment and amplification status were 1.79 (95 percent confidence interval, 1.08 to 2.96;  $P=0.02$ ) for relapse-free survival and 1.66 (95 percent confidence interval, 0.97 to 2.85;  $P=0.07$ ) for overall survival. The hazard ratio for the interaction between treatment and amplification status, after adjustment for age, nodal status, estrogen-recep-



tor levels, type of surgery, and tumor size, was 1.96 (95 percent confidence interval, 1.15 to 3.36;  $P=0.01$ ) for relapse-free survival and 2.04 (95 percent confidence interval, 1.14 to 3.65;  $P=0.02$ ) for overall survival.

Similar analyses were performed with the amplification of *HER2* obtained by PCR analysis and measurements of the overexpression of *HER2* performed by immunohistochemical analysis with CB11 and TAB 250 antibodies. For each of these methods, the adjusted hazard ratio for the interaction between treatment and amplification status was significant for relapse-free survival. Ad-

justed hazard ratios for the interaction reached significance for overall survival in the FISH analysis and in the immunohistochemical analysis with the use of the CB11 antibody but not with the use of the TAB 250 antibody or in the PCR analysis (Table 3).

## DISCUSSION

Previous analyses have suggested that women with node-positive breast cancer who receive adjuvant chemotherapy with CEF, as compared with CMF, have an increase in relapse-free survival of approximately 30 percent and in overall survival of 18 percent.<sup>5,6</sup> Our correlative analysis indicated that the increase in benefit attributable to CEF is confined almost completely to women whose tumors exhibit amplification or overexpression of *HER2*.

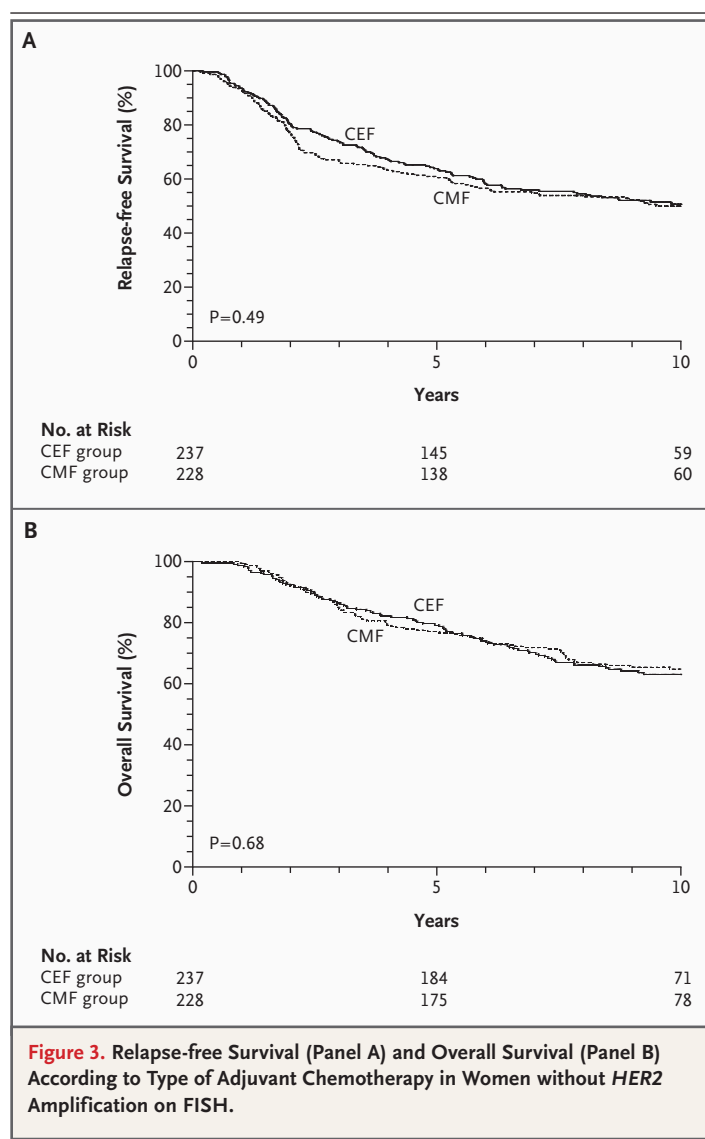
Other investigators have examined the effect of *HER2* amplification or overexpression on outcome in trials comparing regimens that contained an anthracycline with those that did not contain an anthracycline.<sup>20-29,37</sup> Although most of these trials suggested a trend toward greater benefit with the anthracycline-containing regimen in women whose tumors overexpress *HER2* or amplify *HER2*, tests for interaction were significant in only one study, the National Surgical Adjuvant Breast and Bowel Project protocol B-11 trial,<sup>22</sup> in which a regimen of melphalan and fluorouracil was compared with a regimen of melphalan, doxorubicin, and fluorouracil. In that trial, more than 90 percent of the tumors were tested for *HER2* status, whereas in the other studies, tumor specimens were evaluated in no more than 60 percent of the patients.<sup>20,21,23-29</sup> Most of these other studies lacked the statistical power to detect predictive interactions, unless the interactions were extremely strong.

Pegram et al.<sup>38</sup> investigated whether *HER2* overexpression or *HER2* amplification conferred a sensitivity to doxorubicin in four breast-cancer cell lines that were transfected with the *HER2* gene and then exposed to doxorubicin. No alteration in chemosensitivity was observed in any of the lines. An in vitro study of breast-cancer cells obtained from 140 patients who had not undergone chemotherapy showed no association between *HER2* overexpression or *HER2* amplification and resistance to CMF or fluorouracil, epirubicin, and cy-

clophosphamide and no preferential benefit of the latter regimen in tumors positive for HER2.<sup>39</sup> These findings argue against a direct role of HER2 amplification or HER2 overexpression in the sensitivity of breast cancer to anthracyclines.

The association between HER2 overexpression and HER2 amplification and sensitivity to anthracycline may be related to topoisomerase II $\alpha$ . There is evidence<sup>40</sup> that amplification of the topoisomerase II $\alpha$  gene (*TOP2A*) is associated with sensitivity of metastatic breast cancer to anthracyclines. Anthracyclines are topoisomerase inhibitors, and *TOP2A* is close to the *HER2* gene on chromosome 17. Jarvinen et al.<sup>41</sup> have shown, however, that *HER2* and *TOP2A* are not on the same amplicon and that when *HER2* is amplified, *TOP2A* is deleted as often as it is amplified. Nevertheless, changes in topoisomerase II $\alpha$ , at the level of the gene or the protein, may increase sensitivity to anthracyclines, suggesting that measurement of topoisomerase II $\alpha$  in the tumor could be useful in selecting treatment for a woman with breast cancer. A number of groups are exploring this hypothesis.<sup>42</sup> Three recent reports of randomized trials have suggested that deletion or amplification of *TOP2A* is indicative of a poor outcome and predictive of a greater differential response to anthracycline-containing regimens.<sup>37,43,44</sup> In two of the three studies, all tumors had amplified *HER2*,<sup>43,44</sup> but in the other trial comparing CMF and CEF, which was very similar to the MA.5 trial, *HER2* was not predictive of a differential response to CEF even in a univariate analysis, whereas deletion or amplification of *TOP2A* seemed to be predictive of a differential response.<sup>37</sup>

We believe that our analysis of the association between *HER2* amplification or overexpression and responsiveness to CEF, as compared with CMF, is particularly robust, for several reasons. We proposed this analysis before we began collecting tumor specimens; our a priori hypothesis was that women whose tumors exhibited amplification or overexpression of *HER2* would benefit more from CEF than from CMF. We were able to collect tumor specimens from 90 percent of the eligible patients and to measure *HER2* amplification or overexpression of the *HER2* protein with the use of all four of the proposed methods in 88 percent or



more of the tumor samples obtained from these patients.

In conclusion, whether it plays a direct or indirect role, *HER2* amplification or overexpression in breast cancer is associated with a larger benefit from CEF than from CMF. Patients whose tumors do not amplify or overexpress *HER2* receive virtually no benefit from CEF, as compared with CMF. These data suggest that patients whose tumors do not exhibit amplification or overexpression of *HER2* could be treated with the less toxic regimen of CMF, whereas those with tumors that show

**Table 3. Hazard Ratios for Interaction between Treatments and Amplification of *HER2* or Overexpression of *HER2*.\***

Results	FISH	Immunohistochemical Analysis		PCR
		TAB 250 Antibody	CB11 Antibody	
<b>Unadjusted results</b>				
Relapse-free survival				
Hazard ratio (95% CI)	1.79 (1.08–2.96)	1.68 (0.98–2.91)	1.70 (1.00–2.90)	1.62 (1.00–2.63)
P value	0.02	0.06	0.05	0.05
Overall survival				
Hazard ratio (95% CI)	1.66 (0.97–2.85)	1.47 (0.83–2.61)	1.79 (1.12–2.67)	1.33 (0.79–2.24)
P value	0.07	0.18	0.04	0.29
<b>Adjusted results</b>				
Relapse-free survival				
Hazard ratio (95% CI)	1.96 (1.15–3.36)	1.67 (0.94–2.96)	1.81 (1.02–3.21)	1.73 (1.03–2.91)
P value	0.01	0.08	0.04	0.04
Overall survival				
Hazard ratio (95% CI)	2.04 (1.14–3.65)	1.51 (0.83–2.76)	2.09 (1.14–3.83)	1.53 (0.87–2.69)
P value	0.02	0.18	0.02	0.14

\* CI denotes confidence interval.

amplified *HER2* or overexpressed *HER2* should receive dose-intensive anthracycline-containing regimens such as CEF.

Supported by grants from the Canadian Breast Cancer Research Alliance (10032, to Dr. Pritchard) and the Canadian Cancer Society (015469, through the National Cancer Institute of Canada).

Presented as a preliminary analysis at the 38th Annual Meeting of the American Society of Clinical Oncology, Orlando, Fla., May 18–21, 2002.

Dr. Pritchard reports having received consulting fees and speaking fees from Pharmacia (Pfizer). No other potential conflict of interest relevant to this article was reported.

We are indebted to Rosemary Mueller, director of the Cytogenetics Laboratory, Capital Health, University of Alberta Hospital, Edmonton; to Eleanor Latta, M.D., for assistance with FISH and immunohistochemical readings for all specimens; to Tania Molinaro and Suzanna Tjan for technical assistance; to Elizabeth Ramage and Charlene Wainwright for assistance in the preparation of the manuscript; to Vysis for providing FISH kits at a reduced price; and to Pfizer for providing epirubicin.

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