HER2 Gene Amplification Testing by Fluorescent In Situ Hybridization (FISH): Comparison of the ASCO-College of American Pathologists Guidelines With FISH Scores Used for Enrollment in Breast Cancer International Research Group Clinical Trials

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ABSTRACT

Purpose
ASCO and the College of American Pathologists (ASCO-CAP) recently recommended further changes to the evaluation of human epidermal growth factor receptor 2 gene (HER2) amplification by fluorescent in situ hybridization (FISH). We retrospectively assessed the impact of these new guidelines by using annotated Breast Cancer International Research Group (BCIRG) -005, BCIRG-006, and BCIRG-007 clinical trials data for which we have detailed outcomes.

Patients and Methods
The HER2 FISH status of BCIRG-005/006/007 patients with breast cancers was re-evaluated according to current ASCO-CAP guidelines, which designates five different groups according to HER2 FISH ratio and average HER2 gene copy number per tumor cell: group 1 (in situ hybridization [ISH]–positive): HER2-to-chromosome 17 centromere ratio $\geq 2.0$, average HER2 copies $\geq 4.0$; group 2 (ISH-positive): ratio $\geq 2.0$, copies $< 4.0$; group 3 (ISH-positive): ratio $< 2.0$, copies $\geq 6.0$; group 4 (ISH-equivocal): ratio $< 2.0$, copies $< 4.0$ and $< 6.0$; and group 5 (ISH-negative): ratio $< 2.0$, copies $< 4.0$. We assessed correlations with HER2 protein, clinical outcomes by disease-free survival (DFS) and overall survival (OS) and benefit from trastuzumab therapy (hazard ratio [HR]).

Results
Among 10,468 patients with breast cancers who were successfully screened for trial entry, 40.8% were in ASCO-CAP ISH group 1, 0.7% in group 2; 0.5% in group 3, 4.1% in group 4, and 53.9% in group 5. Distributions were similar in screened compared with accrued subpopulations. Among accrued patients, FISH group 1 breast cancers were strongly correlated with immunohistochemistry 3+ status ($P < .0001$), whereas groups 2, 3, 4, and 5 were not; however, groups 2, 4 and, 5 were strongly correlated with immunohistochemistry 0/1+ status (all $P < .0001$), whereas group 3 was not. Among patients accrued to BCIRG-005, group 4 was not associated with significantly worse DFS or OS compared with group 5. Among patients accrued to BCIRG-006, only group 1 showed a significant benefit from trastuzumab therapy (DFS HR, 0.71; 95% CI, 0.60 to 0.83; $P < .0001$; OS HR, 0.69; 95% CI, 0.55 to 0.85; $P = .0006$), whereas group 2 did not.

Conclusion
Our findings support the original categorizations of HER2 by FISH status in BCIRG/Translational Research in Oncology trials.

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INTRODUCTION

Amplification and overexpression of the human epidermal growth factor receptor type 2 gene (HER2/ERBB2) is an established therapeutic target in breast and gastric carcinomas.1,5 Because this alteration is found in other carcinomas at varying prevalence,6-8 the alteration may also prove therapeutically useful in some of these cancers. Although not associated with overexpression,9 activating mutations in extracellular and tyrosine kinase domains of HER2/ERBB2 in breast cancer respond to small-molecule inhibitors, such as lapatinib and neratinib, but to date, these findings have been restricted to preclinical model systems.10

As humanized anti-HER2 monoclonal antibodies2-5,11,12 and small-molecule kinase inhibitors13,14 of HER2 are established as effective only in cancers with amplification and overexpression, the US Food and Drug Administration (FDA) has required a companion diagnostic to select patients for these treatments. Because of reported discrepancies in HER2 testing results using HER2 companion diagnostics, ASCO and College of American Pathologists (ASCO-CAP) convened a panel to standardize performance and interpretation of these HER2 diagnostic assays.15,16 This panel was recently reconvened, and new guidelines were once again issued for HER2 test results.17,18 Because these recommendations differ from past ASCO-CAP and FDA recommendations—and given the fact that HER2 status by fluorescent in situ hybridization (FISH) assay was an entry criterion for the Breast Cancer International Research Group (BCIRG)/Translational Research in Oncology (TRIO) clinical trials of trastuzumab and lapatinib in the treatment of breast and gastric cancers, respectively, in the adjuvant and advanced disease settings,4,5,13,14,19-23—we decided to retrospectively re-evaluate our interpretations of the HER2 FISH assays from three BCIRG clinical trials.4,19,24 These trials now have long-term clinical follow-up data available1,19,25 that facilitate determination of whether the new HER2 guidelines for FISH are clinically useful and predictive of known outcomes. In the current study, we compared the original FDA-approved criteria for HER2 gene amplification with current ASCO-CAP guidelines, assessed the number of cases in each guidelines group, and determined whether new ASCO-CAP FISH testing criteria used to define each of the five HER2 FISH groups are correlated with characteristics known to be associated with HER2 gene amplification, such as HER2 protein overexpression, worse clinical outcomes (disease-free survival [DFS] and overall survival [OS]) in the absence of HER2 targeted therapy, and significant improvement in DFS and OS when such patients are treated with HER2-targeted therapy.

Patients and Clinical Trials

Patients in BCIRG-005/006/007 trials were screened for enrollment in one of two central laboratories by using HER2 gene amplification status determined by FISH as an enrollment criterion4,19,21 (Fig 1). Those patients whose breast cancers were HER2 amplified were eligible for BCIRG-006 or 007, whereas those whose breast cancers were not HER2 amplified were eligible for BCIRG-005 (Fig 1). Criteria for amplified and not amplified that were initially used to screen for entry to these trials are summarized below and in the Data Supplement.

BCIRG-006 trial (n = 3,222) is a randomized, three-arm study of adjuvant chemotherapy with or without trastuzumab in patients with HER2-amplified stage I to III breast cancer who were accrued between April 2001 and March 2004.5 Therapy in the control arm was adjuvant docetaxel, and cyclophosphamide. TCH, docetaxel, carboplatin, and trastuzumab.
Fig 2. Schematic diagram of the ASCO and College of American Pathologists (ASCO-CAP) algorithm for human epidermal growth factor receptor 2 (HER2) testing by fluorescent in situ hybridization (FISH) as published by the ASCO-CAP guidelines committee, modified here by introduction of the numbers 1 to 5 to identify the various ASCO-CAP FISH groups categorized, followed by FISH and immunohistochemistry (IHC) photomicrographs of representative cases from each of.
anthracycline, cyclophosphamide, and docetaxel (AC-T) with or without hormonal therapy depending on tumor estrogen receptor and progesterone receptor status at site investigator discretion. Therapy in the two experimental arms involved trastuzumab (H) with patients randomly assigned to either standard AC-T adjuvant chemotherapy or nonanthracycline chemotherapy with docetaxel and a platinum salt, again, with or without hormonal therapy depending on tumor estrogen receptor and progesterone receptor status. This trial demonstrated significant improvement in DFS for both trastuzumab-containing treatment arms compared with control AC-T adjuvant chemotherapy alone. Outcomes are summarized in the Data Supplement and reported elsewhere.18,20

BCIRG-005 clinical trial (n = 3,298) is a randomized study of concurrent (taxotere, adriamycin, and cyclophosphamide) or sequential (AC-T) adjuvant anthracycline-containing chemotherapy in patients with HER2-normal (nonamplified) stage II and III breast cancer who were accrued from August 2000 to February 2003. This trial demonstrated that sequential and combination regimens that incorporated three drugs were equally efficacious but differed significantly in toxicity profile. Clinical outcomes are summarized in the Data Supplement, and trial details are reported elsewhere.18,20

BCIRG-007 trial (n = 263), a randomized phase III trial of docetaxel and trastuzumab compared with docetaxel, carboplatin, and trastuzumab in women with HER2-amplified metastatic breast cancer, was screened for HER2 status by PCR concurrently with BCIRG-005 and BCIRG-006. Data for HER2 gene amplification and expression are included in the current study; however, outcome information is not included as this trial had no control, nontrastuzumab treatment arm (Data Supplement).

Laboratory Methods

HER2 gene amplification status was determined by using FISH as described in the Data Supplement. Patients whose breast cancers were HER2 amplified—HER2-to-chromosome 17 centromere (CEP17) FISH ratio $\geq 2.0$—without regard to the average HER2 gene copy number as approved by the FDA met an eligibility criterion for BCIRG-006 and BCIRG-007, whereas those whose breast cancers were HER2 nonamplified by FDA-approved criteria met the eligibility criterion for BCIRG-005 (Fig 1). HER2 protein expression was evaluated in a blinded fashion by using the HercepTest (DAKO, Carpenteria, CA) immunohistochemical (IHC) assay (Data Supplement); however, only FISH was used for enrollment.

Breast cancers screened for enrollment into these BCIRG/TRIO trials were simultaneously screened for all three clinical trials: BCIRG-005, BCIRG-006, and BCIRG-007. As personnel in central laboratories had no knowledge of which cases were potential participants for any of the studies, all screened cases were handled in the same fashion without any distinction related to trial design. As previously described,21 only 5% of these specimens had prior assessment for HER2 status by FISH in local laboratories, whereas approximately 60% had been previously assessed by some HER2 IHC assay. Because of a relatively high false-positive rate (22%) among outside IHC+ cases, outside IHC assays were not considered sufficiently accurate for accrual to or exclusion from any of the trials.21 For current comparisons of FISH to IHC, these cases were all analyzed in the same fashion as they were initially processed; as such, we have no reference to these specific trials for this particular trial.

We consider this the most appropriate way to avoid introducing bias into the comparison of HER2 gene amplification by FISH with HER2 protein expression by IHC.

(continued) the five groups. (A) Breast cancers with HER2-to-chromosome 17 centromere (CEP17) ratios $\geq 2.0$ are divided in two groups, one with an average HER2 gene copy number per tumor cell $\geq 4.0$ in situ hybridization (ISH) positive; our group 1) and one with an average HER2 gene copy number per tumor cell $< 4.0$ (ISH positive; our group 2).

Breast cancers with HER2-to-CEP17 ratios $< 2.0$ are separated into three additional groups: one with average HER2 gene copy number per tumor cell $\geq 6.0$ (ISH positive; our group 3), another with average HER2 gene copy number per tumor cell $\geq 4.0$ but $< 6.0$ (ISH equivocal; our group 4), and one with breast cancers that contained an average HER2 gene copy number per tumor cell $< 4.0$ (ISH negative; our group 5). Therefore, according to the ASCO-CAP guidelines,7,18 breast cancers in groups 1, 2, and 3 are interpreted as ISH positive, group 4 as ISH equivocal, and group 5 as ISH negative. (B-M) ASCO-CAP guidelines algorithm ISH groups compared with observed HER2 gene amplification status by FISH and HER2 protein expression status by IHC staining using the DAKO HercepTest IHC assay. ASCO-CAP guidelines algorithm identification of subversions by HER2 FISH ratios and average HER2 gene copy number into group 1 is categorized as ISH positive, with results as illustrated in panels B (FISH) and C (IHC); group 2 is also categorized as ISH positive, but with contradictory results as illustrated in panels D and E (FISH and IHC), group 3 is categorized as ISH equivocal, but with contradictory results as illustrated in panels F (ISH) and G (IHC); and group 4 is categorized as ISH negative, with confirmatory results as illustrated in panels J (FISH) and K (IHC); (B) ASCO-CAP group 1 breast cancer with HER2 gene amplification by FISH, consistent with the ASCO-CAP guidelines designation of ISH positive (and Breast Cancer International Research Group [BCIRG] designation of HER2 amplified). Average HER2 gene copy number for this case was 16.85 copies per tumor cell, and the CEP17 copy number per cell was 2.28 with a HER2-to-CEP17 FISH ratio of 7.38. HER2 signals are sufficiently numerous and are not captured in a single plane of focus in this photomicrograph so that some appear out of focus. Computer enhancement was not used for any image (BCIRG01681, original photomicrograph at 1,000×). (C) ASCO-CAP group 1 breast cancer case with HER2 protein overexpression, IHC3+ by the HercepTest IHC assay (BCIRG01706, original magnification, ×400). (D) ASCO-CAP group 2 breast cancer. Average HER2 gene copy number for this breast cancer was 3.75 copies per tumor cell, with a CEP17 copy number per cell of 2.08. This breast cancer was reported as ISH positive with an HER2-to-CEP17 FISH ratio of 1.89. The patient was accrued to the BCIRG-005 trial (n = 263), a randomized phase III trial of docetaxel and trastuzumab compared with docetaxel, carboplatin, and trastuzumab in women with HER2-amplified metastatic breast cancer, was screened for HER2 status by PCR concurrently with BCIRG-005 and BCIRG-006.
Interpretation of FISH Assays According to ASCO-CAP Guidelines

We re-evaluated HER2 status of all samples for the current study by using FISH according to the new ASCO-CAP guidelines, which separates in situ hybridization (ISH) into five groups (Fig 2). Three of these groups identify breast cancers that are ISH positive, one ISH equivocal, and one ISH negative. Breast cancers with HER2-to-CEP17 ratios of ≥ 4.0 are divided into two groups, one with an average HER2 gene copy number of ≥ 4.0/tumor cell (our group 1) and one with an average HER2 gene copy number of < 4.0/tumor cell (our group 2). Breast cancers with HER2-to-CEP17 ratios of < 2.0 are divided into three additional groups: one with average HER2 gene copy number of ≥ 6.0/tumor cell (our group 3), which is also classified as ISH positive; another with average HER2 gene copy number of ≥ 4.0 but < 6.0/tumor cell (our group 4), which is classified as ISH equivocal; and one with breast cancers that contain an average HER2 gene copy number of < 4.0/tumor cell (our group 5), which is classified as ISH negative. According to the newly proposed ASCO-CAP guidelines, breast cancers in groups 1, 2, and 3 are interpreted as ISH positive, group 4 as ISH equivocal, and group 5 as ISH negative (Fig 2).

Statistical Methods

Standard statistical methods (Data Supplement) were used to assess significance for associations between ASCO-CAP FISH groups and HER2 protein expression (Friedman tests and χ² tests) and clinical outcomes (log-rank tests) in BCIRG-00535, BCIRG-006, and BCIRG-007—and reclassified all screened cases into five groups according to the new ASCO-CAP guidelines (Table 1 and Fig 2).

The distribution by ASCO-CAP ISH group among the 10,468 patients whose breast cancers were successfully screened for enrollment into the three BCIRG/TRIO trials demonstrates that 40.8% were in group 1, 0.7% in group 2, 0.5% in group 3, 4.1% in group 4, and 53.9% in group 5 (Table 1 and Fig 3). A similar distribution was observed among randomly assigned patients whose cancers had FISH assay results available for analysis (Table 1) as well as those randomly assigned whose breast cancers were also evaluated by the HercepTest for HER2 protein expression (Table 1).

As expected, there was a significant association between increasing HER2 FISH ratios and increasing IHC scores among those breast cancers for which both an HER2 FISH assessment and an HER2 protein expression assessment by HercepTest IHC assay were available (P < .0001; Table 2). Similarly, an association was also observed between increasing average HER2 gene copy number per tumor cell and increasing IHC scores (P < .0001; Table 2). Assessment of HER2 gene amplification status typically involves an evaluation of both average HER2 gene copy number per tumor cell and HER2-to-CEP17 ratio. The new ASCO-CAP guidelines have formalized this evaluation to create five different groups (Table 1 and Fig 2), which we evaluated by group to determine if HER2 protein—either low expression or overexpression—is associated with each ASCO-CAP FISH group (Table 2).

HER2 Protein Expression by IHC in Each ASCO-CAP FISH Group

We determined whether HER2 ISH-positive breast cancers, categorized by the new ASCO-CAP guidelines as groups 1, 2, and 3, were correlated with HER2 protein overexpression or, alternatively, low expression. As described in the Data Supplement, we found that only breast cancers in group 1 (FISH ratio ≥ 2.0, average HER2 copy number/cell ≥ 4.0) were significantly associated with HER2 overexpression (IHC3+), with 75% of these showing either IHC2+ (28%) or IHC3+ (47.3%) immunostaining (P < .0001; Table 2). In contrast, breast cancers from group 2 (FISH ratio ≥ 2.0, average HER2 copy number/cell < 4.0) were associated with low HER2 expression, not overexpression (P = .007), as > 90% showed either IHC0 or IHC1+ immunostaining (Table 2), whereas breast cancers in group 3 (FISH ratio < 2.0, average HER2 copy number/cell ≥ 6.0) were not significantly (P = .3881) associated with either
overexpression or low expression. Breast cancers in ASCO-CAP ISH groups 4 and 5—ISH equivocal and ISH negative, respectively—were also significantly associated with low HER2 expression (both \( P < .0001 \); Table 2).

Breast cancers of group 3 (FISH ratio \( < 2.0 \), average HER2 copy number/cell \( \geq 6.0 \)) were composed of two different groups of breast cancers, a substantial majority (76%) of which were associated with low HER2 expression, whereas a minority (Data Supplement, Table S1 and Fig S2) showed HER2 overexpression.

**Clinical Outcomes by ASCO-CAP ISH Groups**

Because HER2 amplification is a known adverse prognostic marker for shorter DFS and OS and predictive of improved outcomes with trastuzumab therapy, we used these outcomes to determine whether ASCO-CAP FISH groups were associated with particular end points, as expected for either HER2-positive disease or HER2-negative disease. Because the natural history of HER2 gene amplification and overexpression in patients with breast cancer is associated with worse DFS and OS in the absence of HER2-targeted therapy, \( 2-5,20,29,30 \) we have used these clinical outcomes to support the assignment of the various FISH groups as either amplified or not amplified as summarized below.

ASCO-CAP group 1 (ISH positive), HER2-to-CEP17 ratio \( \geq 2.0 \) and average HER2 copy number \( \geq 4.0 \) per tumor cell. As expected, those patients whose breast cancers were HER2 amplified, with HER2 FISH ratios of \( \geq 2.0 \) and average HER2 copy

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**Fig 3.** Distribution of average human epidermal growth factor receptor 2 gene (HER2) copy number and HER2 FISH ratios among breast cancers successfully screened for enrollment into Breast Cancer International Research Group trials from 2000 to 2004. (A) Plot of average HER2 gene copy number per tumor cell nucleus from lowest to highest, with cases identified according to the ASCO and College of American Pathologists (ASCO-CAP) guidelines as groups 1 (blue), 2 (purple), 3 (green), 4 (orange), and 5 (yellow; \( N = 10,468 \)). (B) Plot of HER2 FISH ratios from lowest to highest, as in panel A, with identification of ASCO-CAP groups 1 (blue), 2 (purple), 3 (green), 4 (red), and 5 (yellow; \( N = 10,468 \)).
number of ≥ 4.0, had improved DFS and OS when treated with trastuzumab compared with those treated with conventional (AC-T) chemotherapy alone (n = 3,109; DFS: HR, 0.71; 95% CI, 0.60 to 0.83; P < .0001; and OS: HR, 0.69; 95% CI, 0.55 to 0.85; P = .0006; Tables 3 and 4).

**ASCO-CAP group 2 (ISH positive), HER2-to-CEP17 ratio ≥ 2.0 and average HER2 copy number < 4.0.** Among patients who were randomly assigned to BCIRG-006 trial of adjuvant trastuzumab whose breast cancers had an HER2 FISH ratio of ≥ 2.0 but average HER2 copy number of < 4.0/tumor cell, there was no apparent benefit from trastuzumab therapy, either in terms of DFS (n = 46; HR, 1.10; 95% CI, 0.31 to 3.89; P = .886) or OS (HR, 3.15; 95% CI, 0.35 to 28.63; P = .284; Tables 3 and 4).

**ASCO-CAP group 3 (ISH positive), HER2-to-CEP17 ratio < 2.0 and average HER2 copy number ≥ 6.0.** Overall, patients with breast cancer in this FISH group who were accrued to BCIRG-006 had a worse DFS (HR, 2.50; P = .0252) and OS (HR, 2.35; P = .0885; Tables 3 and 4) than did the comparator group, group 5. However, during central laboratory FISH screening, patients whose breast cancers had HER2 ratios of < 2.0 and average HER2 copy numbers of ≥ 6.0/tumor cell were considered to consist of a minority of HER2-amplified breast cancers within a majority pool of HER2-nonamplified breast cancers. These cases were distinguished from one another by additional analyses.²¹,²⁶,³¹,³² (Data Supplement). Most patients in this HER2 FISH group were accrued to BCIRG-005 as not amplified, whereas few were accrued to BCIRG-006 through protocol amendment as amplified. This approach with separation into two subgroups is supported by HER2 IHC assay results (Data Supplement). Although we had divided group 3 breast cancers into two different subgroups—one eligible for BCIRG-005 with an average of 7.43 HER2 gene/tumor cell and the other eligible for BCIRG-006 with an average of 16.38 HER2 gene/tumor cell—we considered the small numbers insufficient for definitive evaluation of this group in either BCIRG-005 or BCIRG-006.

**ASCO-CAP group 4 (ISH equivocal), HER2-to-CEP17 ratio < 2.0 and average HER2 copy number ≥ 4.0 and < 6.0/tumor cell.** Because patients with breast cancers that had a ratio of < 2.0 were considered HER2 not amplified, these patients were accrued to the BCIRG-005 trial of sequential (AC-T) or concurrent (taxotere, adriamycin, and cyclophosphamide) chemotherapy.²⁹ Outcomes among these 176 patients did not differ significantly from outcomes in group 5 (DFS: HR, 0.923;
New ASCO-CAP Guidelines for HER2 Testing by FISH in Breast Cancer

95% CI, 0.70 to 1.22; P = 0.58; and OS: HR, 0.88; 95% CI, 0.61 to 1.27; P = 0.49; Tables 3 and 4).

ASCO-CAP group 5 (ISH negative), HER2-to-CEP17 ratio < 2.0 and average HER2 copy number < 4.0/tumor cell. HER2 status by FISH for these patients with breast cancer was considered HER2 not amplified or ISH negative and served as the baseline comparison group for DFS and OS in the BCIRG-005 trial.

### DISCUSSION

The most recent ASCO-CAP guidelines have again redefined HER2 gene amplification as determined by ISH in a fashion that is different from prior definitions, particularly the FDA-approved package inserts for HER2 FISH companion diagnostic assays, which includes criteria used for BCIRG/TRIO clinical trials, as well as prior 2007 ASCO-CAP guidelines. Originally, HER2 gene amplification was assessed by Southern blot using hybridization of a radiolabeled HER2 gene probe compared with hybridization of a control gene for a control probe, for example, arginase (ARG1), myeloperoxidase (MPO), or TP53, as an internal control for amplification. A ratio between HER2 and control signals ≥ 2.0 was evaluated as amplification. Subsequently, gene amplification was assessed by FISH using either CEP17 or another gene on the same chromosome as an internal control, again with a ratio of ≥ 2.0 being considered as evidence for HER2 amplification. Therefore, similar strategies have been used over a 30-year period to assess breast cancers as either amplified or not amplified. These criteria were used for enrollment in all major trials of trastuzumab, lapatinib, and, more recently, pertuzumab and trastuzumab emtansine, which demonstrated a clinical benefit for HER2-targeted therapies.

ASCO-CAP guidelines changed the HER2-to-CEP17 ratio used for amplification from ≥ 2.0 to ≥ 2.2 in 2007, then changed the ratio back to ≥ 2.0 in 2013 and 2014 with the addition of formalized categories using average HER2 copy numbers per tumor cell. Because these new criteria for amplification by ISH are likely to select somewhat different patient populations for HER2-targeted therapies, we retrospectively re-evaluated these issues with breast cancers that had annotated long-term clinical outcomes from our clinical trials. Because HER2 amplification is accepted as directly associated with protein overexpression, a worse DFS and OS in the absence of HER2-targeted therapy, and with improved outcomes with HER2-targeted therapy, we used these as criteria for assessment of each newly defined ASCO-CAP group (Table 5). In these analyses, most patients experienced no change in HER2 amplification status as determined by FISH, as ASCO-CAP groups 1 and 5 represent the vast majority of patients (approximately 95%) and because the status as amplified (group 1) and not amplified (group 5) is not changed by the new guidelines (Table 5). Although we find only a small minority of patients (approximately

### Table 3. Comparison of HER2 Ratio and Average HER2 Gene Copy Number and ASCO-CAP Groupings With Clinical Outcomes in BCIRG-005

<table>
<thead>
<tr>
<th>HER2 FISH (HER2/CEP17) Ratio</th>
<th>HER2 Copies per Cell</th>
<th>No. of Subjects</th>
<th>DFS, No. of Events</th>
<th>OS, No. of Events</th>
<th>DFS HR (95% CI) and P for Log-Rank Test*</th>
<th>OS HR (95% CI) and P for Log-Rank Test*</th>
<th>ASCO-CAP FISH Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 2.0</td>
<td>&lt; 4.0</td>
<td>3,079</td>
<td>971</td>
<td>606</td>
<td>1.0 (reference)</td>
<td>1.0 (reference)</td>
<td>Group 5</td>
</tr>
<tr>
<td>4.0-6.0</td>
<td>4.01-6.0</td>
<td>176</td>
<td>51</td>
<td>30</td>
<td>0.923 (0.697 to 1.224)</td>
<td>0.878 (0.609 to 1.267)</td>
<td>Group 4</td>
</tr>
<tr>
<td>≥ 6</td>
<td>6</td>
<td>11</td>
<td>6</td>
<td>4</td>
<td>2.502 (1.121 to 5.583)</td>
<td>2.351 (0.879 to 6.284)</td>
<td>Group 3</td>
</tr>
</tbody>
</table>

NOTE. The hazard ratios are for each ASCO group compared with ASCO Group 5 taken as the reference. There were too few patients accrued to BCIRG-005 with a HER2 FISH ratio ≤ 2.0 for analysis of DFS or OS.

**Table 4. Comparison of HER2 Ratio and Average HER2 Gene Copy Number and ASCO-CAP Groupings With Clinical Outcomes in BCIRG-006**

<table>
<thead>
<tr>
<th>HER2 FISH (HER2/CEP17) Ratio</th>
<th>HER2 Copies per Cell</th>
<th>No. of Subjects</th>
<th>DFS, No. of Events/No. of Subjects</th>
<th>DFS Trastuzumab, No. of Subjects</th>
<th>DFS, HR (95% CI)*</th>
<th>DFS P for Log-Rank Test*</th>
<th>OS Control</th>
<th>OS Trastuzumab</th>
<th>OS, HR (95% CI)*</th>
<th>OS P for Log-Rank Test*</th>
<th>ASCO-CAP FISH Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 2.0</td>
<td>&lt; 4.0</td>
<td>46</td>
<td>4/18</td>
<td>6/28</td>
<td>1.10 (0.31 to 3.89)</td>
<td>.8860 2/18</td>
<td>4/28</td>
<td>138/1,031</td>
<td>202/2,078</td>
<td>3.15 (0.35 to 29.63)</td>
<td>.2839 Group 2</td>
</tr>
<tr>
<td>≥ 4</td>
<td>3,109</td>
<td>251/1,031</td>
<td>391/2,078</td>
<td>1.0 (0.60 to 0.93)</td>
<td>.0001</td>
<td>202/2,078</td>
<td>0.69 (0.55 to 0.95)</td>
<td>.0006 Group 1</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Total 3,155

NOTE. The HRs are for trastuzumab treatment arms compared with control chemotherapy-only arm. There were too few patients (n = 5) accrued to BCIRG-006 with a HER2 FISH ratio < 2.0 and ≥ 4.0 average HER2 gene copy number/tumor cell for analysis of the HR.

Abbreviations: BCIRG, Breast Cancer International Research Group; CAP, College of American Pathologists; DFS, disease-free survival; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; OS, overall survival.

*Trastuzumab-containing treatment arms compared with control (chemotherapy alone) treatment arm.
Table 5. Comparison of FISH Groups, FDA Guidelines Status, and ASCO-CAP Guidelines Status, and Associations With Outcomes in BCIRG Clinical Trials

<table>
<thead>
<tr>
<th>FISH Group</th>
<th>Frequency, %</th>
<th>FDA Status†</th>
<th>ASCO-CAP Guidelines</th>
<th>HER2 Protein Expression</th>
<th>Prognosis (BCIRG-005 trial)</th>
<th>Response to HER2-Targeted Therapy (BCIRG-006)</th>
<th>BCIRG/TRIO Study Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 2.0, ≥ 4.0</td>
<td>40.8</td>
<td>Amplified</td>
<td>ISH positive</td>
<td>HER2 overexpression (P &lt; .0001; IHC3+)</td>
<td>Not included in trial</td>
<td>Significantly improved outcomes</td>
<td>HER2 amplified</td>
</tr>
<tr>
<td>≥ 2.0, &lt; 4.0</td>
<td>0.7</td>
<td>Amplified</td>
<td>ISH positive</td>
<td>HER2 low expression (P &lt; .0001; IHC0/1+)</td>
<td>Not included in trial</td>
<td>No significant benefit</td>
<td>HER2 not amplified</td>
</tr>
<tr>
<td>&lt; 2.0, ≥ 6.0</td>
<td>0.5</td>
<td>Not amplified</td>
<td>ISH positive</td>
<td>Combination of HER2 low and overexpression</td>
<td>Indeterminate mixed category</td>
<td>Indeterminate, mixed category</td>
<td>Mixed HER2 not amplified and amplified, on the basis of expression</td>
</tr>
<tr>
<td>&lt; 2.0, ≥ 4.0, &lt; 6.0</td>
<td>4.1</td>
<td>Not amplified</td>
<td>ISH equivocal</td>
<td>HER2 low expression (P &lt; .0001; IHC0/1+)</td>
<td>Not associated with worse outcomes</td>
<td>Not included in trial</td>
<td>HER2 not amplified</td>
</tr>
<tr>
<td>&lt; 2.0, &lt; 4.0</td>
<td>53.9</td>
<td>Not amplified</td>
<td>ISH negative</td>
<td>HER2 low expression (P &lt; .0001; IHC0/1+)</td>
<td>Not associated with worse outcomes</td>
<td>Not included in trial</td>
<td>HER2 not amplified</td>
</tr>
</tbody>
</table>

Abbreviations: BCIRG, Breast Cancer International Research Group; CAP, College of American Pathologists; FDA, US Food and Drug Administration; FISH, fluorescent in situ hybridization; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, in situ hybridization; TRIO, Translational Research in Oncology.

*Frequencies are based on screened population in Table 1.
†FDA HER2 status is based on the 1997 HER2 INFORM-HER assay approval and the 2002 FDA package insert related to the HER2 PathVysion FISH assay (Abbott Laboratories).
New ASCO-CAP Guidelines for HER2 Testing by FISH in Breast Cancer

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AUTHORS’ DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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New ASCO-CAP Guidelines for HER2 Testing by FISH in Breast Cancer

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

HER2 Gene Amplification Testing by Fluorescent In Situ Hybridization (FISH): Comparison of the ASCO-College of American Pathologists Guidelines With FISH Scores Used for Enrollment in Breast Cancer International Research Group Clinical Trials

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