

# Controversies in *HER2* Oncogene Testing: What Constitutes a True Positive Result in Patients With Breast Cancer?

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## Abstract

*HER2* gene amplification is directly related to *HER2* protein overexpression in human breast cancers. This somatically acquired genetic alteration is associated with shorter disease-free and overall survival of patients in the absence of *HER2*-targeted therapy. Because *HER2*-targeted therapies have significantly improved outcomes for patients whose cancers have this alteration, accurate assessment of the alteration with companion diagnostics has become critically important. US Food and Drug Administration (FDA)-approved companion diagnostics assess either *HER2* gene amplification using fluorescence in situ hybridization (FISH) or *HER2* protein overexpression using immunohistochemistry (IHC) assays. In an effort to standardize these evaluations of *HER2* status, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have convened committees to establish guidelines for evaluation of *HER2* status. Although results with *HER2* IHC assays have been more problematic, our focus in this perspective is an overview of current issues related to *HER2* assessment by FISH. Current ASCO-CAP guidelines for *HER2* FISH assay interpretation designate 5 different groups according to *HER2* FISH ratio and average *HER2* gene copy number per tumor cell. These ASCO-CAP FISH groups are "group 1," designated in situ hybridization [ISH]-positive, which has a *HER2*-to-chromosome 17 centromere (CEP17) ratio  $\geq 2.0$  and an average *HER2* gene copy number per tumor cell  $\geq 4.0$ ; FISH "group 2," also currently designated as "ISH-positive," which has cancer cells with *HER2*-to-CEP17 ratio  $\geq 2.0$  but an average *HER2* gene copy number per tumor cell  $< 4.0$ ; FISH "group 3," also currently designated as "ISH-positive," has cancer cells with *HER2*-to-CEP17 ratio  $< 2.0$  and an average *HER2* gene copy number per tumor cell  $\geq 6.0$ ; FISH "group 4," currently designated as "ISH-equivocal," which has cancer cells with *HER2*-to-CEP17 ratio  $< 2.0$  and an average *HER2* gene copy number per tumor cell  $\geq 4.0$  and  $< 6.0$ ; FISH "group 5," designated as ISH-negative, which has cancer cells with *HER2*-to-CEP17 ratio  $< 2.0$  and an average *HER2* gene copy number per tumor cell  $< 4.0$ . At the time when these guidelines were published, there were no studies using this interpretative strategy and, therefore, no available data related to prevalence rates of each FISH group, correlation of each FISH group with *HER2* protein expression, or correlation of each group with clinical outcomes, either with or without *HER2*-targeted therapies. We and others have assessed these prevalence rates and correlations. These findings are summarized in this overview. *AJHO*. 2017;13(9):18-28

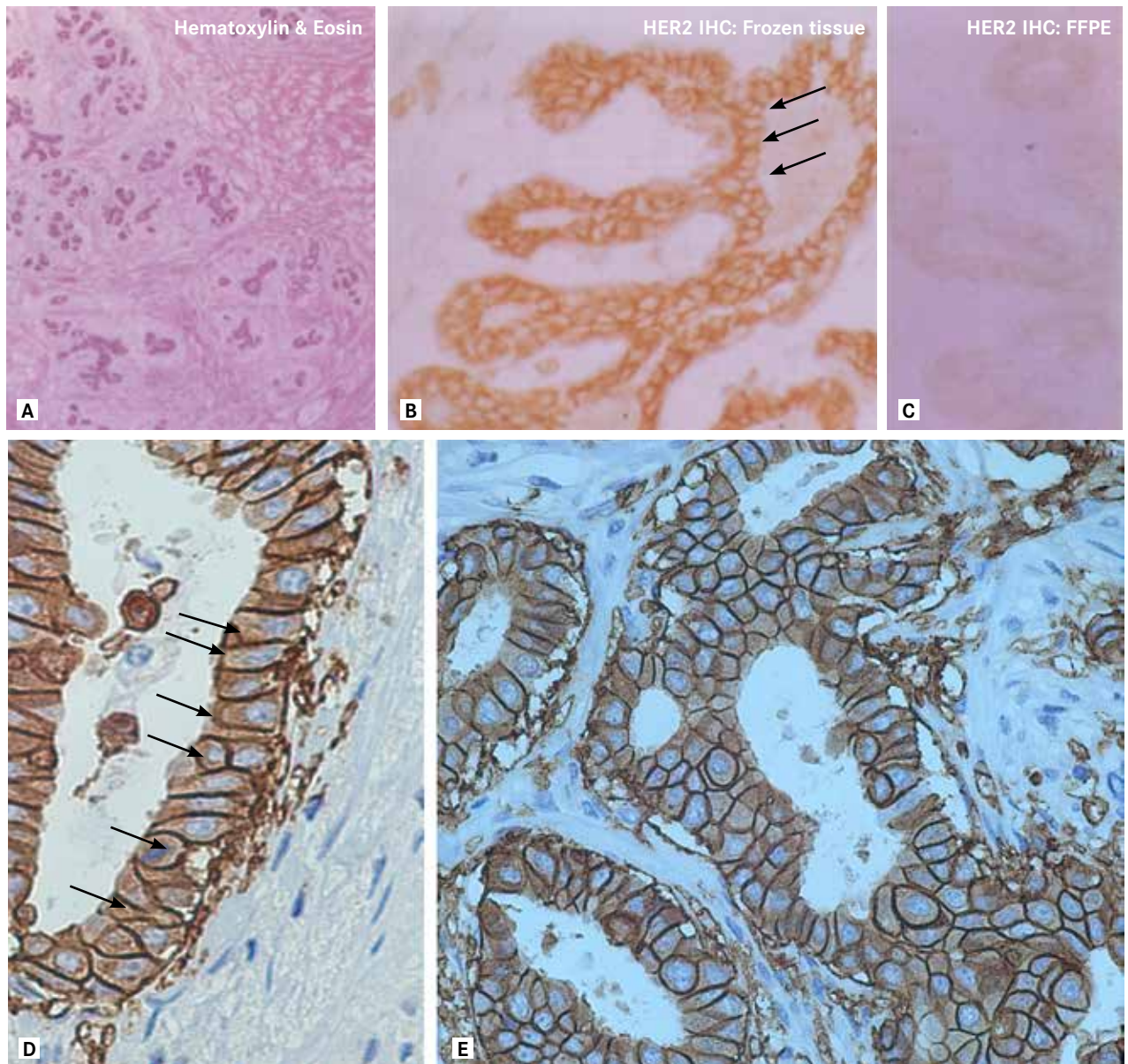
## Introduction

The human epidermal growth factor receptor type 2 gene (*HER2*), also known as *ERBB2*, is amplified and overexpressed in approximately 20% of unselected breast cancers.<sup>1,2</sup> The gene encodes a membrane receptor protein expressed at relatively low levels on lateral and basal surfaces of virtually all normal epithelial cells<sup>3</sup> including normal breast epithelium (Figures 1A, 1B, 1C). Amplification of this gene leads to high levels of protein expression, referred to as overexpression (Figures 1D, 1E), which is associated with shorter disease-free survival (DFS) and overall survival (OS) in patients with breast cancer.<sup>2,4</sup> *HER2* overexpression has now been effectively targeted for therapeutic intervention using humanized monoclonal antibodies to the extracellular domain (trastuzumab,<sup>5,7</sup> pertuzumab,<sup>8</sup> and T-DM1<sup>9</sup>) and small molecular inhibitors to the intracellular kinase domain (lapatinib,<sup>10</sup> neratinib<sup>11</sup>). These *HER2*-targeted agents have substantially improved both DFS and OS in both the adjuvant and metastatic settings for *HER2*-positive breast cancer patients.

However, to achieve these benefits, correct recognition of which breast cancers have the alteration and which do not is critically important for appropriate patient selection. The expense and potential adverse effects of these therapeutics should preclude their use in patients who are not likely to benefit. Equally problematic is the possibility of withholding an effective therapy from someone who may potentially benefit. Given this, the accuracy of the testing methodology as well as the scoring criteria used for reporting a cancer as *HER2*-positive or *HER2*-negative is of paramount importance.

The 2 most common modalities used for testing breast cancer specimens for the presence or absence of the *HER2* alteration are immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). Based on the variabilities

**FIGURE 1. Expression of HER2 Protein in Normal Breast Epithelium With Normal Diploid *HER2* Gene Copy Number and in Breast Adenocarcinomas With *HER2* Gene Amplification.**



A) Histology of normal breast ducts and lobular epithelium in frozen tissue section from reduction mammoplasty specimen. Hematoxylin-and-eosin stained tissue section. Original magnification: 150x.

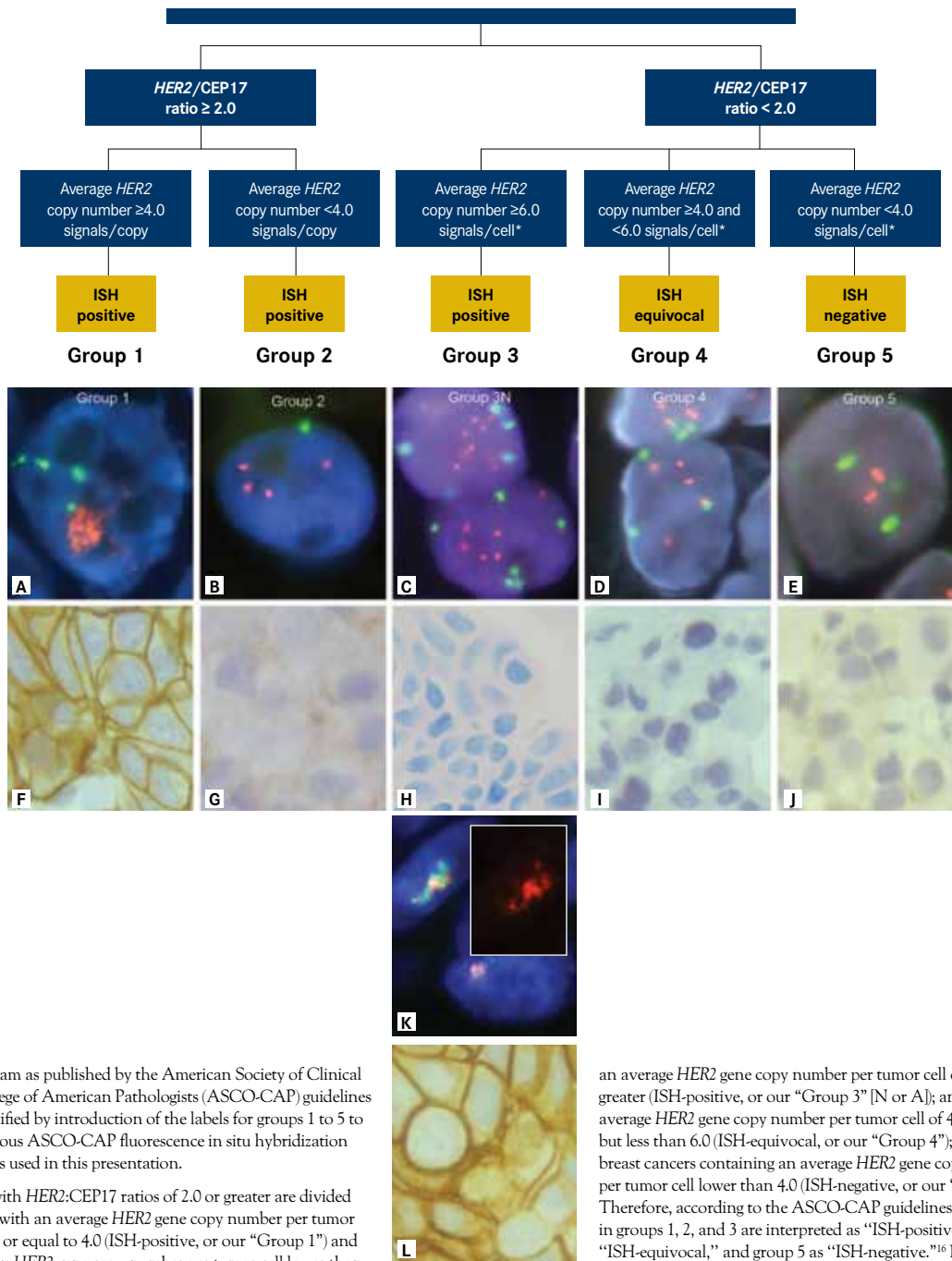
B) Serial section from same normal breast after immunohistochemical staining for HER2 protein at higher magnification showing lateral and basal membranes with HER2 protein by IHC and lumen membrane surfaces lacking HER2 protein expression in normal breast ducts and terminal ducts. HER2 by IHC in frozen section. Original magnification: 1450x.

C) Normal breast tissue processed by formalin-fixation and paraffin-embedding (FFPE), followed by immunohistochemical staining for HER2 protein as in B. Lack of detectable membrane staining using the same IHC antibody and methods used in B. HER2 by IHC in FFPE tissue. Original magnification: 1450x.

D) Luminal membranes of breast adenocarcinomas lack HER2 protein immunostaining, and E) *HER2*-amplified breast adenocarcinoma demonstrates strong membrane immunostaining (IHC 3+) of lateral and basal membranes in an FFPE breast cancer. HerpTest for HER2 by IHC. Original magnification: 400x. BETH2369, HER2 IHC by Herceptest = 3+, HER2 by FISH: FISH ratio = 11.70/1.45 = 8.07.

A, B, and C reproduced with permission from Press MF et al, 1990,<sup>3</sup> with addition of text modifications. FISH indicates fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

**FIGURE 2. Schematic Diagram of the “Algorithm for Evaluation of Human Epidermal Growth Factor Receptor 2 (HER2) Gene Amplification by In Situ Hybridization (ISH) Assay of the Invasive Component of a Breast Cancer Specimen Using a Dual-Signal (HER2 gene) Assay (dual-probe ISH)”**



This is the diagram as published by the American Society of Clinical Oncology /College of American Pathologists (ASCO-CAP) guidelines committee, modified by introduction of the labels for groups 1 to 5 to identify the various ASCO-CAP fluorescence in situ hybridization (FISH) categories used in this presentation.

Breast cancers with *HER2:CEP17* ratios of 2.0 or greater are divided into 2 groups: 1 with an average *HER2* gene copy number per tumor cell greater than or equal to 4.0 (ISH-positive, or our “Group 1”) and 1 with an average *HER2* gene copy number per tumor cell lower than 4.0 (ISH-positive, or our “Group 2”). Breast cancers with *HER2:CEP17* ratios lower than 2.0 are separated into 3 additional groups, 1 with

an average *HER2* gene copy number per tumor cell of 6.0 or greater (ISH-positive, or our “Group 3” [N or A]); another with an average *HER2* gene copy number per tumor cell of 4.0 or greater but less than 6.0 (ISH-equivocal, or our “Group 4”); and 1 with breast cancers containing an average *HER2* gene copy number per tumor cell lower than 4.0 (ISH-negative, or our “Group 5”). Therefore, according to the 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.”<sup>16</sup> Fluorescence in situ hybridization (FISH) for *HER2* gene status (A-E, and K) and immunohistochemical staining for HER2 protein status (F-J, and L) are illustrated with cases representing each of the ASCO-CAP

**FIGURE 2. Schematic Diagram of the “Algorithm for Evaluation of Human Epidermal Growth Factor Receptor 2 (*HER2*) Gene Amplification by In Situ Hybridization (ISH) Assay of the Invasive Component of a Breast Cancer Specimen Using a Dual-Signal (*HER2* gene) Assay (dual-probe ISH)” (Continued)**

guidelines for ISH algorithm groups 1 to 5. The ASCO-CAP guidelines’ algorithm ISH groups are compared with observed *HER2* gene amplification status by FISH, and *HER2* protein expression status by immunohistochemical staining with either the Dako HercepTest or our laboratory-developed 10H8 immunohistochemistry (IHC) assay.

A) ASCO-CAP group 1 breast cancer with *HER2* gene amplification by FISH, consistent with the ASCO-CAP guidelines’ designation of “ISH-positive” (and consult practice designation of “*HER2*-amplified”). The average *HER2* gene copy number for this case was 28.8 *HER2* copies per tumor cell, with an average of 4.45 chromosome 17 centromere (CEP17) copies per tumor cell and a *HER2*:CEP17 FISH ratio of 6.47. The *HER2* signals are sufficiently numerous and are not captured in a single plane of focus in this photomicrograph, so they appear here as orange clusters of intranuclear signals. Consultation case number C17984. *HER2* gene (orange) and CEP17 (green) are identified using the Abbott-Molecular PathVysion *HER2* DNA probe kit (Vysis LSI *HER2*-/neu SpectrumOrange/CEP17 SpectrumGreen) FISH assay.

B) ASCO-CAP group 2 breast cancer, previously reported in consultation with a lack of *HER2* gene amplification by FISH, contradicts the ASCO-CAP guidelines’ designation of “ISH-positive.” The average *HER2* gene copy number for this breast cancer was 3.4 copies per tumor cell, with an average of 1.2 CEP17 copies per tumor cell and a *HER2*:CEP17 FISH ratio of 2.8. Consultation case number C20890. *HER2* gene (orange) and CEP17 (green) are identified using the Abbott-Molecular PathVysion *HER2* DNA probe kit (Vysis LSI *HER2*-/neu SpectrumOrange/CEP17 SpectrumGreen) FISH assay.

C) ASCO-CAP group 3 breast cancer, 1 of our “group 3N” cases, was reported to have a lack of *HER2* gene amplification by FISH in consultation, contrary to the ASCO-CAP guidelines’ designation of “ISH-positive.” This breast cancer had an average of 6.6 *HER2* gene copies per tumor cell and an average of 3.9 CEP17 copies, with a *HER2*:CEP17 FISH ratio of 1.69. *HER2* gene (orange) and CEP17 (green) are identified using the Abbott-Molecular PathVysion *HER2* DNA probe kit (Vysis LSI *HER2*-/neu SpectrumOrange/CEP17 SpectrumGreen) FISH assay. Consultation case number C18756.

D) ASCO-CAP group 4 breast cancer, designated as “ISH-equivocal” by ASCO-CAP but reported in our consultation practice as “*HER2*-not-amplified” by FISH. This breast cancer had an average *HER2* gene copy number of 5.3 *HER2* gene copies per tumor cell, an average CEP17 copy number of 3.0 per tumor cell, and therefore had a *HER2*:CEP17 FISH ratio of 1.77. The use of retinoic acid receptor alpha gene (*RARA*) probe as an alternative CEP17 control demonstrated an average of 3.3 *RARA* copies per tumor cell, providing a *HER2*:*RARA* ratio of 1.6. Similarly, using the Smith-Magenis Syndrome (*SMS*) region FISH probe as an alternative control, there were 2.9 copies per tumor cell, providing a *HER2*:*SMS* ratio of 1.8. Consultation case number C18137. *HER2* gene (orange) and CEP17 (green) are identified using the Abbott-Molecular PathVysion *HER2* DNA probe kit (Vysis LSI *HER2*-/neu SpectrumOrange/CEP17 SpectrumGreen) FISH assay.

E) ASCO-CAP group 5 breast cancer, consistent with the guidelines’ designation of “ISH-negative,” which was reported as “*HER2*-not-amplified” by FISH in our consultation practice. The case had an average *HER2* gene copy number of 2.65 per tumor cell, a CEP17 average of 2.05 copies per tumor cell, and a *HER2*:CEP17 ratio of 1.29. Consultation case number C18066. *HER2* gene (orange) and CEP17 (green) are identified using the Abbott-Molecular PathVysion *HER2* DNA probe kit (Vysis LSI *HER2*-/neu SpectrumOrange/CEP17 SpectrumGreen) FISH assay.

F) ASCO-CAP group 1 breast cancer case with *HER2* protein overexpression, IHC 3+, both by the Dako HercepTest (illustrated) and our laboratory-developed 10H8-*HER2* (data not shown) immunohistochemical assays. This breast cancer, corresponding to A above, is an ASCO-CAP FISH group 1 case consistent with the ASCO-CAP guidelines’ designation of “ISH-positive.” Consultation case number C17984.

G) ASCO-CAP group 2 breast cancer, corresponding to the breast cancer in B above, with *HER2* protein expression determined as IHC 1+ with the HercepTest (illustrated) and the 10H8-*HER2* IHC assay (not shown), contradicts the ASCO-CAP guidelines’ designation of “ISH-positive.” Consultation case number C20890.

H) ASCO-CAP group 3 breast cancer with low *HER2* protein expression by the *HER2* 10H8-IHC (IHC 0) immunohistochemical assay. This breast cancer, corresponding to C above, was reported as not amplified, contrary to the ASCO-CAP guidelines’ designation of “ISH-positive.” Consultation case number C18756.

I) ASCO-CAP group 4 breast cancer, corresponding to D above, had low *HER2* protein expression by both the 10H8-IHC *HER2* assay (IHC 0, data not shown) and the Dako HercepTest (IHC 1+), as illustrated. Consultation case number C18137.

J) ASCO-CAP group 5 breast cancer, corresponding to E above, with low *HER2* protein expression by IHC with both the Dako HercepTest (IHC 1+, as illustrated) and 10H8-IHC (IHC 0, data not shown), consistent with the ASCO-CAP guidelines’ designation of “ISH-negative.” Consultation case number C18066.

K) A minority of American Society of Clinical Oncology/College of American Pathologists (ASCO-CAP) group 3 breast cancers, referred to here as “group 3A,” show *HER2* gene amplification and *HER2* protein overexpression. K, an ASCO-CAP group 3 breast cancer, 1 of our group 3A cases, has an average *HER2* gene copy number of 23.2 *HER2* copies per tumor cell and an average CEP17 copy number of 15.75 per tumor cell. Therefore, it has a *HER2* FISH ratio of only 1.47. This illustration with a triple bandpass image shows the composite (blue/orange/green) image with *HER2* gene copies (orange) and CEP17 copies (green) co-localized together in a limited geographic area of tumor cell nuclei (blue). Please note that the *HER2* gene signals (orange) and CEP17 signals (green) are aggregated together in the same limited geographic area of the nucleus, making assessment of individual signals challenging without the aid of single bandpass filters, as illustrated by the inset for the *HER2* gene (orange) signals which are otherwise partially obscured by the CEP17 signals. Abbott-Molecular PathVysion *HER2* DNA probe kit (Vysis LSI *HER2*-/neu SpectrumOrange/CEP17 SpectrumGreen) FISH assay. Consultation case number C20906. FISH with alternative control probes located on chromosome 17, remote from the *HER2* locus, such as retinoic acid receptor alpha gene (*RARA*) in this breast cancer, demonstrated an average of 2.55 *RARA* copies per tumor cell by FISH, providing a *HER2*:*RARA* ratio of 9.1. Similarly, using the *SMS* region FISH probe as an alternative control gene probe, this breast cancer demonstrated 1.85 copies per tumor cell, providing a *HER2*:*SMS* ratio of 12.54. This breast cancer was reported as “*HER2*-amplified” in our consultation practice, consistent with the ASCO-CAP guidelines’ designation of “ISH-positive.”

L) ASCO-CAP group 3 breast cancer, corresponding to our “group 3A” cases, with *HER2* protein overexpression by immunohistochemistry (IHC; IHC 3+ by HercepTest) consistent with the ASCO-CAP guidelines’ designation of “ISH-positive.” Similar results were obtained with our 10H8-IHC assay (IHC 3+, data not shown). Consultation case number C20906 (original magnifications, 1000x [A-E, and K] and 400x [F-J, and L]). A normal immunoglobulin G–negative control was performed for each of the immunohistochemical assays used in F-J and L, which showed a lack of any staining; however, these have not been illustrated.

**Note:** This figure has been modified from Figures 1-3 of a previously published article by Press MF et al, 2016 (citation 21), with permission from the *Archives of Pathology & Laboratory Medicine*. Copyright 2014 and 2016 by the College of American Pathologists.

in accuracy for HER2 testing that have already been previously reported,<sup>12-14</sup> particularly using IHC,<sup>12</sup> the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) convened a panel to standardize approaches to HER2 testing.<sup>13</sup> Subsequently, the ASCO-CAP guidelines panel for HER2 testing was reconvened to modify the initial recommendations.<sup>15,16</sup> While we have already reported on many of the contentious aspects of HER2 testing by IHC,<sup>12</sup> here we summarize some of the issues related to the current ASCO-CAP guidelines for HER2 testing by FISH.<sup>15,16</sup>

### The 2007 ASCO-CAP Guidelines

The primary purposes of the initial ASCO-CAP guidelines for HER2 testing were multifold and aimed at improving accuracy of HER2 testing through standardization of preanalytic tissue-processing procedures (eg, anoxia tissue time, fixative type [formalin] and duration [6-48 hours], and methods of tissue processing), analytical procedures, and postanalytical procedures when testing was performed in a CAP-accredited laboratory environment. This included guidelines for interpretation with algorithms for scoring based on a ratio of the average *HER2* gene copy number-to-average CEP17 copy number per tumor cell. The 3 categories were defined as negative with a ratio of <1.8, equivocal when the ratio is 1.8 to 2.2, and positive when the ratio is >2.2. Prior to that, the US FDA had approved clear criteria for defining HER2-positive disease as cancers with a FISH ratio  $\geq 2.0$  and HER2-negative cancers as those with a ratio <2.0; the criteria included a method for resolution of cases when ratios are within 10% of the 2.0 cut-off—ie, 1.8 to 2.2—without further testing.<sup>4,17</sup> Despite this, the ASCO-CAP guidelines identified a new “equivocal” category and recommended additional assessment for resolution. Of note, these “equivocal” cases represented only 2% of all breast cancers.<sup>12</sup> In the subsequent 2013/2014 ASCO-CAP guidelines, the designation of “equivocal” was retained; however, the definition of what constituted a “HER2-equivocal” breast cancer was modified and the number of cases increased to between 4% and 12%.<sup>18-25</sup>

### The 2013<sup>15</sup>/2014<sup>16</sup> ASCO-CAP Guidelines and Associations with Protein Expression and Clinical Outcomes

According to the new and current ASCO-CAP guidelines for HER2 testing, in situ hybridization (ISH) assay results, including FISH, should now be divided into 5 groups based on a formalized assessment of both average *HER2* gene copy number and *HER2* FISH ratio (Figure 2). Three of these groups define breast cancers that are “ISH-positive,” 1 that is “ISH-equivocal,” and 1 that is “ISH-negative.” Breast cancers with *HER2*-to-CEP17 ratios  $\geq 2.0$  are composed of 2 groups: 1 with an average *HER2* gene copy number  $\geq 4.0$  per tumor cell (our “group 1”) and 1 with an average *HER2* gene copy number <4.0 per tumor cell (our “group 2”). Breast cancers with *HER2*-to-CEP17 ratios <2.0 are composed of 3 additional groups: 1 with average *HER2* gene copy number  $\geq 6.0$  per tumor cell (our “group 3”), which is also classified as “ISH pos-

itive;” another with average *HER2* gene copy number  $\geq 4.0$  but <6.0 signals/tumor cell (our “group 4”), which are then classified as the new “ISH-equivocal” cases; and 1 with breast cancers containing an average *HER2* gene copy number <4.0 signals/tumor cell (our “group 5”), which is classified as “ISH-negative.” According to these ASCO-CAP guidelines,<sup>15,16</sup> breast cancers in groups 1, 2, and 3 are interpreted as “ISH-positive,” group 4 as “ISH-equivocal,” and group 5 as “ISH-negative” (Figure 2).

At the time these guidelines were published, no clinical or demographic data were available using this classification schema, and basic information such as the prevalence of each FISH group in the general breast cancer population was not known. Moreover, data regarding whether these new ASCO-CAP groups correlated with HER2 protein expression or, more importantly, clinical outcomes, were also not available. To better address these questions, we conducted 2 retrospective studies of breast cancer specimens previously characterized for HER2 status in our laboratories: one set was from a cohort of an academic consultation practice,<sup>21</sup> and the other set was from breast cancers screened for entry to Breast Cancer International Research Group (BCIRG)/Translational Research In Oncology (TRIO) clinical trials.<sup>20</sup>

Eligibility requirements for inclusion in the cohort study and BCIRG/TRIO trials are described in detail elsewhere.<sup>20,21</sup> In brief, all consecutive, primary, invasive breast carcinomas submitted to the clinical consultation practice of one of us (MFP) from April 1999 until September 2015 that had both *HER2* gene amplification status determined by FISH and *HER2* protein level determined by IHC were eligible for inclusion in the cohort study of HER2 status by FISH (n = 7526).<sup>21</sup> The study of BCIRG/TRIO clinical trials breast carcinoma samples included primary invasive breast carcinomas from 10,468 patients who were screened for enrollment in either of 2 central laboratories (MFP and GS) for *HER2* gene amplification status determined by FISH as an enrollment criterion for eligibility to 3 different trials: BCIRG-005,<sup>26</sup> BCIRG-006,<sup>7</sup> and BCIRG-007.<sup>27</sup>

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. The BCIRG-006 trial (n = 3222) is a randomized, 3-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.<sup>7</sup> Therapy in the control arm was adjuvant 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 2 experimental arms involved trastuzumab 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

**TABLE 1. Prevalence of Breast Cancer for Each ASCO-CAP *HER2* FISH Group<sup>20,21a</sup>**

| Group  | Description of FISH Category                          | BCIRG/TRIO Trials |           | Consultation Practice |           |
|--------|---|-------------------|-----------|-----------------------|-----------|
|        |   | Number of Cases   | Overall % | Number of Cases       | Overall % |
| 1      | Ratio $\geq 2.0$ ; <i>HER2</i> average $\geq 4.0$     | 4269              | 40.8%     | 1328                  | 17.7%     |
| 2      | Ratio $\geq 2.0$ ; <i>HER2</i> average $< 4.0$        | 71                | 0.7%      | 31                    | 0.4%      |
| 3      | Ratio $< 2.0$ ; <i>HER2</i> average $\geq 6.0$        | 55                | 0.5%      | 48                    | 0.6%      |
| 4      | Ratio $< 2.0$ ; <i>HER2</i> average $\geq 4.0, < 6.0$ | 432               | 4.1%      | 345                   | 4.6%      |
| 5      | Ratio $< 2.0$ ; <i>HER2</i> average $< 4.0$           | 5641              | 53.9%     | 5774                  | 76.7%     |
| Totals |   | 10,468            | 100%      | 7526                  | 100%      |

<sup>a</sup>Reproduced as a combined single table using data from Table 1 in each of the 2 cited studies.

ASCO indicates American Society of Clinical Oncology; BCIRG, Breast Cancer International Research Group; CAP, College of American Pathologists; FISH, fluorescence in situ hybridization; *HER2*, human epidermal growth factor receptor 2; TRIO, Translational Research in Oncology.

trial demonstrated significant improvement in DFS for both trastuzumab-containing treatment arms compared with control AC-T adjuvant chemotherapy alone. BCIRG-005 clinical trial (n = 3298) is a randomized study of concurrent (taxotere, adriamycin, and cyclophosphamide) or sequential (AC-T) adjuvant anthracycline-containing chemotherapy in patients with *HER2*-not-amplified, 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 3 drugs were equally efficacious but differed significantly in toxicity profile.<sup>26,28</sup> The 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 FISH concurrently with BCIRG-005 and BCIRG-006. Data for *HER2* gene amplification and expression are included in the study<sup>20</sup>; however, outcome information is not included as this trial had no control, nontrastuzumab treatment arm.<sup>27</sup>

We performed analyses of prevalence by FISH group, association with *HER2* expression by IHC, and clinical outcomes. 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 or not the new ASCO-CAP FISH testing criteria used to define each of the 5 *HER2* FISH groups are correlated with those characteristics already known to be associated with *HER2* gene amplification, such as *HER2* protein overexpression, poorer clinical outcomes (DFS/OS) in the absence of *HER2*-targeted therapy, and significant improvement in DFS and OS when such patients are treated with *HER2*-targeted therapy.

#### Prevalence of Each ASCO-CAP *HER2* FISH Group Within the Breast Cancer Population

As expected, in both study cohorts the majority of breast cancers had a *HER2* FISH ratio  $< 2.0$  with an average *HER2* gene copy

number  $< 4.0$  (group 5) and the second largest group were those with a *HER2* ratio  $\geq 2.0$ , with an average *HER2* gene copy number  $\geq 4.0$  (group 1) (Table 1). These are the breast cancers traditionally considered “*HER2*-negative” and “*HER2*-positive,” respectively, by FISH assay. Groups 2 and 3 each represented less than 1% of the study population and ASCO-CAP Group 4, the “equivocal” breast cancers, represented 4% to 5% of each study population.<sup>20,21</sup>

#### Association Between Each ASCO-CAP FISH Group With *HER2* Protein Expression Level

Because only ASCO-CAP groups 1 and 5, designated respectively as ISH-positive and ISH-negative, corresponded to the interpretations we assigned in our consultation practice and in our central laboratory for entry to BCIRG/TRIO trials, we also wanted to evaluate association with *HER2* protein expression levels by IHC to determine agreement between ASCO-CAP FISH guidance and protein expression category by IHC. Contrary to the ASCO-CAP designations, we found that ASCO-CAP groups 2 and 4 were significantly associated with low *HER2* protein expression, not overexpression. ASCO-CAP group 3 appeared to be composed of 2 different subgroups: a larger subgroup (our group 3N) *HER2*-negative with low expression, and a smaller subgroup (our group 3A) *HER2*-amplified that had protein overexpression (Tables 2 and Table 3).

#### Association With Clinical Outcomes

Given the fact that the findings with IHC in 3 new ASCO-CAP FISH groups (groups 2, 3, and 4) appeared to contradict the assigned designation of the ASCO-CAP guidelines for *HER2* testing, we evaluated known clinical outcomes in the BCIRG/TRIO clinical trials. These trials have long-term clinical follow-up data available<sup>7,26,28</sup> that allow for a determination of whether or not the new *HER2* guidelines for FISH/ISH testing are predictive of known clinical outcomes and, therefore, clinically useful.

As described above, we found that breast cancers in ASCO-CAP FISH group 1 had *HER2* protein IHC levels

**TABLE 2. American Society of Clinical Oncology/College of American Pathologists (ASCO-CAP) Fluorescence In Situ Hybridization (FISH) Groupings Compared with HER2 Protein by Immunohistochemistry (IHC) Scores<sup>20,21\*</sup>**

| 2A. Academic Consultation Practice Cohort |                    |                                   |              |               |               |               |        |        |                                      |
|---|--------------------|-----------------------------------|--------------|---------------|---------------|---------------|--------|--------|--------------------------------------|
| HER2 Protein Expression by IHC            |                    |                                   |              |               |               |               |        |        |                                      |
| ASCO-CAP FISH Group                       | HER2 / CEP17 Ratio | Average HER2 Copy Number per Cell | IHC 0, n (%) | IHC 1+, n (%) | IHC 2+, n (%) | IHC 3+, n (%) | Totals | P      | Association with Level of Expression |
| Group 1                                   | ≥2.0               | ≥4.0                              | 123 (9.3%)   | 167 (12.6%)   | 427 (32.2%)   | 611 (46%)     | 1328   | <.001  | Overexpression                       |
| Group 2                                   | ≥2.0               | <4.0                              | 13 (41.9%)   | 10 (32.3%)    | 8 (25.8%)     | 0             | 31     | .02    | Low Expression                       |
| Group 3                                   | <2.0               | ≥6.0                              | 15 (31.3%)   | 22 (45.8%)    | 7 (14.6%)     | 4 (8.3%)      | 48     | <.001  | Low Expression                       |
| Group 4                                   | <2.0               | ≥4.0 to <6.0                      | 139 (40.3%)  | 168 (48.7%)   | 35 (10.1%)    | 3 (0.9%)      | 345    | <.001  | Low Expression                       |
| Group 5                                   | <2.0               | <4.0                              | 3599 (62.3%) | 1967 (34%)    | 197 (3.4%)    | 11 (0.2%)     | 5774   | <.001  | Low Expression                       |
| Total                                     |                    |                                   |              |               |               |               | 7526   |        |                                      |
| 2B. BCIRG / TRIO Clinical Trials          |                    |                                   |              |               |               |               |        |        |                                      |
| HER2 Protein Expression by HercepTest IHC |                    |                                   |              |               |               |               |        |        |                                      |
| ASCO-CAP FISH Group                       | HER2 / CEP17 Ratio | Average HER2 Copy Number per Cell | IHC 0, n (%) | IHC 1+, n (%) | IHC 2+, n (%) | IHC 3+, n (%) | Totals | P      | Association with Level of Expression |
| Group 1                                   | ≥2.0               | ≥4.0                              | 240 (11.8%)  | 264 (12.9%)   | 571 (28%)     | 965 (47.3%)   | 2040   | <.0001 | Overexpression                       |
| Group 2                                   | ≥2.0               | <4.0                              | 24 (68.6%)   | 8 (22.9%)     | 3 (8.6%)      | 0 (0%)        | 35     | <.0007 | Low Expression                       |
| Group 3                                   | <2.0               | ≥6.0                              | 5 (55.5%)    | 2 (22.2%)     | 1 (11.1%)     | 1 (11.1%)     | 9      | .388   | Not Significant                      |
| Group 4                                   | <2.0               | ≥4.0 to <6.0                      | 105 (78.4%)  | 21 (15.7%)    | 7 (5.2%)      | 1 (0.7%)      | 134    | <.0001 | Low Expression                       |
| Group 5                                   | <2.0               | <4.0                              | 1988 (94.1%) | 114 (5.4%)    | 10 (0.5%)     | 1 (0.05%)     | 2113   | <.0001 | Low Expression                       |
| Total                                     |                    |                                   |              |               |               |               | 4331   |        |                                      |

\*Reproduced as a combined single table using data from Table 2 and Table 3, respectively, from the cited studies.

BCIRG indicates Breast Cancer International Research Group; FISH, fluorescent in situ hybridization; HER2, human epidermal growth factor receptor 2; TRIO, Translational Research in Oncology.

that correlated with HER2 overexpression (Tables 2A and 2B). Those patients whose cancers were in this group were accrued to the BCIRG-006 trial of adjuvant trastuzumab, and those randomly assigned to a trastuzumab-plus-chemotherapy treatment arm experienced significant improvements in DFS (hazard ratio [HR], 0.71; 95% CI, 0.60-0.83;  $P < .0001$ ) and OS (HR, 0.69; 95% CI, 0.55-0.85;  $P < .0006$ ) compared with similar patients assigned to standard chemotherapy alone.<sup>20</sup>

It is worth noting that approximately 20% of ASCO-CAP FISH group 1 (HER2-amplified) breast cancers were IHC-negative (IHC 0/1+) (Table 2A). We have previously used a variety of approaches to confirm that IHC-negative, HER2-amplified breast cancers are predominantly the result of tissue processing artifacts that impact IHC, but not FISH.<sup>29</sup> For example, we assessed HER2 gene amplification status, HER2 mRNA expression, and HER2 protein expression by western immuno-

blot; assessed HER2 protein expression by frozen section IHC in frozen breast cancer samples; and compared HER2 status with HER2 protein expression by IHC in the corresponding formalin-fixed, paraffin-embedded (FFPE) breast cancers. We found that a significant number of HER2-amplified, overexpressed breast cancers were IHC 0 in the corresponding FFPE tissue sections.<sup>2</sup> Although the percentage of IHC false-negative breast cancers varies depending on tissue processing and IHC assay methods used, this is a recurring observation, not only in our own studies,<sup>2,12,30-32</sup> but in the published literature comparing IHC with FISH.<sup>33-36</sup> As expected, these patients with IHC false-negative, FISH-amplified HER2 status currently do not receive trastuzumab or other anti-HER2 therapy and have been shown to experience a statistically significantly worse distant DFS compared with similarly treated patients with IHC-negative, FISH-not-amplified breast cancers.<sup>32</sup>

**TABLE 3.** Comparison of *HER2* Protein Expression by IHC Scores Among Patients Whose Breast Cancers Were ASCO-CAP Group 3, Separated According to *HER2* Gene Amplification Status as Previously Evaluated in Either a Consultation Practice or in BCIRG Clinical Trials<sup>20,21c</sup>

| Academic Consultation Practice            |                    |                                      |              |               |               |                |          |                    |                                      |
|---|--------------------|--------------------------------------|--------------|---------------|---------------|----------------|----------|--------------------|--------------------------------------|
| HER2 Protein Expression by IHC            |                    |                                      |              |               |               |                |          |                    |                                      |
| ASCO-CAP FISH Group                       | HER2/CEP17 Ratio   | Mean HER2 Copy Number per Cell, ± SD | IHC 0, n (%) | IHC 1+, n (%) | IHC 2+, n (%) | IHC 3+, n (%)  | Totals   | P                  | Association with Level of Expression |
| Group 3A                                  | <2.0               | 12.3 ± 6.6                           | 1 (12.5%)    | 1 (12.5%)     | 2 (25%)       | 4 (50%)        | 8 (100%) | <.001 <sup>a</sup> | Overexpression                       |
| Group 3N                                  | <2.0               | 6.8 ± 0.9                            | 14 (35%)     | 21 (52.5%)    | 5 (12.5%)     | 0 (0%)         | 40       |                    | Low expression                       |
| Total                                     |                    |                                      |              |               |               |                | 48       |                    |                                      |
| BCIRG / TRIO trials                       |                    |                                      |              |               |               |                |          |                    |                                      |
| HER2 Protein Expression by HercepTest IHC |                    |                                      |              |               |               |                |          |                    |                                      |
| ASCO-CAP FISH Group                       | HER2 / CEP17 Ratio | Mean HER2 Copy Number per Cell       | IHC 0, n (%) | IHC 1+, n (%) | IHC 2+, n (%) | IHC 3+, n. (%) | Totals   | P                  | Association with Level of Expression |
| Group 3A                                  | <2.0               | 16.38                                | 1 (17%)      | 0 (0%)        | 3 (50%)       | 2 (33%)        | 6        | .002 <sup>b</sup>  | Overexpression                       |
| Group 3N                                  | <2.0               | 7.43                                 | 8 (42%)      | 9 (47%)       | 2 (11%)       | 0 (0%)         | 19       |                    | Low expression                       |
| Total                                     |                    |                                      |              |               |               |                | 25       |                    |                                      |

ASCO indicates American Society of Clinical Oncology; BCIRG, Breast Cancer International Research Group; CAP, College of American Pathologists; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor 2; TRIO, Translational Research in Oncology.

<sup>a</sup>There is a significant difference between Group 3A and Group 3N in terms of IHC staining, with 75% of Group 3A being IHC 2+/3+ compared with 87.5% of Group 3N being 0/1+ ( $P < .001$ ; Fisher's exact test).

<sup>b</sup>There is a significant difference between Group 3A and Group 3N in terms of IHC staining, with 83% of Group 3A being IHC 2+/3+ compared with 89% of Group 3N being 0/1+ ( $P = .002$ ; Fisher's exact test).

<sup>c</sup>Reproduced as a combined single table using data from Table 3 and Supplemental Table S1, respectively, from each of the cited studies.

Although few breast cancers are in ASCO-CAP FISH group 2, these cases have *HER2* IHC scores indicating low *HER2* protein expression in our clinical consultation practice and BCIRG trials cohort (Tables 2A and 2B). Nevertheless, in BCIRG trials, the majority of these patients were accrued to BCIRG-006 due to our use of the FDA-approved FISH criteria for *HER2* gene amplification (ratio >2.0 without regard for the average *HER2* gene copy number per tumor cell). When applying the new ASCO-CAP FISH guidelines to patients ( $n = 46$ ) randomized to receive adjuvant trastuzumab in BCIRG-006, no significant improvement in either DFS (HR, 1.1; 95% CI, 0.31-3.89;  $P = .89$ ) or OS (HR, 3.15; 95% CI, 0.35-28.63;  $P = .28$ ) was observed when compared with patients randomized to receive standard anthracycline-cyclophosphamide followed by taxane chemotherapy alone.

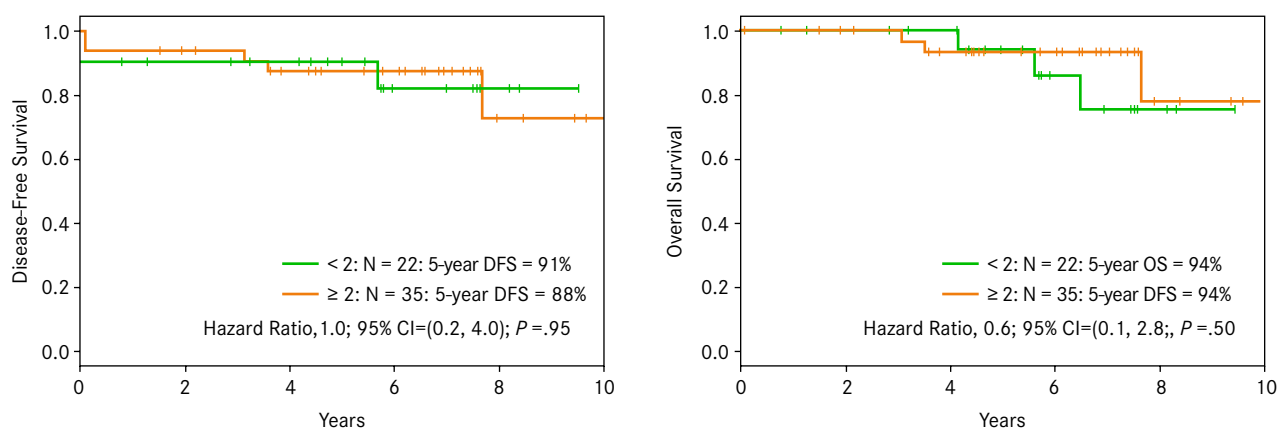
Interestingly, and as expected, the small number of patients in ASCO-CAP group 3 (Table 1) appears to be not a single group of "ISH-positive" breast cancers as specified by the ASCO-CAP guidelines, but a group with at least 2 subgroups, which we have referred to as subgroup 3N (not amplified) and subgroup 3A (amplified). In our consultation practice as well as in the BCIRG clinical trials cohort, the larger 3N subgroup of breast cancers (Table 3) have relatively modest increases in average *HER2* gene copy number per tumor cell of 6.8 and 7.4,

respectively. As described above, these breast cancers are associated with low *HER2* protein expression, while the members of the other, even-less-numerous 3A subgroup have substantially higher average *HER2* gene copy numbers per tumor cell of 12.3 and 16.3, respectively. In our pathology consultation practice as well as in the BCIRG trials cohorts, there is a clear association with *HER2* protein overexpression<sup>20,21</sup> only in the group 3A breast cancers (Table 3). Based on this latter association, we expect the ASCO-CAP FISH 3A subgroup to be associated with worse OS in the absence of *HER2*-targeted therapy, and, conversely, improved DFS and OS with such treatment.

The ASCO-CAP FISH group 4 (*HER2* FISH ratio <2.0; average *HER2* gene copy number per tumor cell  $\geq 4.0$  to <6.0) breast cancers, currently labeled "ISH-equivocal," are associated with low *HER2* protein expression, and, in the absence of trastuzumab treatment, have clinical outcomes that are not significantly worse than those of other patients whose breast cancers lack *HER2* gene amplification and have low *HER2* protein expression (IHC 0/1+).<sup>20</sup> When outcomes of these "ISH-equivocal" patients, our ASCO-CAP FISH group 4, are compared with outcomes of ASCO-CAP FISH group 5 patients, who are those considered *HER2*-not-amplified or "ISH-negative," there is no significant difference in either DFS (HR, 0.92; 95% CI, 0.679-1.224;  $P = .58$ ) or OS (HR, 0.88; 95% CI, 0.609-1.267;



**FIGURE 3. Outcomes for Patients with “HER2 Equivocal” FISH Assay Results Using *HER2*/CEP17 Ratios According to ASCO-CAP Guidelines and After Reassessment Using Alternative Control Probes**



A) Kaplan-Meier DFS according to the *HER2*/alternative chromosome 17 gene ratios in 57 patients with ISH-equivocal *HER2* results using the standard *HER2*-to-CEP17 ratio assessment.

B) Kaplan-Meier OS curves according to the *HER2*/alternative chromosome 17 gene ratios in 57 patients with ISH-equivocal *HER2* results (22 with ratios <2 and 35 with ratios ≥2).

**Note:** Reproduced with permission from Sneige N et al, 2017 (citation 24).

ASCO indicates American Society of Clinical Oncology; CAP, College of American Pathologists; DFS, disease-free survival; FISH, fluorescence in situ hybridization; *HER2*, human epidermal growth factor receptor 2; ISH, in situ hybridization; OS, overall survival.

$P = .49$ ). Similar observations were made by Sneige et al in a study of 3630 patients analyzed by FISH for *HER2* status.<sup>24</sup>

Confirmation of this “*HER2*-not-amplified” status in ASCO-CAP FISH group 4 or “ISH-equivocal” breast cancers can be supported by using alternative control probes in addition to the chromosome 17 centromere control routinely used to calculate the *HER2* FISH ratio.<sup>24,37</sup> However, this approach also has some important shortcomings. The most important pitfall is the lack of recognition that these alternative control genomic regions, especially those on the p-arm of chromosome 17, may show heterozygous deletion, which leads to an increased *HER2*-to-control probe ratio >2.0 based exclusively on heterozygous deletion of the control genomic site rather than true gene amplification<sup>38</sup> (Joshi H, Press MF; unpublished data). An independent study from other investigators has shown that those breast cancer cases converted from “ISH-equivocal” to “ISH-positive” based on the use of p-arm alternative controls for conversion of a *HER2* FISH ratio from <2.0 to >2.0 demonstrate DFS and OS rates similar to those of patients whose cancers continued to have a *HER2* FISH ratio <2.0 after evaluation with these same alternative controls<sup>24</sup> (Figure 3).

### Conclusions

*HER2* gene amplification status is critically important to select the most appropriate patients with breast cancer for *HER2*-targeted

therapies, such as trastuzumab, pertuzumab, TDM1, neratinib or lapatinib treatments. The current ASCO-CAP guidelines for *HER2* testing are widely accepted by pathologists and clinicians for assessment of this status, yet there are inconsistencies with the available data for at least 5% of patients, based on either correlative expression data or actual clinical outcome data or both. These data suggest that these patients should be assigned differently than currently recommended by the most recent guidelines. We have reviewed these inconsistencies and suggested appropriate remedies based on currently available data.

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