HER2 status in advanced or metastatic gastric, esophageal, or gastro-esophageal adenocarcinoma for entry to the TRIO-013/LOGiC trial of lapatinib

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Abbreviations List: CAP, College of American Pathologists; CI, confidence interval; CIRG, Cancer International Research Group; ERBB2, avian erythroblastosis oncogene B2; FISH, fluorescence in situ hybridization; HR, hazard ratio; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; LOGiC, Lapatinib Optimization Study in ErbB2 {HER2} Positive Gastric Cancer; OS, overall survival; PEP, primary efficacy population; PFS, progression-free survival; ToGA, Trastuzumab for Gastric Cancer; TRIO, Translational Research In Oncology; UGI, upper gastro-intestinal

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Lapatinib has become the property of Novartis Pharma AG as of March 1, 2015.

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Abstract (Word count 245, *MCT* 250 word limit)

HER2/*ERBB2* status is used to select patients for HER2-targeted therapy. *HER2*/*ERBB2* amplification/overexpression of upper gastrointestinal (UGI) adenocarcinomas was determined locally or in two central laboratories to select patients for the TRIO-013/LOGiC trial of chemotherapy with or without lapatinib. Patients selected locally had central laboratory confirmation of *HER2*-amplification for inclusion in the primary efficacy population. HER2 was assessed with PathVysion or IQ PharmDx fluorescence in situ hybridization (FISH) and HercepTest immunohistochemistry assays. Associations with outcomes were retrospectively evaluated. Overall, HER2 status was determined in UGI cancers from 4674 patients in a central laboratory for eligibility (1995 cases) and for confirmation of local HER2 results (333 cases). Of 1995 adenocarcinomas screened centrally, 322 (16.1%) had *HER2* amplified disease with 29 (1.5%) showing *HER2* genomic heterogeneity. Men and older patients had higher rates of amplification. Of 545 patients accrued to the trial (gastric, 87.3%; GEJ, 8.3% and esophageal cancer, 4.4%) 487 patients (89%) were centrally confirmed as having *HER2* amplified disease. Concordance between central and local HER2 testing was 83%. Concordance between PathVysion and IQ PharmDx FISH assays was 99% and FISH in the two central laboratories was 95%. Lapatinib-treated Asian participants and those less than 60 years had significant improvement in progression-free survival (PFS), particularly among those whose cancers had 5.01-10.0 and >10.0-fold amplification of *HER2*. In conclusion, *HER2* is commonly amplified in UGI adenocarcinomas with amplification highly correlated to overexpression, and *HER2* amplification levels correlated with PFS. While *HER2* genomic heterogeneity occurs, its prevalence is low.

Introduction

The human epidermal growth factor receptor (EGFR) type 2 (*HER2*) gene (aka *ERBB2*) is commonly amplified(1-3) and overexpressed in adenocarcinomas of the stomach, gastroesophageal junction (GEJ) and esophagus and is a potential target for therapeutic intervention. Although the role of HER2 as a prognostic marker in gastric cancer remains an issue of debate, *HER2* amplification/overexpression is an important therapeutic target for trastuzumab and is associated with significant improvements in progression-free(PFS) and overall survival(OS)(4). Routine diagnostic testing is now recommended in gastric and GEJ adenocarcinomas based on these findings that led to approval of the drug by the U.S. Food and Drug Administration(FDA)(5) and European Medicines Agency (EMA).

Here we report on assessments of HER2 status conducted for patient entry to a trial of lapatinib, a dual tyrosine kinase inhibitor of HER2 and EGFR, in combination with chemotherapy in patients with advanced HER2-positive upper gastrointestinal(UGI) adenocarcinomas. The TRIO-013/LOGiC study was based on efficacy of lapatinib in HER2-positive breast cancer patients(6-9) and activity both in vitro and in vivo with UGI cancer cell lines exhibiting *HER2* gene amplification(10). In addition, modest single-agent activity had been observed in patients with UGI cancers. At the time this trial was initiated no anti-HER2 agent had demonstrated activity in a systematic study of this disease. This retrospective study of *HER2* gene amplification and overexpression in UGI adenocarcinomas was initiated to explore the potential roles of *HER2* amplification levels and *HER2* genomic heterogeneity in lapatinib treatment responsiveness, as measured by improved PFS or improved OS in the LOGiC clinical trial.

Materials and Methods

TRIO-013/LOGiC clinical trial. The TRIO-013/LOGiC clinical trial (Lapatinib Optimization Study in ErbB2 {HER2} Positive Gastric Cancer: A Phase III Global, Blinded Study Designed to Evaluate Clinical Endpoints and Safety of Chemotherapy Plus Lapatinib) was a double-blind, randomized, multi-center, phase III study (ClinicalTrials.gov: NCT00680901) of capecitabine and oxaliplatin (CapeOx) without or with lapatinib conducted in patients with metastatic HER2-positive gastric, GEJ, and esophageal adenocarcinoma between June 2008 and January 2012 at 186 centers in 22 countries. The details of patient accrual, treatment, toxicities and outcomes are described separately(11). Here we describe HER2 testing issues and their associations with outcomes. The study was conducted in accordance with the current ethical principles outlined in the Declaration of Helsinki guidelines(12).

HER2 Status for Enrollment in the TRIO-013/LOGiC clinical trial. Patients enrolled in this trial had advanced, HER2-positive UGI adenocarcinomas as evaluated in local laboratories (Immunohistochemistry {IHC} 2+ and fluorescence in situ hybridization (FISH)-amplified, or IHC3+, or FISH-amplified) or in a central laboratory (FISH-amplified). Patients enrolled in the trial with a positive HER2 local test result were required to have tumor tissue submitted to one of two central laboratories for assessment of *HER2* gene amplification by the HER2 PathVysion FISH assay (Abbott Molecular, Inc., Des Plaines, Illinois) and expression by IHC (HercepTest, Dako Biotechnology, Carpinteria, CA) for confirmation of the local result. Although patients could be enrolled to the trial based on HER2-positive status in a local laboratory, patient inclusion in the primary efficacy population (PEP) required *HER2* gene amplification confirmed by a central laboratory. The PEP was composed of all patients whose tumors were centrally

determined as *HER2*-amplified. The primary endpoint was OS in the PEP. PFS was a secondary endpoint.

Conduct of the trial. Patients whose UGI cancers were assessed as HER2-positive by either IHC or in situ hybridization assay and otherwise eligible for the trial(11) were stratified according to history of prior adjuvant or neoadjuvant therapy and region (Asia, North America and rest of the world) then randomized to receive oxaliplatin (130 mg/m2 on day one) and capecitabine (850 mg/m2, bid, days 1-14) either with lapatinib (1250 mg daily, day 1-21) or with placebo (daily, day 1-21).

Identification of PEP. As no HER2 tests were approved for use in UGI cancers at the start of the trial, Translational Research In Oncology (TRIO) / Cancer International Research Group (CIRG) selected the PathVysion dual-color FISH assay (HER2 PathVysion FISH assay, Abbott Molecular, Inc.) as the primary method for assessment of *HER2*-amplification in patients' cancers to establish a consistent standard for HER2-positivity across the trial based on previous approval as a companion diagnostic for breast cancer patient selection to HER2-targeted therapies and experience with this assay method(8, 13-18). Formalin-fixed paraffin-embedded (FFPE) tissues or unstained sections were submitted to a central laboratory for assessment of HER2 status(Figure 1A).

Laboratory Assessment of HER2 status. *HER2* amplification was determined by FISH, as described(15-17) (Supplementary Data), in one of two central laboratories, one at the University of Southern California (Los Angeles, California) and the other at the University Medical Center Hamburg-Eppendorf (Hamburg, Germany). The entire processed tissue section was routinely screened for focal areas of carcinoma with HER2 status that differed from the remaining tumor both by IHC and FISH (*HER2* genomic heterogeneity). When such areas of disparate *HER2* gene

copy number were identified they were scored separately by FISH and the tumor area estimated. *HER2* amplification was defined as a *HER2* gene-to-CEP17 ratio of 2.0 or greater, the U.S. FDA–approved ratio; an average *HER2* gene copy number of 4.0 or greater was not required(19-21). Similarly for IHC, high geographic variability in HER2 immunostaining (e.g. areas of IHC 3+ and other areas of IHC 0/1+ that are consistent with *HER2* genomic heterogeneity) were noted and the percent of each area was estimated (see Figure 2 and Supplementary Figures S1 and S2).

HercepTest IHC Assay. During the conduct of this trial it became apparent, based on reports from ToGA trial investigators(4, 22), that there may be some controversy related to HER2 testing in gastric and GEJ adenocarcinomas. Therefore, TRIO/CIRG investigators and the trial sponsor decided to also implement immunohistochemistry (IHC) testing for HER2 protein in the central laboratories. The HercepTest(Dako, Inc.), a companion diagnostic initially approved for breast cancer assessments and subsequently, approved for gastric and GEJ adenocarcinomas, was used to determine HER2 protein expression as described(15-17) and summarized in Supplementary Methods.

Concordance Study. The HER2 PathVysion FISH assay was selected as the primary testing method for assessment of HER2 status; however, during the trial questions were raised about assay comparability with the HER2 IQFISH pharmDx method (Dako Corporation) which became FDA-approved for HER2 testing in gastric cancer for selection of patients to trastuzumab therapy. In order to assess agreement rates between assay methods and agreement rates in the two central laboratories, a separate cohort of 488 UGI adenocarcinoma cases, 419(86%) gastric, 43(8.8%) gastroesophageal junction(GEJ), and 26(5.3%) esophageal cancers, were procured from commercial providers (Asterand Bioscience, Analytical Biological Services,

Inc., Proteogenex, Inc., Individumed GmbH) as FFPE blocks and screened by HercepTest to ensure representation of all four IHC staining intensities (Figure 1B). All IHC3+ and IHC2+ cases were included in the concordance study. Whereas IHC1+ and IHC 0 cases were grouped by primary tumor site (gastric, GEJ and esophageal) with inclusion of all GEJ and esophageal carcinomas, then gastric carcinomas were randomly selected. The 159 selected cases were masked and sent to two central laboratories for *HER2* FISH testing, masked to all information.

Data Analyses. Statistical aspects of the LOGIC study design and statistical methods for analyses are summarized elsewhere(11). Briefly, we performed a Cox proportional hazards model on treatment for each of the subgroups to compute a hazard ratio and 95% confidence interval. Then using the unstratified log rank test, we computed p-values, as well as median survival times and 95% confidence intervals for both PFS and OS. Although the analyses between FISH results and outcomes reported here are exploratory and hypothesis generating, the differences observed retrospectively in a clinical trial were still considered in our analyses as potentially meaningful, namely, they warrant further exploration in a subsequent study, or not. We chose a p-value of ≤ 0.05 as our threshold to suggest a marker / analysis was meaningful. For concordance analyses, positive percent agreement (PPA), negative percent agreement (NPA) and overall percent agreement (OPA) with 95% confidence intervals were determined as described(23) and summarized in Supplementary Methods.

Results

HER2 Testing in the TRIO-013/LOGiC Trial. Overall, 4674 patients were registered in the central database and underwent eligibility screening for the TRIO-013/LOGiC trial(Figure 1A). Central FISH testing was performed in 1995 patients for eligibility and 333 for confirmation of a

local HER2 result; one patient enrolled and deemed HER2 positive by a local laboratory did not have tumor tissue remaining for central confirmation of HER2 status and FISH results were not obtained from six additional samples(Figure 1A). Of the 1995 carcinomas screened by a central laboratory for trial enrollment, 322(16.1%) had *HER2* amplification(Figure 1A), 29(1.5%) of which showed *HER2* genomic heterogeneity(Figure 2). The adenocarcinomas with genomic heterogeneity represent 9% of identified *HER2*-amplified cases primarily screened in the central laboratories. An additional 33 cases with *HER2* genomic heterogeneity were identified among those screened in local laboratories and confirmed in a central laboratory. Demographic data for these patients demonstrates a higher frequency of disease among men and shows that men and older patients have higher rates of *HER2* gene amplification (Table 1).

In patients whose cancers were centrally assessed, either for primary assessment or confirmation of local laboratory results, *HER2* status by both FISH and IHC is known for 1250 with 457/547(83.5%) amplified cases IHC2+ or IHC3+ and 680/703(96.7%) non-amplified cases either IHC1+ or IHC0 (Table 2). The overall concordance rate between FISH and IHC results for HER2 status was 91% (95% confidence interval{CI}: 89.2%, 92.4%). In our dataset, there were no cases that were IHC3+/FISH-negative. The majority (84%) of IHC2+ cases were *HER2* amplified, while relatively few IHC0(n=33, 6%) or IHC1+(n=57, 26%) cancers were *HER2* amplified(Table 2). The distribution across the various IHC immunostaining categories progressively transitions from predominantly IHC0 (515/703, 73.3%) without amplification (FISH ratio <2.0), to a more even distribution (IHC0{18%}, IHC1+{28%}, IHC2+{35%}, IHC3+{19%}) with low-level amplification (FISH ratio of 2.0-5.0) to progressively higher distributions of stronger IHC staining (IHC0{2%}, IHC1+{6%}, IHC2+{24%}, IHC3+{67%})

for FISH ratios >5.0-10.0 and the highest distribution (IHC3+{91%}) for FISH ratios >10.0 (Table 2).

Of 333 patients with trial enrollment based on local laboratory testing, 332 had tumor tissue available for subsequent re-assessment in a central laboratory (Supplementary Table S1). Concordance between central and local HER2 testing was 84.4% (275/326, Figure 1A). Since samples were not required for re-evaluation in the central laboratory for patients not enrolled to the trial, overall concordance in this population could not be assessed.

The agreement rate between local and central laboratory HER2 testing was dependent on the HER2 testing method used in the local laboratory and concordance varied accordingly. Among the 332 cancers evaluated both locally and centrally, 259 cases were tested in local laboratories by ISH alone, 33 by ISH and IHC, and 40 by IHC alone. Among the 292 cases tested locally by ISH, the central laboratory determined the *HER2* gene amplification status in 286 of the cases. *HER2* amplified status was confirmed for 250; however, 36 were assessed as not amplified for an ISH concordance rate of 87% (95% CI: 83.1%, 90.8%)(Supplementary Table S2). Seven cases had been evaluated by CISH in local laboratories and five of the cases were confirmed as amplified by central laboratory FISH. Agreement between local laboratory and central laboratory for HER2 IHC assays was even less (58%) (Supplemental Supplementary Table S3), although the number of cases analyzed was limited and different assays may have been used locally (n=73).

Concordance Study for Different HER2 FISH assay methods between the two Central Laboratories. Before accrual to the LOGiC trial was complete, the ToGA trial was completed and the FDA, subsequently, approved two companion diagnostic assays, the Dako HercepTest IHC assay (October, 2010) and the Dako HER2 IQFISH pharmDx FISH assay (February, 2013)

for selection of gastric and GEJ cancer patients to trastuzumab plus chemotherapy(4). Therefore, we performed a concordance study to demonstrate agreement between the PathVysion *HER2* FISH assay and the Dako HER2 IQFISH pharmDx *HER2* assay (Figure 1B and Supplementary Results and Supplementary Tables S4 and S5). UGI adenocarcinomas from 488 patients who were not enrolled in the TRIO-013/LOGiC trial were used for the concordance studies (Figure 1B). In order to ensure the full spectrum of HER2 expression was more evenly represented in the concordance comparison rather than a predominance of HER2 low-expression, not-amplified cases, the tumors were initially processed and scored by IHC with the selection of 159 cases for further analysis by FISH. The central laboratory was blinded to the prior IHC status during FISH analyses performed in the central laboratories. The initial IHC results from all 488 UGI cancers showed highly variable immunostaining in 17 cases (3.5%) that was considered consistent with potential *HER2* genomic heterogeneity (Supplementary Figures S1 and S2). The central masking/randomization center included 14 of these IHC-variable cases that had sufficient tumor for inclusion in the concordance study thus increasing the rate of potential "*HER2* heterogeneity" from 3.5%(17/488) to 8.8%(14/159). Of the 159 cases selected for inclusion in the concordance study: 31 exhibited HER2 staining indicative of IHC3+, 20 IHC2+, 55 IHC1+ and 53 IHC0(Figure 1B).

Overall concordance between LOGiC central laboratories for 151 cases successfully tested by PathVysion HER2 FISH assay in both laboratories was 94.7% (95%CI:89.9%, 97.3%) with 93.8% PPA (95%CI:83.2%, 97.9%) and 95.1% NPA (95%CI:89.1%, 97.9%)(Supplementary Table S5). Discordance between the two central laboratories was primarily related to differences in scoring of 14 samples with highly variable HER2 immunostaining, consistent with *HER2* genomic heterogeneity (Supplementary Results, Supplementary Figures S1 and S2).

Summary of clinical outcomes in the TRIO-013/LOGiC trial. In total, among the 545 patients accrued to the trial, 487 patients had central-laboratory-confirmed *HER2* amplified disease to comprise the primary efficacy population (PEP); while 51 had *HER2* non-amplified disease and *HER2* amplification status was not determined in seven patients (Table 3 and Figure 1A).

Among the 487 patients with *HER2*-amplified cancers in the PEP, 424(87.1%) had gastric, 43(8.8%) GEJ and 20(4.1%) esophageal cancers. In the PEP 297 had cancers that were HER2 IHC3+ (61%), 108 were IHC2+ (22%), 54 were IHC1+ (11%), 27 were IHC0 (6%), and one was IHC unknown. As reported separately in detail(11), the LOGiC trial did not meet its primary end-point of a significant improvement in OS among those patients randomized to receive chemotherapy with lapatinib (HR=0.91 {95%CI: 0.73, 1.12}, p-value=0.35); however, a significant improvement in PFS was observed (HR=0.82 {95%CI: 0.68, 1.00}, p-value=0.038). Subset analyses demonstrated meaningful improvements in both PFS and OS for selected subsets of patients, such as those accrued in Asian countries and those patients less than 60 years of age at trial enrollment (Table 3 and Supplementary Table S6). Among Asian participants and those less than 60 years of age we observed greater PFS related to higher levels of *HER2* gene amplification(Table 3). In the overall trial population (>10.0-fold) as well as among the Asian population (5.01-10.0 and >10.0-fold) and in the population less than 60 years of age the hazard ratio for PFS became more pronounced only at higher levels of *HER2* gene amplification (>5.0-10.0 and >10.0 fold)(Table 3). *HER2* ratio was not associated with OS in the overall population or in the sub-populations analyzed for PFS (Table 3)(p value >0.05), with the exception of Asian participants whose cancers had 5.01-10.0 *HER2* gene amplification levels.

Among the 81 cases included in the trial with *HER2* gene amplification by FISH with only IHC0/1+ for HER2 protein, hazard ratios for PFS were (HR=1.12, p-value=0.65) and OS (HR=0.91, p-value = 0.71). In contrast, those patients whose *HER2*-amplified UGI cancers were also IHC2+/3+ had greater improvement in PFS (HR=0.76, {95%CI: 0.62, 0.94}, p-value=0.0096) but not OS (HR=0.86 {95%CI: 0.68, 1.09}, p-value=0.21). Among the 148 participants from Asian countries with *HER2*-amplified/IHC2+/3+ UGI adenocarcinoma, PFS (HR=0.59; p-value = 0.0020) and OS (HR=0.60, p-value=0.0108) were greatly improved by the addition of lapatinib to chemotherapy. These results were highly intriguing and will be proposed for further prospective assessments in subsequent studies.

Discussion

Gastric cancer remains the third leading cause of cancer-related mortality in the world(24). Treatment typically involves gastric resection; however, surgery alone is generally only curative for patients with early-stage disease. For patients with advanced resectable disease, survival rates remain poor but are improved by chemotherapy and possibly radiotherapy(25-27).

HER2 amplification/overexpression as a prognostic marker. The majority of gastric cancer studies do not show an association of HER2 status with clinical outcomes in the absence of chemotherapy and/or radiation therapy(28-37); however, studies of patients treated with chemotherapy and/or radiation therapy demonstrate a survival advantage for patients whose gastric cancers lack *HER2* gene amplification/overexpression(32, 38-41). An additional large study of patients with adenocarcinomas of the gastric cardia, gastroesophageal junction and esophagus treated by surgical resection (1980-1997) showed no difference in either disease-specific survival or OS among patients whose cancers had *HER2* gene amplification(42). The

potential role of HER2 as a predictive marker of responsiveness to chemotherapy and radiation therapy is supported by a retrospective analysis of *HER2* gene amplification and overexpression in gastric cancers from patients in the INT-0116/SWOG9008 phase III gastric cancer clinical trial comparing surgery alone with surgery and chemotherapy (5-fluorouracil)/radiation therapy(13). Patients whose gastric cancers were HER2-negative and were treated with chemoradiation therapy had a longer disease-free and overall survival than similar patients treated with gastrectomy alone. In contrast, no benefit from chemotherapy and radiation therapy was observed among the subset of patients whose gastric cancers had *HER2* amplification/overexpression(13).

HER2 amplification/overexpression as a target for therapy. *HER2* amplification/overexpression is an important therapeutic target for trastuzumab in metastatic gastric and gastroesophageal cancer(4), as it is in metastatic breast cancer(9, 18, 43). Both the Trastuzumab for Gastric Cancer (ToGA) and LOGiC trials were initiated because of medical need in gastric/gastroesophageal/esophageal carcinoma, a high HER2 positivity rate and an already established toxicity profile in breast cancer patients. The ToGA clinical trial assessed efficacy and safety of trastuzumab added to capecitabine or fluorouracil and cisplatin chemotherapy for first-line treatment of advanced gastric or gastro-esophageal junction cancers with overexpression of HER2(4). Among patients assigned to chemotherapy with trastuzumab in the ToGA trial, median OS was 13.8 months (95% CI 12–16) compared with 11.1 months for those assigned to chemotherapy alone (HR 0·74; 95%CI: 0·60–0·91; p=0·0046). In the LOGiC trial the median OS was 12.2 months (95%CI: 10.6, 14.2) for patients assigned to capecitabine and oxaliplatin chemotherapy with lapatinib compared with 10.5 months (95%CI: 9.0, 11.3) for patients assigned to chemotherapy and placebo (HR: 0.91 {95%CI: 0.73, 1.12}; p=NS)(11).

Although differences in survival among the overall LOGiC trial population were not observed, Asian patients and patients under 60 years of age did show noticeable improvements in OS(44). However, we did not find any systematic difference in HER2 status by either immunohistochemistry or FISH among these subgroups that could account for the differences in outcome.

HER2 testing in UGI cancers. Routine diagnostic HER2 testing is recommended in gastric and GEJ adenocarcinomas based on findings from the ToGA trial that led to regulatory approval of trastuzumab for this indication in 2010. We found a high concordance rate for *HER2* gene amplification status determined with two different FISH assays (99% agreement) and between two different central laboratories when the same FISH assay is used (95% agreement), even though the comparison samples were enriched for cases with *HER2* genomic heterogeneity (8.8%). Agreement between local laboratory and central laboratory for *HER2* FISH assays was only 87%. Agreement between local laboratory and central laboratory for HER2 IHC assays was less (58%)(Supplementary Table S3); however, the number of cases available for this analysis was quite limited. Overall agreement between HER2 IHC and FISH assays in the central laboratory was approximately 90%, 91% in the LOGiC clinical trial cases (n=1250) and 88% in the concordance cohort cases (n =155). The discrepancies identified for HER2 status determined by IHC compared to FISH, as well as disagreements between local and central laboratory testing, have the potential to inappropriately select some patients for HER2 targeted therapy as well as the potential to inappropriately deny other patients such treatment. Our findings suggest this is of particular concern when IHC is used as the primary test, as addressed elsewhere in detail(20).

There has been some concern that UGI endoscopic biopsies may not provide sufficient tissue for adequate identification of all cancers with this alteration. Although we did not have

both endoscopic biopsy samples and resection specimens from the same patients for a direct case-by-case comparison, our *HER2* amplification rates among patients with endoscopic biopsy specimens (18.3%) compared to patients who had surgical resection specimens (15.0%) suggests that endoscopic biopsies are likely to be representative of the *HER2* amplification status (Supplementary Results and Supplementary Table S7).

HER2 genomic heterogeneity. *HER2* genomic heterogeneity was observed overall in approximately 1%-2% of the UGI adenocarcinomas in this study (Table 1, Figure 2 and Supplementary Figures S1 and S2). The rate did not vary significantly by patient age, gender, country, or anatomical site. Although the total number of these cases identified by the central laboratory was modest (n=62), 51 of the cases were identified in patients who were subsequently randomly assigned to study treatment, 24 to the lapatinib treatment arm and 27 to the control arm, no significant differences were observed in PFS or OS. It should be noted that our definition(15) of *HER2* genomic heterogeneity differs from the definition used in CAP guidelines(45).

The definition of *HER2* genomic heterogeneity used in the LOGiC trial was the same as used for trials of breast cancer screened in the same two TRIO/BCIRG central laboratories(16) for the BCIRG-005(46), BCIRG-006(8) and BCIRG-007(47) trials. We consider *HER2* genomic heterogeneity to exist when at least one geographically defined area of contiguous tumor cells are identified within a cancer that differs from the remaining tumor cells by *HER2* amplification status (i.e. *HER2* amplified versus *HER2* not amplified). This differs from the definition established by the CAP guidelines for breast cancer(45). The CAP criteria do not distinguish or require a geographically defined area but simply that the scores of 20 or more cells have at least 5% of scored cells with a *HER2*-to-CEP17 ratio that is $\geq$2.2 and less than 50% of the tumor cells have such a ratio. The guidelines state "HER2 genetic heterogeneity (GH) exists if there are

more than 5% but less than 50% of infiltrating tumor cells with a ratio higher than 2.2." We also differ from the guidelines in our approach to scoring *HER2* heterogeneity. According to the guidelines, "if 20 cells are counted and at least one cell is identified with a HER2/CEP17 signal ratio higher than 2.2, this specimen contains GH. Likewise, if 60 cells are examined and 3 or more cells have a ratio higher than 2.2, HER2 GH is present." Using this definition leads to substantially higher rates of *HER2* heterogeneity by FISH ranging from 14% to 26% in breast cancers(48, 49). However, use of the same definition in gastric cardia, gastroesophageal junction and esophagus adenocarcinomas demonstrated only 20 of 675(2.9%) have *HER2* genetic heterogeneity(42). These studies contrast with our previously reported lower rates (0.5% and 1.1%) in breast cancer(15, 16, 21) and similarly low rate for UGI cancers reported here. The CAP definition(45) may lead to the inclusion of a number of cases that do not contain any *HER2* gene amplification even in a portion of the tumor. For example, we would consider some of the results generated by the CAP definition to reflect inclusion of (scattered) cancer cells in which a proportion of the cells (exceeding 5%) are in G2- or M-phase of the cell cycle where DNA has been duplicated leading to doubling of the number of copies of *HER2* gene but without a doubling of chromosome 17 centrosomes since the centromeres remain fused until late metaphase. These (scattered) individual cells have a *HER2* FISH ratio greater than 2.0, are not amplified, but contribute to "genetic heterogeneity" by the CAP definition. These tumor cells are, likely, simply proliferating (Campeau A and Press MF, unpublished data).

As illustrated (Figure 2, Supplementary Figures S1 and S2), we have required that the differing geographic tumor areas be scored separately and that one area of contiguous tumor cells have an overall FISH ratio less than 2.0 and the other area have a ratio greater than 2.0 with distinctly aggregated or clustered *HER2* gene signals in the amplified cancer cells. Accordingly,

62 cases with *HER2* genomic heterogeneity were identified in this study with 51 accrued to the trial. Only two of these cases had genomic heterogeneity in a subpopulation of tumor cells representing less than 5% of the total area of the tumor. A limitation of our estimates of *HER2* genomic heterogeneity is that we seldom had more than a single tissue block from the primary carcinoma from which to make these evaluations. Therefore, our frequencies should be considered from this perspective.

Association of Lapatinib responsiveness with HER2 amplification level. In breast cancer clinical trials of lapatinib we have found a relatively uniform hazard ratio for PFS that was independent of *HER2* amplification level in *HER2*-amplified breast cancer patients(15). Only patients whose metastatic disease lacked *HER2* amplification (*HER2* FISH ratio <2.0) showed no improvement in PFS with lapatinib treatment (HR = 1.09, 95%CI:0.86-1.37; n=390), while patients whose metastatic disease with low-level (2.0-5.5-fold) amplification (HR=0.48, 95%CI: 0.28-0.83; n=82), moderate (5.5-7.6-fold)(HR=0.35, 95%CI: 0.18-0.69; n=89), high (7.6-10.1-fold) (HR=0.58, 95%CI: 0.33-1.05; n=87) or very high-level (>10.1-fold) amplification (HR=0.42, 95%CI: 0.24-0.74; n=88) levels all showed improved PFS associated with lapatinib treatment.

In contrast, this trial of lapatinib in UGI cancer patients with advanced disease showed an association between lapatinib treatment and improved PFS only at higher levels of *HER2* amplification. In the entire trial population of UGI cancer patients, those without amplification (FISH ratios <2.0; HR=0.89, 95%CI: 0.50-1.59; n=51), with low-level amplification (FISH ratios 2.0-5.0; HR=0.90, 95%CI: 0.63-1.29; n=140) and with moderate-level amplification (FISH ratios 5.01-10.0; HR=0.92, 95%CI: 0.66-1.28; n=171) showed no significant improvement in PFS, while UGI patients whose metastatic cancers had high-level amplification (FISH ratios >10.0;

HR=0.64, 95%CI: 0.47-0.88; n=176) showed significant improvements in PFS with lapatinib treatment. Similarly, in the trial population of Asian UGI cancer patients, those whose cancers lacked *HER2* amplification (FISH ratios <2.0; HR=0.99, 95%CI: 0.37-2.63; n=21) as well as those whose cancers had low-level amplification (FISH ratios 2.0-5.0; HR=0.77, 95%CI: 0.44-1.37; n=56) showed no significant PFS improvement, while those whose cancers had moderate-level amplification (FISH ratios 5.01-10.0; HR=0.62, 95%CI: 0.66-1.02; n=84) and high-level amplification (FISH ratios >10.0; HR=0.59, 95%CI: 0.33-1.04; n=53) showed significant improvements in PFS with lapatinib treatment. Similar observations were made among UGI cancer patients less than 60 years of age at trial entry where only patients whose adenocarcinomas with low-level *HER2* amplification did not show improvements in PFS with lapatinib treatment.

Other investigators have reported similar associations between improved clinical outcomes and moderate or high levels of *HER2* gene amplification in gastric cancer patients treated with trastuzumab(50, 51). In a cohort of 90 patients Gomez-Martin et al. report a mean *HER2*/CEP17 ratio of 4.7 as the optimal cutoff value discriminating sensitive and refractory patients (P = 0.005); a ratio of 4.45 as the optimal cutoff for survival >12 months (P = 0.005), and a ratio of 5.15 as the optimal cut-off for survival >16 months (P = 0.004)(50). Although there were only 66 patients in the Ock et al study with *HER2* gene amplification status determined by FISH, the conclusions were similar(51). Neither of these studies have a control, untreated group to confirm that patients with a *HER2*/CEP17 ratio less than 4.7 lack significant improvement in outcomes; however, patients whose cancers have ratios more than approximately 5 show improved outcomes compared to those whose cancers have lower levels of gene amplification. These findings are consistent with our own findings of greater benefit among patients whose cancers

have higher levels of amplification (ratio 5.0-10 and ≥10).

The LOGiC trial did not show a significant association with OS(44), while ToGA did(4). LOGiC demonstrated a significant association with PFS but not with the primary end-point of OS. We consider this to be potentially influenced by several issues including 1.) sample size and its influence on statistical power, 2.) differential drug efficacy between lapatinib and trastuzumab and 3.) treatment compliance. Design of the original LOGiC trial was probably slightly under-powered (n=545 in LOGiC compared to 594 in ToGA), since LOGiC was originally based on PFS as the initial primary end-point, which was later changed to an OS primary end-point. After exclusion of patients whose disease was *HER2*-not-amplified, the PEP was limited to 487 participants. LOGiC might have benefited from enrollment of additional participants to gain statistical power for an association with OS. Second, the differential in median OS favored the ToGA trial (ToGA: 13.8 versus 11.1 months; compared to LOGiC: 12.2 versus 10.5 months). However, it is unknown what role treatment compliance may have played in the clinical outcomes. LOGiC may have had a lower rate of compliance with experimental drug treatment. In contrast to trastuzumab with the requirement for intravenous administration, lapatinib, an oral medication, was self-administrated by most participants. This introduces the potential for reduced compliance in the lapatinib treatment arm, especially among patients who may have experienced gastrointestinal side-effects. These issues are compounded by our observations, reported here, that *HER2* gene amplification levels may impact clinical outcomes.

Finally, we have not analyzed the potential for other concurrent alterations to effect treatment resistance. For example, co-amplification of *HER2* and *MET* is relatively common in esophago-gastric carcinomas(1, 52) and is associated with lack of treatment response to either MET kinase inhibitor (crizotinib) or HER2 inhibitor (trastuzumab) treatment but is associated with therapeutic response to combined treatment in patients with advanced esophago-gastric carcinomas(52).

A comprehensive assessment of genetic alterations in cancers from patients in the LOGiC trial would potentially provide important information about co-occurring alterations associated with *HER2* amplification and lack of treatment response.

In summary, HER2 status determined by IHC and FISH was highly correlated in UGI cancers. The prevalence of *HER2* gene amplification was slightly higher in UGI cancers from men compared to women and was also higher among older patients. *HER2* genomic heterogeneity was relatively infrequent, approximately 1.6% overall. The level of *HER2* amplification was also correlated with responsiveness to lapatinib treatment in UGI cancer patients.

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Note. Lapatinib has become the property of Novartis Pharma AG as of March 1, 2015.

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Table 1. Demographic information for 1995* patients whose cancers were assessed initially for HER2 status in the central laboratories for eligibility.

Characteristic	*HER2*-amplified	*HER2*-not-amplified	*HER2* Genomic Heterogeneity	Totals	P-value**
Age (years)					
≤39 (n = 92)	11 (11.9%)	80 (87%)	1 (1.1%)	92	
40-49 (n = 224)	29 (12.9%)	192 (85.8%)	3 (1.3%)	224	
50-59 (n = 529)	75 (14.2%)	449 (84.9%)	5 (0.9%)	529	0.0038
60-69 (n = 585)	105 (17.9%)	471 (80.6%)	9 (1.5%)	585	
70-79 (n = 320)	63 (19.7%)	246 (76.9%)	11 (3.4%)	320	
≥80 (n = 27)	9 (33%)	18 (67.0%)	0 (0%)	27	
NA (n = 30)	1	29	0	30	
Total:	293 (16.2%)	1485 (82.2%)	29 (1.6%)	1807	
Gender					
Male (n = 1207)	211 (17.5%)	974 (80.7%)	22 (1.8%)	1207	
Female (n = 592)	82 (13.9%)	503 (85%)	7 (1.1%)	592	0.0262
NA (n=8)	0	8	0	8	
Total:	293 (16.2%)	1485 (82.2%)	29 (1.6%)	1807	
Country of Origin					
Brazil	41 (18.1%)	177 (78.0%)	9 (3.9%)	227	
Canada	8 (13.6%)	50 (84.7%)	1 (1.7%)	59	
Chile	22 (16.2%)	113 (83.1%)	1 (0.7%)	136	
Estonia	8 (14.3%)	48 (85.7%)	0	56	
Hong Kong	4 (22.2%)	14 (77.8%)	0	18	
Hungary	9 (13.6%)	56 (84.8)	1 (1.5)	66	
Israel	5 (13.2%)	33 (86.8%)	0	38	
Italy	23 (20.7%)	87 (78.4%)	1 (0.9%)	111	
Korea	2 (18.2%)	9 (81.8%)	0	11	
Mexico	4 (9.5%)	37 (88.1%)	1 (2.4%)	42	
Netherlands	4 (28.6%)	7 (50%)	3 (21.4%)	14	
Peru	4 (21.1%)	14 (73.7%)	1 (5.3%)	19	
Poland	52 (17.1%)	250 (82.2%)	2 (0.7%)	304	
Russia	29 (20.6%)	111 (78.7%)	1 (0.7%)	141	
Taiwan	0	1	0	1	
Thailand	1 (4.5%)	19 (86.4%)	2 (9.1%)	22	
Turkey	3 (14.3%)	18 (85.7%)	0	21	
United States	11 (23.4%)	35 (74.5%)	1 (2.1%)	47	
Ukraine	62 (13.1%)	406 (85.7%)	6 (1.2%)	474	
Totals: n = 1807				1807	

*Of 1995 cases centrally assessed for HER2 status, a FISH result was not available for analysis in188 of the cases. **P-value was determined using Chi-sqare statistical test. It should also be noted that many Asian sites used a local laboratory, e.g. in China, and provided tissue sections in the central laboratory only for confirmation of their status (primary efficacy population) after accrual to the trial (see Figure 1A). NA, not available.

Table 2. TRIO-013/LOGiC Clinical Trial: Comparison of HER2 testing by FISH and IHC in two central laboratories.

		Herceptest IHC score				
		IHC 0 n	**IHC 1+ n**	**IHC 2+ n**	**IHC 3+ n**	Totals
PathVysion FISH assay	*HER2* not ampl	515 (94%)	165 (74.3%)	23 (16%)	0	703
	HER2 amplif*	33 (6%)	57 (25.7%)	121 (84%)	336 (100%)	547
	Totals	548	222	144	336	1250
		Herceptest IHC score				
PathVysion FISH assay	**FISH Ratios**	**IHC 0**	**IHC 1+**	**IHC 2+**	**IHC 3+**	**Totals**
	<2.0, n (%)	515 (73%)	164 (23%)	24 (3%)	0 (0%)	703
	2.0-5.0, n (%)	29 (18%)	45 (28%)	57 (35%)	31 (19%)	162
	>5.0-10.0, n (%)	4 (2%)	12 (6%)	45 (24%)	124 (67%)	185
	>10.0, n (%)	0 (0%)	1 (<1%)	18 (9%)	181 (91%)	200
		548	222	144	336	**1250**

Of the 547 *HER2* amplified UGI adenocarcinomas 90 (16.5%) had only IHC 0 or 1+ immunostaining for HER2 protein.

Overall agreement between FISH and IHC results for HER2 status was 91% (1,137/1250)(95% confidence interval: 89.2%, 92.4%). Other investigators have reported lower concordance rates between IHC and FISH, especially in the IHC 2+ group where less than 40% have been *HER2* amplified by FISH, in esophageal / GE junction or gastric adenocarcinomas(3, 53).

*For the 1250 cases with results summarized here *HER2* genomic heterogeneity was identified in 69 cases (4.1%) as follows by IHC group:

Among IHC 0 there were 5 cases (0.9%) showing *HER2* genomic heterogeneity.
Among IHC 1+ there were 4 cases (1.8%) showing *HER2* genomic heterogeneity.
Among IHC 2+ there were 13 cases (9%) showing *HER2* genomic heterogeneity.
Among IHC 3+ there were 39 cases (11.6%) showing *HER2* genomic heterogeneity.

All 61 cases were tabulated as "*HER2* amplified"; IHC was not available for one case with *HER2* genomic heterogeneity demonstrated with FISH.

Table 3. Correlation of PFS and OS with Responsiveness to Lapatinib in the TRIO-013 Trial for Central-Laboratory-Determined *HER2* Gene Amplification Status[#].

All Trial Participants					
HER2 Status	**Number***	**PFS, HR (95% CI)**	**PFS, Non-stratified log-rank, P-value**	**OS, HR (95% CI)**	**OS, Non-stratified log-rank, P-value**
Negative	51	0.88 (0.48, 1.59)	0.6628	1.02 (0.54,1.92)	0.9617
Positive (PEP)	487	0.81 (0.67, 0.99)	0.0350	0.88 (0.71, 1.09)	0.2319
FISH Ratios	**Number**	**PFS, HR** (95% CI)	**P-value**	**OS, HR (95% CI)**	**OS, P-value**
<2.0	51	0.89 (0.49, 1.61)	0.6868	1.04 (0.55, 1.20)	0.9172
2.0-5.0	140	0.89 (0.62, 1.28)	0.5439	0.94 (0.64, 1.39)	0.7559
5.01-10.0	171	0.92 (0.66, 1.28)	0.6124	0.89 (0.62, 1.30)	0.5556
>10.0	176	0.62 (0.45, 0.86)	0.0033	0.86 (0.61, 1.21)	0.3742
	538*				
Asian Trial Participants					
HER2 Status	**Number**	**HR (95% CI)**	**P-value**	**OS, HR (95% CI)**	**OS, P-value**
Negative	21	1.00 (0.37, 2.70)	0.9794	1.33 (0.47,3.72)	0.5995
Positive	193	0.66 (0.49, 0.90)	0.0080	0.68 (0.48, 0.96)	0.0261
FISH Ratios	**Number**	**HR (95% CI)**	**P-value**	**OS, HR (95% CI)**	**OS, P-value**
<2.0	21	1.00 (0.37, 2.70)	0.9794	1.33 (0.47, 3.72)	0.5995
2.0-5.0	56	0.77 (0.44, 1.35)	0.3616	0.81 (0.43, 1.52)	0.5149
5.01-10.0	84	0.62 (0.38, 1.01)	0.0511	0.56 (0.33, 0.97)	0.0375
>10.0	53	0.57 (0.32, 1.00)	0.0479	0.77 (0.42,1.44)	0.4191
Total	**214**				
Trial Participants <60 years of age					
HER2 Status	**Number**	**HR (95% CI)**	**P-value**	**OS, HR (95% CI)**	**OS, P-value**
Negative	28	0.43 (0.18, 1.01)	0.0494	0.49 (0.20,1.22)	0.1210
Positive	236	0.57 (0.44, 0.76)	<0.0001	0.69 (0.51, 0.93)	0.0141
FISH Ratios	**Number**	**HR(95% CI)**	**P-value**	**OS, HR (95% CI)**	**OS, P-value**
<2.0	28	0.43 (0.18, 1.02)	0.0494	0.51 (0.20,1.26)	0.1381
2.0-5.0	64	0.73 (0.43, 1.24)	0.2436	0.67 (0.37,1.18)	0.1626
5.01-10.0	81	0.57 (0.35, 0.93)	0.0224	0.59 (0.34,1.02)	0.0566
>10.0	91	0.42 (0.26, 0.68)	0.0003	0.81 (0.51,1.30)	0.3844
Total	**264**				

[#]Findings for the primary endpoint overall survival (OS) are also reported elsewhere (Hecht et al, *JCO* 34(5): 443-51, 2016);findings for progression-free survival (PFS) are reported here. *The HER2 FISH status was not confirmed for 7 patients enrolled in the trial (see Figure 1A). **Analyses for participants from the rest of the world and participants ≥60 years of age are included in Supplementary Table S6.

HR, hazard ratio; PEP, primary efficacy population; ITTP, intent to treat population.

Figure Legends

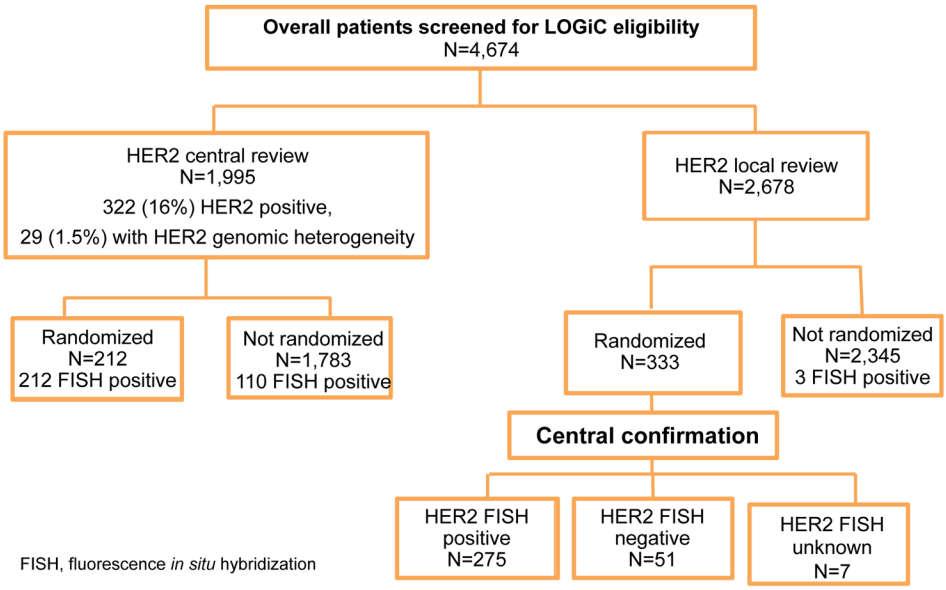
Figure 1. Schematic diagrams illustrating disposition of TRIO-013/LOGiC and UGI Cancer Concordance Cohort. (A.) HER2 testing in the TRIO-013/LOGiC trial. Patients' gastric, GE junction or esophageal adenocarcinomas were evaluated by FISH assay for HER2 status primarily either in a central laboratory (N = 1,995) or initially in a local laboratory (N = 2,678) followed by central laboratory re-evaluation of those cancers considered to be HER2-positive in the local laboratory (N = 333) for *HER2* gene amplification status. (B.) Specimen selection process for the concordance study in the central laboratories of *HER2* testing by FISH to ensure representation of GEJ and esophageal adenocarcinomas. The UGI Cancer Concordance Cohort was composed of 488 UGI adenocarcinomas initially screened by the HER2 HercepTest to identify subgroups with strong (IHC 3+), moderate (IHC 2+), weak (IHC 1+) and absent (IHC 0) HER2 immunostaining. Adenocarcinomas originating from all three anatomical sites (esophagus, gastro-esophageal junction and stomach) are represented in each IHC immunostaining category (see Supplementary Table S4). [1]One case was excluded as no tumor tissue was identified; sufficient variability in HER2 immunostaining was noted in 17 cases to suggest the possibility of "*HER2* genomic heterogeneity".

Figure 2. *HER2* genomic heterogeneity. (A.) Low power photomicrograph of a gastric carcinoma with highly variable HER2 immunostaining that demonstrates geographically distinguishable regions of high (IHC 3+) and low (IHC 0) immunohistochemical staining. Two regions, one with high (IHC 3+)(left box) and one with low (IHC 0)(right box) levels of HER2 immunostaining, are identified.

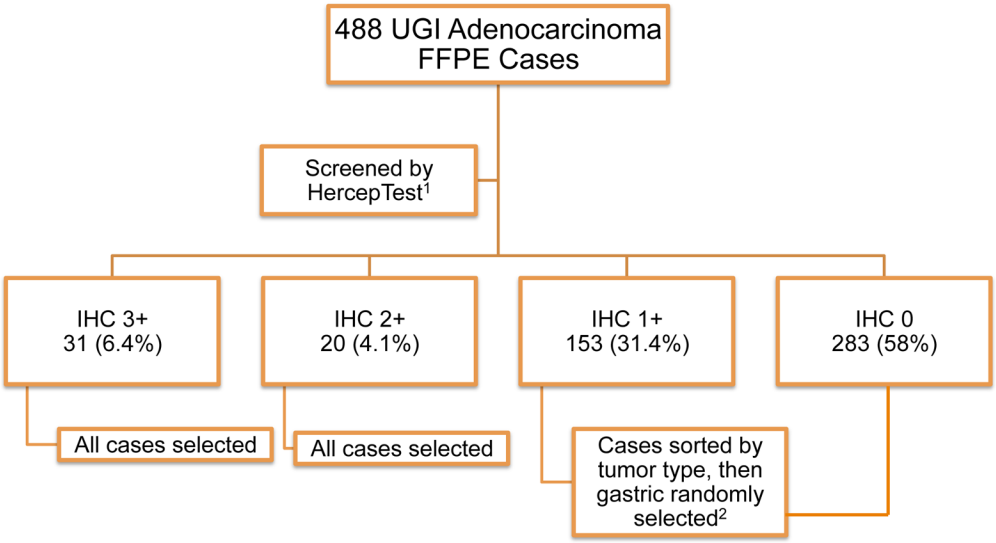
Original magnification: 40x. (B. and C.) The two boxed areas from A are illustrated at higher magnification, one (B) showing HER2 overexpression (IHC 3+) and the other (C) showing low HER2 expression (IHC 0). Original magnification: 400x. (D. and E.) Fluorescence *in situ* hybridization (FISH) of *HER2* gene copies (red) and chromosome 17 centromeres (green) of the same two regions showing *HER2* gene amplification (D) in the region with IHC 3+ immunostaining and a LACK of *HER2* gene amplification (E) in the region with IHC 0 immunostaining. Original magnification: 1000x. TRIO-013/LOGiC case number 595.

Figure 1

A. HER2 testing in the TIRO-013/LOGIC trial.



B. Specimen selection process for the HER2 concordance study in Central Laboratories



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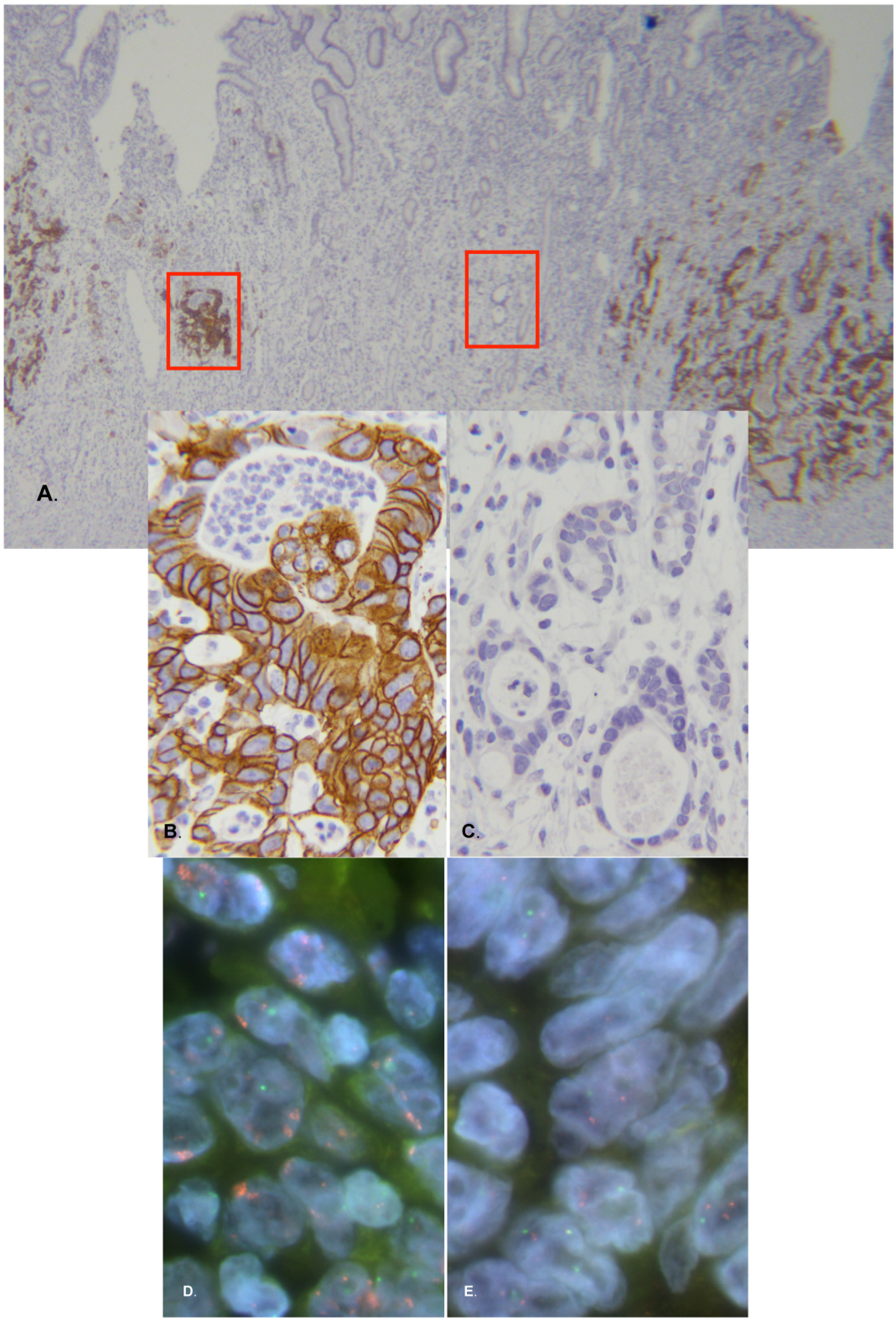
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 2. HER2 genomic heterogeneity



AACR American Association for Cancer Research

Molecular Cancer Therapeutics

HER2 status in advanced or metastatic gastric, esophageal, or gastro-esophageal adenocarcinoma for entry to the TRIO-013/LOGiC trial of lapatinib

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