

Biomarker Analysis of the Phase III NALA Study of Neratinib + Capecitabine versus Lapatinib + Capecitabine in Patients with Previously Treated Metastatic Breast Cancer



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ABSTRACT

Purpose: Neratinib plus capecitabine (N+C) demonstrated significant progression-free survival (PFS) benefit in NALA (NCT01808573), a randomized phase III trial comparing N+C with lapatinib + capecitabine (L+C) in 621 patients with HER2-positive (HER2⁺) metastatic breast cancer (MBC) who had received ≥ 2 prior HER2-directed regimens in the metastatic setting. We evaluated correlations between exploratory biomarkers and PFS.

Patients and Methods: Somatic mutations were evaluated by next-generation sequencing on primary or metastatic samples. HER2 protein expression was evaluated by central IHC, H-score, and VeraTag/HERmark. p95 expression (truncated HER2) was measured by VeraTag. HRs were estimated using unstratified Cox proportional hazards models.

Results: Four hundred and twenty samples had successful sequencing: 34.0% had *PIK3CA* mutations and 5.5% had *HER2* (*ERBB2*) mutations. In the combined patient populations, *PIK3CA* mutations

trended toward shorter PFS [wild-type vs. mutant, HR = 0.81; 95% confidence interval (CI), 0.64–1.03], whereas *HER2* mutations trended toward longer PFS [HR = 1.69 (95% CI, 0.97–3.29)]. Higher HER2 protein expression was associated with longer PFS [IHC 3+ vs. 2+, HR = 0.67 (0.54–0.82); H-score ≥ 240 versus < 240 , HR = 0.77 (0.63–0.93); HERmark positive vs. negative, HR = 0.76 (0.59–0.98)]. Patients whose tumors had higher HER2 protein expression (any method) derived an increased benefit from N+C compared with L+C [IHC 3+, HR = 0.64 (0.51–0.81); H-score ≥ 240 , HR = 0.54 (0.41–0.72); HERmark positive, HR = 0.65 (0.50–0.84)], as did patients with high p95 [p95 ≥ 2.8 relative fluorescence (RF)/mm², HR = 0.66 (0.50–0.86) vs. p95 < 2.8 RF/mm², HR = 0.91 (0.61–1.36)].

Conclusions: *PIK3CA* mutations were associated with shorter PFS whereas higher *HER2* expression was associated with longer PFS. Higher HER2 protein expression was also associated with a greater benefit for N+C compared with L+C.

Introduction

HER2 overexpression occurs in 15% to 20% of breast cancers (1). Before the approval of trastuzumab, HER2-overexpressing breast cancer was the subtype with the worst prognosis (2). Multiple additional HER2-targeted therapies have subsequently been developed, two of which are the small-molecule tyrosine kinase inhibitors (TKI) neratinib and lapatinib, the focus of comparison in this analysis. Neratinib and lapatinib target the adenosine triphosphate-binding domain of HER proteins. Their distinguishing features include their binding mechanisms, preclinical cytotoxicity, and impact on HER family dimerization and downstream signaling and gene expression (3). Neratinib binds covalently/irreversibly to HER2 (4), whereas lapatinib binds noncovalently/reversibly (5). Neratinib binds EGFR, HER2, and HER4 with high affinity; lapatinib targets only EGFR and HER2 with high affinity (3). Both have demonstrated *in vitro* and *in vivo* inhibition of growth and proliferation in HER2- or EGFR-amplified preclinical breast cancer models, with lower median inhibitory concentration values for neratinib (6). Finally, neratinib, but not lapatinib, inhibits tumors with a wide spectrum of somatic *HER2* mutations (7–9).

Multiple mechanisms of resistance to HER2-targeted therapies have been reported. These include upregulation of alternative receptor tyrosine kinases (10), upregulation of the estrogen receptor (ER) pathway (11), dysregulation of

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Translational Relevance

Neratinib is an irreversible pan-HER tyrosine kinase inhibitor (TKI), whereas lapatinib is a reversible, HER2-specific TKI. The phase III NALA trial of neratinib + capecitabine (N+C) versus lapatinib + capecitabine (L+C) in patients with HER2-positive metastatic breast cancer (MBC) who had previously received two or more HER2-targeted therapies in the metastatic setting was the basis for FDA approval of neratinib in that setting. Our retrospective biomarker analysis reports that within this HER2-positive group, those with tumors expressing higher HER2 levels had longer progression-free survival (PFS), and greater PFS benefit from N+C vs. L+C. This predictive effect was more pronounced in patients with hormone receptor–negative disease. *PIK3CA* mutation was associated with shorter PFS, whereas *HER2* mutation was associated with longer PFS.

downstream PIK3K/Akt signaling (12), increased expression of p95, a highly active truncated form of HER2 that lacks the extracellular trastuzumab-binding domain (13), and additional upregulation of HER2 via acquired amplifications and/or somatic mutations (14, 15).

The phase III NALA study compared neratinib (240 mg once a day) plus capecitabine (750 mg/m² twice a day on days 1–14 of a 21-day cycle; N+C) with lapatinib (1,250 mg once a day) plus capecitabine (1,000 mg/m² twice a day on days 1–14 of a 21-day cycle; L+C) in patients with HER2-positive (HER2⁺) metastatic breast cancer (MBC) who had received ≥2 prior HER2-directed regimens in the metastatic setting (16). There was a statistically significant benefit in progression-free survival (PFS) favoring N+C (HR = 0.76; 12-month PFS, N+C 29% vs. L+C 15%), translating to a 2.2-month mean PFS improvement without significant benefit in overall survival. Patients treated with N+C had significantly fewer interventions for symptomatic central nervous system disease compared with L+C (cumulative incidence 22.8% vs. 29.2%; *P* = 0.043). Based on these results, the FDA approved N+C for adult patients with advanced or metastatic HER2⁺ breast cancer who have received ≥2 prior anti-HER2-based regimens in the metastatic setting (17).

The high rate of early events in both arms and the largely indistinguishable PFS curves up until 6 months in NALA suggest that the trial enrolled a group of patients resistant to HER2-directed therapies, capecitabine, or both. In this exploratory analysis, we assessed associations of biomarkers with prognostic or predictive PFS benefit (N+C over L+C) in biomarker-defined subgroups in NALA.

Patients and Methods

Study design

NALA (NCT01808573) was a randomized, international, multicenter, parallel-group, active-controlled phase III trial in patients with HER2⁺ MBC. Patient characteristics, eligibility criteria, and treatment have been described (16). The protocol was approved by national/institutional ethics committees at participating sites and conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent. HER2 positivity was centrally confirmed before enrollment as IHC 3+ or IHC 2+/FISH positive (18, 19).

Specimen characteristics

Tumor samples were collected at screening before randomization and classified by study sites as originating from primary or metastatic tumor, and as archival or fresh biopsy. Sites were instructed to send either one formalin-fixed, paraffin-embedded (FFPE) block or 10 unstained, freshly cut 4-μm FFPE slides, and to utilize tissue from the time of metastasis whenever possible.

Assay methods

Biomarkers and assays were selected based on documented evidence of association with response or resistance to HER2-targeted therapies, and, because of limitations in tissue availability, ability to reliably test minimal amounts of tissue. Hormone receptor status was established via central IHC, with Allred total score ≥3 considered ER/progesterone receptor (PR) positive (ER/PR PharmDx Kit, Dako). All assays were performed blinded to study endpoint and treatment assignment. Additional candidate biomarkers that could not be performed due to lack of sufficient tissue included RNA expression, including but not limited to molecular subtype categorization, and retrospective FISH evaluation of all samples.

Somatic mutation analysis

The spectrum of somatic mutations was evaluated by whole-exome next-generation sequencing (NGS; ref. 20). DNA extraction was performed from FFPE slides (1–3 × 4 μm) using Maxwell FFPE Tissue LEV DNA Purification Kit (Promega). All slides were pathologist-evaluated to determine ≥20% of tumor area content. If required, microdissection was performed to enrich for tumor content. DNA quality and concentration were measured with double-stranded DNA (dsDNA) Qubit broad-range assay kit (Invitrogen). A minimum of 4 ng of extracted DNA was input for amplicon-based NGS using VHIO-Card, an in-house developed panel of more than 800 primer pairs targeting hotspot mutations in 55 genes (Supplementary Table S1). PCR products were pooled, and libraries prepared with NEBNext Ultra DNA Library Prep Kit for Illumina (New England BioLabs). Indexed libraries were pooled, loaded onto a HiSeq 2500 System (Illumina), and sequencing performed (2 × 100) at average coverage of more than 1000×.

Somatic mutations identified in *PIK3CA*, *ESR1*, *HER2*, *AKT1*, and *KRAS* were confirmed by droplet digital PCR (ddPCR; QX200 system Bio-Rad Laboratories). These were of special interest because of involvement in HER2 signaling. Custom Taqman SNP genotyping assays for ddPCR were designed and validated with in-house available mutant samples. A black-hole quencher was used with fluorescent dye reporters, fluorescein amidite (FAM) for mutant probes, and 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC) for wild-type (WT) probes (Thermo Fisher Scientific).

HER2 protein expression

HER2 IHC and FISH scores were determined according to manufacturer specifications (HercepTest and IQFISH pharmDX, respectively; Dako). To better understand the spectrum of HER2 protein expression, HER2 was retrospectively evaluated by two additional methods: H-score (Targos GMBH) and HERmark (Monogram Biosciences). H-scores were evaluated from original IHC images. The HERmark assay, which quantitatively measures HER2 receptors using a dual-antibody, proximity-based, fluorescence assay (VeraTag technology; refs. 21, 22), was performed on an adjacent section. p95 levels were evaluated using VeraTag technology and categorized as high or

low based on previously established cut-off points (23, 24). VeraTag measurements were expressed in units of relative fluorescence per square millimeter of invasive tumor (RF/mm²; ref. 21).

Statistical analysis

Efficacy endpoints were assessed in individual biomarker populations, defined as any randomized patient with that biomarker result. The primary analysis methods were log-rank test for hypothesis testing and Cox proportional hazards model to estimate HRs and 95% confidence intervals (CI). Multivariable Cox proportional hazard models were used with the following covariates: treatment, time from diagnosis to randomization, time from metastasis to randomization, Eastern Cooperative Oncology Group (ECOG) performance score, previous lines of HER2 therapy, HER2 IHC, histologic grade at diagnosis, region, ER/PR status, and disease location. Kaplan–Meier methodology was used for time-to-event endpoints. Subpopulation treatment effect pattern plot (STEPP) was used to assess patterns of effect size for continuous HER2 protein variables. Analyses were

performed using SAS (version 9.1; SAS Institute Inc. RRID: SCR_008567) and R (RRID:SCR_001905; ref. 25).

Results

Patients and samples

As previously reported (16), 621 patients with HER2-positive MBC were randomized to N+C or L+C. Most samples originated from primary tumors ($n = 393$; 63%), with the remainder classified as metastatic ($n = 184$; 30%) or otherwise uncharacterized ($n = 63$; 10%). Across the 621 available samples, NGS was successfully performed on 420 patients (68%), H-scoring was possible for 615 (99%) of HER2 IHC-stained slides, HERmark score was reported for 526 patients (85%), and VeraTag p95 was reported for 451 patients (73%; Supplementary Fig. S1). Biomarker-expression characteristics of patients with biomarker analysis results were generally comparable across treatment arms (Table 1). Biomarker-analysis populations were generally comparable with the intent-to-treat population (Supplementary Table S2). HER2 levels were not

Table 1. Biomarker expression by treatment arm for the intention-to-treat population.

Biomarker	N+C (n = 307)	L+C (n = 314)	Total (n = 621)
HER2 IHC (Targos), n (%)			
2+	80 (26.1%)	115 (36.6%)	195 (31.4%)
3+	227 (73.9%)	199 (63.4%)	426 (68.6%)
HERmark, n (%)			
Low	56 (18.2%)	70 (22.3%)	126 (20.3%)
Equivocal	33 (10.7%)	30 (9.6%)	63 (10.1%)
High	174 (56.7%)	163 (51.9%)	337 (54.3%)
Missing	44 (14.3%)	51 (16.2%)	95 (15.3%)
HERmark, RF/mm ²			
Number	263	263	526
Mean	91.5	95.0	93.2
SD	126.8	147.4	137.3
Median	39.0	39.1	39.1
Minimum, maximum	1.3, 764	0.9, 1,179	0.9, 1,179
HER2 H-score, n (%)			
≥240 (median)	164 (53.4%)	146 (46.5%)	310 (49.9%)
<240 (median)	142 (46.3%)	163 (51.9%)	305 (49.1%)
Missing	1 (0.3%)	5 (1.6%)	6 (1.0%)
HER2 H-score			
Number	306	309	615
Mean	229.5	220.2	224.8
SD	57.4	64.8	61.3
Median	240	230	240
Minimum, maximum	42, 300	35, 300	35, 300
p95, RF/mm ²			
Number	224	227	451
Mean	5.0	6.1	5.6
SD	4.6	7.5	6.2
Median	4.0	4.1	4.0
Minimum, maximum	0.5, 30.5	0.5, 76.1	0.5, 76.1
≥2.8	153 (68.3%)	149 (65.6%)	302 (67.0%)
HER2 mutation			
Mutant	14 (4.6%)	9 (2.9%)	23 (3.7%)
WT	203 (66.1%)	194 (61.8%)	397 (63.9%)
Missing	90 (29.3%)	111 (35.4%)	201 (32.4%)
PIK3CA mutation			
Mutant	77 (25.1%)	66 (21.0%)	143 (23.0%)
WT	140 (45.6%)	137 (43.6%)	277 (44.6%)
Missing	90 (29.3%)	111 (35.4%)	201 (32.4%)

distributed evenly between hormone receptor–negative and –positive cohorts; higher HER2 was more common in patients who were hormone receptor negative (Supplementary Table S3), consistent with prior reports (26).

Somatic mutations

After NGS identification, *PIK3CA*, *HER2*, *HER3*, *AKT1*, and *ESR1* activating mutations were orthogonally confirmed by ddPCR for cases with sufficient material ($n = 150/176$; 86%); 100% concordance was observed. Mutation prevalence was: *PIK3CA* 34% (143/420); *HER2* 5% (23/420); *HER3* 1% (4/420), *AKT1* 1% (3/420), and *ESR1* 2% (7/420). *PIK3CA* and *HER2* mutations were balanced between treatment arms (Table 1); mutation frequencies were consistent with prior MBC datasets (27).

PIK3CA

Most *PIK3CA* mutations were kinase-domain hotspots, e.g., H1047R/L ($n = 83$; 56%), or helical-domain hotspots, e.g., E545K/A ($n = 32$; 22%) and E542K ($n = 24$; 16%); patients could have more than one *PIK3CA* mutation (Supplementary Fig. S2A). Ninety-five of the 143 mutations (66%) were identified from primary tissue, 29 (20%) were from metastases, and 19 (13%) were from otherwise unclassified lymph node tissue. *PIK3CA* mutations were distributed similarly in hormone receptor–positive and hormone receptor–negative disease: they were detected in samples from 58 of 180 patients (32.2%) with hormone receptor–negative disease and 85 of 240 patients (35.4%) with hormone receptor–positive disease. *PIK3CA* mutations were also distributed similarly between lower and higher HER2 expression levels (Supplementary Table S4).

The presence of a *PIK3CA* mutation trended toward shorter PFS, independent of treatment [Fig. 1A; WT vs. mutant *PIK3CA*: HR = 0.81 (95% CI, 0.64–1.03); median PFS 5.55 vs. 4.34 months, respectively].

HER2

HER2 gene mutations ($n = 23$) included kinase-domain hotspots L755S ($n = 5$; 22%), D769Y ($n = 5$; 22%), V777 L ($n = 5$; 22%), G776V ($n = 1$; 4%), I767M ($n = 1$; 4%), and L841V ($n = 1$; 4%); extracellular-domain hotspot S310F/Y ($n = 2$; 9%); exon 20 insertion G778_P780dup ($n = 1$; 4%); transmembrane-domain hotspot R678Q ($n = 1$; 4%); and non-hotspot mutations L841V ($n = 1$; 4%) and V669 L ($n = 1$; 4%; Supplementary Fig. S2B). Fifteen of the 23 *HER2* mutations (65.2%) were identified from primary tumor tissue, 5 were from metastases (22%), and 3 were from lymph node tissue (13%). Median PFS was 5.45 months in patients with WT *HER2* tumors and 5.72 months in those with mutant *HER2* tumors [Fig. 1B; WT vs. mutant; HR = 1.686 (95% CI, 0.967–3.288)].

HER2 mutations were not predictive of PFS benefit for N+C over L+C (P -value_{inter} = 0.502; Fig. 1C). After adjusting for other prognostic factors in a multivariable Cox model, *PIK3CA* mutation maintained a trend toward shorter PFS [HR = 0.82 (95% CI, 0.65–1.05, WT vs. mutant)]. *PIK3CA* mutations were not predictive of PFS benefit for N+C over L+C (P -value_{inter} = 0.995; Fig. 1C). Too few *AKT1*, *HER3*, and *ESR1* mutations occurred for meaningful evaluation.

HER2 protein expression

Among 621 patients in NALA, 426 (69%) had IHC 3+, and 195 (31%) had IHC 2+/FISH-positive breast cancer. HER2 protein expression orthogonally measured by H-score and HERmark was well

balanced between treatment arms (Table 1). Differences were observed in the extent of quantification dependent on methodology (Fig. 2A): HERmark reported across a larger dynamic range, particularly at highest HER2 protein levels; H-scores ranged from 35 to 300 (median 240), and HERmark scores ranged from 0.9 to 1,179 RF/mm² (median 39 RF/mm²).

Higher HER2 protein expression was prognostic of longer PFS when both treatment arms were combined, using all three methods (Fig. 2B). Higher HER2 protein level also significantly predicted PFS benefit for N+C versus L+C [Fig. 2C; IHC 3+: HR = 0.64 (95% CI, 0.51–0.81); H-score ≥ 240 : HR = 0.54 (95% CI, 0.41–0.72); HERmark positive: HR = 0.65 (95% CI, 0.50–0.84); P -value_{inter} < 0.001 for IHC and H-score and $P = 0.061$ for HERmark]. Evaluation of HER2 protein level as a continuous variable using STEPP analysis of H-score and HERmark revealed that increasing HER2 protein levels was associated, although not statistically significantly, with HRs favoring the neratinib combination (Fig. 2D).

p95

High p95 was associated with PFS benefit favoring N+C over L+C [Fig. 2C; high p95 (≥ 2.8 RF/mm²): HR = 0.66 (95% CI, 0.50–0.86) vs. low p95 (<2.8 RF/mm²): HR = 0.91 (95% CI, 0.61–1.36); P -value_{inter} = 0.236]. N+C benefit versus L+C was shown in patients with HERmark-positive/p95-high [HR = 0.63 (95% CI, 0.46–0.86)] and HERmark-positive/p95-low [HR = 0.55 (95% CI, 0.29–1.04)] tumors; P -value_{inter} for HERmark/p95 was 0.063 (Fig. 2C). This supports the hypothesis that patients with high levels of active HER2, whether full-length or truncated, derive greater benefit from N+C compared with L+C.

Hormone receptor status

In the intent-to-treat population, PFS benefit from N+C versus L+C was seen in the hormone receptor–negative subgroup [HR = 0.42 (95% CI, 0.31–0.57)] but not in the hormone receptor–positive subgroup [HR = 1.08 (95% CI, 0.84–1.40); ref. 16]. A multivariable Cox model indicated a significant interaction between treatment and hormone receptor status ($P = 0.0038$); the effect of HER2 protein levels was therefore investigated by hormone receptor status (Fig. 3). The predictive effect of HER2 level appeared more pronounced in patients with hormone receptor–negative tumors. Patients whose tumors were both hormone receptor–negative and high in HER2 protein expression derived the greatest PFS benefit from N+C compared with L+C [IHC 3+: HR = 0.35 (95% CI, 0.25–0.49); H-score ≥ 240 : HR = 0.32 (95% CI, 0.22–0.46); HERmark-positive: HR = 0.35 (95% CI, 0.24–0.51)].

Discussion

This unplanned, exploratory analysis examined somatic mutations, HER2 protein expression, and hormone receptor status and their association with outcomes in NALA, providing meaningful information on the potential role of biomarkers in clinical outcomes for this patient population.

PIK3CA mutation has been described as a biomarker of resistance to multiple HER2-targeted therapies in MBC (28–30). The trend observed for shorter PFS with *PIK3CA* mutation in NALA is therefore as expected. In contrast, no associations between *PIK3CA* mutations and prognosis have been reported for patients with HER2-positive breast cancer undergoing adjuvant treatment with targeted agents, including neratinib in the extended adjuvant setting (31–33).

A

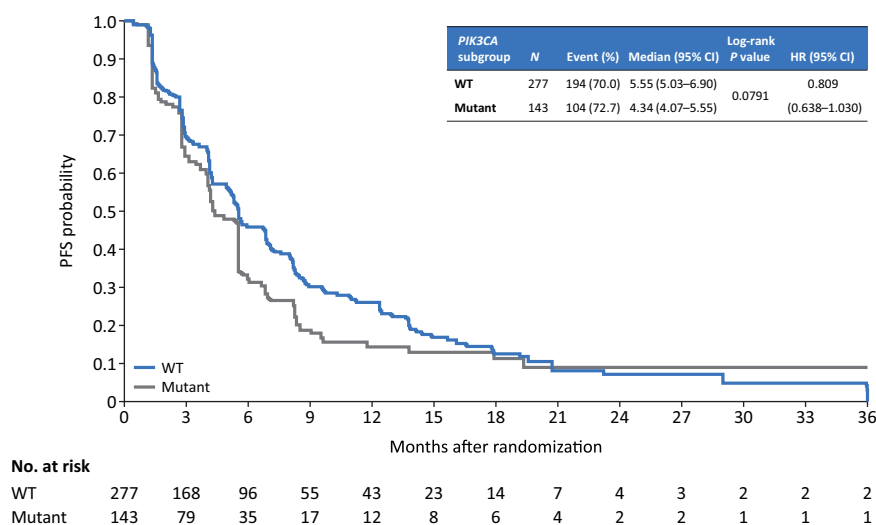
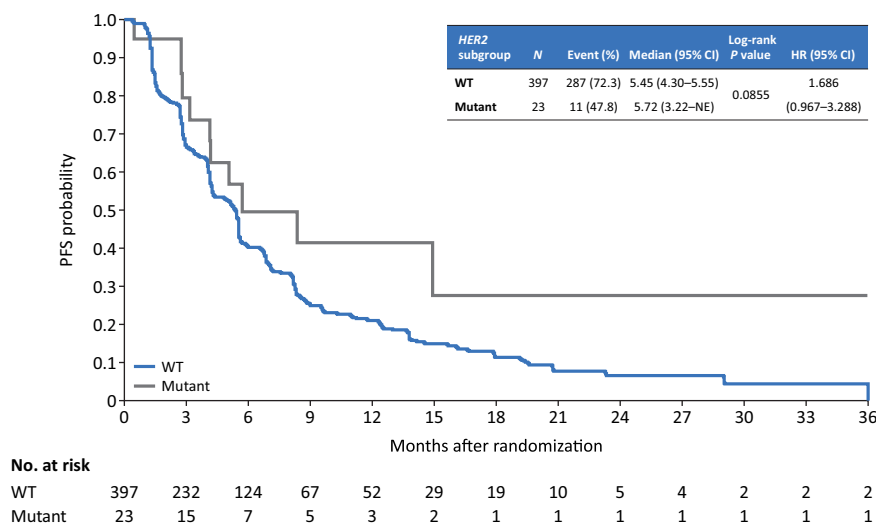


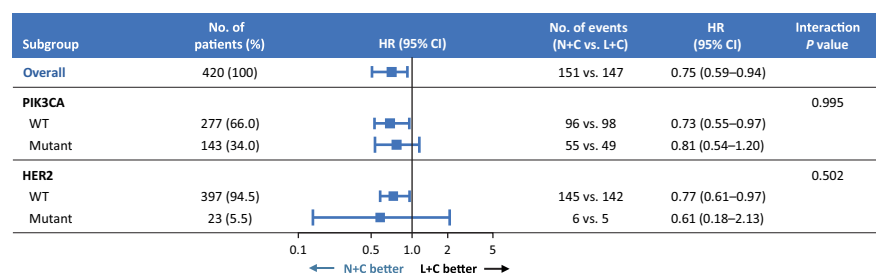
Figure 1.

A, *PIK3CA* mutations were associated with worse PFS outcome, independent of treatment arm. **B**, *PIK3CA* mutations and *HER2* mutations were not predictive of PFS benefit for neratinib versus lapatinib. **C**, *HER2* mutations were associated with a trend toward better PFS outcome, independent of treatment arm. NE, not evaluable.

B



C



HER2 somatic mutations have been adversely associated with prognosis in the absence of *HER2*-targeted therapy (34). In contrast, *HER2* mutations in patients in NALA tended to be associated with better prognosis, although this interpretation is limited by a small sample size ($n = 23$). This may be due to prior exposure to *HER2*-targeted therapy in NALA, although neither trastuzumab nor pertuzumab—the most common therapies received before study entry—has

proven activity against *HER2*-mutant MBC (35). Preclinically, tumors with *HER2* mutations are more sensitive to neratinib than lapatinib (7–9). In patients with MBC whose tumors harbored somatic *HER2* mutations in the absence of *HER2* amplification, the SUMMIT trial (NCT01953926) reported responses to neratinib monotherapy or in combination with fulvestrant (15). *HER2* mutations coincident with amplification are less well characterized, although *HER2* amplification

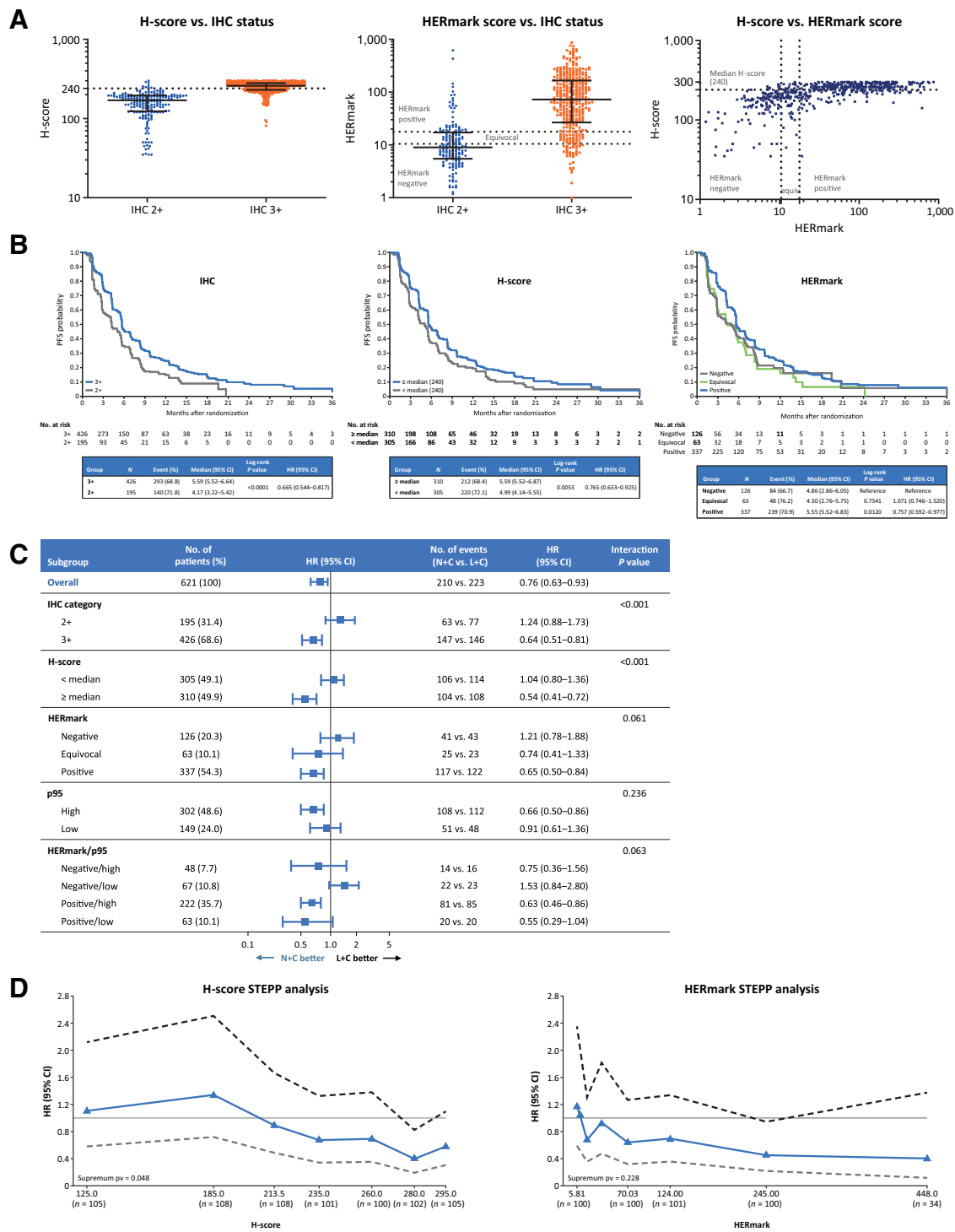
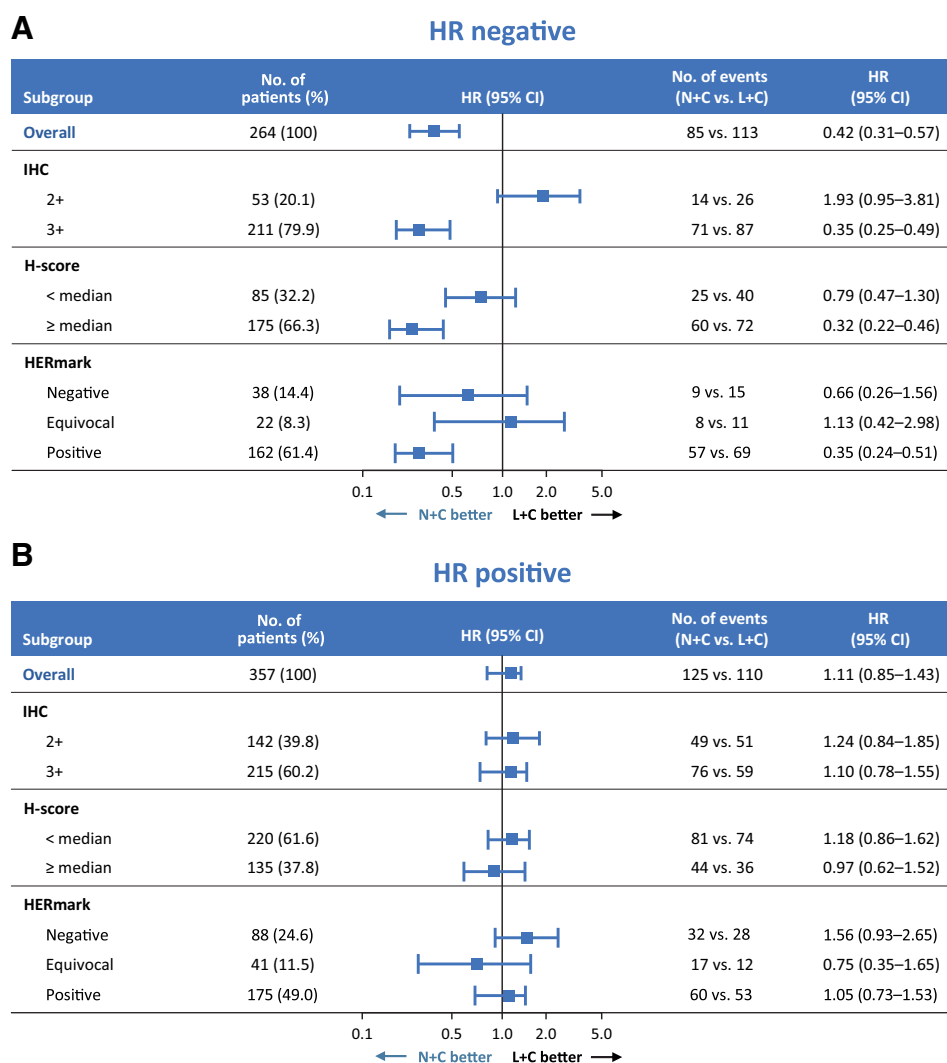


Figure 2. HER2 protein expression. **A**, Comparison of test methods. All three methods detect HER2 protein expression but differ in the extent of their quantification. HERmark has a wider dynamic range than H-score, especially at the highest levels of protein expression. **B**, Orthogonal methods show association of increased HER2 with better PFS, independent of treatment. **C**, Forest plot of PFS according to HER2 testing methods. HERmark-negative, -equivocal, and -positive categories, and p95 high and low, were based on published cutpoints (22, 23). Higher HER2 protein expression measured by all three methods correlated with better response to neratinib over lapatinib. **D**, STEPP analysis of H-score and HERmark results showing PFS of N+C versus L+C for different HER2 levels. pv, P value.

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**Figure 3.**

Biomarker expression according to hormone receptor status: hormone receptor-negative (**A**) and hormone receptor-positive (**B**) tumors.

has been described as an acquired resistance mechanism to neratinib in *HER2*-mutated MBC (15). Notably, *HER2* mutations in NALA were identified in tissues from both primary and metastatic samples, suggesting that *HER2* mutation and amplification can potentially, albeit very rarely, emerge together.

HER2 protein level was a clear response biomarker in NALA. Not only was higher *HER2* expression prognostic of PFS, but patients with tumors expressing higher *HER2* levels also benefited more from N+C compared with L+C. This association may help explain the late separation of PFS Kaplan–Meier curves in the primary clinical analysis, as tumors with lower *HER2* protein level may simply be less sensitive to *HER2*-targeted therapies. Notably, because of differences in the maximum tolerated dose of capecitabine with neratinib and lapatinib (36), the capecitabine dose was lower in the N+C arm (750 mg/m² twice a day) than in L+C (1,000 mg/m² twice a day) in NALA; consequently, the capecitabine component in NALA regimens may have been responsible for response to treatment among patients whose tumors were not *HER2* driven, including those with low *HER2* expression.

Neratinib and lapatinib are both small-molecule TKIs; however, neratinib is considered a more potent inhibitor (3). Our results are consistent with prior phase III clinical trials reporting association of

higher *HER2* levels with better response to the stronger *HER2*-targeting regimen in *HER2*⁺ MBC: CLEOPATRA, EMILIA, and TH3RESA all reported increased PFS favoring the more potent regimen in patients with greater *HER2* mRNA expression (28, 29, 37). In the adjuvant setting, APHINITY reported increased benefit with the stronger regimen in patients with higher *HER2* protein levels, as measured by IHC (38). Finally, in the neoadjuvant NeoALTTO trial, the predicted probability of pathologic complete response to lapatinib + trastuzumab versus either agent alone was associated with increasing *HER2* levels as measured by HERmark (39). Although beyond the scope of this manuscript, it would be interesting to understand whether the same trend holds for the novel *HER2*-specific, reversible TKI tucatinib, which has been reported to be less potent than neratinib when comparing IC₅₀ values in the context of cell-line models (40). Within the context of *HER2* positivity, p95 quantitation did not provide additional predictive or prognostic information, perhaps due to the fact that p95 trended with HERmark score in patients who were *HER2* positive, and/or that both neratinib and lapatinib bind to the intracellular domain of *HER2*.

Negative hormone receptor status in NALA was associated with a trend toward better prognosis and increased response to N+C compared with L+C. The ExteNET extended adjuvant trial, in contrast,

reported an association of neratinib with improved invasive disease-free survival in patients with hormone receptor–positive status (41). Cross-talk between the ER and HER2 signaling pathways, which has been well described (42, 43), may explain this apparent discrepancy. Patients with hormone receptor–positive disease received coincident hormonal therapy in ExteNET; however, consistent with standard of care and with other MBC trials in similar populations, hormonal therapy was not permitted in NALA. Preclinical models of ER-positive/HER2⁺ breast cancer evaded HER2 blockade with neratinib via upregulation of ER-pathway signaling, and conversely, inhibition with hormonal therapy directed upregulation of HER2 signaling (44). Dual inhibition with endocrine therapy and neratinib may warrant evaluation in patients with hormone receptor–positive, HER2⁺ MBC.

A primary limitation of this study is the lack of a prerandomization biopsy, which necessitated use of a combination of primary and metastatic samples, resulting in an analysis that may not reflect all the molecular changes acquired due to anticancer therapies administered after biopsy. A second consideration is the limited number of slides that were available for each patient (2–4 slides per patient in general). This restricted the analyses that could be performed, e.g., gene-expression profiling was technically unfeasible for such limited tissue.

In conclusion, meaningful biomarker associations were identified in this analysis of tumor samples from patients included in the NALA study. Patients with MBC with high HER2 protein expression regardless of p95 level may benefit most from N+C. Exploration of alternative therapies in patients with low HER2 may be warranted, and patients with high HER2 and hormone receptor–positive disease, and/or *PIK3CA* mutations, may derive greater benefit from addition of endocrine therapy or PI3K pathway inhibitors.

Data Availability Statement

The authors declare that the data supporting the findings of this study are available within the article. The authors may be contacted for further data sharing.

Authors' Disclosures

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