HER2 Copy Number and Resistance Mechanisms in Patients with HER2-positive Advanced Gastric Cancer Receiving Initial Trastuzumab-based Therapy in JACOB Trial

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ABSTRACT

Purpose: In JACOB trial, pertuzumab added to trastuzumab-chemotherapy did not significantly improve survival of patients with HER2-positive metastatic gastric cancer, despite 3.3 months increase versus placebo. *HER2* copy-number variation (CNV) and AMNESIA panel encompassing primary resistance alterations (*KRAS/PIK3CA/MET* mutations, *KRAS/EGFR/MET* amplifications) may improve patients' selection for HER2 inhibition.

Experimental Design: In a *post hoc* analysis of JACOB on 327 samples successfully sequenced by next-generation sequencing (NGS; Oncomine Focus DNA), *HER2* CNV, HER2 expression by IHC, and AMNESIA were correlated with overall response rate (ORR), progression-free survival (PFS), and overall survival (OS) by univariable/multivariable models.

Results: Median *HER2* CNV was 4.7 (interquartile range, 2.2–16.9). *HER2* CNV-high versus low using the median as cutoff was associated with longer median PFS (10.5 vs. 6.4 months; HR = 0.48; 95% confidence interval: 0.38–0.62; $P < 0.001$) and OS (20.3 vs. 13.0 months; HR = 0.54; 0.42–0.72; $P < 0.001$). Combining *HER2* CNV and IHC improved discriminative ability, with better outcomes restricted to *HER2*-high/HER2 3+ subgroup. AMNESIA positivity was found in 51 (16%), with unadjusted HR = 1.35 (0.98–1.86) for PFS; 1.43 (1.00–2.03) for OS.

In multivariable models, only *HER2* CNV status remained significant for PFS ($P < 0.001$) and OS ($P = 0.004$). Higher ORR was significantly associated with IHC 3+ [61% vs. 34% in 2+; OR = 3.11 (1.89–5.17)] and *HER2*-high [59% vs. 43% in *HER2*-low; OR = 1.84 (1.16–2.94)], with highest OR in the top CNV quartile. These biomarkers were not associated with treatment effect of pertuzumab.

Conclusions: *HER2* CNV-high assessed by NGS may be associated with better ORR, PFS, and OS in a JACOB subgroup, especially if combined with HER2 3+. The negative prognostic role of AMNESIA requires further clinical validation.

Introduction

In patients with HER2-positive metastatic gastric cancer or gastroesophageal junction cancer (GEJC), trastuzumab plus platinum/fluoropyrimidine first-line chemotherapy has remained the standard of care for over 10 years based on the ToGA trial (1) and HER2 testing by means of IHC and ISH has been the main driver of initial treatment decision-making for trastuzumab treatment in the clinical practice. Several pivotal studies with other anti-HER2 strategies have failed during subsequent years (2–5), whereas newer agents or combinations such as trastuzumab deruxtecan and pembrolizumab/trastuzumab plus chemotherapy showed promising activity that led to their FDA approval pending survival data (6, 7). Among negative studies, the JACOB trial failed to demonstrate a significant improvement in overall survival (OS) with the addition of pertuzumab to trastuzumab and chemotherapy in the first line setting, even though a 3.3-month increase in median OS (mOS) was reported (2).

Long-term benefit from trastuzumab-based first-line therapy is observed in a minority (about 15%) of patients and the potential biological explanations are multiple. First, research showed that higher *HER2* copy-number variation (CNV) in tumor cells is associated with superior outcomes after HER2-targeting treatments (8, 9), because HER2 "hyperamplification" may be a surrogate of HER2 addiction and is clearly associated with long-term responses to trastuzumab. Similar results have been reported for HER2 overexpression assessed by IHC or mass spectrometry (1, 10, 11).

In terms of mechanisms of primary resistance, we showed the clinical validity and negative prognostic role of candidate genomic alterations, grouped together in the so-called AMNESIA panel: *EGFR/MET/KRAS/PI3K* mutations and *EGFR/MET/KRAS* amplifications (12).

On the basis of these considerations, we hypothesized that optimized patients' positive selection based on *HER2* CNV and HER2 IHC and/or negative selection based on primary resistance mechanisms

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Translational Relevance

In this *post hoc* analysis of the JACOB trial, *HER2* copy-number variation (CNV), HER2 expression, and AMNESIA were correlated with treatment outcomes. HER2 CNV assessed by next-generation sequencing may be a new biomarker associated with HER2 addiction and exceptional responsiveness to HER2 inhibition and should be implemented in future trials.

could lead to the identification of patients with long-term benefit from trastuzumab-based therapy or to the identification of those with benefit from dual HER2 blockade strategies. Therefore, we performed a translational study with next-generation sequencing (NGS) aimed at assessing the prognostic and predictive role of the above-mentioned biomarkers in a subset of patients with HER2-positive metastatic gastric cancer/GEJC enrolled in the JACOB trial and receiving trastuzumab and chemotherapy with or without pertuzumab.

Materials and Methods

Patients

JACOB was a double-blind, placebo-controlled Phase III trial that investigated the addition of pertuzumab to trastuzumab and chemotherapy as first-line treatment of patients with HER2-positive metastatic or unresectable gastric cancer/GEJC. HER2 positivity was centrally confirmed for eligibility and defined as IHC 3+ or IHC 2+ and ISH positive by using PATHWAY anti-HER2/neu (4B5) IHC and the INFORM HER2 Dual ISH assays (Ventana Medical Systems). The data generated in the current study are a *post hoc* translational analysis conducted in 580 of 780 patients who consented to future research and had available extracted leftover DNA after tumor tissue prescreening. The study was carried out in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. This translational study was approved by the Ethic Committee of Fondazione IRCCS Istituto Nazionale dei Tumori (INT 111/19) and all trial patients had signed an informed consent for future research.

NGS

Tumor DNA was extracted from all samples at the central lab after wet macrodissection according to the DNA Sample Preparation Kit (Roche). A total of 20 ng of DNA was used to build the Oncomine Focus DNA Assay panel libraries (Thermo Fisher Scientific, Inc.), using the Ion AmpliSeq Library kit 2.0 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A total of 30 uniquely barcoded library samples were pooled for sequencing per run on an Ion 530 chip (Thermo Fisher Scientific, Inc.) for an expected mean read depth of 300×.

BAM files derived from processed raw data were generated with the Ion Reporter Software (v. 5.10.5.0; Thermo Fisher Scientific, Inc.) and analyzed for single-nucleotide variants, indels [variant allele frequency (VAF) > 10%], and CNVs [for sample with a median absolute pairwise difference (MAPD) ≤ 0.5] by the Oncomine Focus w2.4 - DNA - Single Sample (v. 5.10) pipeline. Finally, a custom filter chain was applied to report only likely somatic mutations with a VAF ≥ 0.1 and a minor allele frequency or global allele frequency in ExAC or 5,000 exomes databases ≤1.0E-6. Mutations must also be nonsynonymous and occur in exonic or splice-site regions. *MET*, *EGFR*, and *KRAS* amplification were defined by the presence of CNV ≥ 4.

Statistical analysis

Progression-free survival (PFS), OS, and overall response rate (ORR) were defined as in the original publication. This study was a *post hoc* exploratory analysis without a formal statistical hypothesis. Interquartile ranges (IQR) were used to report distribution of continuous variables. Confidence intervals (CI) were calculated at a 95% level. Categorical data distribution was tested with χ^2 and Fisher exact tests as appropriate. Mann–Whitney *U* test was used for the comparisons of continuous nonparametric data. Multivariate logistic regression was used to model categorical data. Right-censored variables were modeled with univariable and multivariate Cox regressions; Schoenfeld residuals were used to test the assumption of linearity of the hazard over time; symmetricity of the residuals deviance over linear predictions was inspected to check the presence of outliers; performance of the Cox models was measured with the concordance index (Harrel C-index); and the precision of prognostication was evaluated by the 95% CIs of the ORs and HRs. Univariate spline regression with 2 degrees of freedom was used to investigate the presence of nonlinear interplays of variables of interest with OS. To test the predictive value of each biomarker for the benefit from the addition of pertuzumab, Cox regression with the interaction term between the treatment arm and the respective variable was used.

Data were imported and handled in R v4.1.2, using ggplot2, dplyr, survminer, survival, finalfit, and ComplexHeatmap packages (11).

Data availability statement

A specific data sharing agreement with Roche and Fondazione IRCCS Instituto Nazionale dei Tumori, Milan will be needed. Also, requests for data should be directed to the corresponding author.

Results

Patient population

As shown in Supplementary Fig. S1, the biomarker-evaluable population included a subset of 327 of 780 patients from the JACOB trial (42% of the intention-to-treat population) with available DNA derived from tumor tissue and successful sequencing data. **Table 1** shows the main patients and disease baseline characteristics including treatment arm by median *HER2* CNV and HER2 IHC status. The median value of *HER2* CNV was 4.7 (IQR, 2.2–16.9). HER2 score 3+ status was detected in 212 (64.8%) patients, whereas 51 (15.6%) patients had at least one genetic alteration included in AMNESIA panel. The investigated biomarkers were well balanced in the two treatment arms.

In **Table 1**, the median values of *HER2* CNV, HER2 IHC, and AMNESIA status are also reported and compared in each baseline subgroup. Notably, the *HER2* CNV was significantly increased in patients bearing HER2 IHC score 3+ tumors. The heatmap in **Fig. 1** shows the distribution of the AMNESIA panel alterations along with relevant clinical features and other investigated biomarkers. Notably, these putative resistance alterations were enriched in the *HER2* CNV-low versus CNV-high subgroup using the median as cutoff (21.3% vs. 9.8%, $P = 0.007$).

Survival analysis

Supplementary Figure S2 shows PFS and OS according to treatment arm in the biomarker-evaluable population, with lack of significant differences between the study arms. We first explored the prognostic impact of *HER2* CNV using the median value of 4.7 as the cutoff. Patients with *HER2* CNV-high status had significantly superior PFS [median PFS (mPFS): 10.5 vs. 6.4 months; HR = 0.48; 95% CI:

Table 1. Patients' and disease baseline features in the overall study population. Distribution of selected biomarkers according to baseline features.

Baseline variables	Overall	Median *HER2* CNV (IQR)	*P*	HER2 IHC 2+	HER2 IHC 3+	*P*	AMNESIA−	AMNESIA+	*P*
Overall	327 (100%)	4.7 (2.2–16.9)	—	115 (35.2)	212 (64.8)	—	276 (84.4)	51 (15.6)	—
Age			0.228			0.236			0.107
<65	178 (54.4)	5.8 (2.2–18.0)		57 (49.6)	121 (57.1)		156 (56.5)	22 (43.1)	
≥65	149 (45.6)	3.8 (2.1–15.8)		58 (50.4)	91 (42.9)		120 (43.5)	29 (56.9)	
Sex			0.300			0.541			1
Female	76 (23.2)	4.2 (2.0–15.8)		24 (20.9)	52 (24.5)		64 (23.2)	12 (23.5)	
Male	251 (76.8)	5.2 (2.2–17.3)		91 (79.1)	160 (75.5)		212 (76.8)	39 (76.5)	
ECOG PS			0.176			0.093			0.499
0	158 (48.5)	6.3 (2.3–17.6)		48 (41.7)	110 (52.1)		136 (49.5)	22 (43.1)	
1	168 (51.5)	3.8 (2.1–15.1)		67 (58.3)	101 (47.9)		139 (50.5)	29 (56.9)	
Histology			0.059			0.787			0.309
Diffuse/mixed	28 (8.6)	3.1 (2.4–5.4)		11 (9.6)	17 (8.0)		26 (9.4)	2 (3.9)	
Intestinal	299 (91.4)	5.6 (2.2–17.9)		104 (90.4)	195 (92.0)		250 (90.6)	49 (96.1)	
Primary tumor			0.424			0.642			0.77
GEJ	79 (24.2)	4.2 (2.2–23.8)		30 (26.1)	49 (23.1)		68 (24.6)	11 (21.6)	
Stomach	248 (75.8)	4.7 (2.2–15.8)		85 (73.9)	163 (76.9)		208 (75.4)	40 (78.4)	
Gastrectomy			0.527			0.255			0.409
No	211 (64.5)	4.2 (2.2–14.9)		69 (60.0)	142 (67.0)		175 (63.4)	36 (70.6)	
Yes	116 (35.5)	6.1 (2.1–22.0)		46 (40.0)	70 (33.0)		101 (36.6)	15 (29.4)	
Metastatic sites			0.844			0.874			0.107
1–2	250 (76.5)	4.7 (2.2–16.5)		89 (77.4)	161 (75.9)		216 (78.3)	34 (66.7)	
>2	77 (23.5)	4.2 (2.1–17.3)		26 (22.6)	51 (24.1)		60 (21.7)	17 (33.3)	
HER2 IHC			<0.001			—			0.255
2+	115 (35.2)	2.1 (1.8–2.6)		—	—		93 (33.7)	22 (43.1)	
3+	212 (64.8)	10.4 (3.9–26.0)		—	—		183 (66.3)	29 (56.9)	
Treatment arm			0.737			0.743			0.928
Trastuzumab plus placebo	168 (51.4)	4.9 (2.1–17.8)		61 (53.0)	107 (50.5)		141 (51.1)	27 (52.9)	
Trastuzumab plus pertuzumab	159 (48.6)	4.6 (2.2–14.9)		54 (47.0)	105 (49.5)		135 (48.9)	24 (47.1)	

Abbreviations: CNV, copy-number variation; ECOG, Eastern Cooperative Oncology Group; GEJ, gastroesophageal junction; IHC, immunohistochemistry; IQR, interquartile range; PS, performance status.

0.38–0.62; $P < 0.001$] and OS (mOS: 20.3 vs. 13.0 months; HR = 0.54; 95% CI: 0.42–0.72; $P < 0.001$) compared with *HER2* CNV-low (**Fig. 2A** and **B**). Similarly, patients with IHC 3+ status had significantly superior PFS (mPFS: 9.5 vs. 6.3 months; HR = 0.55; 95% CI: 0.43–0.71; $P < 0.001$) and OS (mOS: 18.6 vs. 13.0 months; HR = 0.64; 95% CI: 0.49–0.85; $P = 0.002$) compared with HER2 2+ (**Fig. 2C** and **D**). On the opposite, patients with AMNESIA positivity had a nonsignificantly inferior PFS (mPFS: 6.3 vs. 8.3 months; HR = 1.35; 95% CI: 0.98–1.86; $P = 0.066$) and significantly shorter OS (mOS: 12.7 vs. 16.9 months; HR = 1.43; 95% CI: 1.00–2.03; $P = 0.047$) compared with those with AMNESIA negative status (**Fig. 2E** and **F**). Supplementary Table S1 shows the prognostic effect of each individual genomic

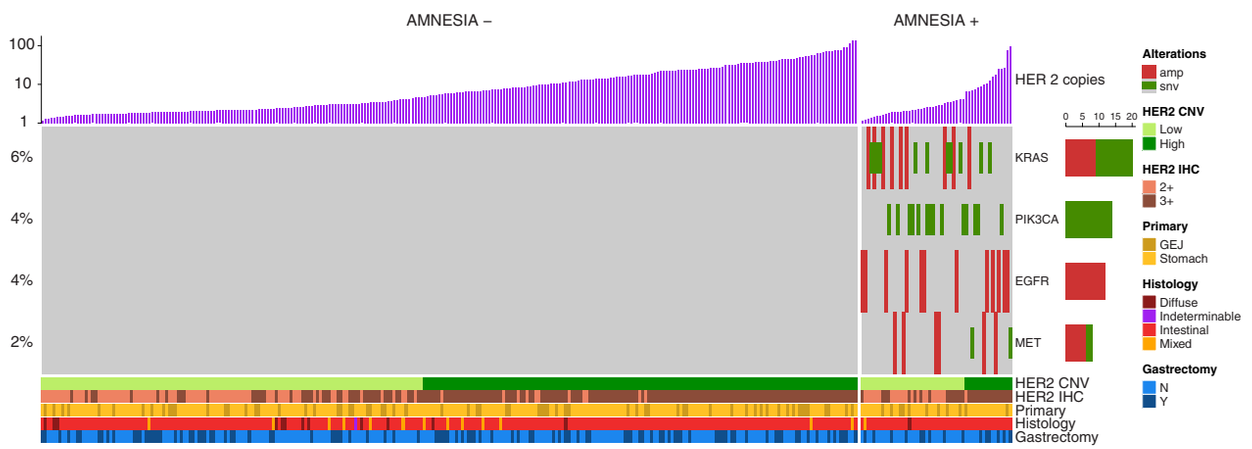

Figure 1.
Heatmap showing the distribution of the AMNESIA panel alterations along with the other investigated biomarkers and clinically relevant tumor features in the study cohort.

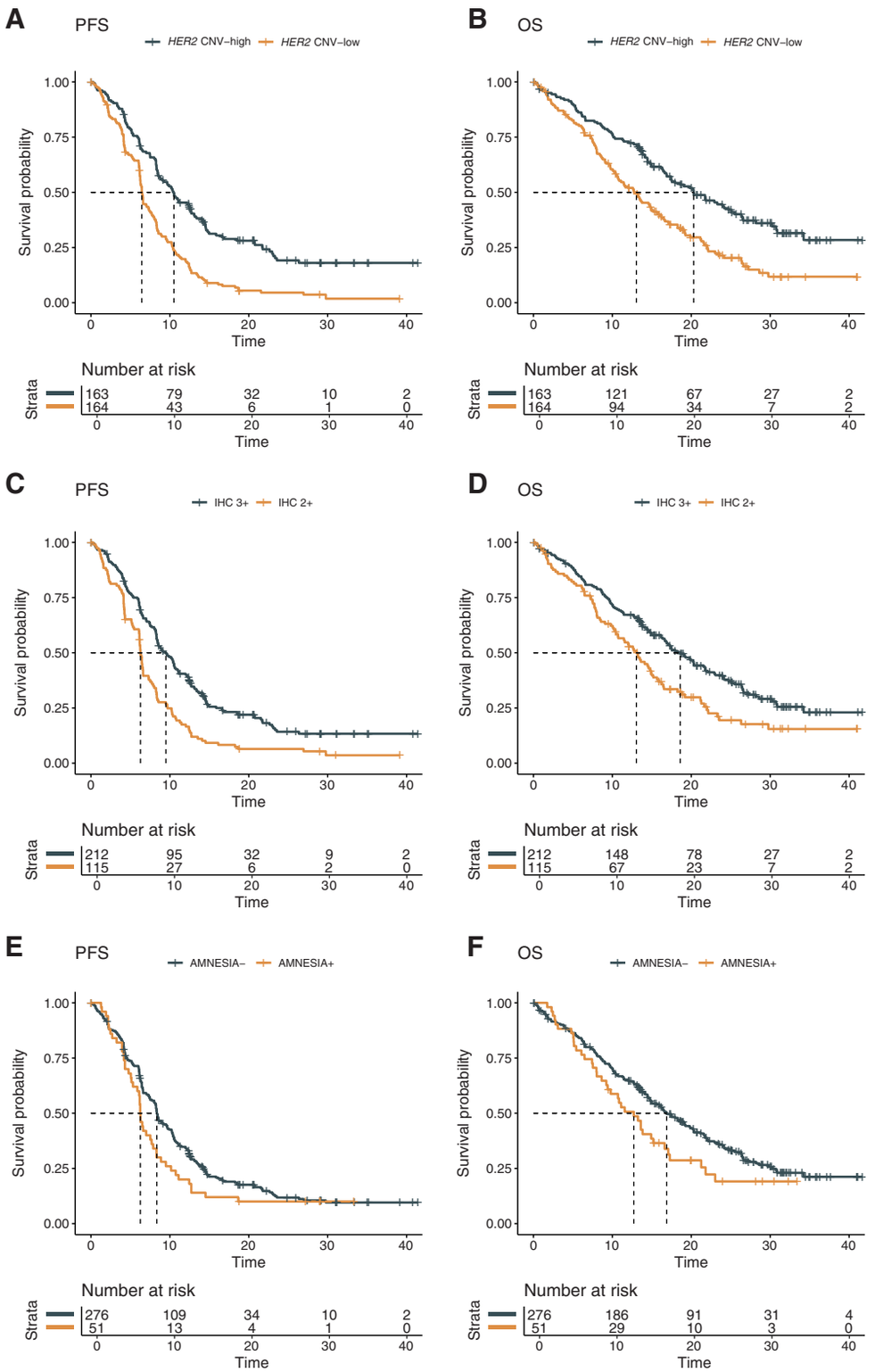

Figure 2.

Kaplan–Meier curves of PFS and OS according to *HER2* CNV-high versus -low status (**A** and **B**), HER2 IHC 3+ versus 2+ (**C** and **D**), and AMNESIA panel positive versus negative status (**E** and **F**).

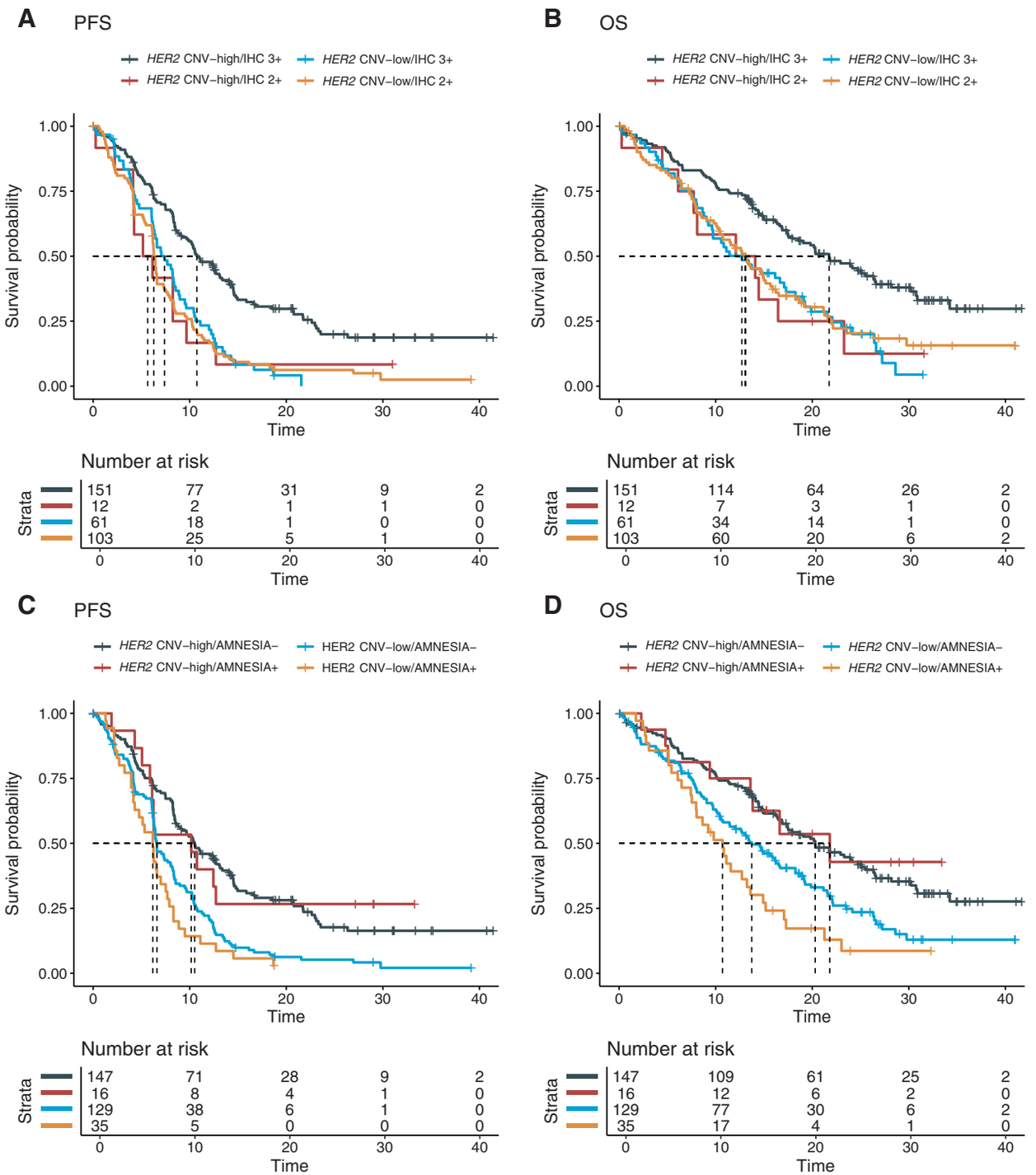

Figure 3:
Kaplan–Meier curves of PFS and OS according to the combined assessment of *HER2* CNV status and HER2 IHC (**A** and **B**) to the combined assessment of *HER2* CNV status and AMNESIA panel status (**C** and **D**).

alteration included in the AMNESIA panel. Specifically, after *P*-value adjustment, only KRAS mutations and MET coamplifications were significantly associated with worse outcomes.

We then performed a combined assessment of *HER2* CNV with HER2 IHC or AMNESIA status (Supplementary Table S2). The coexistence of *HER2* CNV-high with HER2 IHC 3+ status identified the only subgroup of patients with a remarkably longer PFS and OS (**Fig. 3A** and **B**), therefore the combined use of HER2 IHC and HER2 CNV ameliorated the prognostic stratification, whereas the AMNESIA panel was associated with inferior outcomes only in the HER2 CNV-low subgroup (**Fig. 3C** and **D**). When considering the number of *HER2* gene copies as a continue variable, we observed a nonlinear correlation with OS only in the HER2 IHC 3+ subgroup (Supplementary Fig. S3; $P = 0.001$ for the nonlinear term) but not for the HER2 IHC 2+ ($P = 0.21$ for the nonlinear term).

Finally, we built univariate and multivariable Cox proportional hazards regression models for both PFS and OS (**Table 2**). Notably, *HER2* CNV status was significantly associated with both PFS ($P < 0.001$) and OS ($P = 0.004$) in the multivariable analyses, whereas HER2 IHC or AMNESIA status were not.

Activity analysis

In the subgroup of patients with measurable disease ($n = 292$), we then investigated the impact of *HER2* CNV, HER2 IHC, and AMNESIA status on the ORR according to RECIST v1.1 (**Fig. 4**). *HER2* CNV-high status was significantly associated with higher ORR versus *HER2* CNV-low (59.0% vs. 43.9%, OR = 1.83; 95% CI: 1.13–3.01; $P = 0.010$), as well as HER2 IHC 3+ vs. 2+ (61.2% vs. 33.7%; OR = 3.09; 95% CI: 1.83–5.30; $P < 0.001$), whereas AMNESIA negativity was not (52.8% vs. 43.5% in AMNESIA positive; OR = 1.45; 95% CI: 0.73–2.91; $P = 0.264$).

Treatment effect

We then investigated the differential efficacy and activity of the treatment effect (pertuzumab vs. placebo) according to *HER2* CNV, HER2 IHC, and AMNESIA status. No significant interaction between treatment arm and specific subgroups (*HER2* CNV-high vs. -low, HER2 IHC 3+ vs. 2+, AMNESIA positive vs. negative) was observed in terms of OS, PFS, and ORR (Supplementary Fig. S4). This was consistent with the treatment effect by HER2 CNV quartiles (Supplementary Fig. S5).

Table 2. Univariate and multivariable Cox proportional hazards regression models for PFS and OS.

	PFS		OS	
	Univariate HR (95% CI, *P*)	**Multivariate HR (95% CI, *P*)**	**Univariate HR (95% CI, *P*)**	**Multivariate HR (95% CI, *P*)**
Age				
<65	—	—	—	—
≥65	0.99 (0.78–1.26, $P = 0.945$)	—	0.89 (0.68–1.16, $P = 0.392$)	—
Sex				
Female	—	—	—	—
Male	0.88 (0.66–1.17, $P = 0.375$)	—	0.77 (0.57–1.05, $P = 0.101$)	—
ECOG PS				
0	—	—	—	—
1	1.28 (1.01–1.63, $P = 0.042$)	1.26 (0.99–1.60, $P = 0.061$)	1.79 (1.37–2.35, $P < 0.001$)	1.75 (1.33–2.29, $P < 0.001$)
Histology				
Diffuse/mixed	—	—	—	—
Intestinal	0.64 (0.42–0.97, $P = 0.035$)	0.74 (0.48–1.12, $P = 0.155$)	0.56 (0.36–0.87, $P = 0.010$)	0.63 (0.40–1.00, $P = 0.049$)
Primary				
GEJ	—	—	—	—
Stomach	0.95 (0.72–1.26, $P = 0.719$)	—	1.18 (0.84–1.64, $P = 0.339$)	—
Gastrectomy				
No	—	—	—	—
Yes	0.71 (0.55–0.92, $P = 0.010$)	0.72 (0.55–0.94, $P = 0.017$)	0.80 (0.60–1.07, $P = 0.129$)	—
Metastatic sites				
1–2	—	—	—	—
>2	1.39 (1.05–1.83, $P = 0.020$)	1.32 (1.00–1.76, $P = 0.053$)	1.45 (1.07–1.96, $P = 0.016$)	1.43 (1.05–1.95, $P = 0.022$)
HER2 IHC				
2+	—	—	—	—
3+	0.55 (0.43–0.71, $P < 0.001$)	0.78 (0.57–1.07, $P = 0.129$)	0.64 (0.49–0.85, $P = 0.002$)	0.93 (0.66–1.31, $P = 0.664$)
HER2 CNV				
≤4.7	—	—	—	—
>4.7	0.48 (0.38–0.62, $P < 0.001$)	0.56 (0.41–0.77, $P < 0.001$)	0.55 (0.42–0.72, $P < 0.001$)	0.60 (0.42–0.85, $P = 0.004$)
AMNESIA				
Negative	—	—	—	—
Positive	1.35 (0.98–1.86, $P = 0.066$)	—	1.43 (1.00–2.03, $P = 0.047$)	1.19 (0.83–1.71, $P = 0.346$)
Treatment arm				
Trastuzumab plus placebo	—	—	—	—
Trastuzumab plus pertuzumab	0.93 (0.73–1.18, $P = 0.545$)		0.99 (0.76–1.29, $P = 0.928$)	

Note: Harrell C-Indices for the PFS and the OS multivariate models were, respectively, 63.1% ± 1.7% and 64.0% ± 1.8%.
Abbreviations: CNV, copy-number variation; ECOG, Eastern Cooperative Oncology Group; GEJ, gastroesophageal junction; HR, hazard ratio; IHC, immunohistochemistry; OS, overall survival; PFS, progression-free survival; PS, performance status.

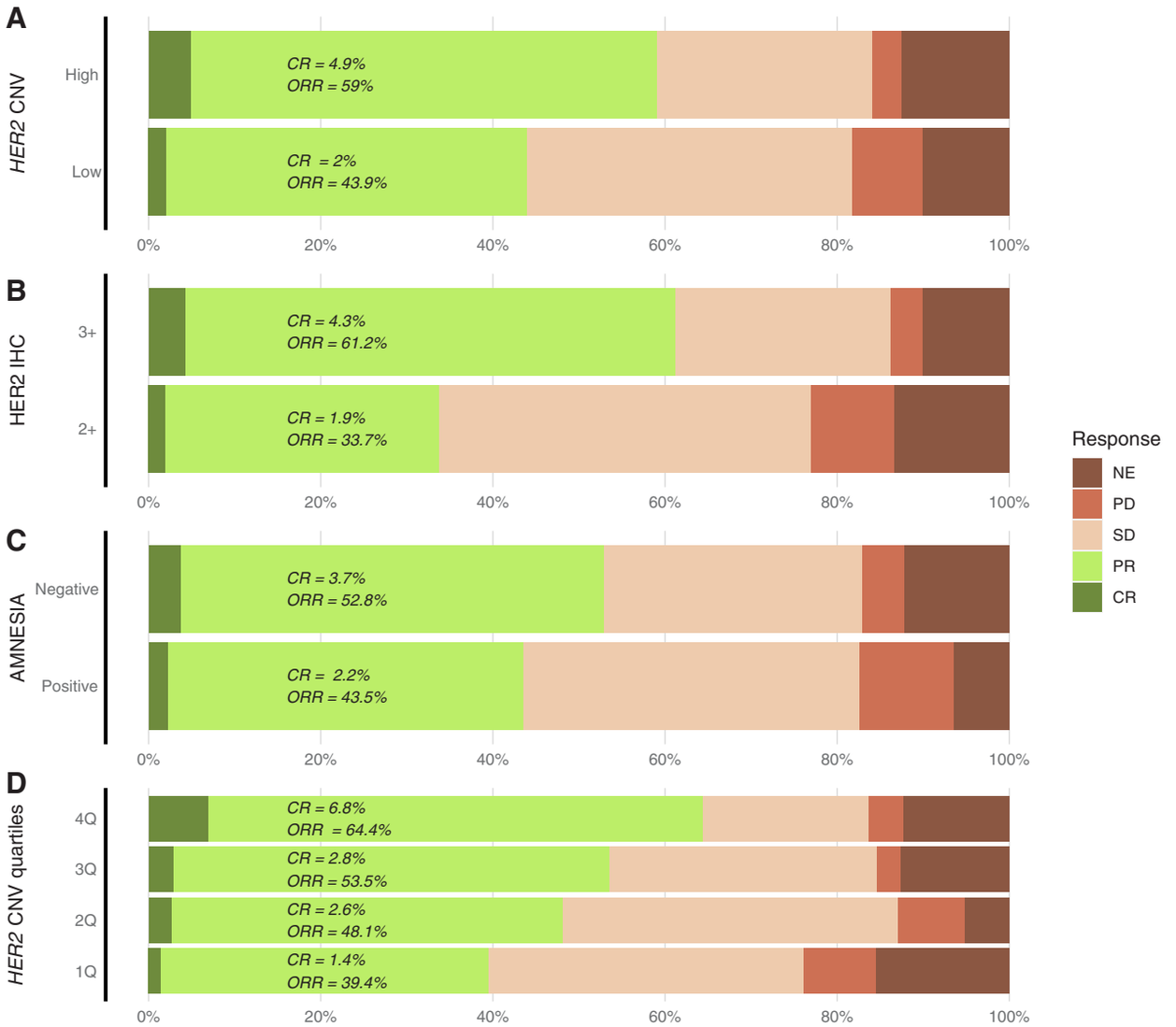

Figure 4.
Tumor response based on RECIST v1.1 and according to *HER2* CNV-high versus -low status (**A**), HER2 IHC 3+ versus 2+ (**B**), AMNESIA panel positive versus negative status (**C**), and *HER2* CNV quartiles (**D**).

Discussion

In this *post hoc* translational analysis carried out in a subset of patients with HER2-positive metastatic gastric cancer/GEJC enrolled in the JACOB trial and treated with trastuzumab plus chemotherapy with or without pertuzumab, we showed that *HER2*-high CNV assessed by NGS was associated with better ORR, PFS, and OS, especially if combined with HER2 3+ expression by IHC.

The JACOB study failed to meet its primary endpoint of improved OS with the addition of pertuzumab to standard trastuzumab-containing therapy (2). However, the end-of-study analysis recently reported a potentially clinically meaningful absolute gain of mOS of 3.9 months, with a median follow-up exceeding 44.4 months (13). This result clearly paved the way to the hypothesis that a subgroup of patients may benefit from dual HER2 blockade in the first-line setting. Thus, despite the lack of signals in clinically relevant subgroups investigated in the trial, refining the molecular selection for HER2 inhibition strategies thanks to biomarkers may help to identify patients with HER2-addicted cancers and potential benefit from boosted HER2 blockade. Drawing from these considerations, we focused on prespecified biomarkers which had been previously associated with the efficacy of standard first-line trastuzumab plus chemotherapy.

From a translational perspective, retrospective studies showed the impact of *HER2* "hyperamplification" (i.e., higher *HER2* CNV or its values greater than a specific cutoff) on better outcomes of trastuzumab or even long-term response in patients with HER2-positive metastatic gastric cancer/GEJC, because higher level of *HER2* amplification assessed by ISH or NGS may be a surrogate of HER2 addiction (8, 9, 14–16). Also, patients with higher amounts of HER2 in their tumors assessed by IHC or mass spectrometry derive greater benefit from trastuzumab-based therapy (1, 10, 11). In the JACOB trial,

HER2 IHC was associated with a clear prognostic effect, because patients with IHC score 3+ expression showed better outcomes than those with score 2+, independent from the treatment arm (2). However, in this analysis, only *HER2* CNV was independently prognostic, but not HER2 IHC. This observation may be related to the strong association between *HER2* CNV and HER2 IHC status and to the possibility to achieve a more accurate stratification of outcomes with *HER2* CNV compared with HER2 IHC as a two-category factor. Finally, we and others showed the negative prognostic impact of candidate genomic alterations of primary resistance to trastuzumab-based therapy (12, 14, 17). Our AMNESIA panel included *EGFR/MET/KRAS/PI3KCA* mutations and *EGFR/MET/KRAS* amplifications, allowing us to predict primary resistance in 55% of patients included in a prospective case–control study. Our approach also allowed the simultaneous assessment of multiple resistance mechanisms with an individual low frequency, thus providing a greater chance of validating the whole AMNESIA panel as opposed to attempts of investigating just one biomarker at a time.

However, most of the above-mentioned studies on positive and negative biomarkers have a small sample size and several potential selection biases. In the current work, the availability of a large dataset allowed us to perform a multivariable analysis, reliably showing that only *HER2* CNV status had an independent prognostic impact. Moreover, the combined assessment of both *HER2* CNV by NGS and HER2 IHC potentially helped to further refine the selection of patients with increased benefit, that is, those with higher *HER2* amplification and expression levels. Patients with AMNESIA+ and *HER2* CNV-low status had an extremely worse outcome, but the combined assessment of AMNESIA and *HER2* CNV-low increased with lower extent the discriminative ability of outcomes. The possible reasons may rely in the low numbers of patients with AMNESIA alterations and in the differential effect of specific alterations, considering that only *KRAS* alterations and *MET* amplifications had a significant adverse impact on survival endpoints. This specific effect restricted to *KRAS* or *MET* alterations may be primarily related to their strong poor prognostic effect, rather than a potential negative predictive role for the efficacy of trastuzumab-based therapy.

Regarding the treatment effect according to the investigated biomarkers, several preclinical works showed that dual HER2 blockade with trastuzumab plus pertuzumab or lapatinib is more effective than single-agent trastuzumab, especially in *HER2* "hyperamplified" models, whereas the presence of codrivers such as *MET, EGFR*, or *KRAS* amplifications is associated with cross-resistance to either single-agent or dual HER2-targeted strategies (14, 17–21). Therefore, there is a strong rationale to refine both the positive selection of HER2-addicted cancers by means of *HER2* CNV-high status with or without HER2 overexpression (score 3+) and the negative selection with the exclusion of patients with primary resistance alterations. Indeed, the strong association of *HER2* CNV-high status and lack of primary resistance alterations may be *per se* an indicator of progressively increased HER2 addiction with increase of the levels of *HER2* amplification. However, despite our aim of potentially identifying a molecular subgroup of patients with benefit from the addition of pertuzumab to trastuzumab-based therapy, none of the investigated biomarkers allowed to show significantly improved outcomes in the experimental arm and especially *HER2* CNV did not seem to be predictive of the efficacy or activity of pertuzumab. Therefore, the increased heterogeneity of HER2 status in gastric cancer/GEJC compared with breast cancer and the increased complexity of the genomic landscape of gastric cancer/GEJC suggest that HER2 signaling may not be the only actionable driver in some of the patients.

Regarding the potential applications of our work, *HER2* CNV assessed by NGS or ISH appears to be a potentially important biomarker in patients receiving anti–HER2-based strategies, because it seems to enrich patients with greater benefit. Despite demonstration of the clinical validity of *HER2* CNV, this biomarker should be reassessed in the context of the current standard of care in the United States, which is represented by pembrolizumab/trastuzumab-based chemotherapy. Most importantly, the clinical usefulness of *HER2* CNV and NGS testing to potentially drive patients' management in a cost-effective fashion has not yet been formally demonstrated. On the contrary, it should be clearly pointed out that patients with *HER2* CNV-low status may still benefit from HER2 inhibition strategies, because we demonstrated that *HER2* CNV is a prognostic biomarker in patients receiving trastuzumab-based therapy, but a potential predictive role cannot be hypothesized on the basis of the available data. Regarding clinical applicability of *HER2* CNV, the association of *HER2* CNV-high status with both long-term survival outcomes and complete responses to first-line trastuzumab-based therapy may allow the potential design of personalized treatment strategies. For instance, considering the recent FDA approval of pembrolizumab plus trastuzumab and chemotherapy in the first-line setting (6), coupled with the proof of evidence that one cycle of chemo-free pembrolizumab plus trastuzumab can induce radiological responses (22), the omission of chemotherapy or the lightening of its burden could be investigated in a molecularly selected population with predicted HER2 addiction (23). In parallel, *HER2* CNV may be an important biomarker also for patients treated with novel anti-HER2 agents such as the antibody–drug conjugate trastuzumab deruxtecan. Indeed, the recent *post hoc* analysis of the DESTINY-Gastric-01 showed that patients treated with trastuzumab deruxtecan and bearing *HER2* amplification or higher *HER2* CNV in baseline circulating tumor DNA had better outcomes, but a predictive role of *HER2* CNV for the efficacy of trastuzumab deruxtecan has not been investigated yet (24). Finally, the increased response rate (including the complete response rate) observed in patients with higher HER2 levels is clearly important for the translation of anti-HER2 strategies in the neoadjuvant treatment of patients with early-stage disease.

Compared with the assessment of *HER2* amplification levels by standard ISH testing, NGS has several advantages, including the reduced interobserver and intraobserver subjectiveness, automatization and widespread use, at price of higher—but constantly lowering—costs. On top of this, NGS allows to concomitantly assess several genes beyond *HER2* itself, thus investigating the role of potential drivers of treatment resistance. On the contrary, bulk analysis without a microdissection-based enrichment of tumor cells could lead to an underestimation of the *HER2* CNV by stromal dilution. This is consistent with the results of our study showing a non-negligible proportion of samples without *HER2* amplification at NGS, despite the presence of centrally confirmed HER2 positivity by IHC ± ISH as an inclusion criterion of the trial. While ISH testing may allow to spatially resolve the levels of *HER2* amplification and discriminate tumor versus stromal cells, the spatial heterogeneity and/or subclonality of the *HER2* amplification may be a critical challenge with both assays. From this point of view, the use of liquid biopsy and the assessment of *HER2* CNV in blood may overcome such limitations and further improve patients' selection.

Our study has limitations. First, it is a *post hoc* study conducted in only 42% of trial patients consenting to future research and with available and successfully analyzed DNA. In this biomarker-evaluable population, the efficacy observed in the two treatment arms was not reflecting the intention-to-treat population. Second, the use of NGS

may have underestimated the prevalence of *MET, EGFR*, or *KRAS* coamplifications and therefore the proportion of AMNESIA positivity could have been higher with availability of ISH testing. Moreover, other putative resistance biomarkers such as *CCND1* and *CCNE1* amplifications may be important in patients receiving trastuzumab-based therapy, and the prognostic role of these alterations should be investigated by means of more comprehensive NGS panels and larger datasets (19). Finally, the use of *HER2* CNV assessed by NGS, as a selection or stratification factor in clinical trials or even in the standard practice, will require harmonization between different sequencing platforms and further prospective investigation on the optimal cutoffs.

In conclusion, in this large subset of patients with HER2-positive gastric cancer/GEJC enrolled in the JACOB trial, we highlighted the potential role of NGS in identifying patients with *HER2*-high tumors and addiction to HER2 signaling, with clinical relevance for ongoing trials and for the design of future studies.

Authors' Disclosures

F. Pietrantonio reports personal fees from Amgen, Merck-Serono, Pierre-Fabre, Servier, Bayer, MSD, and Lilly; grants and personal fees from AstraZeneca and BMS; and grants from Incyte outside the submitted work. A. Raimondi reports personal fees from Elma Academy and Servier, as well as other support from Amgen outside the submitted work. F. Morano reports personal fees from Servier and Lilly, as well as grants from Incyte outside the submitted work. M. Niger reports personal fees from Incyte, EMD Serono, Basilea Pharmaceutical, MSD Italia, Servier, Sandoz, MedPoint SRL, and Accademia della Medicina outside the submitted work. C. Marchiò reports personal fees from Bayer, Roche, AstraZeneca, and Daiichi Sankyo outside the submitted work. E. Restuccia reports personal fees from F. Hoffmann-La Roche, as well as other support from F. Hoffmann-La Roche during the conduct of the study. C. Lambertini reports personal fees from F. Hoffmann-La Roche LTD during the conduct of the study. J. Tabernero reports personal fees from Array Biopharma, AstraZeneca, Bayer, Boehringer Ingelheim, Chugai, Daiichi Sankyo, F. Hoffmann-La Roche Ltd, Genentech Inc, HalioDX SAS, Hutchison MediPharma International, Ikena Oncology, Inspirna Inc, IQVIA, Lilly, Menarini, Merck Serono, Merus, MSD, Mirati, Neophore, Novartis, Ona Therapeutics, Orion Biotechnology, Peptomyc, Pfizer, Pierre Fabre, Samsung Bioepis, Sanofi, Scandion Oncology, Scorpion Therapeutics, Seattle Genetics, Servier, Sotio Biotech, Taiho, Tessa Therapeutics and TheraMyc, Imedex/HMP, Medscape Education, MJH Life Sciences, PeerView Institute for Medical Education and Physicians Education Resource (PER), and Oniria Therapeutics outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

F. Pietrantonio: Conceptualization, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. **P. Manca:** Formal analysis. **S.E. Bellomo:** Data curation, software, formal analysis. **S. Corso:** Data curation, funding acquisition, validation, writing–review and editing. **A. Raimondi:** Investigation, visualization, project administration, writing–review and editing. **E. Berrino:** Software, formal analysis. **F. Morano:** Validation, investigation, visualization. **C. Migliore:** Software, formal analysis, validation, investigation, visualization, writing–review and editing. **M. Niger:** Software, validation, writing–original draft, project administration, writing–review and editing. **L. Castagnoli:** Validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing. **S.M. Pupa:** Funding acquisition, validation, visualization, writing–original draft, writing–review and editing. **C. Marchiò:** Resources, data curation, software, supervision, funding acquisition, writing–review and editing. **M. Di Bartolomeo:** Validation, investigation, visualization, writing–review and editing. **E. Restuccia:** Resources, data curation, writing–review and editing. **C. Lambertini:** Resources, data curation, writing–review and editing. **J. Tabernero:** Supervision, validation, investigation, visualization, methodology, writing–review and editing. **S. Giordano:** Conceptualization, resources, supervision, funding acquisition, writing–review and editing.

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Note

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