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Original Article

HER2 copy number and resistance mechanisms in patients with HER2-positive advanced gastric cancer receiving initial trastuzumab-based therapy in JACOB trial

Running title: biomarkers study 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 NGS (Oncomine Focus DNA), *HER2* CNV, HER2 expression by IHC and AMNESIA were correlated with ORR, PFS and OS by uni/multivariable models.

Results: Median *HER2* CNV was 4.7 (IQR 2.2-16.9). *HER2* CNV-high vs low using the median as cut-off was associated with longer median PFS (10.5 vs 6.4 months; HR=0.48, 95%CI: 0.38-0.62; p<.001) and OS (20.3 vs 13.0 months; HR=0.54, 0.42-0.72; p<.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<.001) and OS (p=.004). Higher ORR was significantly associated with IHC 3+ [61% vs 34% in 2+; odds ratio (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, OS in a JACOB subgroup, especially if combined with HER2 3+. The negative prognostic role of AMNESIA requires further clinical validation.

Keywords: gastric cancer, HER2, next-generation sequencing, pertuzumab, trastuzumab.

Statement of translational relevance

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

Introduction

In patients with HER2-positive metastatic gastric cancer (GC) 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], since HER2 "hyper-amplification" 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 immunohistochemistry (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].

Based on these considerations, we hypothesized that optimized patients' positive selection based on *HER2* copy number variation (CNV) and HER2 IHC and/or negative selection based on primary resistance mechanisms 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 GC/GEJC enrolled in the JACOB trial and receiving trastuzumab and chemotherapy with or without pertuzumab.

Patients and Methods

Patients

JACOB was a double-blind, placebo-controlled phase 3 trial that investigated the addition of pertuzumab to trastuzumab and chemotherapy as first-line treatment of patients with HER2-positive metastatic or unresectable GC/GEJC. HER2 positivity was centrally confirmed for eligibility and defined as IHC3+ or IHC2+ and ISH-positive by using PATHWAY anti-HER2/neu (4B5) IHC and the INFORM HER2 Dual ISH assays (Ventana Medical Systems, Tucson, AZ, USA). The data generated in the present study are a post-hoc translational analysis conducted in 580 out 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.

Next-generation sequencing

Tumor DNA was extracted from all samples at the central lab after wet macro-dissection 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 300X.

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 SNVs, indels (VAF > 10%) and CNVs (for sample with a MAPD $\leq$ 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 $\geq$ 0.1 and a minor allele frequency or global allele frequency in ExAC or 5000 exomes databases $\leq$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 $\geq$ 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 were used to report distribution of continuous variables. Confidence intervals were calculated at a 95% level. Categorical data distribution was tested with $\chi 2$ 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 uni- 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's 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 non-linear 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, Basel and Fondazione IRCCS Instituto Nazionale dei Tumori, Milan will be needed. Also, requests for data should be directed to the corresponding author.

Results

Patients' population

As shown in **Supplementary Figure 1**, the biomarker evaluable population included a subset of 327 out 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 1shows 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 **Figure 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 vs CNV-high subgroup using the median as cut-off (21.3% vs 9.8%, p=0.007).

Survival analysis

Supplementary Figure 2 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 cut-off. Patients with *HER2* CNV-high status had significantly superior PFS (median PFS (mPFS): 10.5 vs 6.4 months; Hazard Ratio (HR)=0.48, 95%CI: 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 to *HER2* CNV-low (**Figure 2A-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 to HER2 2+ (**Figure 2C-D**). On the opposite, patients with AMNESIA positivity had a non-significantly 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 to those with AMNESIA negative status (**Figure 2E-F**). **Supplementary Table 1** shows the prognostic

effect of each individual genomic alteration included in the AMNESIA panel. Specifically, after p values adjustment, only KRAS mutations and MET co-amplifications were significantly associated with worse outcomes.

We then performed a combined assessment of *HER2* CNV with HER2 IHC or AMNESIA status (**Supplementary Table 2**). The co-existence of *HER2* CNV-high with HER2 IHC 3+ status identified the only subgroup of patients with a remarkably longer PFS and OS (**Figure 3A-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 (**Figure 3C-D**). When considering the number of *HER2* gene copies as a continue variable, we observed a non-linear correlation with OS only in the HER2 IHC 3+ subgroup (**Supplementary Figure 3**) (p=0.001 for the non-linear term) but not for the HER2 IHC 2+ (p=0.21 for the non-linear term).

Finally, we built univariate and multivariable Cox proportional hazard 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 overall response rate (ORR) according to RECIST v1.1 (**Figure 4**). *HER2* CNV-high status was significantly associated with higher ORR vs *HER2* CNV-low (59.0% vs 43.9%, Odds Ratio (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 versus 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 Figure 4**). This was consistent with the treatment effect by HER2 CNV quartiles (**Supplementary Figure 5**).

Discussion

In this post-hoc translational analysis carried out in a subset of patients with HER2-positive metastatic GC/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, 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 pre-

specified 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* "hyper-amplification" (i.e. higher *HER2* CNV or its values greater than a specific cut-off) on better outcomes of trastuzumab or even long-term response in patients with HER2-positive metastatic GC/GEJC, since 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, since 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 to HER2 IHC as a 2-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 present 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, i.e. 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* "hyper-amplified" models, whereas the presence of co-drivers 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 GC/GEJC compared to breast cancer and the increased complexity of the genomic landscape of GC/GEJC suggest that HER2 signaling may not be the only actionable driver of 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, since 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 US, 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, since we demonstrated that *HER2* CNV is a prognostic biomarker in patients receiving trastuzumab-based therapy, but a potential predictive role cannot be hypothesized based on 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 1 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 to the assessment of *HER2* amplification levels by standard ISH testing, NGS has several advantages, including the reduced inter- and intra-observer 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 were not reflecting the intention-to-treat population. Second, the use of NGS may have underestimated the prevalence of *MET, EGFR* or *KRAS* co-amplifications 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 cut-offs.

In conclusion, in this large subset of patients with HER2-positive GC/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.

References

1. Bang YJ, Van Cutsem E, Feyereislova A et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet 2010; 376: 687-697.
2. Tabernero J, Hoff PM, Shen L et al. Pertuzumab plus trastuzumab and chemotherapy for HER2-positive metastatic gastric or gastro-oesophageal junction cancer (JACOB): final analysis of a double-blind, randomised, placebo-controlled phase 3 study. Lancet Oncol 2018; 19: 1372-1384.
3. Hecht JR, Bang YJ, Qin SK et al. Lapatinib in Combination With Capecitabine Plus Oxaliplatin in Human Epidermal Growth Factor Receptor 2-Positive Advanced or Metastatic Gastric, Esophageal, or Gastroesophageal Adenocarcinoma: TRIO-013/LOGiC--A Randomized Phase III Trial. J Clin Oncol 2016; 34: 443-451.
4. Satoh T, Xu RH, Chung HC et al. Lapatinib plus paclitaxel versus paclitaxel alone in the second-line treatment of HER2-amplified advanced gastric cancer in Asian populations: TyTAN--a randomized, phase III study. J Clin Oncol 2014; 32: 2039-2049.
5. Thuss-Patience PC, Shah MA, Ohtsu A et al. Trastuzumab emtansine versus taxane use for previously treated HER2-positive locally advanced or metastatic gastric or gastro-oesophageal junction adenocarcinoma (GATSBY): an international randomised, open-label, adaptive, phase 2/3 study. Lancet Oncol 2017; 18: 640-653.
6. Janjigian YY, Kawazoe A, Yañez P et al. The KEYNOTE-811 trial of dual PD-1 and HER2 blockade in HER2-positive gastric cancer. Nature 2021; 600: 727-730.
7. Shitara K, Bang YJ, Iwasa S et al. Trastuzumab Deruxtecan in Previously Treated HER2-Positive Gastric Cancer. N Engl J Med 2020; 382: 2419-2430.
8. Gomez-Martin C, Plaza JC, Pazo-Cid R et al. Level of HER2 gene amplification predicts response and overall survival in HER2-positive advanced gastric cancer treated with trastuzumab. J Clin Oncol 2013; 31: 4445-4452.
9. Zhang L, Hamdani O, Gjoerup O et al. ERBB2 Copy Number as a Quantitative Biomarker for Real-World Outcomes to Anti-Human Epidermal Growth Factor Receptor 2 Therapy in Advanced Gastroesophageal Adenocarcinoma. JCO Precis Oncol 2022; 6: e2100330.
10. Catenacci DVT, Liao WL, Zhao L et al. Mass-spectrometry-based quantitation of Her2 in gastroesophageal tumor tissue: comparison to IHC and FISH. Gastric Cancer 2016; 19: 1066-1079.
11. An E, Ock CY, Kim TY et al. Quantitative proteomic analysis of HER2 expression in the selection of gastric cancer patients for trastuzumab treatment. Ann Oncol 2017; 28: 110-115.
12. Pietrantonio F, Fuca G, Morano F et al. Biomarkers of Primary Resistance to Trastuzumab in HER2-Positive Metastatic Gastric Cancer Patients: the AMNESIA Case-Control Study. Clin Cancer Res 2018; 24: 1082-1089.
13. Tabernero J, Hoff PM, Shen L et al. 1423MO End-of-study analysis from JACOB: A phase III study of pertuzumab (P) + trastuzumab (H) and chemotherapy (CT) in HER2-positive metastatic gastric or gastro-esophageal junction cancer (mGC/GEJC). Annals of Oncology 2020; 31: S900-S901.

14. Janjigian YY, Sanchez-Vega F, Jonsson P et al. Genetic Predictors of Response to Systemic Therapy in Esophagogastric Cancer. Cancer Discov 2018; 8: 49-58.
15. Stein SM, Snider J, Ali SM et al. Real-world association of HER2/ERBB2 concordance with trastuzumab clinical benefit in advanced esophagogastric cancer. Future Oncol 2021; 17: 4101-4114.
16. Ock CY, Lee KW, Kim JW et al. Optimal Patient Selection for Trastuzumab Treatment in HER2-Positive Advanced Gastric Cancer. Clin Cancer Res 2015; 21: 2520-2529.
17. Ughetto S, Migliore C, Pietrantonio F et al. Personalized therapeutic strategies in HER2-driven gastric cancer. Gastric Cancer 2021; 24: 897-912.
18. Sanchez-Vega F, Hechtman JF, Castel P et al. EGFR and MET Amplifications Determine Response to HER2 Inhibition in ERBB2-Amplified Esophagogastric Cancer. Cancer Discov 2019; 9: 199-209.
19. Kim J, Fox C, Peng S et al. Preexisting oncogenic events impact trastuzumab sensitivity in ERBB2-amplified gastroesophageal adenocarcinoma. J Clin Invest 2014; 124: 5145-5158.
20. Kwak EL, Ahronian LG, Siravegna G et al. Molecular Heterogeneity and Receptor Coamplification Drive Resistance to Targeted Therapy in MET-Amplified Esophagogastric Cancer. Cancer Discov 2015; 5: 1271-1281.
21. Shimozaki K, Shinozaki E, Yamamoto N et al. KRAS mutation as a predictor of insufficient trastuzumab efficacy and poor prognosis in HER2-positive advanced gastric cancer. J Cancer Res Clin Oncol 2022.
22. Janjigian YY, Maron SB, Chatila WK et al. First-line pembrolizumab and trastuzumab in HER2-positive oesophageal, gastric, or gastro-oesophageal junction cancer: an open-label, single-arm, phase 2 trial. Lancet Oncol 2020; 21: 821-831.
23. Trapani D, Franzoi MA, Burstein HJ et al. Risk-adapted modulation through de-intensification of cancer treatments: an ESMO classification. Ann Oncol 2022.
24. Shitara K, Bang Y, Iwasa S et al. O-14 Exploratory biomarker analysis of trastuzumab deruxtecan in DESTINY-Gastric01, a randomized, phase 2, multicenter, open-label study in patients with HER2-positive or -low advanced gastric or gastroesophageal junction adenocarcinoma. Annals of Oncology 2021; 32: S224.

Figure legends

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 (panels A and B), HER2 IHC 3+ versus 2+ (panels C and D) and AMNESIA panel positive versus negative status (panels E-F).

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

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

Acknowledgements. We are grateful to Thermo Fisher Scientific for support to NGS analysis and Chiara Olivieri, MD student, for the review.

Funding: AIRC IG 23624 to FP, AIRC IG 20210 to SG, AIRC IG 21770 to SC. Roche owns the data of the whole JACOB trial, but the present investigator initiated study was conducted without funding from Roche and the intellectual property of the study results is attributed to Fondazione IRCCS Istituto Nazionale dei Tumori.

Declaration of Potential Conflicts of Interest:

- **FP:** honoraria from Amgen, Bayer, Servier, Merck-Serono, Lilly, MSD, BMS, Astrazeneca, Pierre-Fabre; research grants from Bristol-Myers Squibb, AstraZeneca and Incyte.
- **FM:** honoraria from SERVIER, research grant from Incyte.
- **MN:** Travel expenses from Celgene, speaker honorarium from Accademia della Medicina; honoraria from Sandoz, Medpoint SRL for editorial collaboration. Consultant honoraria from EMD Serono, Basilea Pharmaceutica, Incyte and MSD Italia
- **MdB:** honoraria from Lilly, MSD Oncology, Servier, consulting/advisory role with Lilly, MSD oncology, research grant from Lilly, travel/accommodation expenses from Roche and Sanofi.
- **JT:** consulting fees from Array Biopharma, AstraZeneca, Avvinity, Bayer, Boehringer Ingelheim, Chugai, Daiichi Sankyo, F. Hoffmann-La Roche Ltd, Genentech Inc., HalioDX SAS, Hutchison MediPharma International, Ikena Oncology, IQVIA, Lilly, Menarini, Merck Serono, Merus, Merck Sharp & Dohme (MSD), Mirati, Neophore, Novartis, Orion Biotechnology, Peptomyc, Pfizer, Pierre Fabre, Samsung Bioepis, Sanofi, Scandion Oncology, Seattle Genetics, Servier, Taiho, Tessa Therapeutics, and TheraMyc; payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from F. Hoffmann-La Roche, Imedex, Medscape Education, MJH Life Sciences, PeerView Institute for Medical Education, and Physicians Education Resource (PER); and institutional financial support for clinical trials from Amgen Inc., Array Biopharma Inc., AstraZeneca Pharmaceuticals LP, BeiGene, Boehringer Ingelheim, Bristol Myers Squibb (BMS), Celgene, Debiopharm International SA, F. Hoffmann-La Roche Ltd, Genentech Inc, HalioDX SAS, Hutchison MediPharma International, Janssen-Cilag SA , MedImmune, Menarini, Merck Health KGAA, Merck Sharp & Dohme, Merus NV, Mirati, Novartis Farmacéutica SA, Pfizer, PharmaMar, Sanofi Aventis Recherche & Développement, Servier, Taiho Pharma USA Inc., Spanish Association Against Cancer Scientific Foundation, and Cancer Research UK.

All other authors declared no conflicts of interest.

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)	

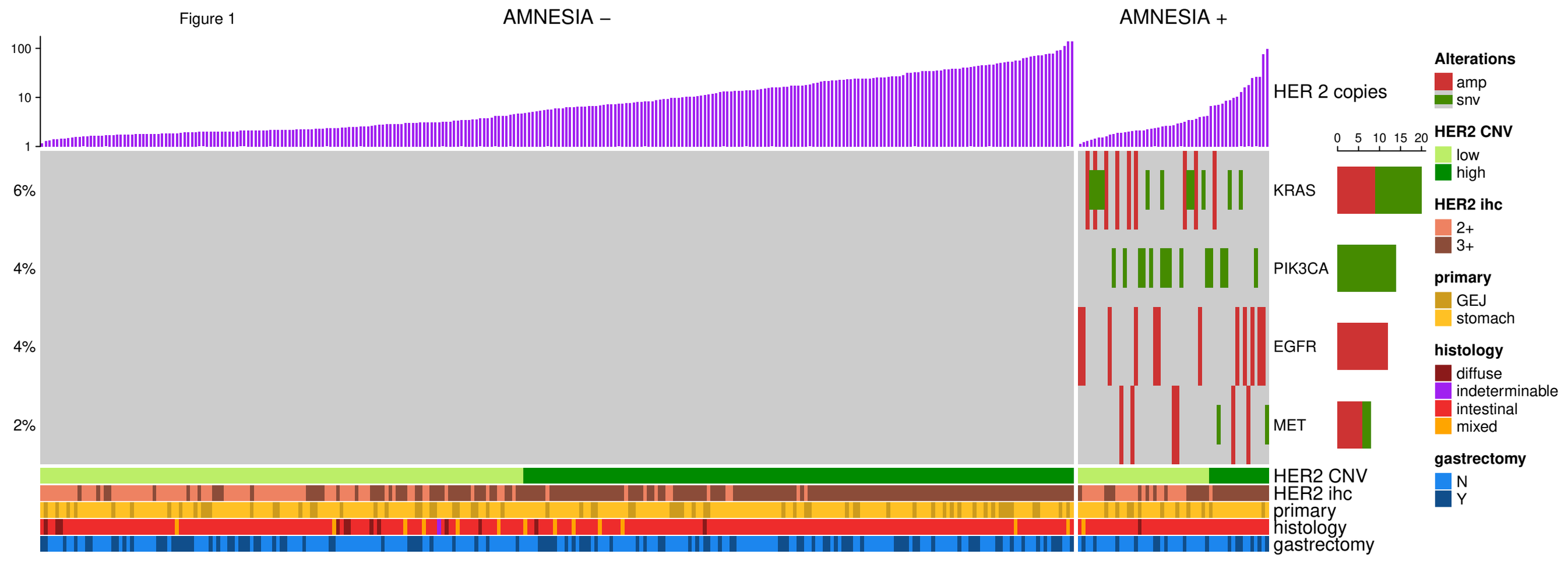
CNV, copy number variation; IQR, interquartile range; ECOG, Eastern Cooperative Oncology Group; PS, performance status; GEJ, gastroesophageal junction; IHC, immunohistochemistry.

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

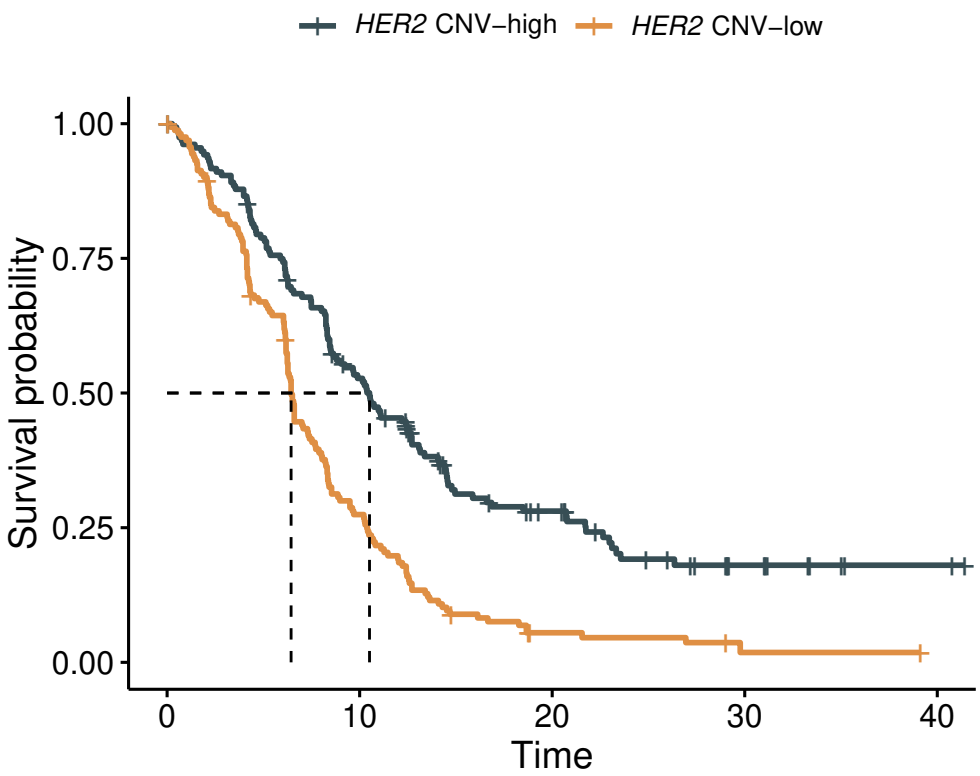
	PFS		OS	
	Univariate HR (95%CI, *p* value)	**Multivariate HR (95%CI, *p* value)**	**Univariate HR (95%CI, *p* value)**	**Multivariate HR (95%CI, *p* value)**
Age				
<65	-	-	-	-
≥65	0.99 (0.78-1.26, *p*=0.945)	-	0.89 (0.68-1.16, *p*=0.392)	-
Sex				
Female	-	-	-	-
Male	0.88 (0.66-1.17, *p*=0.375)	-	0.77 (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)	
Harrell C-Indeces for the PFS and the OS multivariate models were, respectively, 63.1 ± 1.7% and 64.0 ± 1.8%				

List of abbreviations: HR, hazard ratio; PFS, Progression Free Survival; OS, Overall Survival; ECOG, Eastern Cooperative Oncology Group; PS, performance status, GEJ, gastroesophageal junction; IHC, immunohistochemistry; CNV, copy number variation.

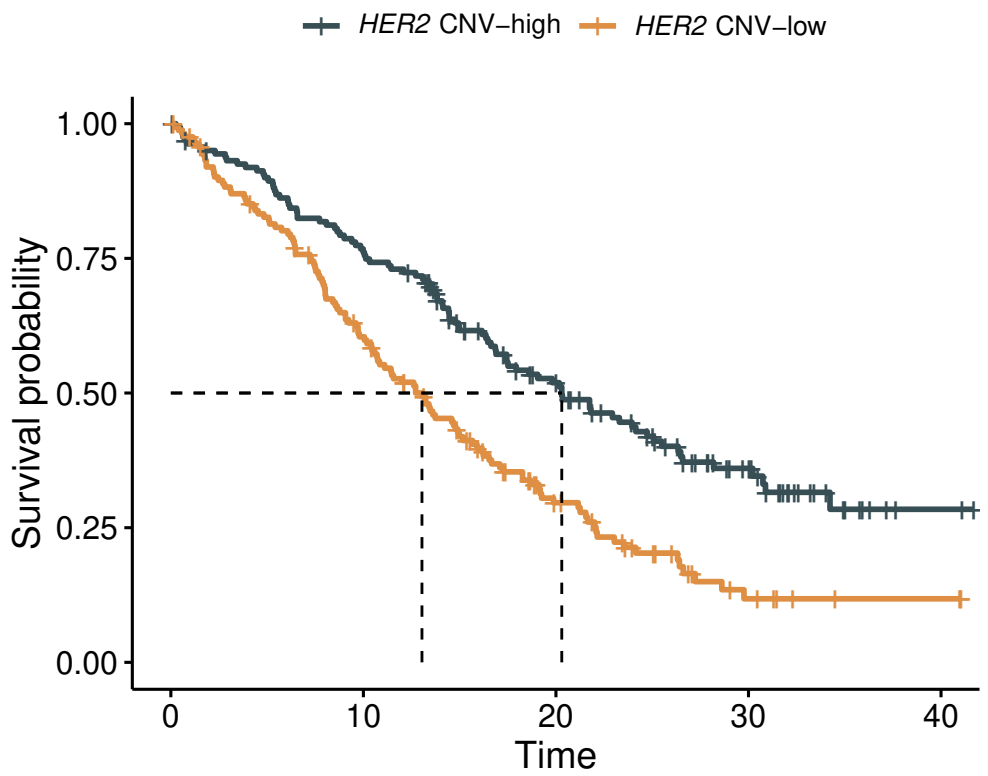
Figure 1


AMNESIA −
AMNESIA +
100
10
1
HER 2 copies
0 5 10 15 20
6%
KRAS
4%
PIK3CA
4%
EGFR
2%
MET
HER2 CNV
HER2 ihc
primary
histology
gastrectomy
Alterations
amp
snv
HER2 CNV
low
high
HER2 ihc
2+
3+
primary
GEJ
stomach
histology
diffuse
indeterminable
intestinal
mixed
gastrectomy
N
Y

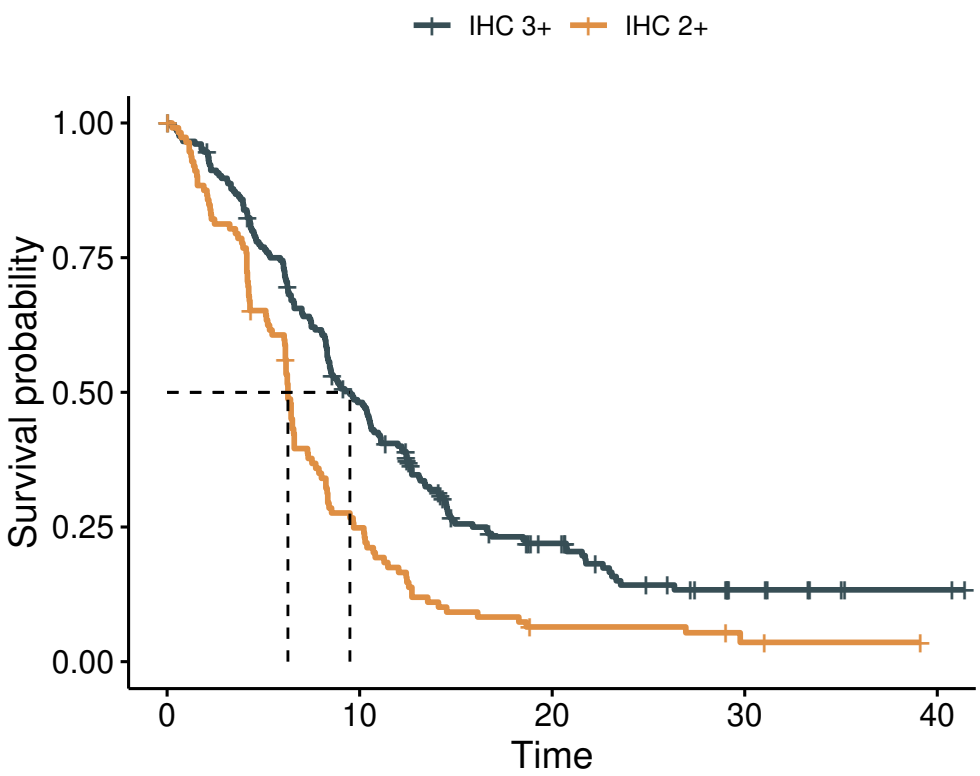
A PFS



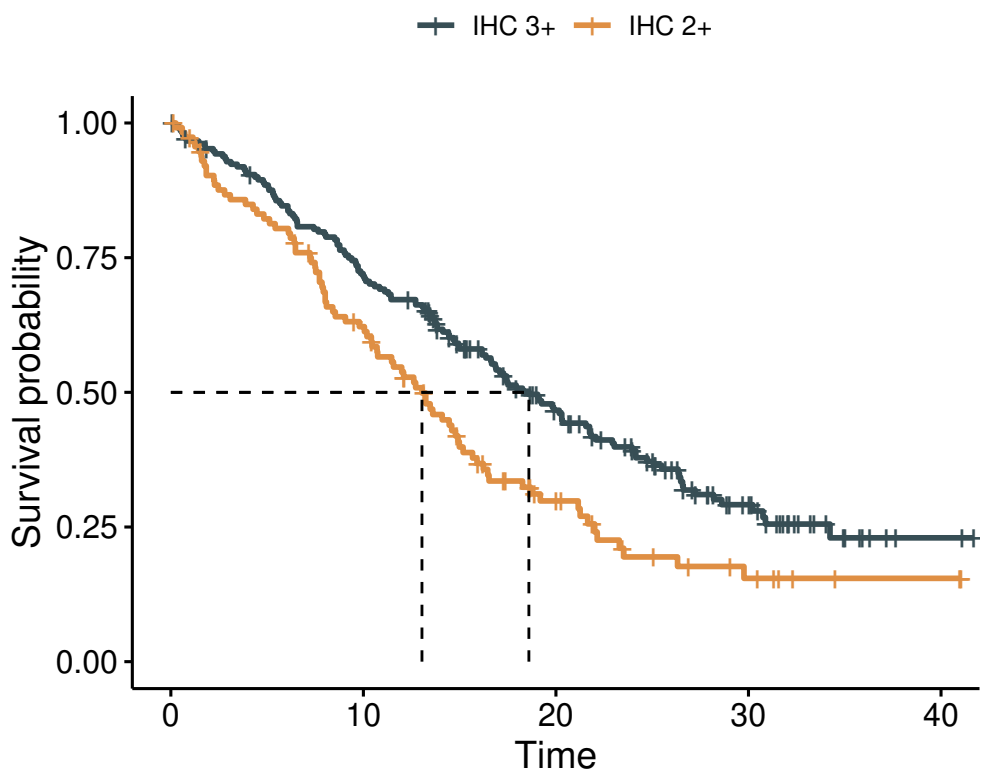
B OS



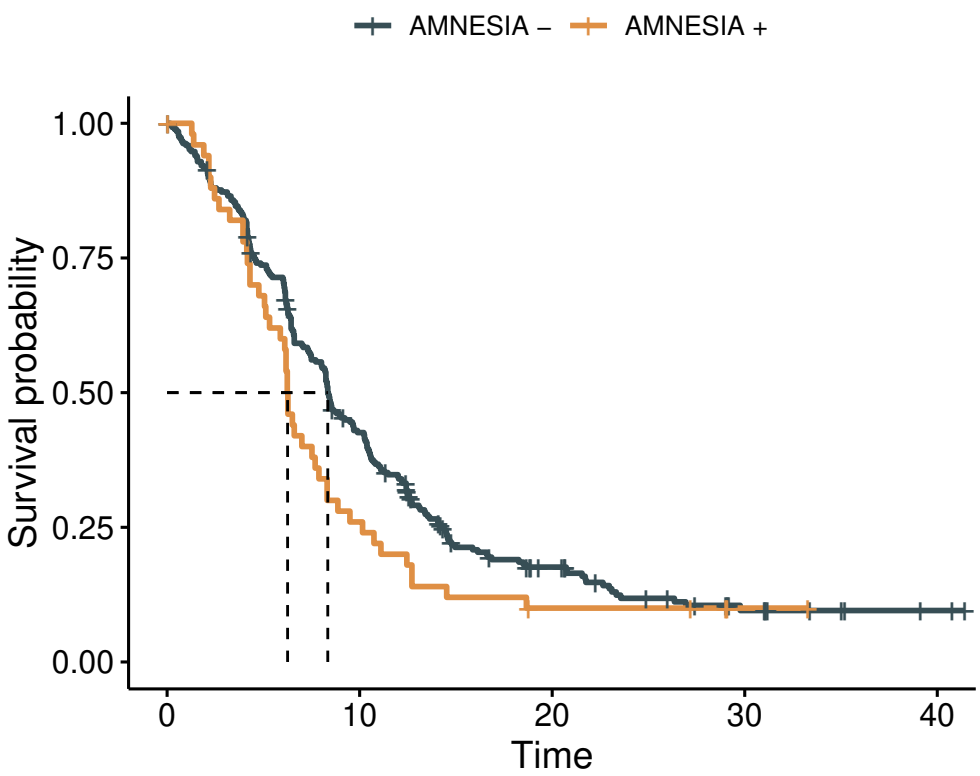
C PFS



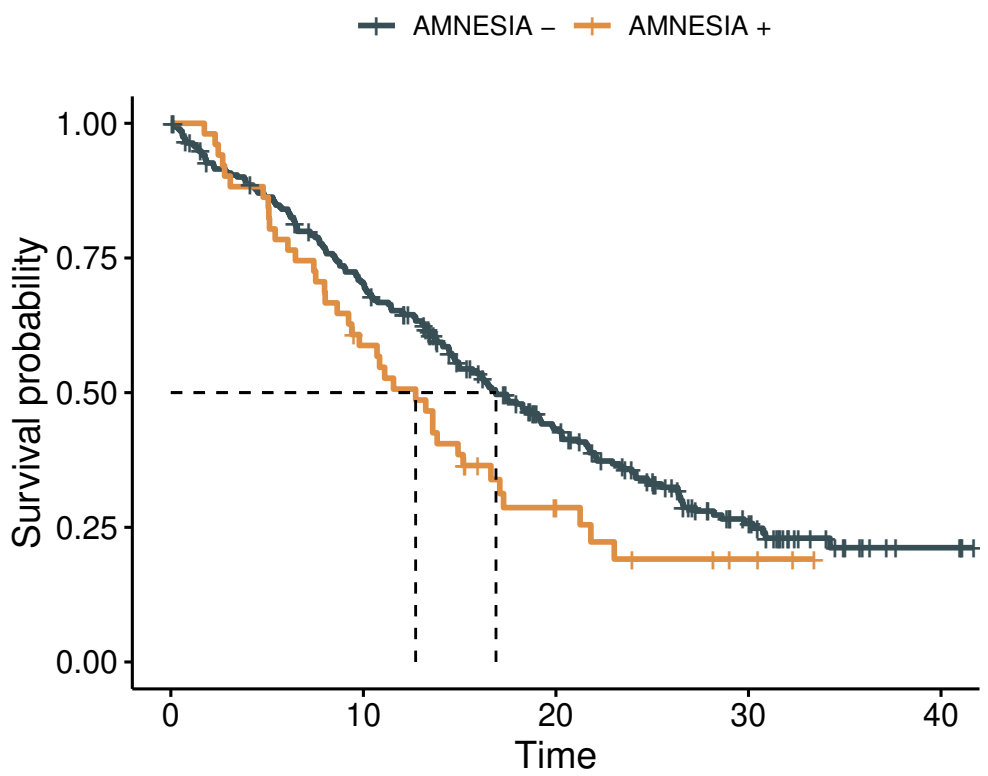
D OS

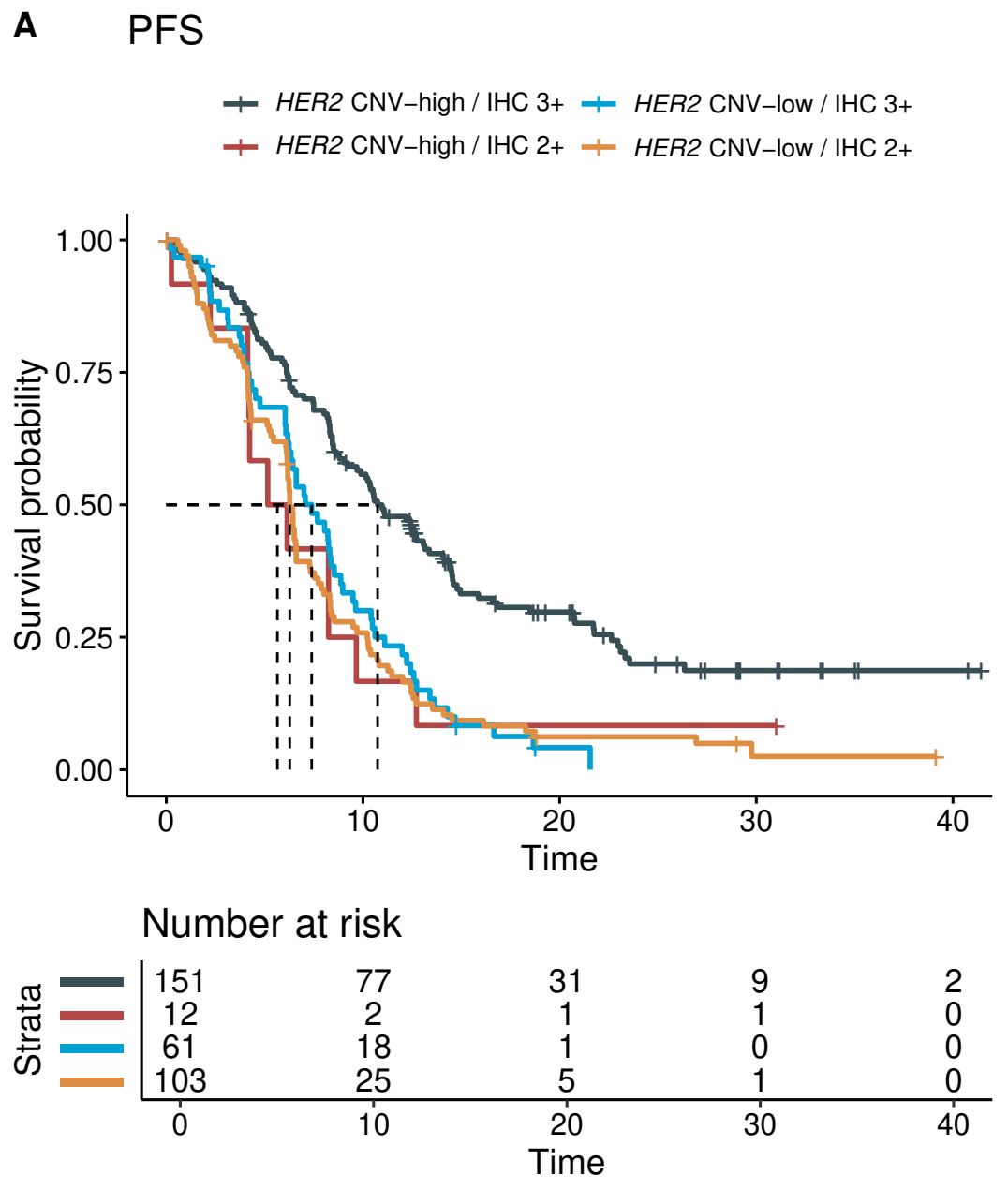


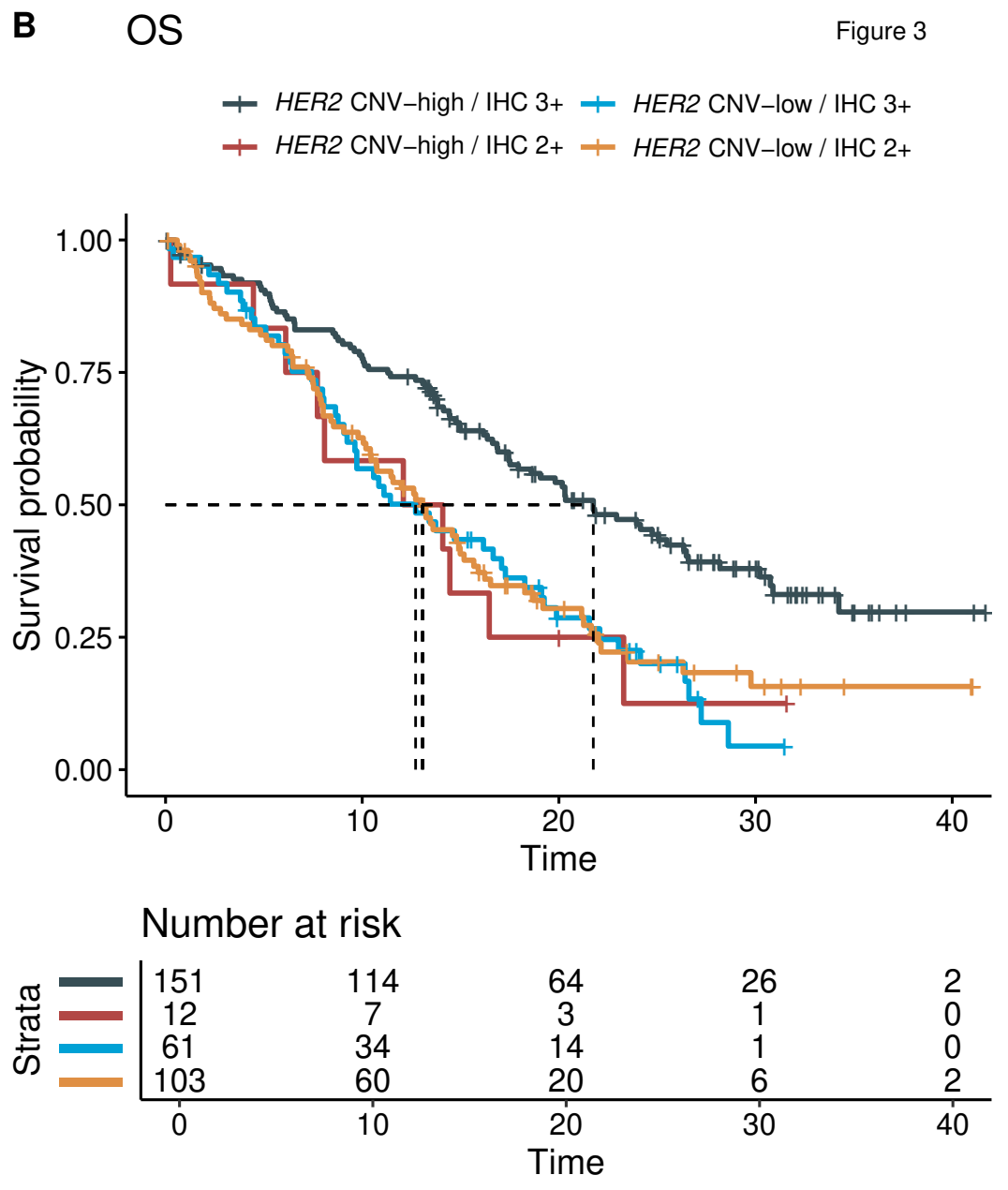
E PFS

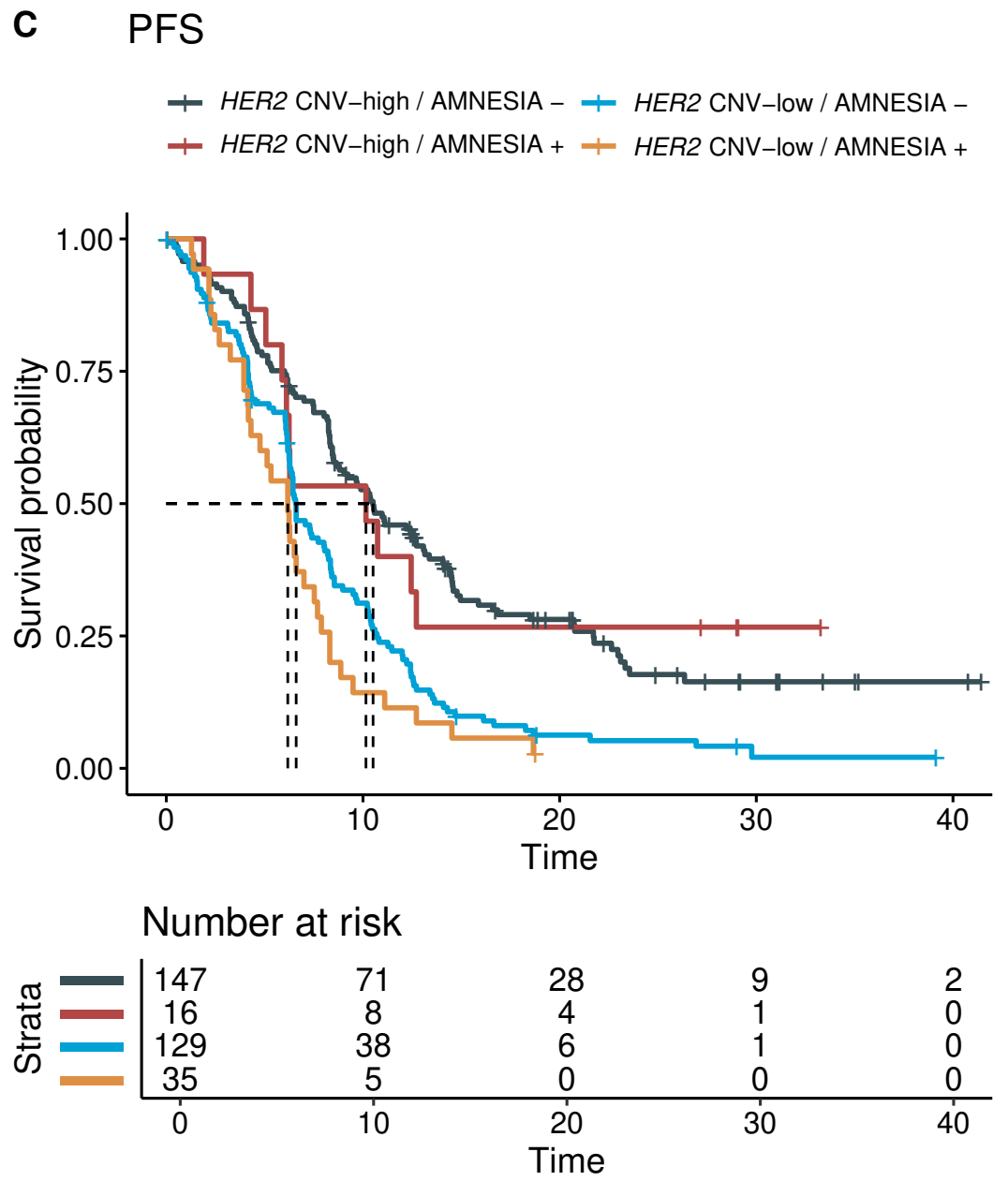


F OS




A
PFS
HER2 CNV−high / IHC 3+
HER2 CNV−low / IHC 3+
HER2 CNV−high / IHC 2+
HER2 CNV−low / IHC 2+
Survival probability
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
Time
Number at risk
Strata
151 77 31 9 2
12 2 1 1 0
61 18 1 0 0
103 25 5 1 0
0
10
20
30
40
Time


B
OS
HER2 CNV−high / IHC 3+
HER2 CNV−low / IHC 3+
HER2 CNV−high / IHC 2+
HER2 CNV−low / IHC 2+
Survival probability
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
Time
Number at risk
Strata
151 114 64 26 2
12 7 3 1 0
61 34 14 1 0
103 60 20 6 2
0
10
20
30
40
Time


C
PFS
HER2 CNV−high / AMNESIA −
HER2 CNV−low / AMNESIA −
HER2 CNV−high / AMNESIA +
HER2 CNV−low / AMNESIA +
Survival probability
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
Time
Number at risk
Strata
147 71 28 9 2
16 8 4 1 0
129 38 6 1 0
35 5 0 0 0
0
10
20
30
40
Time

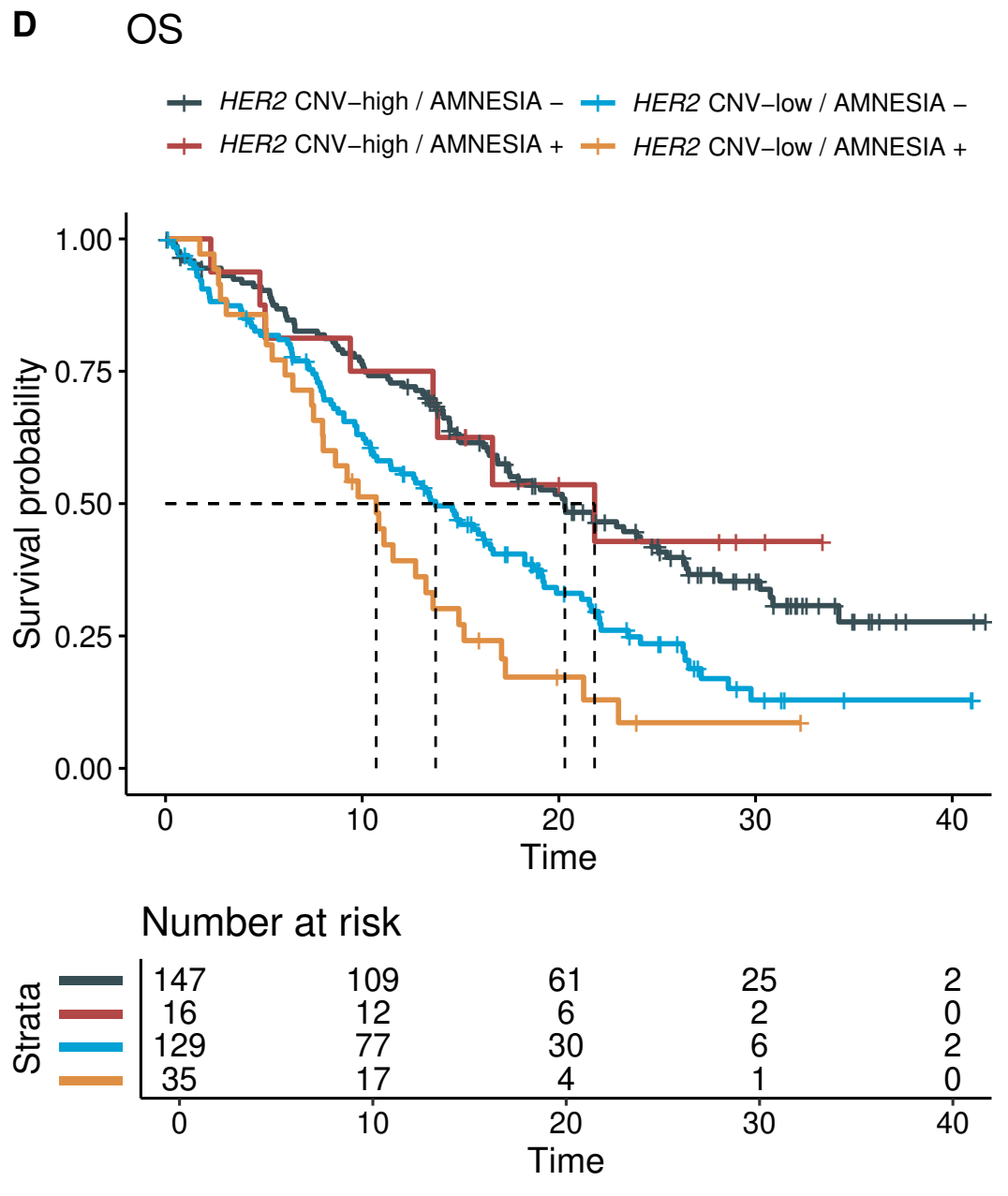
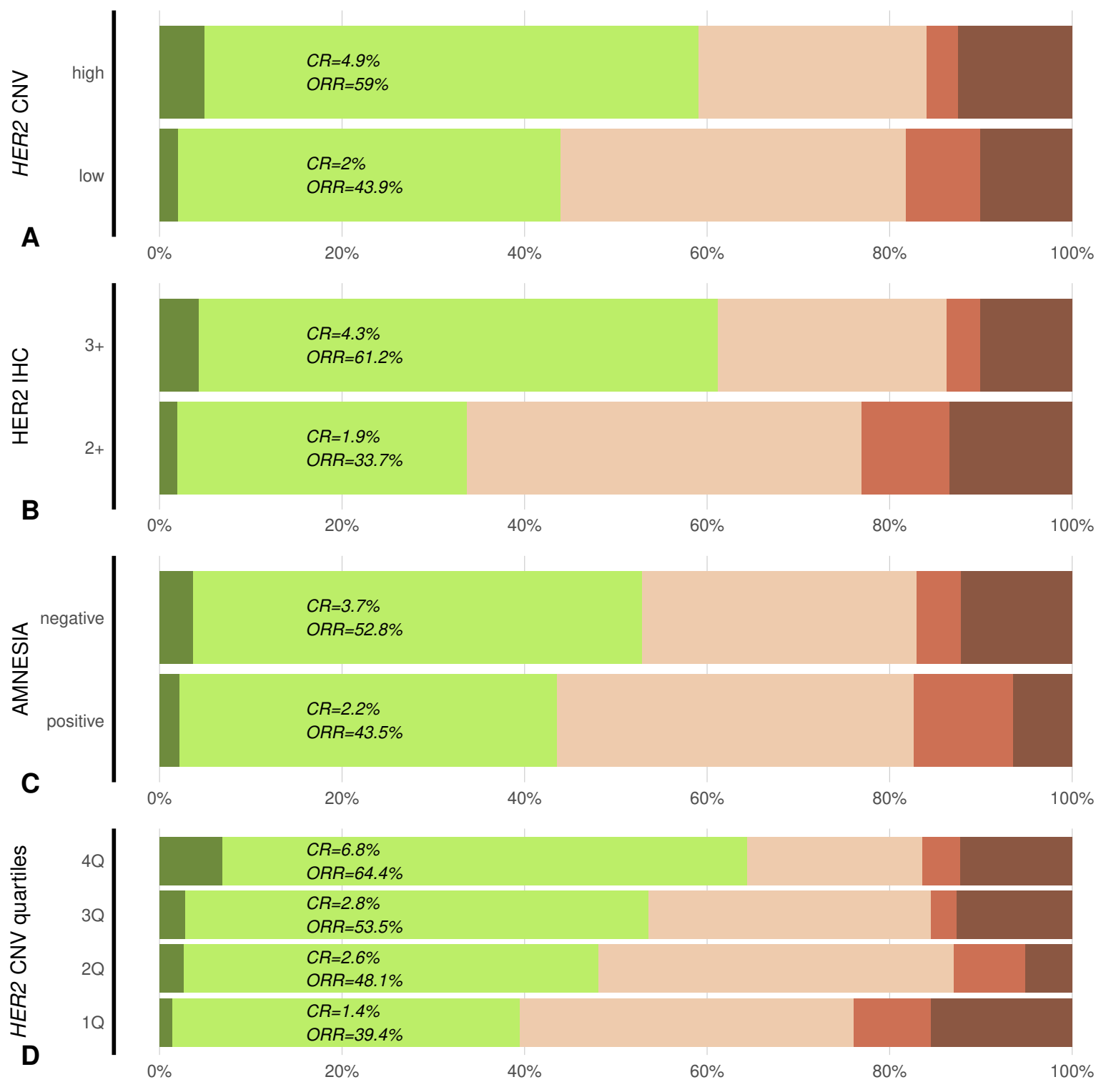

D
OS
HER2 CNV−high / AMNESIA −
HER2 CNV−low / AMNESIA −
HER2 CNV−high / AMNESIA +
HER2 CNV−low / AMNESIA +
Survival probability
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
Time
Number at risk
Strata
147 109 61 25 2
16 12 6 2 0
129 77 30 6 2
35 17 4 1 0
0
10
20
30
40
Time

Figure 4


HER2 CNV
high
low
CR=4.9%
ORR=59%
CR=2%
ORR=43.9%
A
0%
20%
40%
60%
80%
100%
HER2 IHC
3+
2+
CR=4.3%
ORR=61.2%
CR=1.9%
ORR=33.7%
B
AMNESIA
negative
positive
CR=3.7%
ORR=52.8%
CR=2.2%
ORR=43.5%
C
HER2 CNV quartiles
4Q
3Q
2Q
1Q
CR=6.8%
ORR=64.4%
CR=2.8%
ORR=53.5%
CR=2.6%
ORR=48.1%
CR=1.4%
ORR=39.4%
D
response
NE
PD
SD
PR
CR