nature portfolio

Peer Review File

HER2-related biomarkers predict clinical outcomes with trastuzumab deruxtecan treatment in patients with HER2-expressing metastatic colorectal cancer: biomarker analyses of DESTINY-CRC01

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The manuscript mostly presents descriptive statistics for various types of HER2 measurements. Given the exploratory nature, it is difficult to determine statistical or clinical relevance. Also, without information of patient characteristic in the biomarker data cohort, it becomes challenging to follow the biomarker analysis. Detailed comments are listed below.

1. “Numerically higher” or “trend” are constantly used in the content (e.g., numerically higher objective response rate; longer progression-free and overall survival). Lack of information of statistical significance raises the concern of where the chance is due to randomness.
2. Biomarker threshold is based on optimal cut-offs (e.g., maximum value of the Youden index or minimal p value in log-rank test). Such strategy could lead to biased results.
3. Most results are descriptive without clear conclusion. Some are even lengthy without focus, such as “Among the biomarkers investigated, the ORR, median PFS (mPFS), and median OS (mOS) were 7.7% and 57.5%, 4.1 months and 8.3 months, and 11.0 months and 19.9 months, respectively, in patients with HER2 IHC 2+/ISH+ and IHC 3+ tumors; …….”
4. More details are needed to better understand baseline characteristics between biomarker dataset and each cohort. The current description is difficult for evaluation: “Baseline characteristics of the patients in each biomarker dataset were similar to the baseline characteristics of each cohort overall23.”
5. Since response is one primary outcome for biomarker evaluation, distribution of response status in biomarker cohort will be informative for downstream biomarker analysis.
6. While the Supplementary Figure S1 is useful, it would be more informative have the number of patients with both ctDNA and HER2ECD datasets, and the number of patients with a complete collection of ctDNA (C1D1, C4D1, and EOT), and partial collection of ctDNA (e.g., C1D1 and C4D1 or C1D1 and EOT).
7. Multiple variations of HER2 biomarker were evaluated and showed associated with clinical outcomes. Are they highly correlated? Please comment which one is recommended for clinical use. Will combination of all these variations or subset of the variations give better prediction performance?
8. Histological grade at baseline is negatively associated with clinical outcomes (e.g., well differentiation with lowest ORR and shortest survival in contrast to poor differentiation). Clarification is needed.
9. Longer interval from last irinotecan treatment tended to have high ORR (Q3+Q4 >Q1+Q2). However, Q1 seems to have a longer survival than Q2. Little discussion is

presented in this issue.

Reviewer #2:
Remarks to the Author:
I thank the editor for the invitation to review this work, and congratulate the authors with the trial results and the translational work.

The manuscript holds the results of tissue and blood analysis on the material, and the focus for the translational studies seem to be the importance of various HER2 related measures for outcome, the potential of efficacy in patients with known activating mutations and an explorative analysis of the changes in ctDNA measures. Also, results of mutations at progression is described. Most of the presentation is descriptive due to the limited sample size, but with a resonable balance between analysis and conclusion based on the dataset.

The number of clinical studies of HER2 targeted therapies in mCRC is limited but increasingly ongoing, and translational studies are needed to improve the biological knowledge and clinical implication. Most studies are of limited sample size, which hampers strong evidence, and consequently the present data set is valuable in this context, despites the limited number in earch subgroup for translational analysis.

the methods section is relevantly desribed, and the clinical trials previously published.

The introduction is very well written, but since the primary data is already published I would have liked to see a bit of background for the choice of biomarkers in the introduction, rather than focus on the treatment regimen only.

Results; the figure 2 is widely described in the results section, which is rather difficult to read without loosing the overview. This is repeated from the figure and could perhaps be reduced with focus on statistically relevant differences ?

General comment, through out the text it would be useful if it was pointed out what is measured at baseline and what is in tissue and blood. This would make the text easier to understand for the readers. aslo,I would have liked the flow-chart of samples as part of the manu rather than supplementary.

Results of "activating" mutations, and fig 3, please add to the figure heading that this is

measured in tumor or ctDNA. Please also consider additing if there were overlapping between the mutations/alterations .

Figure 4. appologies, but it is somehow difficult to extract what is ment by baseline status .. at progression .... please consider rephrasing.

In general, it could be considered not to repeat the results fully both in text and tables.

The section of ctDNA/VAF changes could have deserved more attention, and would form basis for future studies ? the novelty is the ctDNA variations during therapy in patients treated with this class if drugs rather than chemotherapy alone, and the potential of HER2 related measurements. Some presentation in the main rather than supplementary would have been interesting.

It is not entirely clear why the seperate paragraph on HER2 apmlification at progression is prioritized. Please consider adding some clinical relevance.

Figure 5 , consider to use another wording than the aquired mutations, this could be a sensitivity aspect rather than clonal evolution adding to resistance.

Discussion
Please add to the importance of the last comment in 267-268. is this clinically relevant? would it add to therapy?

It could be suggested to underline exactly the novelty of the current findings and the clinical relevance of these in a brief seperate section.

The finding of RAS mutations in plasma in tumor negative cases is discussed. It is suggested that the RAS mutated are aquired . please consider/discuss that RAS mutations in low concentration in the plasma may not be clinically important ? and if this is analysed in the other mentioned studies of HER2 targeted treatments. Also please add to the presentation early in the text if the RAS status was done on archival old tissue or newly added (since the methods section is last), if cases overlap.

It could be added overall, that a true predictive value of potential biomarkers must be statistically established in a randomised setting, but that the present results adds to the hypothesis of a potential clinical relevance of HER2 markers and efficacy across mutational status.

Conclusion, I would add a "detected in ctDNA" to the last sentence in abstract as well as in the text.

Reviewer #3:
Remarks to the Author:
This is an important biomarker analysis from the Destiny CRC 01 trial. There were 86 patients in 3 cohorts which were as following the IHC 3+ or 2+/ISH positive patients (high), intermediate (IHC2+/ISH+) and low patients (ICH1+) 53 were 3+.

The authors looked at both blood and tissue based biomarkers including HER2 status (IHC/ISH), HER2/CEP17 ratio, HER2 ISH signals, HER2 H-score, plasma HER2 amplification status, HER2 plasma copy number, and HER2 extracellular domain

These were are exploratory analyses and ultimately in small numbers of patients but provocative particularly in so far as there were plasma RAS/PI3K mutated patients benefited and sets the stage for possible other biomarkers of non response too.

Some of the limitations include sample size. For example,for ctDNA cohort B was only 15 patients of which 11 had EOT samples. Cohort C app 20. There were more for ECD, IHC/ISH. Notably in cohort A, ctDNA correlated well with tissue based amplifications (47/52) and 6, 8, 6patients actually had RAS mutations, Her2 mutations and PI3K mutations from Cohort A on ctDNA.

Focal plasma amplification correlated with tissue IHC3 plus, less in intermendiate and reassuringly none on the low patients. Copy number, HER2/CEP17 ratio and HER2 ECD all correlated with the IHC3+ tumor patients.

Across tissue and blood, albeit again small numbers, there were trends towards the biomarkers examined and response such as plasma HER2 focal amplification, higher levels of HER2 ApCN and
HER2ECD in liquid biopsy samples at baseline

Notable at time of PD, most patients who responded maintained amplification with several acquired resistance mechanisms but without clear patterns emerging of resistance.

Some questions for the authors:
1) How were cut offs of VAF to predict response correlation determined. How was 16.4% determined as the biomarker cutoff of VAP response?

2) Any suggestions of response for both tissue AND blood rather than OR
3) any suggestion of non response for those who were focally amplified, IHC3+, higher HEr2 ApCN and her2 ECD in liquid who did not respond?

The authors rightly point out tissue based testing seems to still be the best biomarkers but reassuring if tissue limited, plasma may be okay. Furthermore, the findings of RAS/HER2/PI3K mut in the blood and some response is important.

March 12, 2024

Response to reviewer comments for the manuscript "HER2-related biomarkers are predictive of clinical outcomes with trastuzumab deruxtecan treatment in patients with HER2-expressing metastatic colorectal cancer: biomarker analyses of the phase 2 DESTINY-CRC01 trial." (NCOMMS-23-50923-T).

Author Responses to Reviewer Comments

Reviewer 1 Comments

COMMENT:
"Numerically higher" or "trend" are constantly used in the content (e.g., numerically higher objective response rate; longer progression-free and overall survival). Lack of information of statistical significance raises the concern of where the chance is due to randomness.

> **RESPONSE:** Thank you for this suggestion. We have removed the terms "numerically higher" and "apparent trend" throughout the manuscript.
>
> *P* values were calculated for this analysis using the Fisher exact test for ORR and log-rank test for PFS and OS. We have updated Figures 3A and 4A and Supplementary Figure S6 to show *P* values for the comparison of ORR, PFS, and OS, and we have updated the statistical analysis methods on page 20, lines 431-432, accordingly.

COMMENT:
Biomarker threshold is based on optimal cut-offs (e.g., maximum value of the Youden index or minimal p value in log-rank test). Such strategy could lead to biased results.

> **RESPONSE:** Thank you for the comment. Before calculating optimal cutoff values, we assessed how median cutoff values were associated with outcomes; these data are shown in Supplementary Figure S6 and demonstrate that a consistent trend was observed across all HER2 biomarkers, as expected given that T-DXd is a HER2-directed therapy. Optimal cutoff thresholds were used in the current analysis in order to use values beyond those identified in the median value analyses.

COMMENT:

Most results are descriptive without clear conclusion. Some are even lengthy without focus, such as "Among the biomarkers investigated, the ORR, median PFS (mPFS), and median OS (mOS) were 7.7% and 57.5%, 4.1 months and 8.3 months, and 11.0 months and 19.9 months, respectively, in patients with HER2 IHC 2+/ISH+ and IHC 3+ tumors; ……."

RESPONSE: Thank you for this suggestion. We have revised the lengthy descriptions in the results section to improve readability. Because the sample size was small and the analyses were performed retrospectively, we have been careful to only present the data in the results and avoid using conclusive statements in the results text.

COMMENT:

More details are needed to better understand baseline characteristics between biomarker dataset and each cohort. The current description is difficult for evaluation: "Baseline characteristics of the patients in each biomarker dataset were similar to the baseline characteristics of each cohort overall[23]."

RESPONSE: Thank you for your suggestion. Because most patients were included in each biomarker set across all subgroups, baseline characteristics of the patients in the biomarker dataset were similar to those of patients in the overall cohort. We have added a new Supplementary Table S1 to the supplementary materials to demonstrate this.

In addition, we summarized baseline characteristics (sex, age, region, ECOG PS, and side of primary tumor site) in each subgroup (HER2 biomarker higher vs lower) and found no clear difference in baseline characteristics except for ECOG PS (lower ECOG PS was observed in the HER2 biomarker low subgroups). This is shown in the table below for the reviewer's information; however, we have not added this to the manuscript.

[Redacted]

COMMENT:

Since response is one primary outcome for biomarker evaluation, distribution of response status in biomarker cohort will be informative for downstream biomarker analysis.

RESPONSE: Thank you for your suggestion. As shown in the table below, confirmed clinical outcomes (ORR, mPFS, mOS) were comparable across biomarker cohorts. However, we did not consider it necessary to include this information in the manuscript because the majority of patients were included in each biomarker cohort.

[Redacted]

COMMENT:

While the Supplementary Figure S1 is useful, it would be more informative have the number of patients with both ctDNA and HER2ECD datasets, and the number of patients with a complete collection of ctDNA (C1D1, C4D1, and EOT), and partial collection of ctDNA (e.g., C1D1 and C4D1 or C1D1 and EOT).

RESPONSE: Thank you for your comments. Please find the information requested summarized in the tables below for information. We have not added this information to the

manuscript; however, Supplementary Figure S1 has been moved to the main manuscript (now Figure 1) as requested by Reviewer 2.

[Redacted]

COMMENT:

Multiple variations of HER2 biomarker were evaluated and showed associated with clinical outcomes. Are they highly correlated? Please comment which one is recommended for clinical use. Will combination of all these variations or subset of the variations give better prediction performance?

RESPONSE: As shown in Figures S3, S4, and S5, biomarkers were well correlated. Because patients were selected based on HER2 IHC status and the number of patients in this analysis was small, we cannot reliably comment on which HER2 biomarker would be recommended for clinical use based on the current data. However, we have noted in the discussion on page 14, lines 288-291, that plasma *HER2* amplification (and HER2ECD) might be potential alternative biomarkers for patients without adequate tumor tissue for assessment of HER2 IHC status. Because these HER2 biomarkers are well correlated with each other, we do not think that biomarker combinations would provide better predictions. We have summarized the results showing clinical outcomes in patients with HER2 IHC 3+ status and *HER2* focal plasma amplification in the table below. These data show that outcomes are comparable to those in patients with focal plasma amplification or HER2 IHC 3+ status. Because these

biomarkers are well correlated, we do not believe they would add additional information to the manuscript, but we include them here for the reviewer's information.

[Redacted]

COMMENT:
Histological grade at baseline is negatively associated with clinical outcomes (e.g., well differentiation with lowest ORR and shortest survival in contrast to poor differentiation). Clarification is needed.

RESPONSE: Because the numbers of patients with poorly differentiated and well differentiated tumors are very small, it cannot be reliably concluded that histologic grade at baseline is negatively associated with clinical outcomes. This would need to be verified in future studies; therefore, we respectfully request to keep the current statement as it is and not draw conclusions on association between histologic grade and outcomes.

COMMENT:
Longer interval from last irinotecan treatment tended to have high ORR (Q3+Q4 >Q1+Q2). However, Q1 seems to have a longer survival than Q2. Little discussion is presented in this issue.

RESPONSE: As shown on pages 12-13 and Supplementary Figure S11, the key finding from this analysis was that response to T-DXd was observed regardless of the time since the last irinotecan treatment. Therefore, we have not added any further discussion to the manuscript.

Reviewer 2 Comments

COMMENT:
The introduction is very well written, but since the primary data is already published I would have liked to see a bit of background for the choice of biomarkers in the introduction, rather than focus on the treatment regimen only.

RESPONSE: The biomarkers selected were those that are well-known biomarkers in CRC, such as *RAS* or *BRAF* mutation, and biomarkers with known relevance to the mechanism of

action of T-DXd as a HER2-directed antibody-drug-conjugate. We have added this explanation to the last paragraph of the introduction.

COMMENT:

Results: Figure 2 is widely described in the results section, which is rather difficult to read without losing the overview. This is repeated from the figure and could perhaps be reduced with focus on statistically relevant differences?

RESPONSE: As noted in response to Reviewer 1, we have revised the lengthy descriptions in the results section to improve readability and added *P* values to Figure 2A. We have also removed terms such as “numerically higher” and “apparent trend” in response to comments from Reviewer 1.

COMMENT:

General comment, throughout the text it would be useful if it was pointed out what is measured at baseline and what is in tissue and blood. This would make the text easier to understand for the readers.

RESPONSE: The methods section explains the biomarkers that were measured in tissue versus liquid biopsy (serum or plasma) samples. To make this information clearer, we have moved Supplementary Figure 1 (the flow chart mentioned in next comment) into the main part of the manuscript and it is now Figure 1.

COMMENT:

Also, I would have liked the flow-chart of samples as part of the manuscript rather than supplementary.

RESPONSE: Thank you for this suggestion. We have now moved Supplementary Figure 1 (the flow chart) into the main part of the manuscript, and it is now Figure 1.

COMMENT:

Results of "activating" mutations, and fig 3, please add to the figure heading that this is measured in tumor or ctDNA. Please also consider adding if there were overlapping between the mutations/alterations.

RESPONSE:

Thank you. We have added "in ctDNA" to the figure heading (it is now renumbered as Figure 4). Please also note that Figure 2 shows the overlap of several biomarkers, and Table S1 summarizes information on *RAS*, *PIK3CA*, and *HER2*.

COMMENT:

Figure 4. apologies, but it is somehow difficult to extract what is meant by baseline status .. at progression .... please consider rephrasing.

RESPONSE:

Thank you, this figure, now Figure 5, illustrates how plasma *HER2* amplification status changed from baseline. We have simplified the title to: "Plasma HER2 amplification status at baseline and at disease progression."

COMMENT:

In general, it could be considered not to repeat the results fully both in text and tables.

RESPONSE: Thank you for this feedback. We have made some adjustments to the results text based on feedback from Reviewer 1 to avoid using terms such as "numerically higher" and "trend" and just show the values instead; therefore, some of the results are summarized in the text but full details are in the figures or tables. In some instances, including results in the main text is necessary for interpretation, particularly with limited statistical comparison.

COMMENT:

The section on ctDNA/VAF changes could have deserved more attention and would form basis for future studies? The novelty is the ctDNA variations during therapy in patients treated with this class of drugs rather than chemotherapy alone, and the potential of HER2 related measurements. Some presentation in the main rather than supplementary would have been interesting.

RESPONSE: Thank you for this suggestion. We have elaborated further in the discussion on the potential relationship between VAF changes and clinical outcomes during T-DXd treatment (which are presented in Figures S7, S9, and S10). Although there were signs of potential associations—for example, a reduction in VAFs in patients with *RAS* or *PIK3CA*

mutations who achieved tumor shrinkage (Figure S7)—we cannot make robust conclusions because of the small sample size. We have added this discussion on page 14, lines 299-301 and noted on line 329 that conclusions cannot be drawn regarding potential relationships between changes in mVAF and clinical outcomes due to the small number of patients.

COMMENT:

It is not entirely clear why the separate paragraph on HER2 amplification at progression is prioritized. Please consider adding some clinical relevance.

RESPONSE: Because HER2 therapies are approved in other indications and undergoing various stages of development for the treatment of CRC, we considered that information about HER2 status at disease progression would be important information for clinicians regarding ongoing research, which is why we separated out that paragraph.

COMMENT:

Figure 5, consider using another wording than the acquired mutations, this could be a sensitivity aspect rather than clonal evolution adding to resistance.

RESPONSE: Thank you for the suggestion. As shown in the figure below for the reviewer's information, ctDNA shedding was not increased at disease progression in most of the patients who had acquired mutations. Therefore, we did not consider the sensitivity aspect to be a major concern for the current analysis.

[Redacted]

COMMENT:

Discussion: Please add to the importance of the last comment in 267-268.

"Based on the current results, HER2-positive status defined by IHC/ISH appears to remain as the most important biomarker to predict response to T-DXd. However, the exploratory data reported in this study, particularly the blood-based HER2-related biomarkers, also appear to show a correlation with response to T-DXd."

Is this clinically relevant? Would it add to therapy?

> **RESPONSE:** The sample size of the current analysis and limited statistical comparison precludes drawing any conclusions regarding the clinical relevance or treatment recommendations. However, ctDNA analysis of HER2-related biomarkers such as plasma *HER2* amplification and measurement of HER2ECD might become important biomarkers for patients who do not have adequate tumor tissue for determination of HER2 IHC status. We have added a statement at the end of the first paragraph of the discussion on pages 13-14, lines 285-291, noting this.

COMMENT:

It could be suggested to underline exactly the novelty of the current findings and the clinical relevance of these in a brief separate section.

RESPONSE: As noted in the responses above, the sample size of the current analysis limits drawing conclusions on the clinical relevance of our results, and we have been careful not to overstate the findings of this exploratory analysis. However, identification of novel HER2 biomarkers for patients who do not have sufficient tumor tissue sample available for determination of HER2 IHC status is an important finding that warrants further investigation. This has been highlighted in the discussion as noted in the previous reviewer comments.

COMMENT:

The finding of RAS mutations in plasma in tumor negative cases is discussed. It is suggested that the RAS mutated are acquired. Please consider/discuss that RAS mutations in low concentration in the plasma may not be clinically important? And if this is analyzed in the other mentioned studies of HER2 targeted treatments. Also please add to the presentation early in the text if the RAS status was done on archival old tissue or newly added (since the methods section is last), if cases overlap.

RESPONSE: Thank you for your feedback. *RAS* mutation was assessed locally before enrollment; therefore, additional details are unfortunately not available. Due to the lack of available background data, the clinical importance of *RAS* mutations with low VAF in ctDNA is challenging to interpret. As the reviewer suggests, it might not be appropriate to discuss the antitumor activity of T-DXd in tumors with *RAS* mutations using data from patients with lower VAF of *RAS* mutations. We recognize this point in the following statement in the discussion on page 14, lines 299-304: *"Although there were signs of potential associations—for example, a reduction in VAFs in patients with RAS or PIK3CA mutations who achieved tumor shrinkage (Figure S7)—the small number of patients with activating mutations of RAS, PIK3CA, and HER2 and high bTMB means that interpretation of these results is limited and further investigation to validate these findings is warranted in a larger study, such as the DESTINY-CRC02 trial (NCT04744831)."*

Furthermore, regarding this question, it is interesting to note the "change in VAF of *RAS* and *PIK3CAmut*" of patient #1 in our study (please see Fig. S7). The VAF of *RAS* mutation in this patient was 3.05% (ie, not low) at baseline and *RAS* mutation became undetectable by C4D1, even though other somatic gene mutations were still detectable with the comparable level of VAF. Although this is just one case, the observation may suggest antitumor activity of T-DXd

in tumors with *RAS* mutation; however, this would require further investigation in a larger cohort.

COMMENT:

It could be added, overall, that a true predictive value of potential biomarkers must be statistically established in a randomized setting, but that the present results add to the hypothesis of a potential clinical relevance of HER2 markers and efficacy across mutational status.

RESPONSE: Thank you for this suggestion. We have added this to the end of the first paragraph of the discussion on pages 13-14, lines 285-291.

COMMENT:

Conclusion: I would add a "detected in ctDNA" to the last sentence in abstract as well as in the text.

RESPONSE:

Thank you, we have added this wording to the conclusion in the abstract.

Reviewer 3 Comments

COMMENT:

1. How were cut offs of VAF to predict response correlation determined. How was 16.4% determined as the biomarker cutoff of VAP response?

   RESPONSE: The cutoff of 16.4% was an exploratory cutoff value determined according to the minimum *P* value of the log-rank test for PFS. This is mentioned in the results on page 12, lines 245-248, of the manuscript and shown in Supplementary Figure S9.

2. Any suggestions of response for both tissue AND blood rather than OR?

   RESPONSE: We evaluated response for both tissue (HER2 IHC) and blood (*HER2* amplification) as shown in the table below for the reviewer's information. Patients with HER2 IHC 3+ status and *HER2* focal amplification showed comparable responses (no

obvious higher response) to those with HER2 IHC 3+ status and *HER2* focal amplification. This is likely due to a strong correlation between HER2 IHC status and plasma focal amplification status.

[Redacted]

3. Any suggestion of non response for those who were focally amplified, IHC3+, higher HER2 ApCN and her2 ECD in liquid who did not respond?

   RESPONSE: We evaluated baseline characteristics (sex, age, region, ECOG PS, and side of primary tumor site) and ctDNA status (single nucleotide variant [SNV] and copy number variation [CNV]) between responders and nonresponders in subgroups of patients with higher levels of HER2 biomarkers; however, we did not identify any baseline characteristics that clearly explained resistance mechanisms.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors addressed most my questions. A few questions still require clarification.

1. Exploratory cutoff in a series of HER2 biomarkers and VAF.
• The authors attempted to use maximum p value approaches to boost p value. However, the resulted p values were inflated and biased as I mentioned before. In contrast, the median cutoff also showed similar results with $p<0.05$ in most cases. Since the median cutoff is a common practice and more objective, I recommend using the median cutoff as the primary results and considering the optimal cuttoff as exploratory results.
• For Plasma HER2 Amp, it makes sense to group n.d and Ane to compare to Focal due to limited n in n.d.
• For mVAF reduction, will it make more sense to compare reduction vs no reduction/increase, rather than artificial optima cutoff 16% reduction without clear biological or clinical explanation?
• Which clinical outcome was used in Receiver operating characteristic (ROC) analysis?

2. The authors naïvely removed the terms “numerically higher” and “apparent trend” throughout the manuscript without indicating statistical significance level.
3. The lengthy descriptions in the revised results section remains an issue as pointed out by reviewer 2. The authors should put more effort to focus on key findings.
Below is one example.

Reviewer #3:
Remarks to the Author:
All comments appropriately addressed.

Siena S et al.

NCOMMS-23-50923A: HER2-related biomarkers are predictive of clinical outcomes with trastuzumab deruxtecan treatment in patients with HER2-expressing metastatic colorectal cancer: biomarker analyses of the phase 2 DESTINY-CRC01 trial

Author Responses to Reviewer Comments (Round 2)

Reviewer #1 Comments

The authors addressed most of my questions. A few questions still require clarification.

COMMENT:

Exploratory cutoff in a series of HER2 biomarkers and VAF:

- The authors attempted to use maximum *P* value approaches to boost *P* value. However, the resulting *P* values were inflated and biased as I mentioned before. In contrast, the median cutoff also showed similar results with $P<0.05$ in most cases. Since the median cutoff is a common practice and more objective, I would recommend using the median cutoff as the primary results and consider the optimal cutoff as exploratory results.

  RESPONSE: All biomarker analyses were exploratory because this was predefined in the study protocol; therefore, both results are considered exploratory. We have presented the exploratory cutoff values based on the maximum value of the Youden index for ORR in the main part of the manuscript in Figure 3, with supporting evidence from the analysis using median values as the cutoff in Supplementary Figure S6. This approach was adopted because HER2 levels are expected to be associated with outcomes because T-DXd is a HER2-directed therapy. The biomarkers in these analyses are all HER2-related biomarkers and, as expected, a clear association between HER2 status and clinical outcomes was observed using both cutoffs, which also supports the relationship between HER2 status and outcomes reported previously in the primary analysis (Siena S et al. *Lancet Oncol*. 2021;22:779-789). Based on this rationale, we respectfully request to present the results of the exploratory cutoff using the maximum value of the Youden index in the main part of the manuscript to further understanding of the predictiveness of HER2 biomarkers with optimized cutoffs because the conclusions are consistent between the analyses using the optimized and median cutoffs. These findings are being validated in additional trials of T-DXd, which will be reported separately.

COMMENT:

- For plasma HER2 amplification status, it makes sense to group ‘not detected’ and ‘aneuploidy’ together to compare with ‘focal’ due to limited number in the ‘not detected’ group.

RESPONSE: The grouping by not detected/aneuploidy and focal amplification in Figure 3 was determined by the amplification type reported from Guardant Health (aneuploidy amplification is not included as *HER2* amplification). The analysis shown in Supplementary Figure 6 considers *HER2* aneuploidy amplification and it is therefore grouped with *HER2* focal amplification, and samples with no amplification detected are shown separately.

COMMENT:

- For mean variant allele fraction (VAF) reduction, would it make more sense to compare reduction versus no reduction/increase, rather than the “artificial optimal” cutoff 16% reduction without clear biological or clinical explanation?

RESPONSE: We thank the reviewer for this suggestion. We previously evaluated increase versus reduction in VAF as suggested. Data are not included in the manuscript; however, we have shown the ORR [95% CI] below for the reviewer’s information:

Increase: 0% [0–45.9] (0/6)

Reduction: 64.7% [45.6–80.3] (22/34)

Because the number of patients with “increase” was small, we used exploratory mVAF cutoff values instead. Analysis using several mVAF cutoffs for molecular response (complete, major, partial, absent) shown in Supplementary Figure S8 demonstrated consistent trends and, therefore, the cutoff was explored for additional analyses.

COMMENT:

- Which clinical outcome was used in the receiver operating characteristic (ROC) analysis?

RESPONSE: ORR was the clinical outcome used in ROC analysis. We have now clarified this in the statistical methods on page 20, line 416.

COMMENT:

The terms “numerically higher” and “apparent trend” were removed throughout the manuscript without indicating statistical significance level.

RESPONSE: Thank you for your comment. Instead of "numerically higher," which was removed previously, we have now added statistical significance (*P* values) where relevant for differences in clinical outcomes according to HER2-related biomarkers on page 8, lines 160–168.

COMMENT:

Lengthy descriptions in the revised results section remain an issue (as pointed out previously by reviewer #2). The authors should put more effort into focusing on the key findings.

RESPONSE: Thank you for your comment. We have made further edits to remove lengthy description of results.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The rebuttal letter is reasonable. I have no further question.