nature portfolio

Peer Review File

Longitudinal Evaluation of Circulating Tumor DNA in Patients Undergoing Neoadjuvant Therapy for Early Breast Cancer Using a Tumor-Informed Assay

Corresponding Author: Dr Mitchell Elliott

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors investigate a highly sensitive (LoD95%: 0.001%) tumor informed assay in NAC BC patients. Median of 48 variants tracked per patient.

Whilst a smallish cohort, and mixed subtypes, and irregular followup collection times, low number of detectable results in follow up, results are very interesting and highlight the benefits of the higher sensitivity assays. Particularly with regards to those who are positive at baseline, nearly all recurred, suggesting disease biology differences in these patients, though it is unclear to me if some of these did clear mid way. Novelty is the assay itself.

Please add PPV NPV Send and Spec for all timepoints, baseline and mid, and follow up

What is the overlap in patients who recur?
Which is the best timepoints in a multviariate analysis or do they all provide useful information?

Lead time is influenced by sampling frequency- this should be mentioned.
Some discussion of the next generation assays with many 100s-1000s of mutations may also be worth mentioning, with some assays now getting down to 1 in 10x6 LOD95

Please label figures on PDF
Figure 3- please include PPV,NPV, send and see for all plots as well as number of events
Figure 4 A RCB- shouldn't there be 4 lines are two overlapping? Would be interesting to see the differences RCB 0 detected vs not

Does Table 3 refer to the last figure? Detection of ctDNA post op?

Reviewer #2

(Remarks to the Author)
I commend the authors for conducting a well-designed study that provides insights into the use of circulating tumour DNA (ctDNA) in early breast cancer. Despite being a single-centre and retrospective analysis conducted during the challenging circumstances of the pandemic, this study is valuable and contributes to the field.

Major comments:

1. Data Availability: It is imperative that the authors make the underlying data available for review and future research. Sharing the data will enhance the transparency of the study and allow others in the field to build upon these findings.

2. Method Description: The description of the methods is not entirely clear. It appears that the whole-exome sequencing

(WES) methodological steps are included under the ctDNA sequencing subsection. This needs to be carefully reviewed and clarified to ensure that each method is properly described in its respective section. Clear and precise method descriptions are essential for understanding of the study.

I have a few minor comments regarding the figures and data presentation:

1. Figure 1A: It appears that this figure does not contain any meaningful information, as it seems to show the same data twice. If there is additional information that I am missing, clarification would be helpful. Additionally, the colour scheme across the figure panels could be improved. The current use of the same colours for all box plots is confusing and does not effectively differentiate the data. I suggest harmonizing the colours across figure panels, using distinct colours consistently to represent different cancer subtypes, for example. This adjustment would enhance the clarity and interpretability of the figures.

2. Figure 2E: Please consider adding an annotation band for cancer subtypes and the number of patients. Additionally, include the scale for Tumour Mutation Burden (TMB); I assume it is mutations per megabase (mutations/Mb), but this should be clearly indicated.

3. For the next round of reviews, I recommend adding figure numbers directly to the figures and, preferably, including captions with the figures. Moreover, ensure that all abbreviations used in the figures are defined in the figure captions. These changes will significantly aid in the review process and enhance the overall readability of the manuscript.

Overall, I appreciate the effort put into this study and believe that addressing these concerns, particularly the availability of the data, would further strengthen the presentation of the findings.

Reviewer #3

(Remarks to the Author)
In this manuscript, Elliott and colleagues utilize RaDaR, a tumor-informed ctDNA MRD assay, in a cohort of 118 early-stage breast cancer patients who received standard-of-care neoadjuvant chemotherapy in real-world setting. The study includes analysis of serial cfDNA samples at various timepoints, whereas other studies often focus on specific settings (post-operative or on neoadjuvant chemotherapy). The authors describe ctDNA dynamics during different stages of therapy and demonstrate that ctDNA clearance and lack of ctDNA positivity at baseline were associated with significantly better outcomes, while ctDNA positivity during follow-up was associated with recurrence in almost all cases.

The article is timely and has major clinical implications. The manuscript is clear, well-written, and the analyses are sound and appropriate. While ctDNA dynamics during neoadjuvant chemotherapy have previously been described based on the analysis of iSPY2 clinical trials utilizing a 16-mutation bespoke MRD assay (Magbanua et al, Cancer Cell), in my opinion, this study is a significant contribution to the field, demonstrating that such dynamics can be assessed in a real-world setting using an assay theoretically ~10 times more sensitive than the one used in the iSPY2 analysis. Additionally, this study does not have the major limitation of the iSPY2 analysis, which lacked any ctDNA analysis in the post-operative and follow-up settings.

Here are a few comments to be considered by the authors:

Major comments:

• Table 1: In the recurrence section, the numbers exceed the total number of patients. Were there any local recurrences in this cohort, and was the assay able to identify them?

• Figure 2a provides minimal information not presented in the other figures. It could be moved to the supplemental section or removed in its current form.

• Figure 2E: There is an unexpectedly higher rate of BRCA1, BRCA2, and FAT1 mutations in this cohort. Are there other alterations not demonstrated in this oncoprint that could raise similar concerns? The authors need to explain whether this is related to the true characteristics of the cohort or if it could be due to technical issues, such as a lack of matched normal sequencing for tumor WES, inadequate germline and noise filtering, etc. Was there any association between the genomic profile of the tumor and baseline ctDNA detectability after controlling for other clinical parameters such as tumor size and nodal involvement?

• Based on the review of the swimmer plots, there seem to be no patients who were negative at baseline but later became MRD-positive and ctDNA negativity at baseline was associated with a lack of distant recurrence (except for one case). As discussed in the paper, this could be a result of the assay's LOD or the biological features of the tumor. Importantly, baseline ctDNA negativity appears to be prognostic, regardless of neoadjuvant treatment outcomes, as the majority of patients (except one) failed to achieve a pCR. This seems worth further exploration by the authors.

• What was the rationale for excluding patients with no baseline detection from the analyses presented in Figures S2F-2K?

These baseline samples could have been added as “0” in the analysis and visualized as “ND” in the log-scale plots. Overall, these are informative analyses and could potentially be moved to the main figures.

• The authors present outcome analyses stratified by RCB and mid-NAT ctDNA detection, demonstrating the potential added prognostication of ctDNA to RCB. Was ctDNA detection at baseline, or its clearance at mid-NAT or pre-op, associated with RCB?

• The prior iSPY2 analysis did not include post-op ctDNA landmark analysis. What was the association between single post-op sample as landmark analysis and outcomes. It would be informative to present this analysis stratified by RCB and receptor subtypes as well.

• The survival analysis based on future ctDNA detection anytime during follow-up has a major statistical flaw, as it assumes that ctDNA positivity (or its persistent negativity) was known at time zero for KM or Cox analysis. This is a common statistical flaw seen in many ctDNA MRD publications and has unfortunately become the norm for such papers. It would be imperative to perform a statistically appropriate analysis that includes ctDNA positivity as a time-dependent variable and present this either as a main or supplemental figure.

• Was there any evidence of ctDNA in the adjuvant collections for LIB-02-0085 and LIB-02-0093 that was below the threshold for a positive MRD?

• In the discussion section, while the study results are thoroughly analyzed, they should be more closely integrated with the existing literature in each context (e.g., paragraph line 355). It is also important to address the fact that most post-operative samples were negative in patients who ultimately relapsed. This suggests that using this timepoint to escalate or de-escalate treatment could be clinically challenging with the current assay. It is unclear whether assays with higher sensitivity could address this issue.

• The authors state, “The overall baseline detection rate in our analysis was numerically higher than previously reported in a similar cohort, possibly due to the increased technical sensitivity of the assay used.” This is debatable. In Magbanua et al. (Cancer Cell, 2023, and not the 2021 publication), the baseline ctDNA detection rates were 69% for HR+ and 91% for TNBC, which are very similar to this cohort (70% and 89%, respectively). Signatera uses 16 variants, while in this study, the median number of variants for ER+/HER2- tumors was 23. Given the differences in cfDNA analysis technologies (NGS for RaDaR vs. multiplex PCR for Signatera) and the relatively similar number of mutations used, this raises the question of whether this assay offers a clinically meaningful improvement in detection rates compared to Signatera, especially in ER+ tumors.

• There are multiple cases where ctDNA was negative during NAT or follow-up, but later became positive, suggesting that detection of ctDNA could be possible using assays with a lower LoD.

• Lines 355-369: The authors seem to describe LIB-02-0115, which, based on Figure 5A, appears to be a case of cTN0 low-grade ER+. The authors suggest that not competing neiadjuvant chemotherapy could have affected the outcomes. This is speculative, as this patient seems to have indolent disease with undetectable ctDNA at baseline (indicating low-grade tumor with low tumor turnover). It is unclear if this tumor would have benefited from completing chemotherapy. Was it confirmed via sequencing that the metastatic disease was genomically related to the primary tumor used for ctDNA assay development? Was the residual disease histologically and genomically related to the primary biopsy?

• It is curious why two patients in the ER+ cohort (Figure 5A) received anti-HER2 therapy. Were these cases of initial ER+/HER2- disease that later showed HER2+ disease in the residual tumor? If so, this should be explained in the figure legend.

Minor comments:

• The methods section on routine tumor WES can be moved to the supplemental methods and briefly described in the main text.
• Regarding the cfDNA sequencing methods, it would be helpful to reiterate the role of matched tumor DNA sequencing using the panel as a positive control to remove CHIP and sequencing artifacts from ctDNA calls. This section is also too long and could be summarized in the main text and moved to the supplemental methods.
• Line 109: Typo (RaRaR → RaDaR)
• Line 144: CHIP has already been defined.
• Line 282: Typo (“he PPV” → “the PPV”)
• Line 312: Missing references.
• Figure 2E Oncoprint: It would be interesting to plot subtypes to see if the genomic alterations align.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed my comments.

Reviewer #3

(Remarks to the Author)
The authors have addressed all my comments, and the manuscript has significantly improved.
I have no further comments.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Authors investigate a highly sensitive (LoD95%: 0.001%) tumor informed assay in NAC BC patients. Median of 48 variants tracked per patient.

Whilst a smallish cohort, and mixed subtypes, and irregular follow up collection times, low number of detectable results in follow up, results are very interesting and highlight the benefits of the higher sensitivity assays. Particularly with regards to those who are positive at baseline, nearly all recurred, suggesting disease biology differences in these patients, though it is unclear to me if some of these did clear mid way. Novelty is the assay itself.

We thank this reviewer for their comments and for highlighting the novelty of this study. We have added language to the discussion regarding the association between delayed clearance of ctDNA during neoadjuvant therapy and disease recurrence. We have clarified that some patients who did clear ctDNA midway through therapy had subsequent disease recurrence (8/14).

"However, persistent detection of ctDNA mid-NAT was associated with a worse RFI in all participants with 6/14 evaluable patients experiencing disease recurrence (Fig. 3B; HR: 3.59, 95%CI: 0.99-12.87, p=0.0502). While this identified a high-risk population, the positive predictive value (PPV) for clinical recurrence of persistent ctDNA detection mid-NAT was 35% (sensitivity: 43%, specificity: 81%)."

Comments:

1. Please add PPV NPV Send and Spec for all timepoints, baseline and mid, and follow up

   Thank you for this suggestion. We have included a Supplemental Table 5 listing the sensitivity, specificity, PPV and NPV at each of the following timepoints: baseline, mid-NAT, and in follow-up. This has also been reported for all participants, and by HER2 status. We do caution that the "specificity" of detection at timepoints prior to the completion of locoregional and adjuvant therapy should be interpreted as diagnostic parameters for the test, rather than "false positives" of the test (since subsequent clearance may occur with the delivery of effective therapy). We note several occasions where the delivery of adjuvant therapy resulted in clearance, followed by subsequent ctDNA detection and then clinical recurrence. This is highlighted in the 100% PPV of ctDNA detection at followup timepoints with this assay in this study.

2. What is the overlap in patients who recur?

   There is complete overlap in all patients with ctDNA detected in the adjuvant setting and recurrence. We have included time-dependent cox modeling analysis below.

3. Which is the best time points in a multivariate analysis or do they all provide useful information?

We did not perform a multivariate analysis in this study given the number of recurrences and the resulting limited power. Ultimately, validation of these findings and potential prospective intervention would be required in a large clinical trial to develop a use case and clinical utility.

In an additional analysis now included in the manuscript, ctDNA positivity was considered as a time-dependent variable, based on the time interval of subsequent measures until the last follow up. Using a proportional hazards model to assess the impact on relapse-free interval significance was noted at all timepoints (HR=24.8, 95%CI: 9.3-65.7; p<0.0001), timepoints collected prior to surgery (HR=4.6, 95%CI: 1.9-11.5; p=0.001), and timepoints collected after surgery (HR=53.7, 95%CI: 17.4-166.2; p<0.0001). This analysis further highlights that ctDNA persistence or emergence is associated with worse outcomes in this setting.

4. Lead time is influenced by sampling frequency- this should be mentioned.

   Thank you for this suggestion, we have added this in the discussion of lead time to recurrence.

   "Our study has several limitations. Recruitment and sample collection occurred during the COVID-19 pandemic, which impacted the timing of collections, particularly in perioperative and follow-up phases, due to efforts to reduce hospital visits. Consequently, some research samples were missed as more clinical testing was performed outside the primary center. The sampling frequency likely contributed to the lead time observed in this study."

5. Some discussion of the next generation assays with many 100s-1000s of mutations may also be worth mentioning, with some assays now getting down to 1 in 10x6 LOD95

   Thank you for this suggestion, we agree this is an excellent point and we have further elaborated in the discussion.

   "New ctDNA assays that evaluate hundreds or thousands of tumor-specific variants may further improve assay sensitivity in this setting. Evaluation of detection rates and other parameters of such assays will be important to assess potential clinical advantages in the development of ctDNA-based strategies.[21]"

   21. Garcia-Murillas, I. *et al.* Ultra-sensitive ctDNA mutation tracking to identify molecular residual disease and predict relapse in patients with early breast cancer. *J. Clin. Oncol.* 42, 1010–1010

6. Please label figures on PDF

   We have added these labels to the figures in the revision. Thank you for this suggestion.

7. Figure 3- please include PPV,NPV, sens. and spec. for all plots as well as number of events

We have added "Supplementary Table 5", highlighting the PPV, NPV, sensitivity, and specificity of our analyses so they can be reviewed in one section. We have included the results for baseline, mid-NAT, as well as post operative timepoints in addition to stratifying the data by HER2-positive and HER2-negative disease. We have also added the number of events and Median recurrence-free interval to the Kaplan-Meier curves in the main figures, as suggested.

8. Figure 4 A RCB- shouldn't there be 4 lines are two overlapping? Would be interesting to see the differences RCB 0 detected vs not

   Yes - the lines for RCB-0 "Detected" and "Not Detected" are superimposed. We have updated the colors of the figures and added a comment in the figure legend to clarify this point.

9. Does Table 3 refer to the last figure? Detection of ctDNA post op?

   Yes, Table 3 highlights the lead time from ctDNA detection. We have updated the title of this table: "Clinical characteristics and postoperative ctDNA lead time in participants with clinical recurrence."

Reviewer #2 (Remarks to the Author):

I commend the authors for conducting a well-designed study that provides insights into the use of circulating tumour DNA (ctDNA) in early breast cancer. Despite being a single-centre and retrospective analysis conducted during the challenging circumstances of the pandemic, this study is valuable and contributes to the field.

We thank the reviewer for their supportive comments.

Major comments:

1. Data Availability: It is imperative that the authors make the underlying data available for review and future research. Sharing the data will enhance the transparency of the study and allow others in the field to build upon these findings.

   Thank you for this suggestion. We agree that data availability is important to continue to build upon this field. In keeping with the publication policy of Nature Communications, we are uploading the whole-exome sequencing data for the participants included in the study to EGA. All other data underlying the reported analyses are included in supplementary data tables.

2. Method Description: The description of the methods is not entirely clear. It appears that the whole-exome sequencing (WES) methodological steps are included under the ctDNA sequencing subsection. This needs to be carefully reviewed and clarified to ensure that each method is properly described in its respective section. Clear and precise method descriptions are essential for understanding of the study.

We regret that this was not entirely clear. We have edited the methods section to improve their description. We have also included a supplemental methods section to outline the genomic analyses in more detail.

Minor Comments:

1. Figure 1A: It appears that this figure does not contain any meaningful information, as it seems to show the same data twice. If there is additional information that I am missing, clarification would be helpful.

   Thank you for raising this point. We intended to have Figure 2A highlight the number of participants with eVAF<0.01% at baseline. We have edited this figure to better illustrate this result.

2. Additionally, the colour scheme across the figure panels could be improved. The current use of the same colours for all box plots is confusing and does not effectively differentiate the data. I suggest harmonizing the colours across figure panels, using distinct colours consistently to represent different cancer subtypes, for example. This adjustment would enhance the clarity and interpretability of the figures.

   We appreciate these suggestions to improve the interpretability of the figures, and have updated the color schemes accordingly.

3. Figure 2E: Please consider adding an annotation band for cancer subtypes and the number of patients. Additionally, include the scale for Tumour Mutation Burden (TMB); I assume it is mutations per megabase (mutations/Mb), but this should be clearly indicated.

   Thank you for this excellent suggestion. We have updated this figure accordingly including clinical subtypes and the TMB scale.

4. For the next round of reviews, I recommend adding figure numbers directly to the figures and, preferably, including captions with the figures. Moreover, ensure that all abbreviations used in the figures are defined in the figure captions. These changes will significantly aid in the review process and enhance the overall readability of the manuscript.

   We have made these changes to facilitate the review, and apologize for not having done so in the initial submission.

Overall, I appreciate the effort put into this study and believe that addressing these concerns, particularly the availability of the data, would further strengthen the presentation of the findings.

Reviewer #3 (Remarks to the Author):

In this manuscript, Elliott and colleagues utilize RaDaR, a tumor-informed ctDNA MRD assay, in a cohort of 118 early-stage breast cancer patients who received standard-of-care neoadjuvant chemotherapy in real-world setting. The study includes analysis of serial cfDNA samples at various time points, whereas other studies often focus on specific settings (post-operative or on neoadjuvant chemotherapy). The authors describe ctDNA dynamics during different stages of therapy and demonstrate that ctDNA clearance and lack of ctDNA positivity at baseline were associated with significantly better outcomes, while ctDNA positivity during follow-up was associated with recurrence in almost all cases.

The article is timely and has major clinical implications. The manuscript is clear, well-written, and the analyses are sound and appropriate. While ctDNA dynamics during neoadjuvant chemotherapy have previously been described based on the analysis of iSPY2 clinical trials utilizing a 16-mutation bespoke MRD assay (Magbanua et al, Cancer Cell), in my opinion, this study is a significant contribution to the field, demonstrating that such dynamics can be assessed in a real-world setting using an assay theoretically ~10 times more sensitive than the one used in the iSPY2 analysis. Additionally, this study does not have the major limitation of the iSPY2 analysis, which lacked any ctDNA analysis in the post-operative and follow-up settings.

Here are a few comments to be considered by the authors:

Major comments:

1. Table 1: In the recurrence section, the numbers exceed the total number of patients. Were there any local recurrences in this cohort, and was the assay able to identify them?

   Thank you for flagging this issue. We have corrected the table, adding a new row indicating the number of local vs. distant recurrence events. One patient initially treated for ER+/HER2+ disease had an event consisting of pT1a Nx discovered incidentally at reconstructive surgery. This was ER-/HER2+, suggestive of a new primary, and did not have ctDNA detected.

2. Figure 2a provides minimal information not presented in the other figures. It could be moved to the supplemental section or removed in its current form.

   Figure 2A was intended to highlight the number of participants with eVAF<0.01% at baseline, since this is a range particularly relevant to the sensitivity of the assay used in this study. We have edited this figure in an attempt to better illustrate this result, but can certainly move it to the supplement if the added data are felt to be minimally informative.

3. Figure 2E: There is an unexpectedly higher rate of BRCA1, BRCA2, and FAT1 mutations in this cohort. Are there other alterations not demonstrated in this oncoprint that could raise similar concerns? The authors need to explain whether this is related to the true characteristics of the cohort or if it could be due to technical issues, such as a lack of matched normal sequencing for tumor WES, inadequate germline and noise filtering, etc. Was there any association between the

genomic profile of the tumor and baseline ctDNA detectability after controlling for other clinical parameters such as tumor size and nodal involvement?

Thank you for this excellent comment. We agree that the rate of somatic alterations observed is higher than what would be expected based on external datasets (ie. METABRIC). Interpretation of the WES data is limited by the lack of germline sequencing for all 119 participants; however, we did use routine filtering strategies in an effort to remove technical artifacts. We have also included orientation and bias filtering into our standard tumor-only pipeline but this did not result in any substantial changes in the variant frequency.

Methods: "Orientation bias filtering and contamination estimation were applied during variant calling."

We are in the process of uploading the WES files to the EGA in keeping with the data sharing policy of Nature Communications. We have also highlighted this as a limitation in the discussion:

"In addition, although the ctDNA results are derived from a locked assay which includes the filtering of germline variants, our mutation calls for recurrent alterations are limited by the lack of matched germline. This may have led to a higher than expected frequency of some alterations, despite the use of tumor-only filtering strategies."

In an exploratory assessment of TMB (mutations/Mb) and baseline ctDNA detection, those with TNBC and ctDNA not detected at baseline had lower TMB than those with ctDNA detected (Sup. Fig. 2E). In addition, we have added the following paragraph highlighting that the presence of commonly altered genes were not associated with ctDNA detection at baseline:

"Alterations in commonly mutated breast cancer genes were evaluated using a tumor-only filtering strategy (Fig. 2J). The presence of high frequency alterations were not associated with ctDNA detection including TP53 (n=52; p=0.1975), PIK3CA (n=15; p=0.9999), and BRCA2 (n=14; p=0.9999).

4. Based on the review of the swimmer plots, there seem to be no patients who were negative at baseline but later became MRD-positive and ctDNA negativity at baseline was associated with a lack of distant recurrence (except for one case). As discussed in the paper, this could be a result of the assay's LOD or the biological features of the tumor. Importantly, baseline ctDNA negativity appears to be prognostic, regardless of neoadjuvant treatment outcomes, as the majority of patients (except one) failed to achieve a pCR. This seems worth further exploration by the authors.

We appreciate the reviewer's careful review of the swimmer plots, which do suggest some interesting correlations, as noted. However, given the many potential factors that could contribute to these associations, and the relatively small number of events, we prefer to avoid

over-interpreting these signals. We definitely agree that baseline ctDNA detection (or levels) should be considered in future studies as potential independent factors.

We do note that this is different from what reported in the secondary analysis of the iSPY2 cohort using the Signatera assay, where 6 of the patients (Magbanua Cancer Cell 2023) were negative at baseline with subsequent ctDNA detected. Given the variation between assays including input, bioinformatics pipelines, and sequencing platforms, continued exploration of such associations (either detection/not or ctDNA quantity) will be important to the field.

5. What was the rationale for excluding patients with no baseline detection from the analyses presented in Figures S2F-2K? These baseline samples could have been added as "0" in the analysis and visualized as "ND" in the log-scale plots. Overall, these are informative analyses and could potentially be moved to the main figures.

   Thank you for this comment. We have updated the analyses including the samples which were negative at baseline. Based on this feedback, we have also moved the figures from Sup. Fig. 2F-K to the main Figure 2 panel.

6. The authors present outcome analyses stratified by RCB and mid-NAT ctDNA detection, demonstrating the potential added prognostication of ctDNA to RCB. Was ctDNA detection at baseline, or its clearance at mid-NAT or pre-op, associated with RCB?

   Thank you for this question. ctDNA clearance was not associated with residual disease. We have updated the manuscript to read:

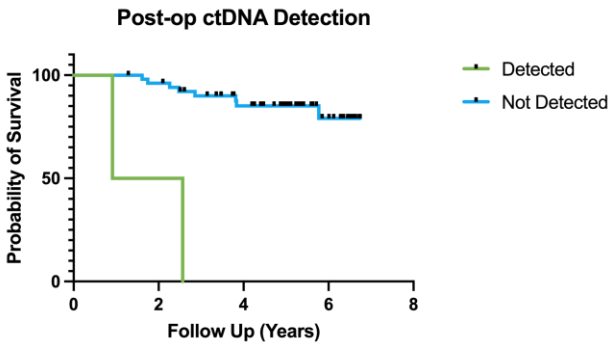
   "However, persistent detection of ctDNA mid-NAT was associated with a worse RFI in all participants with 6/14 evaluable patients experiencing disease recurrence (Fig. 3B; HR: 3.59, 95%CI: 0.99-12.87, p=0.0502); mid-NAT ctDNA clearance was not predictive of RD (p=0.3578)."

   While the detection of ctDNA at baseline is associated with achieving a pCR in the entire group (p=0.0279), this was not consistent when evaluated by individual receptor subtypes, this did not remain significant and is likely biased by the difference in ctDNA detection at baseline between the receptor subtypes. We have not included this analysis in the revised draft, but could discuss this if the reviewer thinks it would be of value for the overall results.

7. The prior iSPY2 analysis did not include post-op ctDNA landmark analysis. What was the association between single post-op sample as landmark analysis and outcomes. It would be informative to present this analysis stratified by RCB and receptor subtypes as well.

   Thank you for this suggestion. We observed a low detection rate at the single postoperative timepoint, and thus this was not explored as a landmark. We have included this result below for which two participants had ctDNA detected in the immediate postoperative period, both

experiencing metastatic recurrence. If the reviewer feels that this analysis would benefit the manuscript, we would be happy to include it as a supplementary figure.



8. The survival analysis based on future ctDNA detection anytime during follow-up has a major statistical flaw, as it assumes that ctDNA positivity (or its persistent negativity) was known at time zero for KM or Cox analysis. This is a common statistical flaw seen in many ctDNA MRD publications and has unfortunately become the norm for such papers. It would be imperative to perform a statistically appropriate analysis that includes ctDNA positivity as a time-dependent variable and present this either as a main or supplemental figure.

   Thank you for highlighting this issue. We performed the main analysis in this fashion, since as noted it has been the convention. To address this important point, we have also performed an additional analysis, now included in the revised manuscript, where ctDNA positivity was considered as a time-dependent variable based on the time interval of subsequent measures until the last follow up and used a proportional hazards model to assess the impact on relapse-free interval. This was significant in an analysis of all timepoints (HR=24.8, 95%CI: 9.3-65.7; p<0.0001), timepoints collected prior to surgery (HR=4.6, 95%CI: 1.9-11.5; p=0.001), and timepoints collected after surgery (HR=53.7, 95%CI: 17.4-166.2; p<0.0001). This analysis further highlights that ctDNA persistence or emergence is associated with worse outcomes in this setting.

9. Was there any evidence of ctDNA in the adjuvant collections for LIB-02-0085 and LIB-02-0093 that was below the threshold for a positive MRD?

   These participants did not have collections after surgery or in follow-up and are thus not evaluable for ctDNA. We have added the following sentence to the table legend for further clarification: "- Participants without evaluable lead times due to a lack of postoperative and follow up collections."

10. In the discussion section, while the study results are thoroughly analyzed, they should be more closely integrated with the existing literature in each context (e.g., paragraph line 355). It is also important to address the fact that most postoperative samples were negative in patients who

ultimately relapsed. This suggests that using this time point to escalate or de-escalate treatment could be clinically challenging with the current assay. It is unclear whether assays with higher sensitivity could address this issue.

Thank you for this comment, with which we agree. The limitation of using a single time point may be due to the sensitivity of the assay, but it is unclear if newer assays with improved LoD95% will perform differently. We have added the following sentence to the discussion which highlights this point:

"However, most participants who recurred did not have detectable ctDNA immediately postoperatively, but rather during follow-up, suggesting that a single landmark timepoint may be insufficient to guide adjuvant therapy. Careful evaluation of assay performance and suitability for the intended purpose is necessary in designing prospective trials, balancing intervention risks with assay effectiveness."

As an extension of this, retrospective analyses of completed adjuvant trials may also have limited ability to identify predictive biomarkers. Instead, strategies that incorporate prospective ctDNA surveillance with subsequent interventions triggered by a positive test require evaluation.

11. The authors state, "The overall baseline detection rate in our analysis was numerically higher than previously reported in a similar cohort, possibly due to the increased technical sensitivity of the assay used." This is debatable. In Magbanua et al. (Cancer Cell, 2023, and not the 2021 publication), the baseline ctDNA detection rates were 69% for HR+ and 91% for TNBC, which are very similar to this cohort (70% and 89%, respectively). Signatera uses 16 variants, while in this study, the median number of variants for ER+/HER2- tumors was 23. Given the differences in cfDNA analysis technologies (NGS for RaDaR vs. multiplex PCR for Signatera) and the relatively similar number of mutations used, this raises the question of whether this assay offers a clinically meaningful improvement in detection rates compared to Signatera, especially in ER+ tumors.

Thank you for this point. We have removed this sentence to reflect the similarity in rates observed.

12. There are multiple cases where ctDNA was negative during NAT or follow-up, but later became positive, suggesting that detection of ctDNA could be possible using assays with a lower LoD.

Thank you for this point. Per Reviewer 1's comments and your previous comment, we have added the following, which highlights this point and potential improvements using next-generation ctDNA assays with improved LoD.

The revised manuscript has been modified to read: "New ctDNA assays that evaluate hundreds or thousands of tumor-specific variants may further improve assay sensitivity in this setting.

Evaluation of detection rates and other parameters of such assays will be important to assess potential clinical advantages in the development of ctDNA-based strategies.[21]"

21. Garcia-Murillas, I. *et al.* Ultra-sensitive ctDNA mutation tracking to identify molecular residual disease and predict relapse in patients with early breast cancer. *J. Clin. Oncol.* 42, 1010–1010

13. Lines 355-369: The authors seem to describe LIB-02-0115, which, based on Figure 5A, appears to be a case of cTN0 low-grade ER+. The authors suggest that not competing neoadjuvant chemotherapy could have affected the outcomes. This is speculative, as this patient seems to have indolent disease with undetectable ctDNA at baseline (indicating low-grade tumor with low tumor turnover). It is unclear if this tumor would have benefited from completing chemotherapy. Was it confirmed via sequencing that the metastatic disease was genomically related to the primary tumor used for ctDNA assay development? Was the residual disease histologically and genomically related to the primary biopsy?

    Thank you for this comment. We have not completed WES sequencing on the patient's surgical specimen nor recurrence and agree that the sentence was overly speculative. We have modified the manuscript to read:

    "An interesting observation in this cohort is that all but one participant who experienced recurrence had ctDNA detected at baseline (20.5%, 18/88 participants). The exception was a participant with low-grade ER+ EBC who received neoadjuvant endocrine therapy; the lack of ctDNA detection may be due to the low cell turnover and minimal ctDNA shed from this tumor."

14. It is curious why two patients in the ER+ cohort (Figure 5A) received anti-HER2 therapy. Were these cases of initial ER+/HER2- disease that later showed HER2+ disease in the residual tumor? If so, this should be explained in the figure legend.

    Thank you for this comment. We have amended the figure legend as outlined below to reflect this point:

    "Figure 5. ctDNA Trajectories and Clinical Events. Participants' clinical timeline and timeline of plasma collection for ctDNA analysis for (A) ER+ (B) TNBC and (C) HER2-positive EBC. Participants who received adjuvant HER2-targeted therapy who were not classified as HER2-positive at baseline had ASCO/CAP-defined HER2+ disease on surgical resection, and received one year of trastuzumab as standard of care. RCB: residual cancer burden, cN: clinical nodal status, cT: clinical tumor size."

Minor comments:

1. The methods section on routine tumor WES can be moved to the supplemental methods and briefly described in the main text.

We have created a new "Supplemental Methods" section and have moved more of these detailed methods.

2. Regarding the cfDNA sequencing methods, it would be helpful to reiterate the role of matched tumor DNA sequencing using the panel as a positive control to remove CHIP and sequencing artifacts from ctDNA calls. This section is also too long and could be summarized in the main text and moved to the supplemental methods.

   Thank you for this suggestion. We have edited the main text to reflect this and put the details in the Supplemental Methods.

3. Line 109: Typo (RaRaR → RaDaR)

   Thank you, this has been updated in the revision.

4. Line 144: CHIP has already been defined.

   Thank you, this has been updated in the revision.

5. Line 282: Typo ("he PPV" → "the PPV")

   Thank you, this has been updated in the revision.

6. Line 312: Missing references.

   Thank you, this has been updated in the revision.

7. Figure 2E Oncoprint: It would be interesting to plot subtypes to see if the genomic alterations align.

   Thank you for this suggestion. We have updated this in the revised version of the manuscript.