

Methylation-based ctDNA monitoring in metastatic breast cancer during CDK4/6 inhibitor therapy

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)

The work presented by Elliott, Fuentes-Antras et al., aimed to evaluate the clinical utility of methylation-based tumor fraction (mTF) measurements using a tumor agnostic ctDNA assay in 57 patients with ER+/HER2 metastatic breast cancer treated with endocrine therapy plus a CDK4/6 inhibitor. More specifically, the epigenomic ctDNA assay Guardant Reveal was tested in baseline prognostication; the Guardant360 Liquid assay was tested for on-treatment ctDNA dynamics as well as the timing of emerging resistance alterations. The authors demonstrate high baseline detection of ctDNA, strong concordance between mTF dynamics and radiographic response, and define the lead time preceding treatment discontinuation and emergence of resistance alterations.

This study addresses a clinical need in the management of ER+/HER2 metastatic breast cancer using a commercially available assay that is already used in clinical practice (i.e. colorectal cancer)

<https://www.guardantcomplete.com/hcp/solutions/guardant-reveal>.

In its current version, this manuscript constitutes a proof-of-concept for the utility of Guardant assays in ER+/HER2-negative metastatic breast cancer, given several limitations, most notably the small sample size and the study design.

Specific comments, not in particular order:

1. Patient cohort is rather small (N=57) and rather heterogeneous as presented in Fig. 1A. As discussed by the authors, a better designed study and perhaps a prospective trial will be needed to make strong conclusions and prove clinical utility. As mentioned above, this study could be seen as a proof of concept that could be used as the basis for further studies.
2. Thresholds used to define mTF response and progression require clearer justification. How were the criteria for a $\geq 50\%$ decline, ctDNA clearance, and molecular progression (absolute mTF $> 0.1\%$ or rise from nadir) selected? The authors should clarify the rationale for these cutoffs, including whether they were pre-specified, informed by prior literature and analysis. For example the authors have previously published a similar study using early stage breast cancer cohort [https://www.esmoopen.com/article/S2059-7029\(25\)01155-X/fulltext](https://www.esmoopen.com/article/S2059-7029(25)01155-X/fulltext).
- Line 398: How was the predefined statistical likelihood threshold determined?
3. ~6-month lead time: The median 5.8-month interval between mTF rise and treatment discontinuation may be substantially influenced by the different sampling intervals and imaging frequency.
4. CHIP-related artifacts may impact maxVAF interpretation. The authors attribute much of the persistent low-level maxVAF signal to CHIP, but the methods for CHIP annotation are proprietary and the data on how many variants were reclassified or excluded are not fully presented. Was CHIP based on Guardant360 Liquid assay data?
6. Potential conflict of interest: The use of a proprietary commercial platform (Guardant Reveal) and presence of company employees among authors necessitates transparency. The manuscript should strengthen its discussion of assay generalizability, including whether similar results could be reproduced with non-commercial or alternative methylation-based platforms. In the current version of the manuscript there is no conflict-of-interest section.
7. The authors report stochastic detection near the 0.01% LoD but do not systematically quantify the false-positive low-level signals, the repeatability of calls at this level and how these influence definitions of progression and analysis and data interpretation of longitudinal samples. Furthermore, the sensitivity of the system to detect 0.01% to 0.1% of mTF without a matched positive control cannot prove whether the signal detected are not simply noise. Is the LoD based on previous work? How may cancer-type specificities influence LoD of the Guardant assay?
8. Data availability section requires further clarification. Authors should indicate whether raw sequencing data (methylation calls, VAF data) are deposited in a public repository or whether restrictions apply given the involvement of GUARDANT in this study.
9. Figures are not of highest quality and could be further improved to help the reader appreciate results and findings. Figure

readability and quality as well as figure labels and legends need to be improved. Longitudinal TF and swimmer plots are dense and could benefit from clearer color schemes, annotations, and standardized axes.

10. Text should be revised providing consistent terms for methylation tumor fraction (mTF), molecular progression (mPD), and clearance across text and figures. It may be advisable to reduce abbreviations whenever possible to help readers that are not from the breast cancer field (i.e. ET could be spelled out).

11. Heterogeneity of CDK4/6 inhibitors: The cohort includes palbociclib, ribociclib, and abemaciclib; while sample size is small, the manuscript should briefly address whether mTF dynamics differed by agent.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

There is a great clinical need for precise tumor-agnostic biomarkers to measure treatment response and correlate this with medical imaging, particularly in the metastatic breast cancer space when there is a desire to monitor disease more frequently without multiple biopsy sampling.

There are several strengths of this manuscript which describe the assessment of ctDNA in a large set of longitudinal samples over a reasonably long time period. Both methylated tumor fraction (mTF) as well as mutational frequency (VAF) was analyzed which is the unique and interesting part of this study in HR+/HER2-mBC. The measurement of both assay types in the same study population is important, since a number of studies have reported that measurement of methylation patterns may be both more specific and sensitive to assessment of tumor progression, and that looking only at specific mutations or the max VAF may be less sensitive. Additionally, not all breast cancer patients may harbor specific mutations to track. The comprehensive and careful analysis results in this manuscript agree with these prior reports. Another strength is the exploratory analysis to look at correlation with radiological imaging and define thresholds for molecular response and progression, something that is not validated or well-defined at present in the field. It is interesting to see the relative timing of mTF vs the emergence of genomic alterations and this warrants much deeper study, outside the scope of this paper.

Small limitations are that these are retrospective sampling in small patient population (N=58) which explains lower power in some of the threshold analyses to assess ctDNA and outcome. Larger study populations and prospective trials will be needed to validate whether mTF is a more accurate, predictive assay with the use of radiological assessment for treatment response measure, (which is well addressed in the discussion), or if in fact it should be used in the context also with genomic changes (which adds cost and sample), and whether the timing relative to an imaging response will be reliable enough to dictate treatment changes. Only recently we have seen reports of treatment switching in the context of rising ESR1 mutation levels.

There are some minor points to address:

1. Why is receipt of abemaciclib or fulvestrant correlated with higher mTF?
Disease biology, size of tumor, high grade? Does this correlate to specific tumor baseline features
2. Cite somewhere that each bar in Fig1 represents a single patient? Also numbers here don't seem to represent what is on Figure 1A eg PIK3CA should be 38% and Tp53 should be 30% if rounded up or down.
3. Fig 1B , 2C and other Kaplan-Meier plots looking a TTD should state Probability of TTD on the Y axis, since the event being measure isn't survival
4. Fig 1B, C- Explain the criteria, thresholds for low vs high mTF for this graph in the figure legend, methods (even though it may be arbitrary).
5. change TF to mTF (methylated Tumor Fraction) in Supp Table 3 in row 1 and 17, and elsewhere in text and legends eg supp Fig 2 for consistency, so the reader can easily distinguish which assay readout is being described, eg as compared to the genomic assay. (ie you can also have a TF for genomic analysis).
6. Fig 3A small point: Since this is a busy figure, please keep asterisks versus crosses consistent for reader is legend is different to swimmer plot end lanes.
7. Fig4A vs Supp 4B. The populations being analyzed in these 2 figures is not well described in the legends or results. Ie assume 4A are the N=175 matched CT events, and Supp 4B is the RECIST each with their own mTF/maxVAF samples and assays. The max VAF tables have different values, but the TF table appears to be the same in both analyses?

Reviewer #4

(Remarks to the Author)

This manuscript describes the correspondence between mTF dynamics and radiological assessment of response, compared mTF with maxVAF in the correlation with TTD and OS, and demonstrated the emergence of genomic alterations with time, consistent with what has been reported in the literature on HR+ mBC. It is an exploratory analysis using a particular epigenomic platform (Guardant Reveal), adding to the growing body of knowledge on ctDNA and its uses in different clinical settings.

Suggest

- state the advantages of using methylation patterns over usual ctDNA genotyping assays
- add in the main text that not all patients were receiving endocrine therapy + CDK4/6i as 1st-line therapy (as in Figure 3), so the reader can gauge the level of ET resistance. Would the authors comment on how the treatment line might have affected the findings of this study?
- line 280: remove 'reducing costs': minimizing cost of scans is negated by the expensive ctDNA analysis that is done repeatedly
- ctDNA clearance: 7 by maxVAF, 18 by mTF – is it a complete overlap?
- Figure 1: was the patient on Giredestrant (presumably switched from Letrozole) a patient from another trial? Does 'Palbociclib/Abemaciclib' mean a switch from Abemaciclib to Palbociclib?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have substantially improved the manuscript and have addressed many of the reviewers' comments. However, several methodological aspects remain insufficiently described. In particular, the authors state that proprietary bioinformatics algorithms were used, which limits the ability of reviewers to assess the methodology. Although the authors indicate that additional references have been added, it is not clear which specific references support these methods, making it difficult to evaluate whether they provide sufficient detail and clarity.

Authors do not provide raw sequencing and genotyping analyses are not deposited in public repositories because they are generated using proprietary Guardant Health assays and bioinformatics pipelines which represent the core intellectual property and are subject to data-use and IP restrictions. Whether this level of data accessibility is acceptable should be carefully evaluated against the journal's data sharing requirements and editorial policies.

It would have been helpful if the authors had provided a tracked-changes version of the revised manuscript and included updated figures in the response letter, to facilitate assessment of the revisions.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

I have no further comments- authors have addressed concerns, and satisfactorily stated limitations.

Reviewer #4

(Remarks to the Author)

I have reviewed the authors' replies and changes to the manuscript and have no further comment.

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Tissue-free methylation-based ctDNA monitoring in ER+/HER2- metastatic breast cancer receiving endocrine and CDK4/6 inhibitor therapy

Response to Reviewers:

Reviewer #1 (Remarks to the Author):

The work presented by Elliott, Fuentes-Antras et al., aimed to evaluate the clinical utility of methylation-based tumor fraction (mTF) measurements using a tumor agnostic ctDNA assay in 57 patients with ER+/HER2 metastatic breast cancer treated with endocrine therapy plus a CDK4/6 inhibitor. More specifically, the epigenomic ctDNA assay Guardant Reveal was tested in baseline prognostication; the Guardant360 Liquid assay was tested for on-treatment ctDNA dynamics as well as the timing of emerging resistance alterations. The authors demonstrate high baseline detection of ctDNA, strong concordance between mTF dynamics and radiographic response, and define the lead time preceding treatment discontinuation and emergence of resistance alterations.

This study addresses a clinical need in the management of ER+/HER2 metastatic breast cancer using a commercially available assay that is already used in clinical practice (i.e. colorectal cancer) <https://www.guardantcomplete.com/hcp/solutions/guardant-reveal>.

In its current version, this manuscript constitutes a proof-of-concept for the utility of Guardant assays in ER+/HER2-negative metastatic breast cancer, given several limitations, most notably the small sample size and the study design.

We appreciate this thoughtful summary of our study and agree with the reviewer's contextualization and limitations.

Specific comments, not in particular order:

1. Patient cohort is rather small (N=57) and rather heterogeneous as presented in Fig. 1A. As discussed by the authors, a better designed study and perhaps a prospective trial will be needed to make strong conclusions and prove clinical utility. As mentioned above, this study could be seen as a proof of concept that could be used as the basis for further studies.

We thank the reviewer for this comment and agree that the cohort size and heterogeneity limit definitive conclusions regarding clinical utility. We also view these findings as proof-of-concept, providing important clinical validity data that support and motivate prospective, well-powered studies to formally evaluate the utility of methylation-based ctDNA monitoring. Given the superior sensitivity and longitudinal performance of methylation-based tumor fraction compared with tumor-agnostic, mutation-based metrics in this study, these data also support prioritizing mTF-based monitoring for evaluation in future prospective clinical trials.

2. Thresholds used to define mTF response and progression require clearer justification. How were the criteria for a $\geq 50\%$ decline, ctDNA clearance, and molecular progression (absolute mTF $> 0.1\%$ or rise from nadir) selected? The authors should clarify the rationale for these cutoffs, including whether they were pre-specified, informed by prior literature and analysis. For example the authors have previously published a similar study using early stage breast cancer cohort [https://www.esmooopen.com/article/S2059-7029\(25\)01155-X/fulltext](https://www.esmooopen.com/article/S2059-7029(25)01155-X/fulltext).

The thresholds used to define molecular response and progression were not pre-specified, but were explored by systematically evaluating multiple candidate cutoffs, as shown in Figure 4A for mTF and Supplementary Figure 4B for maxVAF, where we report sensitivity, specificity, PPV, and NPV across the evaluated definitions. As illustrated in Supplementary Figure 3, we observed fluctuations in several patients with lower levels of ctDNA, which motivated inclusion of an exploratory absolute threshold ($> 0.1\%$ mTF). Accordingly, we present performance metrics both with and without this absolute floor to demonstrate its effect, showing improved discriminative performance when applied. This highlights a consideration for definitions of molecular progression if prospective validation is pursued.

To put this into context, we have added the following language to the revision: *“Given the presence of fluctuating detection in patients with lower levels of ctDNA as determined by the methylation-based assay (mTF), an analytical floor was considered and definitions of response and progression were explored with progression defined as rising above 0.1% (Fig. 4A, Sup. Fig. 4B). In general, this improved sensitivity and NPV across evaluated definitions (Fig. 4A, Sup. Fig. 4B).”*

Consistent with prior Guardant Health analyses, a $\geq 50\%$ decline was used as a pragmatic, exploratory response metric to enable comparability across analyses while explicitly acknowledging the need for further validation.

(Previously described in: Liang, S. I. *et al.* Methylation-based ctDNA tumor fraction changes predict long-term clinical benefit from immune checkpoint inhibitors in RADIOHEAD, a real-world pan-cancer study. *Cancer Res. Commun.* 5, 1384–1395 (2025).)

3. Line 398: How was the predefined statistical likelihood threshold determined?

Guardant Reveal sequences thousands of regions that are differentially methylated in cancer (DMRs) as well as control regions. Sequencing data are analyzed using an analytically validated and locked proprietary bioinformatic classifier trained to detect the presence of ctDNA. Samples are classified as ctDNA detected or not detected based on a pre-defined calling threshold. Detection threshold selection was performed for each tumor region set using a training set of more than 5,000 cancer-free donor samples, ctDNA-free samples, and samples from individuals with cancer.

We have added the following citations to the manuscript which describe the use of Guardant Reveal:

Liang, Samantha I., Zoe Quandt, Sara Wienke, et al. 2025. "Methylation-Based ctDNA Tumor Fraction Changes Predict Long-Term Clinical Benefit From Immune Checkpoint Inhibitors in RADIOHEAD, a Real-World Pan-Cancer Study." *Cancer Research Communications* 5 (8): 1384–1395.

Rolfo, Christian D., Moh'd M. Khushman, Alessandro Russo, et al. 2025. "Chemotherapy Response Monitoring with DNA Methylation-Based ctDNA Tumor Fraction: Evidence from a Real-World Cohort of Patients with Advanced Common Solid Malignancies." *The Journal of Liquid Biopsy* 10 (100442): 100442.

4. ~6-month lead time: The median 5.8-month interval between mTF rise and treatment discontinuation may be substantially influenced by the different sampling intervals and imaging frequency.

We thank the reviewer for this comment and agree that the estimated lead time between molecular progression and treatment discontinuation is influenced by the frequency of blood sampling and radiographic assessments. We have clarified this point in the Discussion, noting that the observed 5.8-month median lead time reflects real-world sampling and imaging intervals in this retrospective cohort and may vary with different surveillance schedules. Accordingly, this estimate should be interpreted as descriptive rather than definitive, and prospective studies with predefined sampling and imaging intervals will be required to more precisely characterize lead time and determine its clinical relevance.

We have added the following sentence to the limitation section of the discussion: *"The estimated lead time from mTF rise to treatment discontinuation is likely influenced by the frequency and timing of blood collection and imaging in this real-world cohort, and may differ under prospective surveillance schedules with standardized sampling intervals."*

5. CHIP-related artifacts may impact maxVAF interpretation. The authors attribute much of the persistent low-level maxVAF signal to CHIP, but the methods for CHIP annotation are proprietary and the data on how many variants were reclassified or excluded are not fully presented. Was CHIP based on Guardant360 Liquid assay data?

Yes, CHIP annotation was performed by the Guardant360 Liquid assay using Guardant Health's proprietary bioinformatics pipeline, which classifies variants as somatic, germline, or CHIP based on variant-level features and large reference datasets. We acknowledge that these methods are proprietary and that variant-level reclassification counts are not fully detailed. We include all baseline and at progression somatic variants (per Guardant360 assay) in the

Supplemental tables. We also annotate all somatic variants over time compared to changes in mTF in the Supplemental Data 1 file.

The CHIP annotation is currently outlined in the methods: *“CHIP annotation was performed using a proprietary bioinformatics algorithm assessing variant-level metrics related to fragmentomics, methylation patterns, and variant allele frequencies, as well as metadata features aggregated from over 250,000 clinical samples and public databases such as COSMIC and GnomAD.”*

We have also added references to this statement in the main text.

6. Potential conflict of interest: The use of a proprietary commercial platform (Guardant Reveal) and presence of company employees among authors necessitates transparency. The manuscript should strengthen its discussion of assay generalizability, including whether similar results could be reproduced with non-commercial or alternative methylation-based platforms. In the current version of the manuscript there is no conflict-of-interest section.

We agree and have copied the COI statement that was uploaded with the manuscript below, emphasizing the Guardant Health authors’ relationships in particular.

“Conflicts of Interest: C.M.W. and G.Y. are employees and stockholders of Guardant Health. E.A. reports honoraria from Sandoz and Seagen and consulting fees from AstraZeneca and Novartis. M.B.N. reports speaker honoraria and consulting fees from Novartis and Exact Sciences, outside the scope of the submitted work. L.L.S. reports participation on scientific advisory boards for Merck, Pfizer, AstraZeneca, Roche, GlaxoSmithKline, Voronoi, Arvinas, Navire, Relay, Daiichi Sankyo, Amgen, Marengo, Medicenna, Tubulis, LTZ Therapeutics, Pangea, and Break Through Cancer; institutional clinical trial support from Novartis, Bristol-Myers Squibb, Pfizer, Boehringer-Ingelheim, GlaxoSmithKline, Roche/Genentech, AstraZeneca, Merck, Celgene, Astellas, Bayer, AbbVie, Amgen, Symphogen, Mirati, BioNTech, 23andMe, and EMD Serono; stock ownership by a spouse in Agios; and leadership roles held by a spouse at Treadwell Therapeutics. P.L.B. reports institutional research funding from Amgen, AstraZeneca, Bayer, Bicara, Bristol Myers Squibb, Genentech/Roche, Gilead, GlaxoSmithKline, Lilly/Loxo, Medicenna, Merck, Nektar, Novartis, PTC Therapeutics, Sanofi, Seagen, Servier, SignalChem Life Sciences, Takeda, and Zymeworks, and uncompensated honoraria or consultancy from Zymeworks, Lilly, Seattle Genetics, Merck, Amgen, Gilead, Janssen, and Repare. M.J.E. reports honoraria or consultancy from Gilead, Knight Therapeutics, and Roche. DWC reports consultancy and advisory relationships with AstraZeneca, Daiichi Sankyo, Exact Sciences, GenomeRx, Gilead, GlaxoSmithKline, Inivata/NeoGenomics, Knight Therapeutics, Lilly, Merck, Novartis, Pfizer, Roche and SAGA; research funding to their institution from AstraZeneca, GenomeRx, Guardant Health, Grail, Gilead, GlaxoSmithKline, Inivata/NeoGenomics, Knight, Merck, PearBio, Pfizer, ProteinQure, RayzeBio and Roche. All other authors declare no competing interests.”

The discussion section has been expanded to draw attention to other methods for tumor fraction quantification, recognizing that direct head-to-head comparisons between different methylation-based platforms and between methylation-based and mutation-based ctDNA assays have not been performed, and the relative performance of these approaches remains uncertain.

“Several additional approaches to tumor fraction quantification have been described, including alternative methylation-based platforms (both commercial and laboratory-developed), mutation-tracking strategies focused on recurrent SNVs, and sWGS-based methods such as ichorCNA. While these assays have demonstrated clinical validity in selected contexts, they differ in pre-analytical handling requirements, input, analytic sensitivity and dynamic range, and no prospective head-to-head comparisons have been performed to determine relative performance or clinical interchangeability across platforms. Future studies would benefit from this approach to nominate ideal candidates for future prospective validation; our results suggest that sensitivity in the range of the assay used here may be important.”

7. The authors report stochastic detection near the 0.01% LoD but do not systematically quantify the false-positive low-level signals, the repeatability of calls at this level and how these influence definitions of progression and analysis and data interpretation of longitudinal samples. Furthermore, the sensitivity of the system to detect 0.01% to 0.1% of mTF without a matched positive control cannot prove whether the signal detected are not simply noise. Is the LoD based on previous work? How may cancer-type specificities influence LoD of the Guardant assay?

The LoD of the Guardant assay has been previously determined through analytical validation studies, where the breast cancer LoD is specific to this cancer type. Variability in detection at low levels occurs in this clinical dataset, but the source of this variability is uncertain (eg. biological, or clinical factors influencing actual ctDNA levels, or pre-analytical factors related to sample collection, processing, or assay performance and analysis when actual levels are at the LoD. We clarify these points and highlight our previous language in the discussion which spoke about this issue:

“Interestingly, when evaluating the mTF in longitudinal samples with low level ctDNA detection, there was fluctuation in positivity (from undetectable to detectable mTF) at levels near the assay lower limit of detection (0.005% for advanced breast cancer), in the absence of radiographic progression. The LoD for this assay has been previously established through analytical validation studies, including breast cancer-specific performance characteristics.^{18,23} However, variability at levels near the LoD in this clinical dataset may reflect biological fluctuation, pre-analytical factors, or assay-related noise, and therefore warrants cautious interpretation. It remains unclear whether these low-level signals are related to the cyclical administration of CDK4/6i, other patient- or tumor-specific factors, or technical variability, and further characterization in both advanced- and early-stage disease will be important. Applying

a definition of molecular progression incorporating both a percentage and absolute value threshold provided considerable improvement in sensitivity for radiographic progression.”

8. Data availability section requires further clarification. Authors should indicate whether raw sequencing data (methylation calls, VAF data) are deposited in a public repository or whether restrictions apply given the involvement of GUARDANT in this study.

The raw sequencing data underlying the methylation-based and genotyping analyses are not deposited in a public repository because they are generated using proprietary Guardant Health assays and bioinformatics pipelines which represent the core intellectual property, and are subject to data-use and IP restrictions. Summary-level data supporting the findings of this study, including all mTF values and all genomic alterations called in the Guardant360 assay, are provided in the Supplementary Materials. Additional derived data may be made available from the corresponding author upon reasonable request, subject to applicable data-sharing agreements.

9. Figures are not of highest quality and could be further improved to help the reader appreciate results and findings. Figure readability and quality as well as figure labels and legends need to be improved. Longitudinal TF and swimmer plots are dense and could benefit from clearer color schemes, annotations, and standardized axes.

We have revised the figures to improve overall quality and readability, including clearer color schemes, enhanced annotations, standardized axes, and expanded figure legends, with particular attention to the longitudinal tumor fraction and swimmer plots to better guide interpretation of the results.

10. Text should be revised providing consistent terms for methylation tumor fraction (mTF), molecular progression (mPD), and clearance across text and figures. It may be advisable to reduce abbreviations whenever possible to help readers that are not from the breast cancer field (i.e. ET could be spelled out).

We have revised the manuscript to ensure consistent terminology and have reduced the use of abbreviations where possible to improve clarity for a broad readership.

11. Heterogeneity of CDK4/6 inhibitors: The cohort includes palbociclib, ribociclib, and abemaciclib; while sample size is small, the manuscript should briefly address whether mTF dynamics differed by agent.

We thank the reviewer for this suggestion. Given the small number of patients treated with ribociclib and abemaciclib in this cohort, we did not have sufficient power to perform a meaningful agent-specific comparison of mTF dynamics, and avoid these comparisons which may yield spurious chance findings. While the similar clinical performance of these agents might

suggest that their ctDNA dynamics would be similar, we acknowledge this as an important question for future prospective characterization

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We are glad to see that the Early Career Researcher had the opportunity to participate in this review, and appreciate the very thorough and constructive feedback provided.

Reviewer #3 (Remarks to the Author):

There is a great clinical need for precise tumor-agnostic biomarkers to measure treatment response and correlate this with medical imaging, particularly in the metastatic breast cancer space when there is a desire to monitor disease more frequently without multiple biopsy sampling.

There are several strengths of this manuscript which describe the assessment of ctDNA in a large set of longitudinal samples over a reasonably long time period. Both methylated tumor fraction (mTF) as well as mutational frequency (VAF) was analyzed which is the unique and interesting part of this study in HR+/HER2-mBC. The measurement of both assay types in the same study population is important, since a number of studies have reported that measurement of methylation patterns may be both more specific and sensitive to assessment of tumor progression, and that looking only at specific mutations or the max VAF may be less sensitive. Additionally, not all breast cancer patients may harbor specific mutations to track. The comprehensive and careful analysis results in this manuscript agree with these prior reports. Another strength is the exploratory analysis to look at correlation with radiological imaging and define thresholds for molecular response and progression, something that is not validated or well-defined at present in the field. It is interesting to see the relative timing of mTF vs the emergence of genomic alterations and this warrants much deeper study, outside the scope of this paper.

Small limitations are that these are retrospective sampling in small patient population (N=58) which explains lower power in some of the threshold analyses to assess ctDNA and outcome. Larger study populations and prospective trials will be needed to validate whether mTF is a more accurate, predictive assay with the use of radiological assessment for treatment response measure, (which is well addressed in the discussion), or if in fact it should be used in the context also with genomic changes (which adds cost and sample), and whether the timing relative to an imaging response will be reliable enough to dictate treatment changes. Only recently we have seen reports of treatment switching in the context of rising ESR1 mutation levels.

We thank the reviewer for this thoughtful and constructive assessment of our work, and for highlighting both the clinical need addressed by this study and the strengths of our longitudinal, dual-assay analysis, while appropriately noting the limitations that motivate further prospective investigation.

There are some minor points to address:

1. Why is receipt of abemaciclib or fulvestrant correlated with higher mTF? Disease biology, size of tumor, high grade? Does this correlate to specific tumor baseline features

We appreciate this interesting observation, noting that at the time this study was performed, availability of different CDK4/6i varied, and reasons for agent selection were not captured. Given the limited number of patients in these subgroups, particularly those receiving abemaciclib, formal adjustment for baseline tumor burden, grade, or other disease features was not feasible, and these findings should be interpreted cautiously as hypothesis-generating rather than indicative of a causal relationship.

2. Cite somewhere that each bar in Fig1 represents a single patient? Also numbers here don't seem to represent what is on Figure 1A eg PIK3CA should be 38% and Tp53 should be 30% if rounded up or down.

We have revised the Figure 1A legend to state that each column represents a single patient (with one patient not shown due to baseline NGS panel failure). We also reviewed the alteration frequencies and updated the percentages in Figure 1A and the corresponding text to ensure they accurately reflect the correct denominator and rounding.

3. Fig 1B , 2C and other Kaplan-Meier plots looking at TTD should state Probability of TTD on the Y axis, since the event being measured isn't survival

We have updated the y-axis labels for all Kaplan-Meier plots.

4. Fig 1B, C- Explain the criteria, thresholds for low vs high mTF for this graph in the figure legend, methods (even though it may be arbitrary).

We have clarified in the Results section that the low versus high mTF groups in Figures 1B and 1C were defined based on the cohort median baseline mTF and those with undetectable ctDNA. We have also added this description to the figure legend.

“An association between higher baseline mTF (defined relative to the cohort median with participants having undetectable ctDNA by this method considered separately) and shorter time to treatment discontinuation (TTD) approached but did not reach statistical significance (Fig. 1B; $p=0.058$), whereas a significant association with overall survival was observed (Fig. 1C; $p=0.0023$).”

5. Change TF to mTF (methylated Tumor Fraction) in Supp Table 3 in row 1 and 17, and elsewhere in text and legends eg supp Fig 2 for consistency, so the reader can easily distinguish which assay readout is being described, eg as compared to the genomic assay. (ie you can also have a TF for genomic analysis).

We have revised Supplementary Table 3 (rows 1 and 17), Supplementary Figure 2, and all relevant text and figure legends have been updated for consistency.

6. Fig 3A small point: Since this is a busy figure, please keep asterisks versus crosses consistent for reader is legend is different to swimmer plot end lanes.

Thanks for this feedback. We have updated Figure 3A to ensure consistent use of symbols between the legend and the swimmer plot end lanes to improve clarity and readability.

7. Fig4A vs Supp 4B. The populations being analyzed in these 2 figures is not well described in the legends or results. I assume 4A are the N=175 matched CT events, and Supp 4B is the RECIST each with their own mTF/maxVAF samples and assays. The max VAF tables have different values, but the TF table appears to be the same in both analyses?

Thank you for this helpful comment. We have revised the figure legends and results to clearly define the analyzed populations, specifying that Figure 4A includes the 175 matched ctDNA-CT events, while Supplementary Figure 4B is restricted to RECIST v1.1-evaluable events with corresponding mTF and maxVAF data. We have also updated Sup. Fig. 4B with the correct values.

Reviewer #4 (Remarks to the Author):

This manuscript describes the correspondence between mTF dynamics and radiological assessment of response, compared mTF with maxVAF in the correlation with TTD and OS, and demonstrated the emergence of genomic alterations with time, consistent with what has been reported in the literature on HR+ mBC. It is an exploratory analysis using a particular epigenomic platform (Guardant Reveal), adding to the growing body of knowledge on ctDNA and its uses in different clinical settings.

Suggest

1. State the advantages of using methylation patterns over usual ctDNA genotyping assays

Thanks for this suggestion. Methylation patterns provide a method to quantify tumor fraction which do not depend on the occurrence of any specific tumor mutations or prior knowledge of genomic alterations, providing a sensitive and tumor-agnostic method for quantification and serial measurement.

“Compared with tumor-agnostic, mutation-based ctDNA assays that rely on tracking putative somatic variants, a methylation-based approach enables tumor fraction quantification which is independent of the mutational status of a patient's cancer and not confounded by CHIP variants. This may provide a more broadly applicable strategy for longitudinal disease monitoring, especially in tumor types with low somatic mutation rates.”

2. Add in the main text that not all patients were receiving endocrine therapy + CDK4/6i as 1st-line therapy (as in Figure 3), so the reader can gauge the level of ET resistance. Would the authors comment on how the treatment line might have affected the findings of this study?

We have clarified the description of the population noting those data in Figure 3, and also that the line of therapy was not uniform. We have also added this line to the discussion.

“Clearance of mTF was associated with the use of letrozole as the endocrine partner suggesting that this is likely to occur in an endocrine-sensitive setting.”

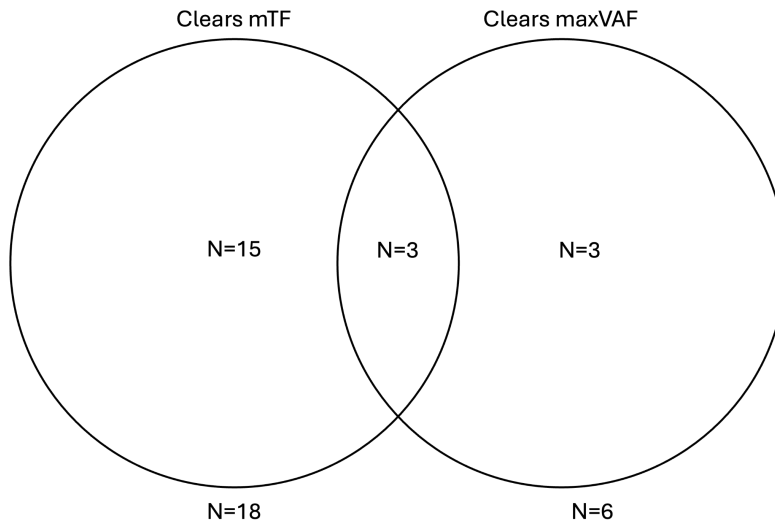
3. Line 280: remove ‘reducing costs’: minimizing cost of scans is negated by the expensive ctDNA analysis that is done repeatedly

We have rephrased this point, which was intended to suggest that blood based monitoring might permit avoidance of many CT scans.

“Future studies could test the impact of monitoring using mTF, potentially decreasing the frequency of imaging in those with an mTF-defined molecular response. Such a clinical paradigm could lessen patient inconvenience and scan related anxiety, while also reducing demands on imaging infrastructure and broader health system resources.”

4. ctDNA clearance: 7 by maxVAF, 18 by mTF – is it a complete overlap?

Thank you for this question. There was not complete overlap in the patients who cleared ctDNA as measured by mTF and maxVAF.



5. Figure 1: was the patient on Giredestrant (presumably switched from Letrozole) a patient from another trial? Does 'Palbociclib/Abemaciclib' mean a switch from Abemaciclib to Palbociclib?

We thank the reviewer for this question. The patient receiving Giredestrant was enrolled on a clinical trial (blinded) and is included here because serial ctDNA sampling occurred during treatment with endocrine therapy plus a CDK4/6 inhibitor. In addition, the label "Palbociclib/Abemaciclib" indicates a treatment switch between CDK4/6 inhibitors during the treatment course due to toxicity, rather than concurrent use or for disease progression.

We have revised Figure 1 in response to reviewer feedback and, to minimize the risk of inadvertent patient identification in this small cohort, removed all patient-level clinical data.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have substantially improved the manuscript and have addressed many of the reviewers' comments. However, several methodological aspects remain insufficiently described. In particular, the authors state that proprietary bioinformatics algorithms were used, which limits the ability of reviewers to assess the methodology. Although the authors indicate that additional references have been added, it is not clear which specific references support these methods, making it difficult to evaluate whether they provide sufficient detail and clarity.

We appreciate this feedback and agree that transparency regarding the bioinformatics methodology is important. We acknowledge that certain components of the Guardant Health bioinformatics pipeline are proprietary; however, the key analytical principles and performance characteristics have been described and validated in peer-reviewed publications that we have now thoroughly cited. In the revised manuscript, we have added references that provide methodological detail for the specific assay components used in this study. These include:

- mTF quantification: Liang et al. (ref 18) and Rolfo et al. (ref 23), which describes the methylation-based tumor fraction algorithm, its analytical validation, and clinical performance across multiple cancer types.
- Analytical validation of the integrated assay platform: refs 26–27, which detail the epigenomic ctDNA detection pipeline, sensitivity, and limit of detection.
- CHIP variant classification: Fiaux et al. (ref 29) and Yen et al. (ref 30), which describe and validate the plasma-only bioinformatics classifier used to distinguish clonal hematopoiesis variants from tumor-derived variants.
- Assay limit of detection and tumor fraction accuracy: Gross et al. (ref 28), which reports pan-cancer performance characteristics.

We have ensured that each methodological claim in the Methods section is now accompanied by the specific supporting reference(s). We recognize that full algorithmic transparency is not possible given the commercial nature of the platform, but we believe these published validation studies provide sufficient detail for the reader to assess the methodology and interpret our findings.

Authors do not provide raw sequencing and genotyping analyses are not deposited in public repositories because they are generated using proprietary Guardant Health assays and bioinformatics pipelines which represent the core intellectual property and are subject to data-use and IP restrictions. Whether this level of data accessibility is acceptable should be carefully evaluated against the journal's data sharing requirements and editorial policies.

We appreciate this important consideration. The raw sequencing data were generated using proprietary Guardant Health assays and pipelines subject to intellectual property restrictions that preclude public deposition, a challenge common to translational research utilizing commercial liquid biopsy platforms. To maximize transparency within these constraints, we have provided all processed ctDNA data (mTF values, maxVAF, variant calls, and longitudinal classifications) in the Source Data and Supplementary

Data files, which we believe represent the minimum dataset necessary to reproduce all analyses and extend the findings of this study, and exceed the level of data granularity typically reported.

It would have been helpful if the authors had provided a tracked-changes version of the revised manuscript and included updated figures in the response letter, to facilitate assessment of the revisions.

We thank the editor for this feedback. We wish to clarify that a tracked-changes version of the manuscript was provided with our previous revision; however, we acknowledge it may not have been clearly labeled among the uploaded files. For the current revision, we have ensured that a clearly labeled tracked-changes version of the manuscript is included, along with updated figures embedded within the point-by-point response letter to facilitate assessment of all changes made.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you for the suggestions and your review of our manuscript, we trust this was a useful experience and appreciate your initiative.

Reviewer #3 (Remarks to the Author):

I have no further comments- authors have addressed concerns, and satisfactorily stated limitations.

Thank you for the suggestions and your review of our manuscript.

Reviewer #4 (Remarks to the Author):

I have reviewed the authors' replies and changes to the manuscript and have no further comment.

Thank you for the suggestions and your review of our manuscript.