We thank the Reviewers for their thoughtful review; the Reviewers’ comments and suggestions clearly improved our manuscript. We hope to have addressed the critiques satisfactorily. We describe these and other changes in more detail in the below point-by-point response and highlighted all changes in the manuscript in blue.

**Reviewer 1**

Thank you for the opportunity to review this manuscript generating additional data around an important and evolving area of translational oncology, the use of circulating tumor DNA (ctDNA) to monitor response to neoadjuvant chemotherapy in early-stage breast cancer. The manuscript is well written and addresses a clinically relevant topic. However, several methodological, interpretive, and structural issues merit further attention before the work can be considered for publication.

**Authors’ reply:** We thank the Reviewer for appreciating the strengths of the study and the detailed feedback.

- The manuscript would benefit from greater clarity around the specific timing of on-treatment plasma sample collection. The manuscript states: “Serial peripheral blood samples were collected from each patient prior to start, during and after completion of NST.” References to "on treatment" or “during” are insufficiently precise (ie. was this always the same timepoint? Was this mid treatment or prior to a specific cycle?). Given the variability in ctDNA dynamics and clearance patterns across different timepoints, accurate temporal context is critical for evaluating the reported findings.

**Authors’ reply:** We thank the Reviewer for highlighting this important point. We have revised the Methods section of the manuscript on Page 11 to explicitly state the timepoints as follows:

“Given the variability in timing and duration of NST regimens in breast cancer, pre-treatment (T0) blood samples were collected prior to the initiation of cycle 1. On-treatment (T1) samples were collected at a planned intermediate timepoint during NST, typically after the mid-cycle. Post-treatment (T2) samples were collected at the end of NST and within two weeks prior to surgery. The median interval between T0 and T1 was 56 days (IQR, 54–64.8), and between T0 and T2 was 119 days (IQR, 98–138.5).”

- The authors should clarify that the baseline cfDNA was numerically higher in those with tumors ≥3 cm (p=0.4).

**Authors’ reply:** We have added this observation to the Results section of the manuscript and noted the lack of statistical significance. The revised text now reads as follows:

“Baseline cfDNA concentration was numerically higher in patients with tumors ≥3 cm compared to smaller tumors (p=0.49), though this did not reach statistical significance.”

- The description around de novo mutations not clear and requires clarification. It appears the authors use of the term “de novo” in the context of somatic mutation calling. According to the manuscript, mutations detected both in tissue and in plasma (e.g., PIK3CA H1047R in patient BC04) are referred to as “de novo,” which is potentially misleading. Traditionally, de novo variants are considered those newly identified in plasma that were not seen in the tumor tissue, raising the possibility of CHIP (clonal hematopoiesis of indeterminate potential) rather than tumor origin. The authors should provide a consistent and biologically appropriate definition of de novo alterations and explicitly discuss the risk of CHIP contamination, especially for variants not cross-referenced with matched tumor sequencing. This looks to be particularly relevant in Figure 1B where the detection of TP53 and other suspicious variants are detected in subsequent samples (albeit the difference between T0, P0, and other labels are not clear in the current version of this manuscript).

**Authors’ reply:** In the initial version of the manuscript, “de novo” was defined as a variant detected outright in a sample by Mutect in the case of an SNV or a combination of VarScan, Strelka, Scalpel and Platypus in the case of an indel. All variants not detected “de novo” were detected by genotyping at the coordinate of a somatic variant known to occur in a related tumor tissue sample.

We have removed all instances of “de novo” in the manuscript and edited Figure 1B of the manuscript accordingly. Variants detected outright are now unlabeled whilst variants detected in a sample by genotyping are labelled “Detected by Genotyping”.

In patients BC08, BC12, BC15, and BC20, no variants were detected outright/ de novo by the somatic mutation calling pipeline in the pre-treatment plasma samples. There was no evidence of supporting reads in the corresponding plasma samples for somatic variants detected in the patient-matched initial biopsies or residual diseases. In patients BC01, BC02, BC03, BC06, BC07, BC09, BC14, and BC18, all variants detected in the pre-treatment plasma samples were only detected by genotyping of somatic variants previously detected in the respective tumor tissue. In patients BC04, BC13, BC17, and BC21, the pre-treatment plasma samples contained a mixture of somatic variants detected outright by the somatic mutation calling pipeline and variants detected by genotyping. In all four cases, the somatic variants detected outright in the pre-treatment plasma samples were also detected outright in either the initial biopsies or residual diseases. There was no evidence to support that these variants are due to clonal hematopoiesis (CH) in these samples.

In patients BC10 and BC16, the pre-treatment plasma samples contained a mixture of somatic variants detected outright by the somatic mutation calling pipeline and variants detected by genotyping. Some of those variants were detected by genotyping in the post-treatment residual disease e.g. TP53 R306\* in BC10 whilst others were never detected in the corresponding tumor tissues e.g. RB1 D507A in BC10 and MED12 Q2100\_Q2101ins\* in BC16 We agree that the latter two examples could be instances of CH. We thank the Reviewer for pointing out this oversight and have removed them along with similar instances from Figure 1B of the manuscript. Additionally, the Methods section on Page 11-12 now reads as follows:

“We considered a mutation as likely clonal hematopoiesis (CH) if the variant was detected outright by the standard pipeline in the baseline plasma sample and was absent from the initial biopsy or residual disease after genotyping. These variants were excluded from further analyses.”

- While the authors note the detection of ctDNA in a majority of patients at baseline, their conclusion that the assay is highly sensitive is not well-supported by the longitudinal results. The low detection rates at on-treatment and pre-surgical timepoints suggest a limited dynamic range and may reflect a floor effect in assay sensitivity or issues with the variant selection strategy. The discussion should acknowledge this discordance and temper conclusions about the assay’s performance accordingly.

**Authors’ reply:** We thank the Reviewer for this excellent comment. We have revised the Discussion to moderate our claims regarding assay sensitivity and to acknowledge the possibility of a floor effect, especially at later timepoints:

“This limitation highlights the dynamic nature of ctDNA clearance and underscores the need for more sensitive assays capable of detecting even minute levels of residual ctDNA. As exemplified by a previous study in triple-negative breast cancer, ctDNA fractions can decrease below the detection limit of commercially available tests during NST. To address this issue, several studies have shown that increasing the number of variants tracked per patient can enhance the sensitivity of ctDNA detection, particularly during and after NST, when ctDNA levels are dynamic and often low. Tumor-informed assays that incorporate a greater number of genomic alterations have demonstrated improved performance in MRD detection and monitoring by leveraging multiple informative variants. This principle becomes even more critical with whole-genome sequencing (WGS)-based approaches, which allow for the tracking of thousands of patient-specific alterations, thereby improving both the signal-to-noise ratio and limit of detection at each timepoint. Moreover, methods using tumor-derived phased variants, defined as multiple somatic mutations co-occurring on the same DNA fragment, offer an orthogonal strategy for enhancing ctDNA detection sensitivity and specificity. On the other hand, tumor-agnostic approaches are also emerging as promising tools for MRD assessment. These include analyses of copy number alterations, fragmentomic features, and methylation patterns in cfDNA, all of which can contribute to improved sensitivity without the need for a tissue-informed variant selection. Together, these evolving technologies underscore the complexity and promise of optimizing ctDNA assays for real-time monitoring of response to NST and for early identification of MRD.”

The conclusions have also been changed accordingly:

“Our study supports the clinical utility of ctDNA analysis as a non-invasive tool for disease management in patients with breast cancer undergoing NST. However, to enable more accurate longitudinal monitoring during both neoadjuvant and adjuvant treatment phases, the development of more sensitive ctDNA assays is essential.”

- An underdeveloped aspect of the discussion section is the implication that larger panels or higher mutation counts inherently improve sensitivity. Recent evidence, including from the Signatera Genome assay (ASCO 2025), underscores that variant quality and biological relevance may be more critical than sheer quantity. The analogy of “shots on goal” is useful but incomplete variants must also be biologically informative and present in sufficient allele frequency in plasma to be actionable. This point should be explored further in the discussion, particularly in light of the panel-based limitations observed in the current study where a limited number of variants ultimately went into the ctDNA/plasma panels where “cancer related” genes from the MSK-IMPACT panel were used.

**Authors’ reply:** We thank the Reviewer for pointing this out and have expanded the Discussion to reflect this nuance. We now include the following:

“This limitation highlights the dynamic nature of ctDNA clearance and underscores the need for more sensitive assays capable of detecting even minute levels of residual ctDNA. As exemplified by a previous study in triple-negative breast cancer, ctDNA fractions can decrease below the detection limit of commercially available tests during NST. To address this issue, several studies have shown that increasing the number of variants tracked per patient can enhance the sensitivity of ctDNA detection, particularly during and after NST, when ctDNA levels are dynamic and often low. Tumor-informed assays that incorporate a greater number of genomic alterations have demonstrated improved performance in MRD detection and monitoring by leveraging multiple informative variants. This principle becomes even more critical with whole-genome sequencing (WGS)-based approaches, which allow for the tracking of thousands of patient-specific alterations, thereby improving both the signal-to-noise ratio and limit of detection at each timepoint. Moreover, methods using tumor-derived phased variants, defined as multiple somatic mutations co-occurring on the same DNA fragment, offer an orthogonal strategy for enhancing ctDNA detection sensitivity and specificity. On the other hand, tumor-agnostic approaches are also emerging as promising tools for MRD assessment. These include analyses of copy number alterations, fragmentomic features, and methylation patterns in cfDNA, all of which can contribute to improved sensitivity without the need for a tissue-informed variant selection. Together, these evolving technologies underscore the complexity and promise of optimizing ctDNA assays for real-time monitoring of response to NST and for early identification of MRD.”

- A more robust discussion of alternate ctDNA detection technologies, such as those leveraging structural variants (ie. SAGA Dx data from the Princess Margaret cohort) and phased variants (ie. Foresight from the MSK cohort) would provide valuable context. Including this perspective would offer a more balanced and comprehensive assessment of the evolving ctDNA landscape.

**Authors’ reply:** We have added a paragraph to the Discussion that compares our approach with other advanced ctDNA detection methods such as structural variant tracking (e.g., SAGA Dx) and phased variant technologies (e.g., MSK’s Foresight):

“This limitation highlights the dynamic nature of ctDNA clearance and underscores the need for more sensitive assays capable of detecting even minute levels of residual ctDNA. As exemplified by a previous study in triple-negative breast cancer, ctDNA fractions can decrease below the detection limit of commercially available tests during NST. To address this issue, several studies have shown that increasing the number of variants tracked per patient can enhance the sensitivity of ctDNA detection, particularly during and after NST, when ctDNA levels are dynamic and often low. Tumor-informed assays that incorporate a greater number of genomic alterations have demonstrated improved performance in MRD detection and monitoring by leveraging multiple informative variants. This principle becomes even more critical with whole-genome sequencing (WGS)-based approaches, which allow for the tracking of thousands of patient-specific alterations, thereby improving both the signal-to-noise ratio and limit of detection at each timepoint. Moreover, methods using tumor-derived phased variants, defined as multiple somatic mutations co-occurring on the same DNA fragment, offer an orthogonal strategy for enhancing ctDNA detection sensitivity and specificity. On the other hand, tumor-agnostic approaches are also emerging as promising tools for MRD assessment. These include analyses of copy number alterations, fragmentomic features, and methylation patterns in cfDNA, all of which can contribute to improved sensitivity without the need for a tissue-informed variant selection. Together, these evolving technologies underscore the complexity and promise of optimizing ctDNA assays for real-time monitoring of response to NST and for early identification of MRD.”

- The manuscript occasionally blurs the boundaries between sections. For instance, depth of sequencing is presented within the Methods but includes interpretive commentary better suited for Results. Similarly, conclusions are sometimes embedded within the Results. The authors should consider a clearer delineation between sections to enhance readability.

**Authors’ reply:** We have revised the manuscript for clearer structural delineation. All interpretive statements have been moved out of the Methods and into the Results or Discussion, and the Results no longer contain premature conclusions.

- Although the authors reference previously published work, key performance metrics for their droplet digital PCR (ddPCR) assays, such as the limit of detection (LoD95), limit of quantification (LoQ), and coefficient of variation, should be restated in the current Methods.

**Authors’ reply:** We have revised the manuscript and included the LoD95 and LoQ metrics for the ddPCR assay in the Methods:

“The ddPCR assays had a limit of detection (LoD95) of 0.01%, limit of quantification (LoQ) of 0.05%, and a coefficient of variation of <10% across replicates, as previously reported.”

- The claim that the “median of the fraction of mutations found in cfDNA... was 71%” is inconsistent with the allele frequency data shown in the figures and supplementary tables. This estimate appears inflated and should be recalculated or clarified. Is this supposed to represent the tumor fraction at baseline?

**Authors’ reply:** This percentage describes the per patient number of variants detected in the pre-treatment plasma sample with the aggregated number of variants detected in the initial biopsies and residual diseases as denominator expressed as a median across the cohort. This percentage can vary from 0% in BC08 to 100% in BC01. This metric was chosen to mitigate under- or overestimates due the varying number of total variants in different patients e.g. n = 1/1 (100%) in BC02 and n = 3/6 (50%) in BC06. We have revised all the calculations and clarified the description in the main text and the methods to avoid misunderstandings. The revised statement now reads:

“The median percentage of mutations detected in the cfDNA at baseline relative to the total number of mutations detected in a patient was 67% (IQR: 25-100%). A higher proportion of cfDNA mutations was detected in the plasma of patients with histological grade 3 (p=0.08; Supplementary Figure S4).”

- The manuscript omits citations to several key studies in the field of neoadjuvant ctDNA monitoring. The authors should reference recent landmark work including Elliott et al., Clinical Cancer Research (SAGA Dx study), iSPY2 ctDNA analyses in Annals of Oncology and Cancer Cell, NeoGenomics RaDaR assay in Nature Communications, Guardant Reveal in ESMO Open, MSK’s Foresight cohort using phased variant tracking etc. Including these references would strengthen the manuscript's positioning within the current literature.

**Authors’ reply:** We have added citations to Elliott et al. (Clinical Cancer Research), iSPY2 ctDNA studies, NeoGenomics RaDaR, Guardant Reveal, and MSK’s Foresight study to the Discussion.

- The authors should consider incorporating Residual Cancer Burden (RCB) classification into Table 1, as it is a standard and clinically validated measure of response to neoadjuvant therapy.

**Authors’ reply:** We thank the Reviewer for this excellent suggestion and have now added the RCB classification to Table 1 for all patients with available data. Additionally, Supplementary Figures S7-S8 compares the cfDNA concentration, Fraction of variants in cfDNA, Max AF, and Mean AF between the different RCB classifications. Table 1 and Supplementary Figures S7-S8 are referenced in the manuscript.

- All abbreviations included in the figures are not listed in the legend which makes it challenging for the reader to know what the authors are referring to.

**Authors’ reply:** We have reviewed all figure legends and ensured that every abbreviation is now defined.

**Reviewer 2**

The authors present a new cohort of patients who received neoadjuvant chemotherapy for breast cancer. They demonstrated their ability to detect circulating tumor DNA (ctDNA) through targeted sequencing of primary and patient-specific droplet digital polymerase chain reaction (ddPCR) in plasma. The detection rate was 83% among the 18 patients with identified mutations.

Authors’ reply: We thank the Reviewer for the helpful critique. Specific points are addressed below.

- A major weakness of this manuscript is the small number of patients included in the study (only 20) and the heterogeneity of the breast cancer subtypes (luminal, triple negative, and HER2 amplified), compared to recent literature. For example, Magbanua et al. demonstrated the prognostic value of ctDNA detection after neoadjuvant chemotherapy, which correlates with outcomes independently of pathological complete response, in a series of 283 patients with high-risk, early-stage, HER2-negative breast cancer (Cancer Cell. 2023;41(6):1091–102 e4). The “tumor-informed” ctDNA detection technology does not appear to be innovative in this study. Additionally, a recent pooled analysis was published on the role of circulating tumor DNA in early-stage triple-negative breast cancer (Zhang et al., Breast Cancer Research, 2025, 27:38, https://doi.org/10.1186/s13058-025-01986-y).

**Authors’ reply:** We acknowledge this limitation and have addressed it more explicitly in the revised Discussion. While our study was designed as a pilot, the inclusion of heterogeneous breast cancer subtypes reflects real-world clinical practice and enables preliminary exploration of ctDNA dynamics across molecular subgroups. We have now added the following to the Discussion:

“First, the relatively small sample size precluded a comprehensive investigation into potential differences in ctDNA kinetics and prognostic significance across breast cancer subtypes in the neoadjuvant setting. Both the limited cohort size and subtype heterogeneity restrict the generalizability of our findings. Validation in larger, subtype-specific cohorts will be essential.”

Additionally, we now position our study as hypothesis-generating and complementary to larger efforts in the field. Relevant citations, including recent large-scale studies, have been added to the Discussion for context.

- The timing of the blood draw during and after neoadjuvant treatment is not precisely given, specifically the delay after surgery.

**Authors’ reply:** We have revised the Methods section to explicitly state the timepoints:

“Given the variability in timing and duration of NST regimens in breast cancer, pre-treatment (T0) blood samples were collected prior to the initiation of cycle 1. On-treatment (T1) samples were collected at a planned intermediate timepoint during NST, typically after the mid-cycle. Post-treatment (T2) samples were collected at the end of NST and within two weeks prior to surgery. The median interval between T0 and T1 was 56 days (IQR, 54–64.8), and between T0 and T2 was 119 days (IQR, 98–138.5).”

- How were the seven cases with ≥1 de novo mutation detected in baseline cfDNA with the standard pipeline, and how were the seven patients with detectable mutations only through genotyping determined? This makes it so that, at baseline, 83% (15/18) of patients had detectable ctDNA by ddPCR.

**Authors’ reply:** We thank the Reviewer for the opportunity to clarify. In the initial version of the manuscript, “de novo” was defined as a variant detected outright in a sample by the standard pipeline. All variants not detected “de novo” were detected by genotyping at the coordinate of a somatic variant known to occur in a related tumor tissue sample. Based on the suggestion of Reviewer #1, we have removed all instances of “de novo” in the manuscript and edited Figure 1B of the manuscript accordingly. Variants detected outright are now unlabeled whilst variants detected in a sample by genotyping are labelled “Detected by Genotyping”.

The baseline plasma is considered ctDNA positive if ≥1 variant, after excluding instances of potential clonal hematopoiesis (CH), was detected either outright by the standard mutation calling pipeline or by genotyping of tumor informed somatic variants. Based on this definition, 4 patients (BC08, BC12, BC15 and BC20) didn’t harbor any somatic mutation. At baseline, therefore, 78% (18-4/18) of patients had detectable ctDNA by targeted panel sequencing. By ddPCR, the ARID1A N935Kfs\*71 variant in patient BC08 was detected in the pre-treatment plasma. BC12, BC15 and BC20 had no detectable allele fraction.

At baseline, therefore 83% (18-3/18) of patients had detectable ctDNA by ddPCR.