**Tracking response to neoadjuvant systemic therapy through circulating tumor DNA analysis in breast cancer**

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**Abstract**

# Neoadjuvant systemic therapy (NST) is a standard treatment approach for patients with early-stage breast cancer, particularly those with stage II-III disease and aggressive subtypes such as HER2-positive and triple-negative breast cancer. While NST improves surgical outcomes and provides prognostic information, accurately assessing preoperative treatment response remains a clinical challenge. Circulating tumor DNA (ctDNA) has emerged as a promising non-invasive biomarker for monitoring disease dynamics and guiding therapeutic decisions. In this study, we aimed to evaluate whether ctDNA analysis in patients with stage II-III breast cancer (n=20) could serve as a surrogate for invasive biopsies in molecular profiling and as a tool for monitoring response to NST. At baseline, ctDNA was detectable in the majority of patients by droplet digital (dd)PCR (15/18, 83%) and all patients with longitudinal follow-up had ctDNA clearance after NST (13/13; 100%). A positive correlation was observed between the allele fraction in cfDNA, histologic grade and molecular subtype, suggesting that ctDNA levels may be influenced by tumor biology. None of the three patients with undetectable baseline ctDNA had distant relapse, regardless of whether they achieved pathologic complete response (pCR), compared to 5/15 (33%) with detectable baseline ctDNA. These findings suggest that ctDNA assessment at baseline may provide additional prognostic information to define the risk of patients after NST. While ctDNA shows promise in capturing tumor burden and biological characteristics, its role in predicting pCR and long-term outcomes requires further investigation.

# **Introduction**

Neoadjuvant systemic therapy (NST) is recommended for patients with stage II-III breast cancer, especially those with aggressive subtypes such as human epidermal growth factor receptor 2-positive (HER2-positive) and triple-negative disease1-3. The goal of NST is not only to induce tumor shrinkage, thereby favoring breast-conserving surgery and avoiding axillary lymph node dissection, but also to assess drug sensitivity of the tumor *in vivo* and to tailor post-NST adjuvant treatment based on the initial tumor response1. Pathologic complete response (pCR) is observed in 10-60% of patients receiving NST, with higher rates seen in triple-negative and HER2-positive subtypes4. The prognostic significance of achieving pCR to NST is well established, with patients achieving pCR experiencing excellent disease-free survival5. In contrast, patients not achieving pCR have a high risk of disease recurrence6,7, highlighting a critical need for innovative strategies to predict and monitor treatment response and disease progression. An active area of study is the development of non-invasive tools, such as liquid biopsies, to monitor response to NST and predict the likelihood of pCR in patients with breast cancer.

The use of liquid biopsy in oncology, through the analysis of circulating tumor-derived DNA (ctDNA) from plasma, may have utility at almost every stage of a patient’s management, including treatment monitoring, detection of minimal residual disease (MRD) and the identification of genetic alterations that can be predictive of response or resistance to specific therapies8,9. ctDNA generally comprises only a small fraction of total DNA in plasma/ circulating cell-free (cf)DNA and has been shown to vary according to cancer type and disease burden with measurably higher quantities detected in advanced tumors compared to early-stage disease10-12.

In breast cancer, ctDNA analysis in clinical practice is currently recommended for patients with metastatic disease to assess the repertoire of actionable genetic alterations13,14 as well as to identify mechanisms of therapeutic resistance (e.g., *ESR1* hotspot mutations)15,16. In patients with breast cancer undergoing NST, some studies have suggested the utility of ctDNA-based methodologies to assess residual disease and potentially identify patients at higher risk of distant relapse17-19.

The detection of ctDNA after NST has emerged as an independent predictor of recurrence, underscoring its potential in tailoring patient-specific therapeutic strategies20,21. Conversely, ctDNA clearance during NST in HER2-negative tumors seem to be linked to favorable long-term outcomes even in patients with residual disease at surgery22. In the current study, we sought to assess if ctDNA analysis of plasma obtained pre-treatment from patients receiving NST could be used as a surrogate to invasive biopsies to detect genetic alterations, and furthermore, could assist in monitoring response to NST.

# **Results**

## ***Patient population***

Twenty patients were included in this study (**Table 1**). Median age at diagnosis was 54 years (IQR: 47- 58 years). Most patients were diagnosed with hormone receptor (HR)-positive/HER2-negative breast cancer (11/20, 55%), while 25% and 20% had HER2-positive and triple-negative disease, respectively. Median tumor size was 3.6 cm (interquartile range [IQR], 2.9-4.6 cm), with 70% (n=14) of patients having T2 tumors. All patients had clinical node-positive disease at baseline (cN1, 80%; cN2, 10%; cN3, 10%). All patients received standard-of-care anthracycline- and taxane-based NST, with dose-dense adriamycin plus cyclophosphamide followed by paclitaxel as the most used regimen (65%). Patients with HER2-positive disease received anti-HER2 therapy (trastuzumab +/- pertuzumab) as per clinical guidelines. At surgery, 30% (6/20) of patients achieved pCR (**Table 2**). After a median follow-up of 89 months (range 18-105 months), 5 patients (25%) experienced distant relapse.

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## ***Cell-free DNA (cfDNA) assessment during NST***

To evaluate changes in total cfDNA throughout the course of NST, plasma samples were collected at three timepoints (baseline, on-treatment and post-treatment, see Methods; **Figures 1A-1B**). For one patient, post-treatment cfDNA collection was not available. 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. No statistically significant differences in baseline cfDNA concentration were observed by histological grade and receptor status (**Supplementary Figure S1**). Longitudinal analysis of blood samples revealed an increment of cfDNA concentration during NST, with both on-NST and post-NST levels significantly higher as compared to baseline (p<0.001 for both; **Figure 1C**), likely reflecting therapy-induced lysis of tumor cells. No differences in cfDNA concentrations, either assessed at on or post-NST, were found when comparing clinical and pathological features with the exception of a negative association between post-NST cfDNA concentration and age at diagnosis (**Supplementary Figures S2** and **S3**). Notably, post-NST cfDNA concentrations were statistically significantly lower in patients who achieved pCR compared to those who did not (p=0.048; **Figure 1D**).

## ***Circulating tumor DNA (ctDNA) assessment by targeted sequencing and patient-specific ddPCR***

To monitor tumor mutations (ctDNA) during NST, primary tumor biopsies, baseline plasma samples and residual disease tissue samples, when available, were subjected to targeted sequencing using the MSK-IMPACT assay (see Methods; **Figures 1A-1B**). Additionally, based on the mutation profile identified in the tissue samples, patient-specific droplet digital (dd)PCR assays were used to assess the baseline as well the on- and post-NST plasma samples (see Methods; **Figure 1A**).

We first assessed the concordance between the tumor biopsy and baseline cfDNA subjected to targeted sequencing. Overall, 14/18 (78%) patients had at least one mutation detected in the baseline cfDNA. In 7 cases ≥1 mutation was detected in baseline cfDNA using our standard pipeline and additional mutations were detected in baseline cfDNA by genotyping of mutations present in the tumor. In the other 7 patients, mutations were detectable only through genotyping (**Figure 1B**). At baseline, 83% (15/18) of patients had detectable ctDNA by ddPCR and a strong correlation of baseline allele fraction (AF) was observed using ddPCR and MSK-IMPACT assays (*R*=0.8, *p*<0.001; **Figure 2A**). 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**). Using the mean or maximum AF of any somatic mutation detected in the baseline cfDNA as a surrogate measure of ctDNA fraction, similar results were obtained (**Supplementary Figures S4-S6**). For instance, using either the maximum AF or the fraction of somatic mutations, triple-negative phenotype had higher levels of baseline ctDNA (**Figures 2B-C**), although the number of cases in each group were small and this difference did not reach statistical significance when using the fraction of somatic mutations. Pairwise comparisons of the different metrics of ctDNA fraction show a high correlation (**Figures 2D-F**) and is consistent with the notion that these alternative definitions capture overlapping but not identical aspects of tumor-derived DNA.

## ***Longitudinal ctDNA tracking by tumor-informed ddPCR during NST and impact on outcome***

In two patients, no mutations were found in the primary tumor by targeted sequencing and, therefore, tumor-informed ddPCR was not attempted. In the remaining 18 cases, ddPCR assays for two variants were developed per case, however in three cases only one mutation could be tracked.

Overall, 15/18 (83%) patients had detectable ctDNA at baseline, and all 15 patients had ctDNA clearance after NST (**Figures 3A-B**). The rates of pCR were 16.7%, 50% and 33.3% for HR-positive/HER2-negative, HER2-positive and triple-negative subtypes, respectively, consistent with the anticipated outcomes for patients treated with regimens used during the time frame of this study4,5. Of the three patients with undetectable baseline ctDNA (2 HR-positive/HER2-negative and 1 HER2-positive), only one with HER2-positive disease achieved pCR (**Supplementary Table S1**). One patient with triple-negative breast cancer, who achieved pCR, experienced tumor relapse. Interestingly, none of the three patients with undetectable baseline ctDNA had distant relapse, regardless of whether they achieved pCR, suggesting that ctDNA assessment at baseline might provide additional prognostic information to define the risk of patients before starting NST.

# **Discussion**

The use of liquid biopsy offers multiple opportunities to improve the management of patients with breast cancer who are candidate to receive NST23. In patients undergoing NST, the application of ctDNA assays is appealing for disease monitoring and identification of patients at higher risk of disease relapse24. In this study, we have explored the potential of ctDNA as a non-invasive tool for disease monitoring during NST as well as its ability to recapitulate the repertoire of somatic genetic alterations acquired by the primary tumor.

We first evaluated the dynamics of cfDNA in our study cohort, showing that cfDNA concentrations tend to increase throughout the course of NST. This observation aligns with the expected therapy-induced release of DNA fragments into the circulation, as tumor and normal cells undergo apoptosis or necrosis in response to treatment25,26. Importantly, we have identified a significant association between elevated post-therapy cfDNA concentrations and the presence of residual disease at surgery, as suggested previously19,22. This finding supports the notion that cfDNA analysis may serve as a surrogate marker for monitoring treatment response and estimating residual disease burden.

Given that cfDNA is primarily composed of normal-derived DNA, we focused our analyses on the role of ctDNA in patients receiving NST. Beyond its role in monitoring treatment response, we have shown that ctDNA levels are influenced by tumor biology. Using different methodologies to estimate the fraction of ctDNA, we observed that high-grade and triple-negative tumors tend to have higher levels of baseline ctDNA. These findings confirm the results of previous studies22,27 that suggested that characteristics of the primary tumor, including its biological and intrinsic aggressiveness, influence the shedding of ctDNA into the bloodstream. Analyses of the I-SPY 2 trial22 have shown that this is also true in HER2-positive EBC. Given that our study included only five HER2-positive tumors, it is likely underpowered to detect such differences. The lower ctDNA fraction in HR-positive EBC compared with HER2-positive and triple-negative might be related to the lower proliferation rate of this subtype, as demonstrated in lung cancer28, as well as to specific molecular profiles that can influence ctDNA shedding29.

We showed a high prevalence of detectable ctDNA at baseline, with the majority of patients exhibiting ctDNA positivity either through tumor-informed ddPCR or targeted sequencing assays. This finding underscores the sensitivity of these ctDNA tools in this setting, providing valuable prognostic information and may aiding in risk stratification and treatment planning8. Even with a limited number of patients, we observed the lack of a significant difference in baseline ctDNA detection between patients with or without pCR. This observation is aligned with previous studies in breast cancer which have found that the detection ctDNA after NST is associated with survival outcomes, regardless of pCR18,27,29, and can therefore be considered a good marker to further stratify the patients at higher risk for treatment escalation. It is important to note that novel ctDNA methodologies, which can track a larger number of tumor mutations in cfDNA, have shown to further improve the sensitivity and specificity of minimal residual disease assessment and monitoring, offering additional opportunities in this setting6,30.

Our study presents several limitations that warrant consideration. 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. Second, the patient cohort enrolled in this study received treatment several years ago and may not fully reflect the current treatment landscape. Recent advances in neo- and adjuvant therapies, such as the incorporation of pre-operative anti-PD-1 immunotherapy and adjuvant CDK4/6 inhibitors for triple-negative and HR-tumors3, respectively, highlight the need for further studies to elucidate the role and opportunities of ctDNA analysis in the context of these newer treatment modalities. Lastly, the observation that all patients in our study cleared ctDNA following neoadjuvant therapy prevented us from confirming previous findings that demonstrated a prognostic role for post-neoadjuvant ctDNA status and its association with pCR. 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 studies in triple-negative breast cancer6,31, 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 detection6,32, 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 variants21,22,29,33,34. 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 timepoint21,35. 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 specificity36,37. 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 selection38-40. 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.

Our findings highlight the potential of incorporating ctDNA into the clinical management of breast cancer patients undergoing NST. ctDNA analysis enables accurate tumor genomic profiling that closely reflects tissue-based profiling while also providing valuable prognostic information based on ctDNA status (negative vs. positive). In the future, ctDNA assessment could be integrated into routine decision-making to optimize treatment escalation and de-escalation strategies in this setting. However, several important questions remain unanswered. The prospective, interventional validation of ctDNA in a large cohort is still missing. The c-TRAK TN trial assessed the utility of prospective ctDNA surveillance in triple-negative breast cancer and the activity of pembrolizumab in patients with positive ctDNA detection41. However, the early amendment to the protocol with the closure of the observation/ctDNA-positive group, does not allow a meaningful statistical comparison. Despite these remaining challenges, ctDNA assessment for minimal residual disease monitoring has the potential to improve the accuracy of treatment response assessment, enable earlier detection of residual disease and disease recurrence, guide the selection of targeted therapies based on tumor molecular profiling, and ultimately to facilitate personalized treatment strategies and improve patient outcomes.

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. Liquid biopsy technologies may be used to improve treatment decision-making, optimize patient outcomes, and ultimately transform the management of EBC. Moving forward, further research is warranted to validate the prognostic significance of ctDNA-based assays and integrate them into routine clinical practice for personalized management of breast cancer.

# **Methods**

# ***Study design and patient selection***

This study is a prospective observational study approved by Memorial Sloan Kettering Cancer Center’s (MSK’s) Institutional Review Board (IRB; #14-251). Patient informed consents were obtained as per IRB protocol and samples were anonymized. Overall, 21 patients with biopsy-confirmed invasive breast cancer screened over an eight-month period (04/2015-12/2015) were initially included; one patient withdrew from the study. All patients were diagnosed with stage IIB-III breast cancer and were candidates to receive NST. Pre-treatment core biopsies were subjected to central review by a breast pathologist (F.P.) to determine the histologic type, grade, and receptor status; HR status, including estrogen receptor (ER) and progesterone receptor (PR), and HER2 status were assessed by immunohistochemistry (IHC), and HER2 fluorescence *in situ* hybridization (FISH), according to the American Society of Clinical Oncology (ASCO)/ College of American Pathologists (CAP) testing guidelines42,43. Tumors were classified as HR-positive/HER2-negative defined as ER IHC >1% and/or PR IHC >1% and HER2 IHC 0/1+ or 2+ without gene amplification by FISH, HER2-positive defined as HER2 IHC 3+ or 2+ with gene amplification by FISH, regardless of the ER status and triple-negative defined as lacking ER, PR and HER2 expression. Clinical data, including demographic information, clinical staging, type of NST, surgical staging, and follow-up were abstracted from the electronic medical records. Following NST all patients underwent surgery and pCR was defined as the absence of residual invasive cancer in the breast and all sampled regional nodes (ypT0/Tis ypN0 in the current American Joint Committee on Cancer staging system)1.

## ***Sample collection and processing***

Tissue samples from the primary tumor were collected at the time of initial biopsy and at the time of surgery after NST. 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). Serial peripheral blood samples from each patient were collected in two Streck cell-free DNA blood collection tubes (BCT; Streck, La Vista, NE). Blood was processed and cell-free DNA (cfDNA) extracted using the QIAsymphony SP system (Qiagen), quantified, and stored following validated standard operating procedures at MSK’s cfDNA extraction laboratory in the Department of Pathology and Laboratory Medicine, as previously described44.

## ***Tumor and cfDNA targeted sequencing***

Formalin-fixed paraffin-embedded pre-treatment tissue samples, pretreatment cfDNA samples and post-treatment residual disease samples (when available) underwent targeted sequencing using the MSK Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) assay at MSK’s Integrated Genomics Organization (IGO), which comprises all coding regions and selected intronic and regulatory regions of up to 410 cancer genes, as previously described45,46. MSK-IMPACT sequencing data were analyzed using a validated bioinformatics pipeline as previously described47-50. The median depth of coverage for tumor samples was 467X (range: 267-841), 604X (range: 199-905) for normal samples and 434X (range: 255-871) for the baseline cfDNA samples. The aggregated set of variants identified in the tissue and baseline cfDNA samples of a given patient were genotyped in all patient-matched samples using *SAMtools mpileup* (htslib 1.2.1)51. 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.

## ***Tumor-guided droplet digital PCR of cfDNA***

To monitor response to NST in the cfDNA, whenever possible, two variants identified in the tumor tissue by MSK-IMPACT sequencing were selected to design personalized ddPCR assays for each patient, as described previously19,52,53. Patient-specific ddPCR assays were then used to track the variant in cfDNA at baseline and in subsequent plasma samples collected on- and post-NST. 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 reported12,19,52,53.

## ***ctDNA positive plasma and ctDNA fraction***

Using the MSK-IMPACT targeted sequencing, a baseline plasma sample 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. Using the tumor-guided ddPCR, w

fraction the the number aggregated number of variants detected in the initial biopsies and residual diseases For the ddPCR assay, the ctDNA fraction was defined as the maximum AF of the two variants being assayed.

## ***Statistical analysis***

All statistical comparisons between groups were performed using the Wilcoxon rank sum test. All tests were two-sided, and all *p*-values are nominal. Statistical associations between continuous variables were evaluated using the Spearman’s rank correlation coefficient. The *p*-values were computed using an asymptotic *t* approximation. All *p*-values are nominal and a *p*-value <0.05 was considered statistically significant. All statistical analyses were performed using R (version 4.1.1).

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**Data availability:** The assembled somatic mutational data of the primary tumor, plasma, residual disease as well as distant/ local relapse from the entire cohort are accessible through cBioPortal at https://www.cbioportal.org/.

**Code availability:** All codes used in this manuscript are available for academic use on GitHub at https://github.com/ndbrown6/MSK-Early-Breast/.

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# **TABLES**

**Table 1**. **Clinicopathologic characteristics of the patients included in this study.**

|  |  |
| --- | --- |
| **Characteristic** | **Cases (n=201)** |
| **Age** (years) | 54 (IQR, 47-58) |
| **Age** |  |
| <50 years | 8 (40%) |
| ≥50 years | 12 (60%) |
| **cStage** |  |
| II | 12 (60%) |
| III | 8 (40%) |
| **cT** |  |
| T2 | 14 (70%) |
| T3 | 3 (15%) |
| T4 | 3 (15%) |
| **cT size** (cm) | 3.6 (IQR, 2.9 - 4.6) |
| **cN** |  |
| N1 | 16 (80%) |
| N2 | 2 (10%) |
| N3 | 2 (10%) |
| **Histology** |  |
| IDC-NST | 20 (100%) |
| **Histologic grade** |  |
| 2 | 7 (35%) |
| 3 | 13 (65%) |
| **Subtype** |  |
| HR-positive/HER2-negative | 11 (55%) |
| HER2-positive | 5 (25%) |
| Triple-negative | 4 (20%) |
| **pCR** | 6 (30%) |
| **pT** **size** (cm) | 1.50 (1.00, 1.95) |
| Not applicable | 6 |
| **pN** |  |
| N0 | 9 (47%) |
| N1 | 6 (32%) |
| N2 | 4 (21%) |
| Unknown | 1 |
| **Neoadjuvant systemic therapy** |  |
| AC-T | 13 (65%) |
| AC-TC | 2 (10%) |
| AC-THP | 4 (20%) |
| AT | 1 (5%) |
| **Surgery** |  |
| BCS | 7 (39%) |
| Mastectomy | 11 (61%) |
| Unknown | 2 |
| **Adjuvant chemotherapy** |  |
| Capecitabine | 2 (10%) |
| CMF | 1 (5%) |
| None | 17 (85%) |
| **Adjuvant anti-HER2** |  |
| HP | 7 (35%) |
| Not Applicable | 13 |
| **Adjuvant endocrine therapy** |  |
| AI | 11 (73%) |
| Tamoxifen | 3 (20%) |
| Tamoxifen-AI | 1 (7%) |
| Not Applicable | 5 |
| **Radiotherapy** | 16 (80%) |
| **Distant** **relapse** | 5 (25%) |

1Median (IQR); n (%). Abbreviations: AC-T, adriamycin, cyclophosphamide, taxane; AC-TC, adriamycin, cyclophosphamide, taxane, carboplatin; AC-THP, adriamycin, cyclophosphamide, taxane, trastuzumab and pertuzumab; AT, adriamycin and taxane; AI, aromatase inhibitor; BCS, breast-conserving surgery; CMF, cyclophosphamide, methotrexate and 5-fluorouracil; HP, trastuzumab and pertuzumab; IDC-NST, invasive breast carcinoma of no special type; pCR, pathological complete response.

**Table 2. Clinicopathologic characteristics according to pathologic complete response at surgery.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Variable** | **N** | **Residual disease** (n=14)1 | **Pathologic complete response** (n=6)1 | **p-value2** |
| **Age** (years; continuous) | 20 | 48 (45, 60) | 57 (54, 58) | 0.3 |
| **Age** | 20 |  |  | 0.042 |
| <50 years |  | 8 (57%) | 0 (0%) |  |
| ≥50 years |  | 6 (43%) | 6 (100%) |  |
| **cStage** | 20 |  |  | >0.9 |
| II |  | 8 (57%) | 4 (67%) |  |
| III |  | 6 (43%) | 2 (33%) |  |
| **cT** | 20 |  |  | 0.8 |
| T2 |  | 9 (64%) | 5 (83%) |  |
| T3 |  | 3 (21%) | 0 (0%) |  |
| T4 |  | 2 (14%) | 1 (17%) |  |
| **cT size** (cm) | 20 | 4.15 (3.38, 5.45) | 2.65 (2.20, 2.95) | 0.006 |
| **cN** | 20 |  |  | >0.9 |
| N1 |  | 11 (79%) | 5 (83%) |  |
| N2 |  | 2 (14%) | 0 (0%) |  |
| N3 |  | 1 (7%) | 1 (17%) |  |
| **Histologic grade** | 20 |  |  | 0.051 |
| 2 |  | 7 (50%) | 0 (0%) |  |
| 3 |  | 7 (50%) | 6 (100%) |  |
| **Subtype** | 20 |  |  | 0.2 |
| HR-positive/HER2-negative |  | 8 (57%) | 1 (17%) |  |
| HER2-positive |  | 4 (29%) | 3 (50%) |  |
| Triple-negative |  | 2 (14%) | 2 (33%) |  |
| **pT size** (cm) | 14 | 1.50 (1.00, 1.95) | NA (NA, NA) |  |
| Not applicable |  | 0 | 6 |  |
| **pN** | 19 |  |  | 0.013 |
| N0 |  | 3 (23%) | 6 (100%) |  |
| N1 |  | 6 (46%) | 0 (0%) |  |
| N2 |  | 4 (31%) | 0 (0%) |  |
| Unknown |  | 1 | 0 |  |
| **Neoadjuvant systemic therapy** | 20 |  |  | 0.003 |
| AC-T |  | 12 (86%) | 1 (17%) |  |
| AC-TC |  | 0 (0%) | 2 (33%) |  |
| AC-THP |  | 1 (7%) | 3 (50%) |  |
| AT |  | 1 (7%) | 0 (0%) |  |
| **Surgery** | 18 |  |  | 0.2 |
| BCS |  | 4 (29%) | 3 (75%) |  |
| Mastectomy |  | 10 (71%) | 1 (25%) |  |
| Unknown |  | 0 | 2 |  |
| **Adjuvant chemotherapy** | 20 |  |  | >0.9 |
| Capecitabine |  | 2 (14%) | 0 (0%) |  |
| CMF |  | 1 (7%) | 0 (0%) |  |
| None |  | 11 (79%) | 6 (100%) |  |
| **Adjuvant anti-HER2** | 20 |  |  | 0.6 |
| HP |  | 4 (100%) | 3 (100%) |  |
| Not Applicable |  | 10 | 3 |  |
| **Adjuvant endocrine therapy** | 20 |  |  | 0.5 |
| AI |  | 8 (57%) | 3 (50%) |  |
| Tamoxifen |  | 2 (14%) | 3 (50%) |  |
| Tamoxifen-AI |  | 3 (21%) | 0 (0%) |  |
| Not Applicable |  | 1 | 0 (0%) |  |
| **Radiotherapy** | 20 | 11 (79%) | 5 (83%) | >0.9 |
| **Distant** **relapse** | 20 | 4 (29%) | 1 (17%) | >0.9 |

1Median (IQR); n (%). 2Wilcoxon rank sum test; Fisher's exact test. Abbreviations: AC-T, adriamycin, cyclophosphamide, taxane; AC-TC, adriamycin, cyclophosphamide, taxane, carboplatin; AC-THP, adriamycin, cyclophosphamide, taxane, trastuzumab and pertuzumab; AT, adriamycin and taxane; AI, aromatase inhibitor; BCS, breast-conserving surgery; CMF, cyclophosphamide, methotrexate and 5-fluorouracil; HP, trastuzumab and pertuzumab; IDC-NST, invasive breast carcinoma of no special type; pCR, pathological complete response.

# **Figure Legends**

**Figure 1: Study design and somatic mutations detected in primary breast cancer tissue and circulating cell-free (cf)DNA.**

(A) Twenty patients were included in the study. Tissue samples from primary tumors were collected at the time of initial biopsy. In patients with residual disease, a post-treatment sample was obtained after surgery. Serial peripheral blood samples were collected from each patient prior to start, during and after completion of neo-adjuvant therapy. All tissue and the baseline plasma samples were sequenced using MSK-IMPACT. All plasma samples were evaluated using patient-specific digital droplet (dd)PCR assays. (B) Oncoprints of somatic mutations found in tissue and circulating cell-free DNA (cfDNA) samples of each patient. Somatic mutations detected in a given sample above filtering thresholds are considered *de novo* (see Methods). All other somatic mutations were detected by genotyping the aggregated set of variants identified in the patient matched tissue and baseline cfDNA samples. Mutation types are color-coded according to the legend. Samples were coded by type and timepoint, where T indicates primary tumor, P indicates plasma, and M indicates metastasis. The accompanying number (e.g., T0, P0, M1) denotes the collection timepoint for each sample type. (C) comparison of cfDNA concentration at different times of plasma collection, and (D) comparison of cfDNA concentration at different times of plasma collection between patients with and without complete pathologic response (pCR). In (C) and (D), the boxes show the median and interquartile range. The whiskers extend to the full range of the data points excluding outliers. *p*-values were calculated using the Wilcoxon rank sum test. All tests were two-sided. Abbreviations: cfDNA, circulating cell-free DNA, ddPCR, digital droplet polymerase chain reaction; ns, not-significant; pg, picogram; SNV, single nucleotide variant;

**Figure 2: Evaluating different metrics of circulating tumor-derived DNA (ctDNA).**

(A) Comparison of Allele Fraction (AF) measured from targeted sequencing using MSK-IMPACT and droplet digital PCR (ddPCR). (B-C) Comparison of surrogate measures of ctDNA fraction at baseline across breast cancer subtypes using (B) the maximum AF of any somatic mutation and (C) the fraction of somatic mutations as a function of the aggregate set of mutations present across all samples of a given patient. (D-E) Cross correlation of different surrogate measures of ctDNA. The orange line shows the best fit linear regression, and the grey line is the identity *y = x*. In (A) and (D) to (F), the Spearman’s correlation was used to assess the association between the two variables, the coefficients and non-parametric *p*-values are displayed. In (B) and (C), the boxes show the median and interquartile range. The whiskers extend to the full range of the data points. In both panels, the *p*-values were calculated using the Wilcoxon rank sum test and all tests were two-sided. Abbreviations: AF, allele fraction; cfDNA, circulating cell-free DNA, ddPCR, digital droplet polymerase chain reaction; HER2, human epidermal growth factor report 2; HR, hormone receptor; ns, not-significant; TN, triple-negative.

**Figure 3: Longitudinal ctDNA tracking by droplet digital PCR (ddPCR).**

Scatter plots showing the evolution of AF of somatic mutations in baseline, on-treatment, and post-treatment plasma samples in (A) patients with no pCR to neo-adjuvant chemotherapy and (B) patients with pCR and no residual disease. For each case, two mutations with the highest AF in the tumor tissue were selected for ddPCR analysis. Abbreviations: AF, allele fraction; pCR, pathological complete response; ddPCR, digital droplet polymerase chain reaction.