**Tracking response to neoadjuvant systemic therapy through cfDNA profiling in early breast cancer**

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# **ABSTRACT**

Neoadjuvant systemic therapy (NST) is the standard-of-care for most patients diagnosed with early breast cancer (EBC). Despite advances in the treatment of EBC, approximately 30% of patients experience disease recurrence after NST, highlighting the need to personalize the treatment in this setting. An active area of study is the use of liquid biopsy to monitor treatment response and predict the likelihood of pathologic complete response (pCR) in patients with EBC. In the current study, we sought to determine whether circulating tumor DNA (ctDNA) in locally advanced breast cancer patients (n=20) could be used as a surrogate to invasive biopsies and guide in monitoring response to NST. We observed a high prevalence of detectable ctDNA at baseline (14/18, 78%) but no significant differences in ctDNA detection rate between patients with or without pCR following NST. There was a positive association between histological grade and molecular subtype with the ctDNA fraction. Lastly, circulating cell free DNA (cfDNA) concentrations tended to increase during NST with a higher cfDNA concentration in patients with residual disease at surgery. These findings underscore the potential clinical utility of ctDNA as a non-invasive tool for monitoring disease progression in EBC patients.

# **Introduction**

Neoadjuvant systemic therapy (NST) is the standard-of-care for most patients diagnosed with stage II-III early breast cancer (EBC), especially those with human epidermal growth factor receptor 2-positive (HER2-positive) and triple-negative disease1-3. The goals of NST include tumor shrinkage to favor breast-conserving surgery, *in vivo* assessment of drug sensitivity and tailoring of post-NST treatment based on the initial tumor’s 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. Despite advances in the treatment of EBC, approximately 30% of patients experience disease recurrence after NST and surgery5, 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 EBC.

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 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 EBC, 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. 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 surgery21.

In the current study, we sought to assess if cfDNA analysis of plasma obtained pre-treatment from patients with locally advanced breast cancer could be used as a surrogate to invasive biopsies to detect genetic alterations, and furthermore, could assist in monitoring response to NST.

# **M****aterials and 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 EBC 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 EBC and were candidates to receive NST. Tumor specimens were subjected to central review by a breast pathologist (F.P.) to determine the histologic type, grade, and receptor status. Hormone receptor (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 guidelines22,23. 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 from the date of patient consent.

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

Tissue samples from the primary tumor were collected at the time of initial biopsy. In patients with residual disease after NST, a post-treatment sample was obtained. pCR was defined as the absence of residual invasive cancer from hematoxylin and eosin stained surgically resected breast specimen and all sampled regional lymph nodes following completion of NST (i.e., ypT0/T is ypN0 in the current AJCC staging system), according to the current guidelines1.

Serial peripheral blood samples were collected from each patient prior to start, during and after completion of NST in two Streck cell-free DNA blood collection tubes (BCT; Streck, La Vista, NE). Blood was processed and 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 described24.

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

Tissue samples and pretreatment cfDNA samples 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 described25,26. MSK-IMPACT sequencing data were analyzed using a validated bioinformatics pipeline as previously described27,28. 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). *De novo* somatic mutations were defined as those that were initially detected in each sample. All other mutations were detected by genotyping.

## ***Tumor-guided digital droplet 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 digital droplet PCR (ddPCR) assays for each patient, as described previously17,29. 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. We considered a plasma sample as ctDNA positive if any variant was observed with an allele fraction (AF) >0%.

## ***Statistical analysis***

The fraction of tumor-derived DNA in the cfDNA (ctDNA) was defined according to the time point and assay under consideration. In the baseline cfDNA sequenced using MSK-IMPACT, we considered three metrics as follows: (1) the maximum AF of any detected variant, (2) the mean AF of all detected variants and (3) the number of variants detected in cfDNA as a fraction of the aggregate set of variants detected in the tissue and cfDNA of a given patient. For ddPCR samples, the ctDNA fraction was defined as the maximum AF of the two variants being assayed.

All statistical comparisons between groups were performed using the Wilcoxon rank sum test. A *p*-value <0.05 was considered statistically significant and all tests were two-sided unless otherwise specified. All statistical analyses were performed using R (version 4.1.1).

## ***Data availability***

The assembled somatic mutational data of the primary tumor, plasma, residual disease as well as distant/ local relapse from the entire cohort will be accessible through cBioPortal at https://www.cbioportal.org/ upon acceptance of the manuscript. All codes used in this manuscript are available on GitHub at https://github.com/ndbrown6/MSK-Early-Breast/.

# **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 HR-positive/HER2-negative EBC (11/20, 55%), while 25% and 20% had HER2-positive and triple-negative disease, respectively. Median tumor size was 3.6 cm (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 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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## ***cfDNA assessment during NST***

To evaluate changes in cfDNA throughout the course of NST, plasma samples were collected at three intervals (baseline, on-treatment and post-treatment; **Figures 1A-1B**). For one patient, post-treatment cfDNA collection was not available. cfDNA concentrations were found to be higher on-NST and post-NST as compared to baseline (p<0.001 for both; **Figure 1C**), as expected from therapy-induced lysis of tumor cells. No differences in cfDNA concentrations, either assessed at baseline or on/post-NST, were found when comparing clinical and pathological features (**Supplementary Figures S1-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**), supporting the hypothesis that elevated post-NST cfDNA levels could be indicative of the presence of residual circulating tumor cells.

## ***ctDNA assessment by targeted sequencing and patient-specific ddPCR***

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

We first assessed the concordance between the tumor biopsy and baseline cfDNA. Overall, 14/18 (78%) patients had at least one mutation detected in the baseline cfDNA. In 7 cases ≥1 mutation was detected *de novo* 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 AF was observed using ddPCR and MSK-IMPACT assays (*R* = 0.8, *p*<0.001; **Figure 2A**). The median of the fraction of mutations found in cfDNA, which can be taken as a crude estimation ctDNA fraction, was 71% (IQR: 46-100%). A higher fraction of mutations in cfDNA was detected in plasma of patients with grade 3 (p=0.07) and ER-negative EBC (p=0.04, **Supplementary Figure S4**). Using the alternative definition of 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**), however the number of cases in each group were small. 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. Overall, 15/18 (83%) patients had detectable ctDNA at baseline, while all patients cleared ctDNA post-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 EBC subtypes, respectively, consistent with previous reports4,5. 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, suggesting that baseline ctDNA does not influence response at the tumor and lymph node level (**Table 3**).

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 detection at baseline might be useful to further stratify patient prognosis prior to starting NST.

# **Discussion**

The use of liquid biopsy offers multiple opportunities to improve the management of EBC34. In patients who are candidates to receive NST, the application of ctDNA assays is appealing for disease monitoring and identification of patients at higher risk of disease relapse35. 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 treatment36,37. Importantly, we have identified a significant association between elevated post-therapy cfDNA concentrations and the presence of residual disease at surgery, as suggested previously20,38. 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 studies38,39 that suggested that characteristics of the primary tumor, including its biological and intrisic agressiveness, influence the shedding of ctDNA into the bloodstream. Analyses of the I-SPY 2 trial38 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 cancer40, as well as to specific molecular profiles that can influence ctDNA shedding22.

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 ctDNA analysis for detecting minimal residual disease and predicting treatment response in patients undergoing NST. The ability to detect ctDNA at baseline provides valuable prognostic information and may aid in risk stratification and treatment planning10. Even with a limited number of patients, we observed the lack of a significant difference in ctDNA detection between patients with or without pCR. This observation is aligned with previous studies in EBC which have found that the detection ctDNA after neoadjuvant systemic therapy is associated with survival outcomes, regardless of pCR19,22,39, 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 setting41,42.

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 various breast cancer subgroups, particularly in the context of neoadjuvant therapy. Second, the patient cohort enrolled in this study received treatment several years ago and may not fully reflect the current treatment landscape for EBC. 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 novel 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 study in triple-negative breast cancer43, ctDNA fractions can decrease below the detection limit of commercially available tests during NST, emphasizing the imperative for ongoing technological advancements in ctDNA analysis.

The findings of our study underscore the potential of ctDNA to revolutionize the management of patients with EBC. 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 detection44. 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 confirms the clinical utility of ctDNA analysis as a non-invasive tool for monitoring disease progression in patients with EBC. 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.

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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%) |
| None | 13 (65%) |
| **Adjuvant endocrine therapy** |  |
| AI | 11 (55%) |
| Tamoxifen | 3 (15%) |
| Tamoxifen-AI | 1 (5%) |
| No | 5 (25%) |
| **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 (29%) | 3 (30%) |  |
| None |  | 10 (71%) | 3 (50%) |  |
| **Adjuvant endocrine therapy** | 20 |  |  | 0.5 |
| AI |  | 8 (57%) | 3 (50%) |  |
| Tamoxifen |  | 2 (14%) | 3 (50%) |  |
| Tamoxifen-AI |  | 3 (21%) | 0 (0%) |  |
| No |  | 1 (7%) | 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.

**Table 3. Detection of ctDNA in baseline plasma by pathologic complete response (pCR) and breast cancer subtype**

|  |  | **Baseline ctDNA detection** | | |
| --- | --- | --- | --- | --- |
| **Breast cancer subtype** | **pCR** | **No (n=3)** | **Yes (n=15)** | **Total (n=18)** |
| **HR-positive/HER2-negative** | No | 2 (100.0%) | 5 (83.3%) | 7 (87.5%) |
|  | Yes | 0 (0.0%) | 1 (16.7%) | 1 (12.5%) |
| **HER2-positive** | No | 0 (0.0%) | 3 (60.0%) | 3 (50.0%) |
|  | Yes | 1 (100.0%) | 2 (40.0%) | 3 (50.0%) |
| **Triple-negative** | No | 0 | 2 (50.0%) | 2 (50.0%) |
|  | Yes | 0 | 2 (50.0%) | 2 (50.0%) |

# **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. (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. *p*-values were calculated using the Wilcoxon rank sum test. All tests were two-sided.

**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.

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

Scatter plots showing the evolution of Allele Fraction (AF) of somatic mutations in baseline, on-treatment, and post-treatment plasma samples (A) in patients with no pathologic complete response (pCR) to neo-adjuvant chemotherapy and (B) in patients with pCR and no residual disease. For each case, mutations with the highest AF in the tumor tissue were selected for ddPCR analysis.