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# Circulating tumor DNA fraction predicts residual cancer burden post-neoadjuvant chemotherapy in triple negative breast cancer

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ABSTRACT

*Purpose:* Pathologic response after preoperative/neoadjuvant chemotherapy (NAC) is a continuum that can range from complete pathologic response (pCR) to extensive residual disease (RD). We hypothesized that post-NAC plasma circulating tumor DNA (ctDNA) fraction (TF) reflect pathologic response as continuum measured by the residual cancer burden (RCB) score.
*Methods:* ctDNA was assessed using the PredicineBEACON assay, that interrogates up to 50 personalized tumor variants and 500 hot-spot mutations, in 3 mL archived plasma isolated from EDTA tubes collected post-NAC but before surgery from 44 patients with stage I/III triple negative breast cancer (TNBC) who received durvalumab and weekly nab-paclitaxel followed by doxorubicin/cyclophosphamide on a clinical trial (NCT02489448). Circulating free tumor DNA methylation profiling was performed using PredicineEPIC assay in paired pre- and post-NAC plasma (N = 30). Youden's J-statistics was used to define optimal thresholds.
*Results:* We observed a significant positive correlation (r = 0.45, $p = 0.004$) between RCB scores and post-NAC TF. The median TF was significantly lower in pCR (RCB0) compared to RD patients (0.06 % vs. 0.3 %, p = 0.02). Using a TF positivity threshold of ≥0.05 %, PredicineBEACON had 58 % sensitivity at 83 % specificity for identifying RD. TF was higher in patients who experienced recurrence (n = 9) compared to those without recurrence (n = 35) (0.17 % vs. 0.05 % TF, $p = 0.029$). There was significant decrease in methylation signal in post-compared to pre-NAC samples, but post-treatment methylation signal was lower in cases with pCR vs RD.
*Conclusions:* Post-NAC plasma tumor fraction and change in tumor-derived methylation signal predict the extent of RD and recurrence in TNBC patients.

## 1. Introduction

Assessing pathologic response after neoadjuvant chemotherapy on a continuous scale allows more accurate prognostic risk stratification [1]. The residual cancer burden (RCB) metric was developed and is now increasingly adopted to quantify the extent of residual cancer after neoadjuvant chemotherapy (NAC) in breast cancer [2]. Studies have demonstrated the prognostic value of RCB score in different subtypes of breast cancers, with higher RCB scores corresponding to more extensive residual cancer and worse event-free survival [3].

The utilization of cell-free circulating tumor DNA (ctDNA) to detect molecular residual disease (MRD) is increasingly explored in the management of solid tumors [4]. ctDNA represents a subset of circulating free DNA (cfDNA) in the plasma that originated from tumor cells. ctDNA results can be reported as number of tumor molecules/mL, or as mutant allele frequency (MAF) of genes of interest, or as tumor fraction (TF) which reflects the ctDNA percent contribution to total circulating free DNA. Several studies demonstrated that ctDNA can be used as a biomarker after neoadjuvant treatment to risk-stratify breast cancer patients [5,6]. Lack of ctDNA clearance is associated with high risk of recurrence, whereas patients with negative ctDNA even with residual disease after therapy have favorable prognosis [7,8]. Next-generation sequencing of cell-free ctDNA from liquid biopsy also provides comprehensive tumor genomic characterization, including detection of actionable mutations [9]. Both ctDNA positivity, that requires a predefined threshold to dichotomize results into positive versus negative,

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and tumor fraction (TF), which is a continuous measure of ctDNA load in the plasma, are predictive of prognosis in early stage disease and also in metastatic cancers [10,11].

The currently most effective NAC for triple negative breast cancer (TNBC) includes 20 weeks of 4-drug chemotherapy concurrent with pembrolizumab which results in pathologic complete response (pCR/RCB0) in over 60 % of cancers and improvement in survival [12]. However, this regimen also causes long lasting immune related adverse effects of various severity in over 30 % of patients and is expensive. It is increasingly clear that many patients achieve a pCR with shorter therapy, and some patients continue to recur despite full treatment. This indicates continued need to develop a diagnostic test to identify patients who may not need the full 20 weeks of neoadjuvant chemotherapy to achieve pCR, and also those who remain at risk for recurrence despite completing treatment and therefore might benefit from further systemic postoperative treatments. In this study we tested the feasibility of performing the PredicineBEACON ultra-deep tumor-informed sequencing assay and the PredicineEPIC cfDNA methylation assay using 3 mL archived plasma collected into EDTA tubes after completion of neoadjuvant chemotherapy but before surgery, and also examined correlation between ctDNA TF and residual cancer burden and recurrence.

## 2. Methods

### 2.1. Patient samples

Sixty-seven patients treated with durvalumab concurrent with weekly nab-paclitaxel followed by doxorubicin/cyclophosphamide for stage I-III TNBC on a single arm neoadjuvant clinical trial (NCT02489448) were eligible for this archived plasma ctDNA study [13]. Patients were accrued to the clinical trial between December 18, 2015 and December 29, 2020. Median patient follow-up time was 35 months (range 25–70 months). Pre- and Post-NAC blood samples were collected into 10 mL EDTA- and heparin-containing vacutainer tubes and plasma was isolated on the same day of collection and aliquoted into 1 mL vials for storage at −80C until further processing. The post-NAC samples were collected after completion of chemotherapy but before surgery. A total of 3 mL stored plasma per patient was used for ctDNA analysis.

Pathologic response was quantified using the RCB method [2]. Based on the continuous RCB scores and previously established thresholds patients were categorized into RCB-0 (pCR, N = 21), RCB-I (minimal residual cancer, N = 7), RCB-II (moderate residual cancer, N = 13), and RCB-III (extensive residual cancer, n = 3) response categories [2]. All patients signed informed consent for research on their donated tissues and ethical approval for this biomarker study was obtained from the Yale Human Investigations Committee.

### 2.2. ctDNA detection and analysis

Whole exome sequencing (WES) data was generated from formalin fixed paraffin embedded (FFPE) tissues of baseline tumor biopsies and from matching buffy coat of 56 participants using HiSeq 4000 at the Yale Center for Genome Analysis. Results of the exome sequencing were previously published [13] and the sequence data is available in dbGaP under bioproject PRJNA558949. Using the publicly available WES data, Predicine Inc has built tumor-informed, personalized ctDNA assays including up to 50 tumor variants per patient. High-confidence somatic variants were identified using the Predicine DeepSea pipeline [14]. The majority of Pre-NAC plasma samples failed ctDNA extraction and therefore only the post-NAC samples were used in the ctDNA analysis. Of the 67 trial participants 19 patients had either missing post-NAC plasma (n = 8) or had no matching primary tumor WES data (n = 11). Plasma from 4 of the remaining 48 patients yielded highly degraded, low quantity DNA that failed to produce meaningful cfDNA results and therefore these samples were excluded as QC failures. The final analysis included 44 patients with post-NAC ctDNA (Fig. 1). Sequencing to detect ctDNA was performed using the PredicineBEACON personalized assay. Personalized variants along with a panel of 500+ cancer gene hotspot regions, including therapeutically targetable mutations, were captured using ultra-deep sequencing (100,000X). Tumor fraction was assessed based on the mutant allele frequency. After filtering variants to include only highest confidence mutations, 37 of the 44 patients had variants that could be used to estimate plasma cfDNA tumor fraction with high confidence. In the 7 lower quality samples, all mutations were used to estimate TF. Plasma ctDNA positivity was determined using a tumor fraction threshold chosen to optimize both sensitivity and specificity to distinguish pCR from RD, or recurrence from no recurrence, by using the Youden's J statistic.

Genome-wide cfDNA methylation profiling was performed using the PredicineEPIC assay [13]. This assay requires substantially lower DNA input than PredicineBEACON and provides an alternative to TF. Methylation signal was assessed by comparing samples against a background model built from a panel of healthy normal plasma samples to obtain genome-wide counts of differentially methylated cfDNA fragments. Pre-NAC and post-NAC cfDNA methylation profiles were successfully obtained for 30 patients.

### 2.3. Statistical analysis methods

Statistical analyses were performed using Graphpad Prism and R (v4.2.0). Median tumor fractions between pathologic response groups,

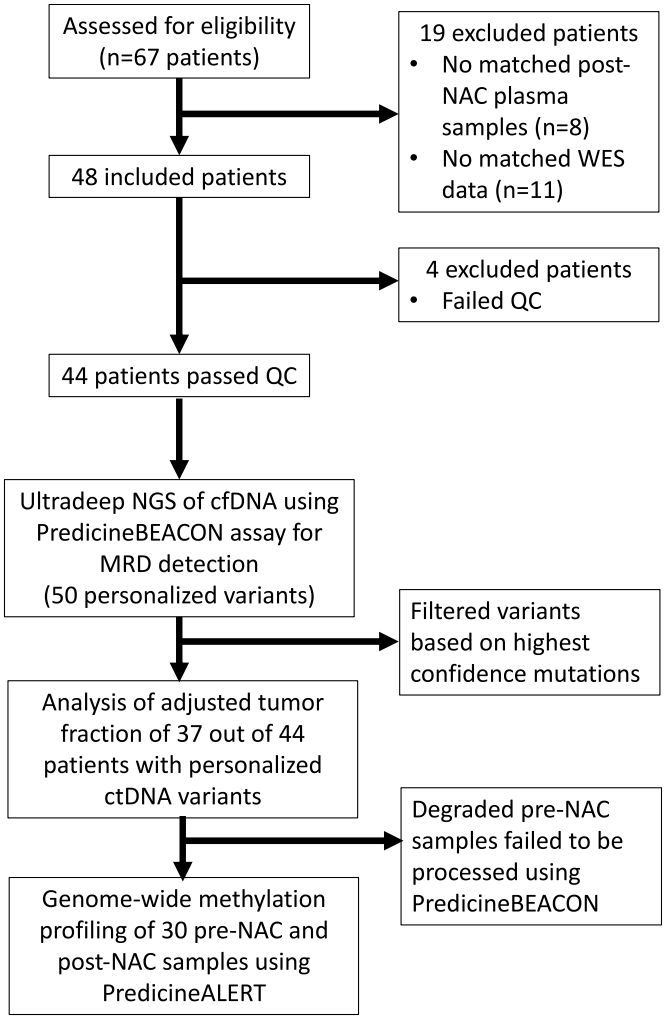


**Fig. 1.** Flow diagram of sample disposition.

and between patients who have or have not recurred were compared using a Wilcoxon test. Tumor fraction threshold to define ctDNA positivity was determined using Youden's J Statistic to maximize accuracy of predicting pCR, and this threshold was used to calculate sensitivity and specificity. Correlation between continuous tumor fraction and continuous RCB score or days to progression were measured using the Spearman correlation coefficient. Normalized RCB score and log tumor fraction were calculated as X – min(X)/max(X)-min(X) for comparability.

## 3. Results

### 3.1. Study population

Median age was 50.5 years (range 27–69), 32 patients (72.7 %) were self-identified as White, 9 patients (20.4 %) as black or African American, 2 patients (4.5 %) as Asian and 1 patient (2.3 %) as Hispanic/Latino. Fourteen patients (31.8 %) had clinical tumor stage 1, 23 (52.8 %) had stage 2, and 7 (15.9 %) had stage 3 disease at diagnosis. Post-NAC, 21 patients (47.7 %) had RCB-0 (RCB score 0), 7 (15.9 %) had RCB-I (RCB score ≥0–1.36), 13 (29.5 %) had RCB-II (RCB score 1.37–3.28) and 3 (6.8 %) had RCB-III (RCB score >3.28) categories. Baseline patient characteristics are summarized in Table 1.

### 3.2. Post-NAC plasma tumor fraction correlates with pathologic response and recurrence

Among the 37 patients who had high-confidence TF estimates, the median plasma TF was significantly lower in samples with pathological complete response (RCB-0, n = 19) compared to those with residual disease (RD, n = 18) 0.06 % vs. 0.3 %, $p = 0.018$ (Fig. 2A). Post-NAC TF had an overall AUC of 0.73 to predict pCR status using the TF positivity threshold of ≥0.05 %, sensitivity was 58 % and specificity 83 % (Fig. S1A). If we used all variants to estimate the TF and included all 44 patients in the analysis, the median TF in patients with pCR (n = 21) was also lower than in those with RD (n = 23), but the difference was not statistically significant, 0.04 % vs. 0.06 %, $p = 0.15$. (Fig. S2A).

There was a strong positive correlation between RCB score and tumor fraction as continuous variables (Spearman ρ = 0.46, Fig. 2B–S2B). Post-treatment plasma TF was significantly higher in patients who experienced local or metastatic recurrence (n = 9) compared to those with no recurrence (n = 35) (0.05 % vs. 0.17 %, Wilcoxon test, $p = 0.029$) (Fig. 2C and S2C). Plasma TF had an overall AUC of 0.76 to predict recurrence and using a Youden-J optimized threshold of ≥0.169 %, TF yielded 64 % sensitivity at 97 % specificity for predicting recurrence (Fig. S1B). Both higher TF and RCB score correlated positively with recurrence (Fig. 2D and S2D).

**Table 1**
Patients characteristics (n = 44).

| Age | Years |
|---|---|
| Median | 50.5 |
| Range | 27–69 |
| Ethnicity | n(%) |
| White | 32(72.7) |
| Black or African | 9(20.4) |
| American | 2(4.5) |
| Asian | 1(2.3) |
| Hispanic/Latino | |
| cT | n(%) |
| 1 | 14(31.8) |
| 2 | 23(52.3) |
| 3 | 7(15.9) |
| cN | n(%) |
| 0 | 24(54.5) |
| 1 | 17(38.6) |
| 2 | 0(0) |
| 3 | 3(6.8) |
| RCB category | n(%) |
| 0 | 21(47.7) |
| I | 7(15.9) |
| II | 13(29.5) |
| III | 3(6.8) |
| Grade | n(%) |
| 1 | 1(2.3) |
| 2 | 9(20.9) |
| 3 | 33(76.7) |
| One case has missing data | |

### 3.3. Post-NAC plasma tumor fraction correlates with extent of residual disease

We defined ctDNA positivity as TF >0.05 % using the optimal threshold for maximizing overall accuracy to predict pCR. TF was significantly higher in cases with residual disease compared to those with complete response (Fig. 3A). Eight ctDNA positive patients had RCB-0 response (42 % of RCB-0 cases), 4 had RCB-I response (80 % of RCB-I cases), 8 had RCB-II response (80 % of RCB-II cases), and 3 had RCB-III response (100 % of RCB-III cases, Fig. 3B).In addition, both higher RCB score, and higher plasma TF were correlated with shorter time to relapse (Fig. 3C).

### 3.4. Hot-spot mutations in plasma and tumor tissue

Tumor WES and plasma ctDNA sequencing identified hotspot mutations in 19 cancer-associated genes including TP53, PIK3CA, ELF3, PTEN, SMAD4, AKT1, PAPPA2, RHOB, MB21D2, HNF1A, RAP2B, TCF7L2, ARID1A, KDM6A, STK11, RB1, PTPRD, C3orf70 and GNAS in 43 patients (Fig. S4). TP53 mutations were the most prevalent in both tissue and plasma. Across all patients and all genes, 21 alterations were seen in both pre-NAC tumor and post-NAC plasma, 49 alterations were only detected in the pre-NAC tumor biopsy, and 3 alterations were observed in the post-treatment plasma of 3 patients. Three novel mutations seen in the plasma; one was a missense mutation in the C3orf70 locus (encoding a gene involved in neurogenesis and neurobehavior and predictor of lymph node metastasis in bladder cancer), another was a missense mutation in GNAS locus (encoding $G_s\alpha$ subunit of G protein that is frequently mutated in colorectal cancer), and the third was a missense mutation in the PTPRD gene (protein tyrosine phosphatase receptor type D, loss of function mutations in this gene are seen in central nervous system tumors).

### 3.5. Methylation changes of cfDNA in paired pre- and post-NAC plasma samples

To assesses treatment induced changes in methylation signal of tumor-derived cfDNA fragments we compared 30 paired pre- and post-NAC plasma samples. In post-NAC plasma, methylation levels were significantly lower in patients who achieved a pCR compared to those with RD (p = 0.007, Fig. 4A). We observed a decrease in methylation signal in post-NAC compared to pre-NAC samples in the majority of patients. The magnitude of decrease in methylation signal was greater in those who had achieved a pCR compared to those with RD (Fig. 4B). Overall, across all samples, 247 cancer-related genes showed differentially methylated genomic regions in the pre-NAC plasma in 2 or more patients (Supplemental Table 1).

## 4. Discussion

Clinical studies showed significant correlation between plasma ctDNA levels and tumor burden, clinical stage, and response to treatment [14]. All current ctDNA detection methods were optimized for plasma isolated from blood collected into special tubes designed to protect DNA (e.g. Streck Cell-Free blood collection tubes). However, many archived blood samples from earlier clinical trials were collected

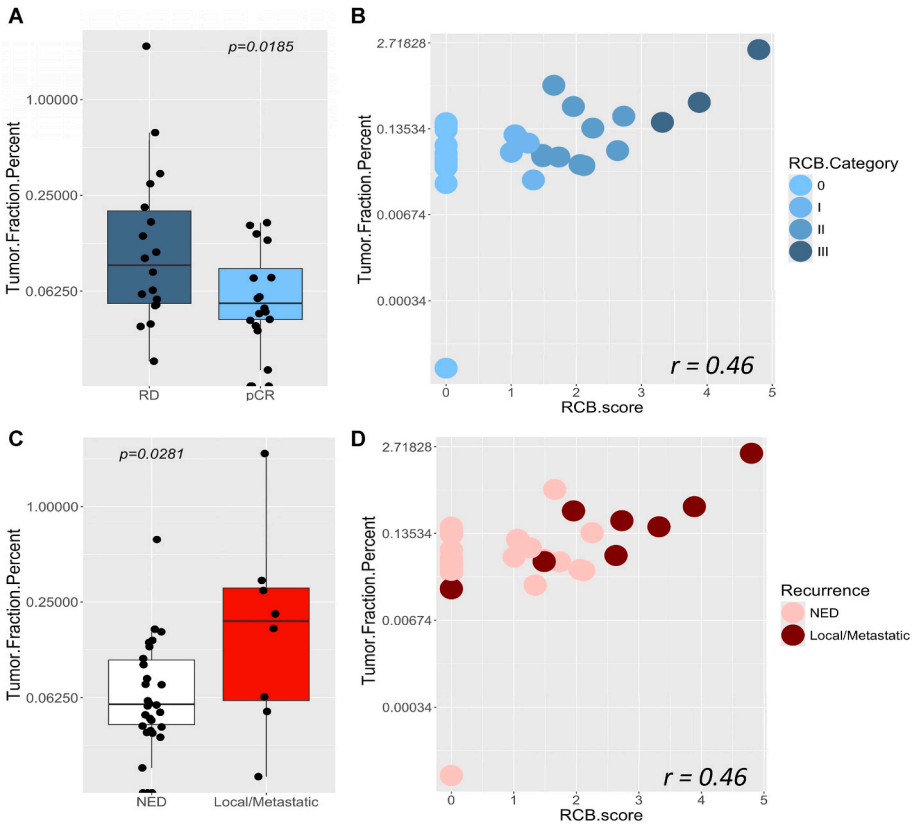


**Fig. 2.** Post-neoadjuvant chemotherapy plasma tumor fraction is associated with residual disease and recurrence. A, Plasma tumor fraction percentage in samples with pathological complete response and residual disease. B, Correlation between RCB score and tumor fraction as continuous variable. $p = 0.004$. C, Plasma tumor fraction in patients with no evidence of recurrent disease and local/metastatic recurrence. D, Correlation of RCB score and tumor fraction in patients with local or metastatic recurrence and no recurrence. $p = 0.004$
RCB = residual cancer burden, NED = no evidence of recurrent disease, RD = residual disease, pCR = pathological complete response.

into EDTA or heparin-tubes and plasma isolated from these samples usually fail to produce high quality ctDNA results because EDTA can inhibit PCR reaction through binding of DNA polymerase co-factors [15]. Our results indicate that the Predicine technology can overcome these technical challenges and can successfully be applied to archived frozen plasma samples from EDTA tubes.

In this study, we examined the performance of the PredicineBEACON minimal residual disease (MRD) detection assay and the PredicineEPIC genome-wide methylation assay in archived plasma samples derived from blood collected into EDTA or heparin-tubes between 2015 and 2020. Tumor-informed personalized ctDNA probes were designed for 48 patients based on publicly available tumor WES data that matched the plasma samples. All patients had pre- and post-NAC plasma samples available. Most ctDNA extracted from pre-NAC plasma failed QC (the majority of these contained heparin preservative) for the PredicineBEACON assay. On the other hand, 44 of 48 post-NAC samples yielded ctDNA that has passed QC and in 37 of the 44 samples, tumor fraction could be called with high confidence. Since the methylation assay could be performed on lower yield cfDNA, we were able to generate PredicineEPIC genome-wide methylation results on 30 paired pre- and post-NAC plasma samples.

The PredicineBEACON assay revealed significantly lower median plasma cfDNA TF in in the post-NAC plasma samples in patients who achieved pCR compared to those with RD. Tumor fraction as continuous variable correlated positively with RCB score as continuous variable indicating that post chemotherapy but pre-surgery ctDNA reflect the extent of residual cancer. The post-NAC TF had an AUC of 0.73 to predict pCR. The Youden j-statistics identified TF $\geq$ 0.05 % as the optimal threshold to distinguish cases with pCR versus RD, with sensitivity and specificity of 58 % and 83 %, respectively. This threshold will need to be validated in future independent studies. Tumor fraction was also significantly higher in patients who experienced recurrence (local or distant) compared to those who remained disease free.

In the clinic, ctDNA analysis is often used to detect tumor-associated mutations that can inform treatment selection [16]. When we assessed hot-spot mutations in the baseline tumor FFPE-derived WES data and in the post-NAC plasma, we found that across all patients and all genes, only 21 alterations were seen in both pre-NAC tumor and post-NAC plasma, whereas 49 alterations were only detected in the pre-NAC tumor biopsy, and 3 alterations were seen only in the post-treatment plasma of 3 patients. Prior studies also showed greater sensitivity of tumor tissue analysis to detect actionable truncal mutations (i.e. mutations that are preserved in primary tumor and metastases) such as PIK3CA in breast cancer [17,18]. The many more variants detected only in the pre-NAC tumor biopsy is likely caused by a combination of technical differences between the FFPE WES and ctDNA sequencing and clonal elimination of variants by 20 weeks of preoperative chemotherapy.

Methylation analysis of cfDNA relies on the principle that tumor-derived ctDNA harbors cancer specific epigenetic alterations that are more numerous and therefore easier to detect than mutations in the highly fragmented ctDNA [19]. Tumor derived methylation signatures can therefore be alternatives to ctDNA mutation detection in finding minimal residual disease or monitoring treatment response [20]. Genome-wide methylation analysis using the PredicineEPIC assay revealed that methylation levels were significantly lower in patients who achieved a pCR compared to those with RD, recapitulating mutation based ctDNA results. We also demonstrated that methylation signal decreased in post-NAC compared to pre-NAC samples consistent with tumor response and the magnitude of decrease was greater in patients

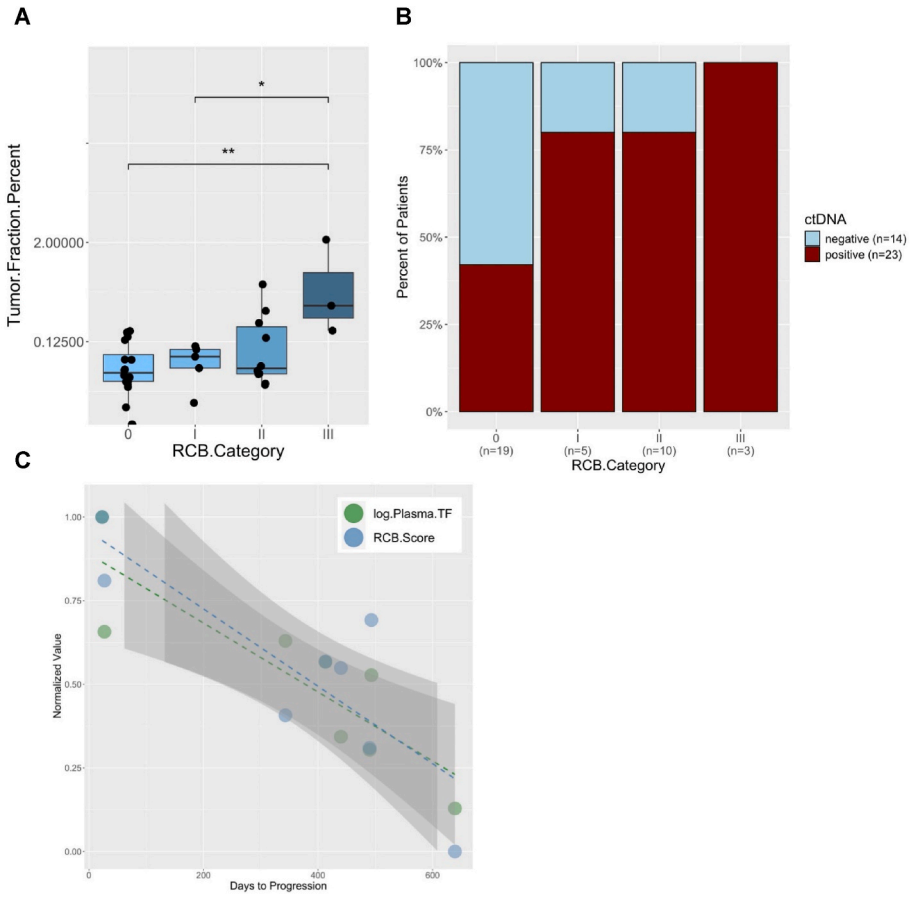


**Fig. 3.** Tumor Fraction and ctDNA positivity are correlated with increasing RCB category. A, Tumor fraction percentages in patients based on RCB category. *, $p < 0.05$; **, $p < 0.01$. B, Percentage of patients based on RCB category. **C,** Correlation between RCB score and tumor fraction vs time to relapse.
RCB = residual cancer burden.

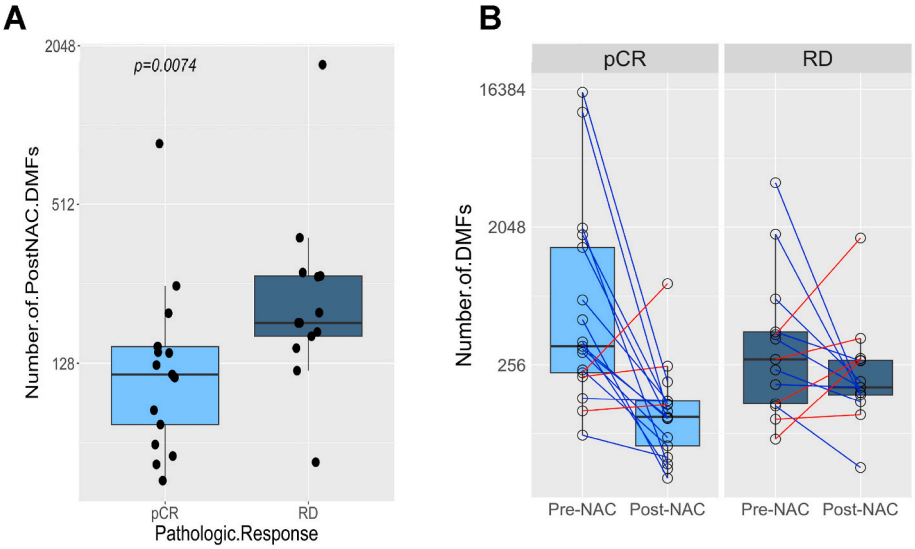


**Fig. 4.** Tumor-derived cfDNA methylation signal pre- and post-NAC. A, Post-NAC methylation levels in patients who achieved pathologic complete response vs patients with residual disease. B, Methylation signal change in post-NAC samples compared to pre-NAC samples in patients who achieved pathologic complete response vs patients with residual disease.
DMF = differentially methylated fragments, pCR = pathological complete response, RD = residual disease.

who experience pCR compared to those who had lesser response. Importantly, methylation data could be successfully generated from samples that were not useable for mutation-based ctDNA analysis. This observation indicates that the two assays could be used in a complimentary manner, particularly in small amounts of degraded archived samples.

Highly sensitive and specific minimal residual disease (MRD) detection assay have the potential to transform care in TNBC, and other breast cancer subtypes. They could allow objective MRD-based de-escalation, and escalation of therapies. Serial monitoring of ctDNA during neoadjuvant therapy coupled with serial imaging could allow individualized stopping of pre-operative cytotoxic therapy when pCR/RCB is achieved with fewer than the current standard of care treatment cycles. This approach is currently prospectively tested in the I-SPY2.2 clinical trial (clinicaltrials.gov ID: NCT01042379). On the other hand, ctDNA surveillance during follow-up after curative intent therapy could identify patients who will recur but still only have microscopic disease (i.e. have ctDNA positive but imaging negative molecular relapse) that is perhaps amenable to 'second line adjuvant therapy" with curative intent. Currently accruing prospective treatment trials that test the clinical utility of ctDNA monitoring during follow in the United States include DARE and LEADER (NCT03285412) for patients with estrogen receptor positive, and ASPIRA (NCT04434040) and PERSEVERE (NCT04849364) for triple-negative breast cancers.

## 5. Conclusions

PredicineBEACON MRD assay and PredicineEPIC genome-wide methylation assays were successfully performed on 3 mL archived plasma derived from blood collected into EDTA and heparin-containing tubes. Post-NAC plasma cfDNA tumor fraction and methylation levels correlated with the extent of residual disease and with recurrence. Paired pre- and post-NAC samples showed significant decrease in methylation signal post-therapy, with greater decrease in cases with pCR.

## Author contribution

Conceptualization: NLS, BG, LP. Data curation: NLS, BG. Formal analysis: NLS, BG. Funding acquisition: LP. Investigation: LP. Methodology: NLS, BG, XW. Project administration: LP. Resources: LP, KB, JF, BG, GEC, MW, GB, ZZ, PD. Supervision: LP. Visualization: BG, NLS. Roles/Writing – original draft: NLS. Writing – review & editing: all authors.

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## Data availability

Patient level ctDNA, tumor fraction and methylation data are included in Supplemental Table 2.

## Ethical approval

This was an investigator-initiated trial, and ethical approval was obtained from the Yale Human Investigations Committee (Yale University, HIC# 1409014537) and its Clinicaltrials.gov identifier was NCT02489448. All patients provide written consent to obtain research biopsies and donate tissues for future research.

## Informed consent

The study was approved and was annually reviewed by the internal institutional review board and all patients provided a written consent form to join the therapeutic study and to use their tissues collected in the context of the trial for future research.

## Declaration of competing interest

NLS, GEC, KB, JF have no relevant financial or non-financial interests to disclose.

Billie Gould: employment—Predicine Inc; stock—Predicine, Freenome, and Myriad.

Pan Du: employment—Predicine, Inc; stock: Predicine, Inc.

Myles Walsh: employment—Predicine, Inc; stock: Predicine, Inc.

Giancarlo Bonara: employment—Predicine Inc, stock: Predicine, Inc.

Xiaohong Wang: employment—Predicine Inc, stock: Predicine, Inc.

Lajos Pusztai: has received consulting fees and honoraria for advisory board participation from Pfizer, Astra Zeneca, Merck, Novartis, Bristol-Myers Squibb, Stemline-Menarini, GlaxoSmithKline, Genentech/Roche, Personalis, Daiichi, Natera, Exact Sciences and institutional research funding from Seagen, GlaxoSmithKline, AstraZeneca, Merck, Pfizer and Bristol Myers Squibb.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jlb.2024.100168.

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