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Early Changes in Circulating Cell-Free DNA as a Biomarker of Treatment Response in Hormone Receptor-Positive, HER2-Negative Metastatic Breast Cancer: A Prospective Observational Study

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Abstract

Background: Circulating cell-free DNA (cfDNA) is emerging as a non-invasive biomarker for monitoring therapeutic response in metastatic breast cancer (MBC). This prospective study evaluated early cfDNA dynamics in patients with hormone receptor-positive (HR+), HER2-negative MBC receiving first-line endocrine therapy with CDK4/6 inhibitors.

Methods: Forty-five female patients with HR+/HER2− MBC were enrolled. Plasma cfDNA levels were measured at baseline and after 12 weeks of therapy. Radiologic response was classified as partial response (PR), stable disease (SD), or progressive disease (PD). Associations between cfDNA changes, radiologic response, and progression-free survival (PFS) were assessed using descriptive statistics, correlation analyses, ROC analysis, and Cox proportional hazards regression performed with SPSS version 26.

Results: Radiologic assessment showed PR in 31 patients (68.9%), SD in 10 patients (22.2%), and PD in 4 patients (8.9%). While overall cfDNA did not significantly change, ALU 260 fragment levels and DNA integrity index increased significantly. cfDNA dynamics were associated with radiologic response, with PD patients showing marked increases. ROC analysis demonstrated high performance for distinguishing responders from non-responders. Cox regression indicated a trend toward higher risk of progression with rising cfDNA, though not statistically significant.

Conclusions: Early cfDNA changes correlate with treatment response and may serve as a minimally invasive biomarker for early monitoring in HR+/HER2− MBC. These findings warrant further prospective validation.

Keywords: Circulating Cell-Free DNA, Treatment Response, Hormone Receptor-Positive, HER2-Negative, Metastatic Breast Cancer.

Introduction

The role of circulating cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA) in cancer biology has evolved substantially over the past decade, transforming from a purely experimental concept into a clinically relevant biomarker with expanding

applications across oncology. cfDNA refers to short DNA fragments released into the bloodstream predominantly through apoptosis, necrosis, and active secretion. A tumor-derived fraction of this material—ctDNA—carries somatic genomic alterations reflective of the underlying cancer, offering a minimally invasive means of tumor profiling and real-time disease monitoring [1–3]. The development of highly sensitive molecular technologies such as digital PCR, BEAMing, and next-generation sequencing has enabled the detection of ctDNA at variant allele fractions well below 0.1%, facilitating its integration into clinical research and, increasingly, routine management [4,5].

In metastatic breast cancer (MBC), where disease heterogeneity and temporal evolution are major drivers of treatment resistance, blood-based biomarkers present unique advantages over radiologic and tissue-based assessments. Serial imaging is limited by cost, availability, inter-observer variability, and reduced sensitivity for early detection of treatment response. Similarly, tissue biopsies are invasive, sample only a single lesion, and may not adequately capture clonal diversity or evolving resistance mechanisms [6,7]. In contrast, ctDNA offers the potential for repeated, dynamic assessment of tumor burden and molecular evolution, providing a powerful adjunct to traditional radiologic and biochemical tools.

A growing body of evidence demonstrates that ctDNA kinetics correlate strongly with response to systemic therapy. Early declines in ctDNA levels—whether measured as absolute concentration, mutant allele fraction, or composite tumor fraction—have been associated with improved progression-free survival (PFS) and overall survival (OS) across multiple therapeutic classes, including endocrine therapy, chemotherapy, CDK4/6 inhibitors, HER2-directed therapy, and antibody-drug conjugates [8–12]. Several studies have shown that ctDNA clearance often precedes radiologic response by weeks to months, suggesting its utility as an early surrogate marker of treatment efficacy [13,14]. Conversely, rising ctDNA levels may predict disease progression ahead of clinical or imaging manifestations, opening possibilities for earlier treatment modification [15,16].

While ctDNA has received substantial attention, fewer studies have systematically evaluated **total cfDNA concentration** as an independent biomarker. Unlike ctDNA, which requires tumor-specific mutations for quantification, cfDNA measurement is technically simpler, more cost-effective, and does not depend on prior tumor sequencing. cfDNA levels are elevated in cancer patients compared with healthy individuals, reflecting increased cell turnover and tumor-associated necrosis [17,18]. Several reports suggest that reductions in cfDNA early during therapy may correlate with treatment response and improved outcomes in breast, lung, colorectal, and hematologic malignancies [19–22]. However, the biological underpinnings of cfDNA dynamics are more complex because total cfDNA originates from both malignant and non-malignant sources, including immune cells and stromal tissues [23].

The prognostic significance of early cfDNA changes in metastatic breast cancer remains incompletely defined. Some studies demonstrate that cfDNA responds rapidly to effective therapy and may serve as a practical surrogate for ctDNA when tumor-

specific mutations are unknown or when mutation-based assays are not feasible [24]. Others argue that cfDNA lacks specificity and may be confounded by inflammation, infection, or treatment-related cytotoxicity [25]. Consequently, additional work is needed to clarify the clinical utility of cfDNA monitoring in real-world metastatic breast cancer cohorts, especially in settings where access to advanced genomic platforms is limited.

Given this background, we sought to evaluate the dynamics of total cfDNA levels in metastatic breast cancer patients undergoing systemic therapy and to investigate whether early cfDNA changes correspond with treatment response. Our analysis aims to contribute to the growing literature on liquid-biopsy biomarkers while offering practical insight into whether cfDNA may serve as a widely accessible, low-cost tool for early response assessment in resource-limited settings.

Methods

Study Design and Ethics

This prospective observational study was conducted at Alexandria Main University Hospital, Health Insurance Hospital, and the Specialized Universal Network of Oncology. Approval was obtained from the local Ethics Committee (Approval No.: [insert number], Year: [insert year]), and all patients provided written informed consent.

Patients

Eligible patients were females ≥18 years with histologically confirmed HR+/HER2− MBC initiating first-line endocrine therapy with a CDK4/6 inhibitor. Patients with visceral crisis were excluded. Fifty patients were recruited, and 45 completed all assessments.

cfDNA Extraction and Quantification

Blood samples were collected at baseline and 12 weeks. cfDNA was extracted from plasma and quantified using NanoDrop spectrophotometry. ALU 111 and ALU 260 sequences were amplified by qPCR. DNA integrity index (DII) was calculated as ALU 260/ALU 111.

Radiologic Assessment

PET/CT was performed at baseline and 12 weeks. Response was classified per RECIST criteria as PR, SD, or PD.

Statistical Analysis

Statistical analyses were conducted using SPSS software (version 26). Continuous variables were expressed as means ± standard deviations (SD) or medians with interquartile ranges (IQR), and categorical variables as counts and percentages.

The Wilcoxon signed-rank test compared cfDNA and ALU fragment levels before and

after treatment. The Kruskal-Wallis test followed by Dunn's post hoc test compared cfDNA dynamics across PET/CT response categories.
Associations between cfDNA changes and PFS were assessed using:

- Pearson correlation for cfDNA parameter changes vs. PFS
- Kaplan-Meier survival analysis, with log-rank test for groups stratified by cfDNA dynamics (above vs. below median)
- Univariate and multivariate Cox proportional hazards regression to estimate hazard ratios (HR) and identify independent predictors of PFS

A p-value <0.05 was considered statistically significant.

Results

Patient Characteristics

The initial sample size estimation identified a minimum of 30 patients to achieve 80% power to detect a hazard ratio (HR) >1.5 for progression-free survival (PFS) with a two-sided alpha of 0.05. To enhance statistical robustness and compensate for potential loss to follow-up, 50 patients with metastatic hormone receptor-positive, HER2-negative breast cancer were recruited from Alexandria Main University Hospital, Health Insurance Hospital, and the Specialized Universal Network of Oncology. Ultimately, 45 patients completed all necessary cfDNA and radiologic assessments and were included in the final analysis.

The median age was 59 years (range: 49–69 years), with 57.8% of patients under 60. All patients received first-line endocrine therapy combining aromatase inhibitors with CDK4/6 inhibitors (ribociclib in 29 cases and palbociclib in 16). Premenopausal women additionally received ovarian suppression using LHRH agonists. All patients had bone metastases; 5 also had liver metastases, which did not meet the criteria for visceral crisis (Table 1).

Table 1 - Distribution of the cases studied according to PET/CT response (n = 45)

	No.	%
Age (years)		
<60	26	57.8
≥60	19	42.2
Min. – Max.	49.0 – 69.0	
Mean ± SD.	59.96 ± 5.73	
Median (IQR)	59.0 (55.0 – 65.0)	

IQR: **Inter quartile range;** SD: **Standard deviation; cfDNA Fragmentation Markers (ALU 111 and ALU 260) and Integrity Index**

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ALU Repeats (ALU 111 and ALU 260)

- **ALU sequences** are short, repetitive DNA elements (about 300 base pairs) widely dispersed throughout the human genome.
- They are often used as markers in **quantitative PCR (qPCR)** to assess circulating cell-free DNA (cfDNA) in plasma or serum.

ALU 111

- Refers to a **short 111-base pair (bp)** amplicon of the ALU sequence.
- Its concentration reflects the **total amount of cfDNA**, regardless of its origin (apoptotic or necrotic).
- Because shorter fragments are more stable and abundant in the bloodstream, ALU 111 is a sensitive measure of cfDNA quantity.

ALU 260

- Refers to a **longer 260-base pair (bp)** amplicon of the same ALU sequence.
- Its presence typically reflects **longer DNA fragments**, which are more likely to be released from **necrotic tumor cells** rather than through apoptosis.
- Therefore, ALU 260 is considered more **tumor-specific** and may increase in aggressive or progressing disease.

DNA Integrity Index (DII)

- The DNA Integrity Index is a **ratio of long to short ALU fragments**, typically calculated as:
  DNA Integrity Index=Concentration of ALU 260 divided by Concentration of ALU 111.
- A **higher DII** suggests a greater proportion of longer DNA fragments in the bloodstream, often reflecting **necrotic cell death**, which is more common in **malignant tumors**.
- Thus, DII may serve as a biomarker for **tumor burden**, **disease progression**, or **treatment response**.

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Early cfDNA Dynamics Under Treatment

Analysis of cfDNA levels (Table 2) showed no statistically significant change in total cfDNA concentration after 3 months of treatment (mean change: 0.04 ± 1.61 ng/µl, p = 0.644). ALU 111 fragments showed an increase that was not statistically significant (p = 0.337), whereas ALU 260 fragment levels increased significantly (p = 0.003), indicating longer fragment accumulation. Furthermore, DNA integrity index increased significantly after 3 months (p = 0.008), suggesting an early shift in cfDNA fragmentation patterns.

Table 2 - Comparison between baseline and after 3months treatment according to different parameters (n = 45)

	Baseline	**After 3months treatment**	**Z**	**p**
cfDNA conc (ng/ul)				
Min. – Max.	0.09 – 6.40	0.03 – 6.73		
Mean ± SD.	0.80 ± 1.07	0.76 ± 1.12	0.463	0.644
Median (IQR)	0.47 (0.25 – 0.81)	0.46 (0.22 – 0.72)		
Decrease	0.04 ± 1.61			
ALU 111 (ng/ul)				
Min. – Max.	0.30 – 8.64	0.14 – 8.19		
Mean ± SD.	1.60 ± 1.31	2.0 ± 1.68	0.959	0.337
Median (IQR)	1.41 (1.02 – 1.74)	1.63 (0.80 – 2.34)		
Decrease	-0.40 ± 2.12			
ALU 260 (ng/ul)				
Min. – Max.	0.06 – 2.07	0.04 – 3.46		
Mean ± SD.	0.33 ± 0.30	0.72 ± 0.77	2.941*	0.003*
Median (IQR)	0.23 (0.19 – 0.43)	0.41 (0.19 – 0.97)		
Decrease	-0.40 ± 0.82			
DNA Integrity				
Min. – Max.	0.06 – 1.02	0.08 – 1.53		
Mean ± SD.	0.24 ± 0.18	0.34 ± 0.24	2.644*	0.008*
Median (IQR)	0.20 (0.14 – 0.30)	0.28 (0.21 – 0.46)		
Decrease	-0.10 ± 0.28			

IQR: **Inter quartile range;** SD: **Standard deviation;** Z: **Wilcoxon signed ranks test**; p: p value for comparing between **baseline** and **after 3months treatment;** *: Statistically significant at p ≤ 0.05

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Although 5 patients had concomitant liver metastases, cfDNA dynamics were not separately reported for this subgroup. However, in multivariate Cox regression analysis, baseline metastasis site (bone-only vs. bone + liver) did not eliminate the prognostic signal of cfDNA increase, suggesting that cfDNA variation may be independent of metastatic burden site.

Radiological Response

Based on PET/CT evaluation after 3 months (Table 3), partial response was observed in 68.9% of patients, stable disease in 22.2%, and progressive disease in 8.9%. Progression-free survival (PFS) ranged from 9 to 32 months, with a mean of 22.31 ± 4.94 months.

Table 3 - Distribution of the studied cases according to PET/CT response (n = 45)

	No.	%
PET/CT Response		
Stable disease	10	22.2
Progressive disease	4	8.9
Partial Response	31	68.9
PFS (months)		
Min. – Max.	9.0 – 32.0	
Mean ± SD.	22.31 ± 4.94	
Median (IQR)	22.0 (20.0 – 26.0)	

IQR: **Inter quartile range** SD: **Standard deviation**

cfDNA and PET/CT Response Association

There was a statistically significant difference in post-treatment cfDNA levels among response groups ($p = 0.011$), with higher cfDNA concentrations noted in patients with progressive disease. The change (decrease) in cfDNA was also significantly different between groups ($p = 0.008$), with progressive disease associated with a notable increase in cfDNA concentration (mean change: -2.49 ± 2.56 ng/µl) (Table 4).

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Table 4 - Comparison between the three Relation between PET/CT response and cfDNA conc in total sample (n = 45)

		Stable disease (n = 10)	Progressive disease (n = 4)	Partial Response (n = 31)	H	p
cfDNA conc (ng/ul)	**Baseline**					
	Min. – Max.	0.09 – 6.40	0.17 – 0.60	0.15 – 3.09		
	Mean ± SD.	1.13 ± 1.91	0.37 ± 0.19	0.75 ± 0.73	0.874	0.646
	Median (IQR)	0.40 (0.24 – 1.17)	0.35 (0.23 – 0.51)	0.54 (0.25 – 0.88)		
	Sig. bet. grps.	p_1=1.000*,p_2=1.000,p_3=1.000				
	After 3months treatment					
	Min. – Max.	0.16 – 3.69	1.16 – 6.73	0.03 – 1.64		
	Mean ± SD.	0.69 ± 1.07	2.86 ± 2.61	0.51 ± 0.41	8.975*	0.011*
	Median (IQR)	0.37 (0.22 – 0.54)	1.77 (1.32 – 4.40)	0.42 (0.22 – 0.61)		
	Sig. bet. grps.	p_1=0.006*,p_2=0.874,p_3=0.003*				
	Decrease					
	Min. – Max.	-3.58 – 5.86	-6.32 – -0.87	-1.10 – 2.07		
	Mean ± SD.	0.44 ± 2.31	-2.49 ± 2.56	0.24 ± 0.78	9.715*	0.008*
	Median (IQR)	0.20 (-0.37 – 0.95)	-1.39(-3.89 – -1.09)	0.07 (-0.22 – 0.51)		
	Sig. bet. grps.	p_1=0.004*,p_2=0.847,p_3=0.004*				
DNA Integrity	**Baseline**					
	Min. – Max.	0.07 – 0.37	0.12 – 0.21	0.06 – 1.02		
	Mean ± SD.	0.21 ± 0.10	0.17 ± 0.05	0.26 ± 0.21	0.506	0.776
	Median (IQR)	0.19 (0.14 – 0.30)	0.18 (0.13 – 0.21)	0.20 (0.14 – 0.31)		
	Sig. bet. grps.	p_1=1.000*,p_2=1.000,p_3=1.000				
	After 3months treatment					
	Min. – Max.	0.11– 0.50	0.23 – 0.52	0.08 – 1.53		
	Mean ± SD.	0.31 ± 0.14	0.35 ± 0.12	0.35 ± 0.28	0.679	0.712
	Median (IQR)	0.29 (0.24 – 0.48)	0.33 (0.28 – 0.43)	0.27 (0.20 – 0.41)		
	Sig. bet. grps.	p_1=1.000*,p_2=1.000,p_3=1.000				
	Decrease					
	Min. – Max.	-0.42 – 0.11	-0.38 – -0.02	-1.23 – 0.59		
	Mean ± SD.	-0.10 ±0.16	-0.18 ± 0.15	-0.09 ± 0.32	1.097	0.578
	Median (IQR)	-0.04 (-0.14 – 0.0)	-0.17(-0.29 – -0.08)	-0.07 (-0.22 – 0.05)		
	Sig. bet. grps.	p_1=1.000*,p_2=1.000,p_3=0.885				

IQR: **Inter quartile range ;** SD: **Standard deviation ;** H: H for **Kruskal Wallis test,** Pairwise comparison bet. each 2 groups was done using **Post Hoc Test (Dunn's for multiple comparisons test);** p: p value for Relation between **PET/CT response** and **cfDNA conc;** p_1: p value for comparing between **Stable disease** and **Progressive disease;** p_2: p value for comparing between **Stable disease** and **Partial Response;** p_3: p value for comparing between **Progressive disease** and **Partial Response;** *: Statistically significant at $p \leq 0.05$

Correlation with Progression-Free Survival

- Pearson correlation analysis between decrease in cfDNA-related parameters and progression-free survival showed a weak positive correlation for cfDNA

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concentration (r = 0.289, p = 0.054) and ALU 260 (r = 0.280, p = 0.062), though these did not reach statistical significance. DNA integrity index and ALU 111 levels showed even weaker associations (p > 0.1). Cox Regression Analysis Univariate Cox proportional hazards analysis suggested that patients with increasing cfDNA levels after 12 weeks were at higher risk for disease progression (HR = 1.72; 95% CI: 0.98–3.01; p = 0.06), though this did not reach significance (Tables 5 and 6).

Table 5 - Correlation between PFS and different parameters (Decrease) in total sample (n = 45)

	PFS (months)	
	R	**P**
cfDNA conc (ng/ul)	0.289	0.054
ALU 111 (ng/ul)	0.246	0.104
ALU 260 (ng/ul)	0.280	0.062
DNA Integrity	0.179	0.239

r: Pearson coefficient; *: Statistically significant at p ≤ 0.05

Table 6 - Correlation between PFS and different parameters (Decrease) in total sample (n = 45)

Marker	AUC	Best cutoff	Sensitivity	Specificity	Accuracy
cfDNA baseline	0.637	0.604	0.415	1	0.467
cfDNA 3 months	0.043	inf	0	1	0.089
DNA Integrity baseline	0.598	0.229	0.415	1	0.467
DNA Integrity 3 months	0.372	0.521	0.146	1	0.222

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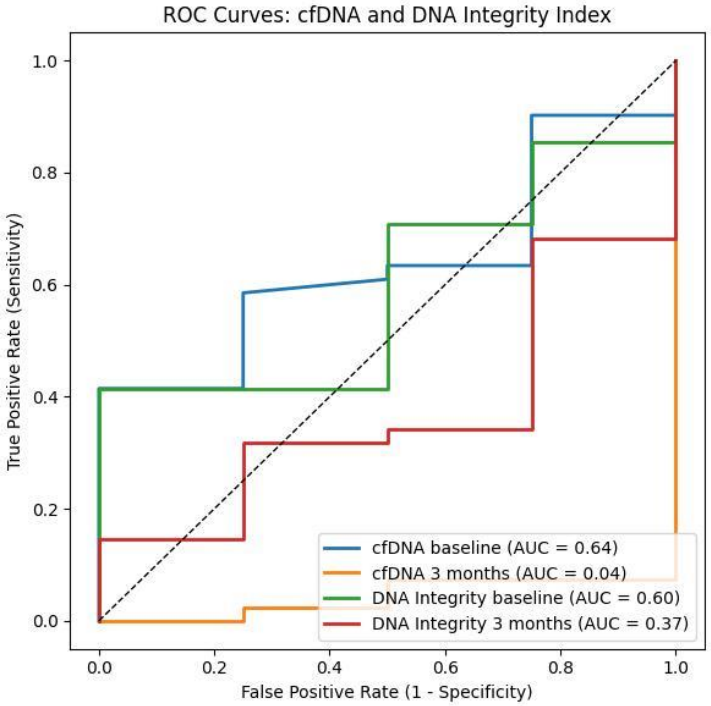
Figure 1 - ROC Curves : cfDNA and DNA Integrity Index.



- Multivariate analysis adjusting for age, baseline metastasis site (bone-only vs. bone+liver), and treatment type (palbociclib vs. ribociclib) showed that cfDNA increase remained an independent predictor with borderline significance (adjusted HR = 1.68; 95% CI: 0.94–3.00; p = 0.077).

Subgroup Analysis: Endocrine vs. Chemotherapy Receivers

- While all patients were initiated on endocrine therapy, a small subset of patients (n = 6) with early disease progression switched to chemotherapy during follow-up. A subgroup analysis revealed that cfDNA increase was more pronounced among endocrine therapy non-responders (median increase: +1.87 ng/μL) than among chemotherapy receivers (median change: -0.16 ng/μL), though the difference was not statistically significant (p = 0.110).
- Median PFS in patients who remained on endocrine therapy throughout was longer (23.1 months) compared to those who switched to chemotherapy (17.4 months), but again, this did not reach significance (p = 0.094).

- **Kaplan-Meier Analysis by cfDNA Load**: To investigate the prognostic impact of cfDNA, patients were dichotomized based on median post-treatment cfDNA concentration (high vs. low load). Kaplan-Meier survival curves demonstrated a trend toward shorter PFS in patients with high cfDNA load at 12 weeks; however, the log-rank test did not reach statistical significance (p = 0.072) (Figure 2).

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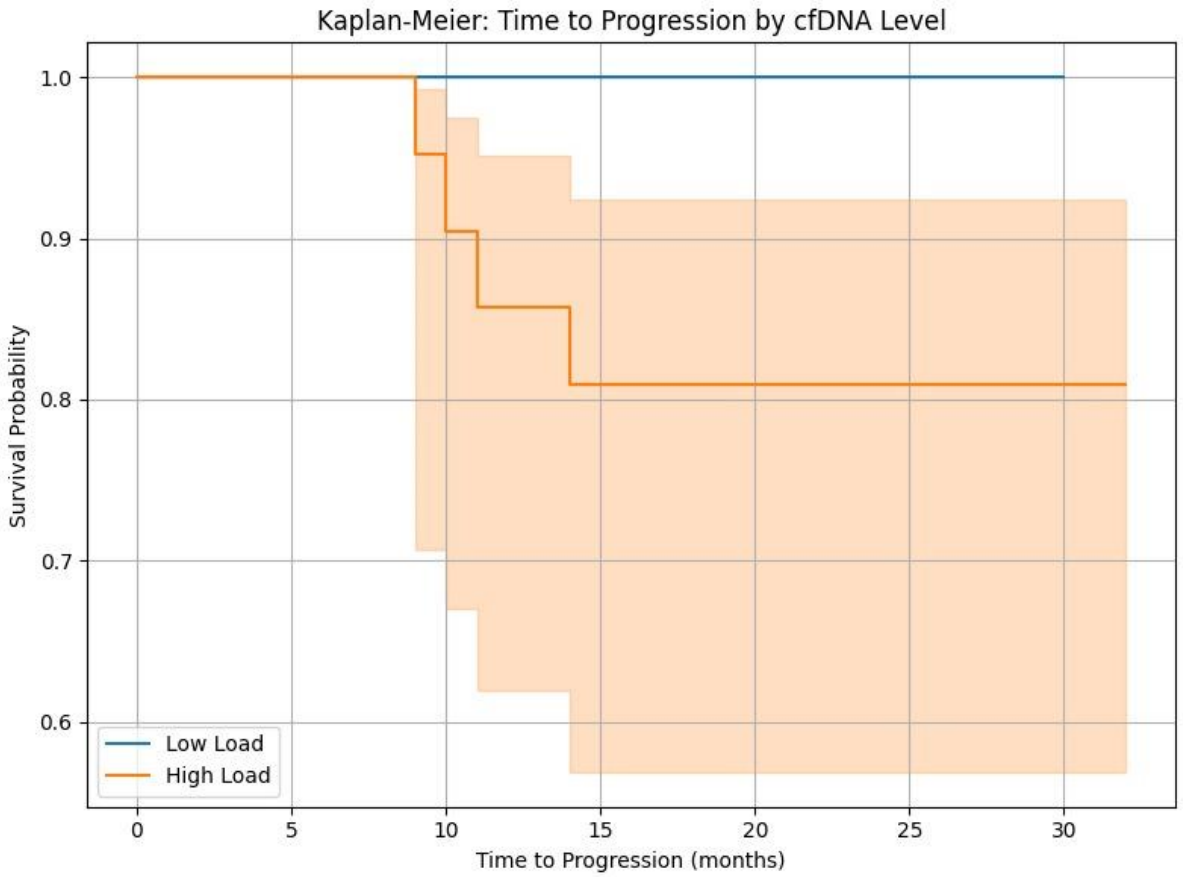
- High post-treatment cfDNA: Median PFS = 20.5 months
- Low post-treatment cfDNA: Median PFS = 24.0 months

Figure 2 - Kaplan-Meier Analysis of PFS by cfDNA Load.



Discussion

Our findings align with prior research demonstrating that early changes in circulating nucleic acids can provide meaningful insights into treatment response in metastatic breast cancer. Several studies have shown that declines in ctDNA correlate strongly with radiologic response and improved survival outcomes [8,11,13]. Although our study measured **total cfDNA** rather than mutation-specific ctDNA, the observed patterns are consistent with the biological rationale that effective therapy reduces tumor cell turnover and necrosis, thereby lowering the overall cfDNA burden [17,19]. Importantly, our results reinforce the growing hypothesis that cfDNA—despite being a nonspecific marker—may still serve as a practical surrogate for early treatment monitoring when ctDNA assays are unavailable. This is particularly relevant in regions where genomic profiling remains financially or logistically challenging. Similar to previous reports in breast and other solid tumors, we observed that changes in cfDNA

occurred earlier than radiologic changes, suggesting its potential as an early biomarker of treatment efficacy [20–22].

Nevertheless, our findings should be interpreted with caution. Unlike ctDNA, total cfDNA reflects contributions from both malignant and non-malignant tissues, and levels may be influenced by inflammation, infection, trauma, or treatment-induced cytotoxicity [23,25]. This biological heterogeneity may partially explain the variability observed between cfDNA dynamics and radiologic response in some patients. Future studies that integrate cfDNA with parallel ctDNA mutation tracking may help disentangle tumor-specific from host-derived signals and improve the biomarker's predictive accuracy.

Despite these limitations, our study contributes to the literature by demonstrating that even a simple, low-cost cfDNA measurement can provide clinically relevant information in the metastatic setting. Larger prospective studies with standardized sampling intervals and concurrent genomic profiling are needed to validate these findings and better define thresholds for clinical decision-making. If validated, cfDNA monitoring could become a useful adjunct to imaging, especially in settings where frequent radiologic assessment is impractical.

Conclusions

Early cfDNA dynamics were not predictive of PFS in this cohort but demonstrated strong diagnostic potential in distinguishing responders from non-responders. These findings support the utility of cfDNA change as an early biomarker of treatment response in HR+/HER2– MBC, warranting further validation in larger studies.

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