original reports

abstract

Circulating Tumor DNA After Neoadjuvant Chemotherapy in Breast Cancer Is Associated With Disease Relapse

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PURPOSE Detection of circulating tumor DNA (ctDNA) after neoadjuvant chemotherapy in patients with early-stage breast cancer may allow for early detection of relapse. In this study, we analyzed ctDNA using a personalized, tumor-informed multiplex polymerase chain reaction–based next-generation sequencing assay.

METHODS Plasma samples (n = 157) from 44 patients were collected before neoadjuvant therapy (baseline), after neoadjuvant therapy and before surgery (presurgery), and serially postsurgery including a last follow-up sample. The primary end point was event-free survival (EFS) analyzed using Cox regression models.

RESULTS Thirty-eight (86%), 41 (93%), and 38 (86%) patients had baseline, presurgical, and last follow-up samples, respectively. Twenty patients had hormone receptor–positive/human epidermal growth factor receptor 2–negative, 13 had triple-negative breast cancer, and 11 had human epidermal growth factor receptor 2–positive disease. Baseline ctDNA detection was observed in 22/38 (58%) patients and was significantly associated with Ki67 > 20% ($P = .036$) and MYC copy-number gain ($P = .0025$, false discovery rate = 0.036). ctDNA detection at presurgery and at last follow-up was observed in 2/41 (5%) and 2/38 (5%) patients, respectively. Eight relapses (seven distant and one local) were noted (median follow-up 3.03 years [range, 0.39-5.85 years]). After adjusting for pathologic complete response (pCR), ctDNA detection at presurgery and at last follow-up was associated with shorter EFS (hazard ratio [HR], 53; 95% CI, 4.5 to 624; $P < .01$, and HR, 31; 95% CI, 2.7 to 352; $P < .01$, respectively). Association between baseline detection and EFS was not observed (HR, 1.4; 95% CI, 0.3 to 5.9; $P = .67$).

CONCLUSION The presence of ctDNA after neoadjuvant chemotherapy is associated with relapse in early-stage breast cancer, supporting interventional trials for testing the clinical utility of ctDNA monitoring in this setting.

INTRODUCTION

Breast cancer is the most common malignancy among women worldwide, with > 275,000 and > 400,000 new cases diagnosed in the United States and Europe, respectively.[1,2]

Most patients are diagnosed with early-stage breast cancer (EBC), which is potentially curable with standard treatments.[3] Although (neo)adjuvant chemotherapies (NAC) reduce the risk of recurrence and death, approximately 30% of patients with EBC will experience recurrence in the first 10 years after surgery.[4] Recent studies have reported that circulating tumor DNA (ctDNA) detection in patients with EBC postdefinitive therapy can identify relapse with a median lead time of 11 months before imaging.[5,6] Treatment of patients at the time of ctDNA relapse may improve cure rates but this hypothesis requires prospective validation.[5,6]

In our study, we aimed to assess the value of ctDNA monitoring using Signatera, a personalized and tumor-informed multiplex-polymerase chain reaction-NGS assay, for the prediction of disease recurrence in patients with EBC, treated with standard NAC.

METHODS

All patients (N = 52) had a histologic diagnosis of EBC and were scheduled to receive NAC. Patients and treating physicians were blinded to ctDNA results, and no treatment decisions were made on the basis of ctDNA detection. This patient cohort is part of an ongoing translational, single-center study at Jules Bordet Institute, Brussels, Belgium (Circulating tumor DNA to monitor tumor evolution in Breast Cancer, Institut Bordet, Ethical Committee, reference CE2557). All patients provided informed consent for participating in this study.

Blood samples were collected before NAC (baseline), after NAC but before surgery (presurgery), and at

CONTEXT

Key Objective

Does circulating tumor DNA (ctDNA) detection after neoadjuvant therapy enable prediction of disease relapse in patients with early-stage breast cancer?

Knowledge Generated

Among 157 plasma samples collected from 44 patients, the presence of ctDNA after neoadjuvant therapy and before surgery was strongly associated with disease recurrence. After adjusting for pathologic complete response, ctDNA detection at presurgery and at the last follow-up was associated with shorter event-free survival.

Relevance

The detection and longitudinal analysis of ctDNA allows for post-therapy risk stratification and prediction of disease recurrence in patients with early-stage breast cancer.

various time points during follow-up. Blood samples collected after surgery, either before disease recurrence or at the last visit, were considered the last follow-up samples. ctDNA testing was performed using a personalized and tumor-informed assay (bespoke multiplex polymerase chain reaction NGS ctDNA assay, Signatera). Detailed methodology is presented in the Data Supplement.

RESULTS

Fifty-two patients were enrolled into this study. Of these, eight were excluded from analysis because of insufficient tumor DNA quantity (one patient) and quality (four patients), failed matched tumor and normal tissue concordance (two patients), and the substitution of an endocrine neoadjuvant regimen in place of NAC (one patient). Of the remaining 44 patients, 157 plasma samples were processed for ctDNA analysis (baseline: 38, pre-surgery: 41, and follow-up: 78; Data Supplement). A wide range of genomic aberrations (from 36 to 669) were observed in the whole-exome sequencing (WES) data of 44 evaluable patients (Data Supplement). Somatic variants with higher variant allele frequency (VAF) were prioritized when designing a patient-specific assay (Data Supplement).

Patient Characteristics and ctDNA Detection

Baseline clinicopathologic characteristics are reported in Data Supplement. Most patients were age ≤ 50 years (57%), and had a ductal histologic subtype (89%), clinical tumor size T1/2 (41%), and nodal stage N1 (73%). Proliferation index (Ki67) was > 20% in 67% of patients. Estrogen receptor–positive (ER+), human epidermal growth factor receptor 2 (HER2)–negative breast cancer was the most represented subtype (45%), followed by triple-negative breast cancer (TNBC; 30%) and the HER2-positive subtype (25%).

Detailed information about the personalized ctDNA assay results is presented in the Data Supplement. ctDNA detection at baseline (before NAC) was observed in 58% (22/38) of patients. High VAF was associated with highly proliferative tumors (P = .018) and TNBC subtype (P = .024; Fig 1).

ctDNA detection before surgery and during follow-up was observed in 5% (2/41) and 13% (5/38) of patients, respectively. For four patients, ctDNA was detected at the last follow-up time point before clinical recurrence (No. 8 and No. 19) or at the time of clinical recurrence (No. 13 and No. 43; Fig 2). For one patient (No. 11), ctDNA was detected at a time point taken 2 years before the last follow-up. This patient presented with hormone receptor–positive disease and was on endocrine therapy, which could have suppressed disease progression.
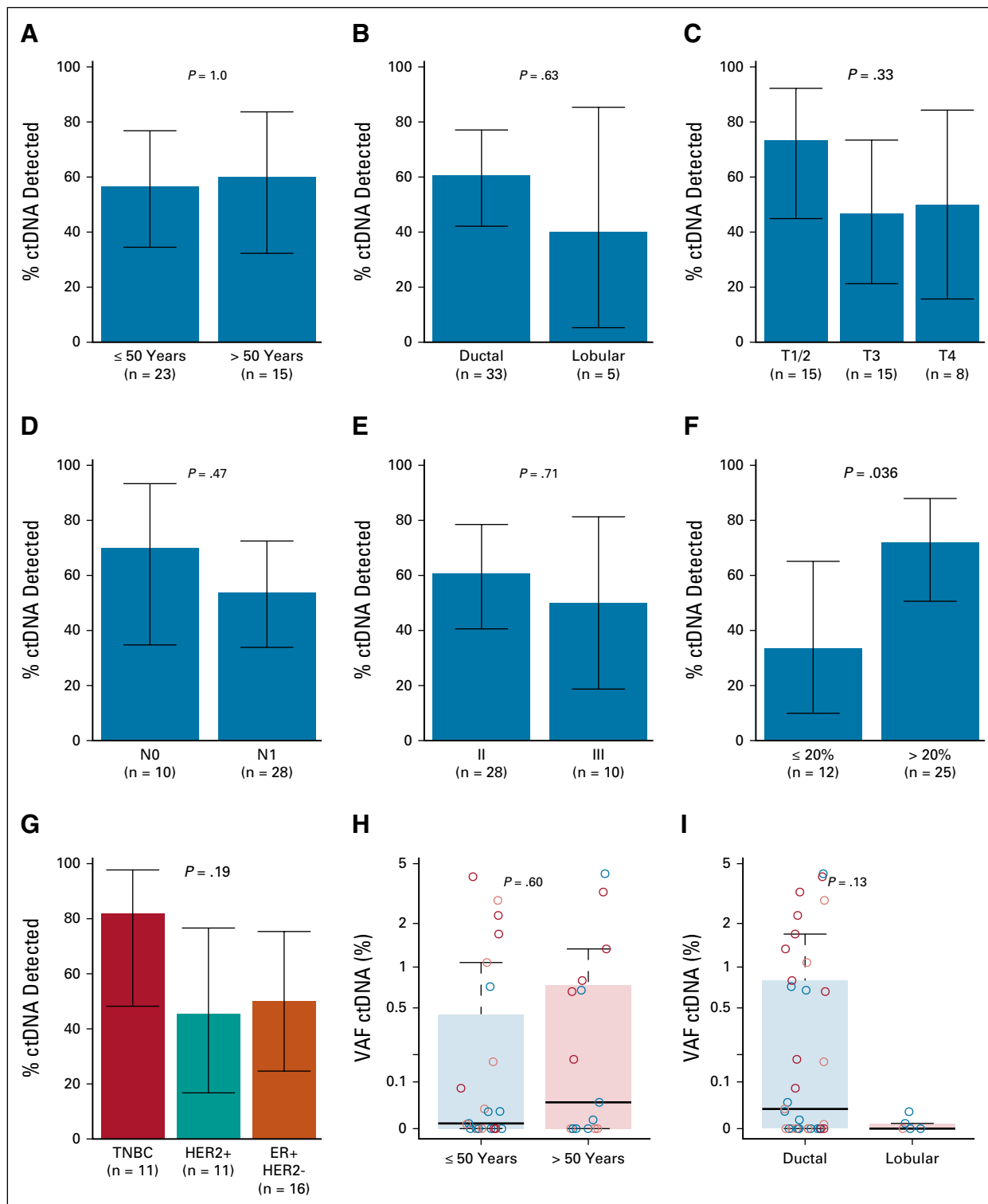
ctDNA Detection and Clinical Outcome

We evaluated associations between ctDNA detection at different time points with event-free survival (EFS). With a median follow-up of 3.03 (range: 0.39-5.85) years, we observed eight disease relapses (seven distant relapse and one local relapse). The presence of ctDNA before surgery and at last follow-up was associated with shorter EFS, after adjusting for pCR (hazard ratio [HR], 53; 95% CI, 4.5 to 624; P < .01, and HR, 31; 95% CI, 2.7 to 352; P < .01, respectively) and in univariate analysis (Figs 3, 4A, and 4B). Higher ctDNA levels expressed as VAF at the last follow-up were associated with shorter EFS in univariate analysis and after correcting for pCR (Figs 4A and 4B). Patient characteristics and pCR were not significantly associated with EFS in this cohort (Fig 4A).

We then evaluated the relationship between ctDNA detection and pCR (Fig 4C). No association was observed between ctDNA status, post-NAC, and pCR. However, none of the patients who achieved pCR had detectable ctDNA at the presurgical time point, and no patients who were ctDNA-positive at the presurgical time point achieved pCR.

Primary Tumor Genomic Aberrations and ctDNA Detection

We also aimed to explore the relationship between primary tumor genomic aberrations and baseline ctDNA detection. We used the primary tumor WES data to derive information for point mutations in the *PIK3CA* and *p53* genes (most frequently mutated genes in breast cancer)[7] as well as

FIG 1. ctDNA detection at baseline and its correlation with clinicopathologic characteristics. (A) age; (B) histological type; (C) clinical tumor size; (D) clinical nodal status; (E) histological grade; (F) Ki67; (G) subtype. The fraction of patients whose ctDNA was detected at baseline as well as the (H-N) VAF of baseline ctDNA (defined as the average across all 16 probes) was evaluated according to baseline clinicopathologic characteristics. Points are colored by immunohistochemistry subtypes (TNBC, hormone receptor–positive/HER2– and HER2+). ctDNA, circulating tumor DNA; ER+, estrogen receptor–positive; HER2–, human epidermal growth factor receptor 2–negative; HER2+, human epidermal growth factor receptor 2–positive; TNBC, triple-negative breast cancer; VAF, variant allele frequency.
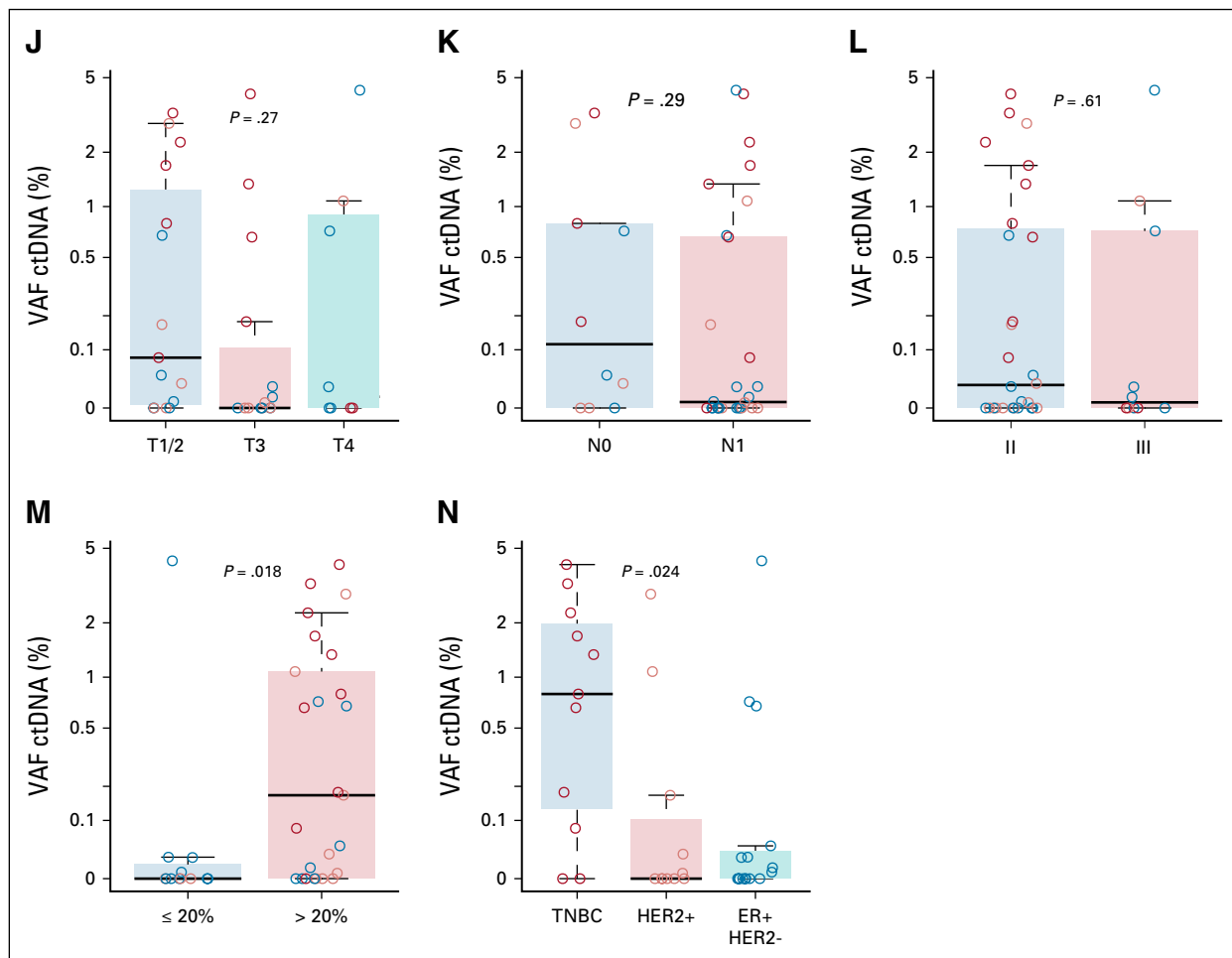
FIG 1. (Continued)

copy-number alterations in genes most frequently altered in breast cancer.[8] Genomic aberrations along with baseline clinicopathologic characteristics, pCR, and disease relapse for each patient are presented in Figure 5. On evaluating the associations between each primary tumor genomic aberration and baseline ctDNA detection levels, we observed that tumors having MYC copy number gains were strongly associated with ctDNA detection at baseline ($P =$ .0025, false discovery rate = 0.036; Data Supplement).

ctDNA Detection and Multifocal/Bilateral Tumors

Next, we asked whether baseline ctDNA detection rate was different in unifocal versus multifocal/bilateral tumors, with the ctDNA assay designed on the basis of a single focus. We observed a nonsignificant difference between baseline ctDNA detection levels in unifocal (60%; 15/25) versus multifocal (46%; 6/13) tumors ($P =$ .77).

Patient 13 presented with bilateral breast cancer: a cT3N1 ER+/HER2– tumor with Ki67 of 10% in the right breast and a cT1N0 ER+/HER2– tumor with Ki67 of 30% in the left breast. The pathology report, following NAC, showed a ypT2N2 residual tumor in the right breast and a ypT0N0 in the left breast. The patient was treated with adjuvant letrozole and presented with metastatic relapse in multiple organs (liver, bone, and lymph nodes). To further evaluate response to therapy, we performed WES on tissue obtained from both lesions (before NAC) and a surgical specimen from one of the tumors. When comparing the point mutations between the three samples, no common variants were found between the baseline samples of the left and right breast, suggesting two different primary breast cancers, whereas the baseline and surgical sample of the right breast tumor shared majority of the point mutations as expected. Personalized ctDNA assays were designed for each of the three tumor specimens separately (Data Supplement). An overlap of 14/16 somatic variants was observed between the ctDNA assays from baseline and the surgical sample of the right breast tumor only. Furthermore, analysis of plasma with corresponding assays revealed the presence of variants derived from the right breast tumor. This is consistent with pCR observed in the left breast and residual disease in the right at the time of surgery (Data Supplement).

FIG 2. Patients' therapy strategy, relapse status, and ctDNA detection in plasma. Personalized and tumor-informed assays were designed using WES data from 44 patients. A total of 157 plasma samples were available for ctDNA analysis. With a median follow-up of 3.03 years (range: 0.39-5.85 years), we observed eight relapses (seven distant and one local). Patients who achieved pathologic complete response at surgery are marked with asterisk (*), and cases with multifocal disease are highlighted in red. After surgery, patients received radiotherapy with/ without hormone or HER2+ therapy on the basis of the subtype. Patient 13 had bilateral tumors that were analyzed independently (13-1 and 13-2). For patient 20, pre-recurrence plasma was collected > 1 year before imaging. ctDNA, circulating tumor DNA; HR+, hormone receptor–positive; HER2–, human epidermal growth factor receptor 2–negative; HER2+, human epidermal growth factor receptor 2–positive; TNBC, triple-negative breast cancer; WES, whole-exome sequencing.
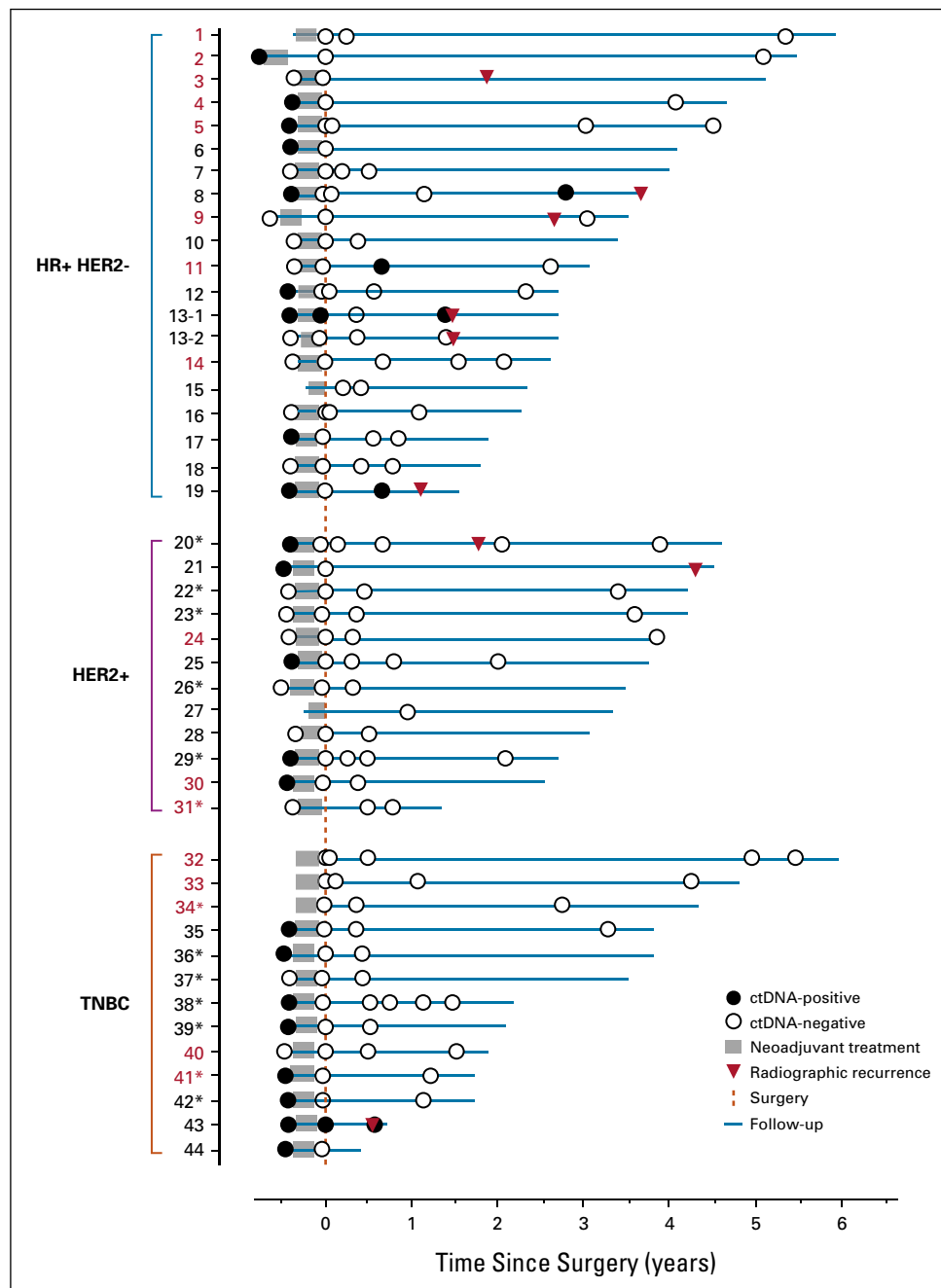
In patients 3 and 9, ctDNA was never detected in any plasma samples including at baseline. Both patients had ER+/HER2– multifocal disease (two lesions in the right breast in patient 3 and three lesions in the left breast in patient 9). Of note, for these two patients, only one focus underwent WES for personalized ctDNA assay design, because of the lack of available tumor tissue from the other focus. Neither patient had samples collected after surgery before recurrence and in the absence of active therapy. In patients 20 and 21 (both had HER2-positive unifocal breast cancer), ctDNA was detected in the baseline sample but not in the presurgery sample following NAC.

The postsurgical sample for patient 20 was collected 13.4 months before recurrence, while undergoing targeted therapy, whereas for patient 21, a follow-up sample after surgery was not available.

DISCUSSION

NAC represents a valuable treatment option for EBC, as it is associated with higher rates of breast conservation and the possibility of direct evaluation of treatment effect.[9] However, many patients develop recurrence after surgery, and biomarkers to predict early recurrence are needed. In this study, we focused on ctDNA monitoring in patients with
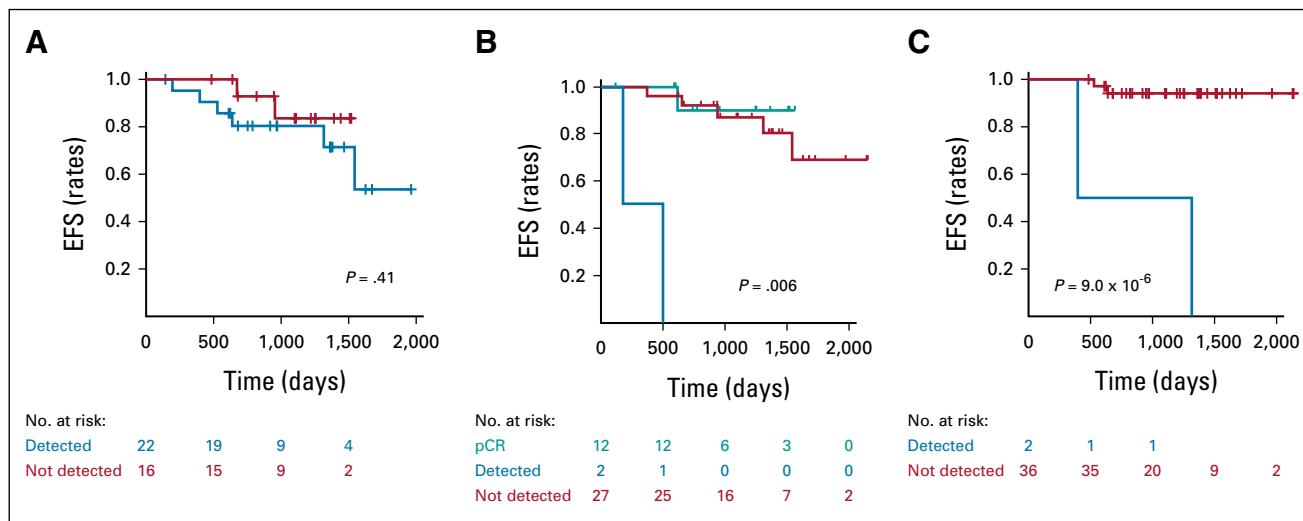
FIG 3. ctDNA detection at different time points and its association with event-free survival. Kaplan-Meier curves depicting the association between ctDNA detection and EFS at (A) baseline, (B) presurgery, and (C) at the last follow-up time point. ctDNA, circulating tumor DNA; EFS, event-free survival; pCR, pathologic complete response.

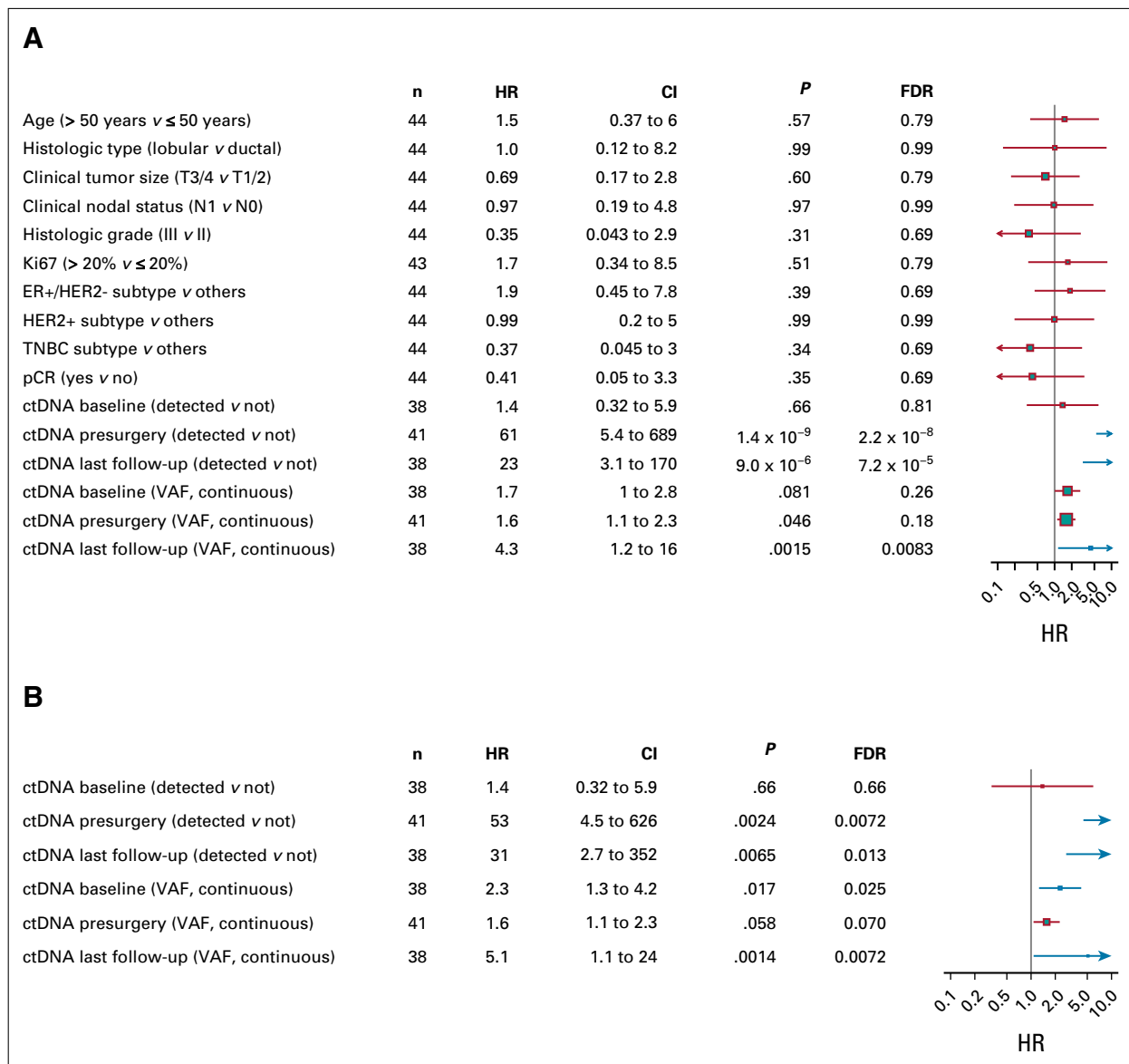EBC, before, during, and after NAC, to detect recurrence early.

We observed a significant association of ctDNA detection at baseline with higher tumor proliferation index and more aggressive subtype (TNBC). This is consistent with published data, showing that highly proliferative and more aggressive tumors have higher ctDNA detection rates.[10] Riva et al[11] observed that baseline ctDNA detection in 46 patients with early-stage TNBC was associated with higher grade (P = .005) and mitotic index (P = .03). Similarly, Zhang et al[12] noted that ctDNA detection rates were elevated in tumors with higher proliferation index (75% in Ki67 $\geq$ 30%, v 59.2% in Ki67 < 30%). Overall, this implies that tumors with high proliferation and increased cell turnover release more tumor DNA fragments in the blood.[11]

We then evaluated the association between ctDNA detection and EFS. The presence of ctDNA after NAC during follow-up was associated with shorter EFS with and without adjusting for pCR. Our results are consistent with those of Magbanua et al,[13] who showed that the lack of ctDNA clearance in patients receiving NAC was a significant predictor of metastatic relapse. Similarly, Garcia-Murillas et al, in a prospective cohort of 55 patients with EBC receiving NAC, demonstrated that ctDNA detection during follow-up was associated with relapse (HR, 25.2; 95% CI, 6.7 to 95.6; P < .001). Detection of ctDNA at baseline or before NAC was also associated with relapse-free survival, but with a lower HR (HR, 5.8; 95% CI, 1.2 to 27.1; P = .01). Interestingly, McDonald et al[14] reported that ctDNA concentrations after NAC were lower in patients who achieved pCR compared with those with residual disease, and patients who achieved pCR showed a greater decrease in ctDNA concentrations during NAC. Taken together, this

suggests that ctDNA detection at a presurgical time point or during follow-up can reliably predict relapse, while the correlation with baseline ctDNA is less clear.

We found that active systemic therapy including chemotherapy and endocrine therapy may influence ctDNA detection and should be considered when selecting time points for ctDNA evaluation. In this study, patients 20 and 9 received systemic therapy at the time of blood collection, which may have influenced the negative ctDNA result. Alternatively, endocrine therapy alone may be effective for a ctDNA-positive patient (No. 11) in suppressing disease progression. These observations deserve further evaluation.

Currently, no data exist on the sensitivity of personalized ctDNA assays in multifocal or bilateral tumors. Presence of genomic heterogeneity in some multifocal tumors suggests that analysis of several foci in bilateral tumors could be useful in designing more successful ctDNA assays. In our study, we used this approach in a patient with bilateral breast cancer, consisting of a node-positive luminal A tumor in the right breast, and a smaller, node-negative, luminal B tumor in the left breast. Both tumors were sequenced at diagnosis, and two different assays were used for ctDNA monitoring during follow-up. The patient underwent NAC and subsequent surgery, and ultimately, developed a distant relapse. ctDNA was detected using the assay created for luminal A-tumor, and not for luminal B-tumor. This helped us trace back the origin of the relapsed tumor to one of the two initially diagnosed breast tumors. Patients 3 and 9 developed recurrences 23 and 37 months after surgery, respectively. Neither had ctDNA detected at baseline or at pre-surgery. Interestingly, both patients had multifocal disease at diagnosis. For these

A

	n	HR	CI	P	FDR
Age (> 50 years v ≤ 50 years)	44	1.5	0.37 to 6	.57	0.79
Histologic type (lobular v ductal)	44	1.0	0.12 to 8.2	.99	0.99
Clinical tumor size (T3/4 v T1/2)	44	0.69	0.17 to 2.8	.60	0.79
Clinical nodal status (N1 v N0)	44	0.97	0.19 to 4.8	.97	0.99
Histologic grade (III v II)	44	0.35	0.043 to 2.9	.31	0.69
Ki67 (> 20% v ≤ 20%)	43	1.7	0.34 to 8.5	.51	0.79
ER+/HER2- subtype v others	44	1.9	0.45 to 7.8	.39	0.69
HER2+ subtype v others	44	0.99	0.2 to 5	.99	0.99
TNBC subtype v others	44	0.37	0.045 to 3	.34	0.69
pCR (yes v no)	44	0.41	0.05 to 3.3	.35	0.69
ctDNA baseline (detected v not)	38	1.4	0.32 to 5.9	.66	0.81
ctDNA presurgery (detected v not)	41	61	5.4 to 689	1.4×10^{-9}	2.2×10^{-8}
ctDNA last follow-up (detected v not)	38	23	3.1 to 170	9.0×10^{-6}	7.2×10^{-5}
ctDNA baseline (VAF, continuous)	38	1.7	1 to 2.8	.081	0.26
ctDNA presurgery (VAF, continuous)	41	1.6	1.1 to 2.3	.046	0.18
ctDNA last follow-up (VAF, continuous)	38	4.3	1.2 to 16	.0015	0.0083

B

	n	HR	CI	P	FDR
ctDNA baseline (detected v not)	38	1.4	0.32 to 5.9	.66	0.66
ctDNA presurgery (detected v not)	41	53	4.5 to 626	.0024	0.0072
ctDNA last follow-up (detected v not)	38	31	2.7 to 352	.0065	0.013
ctDNA baseline (VAF, continuous)	38	2.3	1.3 to 4.2	.017	0.025
ctDNA presurgery (VAF, continuous)	41	1.6	1.1 to 2.3	.058	0.070
ctDNA last follow-up (VAF, continuous)	38	5.1	1.1 to 24	.0014	0.0072

FIG 4. ctDNA detection, baseline clinicopathologic characteristics versus EFS, EFS corrected for pCR, and pCR. (A) Forest plot showing the correlations between EFS and ctDNA detection at baseline, presurgery, and last follow-up as categorical or continuous variables, as well as clinicopathologic characteristics, expressed as HRs with 95% CIs. Forest plot for (B) EFS corrected for pCR and (C) pCR expressed as ORs with 95% CIs. For the ctDNA VAF analysis, the HRs and ORs are on the variable scaled by its standard deviation. ctDNA, circulating tumor DNA; EFS, event-free survival; ER+, estrogen receptor–positive; HER2–, human epidermal growth factor receptor 2–negative; HER2+, human epidermal growth factor receptor 2–positive; HR, hazard ratio; FDR, false discovery rate; OR, odds ratio; pCR, pathologic complete response; TNBC, triple-negative breast cancer; VAF, variant allele frequency.

patients, only the larger tumor focus was sequenced for the ctDNA assay. We were not able to sequence the other focus because of lack of available tissue. We speculate that the lack of ctDNA detection at baseline in these two patients could be attributed to our inability to sequence all tumor foci including the focus with the clone responsible for relapse. These cases suggest that in patients with bilateral or multifocal tumors with different clonal origins, all tumors need to be analyzed for effective ctDNA monitoring.

Additionally, we explored whether ctDNA detection is associated with primary tumor genomic aberrations. We observed that MYC copy number variation gain was associated with ctDNA detection. MYC is a key regulator of cell growth, proliferation, and differentiation, and its deregulation contributes to breast cancer development and progression.[15] In a meta-analysis of 29 studies, MYC amplification in breast cancer was associated with tumor grade (relative risk [RR], 1.61), lymph node metastasis (RR, 1.24), negative
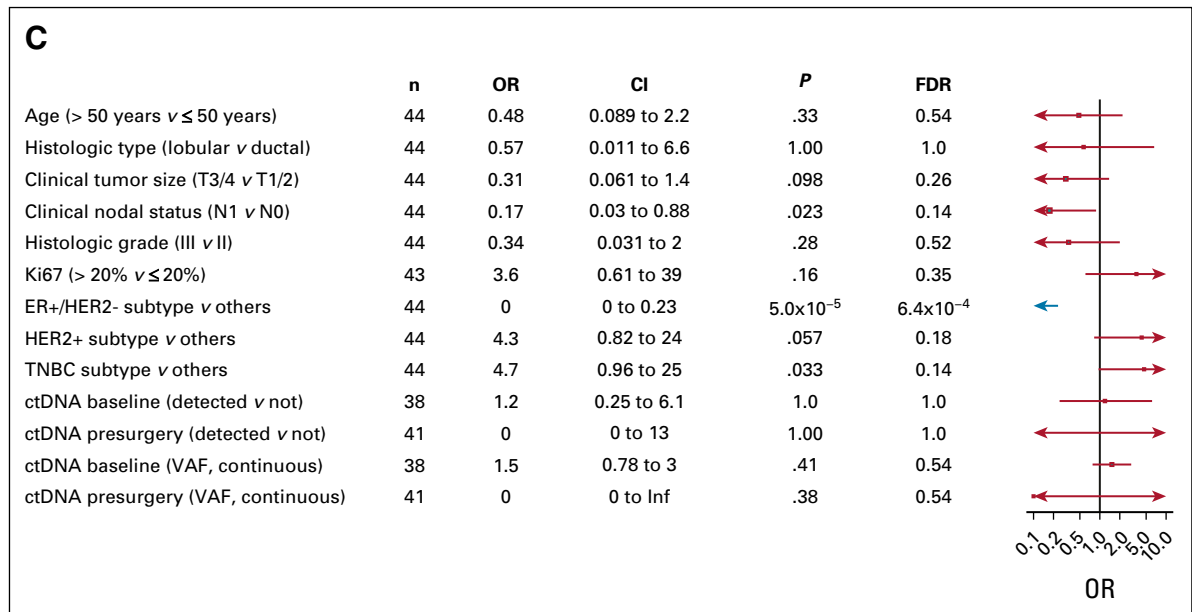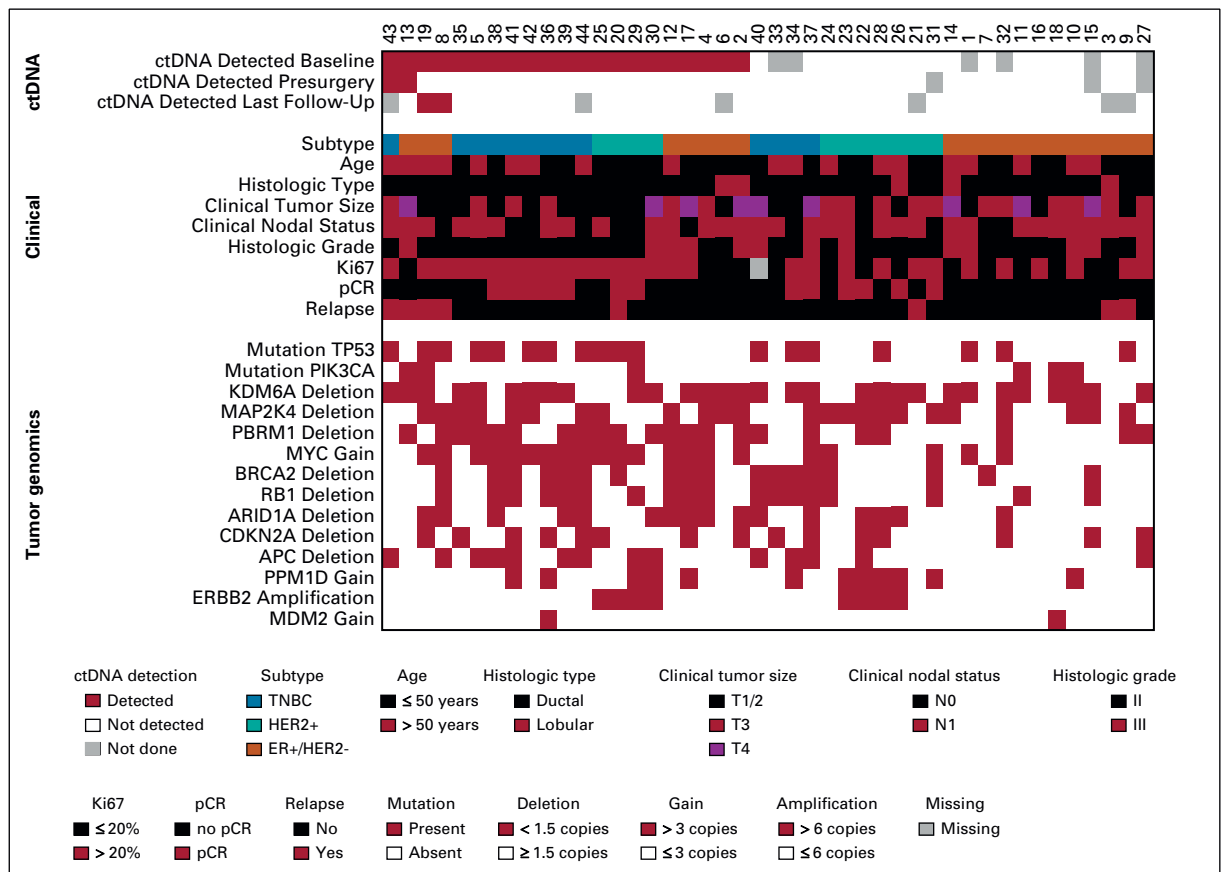
C

	n	OR	CI	P	FDR
Age (> 50 years v ≤ 50 years)	44	0.48	0.089 to 2.2	.33	0.54
Histologic type (lobular v ductal)	44	0.57	0.011 to 6.6	1.00	1.0
Clinical tumor size (T3/4 v T1/2)	44	0.31	0.061 to 1.4	.098	0.26
Clinical nodal status (N1 v N0)	44	0.17	0.03 to 0.88	.023	0.14
Histologic grade (III v II)	44	0.34	0.031 to 2	.28	0.52
Ki67 (> 20% v ≤ 20%)	43	3.6	0.61 to 39	.16	0.35
ER+/HER2- subtype v others	44	0	0 to 0.23	5.0×10^{-5}	6.4×10^{-4}
HER2+ subtype v others	44	4.3	0.82 to 24	.057	0.18
TNBC subtype v others	44	4.7	0.96 to 25	.033	0.14
ctDNA baseline (detected v not)	38	1.2	0.25 to 6.1	1.0	1.0
ctDNA presurgery (detected v not)	41	0	0 to 13	1.00	1.0
ctDNA baseline (VAF, continuous)	38	1.5	0.78 to 3	.41	0.54
ctDNA presurgery (VAF, continuous)	41	0	0 to Inf	.38	0.54

OR axis: 0.1 0.2 0.5 1.0 2.0 5.0 10.0

FIG 4. (Continued)

FIG 5. Oncoplot of patients included in the study. Visualization and summary of common breast cancer–associated genomic aberrations with clinicopathologic characteristics, relapse status, and ctDNA detection at all three time points. All patients with ctDNA detected at baseline are gathered to the left part of the plot (red), while those for whom it was not detected, or data were not available are pulled to the right part of the plot (white and gray, respectively). ctDNA, circulating tumor DNA; ER+, estrogen receptor–positive; HER2–, human epidermal growth factor receptor 2–negative; HER2+, human epidermal growth factor receptor 2–positive; pCR, pathologic complete response; TNBC, triple-negative breast cancer.

progesterone receptor status (RR, 1.27), and poor prognosis.[16] Our findings are therefore consistent with observations that ctDNA is more often detected in aggressive tumors with higher tumor proliferation.

This study has some limitations. We investigated a small cohort (n = 44), with a limited number of recurrences (n = 8). However, our cohort consisted of different breast cancer subtypes homogeneously treated with standard NAC. Interestingly, we noted that primary tumor heterogeneity especially in the case of multifocal or bilateral tumors needs to be considered when designing personalized ctDNA assays.

In summary, the detection of ctDNA post-NAC was associated with disease recurrence in patients with EBC. Interventional trials to evaluate the clinical utility of ctDNA monitoring in this setting are either ongoing or planned.

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DISCLAIMER
The funders had no role in study design, data collection, data analysis, data interpretation, or writing of the manuscript. All authors shared the final responsibility for the interpretation of the study results and for the decision to submit for publication.

EQUAL CONTRIBUTION
F.C. and E.A. contributed equally to this work and share co-first authorship. D.V. and M.I. contributed equally to this work.

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DATA SHARING STATEMENT
Data and results are available at the Institut Jules Bordet in Brussels (Belgium) and can be made available upon reasonable request.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST
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