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ORIGINAL RESEARCH

Blood-based genomics of triple-negative breast cancer progression in patients treated with neoadjuvant chemotherapy☆

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Background: As neoadjuvant chemotherapy (NAC) is increasingly used in triple-negative breast cancer (TNBC), we investigated the value of circulating tumor DNA (ctDNA) for patient monitoring prior, during, and after NAC, and circulating tumor cells (CTCs) for disease characterization at clinical progression.

Materials and methods: Forty-two TNBC patients undergoing NAC were prospectively enrolled. Primary tumor mutations identified by targeted-gene sequencing were validated and tracked in 168 plasma samples longitudinally collected at multiple time-points by droplet digital polymerase chain reaction. At progression, plasma DNA underwent direct targeted-gene assay, and CTCs were collected and analyzed for copy number alterations (CNAs) by low-pass whole genome sequencing.

Results: ctDNA detection after NAC was associated with increased risk of relapse, with 2-year event-free survival estimates being 44.4% [95% confidence interval (CI) 21.4%-92.3%] versus 77.4% (95% CI 57.8%-100%). ctDNA prognostic value remained worthy even after adjusting for age, residual disease, systemic inflammatory indices, and Ki-67 [hazard ratio (HR) 1.91; 95% CI 0.51-7.08]. During follow-up, ctDNA was undetectable in non-recurrent cases with the unique exception of one showing a temporary peak over eight samples. Conversely, ctDNA was detected in 8/11 recurrent cases, and predated the clinical diagnosis up to 13 months. Notably, recurrent cases without ctDNA developed locoregional, contralateral, and bone-only disease. At clinical progression, CTCs presented chromosome 10 and 21q CNAs whose network analysis showed connected modules including HER/PI3K/Ras/JAK signaling and immune response.

Conclusion: ctDNA is not only associated with but is also predictive of prognosis in TNBC patients receiving NAC, and represents an exploitable tool, either alone or with CTCs, for personalized TNBC management.

Key words: circulating tumor DNA, circulating tumor cells, neoadjuvant chemotherapy, triple-negative breast cancer, prognosis

INTRODUCTION

Neoadjuvant chemotherapy (NAC) is a standard part of the multidisciplinary treatment of breast cancer (BC).[1] Initially thought as an approach to allow surgery in locally advanced disease,[2] NAC is increasingly used even when upfront breast conservation is feasible if chemotherapy is inevitable, such as in triple-negative BC (TNBC) lacking both hormone receptor and HER2 expression. In fact, NAC provides prognostic information obtained from tumor response assessment on surgical specimens.[3] Patients with no evidence of invasive cancer in the breast and axillary nodes [pathological complete response (pCR)] have in fact significantly improved disease-free and overall survival. While there is a clear correlation between pCR and prognosis, only poor diagnostic accuracy can be reached when predicting a pCR before surgery by a combination of multiple aspects such as tumor biology, the applied NAC regimen, and breast

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imaging results.[4-6] Thus, a valuable predictive biomarker of NAC response is of paramount importance to assist the clinical decision to continue, change, or stop NAC, and to finally increase the likelihood of achieving pCR. In addition, although the effect of adding further post-surgical treatments in the absence of pCR after NAC is beneficial,[7] not all patients without pCR after NAC experience a BC recurrence, while a portion of patients receiving 'salvage' post-NAC therapies still relapse.[8] The possibility of assessing the molecular residual disease after NAC would allow the prescription of salvage therapies for patients whose tumor has not been eradicated and to de-escalate treatment in those achieving a molecular CR.

Circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) have recently become the focus of research on precision diagnosis and treatment of different tumors, including BC.[9] In addition, thanks to the multiplicity of analyses that can be carried out and the repeatability of the tests, ctDNA and CTCs represent accessible tools to decode both spatial and temporal heterogeneity, and to dynamically monitor cancer progression.[10] Few studies have analyzed patients with non-metastatic BC revealing that almost a quarter of cases have detectable CTCs in the peripheral blood at diagnosis.[11] By comparison, ctDNA is detected in 57%-100% of patients with non-metastatic BC, suggesting it may represent a more broadly applicable biomarker in this setting.[12-15] Intriguingly, studies suggest that residual ctDNA after curative-intent surgery of localized BC is a valuable marker in patients at the highest risk of recurrence. Minimal residual disease can be detected shortly after treatment, and ctDNA changes after treatment may be predictive of long-term outcome.[16,17]

Although these studies have shown the potential of ctDNA and CTCs in early BC management, their systematic application in practice is still hindered by many problems, such as lack of standardization of pre-analytical/analytical procedures, unsatisfactory specificity and sensitivity, confounding with clonal hematopoiesis, requirement of highly specialized and dedicated staff, and elevated economic cost.

Based on this premise and on our previous experience,[18,19] we designed an observational prospective study to explore, in the context of clinical practice, ctDNA in TNBC patients treated with NAC with the aims (i) to explore ctDNA as a longitudinal test for earlier detection of relapse in TNBC patients, and (ii) to challenge high-depth plasma sequencing and single-cell CTC analysis from blood drawn as means to identify druggable molecular features in relapsed cases.

MATERIALS AND METHODS

Patients and samples

Forty-two patients were prospectively recruited from Fondazione IRCCS Istituto Nazionale dei Tumori (INT), Milan, between April 2013 and December 2017. The study was approved by the Institutional Review Board and the Ethics Committee of INT. Eligible cases were 18 years or older, had primary TNBC, i.e. estrogen receptor (ER) and progesterone receptor (PR) immunohistochemical (IHC) staining of fewer than 1% of tumor cell nuclei, and HER2 IHC score of 0-1 or 2+ with a negative chromogenic *in situ* hybridization result, displayed no clinical evidence of metastatic disease, were willing to receive or had already received NAC, and were willing to undergo serial blood drawing for biomarker analyses. Surgery was planned within 6 weeks from completion of NAC. Written informed consent was obtained from all participants.

Sample collection

Formalin-fixed paraffin-embedded (FFPE) tumor samples obtained during routine diagnostic biopsy and/or resection of primary tumor were used to identify the prevalent mutation(s) to be traced in the blood. Hematoxylin and eosin slides were carefully reviewed by two pathologists (GP and AV), and tumor areas were marked for subsequent macrodissection. The corresponding areas were scraped from 10 serial FFPE sections of 8-μm thickness, thus guaranteeing a minimum tumor cellularity of 40%. For patients attaining pCR ($n = 4$), only pre-NAC tumor samples were analyzed, while for those with residual disease at surgery, pre-NAC and post-NAC tumor samples were sequenced in 19 and 26 cases, respectively. Blood sampling for cell-free DNA analysis was obtained prior, during, and after NAC and during follow-up, along with semiannual clinical examination and biochemical measurements. Laboratory tests results included levels of neutrophil and lymphocyte and platelet counts.

Sample analysis

Detailed description of the collection of bio-specimens, plasma cell-free DNA isolation and quantification, CTC recovery, enrichment by the marker-independent Parsortix approach and selection through the DEPArray system, and next-generation sequencing (NGS) is provided in the Supplementary Material, available at https://doi.org/10.1016/j.esmoop.2021.100086. Briefly, primary tumor DNA was extracted from the diagnostic biopsy and/or surgical samples and analyzed using the cancer gene panel Ion AmpliSeq Cancer Hotspot Panel v2 CHPv2, (Thermo Fisher Scientific, Waltham, MA), consisting of 207 amplicons covering hotspot mutations of 50 genes (50-gene HS). Cases with no somatic variants detected by the 50-gene HS were further analyzed by the Ion AmpliSeq Comprehensive Cancer Panel (CCP) (Thermo Fisher Scientific), which covers all exons of 409 cancer-related genes. Personalized droplet digital polymerase chain reaction (ddPCR) assays were designed to validate primary tumor tissue somatic variants and to track individual somatic variants in plasma samples. Cases with undetectable ctDNA were further analyzed by introducing a pre-amplification step. At disease progression, sequencing of cell-free DNA and CTCs were carried out by the 52-gene panel Oncomine pan-cancer cell-free assay (Thermo Fisher Scientific) and low-pass whole genome sequencing (lpWGS) (Menarini Silicon Biosystem, Bologna, Italy), respectively. Detailed methods are provided in the

Supplementary Materials and Methods, available at https://doi.org/10.1016/j.esmoop.2021.100086.

Statistical analysis

Descriptive analyses of Ki-67 and the distribution of systemic inflammatory indices [neutrophil to lymphocyte ratio (NLR), lymphocyte-to-monocyte ratio (LMR), and platelets] according to post-NAC ctDNA status was carried out using box plots. The association between post-NAC ctDNA and age, Ki-67, and systemic inflammatory indices was represented using heatmap plots. Analysis of the association between event-free survival (EFS) and ctDNA was carried out by estimating the Kaplan—Meier curves and fitting Cox models. EFS events were defined as post-surgery BC relapse, second primary malignancy, or death for any cause. EFS time was measured from the date of NAC starting to the date of event, whichever occurred first; time was censored at the date of last follow-up for patients alive and without events. Due to the low number of cases and events preventing the acquiring of reliable estimates, the Cox analyses of ctDNA were carried out by applying Firth's penalized likelihood[20]; moreover, the adjustment for age, post-NAC Ki-67, residual disease at surgery, and post-NAC systemic inflammatory indices was operated by means of a score beforehand estimated as the linear predictor from a (non-penalized) Cox model. The analyses were carried out using R software (http://www.r-project.org/).

RESULTS

Patient cohort

A total of 42 patients with TNBC who received NAC were prospectively enrolled from 2013. Seven and four patients were excluded due to the lack of serial blood sampling and primary tumor tissue, respectively (Figure 1). The clinicopathological features of the 31 patients undergoing primary tumor NGS and with at least two serial blood samples representing the study cohort are listed in Table 1, and detailed in Supplementary Tables S1a and S1b, available at https://doi.org/10.1016/j.esmoop.2021.100086. All except two patients received anthracycline/taxane-based chemotherapy before surgery. All patients presented with primary tumors $\geq$2 cm, and 20/31 (64.5%) with initial nodal involvement. The majority of patients were responsive to NAC except four that anticipated surgery due to progressive disease. Four patients attained pCR. At the reporting census date (12 February 2020), 12 of the 31 patients experienced

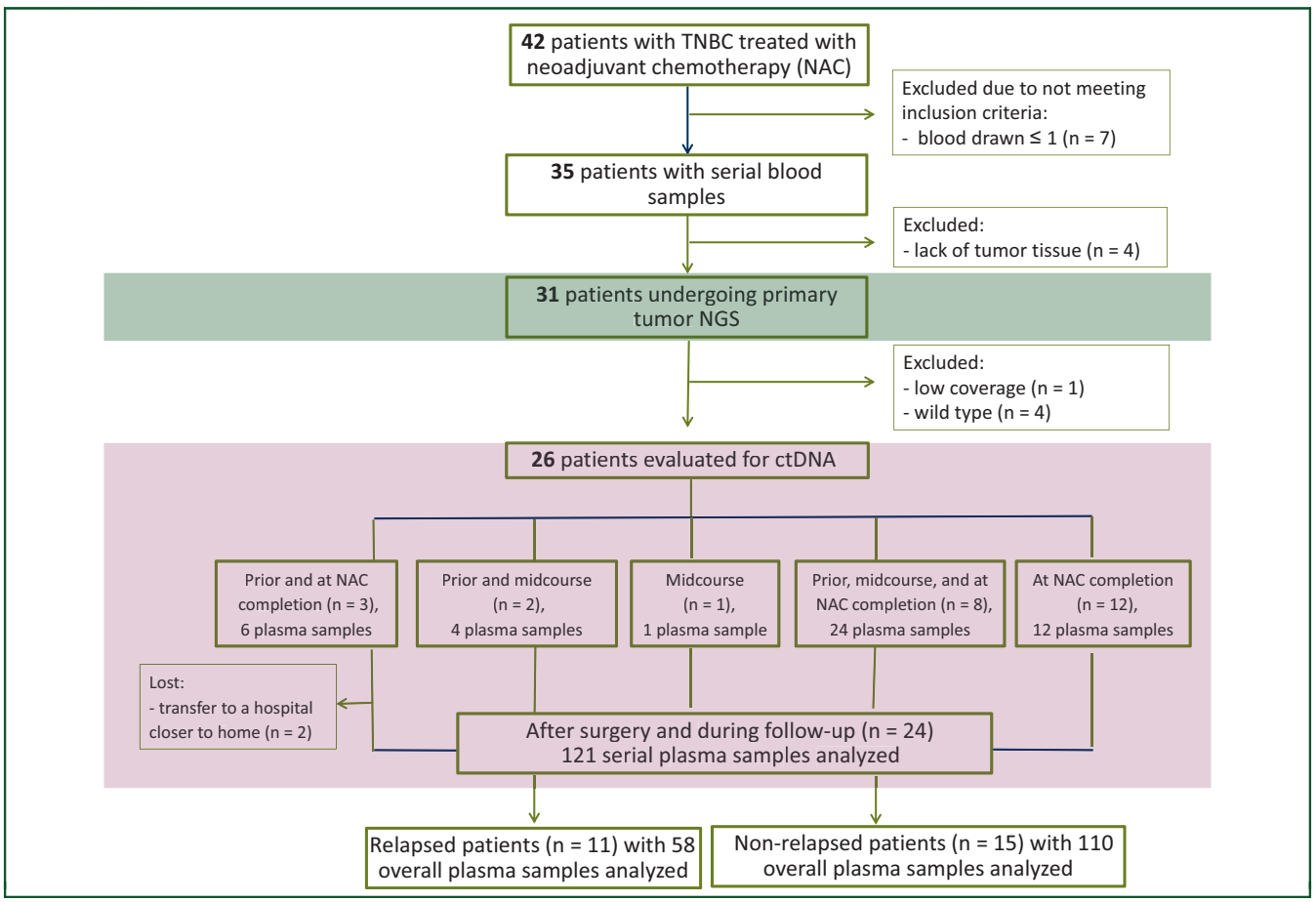

Figure 1. CONSORT diagram showing patients included in each analysis and reasons for their exclusion.
Green shading: patients with primary tumor bearing at least one mutation. Burgundy shading: patients with overall ctDNA assessment: and according to different treatment time points and follow-up.
ctDNA, circulating tumor DNA; NGS, next-generation sequencing; TNBC, triple-negative breast cancer.

Table 1. Clinicopathological characteristics of study patient population ($N = 31$)

	n (%)
Age	
<50 years	19 (61.3)
≥50 years	12 (38.7)
Clinical tumor size	
2-5 cm	22 (71.0)
>5 cm	9 (29.0)
Clinical nodal status	
N0	11 (35.5)
N ≥1	20 (64.5)
Clinical stage	
II	24 (77.4)
III	7 (22.6)
Tumor grade[a]	
G2	1 (3.2)
G3	26 (83.9)
Missing	4 (12.9)
Ki67	
<50%	6 (19.3)
≥50%	22 (71.0)
Missing	3 (9.7)
Type of NAC	
Anthracycline/taxane	25 (80.6)
Anthracycline/taxane plus platins	4 (12.9)
Other	2 (6.5)
Path findings	
ypT0N0 (pCR)	4 (12.9)
ypT1N0	13 (41.9)
ypT1Nx	2 (6.5)
ypT2-3N0	5 (16.1)
ypT1-3N1-3	7 (22.6)
Breast cancer events ($n = 12$)	
Distant metastases	9 (75.0)
Second primary	1 (8.3)
Locoregional relapse	1 (8.3)
Death from any cause	1 (8.3)

NAC, neoadjuvant chemotherapy; pCR, pathological complete response.
[a] Nottingham grading system; American Joint Committee on Cancer, *AJCC Cancer Staging Manual*. 8th ed. New York, NY: Springer; 2018.

an unfavorable event, including one contralateral BC, one locoregional relapse, nine metastatic dissemination, and one death for causes not related to BC and/or treatment (Supplementary Table S1b, available at https://doi.org/10.1016/j.esmoop.2021.100086).

Mutation analysis in tissue

Overall a total of 45 specimens were processed, i.e. diagnostic biopsy ($n = 5$), surgical samples ($n = 12$), and paired diagnostic biopsy/surgical samples ($n = 14$). At least one somatic mutation was found in 22/33 (66.6%) and in 15/21 (71.4%) samples by the 50-gene HS and CCP, respectively. Of note, CCP identified at least one mutation in 3/9 samples missed by the 50-gene HS, and an additional 12 samples were processed upfront. No results were available in six samples with either panel (Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2021.100086). In total, 37 out of 45 (82.2%) cases were found with at least one mutation (range 1-9), and in particular, in 19/37 (51%), multiple mutations were detected. The most frequently mutated gene was *TP53* (20/26, 76.9%), followed by *PI3KCA* (4/26, 15.3%), and *FGFR3* (2/26, 7.7%). A list of all the mutations in the genes of interest and the corresponding variant allele frequency (VAF) is shown in Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2021.100086. Eleven patients had paired pre-NAC, and post-NAC samples analyzed using the same approach. Among the five HS pairs, treatment with NAC tended to decrease (#14, #19, #23, #25) rather than increase (#21) the VAF of predominant baseline mutations, represented by *TP53* ($n = 4$) and *EGFR* ($n = 1$). A similar figure was observed in CCP pairs for *TP53* and *PI3KCA* (#1, #4, #12). Notably, four cases acquired (HS #19 and #23; CCP #12 and #20) and four cases lost (HS #19 and CCP #1, #4, #7) mutations following NAC (Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2021.100086).

Personalized ddPCR assays were developed for 28/58 mutations (48.2%), representing those with the highest values of VAF (median, 27.5%; range 3%-72%), and used to track primary tumor mutations in the plasma of each patient (Figure 2).

Mutation analysis in plasma during NAC

Among the 26 patients evaluated for ctDNA, blood drawings were available prior, during, and after completion of NAC, and after surgery in 13, 11, 23, and 24 patients, respectively, (Supplementary Table S1b, available at https://doi.org/10.1016/j.esmoop.2021.100086, patients 1-26) with a median of 0 days before commencing NAC [interquartile range (IQR) 0-5], 88.5 days from the start of NAC (IQR 44.5-101.25), and 1 day before the scheduled surgery. The first post-operative blood sample was collected a median of 81.5 days from surgery (IQR 35.25-187). Plasma DNA was extracted from 168 samples (median per patient, 6 samples; range 2-13 samples).

The detection rate of ctDNA before commencing NAC was 10 out of 13 evaluable cases (77%) with a median VAF value of 1.36% and 17.55 copies/ml. At mid-course evaluation, half of these cases turned negative, two presented persistent though reduced levels of ctDNA (patients #17 and #4, with corresponding VAF values of 0.3% and 0.24%) and three were missed (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2021.100086). Following NAC, ctDNA was still detectable in 10 out of 23 (11 with initial assessment before commencing NAC, and 12 with initial assessment at the end of NAC) evaluable cases (43%) with a median VAF value of 0.3% and 28.29 copies/ml. Overall, these findings suggest that NAC reduced the levels of ctDNA soon after treatment start and up to undetectable levels in most of the cases at the time of surgery.

ctDNA status, clinicopathological features, and prognosis

No clinicopathological characteristics, including systemic inflammatory indices, were different between patients with detectable or undetectable ctDNA either at baseline or at the end of NAC, though ctDNA detection after NAC appeared more likely in cases with high LMR and low NLR ratios, and high Ki-67 (Supplementary Figure S2, panel A, available at https://doi.org/10.1016/j.esmoop.2021.100086).

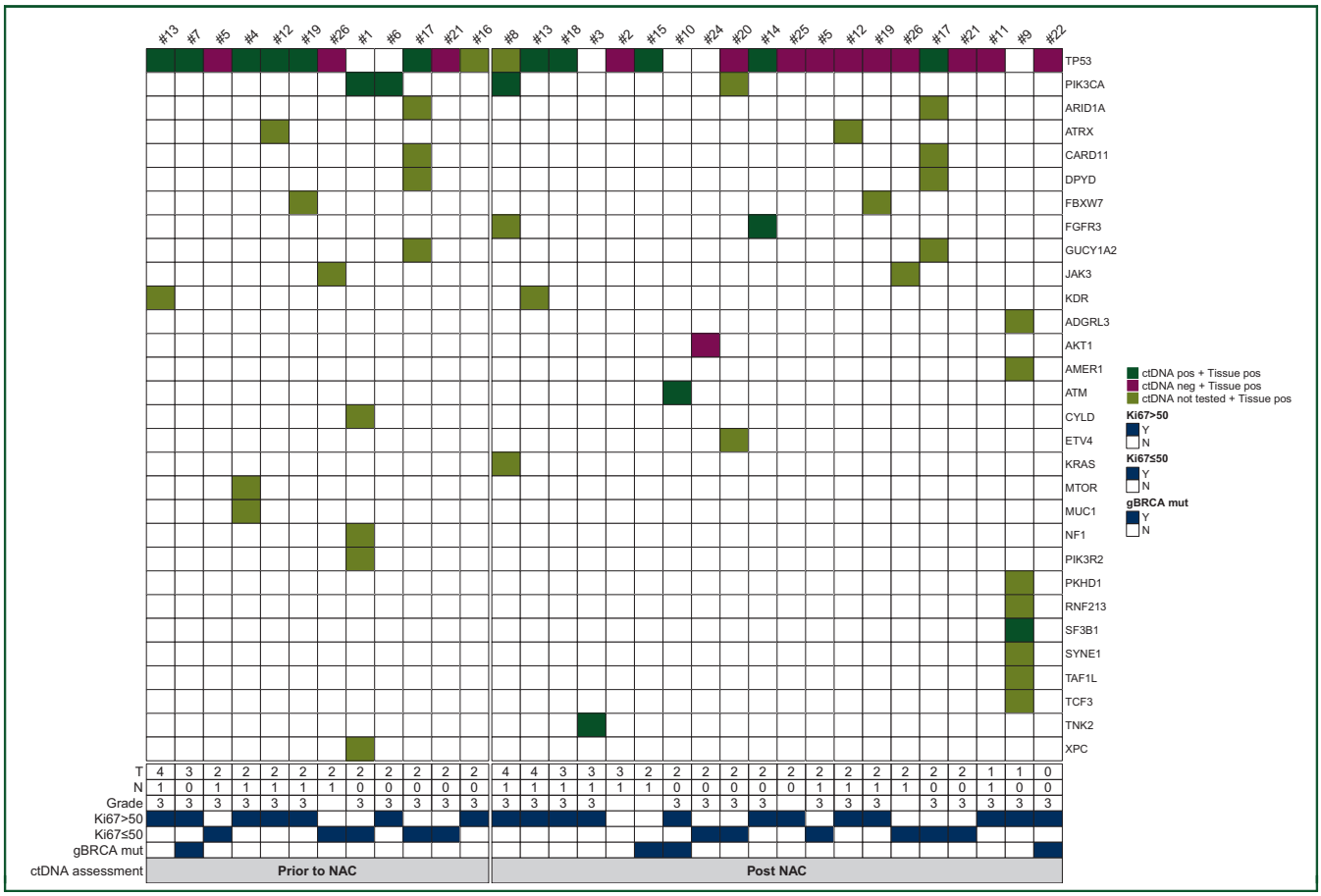

Figure 2. Matched primary tumor and plasma mutations.
The heatmap shows the mutations detected in both primary tumor tissue and plasma samples (green), in just primary tumor tissue (burgundy) or not tested in plasma (olive) for the limited amount of plasma samples. Information on clinical stage, i.e. tumor size (T), and nodal status (N), grade, Ki-67, and germinal BRCA status is also reported. ctDNA, circulating tumor DNA; NAC, neoadjuvant chemotherapy.

The summary of results obtained by the simultaneous consideration of all the variables (ctDNA, systemic inflammatory indices, Ki-67, and patient age) and presence/absence of unfavorable events during follow-up is detailed in the heatmap of Supplementary Figure S2, panel B, available at https://doi.org/10.1016/j.esmoop.2021.100086 .

The conversion of ctDNA status from positive at baseline to negative during and after completing NAC was not associated with primary tumor response, as all 13 evaluable patients with post-NAC undetectable ctDNA levels had residual disease in surgical specimen. Nonetheless, all three evaluable patients (#3, #8, #10) who progressed during NAC still had detectable levels of ctDNA before surgery.

A total of 11 BC events occurred among the 26 patients with detectable ctDNA either pre-NAC or post-NAC. ctDNA detection partially overlapped with higher Ki-67 and LMR values and younger age (Supplementary Figure S2, panel B, available at https://doi.org/10.1016/j.esmoop.2021.100086), even though ctDNA seems to better explain the distribution of BC events in the study population. Specifically, negative ctDNA occurred in five out of six patients without recurrence despite high values of Ki-67, whereas positive ctDNA occurred in one out of three relapsed patients despite low levels of Ki-67.

Survival curves showed no difference in clinical outcome between patients with detectable ctDNA at baseline (pre-NAC, $n = 10$) and those with no detectable ctDNA ($n = 3$), with 2-year EFS estimates of 57.1% [95% Confidence Interval (CI): 32.6%-100%] versus 66.7% (95% CI: 30.0%-100%), respectively. More strikingly, patients with post-NAC ctDNA-positive status ($n = 10$) compared with those with undetectable ctDNA ($n = 13$) had an increased risk of recurrence after surgery, with 2-year EFS estimates of 40.0% (95% CI: 18.7%-85.5%) versus 83.9% (95% CI: 65.7%-100%) [Figure 3, univariable Cox model hazard ratio (HR) 2.65; 95% CI: 0.74-9.44]. Notably, the prognostic value of post-NAC ctDNA remained worthy of consideration even after adjustment for age, Ki-67, residual disease at surgery, and systemic inflammatory indices (HR 1.91; 95% CI: 0.51-7.08).

Serial ctDNA and patient outcome

All the patients with detectable levels of ctDNA at either pre-NAC or post-NAC sampling were longitudinally monitored after surgery with the exception of two (#13 and #16) which had detectable ctDNA before surgery, recurring 7.2 and 9.3 months after surgery, and were not tested during follow-up. The dynamics of ctDNA during follow-up and the lead time of molecular compared with clinical progression is

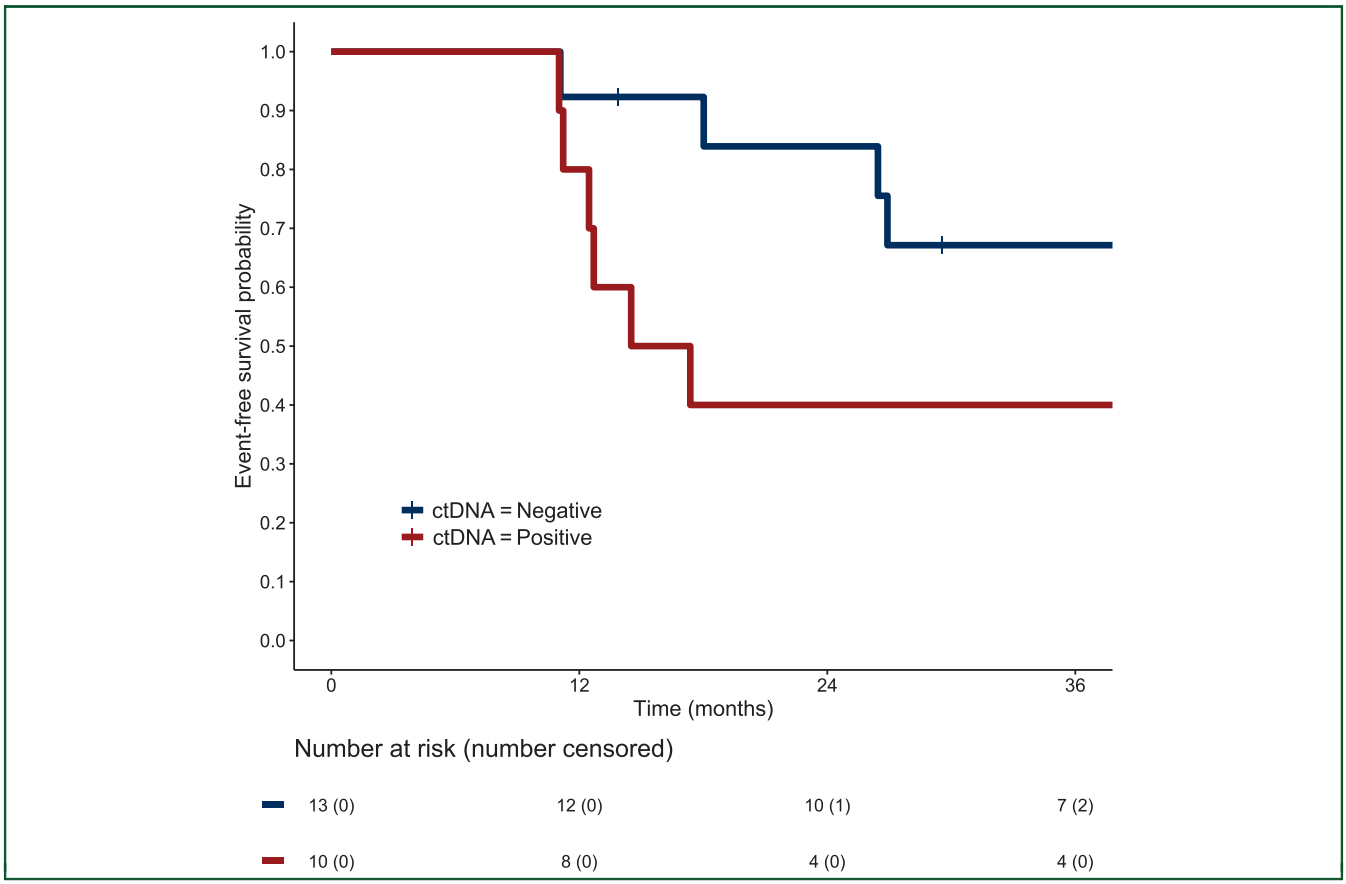

Figure 3. Kaplan–Meier event-free survival.
The curves represent event-free survival according to post-NAC ctDNA status. Number of patients at risk and censored are shown at the bottom of the figure. ctDNA, circulating tumor DNA; NAC, neoadjuvant chemotherapy.

summarized in Figure 4 and detailed for individual patients in Supplementary Figure S3, panels A and B, available at https://doi.org/10.1016/j.esmoop.2021.100086. The first post-operative sample showed undetectable levels of ctDNA in 20 of 24 (83.3%) evaluable cases. Among the patients with persistent levels of ctDNA, three (#14, #17, and #18) experienced a BC event after an average of 6.5 months from surgery (Figure 4 and Supplementary Figure S3, panel A, available at https://doi.org/10.1016/j.esmoop.2021.100086) while patient #5, who showed a transient peak of ctDNA followed by consistently negative levels, is still disease-free up to 4 years from surgery (Supplementary Figure S3, panel B, available at https://doi.org/10.1016/j.esmoop.2021.100086). Three patients (#1, #11, and #12) turned ctDNA-positive during follow-up after an average of 12.7 months after surgery (Figure 4 and Supplementary Figure S3, panel A, available at https://doi.org/10.1016/j.esmoop.2021.100086) and in these patients ctDNA anticipated overt metastases by a mean of 8.9 months (range 6.5-13.1). Thus, the positive predictive value of detectable levels of ctDNA after surgery (either for persistence or reappearance) was evident in 6/7 cases (85.7%).

Among the 17 patients with persistently undetectable ctDNA after NAC, 14 remained disease-free at a median follow-up of 3 years (range, 0.5-6.5; Supplementary Figure S3, panel B, available at https://doi.org/10.1016/j.esmoop.2021.100086). Two patients (#24 and #26) tested negative before and after NAC, and one additional patient (#10) with detectable post-NAC ctDNA was diagnosed with bone metastases, locoregional relapse, and contralateral BC in the absence of detectable ctDNA (Supplementary Figure S3, panel A, available at https://doi.org/10.1016/j.esmoop.2021.100086). Thus, the negative predictive value of undetectable levels of ctDNA after surgery was demonstrated in 14/17 cases (82.3%).

Genomic profiling of ctDNA from recurrent patients

Eight recurrent patients had plasma samples collected following NAC (#13), after surgery (#17), or at the time of clinical relapse (#1, #11, #12, #14, #18, and #24), allowing a further analysis using the NGS panel of 52 cancer-related genes. No mutations were found in three cases. Notably, two of these cases (#13 and #17) had instead detectable ctDNA by ddPCR, though with low VAF values of 0.04% and 0.14%, respectively. In the remaining five cases, a median of two mutations per sample was found, with median higher VAF values of 10.35% (0.7%-80%) (Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2021.100086). All patients retained at least one of the primary tumor

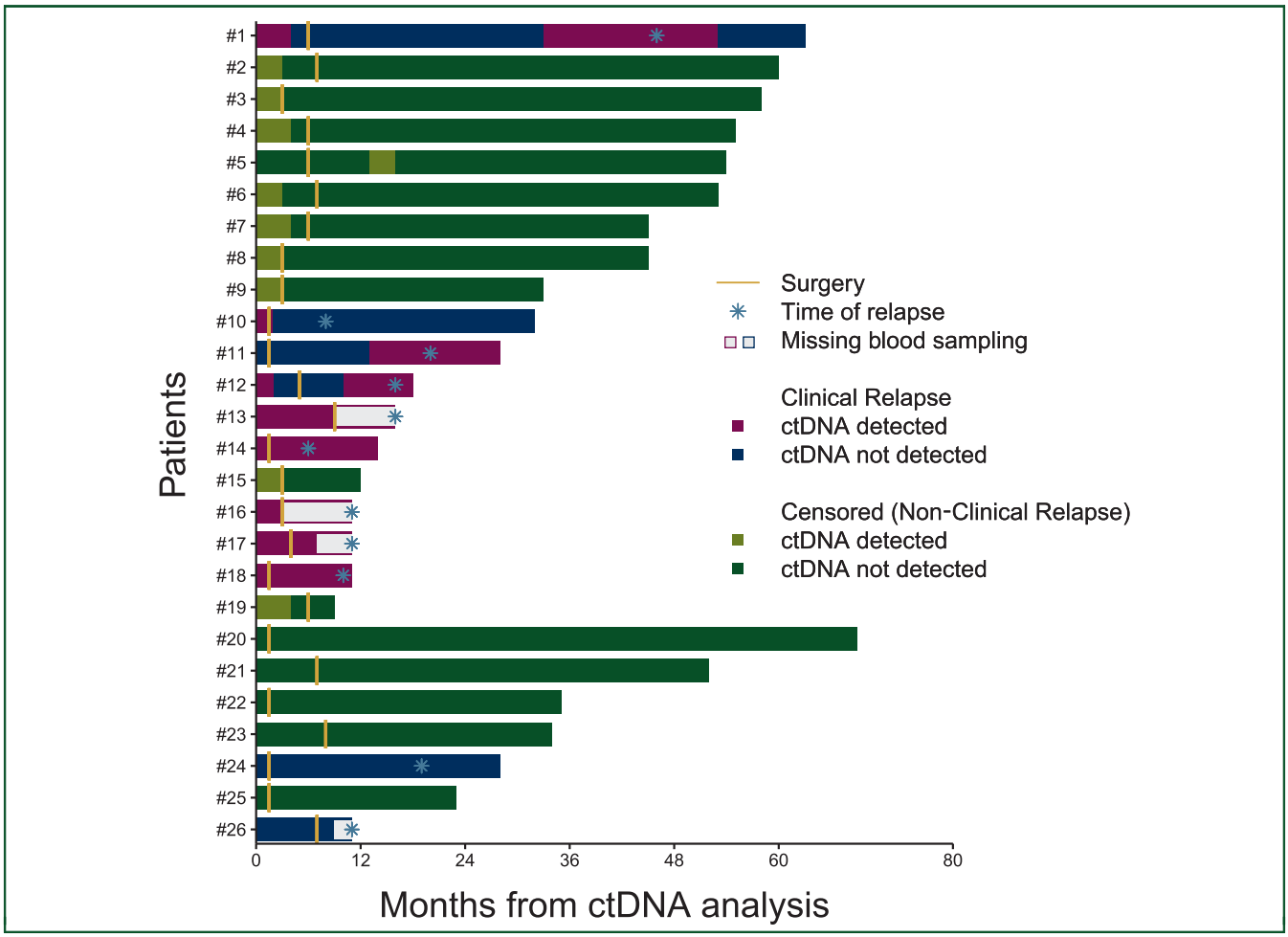

Figure 4. Event-free survival plot among individual patients with or without detectable ctDNA during the study.
For each patient, times of surgical resection and relapse are indicated by a yellow line and a light blue asterisk, respectively. Censored patients did not develop an unfavorable event at the time of data collection.
ctDNA, circulating tumor DNA.

mutations. Aside, patients #12 and #14 lost mutated *ATM* and *TP53* found in surgical samples, patient #11 gained an extra mutation in *TP53* (VAF 80%), and patient #18 a *de novo* mutation in *MAP2K2* (VAF 0.75%). Taken together, these data imply that ctDNA, while retaining the dominant features of the primary tumor, is informative of genetic alterations occurring during progression.

CTC analysis at the time of disease progression

To further characterize liquid biopsies at time of disease progression, CTCs were detected and molecularly profiled in six relapsed patients. Among the 21 CTCs collected, 1 expressed epithelial markers (eCTCs) only, corresponding with the classical CTC definition, and 20 CTCs lacked both epithelial and leukocyte markers or expressed more than one marker. Those latter CTCs that did not meet the classical CTC definition were characterized by aberrant genomes and were considered as non-conventional CTCs (ncCTCs). The molecular characterization of recovered CTCs showed a prevalence of deletions on amplification and included chromosomes 5, 8, and 17 (Figure 5, panel A), which have already been described in the literature as frequently lost in primary TNBCs.[21] Moreover, the 10q and 21q were the most frequently altered chromosomal arms (Figure 5, panel B). A network analysis of physical interaction among BC-related genes from these altered regions identified a module of 28 nodes involved in therapeutically exploitable pathways including mismatch repair, PI3K/Akt, erbB, Raf, platinum-resistence signaling, and regulation of immune response (Supplementary Figure S4, available at https://doi.org/10.1016/j.esmoop.2021.100086).

DISCUSSION

Among the topics of interest for NAC, there is the issue of exploring reliable prognostic and predictive markers at baseline and/or during and after drug exposure as a result of the patient-and-treatment interplay. Herein we challenged the use of ctDNA as a tool to anticipate NAC response, establish distinct prognostic groups after NAC, optimize follow-up by identifying recurrent patients in advance, and the use of CTCs to explore druggable targets at disease progression.

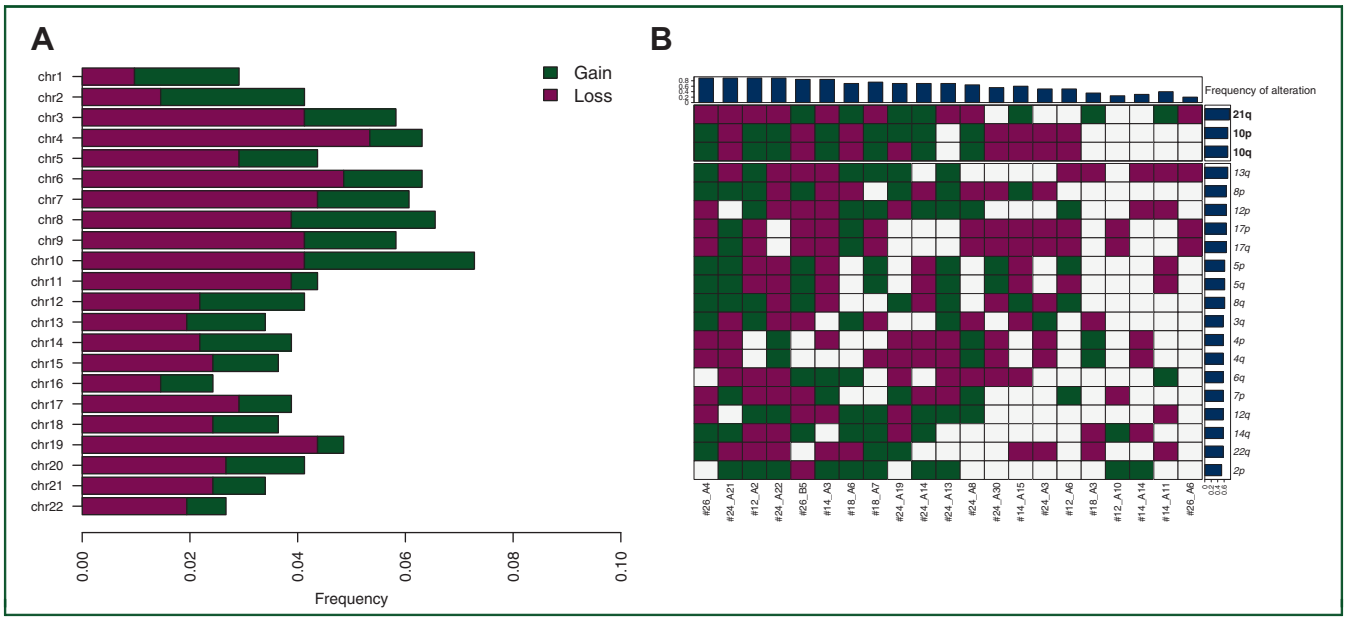

Figure 5. Copy number alterations (CNAs) detected in CTCs from relapsed cases.
(A) The bar plot shows the distribution of CNAs along chromosomes considering all the CTCs collected at time of relapse. Green and burgundy colors refer to amplifications and deletions, respectively. (B) The heatmap reports patients in the column and the top 20 altered chromosome arms on the rows. Green and burgundy colors refer to amplifications and deletions, respectively; 21q (43%), 10p (41%) and 10q (41%) were the most commonly altered arms.

Few studies have evaluated whether ctDNA analysis can be informative of response to NAC. In a prospective cohort of 101 patients with different BC subtypes (84 treated with NAC), ctDNA was detected before any treatment in 41 cases and significantly associated with poor prognosis. Notably, ctDNA was found to anticipate the diagnosis of overt metastases in 16 cases.[12] An ancillary ctDNA analysis of the NeoALTTO trial showed that baseline detection of *PIK3CA* or *TP53* mutations in plasma samples of 28 patients was associated with poor response to anti-HER2 targeted therapy.[19] More recently, ctDNA, alone and in association with CTCs, proved to be associated with disease outcome in more than 100 cases in a preplanned correlative study from a phase II, randomized clinical trial of TNBC patients treated with NAC.[22] In terms of monitoring treatment response, reduced levels of multiple plasma mutations in 22 patients have been recently correlated with increased chances of attaining pCR at NAC completion.[14]

In this study, by implementing a pragmatic approach with primary-tumor-targeted gene sequencing and patient-specific point mutations ddPCR detection, we analyzed a homogeneous cohort of 31 TNBC patients to first assess the clinical value of ctDNA at baseline, during, and after treatment with curative intent. Our pre-NAC ctDNA detection rate of 77% was comparable with the published literature.[12-15] The post-NAC detection rate of 43.4% was, however, higher and likely reflects the fact that we had several patients with large primary tumor size and positive nodal status at initial presentation, which also justifies the low rate (13%) of pCR reported.

A reduction of ctDNA up to undetectable levels occurred mid-treatment and continued until surgery in just over half of the patients. This finding, which is consistent with previous studies,[13,23] is only apparently counter intuitive, as ctDNA is expected to become undetectable only for patients achieving pCR. One plausible mechanism is that chemotherapy kills the dividing cells most likely to contribute to ctDNA release and, because the half-life of ctDNA in the blood is short, leaves behind a tumor less prone to ctDNA release at our mid-course sampling time.[24] Consistent with this hypothesis, the findings reported in the metastatic setting show that patients with stable disease and on treatment presented decreased levels of ctDNA despite the lack of tumor response.[25,26] Hence, the same could happen in the neoadjuvant setting with the important difference that patients with non-metastatic BC present lower ctDNA levels, which instead of reducing, disappeared during treatment. Besides, ctDNA levels were detectable in patients who progressed during treatment, suggesting a role for ctDNA analysis in identifying tumor progression and assaying response to NAC, as increasing ctDNA may be an indicator not only of tumor growth during treatment but of increased risk of recurrence.

Our results suggest that post-NAC ctDNA is a surrogate for the emergence of relapse with 6/10 patients with detectable ctDNA at the end of treatment developing a BC event. Remarkably, despite the small sample size, post-NAC detection of ctDNA retained its prognostic significance even after adjusting for other clinicopathological variables, including Ki-67, whose reduction has been associated with better prognosis in patients who do not obtain pCR response, as 83% of non-relapsed cases despite persistently high Ki-67 levels were negative for ctDNA, and 30% of relapsed patients despite decreased levels of Ki-67 were ctDNA positive. Hence, our data suggest that the evaluation of ctDNA after NAC could act as a clinically available tool that might allow clinicians to stratify patients into those who could benefit from 'complementary' treatment, in

agreement with recently published results.[22] Specifically, TNBC patients with persistent ctDNA levels at the end of NAC could benefit from treatment intensification or alternative therapeutic strategies in an attempt to prevent the development of metastases.

Next, we showed that in 83% of cases, ctDNA preceded clinical detection of distant metastases by 8.9 months (range, 6.5-13.1 months) and with excellent specificity. Therefore, ctDNA is not to be considered another prognostic factor that 'on average' associates with the prognosis, but is rather able to predict at the individual patient level whether the event will happen or not, and the 'lead time' found, which is consistent with other reports, represents a unique window of opportunity for the introduction of non-cross-resistant therapies to prevent overt clinical relapse.

Our ctDNA analysis was restricted to the known mutation profile of the primary tumor, which could be considered a limitation as clonal evolution cannot be studied. However, focusing on the known mutation profile in the tumor may minimize the risk of false positives particularly in light of recent reports identifying plasma somatic mutations arising from clonal hematopoiesis.[27] On the other hand, analysis with the Oncomine pan-cancer cell-free assay showed that primary tumors and metastases exhibit high genomic concordance at the plasmatic level, though additional druggable mutations such as *MAPK* may arise.

In addition to ctDNA detection, using the marker-independent Parsortix approach for CTC-enrichment coupled with positive and negative selection with the DEPArray, we showed that CTCs are non-conventional (i.e. non-epithelial) in most recurrent cases and would not have been detected by any of the commercially available epithelial marker approach, including Cell Search.[28] Notably, analysis for copy number alterations (CNAs) by NGS displayed a unique spectrum of genetic abnormalities, including gain/loss of chromosome 10 and chromosome 21q. Although changes in gene copy number, large and small in scale, contributed to population diversity, our analysis revealed a network among genomic alterations in relapsed cases that defined highly connected modules including HER/PI3K/Ras/JAK signaling and immune response. Altogether, these results provide support to the concept that despite the lack of common CNAs in patients who progressed after NAC, they fell into several and more importantly 'druggable' shared functional categories.

The fact that patients were prospectively recruited for the purpose of these analyses, which were conducted in the same laboratory with uniform methodology, represents the major strengths of our study. However, there are also several limitations to consider, some of which are inherent to the observational design of the study, the small sample size, the number of pre-NAC samples collected, and the variable (i.e. non-standardized) timing for post-surgical blood drawings that could affect the evaluation of relapse/progression anticipation. Importantly, the costs of serial sequencing assays and single-CTC analyses preclude their routine clinical application, which might be overcome by technology improvements. Although the data should be interpreted with caution, emerging findings—including our results—may guide the direction of future studies since risk-adapted treatment strategies continue to be a research priority.

In conclusion, our findings support blood-based genomic analyses as complementary tools to optimize monitoring and to guide therapy in TNBC patients treated with NAC and add to and integrate previous studies demonstrating the clinical validity of ctDNA. Prospective trials are ongoing and will address the clinical utility of incorporating such strategies into routine clinical practice.

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DISCLOSURE

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