Clinical Chemistry 63:3
691–699 (2017)

Patient-Specific Circulating Tumor DNA Detection during Neoadjuvant Chemotherapy in Triple-Negative Breast Cancer

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BACKGROUND: In nonmetastatic triple-negative breast cancer (TNBC) patients, we investigated whether circulating tumor DNA (ctDNA) detection can reflect the tumor response to neoadjuvant chemotherapy (NCT) and detect minimal residual disease after surgery.

METHODS: Ten milliliters of plasma were collected at 4 time points: before NCT; after 1 cycle; before surgery; after surgery. Customized droplet digital PCR (ddPCR) assays were used to track tumor protein p53 (*TP53*) mutations previously characterized in tumor tissue by massively parallel sequencing (MPS).

RESULTS: Forty-six patients with nonmetastatic TNBC were enrolled. *TP53* mutations were identified in 40 of them. Customized ddPCR probes were validated for 38 patients, with excellent correlation with MPS ($r = 0.99$), specificity ($\geq$2 droplets/assay), and sensitivity (at least 0.1%). At baseline, ctDNA was detected in 27/36 patients (75%). Its detection was associated with mitotic index ($P = 0.003$), tumor grade ($P = 0.003$), and stage ($P = 0.03$). During treatment, we observed a drop of ctDNA levels in all patients but 1. No patient had detectable ctDNA after surgery. The patient with rising ctDNA levels experienced tumor progression during NCT. Pathological complete response (16/38 patients) was not correlated with ctDNA detection at any time point. ctDNA positivity after 1 cycle of NCT was correlated with shorter disease-free ($P < 0.001$) and overall ($P = 0.006$) survival.

CONCLUSIONS: Customized ctDNA detection by ddPCR achieved a 75% detection rate at baseline. During NCT, ctDNA levels decreased quickly and minimal residual disease was not detected after surgery. However, a slow decrease of ctDNA level during NCT was strongly associated with shorter survival.

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Triple-negative breast cancers (TNBCs)[10] represent 15%–20% of invasive breast cancers. This subgroup is defined by the absence of estrogen and progesterone receptor expression, no amplification of the erb-b2 receptor tyrosine kinase 2 (*ERBB2*) gene,[11] and frequent tumor protein p53 (*TP53*) inactivating gene mutations *(1, 2)*. At diagnosis, TNBCs tend to display larger tumor size and higher proliferation rate than other breast cancers; in the absence of overt metastasis, TNBCs are often treated by neoadjuvant chemotherapy (NCT) followed by surgery. In addition to breast tumor shrinkage, NCT aims at eradicating any disseminated tumor cell (also known as micrometastasis) that may have spread throughout the body. The persistence of a minimal residual disease at distant sites after the treatment of a localized breast cancer is a key parameter for posttreatment survival but cannot be reliably assessed by the current biological or radiological tools *(3)*. In that context, the detection and quantification of circulating tumor DNA (ctDNA) is a very promising tool that can assess tumor burden, response to therapy, and minimal residual disease *(4, 5)*.

ctDNA corresponds to fragmented DNA released into the blood stream by tumor masses *(6, 7)*. In meta-

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Received July 2, 2016; accepted October 20, 2016.
Previously published online at DOI: 10.1373/clinchem.2016.262337
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[10] Nonstandard abbreviations: TNBCs, triple negative breast cancers; NCT, neoadjuvant chemotherapy; ctDNA, circulating tumor DNA; pCR, pathological complete response; RCB, residual cancer burden; ddcPCR, droplet digital PCR; MAF, mutant allele frequency; cfcDNA, cell-free circulating DNA.
[11] Human genes: *ERBB2,* erb-b2 receptor tyrosine kinase 2; *TP53,* tumor protein p53; *BRCA1,* BRCA1, DNA repair associated; *BRCA2,* BRCA2, DNA repair associated.

static breast cancer patients, ctDNA analysis is an excellent tool to assess the tumor genomic landscape *(8–10)*. We also previously reported that *TP53* mutations can be detected in the plasma of most metastatic TNBC patients *(11)*. ctDNA levels have been repeatedly correlated with the tumor burden *(12–14)*, but other factors influencing ctDNA levels are poorly characterized. Proof-of-concept studies also have suggested the use of ctDNA levels as a dynamic biomarker reflecting the tumor response to therapy in metastatic breast cancer patients *(13, 15)*. However, no data about ctDNA levels and changes during NCT are available for localized breast cancer.

Here, we report a prospective study in which we detected and quantified ctDNA levels before, during, and after NCT and surgery in a homogeneous cohort of nonmetastatic TNBC patients.

Materials and Methods

PATIENTS AND TREATMENT

Patients have been included after written informed consent into the prospective, ethically approved CTC-CEC-DNA study (NCT02220556). Eligibility criteria were patients aged >18 years with nonmetastatic TNBC (stage I–III), treated at Institut Curie (Paris, France) by NCT. All patients underwent a core tumor biopsy to control the triple negative phenotype before NCT. TNBCs were defined as infiltrating tumors with estrogen receptor staining <10%, progesterone receptor staining <10% and with no HER2 (human epidermal growth factor receptor 2) overexpression/amplification according to current guidelines. Ki67 staining (expressed in percent) was assessed on pretreatment tumor biopsies.

For all patients, the routine metastatic workup included full clinical examination, blood sampling, bone scan, and CT (computed tomography)-scan (chest, abdomen, and pelvis, with contrast). After anthracycline/taxane-based NCT, patients underwent lumpectomy or mastectomy, depending on the tumor response, together with axillary lymph node examination (sentinel biopsy or axillary dissection). Pathological complete response (pCR) was defined as the absence of infiltrative carcinoma in breast and in lymph nodes (ypT0/isN0); residual cancer burden (RCB) scores *(16)* were determined retrospectively. Patients received adjuvant radiation therapy whenever indicated. Posttreatment follow up consisted in at least 2 visits per year at Institut Curie and yearly breast mammograms, while BRCA1, DNA repair associated (*BRCA1*), and BRCA2, DNA repair associated (*BRCA2*) mutation carriers had a specific follow up.

TUMOR SAMPLES COLLECTION AND DNA EXTRACTION

Pretreatment tumor biopsies were either fresh frozen and/or formalin fixed. DNA was extracted using a classical phenol chloroform protocol from frozen tissue or the NucleoSpin® formalin-fixed paraffin-embedded DNA kit (Macherey Nagel) for formalin-fixed paraffin-embedded tissue, respectively. Tumor DNA was quantified in each sample using a LINE1 real-time PCR assay, as previously described *(12)*.

MASSIVELY PARALLEL SEQUENCING

A set of 16 specific primer pairs covering all the exons and flanking regions of TP53 and flanked by universal sequences CS1 and CS2 was designed (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue3). Eight nanograms of tumor DNA was amplified with 0.4 μmol/L primers, 0.1 U/μL Phusion Hot Start II High Fidelity (Thermo Scientific), 3% DMSO (dimethyl sulfoxide), and 200 μmol/L deoxynucleotide triphosphates in 1× Phusion HF Buffer in a reaction volume of 20 μL at 98 °C for 30 s, 35 cycles of 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 20 s, followed by 72 °C for 3 min. PCR amplicons were run on a Labchip GX (PerkinElmer) to verify the correct amplicon size. Based on the DNA concentrations provided by Labchip, the 16 amplicons were pooled together in equimolar ratios for each tumor sample. Five microliters of each sample pool was then subjected to another round of amplification with sample indexing primers containing the adapter sequences PE1 and PE2 needed for Illumina sequencing. The amplification reaction was assembled and cycled as above. The products of the second PCR were run on a Labchip GX (PerkinElmer) to verify the amplification size product and the concentration. Then, equimolar pools of tumor samples were made. The entire pool was then purified using AMPure XP PCR purification (Beckman Coulter) according to manufacturer recommendations. The purified library was evaluated using a DNA1000 Bioanalyzer chip (Agilent) and quantified using the Qubit dsDNA HS assay kit (Invitrogen).

Paired-end 150-bp read length sequencing (MiSeq Reagent Kit v3) was performed on an Illumina MiSeq according to manufacturer's instructions. The average sequencing depth was 120 000 reads per sample (range 100 000–150 000).

SEQUENCING DATA ANALYSIS

Reads were extracted from fastq and trimmed to remove adapters and index. Trimmed reads were aligned on the 16 amplicon sequences (extracted from human reference genome hg19) using BWA software, with default parameters. Samtools (v 1.1) was used to sort and index the mapped reads. Local realignment around known indels was done with Genome Analysis Toolkit (v3.1–1) functions: RealignerTargetCreator, IndelRealigner, BaseRecalibrator, and PrintReads. Variant calling was carried out on the mapped reads by Samtools mpileup. Variants

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were annotated using Annovar (v. 2014/11/10) and the following databases: snp138NonFlagged, popfreq_max, ljb23_metasvm, and RefGene annotations. Only nonsynonymous variants with a frequency higher than 1% were identified as possible targetable mutations. A manual indel calling was performed: (*a*) Reads were regrouped according to their sequence forming a cluster of reads with the same sequence. For each sequence, a local alignment was performed to identify the couple of primers allowing the association with a specific amplicon. Reads with more than 1 mismatch on both primers are discarded. (*b*) Sequences with a length different than its associated amplicon are designated as potential indel if the number of clustered reads represents more than 1% of the reads aligned on this amplicon. A local alignment was performed to identify the indel positions. The primer dimers were removed. Indels were annotated using RefGene annotations.

PLASMA SAMPLES COLLECTION AND DNA EXTRACTION

This study allowed for the collection of blood at up to 4 time points, which were chosen as follows: (*a*) at baseline, before the start of NCT; (*b*) before the second cycle of chemotherapy, i.e., 2–3 weeks after the first cycle; (*c*) at the last cycle of NCT, before breast cancer surgery; and (*d*) 2–10 weeks after surgery.

At each time point, to collect 10 mL of plasma, 21 mL of blood was drawn into EDTA tubes (3 tubes) and processed within 1 h at the Circulating Cancer Biomarkers Laboratory, located within the Curie Hospital building. Plasma samples were prepared as described elsewhere *(4)*. Briefly, blood was centrifuged at 820*g* for 10 min. The supernatant was transferred to sterile tubes, centrifuged at 16000*g* for 10 min and the supernatant was stored at −80 °C. Cell-free circulating DNA (cfcDNA) was extracted from 4 mL of plasma using the QIAamp circulating nucleic acid kit (Qiagen), following manufacturer instructions and eluted into 36 μL of buffer. cfcDNA was stored at −20 °C and quantified using the LINE1 real-time PCR assay.

DROPLET DIGITAL PCR

Droplet digital PCR (ddPCR) was performed on a QX100 ddPCR system (Bio-Rad Laboratories). A total volume of 25 μL PCR reaction mixtures was prepared [12.5 μL 2× Supermix for probes without dUTP (Bio-Rad), 1.25 μL 20× target primers/probe, 1.25 μL 20× wild-type primers/probe, and DNA sample/water (variable volume)]. Ten nanograms of DNA was analyzed for each tissue sample and a median of 13 ng of eluted DNA for plasma samples.

The PCR reaction was partitioned into a mean of 14000 droplets per sample using the QX100 Droplet Generator (Bio-Rad) according to the manufacturer's instruction. A thermal gradient experiment on annealing temperatures was done for custom design probes to optimize thermal cycling conditions. Droplets were then transferred to a 96-well PCR plate and thermal cycled as follows: incubation at 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 55 °C for 60 s, followed by 10 min incubation at 98 °C. The temperature increment was 2.5 °C/s for all steps. Samples were then transferred to a Bio-Rad QX-100 droplet reader and analyzed on the basis of fluorescence intensity by QuantaSoft v1.4.0.99 software from Bio-Rad. The threshold distinguishing positive and negative droplets has been determined manually on the Quantasoft software by the operator using tests run on tumor tissue for each patient. To ensure uniformity, the threshold obtained was kept for the analysis of all following samples (plasmas and negative controls).

Each test contained at least 2 negative control wells with no DNA and wild-type DNA. The concentration of mutant DNA and wild-type DNA was estimated from the Poisson distribution. Mutant allele frequency (MAF) was calculated as follows: MAF (in %) = [mutant copy/(wild-type + mutant copy)] × 100.

SENSITIVITY AND SPECIFICITY OF ddPCR ASSAYS

ddPCR probes matching the *TP53* mutations found in tumor tissue were purchased from Bio-Rad: some of the probes were already available with in vitro experiments supporting the claimed sensitivity or with in silico optimization; the remaining probes were custom-designed (see online Supplemental Table 2). Sensitivity was first assessed for each ddPCR probe by running ddPCR on tumor DNA and comparing *TP53* mutant allele fractions estimated by ddPCR with those previously obtained by MPS. As a few patients had no ctDNA detected in plasma at any time point, we undertook systematic sensitivity testing to ensure that the lack of ctDNA detection was not attributable to a lack of sensitivity of the corresponding ddPCR probes. The sensitivity of customized ddPCR assays was assessed on samples with decreasing *TP53* mutant allele fractions (1%, 0.5%, 0.2%, 0.1%, 0.05%) obtained by diluting tumor DNA into approximately 3000 copies of normal DNA (mononucleated blood cells DNA from healthy donors; see online Supplemental Table 2). Specificity was verified and background threshold determined for each ddPCR assay on 10 ng (approximately 3000 copies) of normal DNA; every test was performed and replicated a minimum of 5 times (see online Supplemental Table 2). A threshold of ≥2 positive droplets was determined to eliminate false positive. Because the rate of false positive droplet events is generally independent of the total amount of DNA tested *(17)*, it is important to test the highest possible number of alleles to increase sensitivity. We thus screened all material available to confirm points for which ctDNA was not detected.

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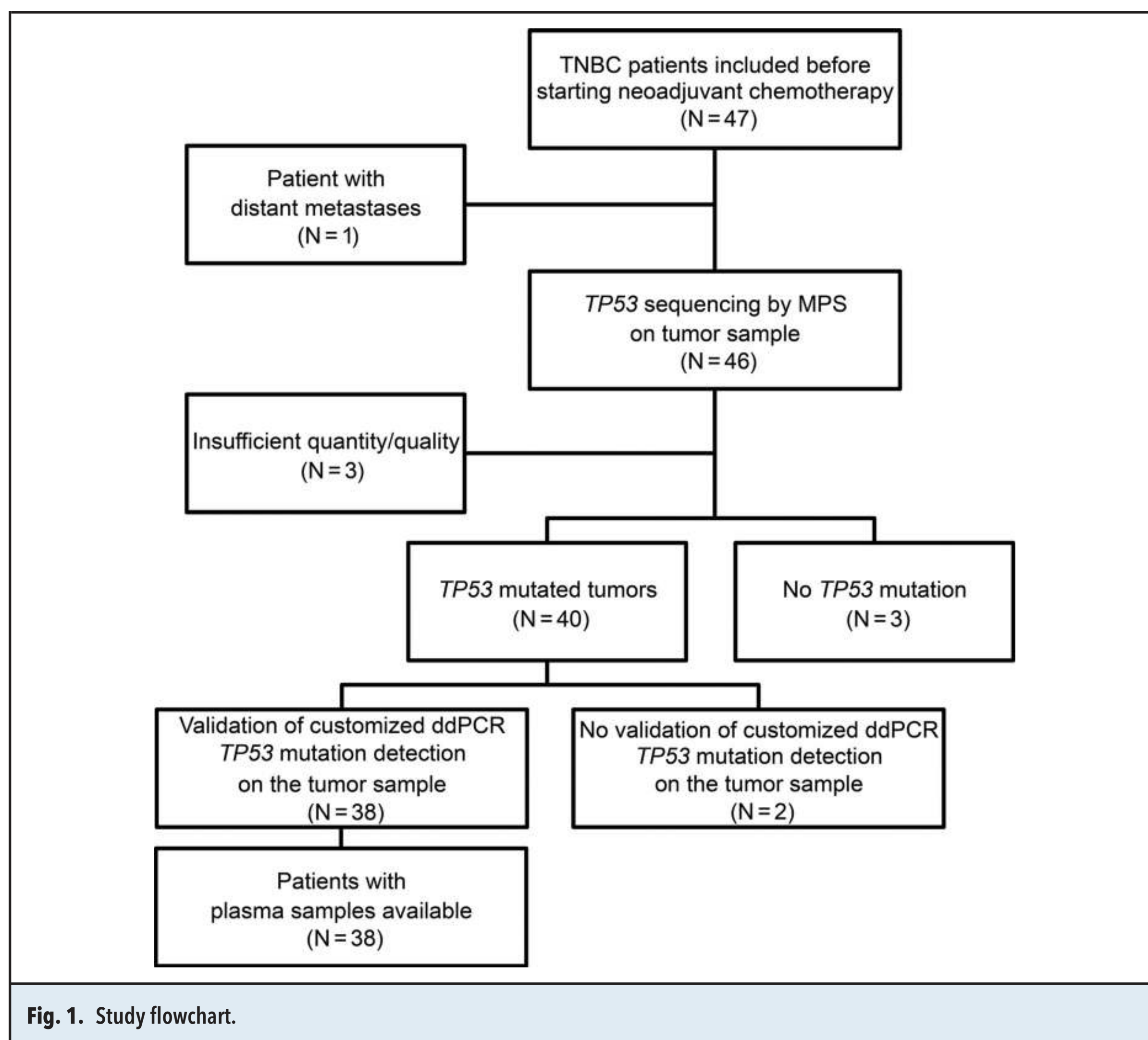

Fig. 1. Study flowchart.

STATISTICAL ANALYSIS

This hypothesis-generating study had no prespecified power. The first objective of the study was to assess the correlation between ctDNA detection at baseline and the breast cancer clinical and pathological characteristics. Secondary objectives were to evaluate ctDNA detection at each time point and the primary tumor response to neoadjuvant chemotherapy, as well as the prognostic impact of ctDNA detection at each time point. Clinical data were obtained from the patient electronic medical files. Pearson correlation coefficients were used to evaluate the relationship of MAF detected by next-generation sequencing and by ddPCR. Relationships between ctDNA status or levels and clinical pathological characteristics were assessed using the Fisher exact test, Student *t*-test, and Pearson and Spearman correlation tests. The Wilcoxon test was used to compare cfcDNA and ctDNA levels at the different time points. Survival analysis was performed using Kaplan–Meier plots with significance tested using the log-rank test. These statistical analyses were executed using GraphPad Prism version 6.0 and R (version 3.2.2). All tests were 2 sided.

Results

DETECTION OF *TP53* MUTATIONS IN TUMORS

From January 2013 to May 2014, 47 TNBC patients were included in the study (study flow chart is shown in Fig. 1). *TP53* mutations were identified in 40 tumor tissues: single nucleotide variations in 29 TNBCs and insertion/deletions in 11 TNBCs (see online Supplemental Table 3). Customized ddPCR assays were then designed to track these *TP53* mutations in plasma samples and detect ctDNA. We first validated customized ddPCR probes on tumor samples. All but 2 *TP53* mutations identified by MPS were retrieved by ddPCR (see

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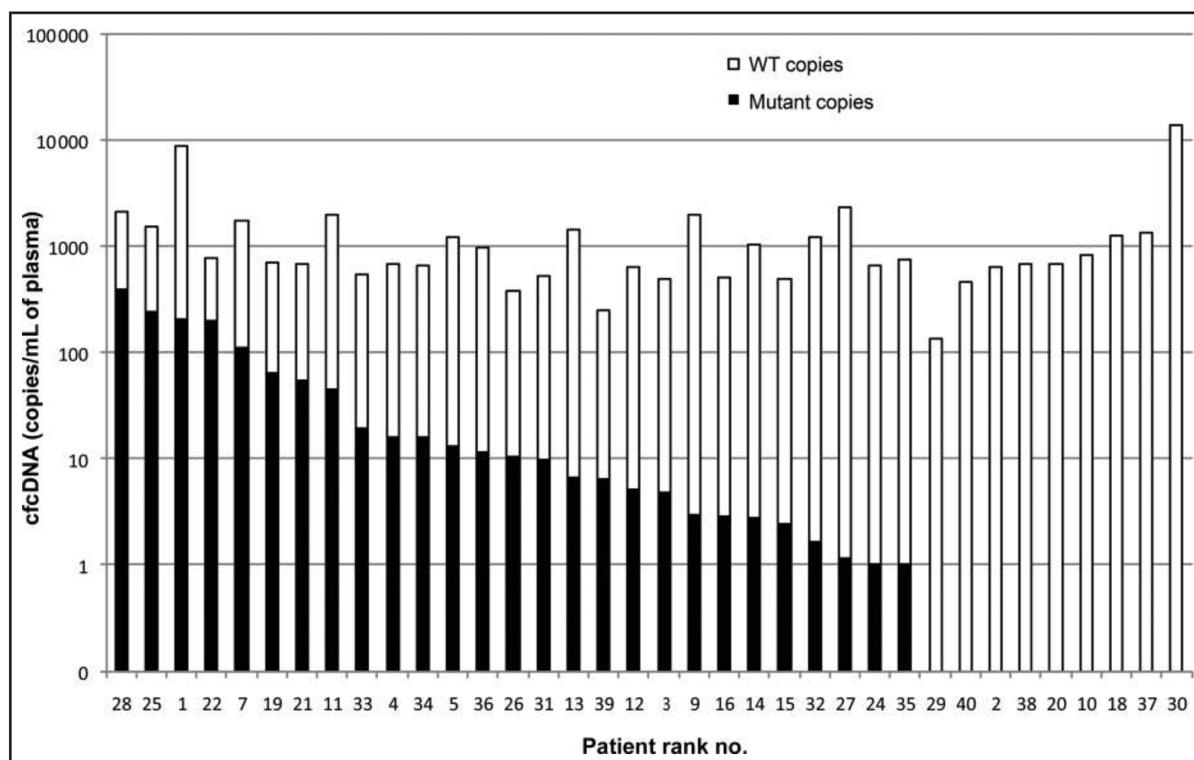

Fig. 2. cfcDNA and ctDNA concentrations before chemotherapy.

Patients are ranked by decreasing ctDNA concentrations. Empty bars represent cfcDNA concentration; filled bars represent ctDNA concentration. WT, wild-type.

online Supplemental Table 3). Plasma samples from the corresponding 2 patients were discarded from further analyses. For all other tumor samples, an excellent correlation was found regarding mutant allele frequencies observed by MPS and ddPCR ($r > 0.99$, $P < 10^{-9}$; see online Supplemental Fig. 1). Additionally, we performed systematic tests to ensure the specificity of each assay (see Materials and Methods and online Supplemental Table 2) and determine the background threshold on nonmutated DNA. We defined a threshold of ≥2 positive droplets for all ddPCR assays.

ctDNA DETECTION

At baseline, plasma samples were available for 36 patients (for 2 patients, blood draw was not performed before starting chemotherapy). Before the start of NCT, concentrations of cell-free circulating DNA (cfcDNA) varied considerably between patients (median 714 copies/mL of plasma, range 133–13750). ctDNA was detected in 27/36 patients [75%, 95% CI, (59%–86%)], with a median concentration of 4.85 copies/mL of plasma (range 1–388). To make sure that undetected ctDNA was not caused by a lack of sensitivity of the corresponding ddPCR, additional sensitivity tests were performed for 8 assays used in patients with no ctDNA detected (see Materials and Methods and online Supplemental Table 2). All tests demonstrated a sensitivity of 0.1% or lower.

No correlation was observed between ctDNA and cfcDNA levels (Fig. 2). ctDNA levels were significantly associated with the clinical tumor size (continuous variable, $P = 0.004$) and tumor stage ($P = 0.03$). Interestingly, ctDNA levels also correlated with high proliferation rate, assessed either by mitotic index ($P = 0.003$) or by tumor grade ($P = 0.003$, Table 1). Ki67 was determined in 26 patients, with no significant correlation with ctDNA levels.

During NCT, cfcDNA and ctDNA showed opposite trends: cfcDNA concentrations increased significantly (Fig. 3A) while those of ctDNA decreased (Fig. 3B). After the first cycle of treatment (T2), a decrease was observed in the ctDNA concentration for all patients but 1 (i.e., only 1 patient had increased ctDNA levels), and residual ctDNA levels were detected in 9/35 patients [26%, 95% CI, (14%–42%), $P < 0.001$]. The patient with increased levels of ctDNA was the only patient exhibiting a progression of her breast tumor during chemotherapy; she was not referred to surgery and was removed from the study. In the remaining patients, the decrease of

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Table 1. Patient characteristics and ctDNA detection at baseline.[a]

Characteristics		Patients, n	Patients with detectable ctDNA at baseline, n (%)	Correlation with ctDNA positivity (Fisher exact test)	Correlation with ctDNA concentration (Pearson correlation test)
Age, years	≤50	19	13 (68)	NS[b]	NS
	>50	17	14 (82)		
cT	1–2	28	20 (71)	NS	NS
	3–4	8	7 (87)		
cN	cN0	22	14 (64)	0.06	NS
	cN1–3	14	13 (93)		
Stage	I	2	0 (0)	0.06	0.03
	II	31	24 (77)		
	III	3	3 (100)		
Tumor grade	1	1	0 (0)	0.005	0.003
	2	4	1 (25)		
	3	31	26 (84)		
Histology	IC-NST	33	24 (73)	NS	NS
	Other	3	3 (100)		
Ki67%	≤20	1	1 (100)	NS[c]	NS
	20 < Ki67 <50	4	4 (100)		
	≥ 50	21	14 (67)		
Mitotic index	Low	3	1 (33)	0.03	0.003
	Intermediate	5	2 (40)		
	High	27	23 (85)		
pCR	RCB 0	16	12 (75)	NS	NS
	RCB ≥1	20	15 (75)		

[a] Baseline plasma sample was available for 36 patients.
[b] NS, not significant; cT, clinical tumor size [tumor, node, metastasis (TNM) classification]; cN, clinical nodal involvement (TNM classification); IC-NST, invasive breast carcinoma of no specific type.
[c] Student *t*-test.

ctDNA persisted during NCT with only 1/24 patients [4%, 95% CI, (0%–20%), Fig. 3B] with detectable ctDNA levels before surgery (T3). After surgery, no patient had detectable ctDNA levels [0/31, 0%, 95% CI, (0%–11%)].

CORRELATION WITH OUTCOME

pCR was achieved in 42% of patients [16/38, 95% CI, (28%–58%)]. We did not find a significant correlation between clinical response, pCR, and ctDNA detection (or changes) at baseline or any time point.

Median follow up was 24 months (range: 9–36). No local recurrence was observed. Four patients (10%) developed distant metastases and 3 died. All the patients who relapsed had detectable ctDNA at baseline (3/3; in the fourth case, a plasma sample was not available at baseline) and after 1 cycle of treatment (4/4), suggesting that ctDNA levels decreased less quickly in these patients than in the others (Fig. 3C). Occurrence of metastatic relapses was not correlated with ctDNA detection at baseline but was significantly correlated with ctDNA positivity after 1 cycle of treatment ($P = 0.002$). Detectable ctDNA at baseline was not related to overall survival or to disease-free survival (Fig. 4). Conversely, ctDNA positivity after 1 cycle of NCT was associated with shorter disease-free survival ($P < 0.001$) and overall survival ($P = 0.006$, Fig. 4).

Discussion

Our study focused on the subgroup of TNBC patients for several reasons. TNBCs, which are often treated by NCT, display high pCR rates but also frequent early metastatic relapses *(1)*. In practice, the very high prevalence of *TP53* mutations in TNBC allowed for targeted sequencing on tumor tissues, in contrast to other breast cancer subtypes—in which no recurrent gene is as frequently mutated *(2)*. Finally, *TP53* mutations are of paramount interest in TNBC because of their driver role in

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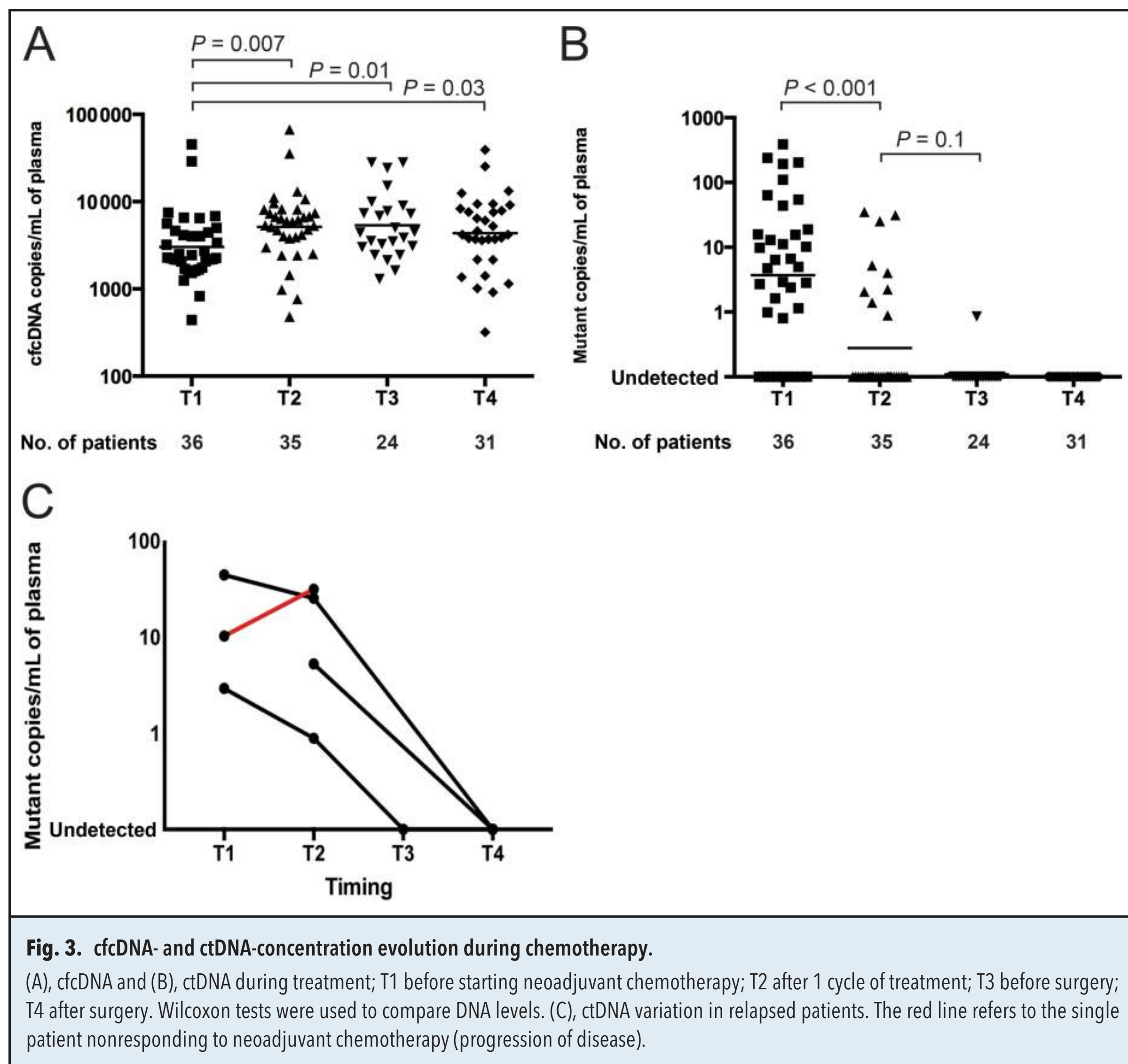

Fig. 3. cfcDNA- and ctDNA-concentration evolution during chemotherapy.
(A), cfcDNA and (B), ctDNA during treatment; T1 before starting neoadjuvant chemotherapy; T2 after 1 cycle of treatment; T3 before surgery; T4 after surgery. Wilcoxon tests were used to compare DNA levels. (C), ctDNA variation in relapsed patients. The red line refers to the single patient nonresponding to neoadjuvant chemotherapy (progression of disease).

tumor cells, leading to clonal distribution and consequently to the highest mutant allelic frequency of all detectable tumor mutations (and in the matched plasma). Note that, in the few *TP53* wild-type tumors, mutations may have been found in other genes than *TP53* and used to track ctDNA during NCT. However, as mutations in distinct genes may be intrinsically associated with distinct prognostic values and outcomes, we chose to focus on *TP53* exclusively.

Screening a large volume of plasma with ddPCR, a widespread technique for detection of rare variants, together with strict sensitivity and specificity controls, we have demonstrated that ctDNA can be detected in 75% [95% CI, (59%–86%)] of nonmetastatic TNBC patients before the start of NCT. As previously reported in patients with metastatic cancer *(12–14)*, ctDNA levels were correlated with tumor burden in our cohort. Interestingly, we found that ctDNA detection was also directly associated with the tumor proliferation rate, measured either by tumor grade ($P = 0.003$) or mitotic index ($P = 0.003$). This is to our knowledge the first direct demonstration that ctDNA levels in humans are intrinsically correlated with the tumor proliferation and turnover in a given cancer type. Necrosis, biologically thought to be a correlative process of high proliferation and ultimately responsible for the release of tumor DNA fragments in the blood, was not assessable on the pretreatment fine needle biopsies used in this study.

Nevertheless, in the setting of primary breast cancer, the clinical utility of ctDNA as a liquid biopsy (i.e., to depict the tumor mutational landscape) could be questionable, as breast tumors are immediately accessible to percutaneous solid biopsies. In fact, the critical clinical questions in the

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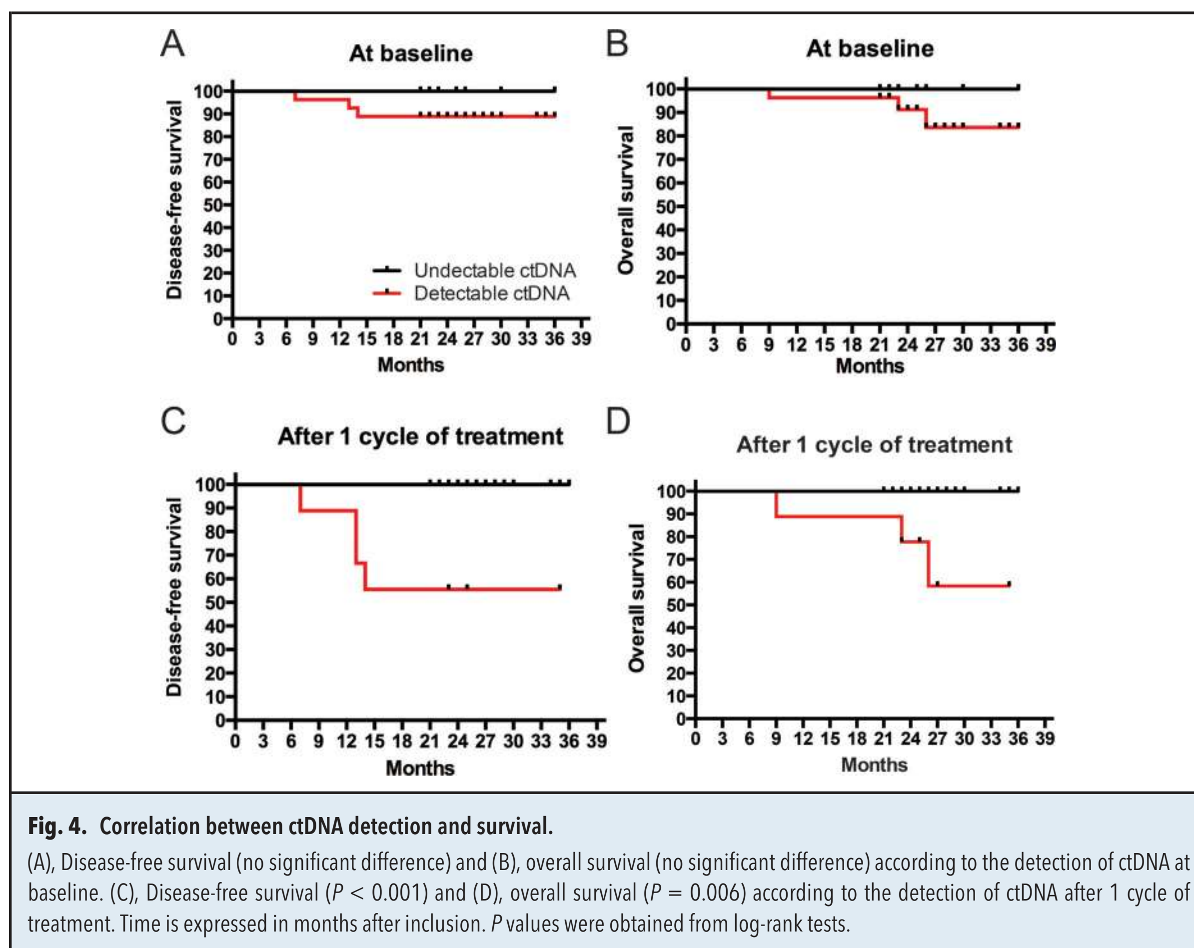

Fig. 4. Correlation between ctDNA detection and survival.

(A), Disease-free survival (no significant difference) and (B), overall survival (no significant difference) according to the detection of ctDNA at baseline. (C), Disease-free survival ($P < 0.001$) and (D), overall survival ($P = 0.006$) according to the detection of ctDNA after 1 cycle of treatment. Time is expressed in months after inclusion. *P* values were obtained from log-rank tests.

setting of nonmetastatic TNBC treated by NCT are as follows: (*a*) can early ctDNA levels or changes at any time point predict the tumor response/resistance to NCT? (*b*) do ctDNA levels or changes at any time point have any prognostic value regarding later metastatic relapse?

The results presented here show a marked decrease of ctDNA levels and positivity rate [34%, 95% CI, (18%–62%)] after a single cycle of NCT. One notable exception of rising ctDNA levels was observed in the only patient whose TNBC progressed during NCT. This highlights the potential of ctDNA to monitor tumor progression. Among the other patients, the decrease of ctDNA levels persisted during the following cycles of chemotherapy, and only 1 patient [4%, 95% CI, (0%–20%)] had detectable *TP53* mutation in her plasma before surgery. Interestingly, this patient had a poor breast tumor response (RCB class II). The observed pCR rate in our cohort [42%, 95% CI, (28%–58%)] is in line with pCR rates observed in TNBC treated by modern antracyclin/taxanes-based NCT, and no significant association between ctDNA levels (at any of the 4 tested time points) and pCR was observed.

A critical finding of our study is that no patient had detectable *TP53* mutation in plasma after surgery, although a few metastatic relapses were observed in the follow up of patients. These results are in contrast with the 19% (n = 7/37 patients) ctDNA detection rate after postneoadjuvant surgery previously reported by Garcia-Murillas et al. *(18)*. Study designs might be partly responsible for this observed difference: our study considered a prospective cohort with systematic collection whereas the study of Garcia-Murillas et al. was a retrospective study on available plasma aliquots from selected patients. We also reported robust sensitivity and specificity controls for each of the customized assays used in our study. More importantly, the absence of ctDNA detection after NCT and surgery may be due to key clinical parameters that contributed to reduce any postsurgical minimal residual disease. First, all patients underwent an extensive metastasis workup at baseline; metastatic cancer patients were excluded from the study. The patient whose tumor progressed during NCT was not included in the postsurgery analysis. Therefore, our results include only patients with proven absence of distant metastases or

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nonoperable locoregional invasion (e.g., supraclavicular lymph node invasion, N3). Second, all patients received antracyclin/taxanes-based chemotherapy regimens, which are more efficient than other regimens which may have been used formerly. Third and last, our cohort was exclusively composed of TNBCs, which frequently respond to NCT. In contrast to micrometastases emanating from e.g., hormone-positive breast cancers, any distant TNBC micrometastases are likely to shrink during neoadjuvant therapy and to be undetectable after breast cancer surgery by current ctDNA detection techniques. More sensitive ctDNA detection approaches are therefore needed to detect minimal residual disease; such approaches may rely on the analysis of a significantly larger volume of plasma combined with more sensitive detection techniques, and targeting multiple mutations in parallel (instead of 1 in our study).

As ctDNA levels were not assessed during the follow up of patients, we were not able to investigate whether rising ctDNA levels can lead to early detection of metastatic relapses after the primary therapy, as reported by Olsson et al. *(19)* and by Garcia-Murillas et al. *(18)*. However, after a median follow up of 2 years, we found that the few patients with remaining detectable ctDNA after the first cycle of chemotherapy were more likely to present with a later metastatic relapse (Fisher $P = 0.003$; log-rank $P < 0.001$). If confirmed by further prospective studies, ctDNA may become a clinically valuable prognostic tool to manage TNBC patients treated by NCT. While minimal residual disease detection remains a key target for TNBC management, additional clinical studies are needed to compare the prognostic value of ctDNA detection and level changes during therapy as well as circulating tumor cells, all of which previously have been demonstrated to have a significant prognostic impact on metastasis-free and overall survivals in early breast cancer *(19–21)*.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: J.-Y. Pierga, Roche, Ipsen, and Novartis.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: J.-Y. Pierga, Roche, Astra Zeneca, and Novartis.
Research Funding: F. Riva; University of Insubria, Italy; J.-Y. Pierga, ITMO "Santé Publique" and Institut Curie SiRIC (grant INCa-DGOS-4654).
Expert Testimony: None declared.
Patents: M.-H. Stern, EP 12305648.3 and US provisional patent application no. 62/331,585.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, and final approval of manuscript.

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