

Genome-wide Copy-number Alterations in Circulating Tumor DNA as a Novel Biomarker for Patients with High-grade Serous Ovarian Cancer



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ABSTRACT

Purpose: High-grade serous epithelial ovarian cancer (HGS-EOC) is defined by high levels of somatic copy-number alterations (SCNA) with marked spatial and temporal tumor heterogeneity. Biomarkers serving to monitor drug response and detect disease recurrence are lacking, a fact which reflects an unmet clinical need.

Experimental Design: A total of 185 plasma samples and 109 matched tumor biopsies were collected from 46 patients with HGS-EOC, and analyzed by shallow whole-genome sequencing (sWGS). The percentage of tumor fraction (TF) in the plasma was used to study the biological features of the disease at the time of diagnosis (T0) and correlated with patients' survival. Longitudinal analysis of TF was correlated with CA-125 levels and radiological images to monitor disease recurrence.

Results: Gain in the clonal regions, *3q26.2* and *8q24.3*, was observed in the 87.8% and 78.05% of plasma samples, suggesting

that plasma sWGS mirrors solid biopsies. At T0, multivariate analysis revealed that plasma TF levels were an independent prognostic marker of relapse ($P < 0.022$). After platinum (Pt)-based treatment, circulating tumor DNA (ctDNA) analysis showed a change in the heterogeneous pattern of genomic amplification, including an increased frequency of amplification, compared with before Pt-based treatment in the *19p31.11* and *19q13.42* regions. TF in serially collected ctDNA samples outperformed CA-125 in anticipating clinical and radiological progression by 240 days (range, 37–491).

Conclusions: Our results support the notion that sWGS is an inexpensive and useful tool for the genomic analysis of ctDNA in patients with HGS-EOC to monitor disease evolution and to anticipate relapse better than serum CA-125, the routinely used clinical biomarker.

See related commentary by Dhani, p. 2372

Introduction

High-grade serous epithelial ovarian cancer (HGS-EOC), the most common and lethal subtype of ovarian cancer, is characterized by a marked anatomic, molecular, and cellular heterogeneity. This feature has so far impaired the identification of reliable predictive biomarkers, which would provide guidance for therapeutic options, monitor patients during treatment, and indicate progression or relapse. Although the majority of patients are initially sensitive to platinum (Pt)-based chemotherapy, many of them eventually relapse within 18 months with the emergence of progressive, resistant disease. The ability to achieve an accurate surveillance strategy to predict patients'

response to chemotherapy and risk of progression continues to constitute an unmet medical need, as standard serum biomarkers, such as CA-125, have proven to have limited sensitivity and specificity (1). To date, Pt-free interval, the measure of time between the end of first-line chemotherapy and relapse, is the only and widely accepted empirical parameter to predict sensitivity to second-line chemotherapy (1). Therefore, novel and reliable molecular biomarkers, which represent the intrinsic molecular complexity of HGS-EOC, are needed to guide second-line therapy and monitor its efficacy. With the rapid progress of high-throughput sequencing and liquid biopsy approach, plasma-based circulating tumor DNA (ctDNA) analysis has emerged as a noninvasive alternative to profile and monitor tumor evolution over time. ctDNA is defined as the fraction of tumor-released DNA fragments in the pool of circulating free DNA (cfDNA) in the plasma and other bodily fluids (2, 3). ctDNA is shed by the tumor cells as a result of apoptosis, necrosis, and active secretion (4), and it can be detected and quantified in cfDNA by tumor-specific genetic alterations. ctDNA has been extensively studied in neoplasms of the colorectum and breast and in melanoma, to understand clonal evolution and uncover genetic determinants of drug resistance (5–10). Moreover, total levels of ctDNA were found to be correlated with tumor burden (10, 11) and capable of anticipating response to anticancer therapy, rendering them a reliable biomarker for cancers of the lungs, colorectum, and skin (12–14).

In contrast to the cancer types mentioned above, HGS-EOC is not characterized by a high prevalence of somatic point mutations, with the exception of clonal *TP53* alterations. Instead, it harbors multiple recurrent chromosomal gains and losses (15, 16). In this setting, we have demonstrated previously the presence of two specific amplified regions in chromosome *3q26.2* and *8q24.3* as clonal events in 90% of HGS-EOCs (17). Although patients with HGS-EOC have detectable

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Translational Relevance

The genome of high grade serous epithelial ovarian cancer (HGS-EOC) is characterized by somatic copy-number alterations that is the prerequisite for the use of untargeted assessment of DNA tumor fraction (TF) in the patients' plasma. In this retrospective, proof-of-principle study, we demonstrated that shallow whole-genome sequencing (sWGS) is an innovative approach to recapitulate the main genomic features of tumor heterogeneity in the plasma of patients with HGS-EOC and thus, can be developed for future clinical decision making. At baseline, the plasma TF percentage was found to be an independent prognostic marker of progression-free survival. Longitudinal monitoring of plasma TF anticipated time of relapse better than the conventional serum biomarker, CA-125, and radiological assessment. Finally, plasma analysis by sWGS allowed to track those genomic evolutionary changes that occurred under treatment and reflect response to therapy and disease progression.

levels of ctDNA (18), the use of liquid biopsy in the clinical setting is currently limited when compared with other tumors, as assessment of region-wide copy-number alterations by ctDNA analysis is more challenging than investigating single hotspot mutations. In addition, high cost and extensive turnaround times render the use of genome-wide sequencing protocols unsuitable for routine implementation and repeated serial sampling over time in the clinical setting.

Recently, genome-wide profiling of copy-number instability (CNI) in solid or liquid biopsy by low-coverage whole-genome sequencing [WGS; shallow WGS (sWGS), $0.1 \times -0.5 \times$] has emerged as a suitable method to quantify chromosomal instability and predict therapeutic response in HGS-EOC, as well as in several other cancers (7, 8, 19). However, the dynamics of how these chromosomal instabilities evolve during Pt-based chemotherapy and follow-up have thus far not been described.

This retrospective study aimed at exploiting ctDNA-based liquid biopsy with sWGS as a noninvasive, inexpensive, and high-throughput tool to study the genomic features of HGS-EOC at the time of diagnosis (T0) and for longitudinal monitoring of disease recurrence. Furthermore, we investigated the effects of first-line therapy on tumor fraction (TF) and CNI in plasma, evaluating their association with patients' survival. Finally, we longitudinally explored tumor heterogeneity after treatment by tracking tumor clonal evolution in serial plasma samples and correlating ctDNA levels with those of the standard serum marker, CA-125.

Materials and Methods

Patient selection

A retrospective cohort of 46 patients with histologically confirmed diagnosis of HGS-EOC was selected by convenience sampling from the Pandora tumor tissue collection located at Istituto di Ricerche Farmacologiche Mario Negri, IRCCS (Milano, Italy). Case selection was performed as depicted in the CONSORT diagram reported in Supplementary Fig. S1, according to the following criteria: (i) confirmed diagnosis of HGS-EOC, (ii) one or more matched plasma samples withdrawn before any treatment and/or at different moments during patient's follow-up (T1, T2, T3, and T4), and (iii) availability of CA-125 levels at T0 and during patients' clinical monitoring. For 41 patients (89%) one or more snap-frozen tumor biopsies obtained at

T0, and thus naïve to chemotherapy, were additionally available. All patients were treated with standard first-line chemotherapy based on six cycles of carboplatin (6 AUC) and taxol (175 mg/m²) before (neoadjuvant chemotherapy, NACT) or after (adjuvant chemotherapy, CT) primary debulking surgery (PDS). As routine clinical practice, all patients were followed both with CA-125 levels every 3 months and computed axial tomography (CAT) scan once a year. CAT scan was anticipated in those cases where CA-125 levels raised or there was a clinical suspect of relapse.

All tumor samples were collected according to a signed informed patient consent prior to surgery, which included their use for genomics profiling.

The study was performed following the principles of the Declaration of Helsinki, the scientific ethical committee of "Brienza" approved the collection and usage of tumor, blood, and plasma samples (N° 1065, on November 10, 2015, emended on February 22, 2018). Written informed consent was obtained from all patients enrolled in the study.

Tumor DNA isolation, plasma preparation, and cfDNA quantification

Tumor DNA (tDNA) was purified from snap-frozen tumor tissues according to the protocol previously standardized in our laboratory (20). Using EDTA Vacutainer Tubes (BD Biosciences), a single tube with 10 mL of whole blood was collected from each patient and kept at 4°C until further processing. Blood samples were collected at T0 and at different timepoints during chemotherapy treatment and follow-up, and plasma fraction was isolated within 24 hours after blood collection through a double-step centrifugation, as follows: 10 minutes at $1,600 (\pm 150) \times g$ at 4°C, followed by 10 minutes at $3,000 (\pm 150) \times g$ at 4°C. The plasma obtained was stored at -80°C. cfDNA was extracted starting from 1 mL of plasma using the Maxwell RSC cfDNA Plasma Kit (Promega) on automatic Purification System (Maxwell, Promega) according to the manufacturer's instructions. cfDNA concentration was measured by Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and its size distribution was evaluated using the TapeStation System Analyzer (Agilent Technologies).

Limit of detection calculation

To test the sensitivity of our analysis (defined below as limit of detection, LOD) we used two approaches. First, we produced a synthetic *in silico* dilution of a sample with known TF by mixing normal reads with tumor reads to generate samples with expected ctDNA content (100%, 50%, 25%, 10%, 5%, 2.5%, and 1%). Precision, recall, and F-score were estimated for each synthetic dilution, both for the whole genome, as well as for the two minimal common regions of gain in chromosomes 3q26.2 and 8q24.3. Next, a sequencing experiment was designed with the same setting described for the *in silico* one. Serial dilutions of cfDNA derived from the same patient spiked into the cfDNA, extracted using the same procedure, of the same healthy donor, in the same proportions, were sequenced. Five nanograms of each dilution were used for library preparation. Precision, recall, and F-score were estimated. Details are reported in the Supplementary Materials and Methods, section S5.

Determination of ctDNA content in cfDNA by sWGS

cfDNA (5–40 ng) and 150 ng of tDNA were used for sWGS analysis. Library constructions of cfDNA and tDNA were performed using the SeqCap EZ HyperCap (Roche), using an automatic liquid handling solution (Bravo B, Agilent Technologies). Libraries were pooled after barcoding procedure and sequenced using 150-bp paired-end runs on NextSeq500 Platform (Illumina) to average genome-wide fold

coverage of $0.5 \times$ (~5 million reads/sample). sWGS reads were aligned to the reference genome (hg38) with BWA (21). Afterwards, an *in silico* size selection method was applied to the reads as described previously (22), by excluding all reads mapping to sequences (“query”) smaller than 90 bp or larger than 180 bp. Prior to sWGS copy-number analysis, the optimal binning size was set to 500 kbp after power calculation (https://gmacintyre.shinyapps.io/sWGS_power/). Somatic copy-number alterations (SCNA) and TF for each sample were detected with ichorCNA (<https://github.com/broadinstitute/ichorCNA>; ref. 23), and manual curation of results was done. As a reference, pooled data from the eleven control samples generated previously were used. Detailed bioinformatics pipeline is described in Supplementary Materials and Methods, section S4. In brief, ichorCNA uses relative sequencing coverage of sWGS data and computational correction of sequencing bias to detect segmental copy-number changes, and a probabilistic framework to estimate, without *a priori* information, TF and copy-number calls. Data have been loaded to the European Genome-Phenome Archive (ID EGAS00001004670).

Statistical analyses

Correlation between SCNA profiles in plasma and synchronous lesions at T0 and longitudinally was estimated with Pearson correlation index (Supplementary Materials and Methods, section S10). Differences among TF and CNI values across time windows and patient groups were assessed with Kruskal–Wallis and Mann–Whitney tests (Supplementary Materials and Methods, section S10). Kaplan–Meier curves with log-rank test and multivariate Cox proportional hazard model (adjusted for age and residual tumor) were used to assess the association of CNI and TF with survival (progression-free survival, PFS; Supplementary Materials and Methods, section S10). Binomial test was used to compare GISTIC call frequencies between plasma and tissue samples and between plasma at T0 and post-treatment (Supplementary Materials and Methods, section S6). All the analyses were performed using R (v.4.0.2) and Prism Software (version 8, GraphPad). TF increase in longitudinal sample was set as the timepoint with a 20% increase compared with the one with the lowest TF for each patient (Supplementary Materials and Methods, section S10).

Results

Cohort description

A total of 185 plasma samples and 109 matched snap-frozen tumor biopsies collected from 46 patients with histologically confirmed diagnosis of HGS-EOC were included in this study. The main clinical, pathologic, and demographic characteristics, as well as therapy responses are summarized in **Table 1**. In detail, patients were classified as Federation Internationale des Gynaecologistes et Obstétristes (FIGO) stage III–IV, and the median age at diagnosis was 62 years (range, 21–81 years), with a median follow-up time of 2 years. Ten of 46 patients (22%) carried *BRCA1/2* pathogenic germline mutations. Patients underwent debulking surgery before CT ($n = 22$, 48%) or after NACT ($n = 24$, 52%). All patients enrolled in this study received first-line chemotherapy, 33 patients (71%) received a second-line chemotherapy, and 20 (43%) patients received more than two lines of chemotherapy. Thirty-four patients experienced relapse and 5 patients had progression of disease during first-line chemotherapy. In particular, 24 of 46 cases (52%) relapsed after 12 months from the end of first-line chemotherapy, and thus were referred to as Pt sensitive. Ten of 46 cases (22%) were in progress during chemotherapy or within 6 months from the end of first-line chemotherapy, and thus

Table 1. Clinical, pathologic, and demographic annotations.

Annotations	No. of patients (%)
Number of patients	46
Median age (years)	62
Age range (years)	21–81
Follow-up time (range) years	2 (0–4)
Histologic type	
Serous	46 (100)
FIGO classification	
III NA	–
III A	–
III B	1 (2)
III C	29 (62)
IV	16 (36)
Pt status	
Sensitive	24 (52)
Partially sensitive	10 (22)
Resistant	5 (11)
Refractory	5 (11)
NA	2 (4)
Chemotherapy	
NACT	24 (52)
Adjuvant chemotherapy (CT)	22 (48)
Line of chemotherapy	
I	46 (100)
II	33 (71)
III	20 (43)
BRCA status	
<i>gBRCA1</i> mut	7 (15)
<i>gBRCA2</i> mut	3 (7)
<i>BRCA</i> wt	13 (28)
NA	23 (50)
CA-125 levels at T0	
CA-125 < 35 U/mL	2
CA-125 > 35 U/mL	38
Interquartile range	264–2581
Total number of biopsies	109
Average biopsies per patient (range)	2 (0–6)
Total number of plasma samples	185
Average plasma samples per patient (range)	4 (2–8)

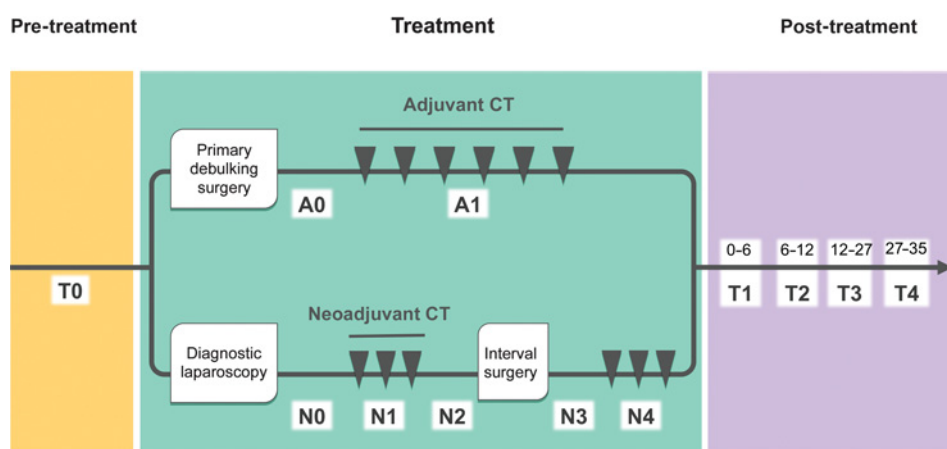
Note: Summary of main clinical, pathologic, and demographic characteristics of patients enrolled in the study.

Abbreviations: Mut, mutant; wt, wild-type.

were defined as Pt-refractory/resistant cases. Ten of 46 cases (22%) relapsed between 6–12 months from the end of first-line chemotherapy, and thus were considered Pt partially sensitive. For two of 46 cases (4%) data were not available, as these patients were not monitored at the same hospital during their follow-up. Serum protein tumor marker, CA-125, was routinely quantified during the treatment course (upper normal cutoff ≤ 35 U/mL; **Table 1**).

Study workflow

Given the retrospective design of the study, longitudinal plasma samples were collected at nonoverlapping timepoints. To make molecular analyses comparable in terms of timepoints and more flexible use of this tool in future prospective clinical trials, samples were grouped into three different “time windows” (**Fig. 1**): (i) “Pre-treatment:” 41 patients had tumor biopsies or plasma samples collected at T0, and were thus naïve to chemotherapy. Of these, only 35 cases had paired ctDNA and tDNA sample ($n = 95$, with two to six synchronous lesions available per patient; Supplementary Table S1). Analysis in this set of

**Figure 1.**

Study design. To render longitudinal plasma analysis comparable, the timeline of sample collection was divided into three different temporal windows: (i) “Pre-treatment:” plasma and matched tumor biopsies were collected at T0, before chemotherapy. (ii) “Treatment:” plasma samples were obtained at different timepoints, after PDS (A0), during first-line chemotherapy (A1), or at different timepoints during NACT protocol (N0–N4). (iii) “Post-treatment:” plasma samples were collected at different time points (months), during patients’ clinical monitoring after first-line Pt-based chemotherapy (T1–T4).

samples evaluated the degree of concordance of genomic alterations between plasma ctDNA and paired tDNA. (ii) “Treatment:” 37 patients had one ($n = 17$) or more ($n = 20$) plasma samples obtained after PDS (A0), during CT (A1), or during the NACT protocol (N0–N4). These samples were used to investigate early effects of Pt-based therapy on overall ctDNA quantity and quality. (iii) “Post-treatment:” 75 longitudinal serial plasma samples were obtained from 26 patients during patients’ surveillance after first-line Pt-based chemotherapy (T1–T4). These samples were used to: (i) verify whether the sWGS approach was able to monitor and anticipate the emergence of relapse and (ii) understand some molecular features of relapsed disease and the effects of Pt-based therapy on the evolution of tumor genomic heterogeneity.

Definition of LOD and assessment of genomic concordance between ctDNA and tDNA at T0

ichorCNA and GISTIC algorithms were used to: (i) reconstruct SCNAs profiles from sequencing data (see Supplementary Materials and Methods section), (ii) establish the percentage of TF within the ctDNA fraction, and (iii) evaluate the percentage of ctDNA affected by CNV. Serial dilution experiments (Supplementary Table S2) defined a TF of 0.25 as the lower LOD of our approach. Using this threshold, 106 of 108 selected plasma samples (98%) resulted to be suitable for downstream analysis.

Focusing on the “pre-treatment” time window (Fig. 1), we initially explored the extent to which the molecular characteristics identified in plasma ctDNA represented HGS-EOC tumor heterogeneity.

The genomic landscape, graphically plotted in Supplementary Fig. S2, highlights extensive copy-number aberrations (both gains and losses) across all chromosomes of the whole genome in plasma and tissue samples of all patients. Using a 500 kbp bin resolution across the genome, concordance analysis revealed that 56.83% of SCNAs (range, 24.3%–91.2%) were common between tDNA and paired ctDNA (Fig. 2A), while the percentages of SCNAs exclusive to tDNA and ctDNA were 25.96% and 14.47%, respectively. The complete list of concordance levels for each case is detailed in Supplementary Table S1.

As tumor spatial heterogeneity can affect the percentage of overlaps between ctDNA and tDNA, we focused further analysis on two genomic regions, namely 3q26.2 and 8q24.3, reported previously to occur in the early steps of malignant transformation of HGS-EOCs, and due to their clonal origin, are shared by all synchronous lesions (17). GISTIC algorithm (99% confidence level and a 5% FDR cutoff) listed the 35 most recurrent genomic regions, with different

length size (ranging from 3.34 to 57.36 Mbp), affected by gain (red squares) or loss (green squares) of genomic material in both tDNA (Fig. 2B) and ctDNA (Fig. 2C). Analysis in The Cancer Genome Atlas database revealed that 33 of the 35 genomic regions (94.28%) were with *bona fide* genomic hallmark of HGS-EOC (Supplementary Table S4). Of these, gains on chromosome 3q26.2 and 8q24.3 were those most frequently observed in tDNA (97% and 89%, respectively; Supplementary Table S3) and in ctDNA (89% and 78%, respectively; Supplementary Table S3). Eleven of 33 genomic regions were significantly less frequently observed in ctDNA than in tDNA (Fig. 2B and C; Supplementary Table S3). A representative case exemplifies the results described here (Supplementary Fig. S3). The vast majority of chromosome alterations observed in solid biopsies (namely A, B, and C), including gain in cytobands 3q26.2 and 8q24.3, were found in their paired plasma sample (PL-1), with a high correlation level ($r = 0.90$ for A vs. PL-1; $r = 0.64$ for B vs. PL-1; and $r = 0.74$ for C vs. PL-1; $P < 0.001$). In conclusion, at T0, ctDNA is likely to contain a wide representation of the genomes from multiple synchronous lesions of HGS-EOC and ctDNA analysis by untargeted approaches, like sWGS, may recapitulate the main features of tumor genome.

Effect of first-line therapy on the amount of TF and CNV

Focusing on ctDNA samples collected within the “treatment” window, we explored the early effects of primary surgery and Pt-based chemotherapy on the percentage of TF and CNV. Data reported in Supplementary Fig. S4 show that TF and CNV in patients who received CT decreased progressively during the course of treatment similarly to patients who received NACT. Considering the CT subgroup, the median percentages of TF at T0 and A0 were 2.13% and 1.05%, respectively ($P < 0.01$; Supplementary Fig. S4A), while the median CNV percentage value decreased from 59% at T0 to 33% at A1 ($P < 0.01$; Supplementary Fig. S4C). Similarly, in the NACT group, the percentage of TF decreased progressively from 2.24% at T0 to 1.24% at N1 ($P < 0.05$; Supplementary Fig. S4B) and the CNV median percentage value decreased from 54.9% at T0 to 37% at A1 ($P < 0.01$; Supplementary Fig. S4D).

Furthermore, when comparing the entire SCNA profile of ctDNA samples collected within the “therapeutic window” with their corresponding tissue samples at T0, we observed a decrease in correlation levels (Pearson correlation index ranging from 0.25 to 0.45, Table 1; Supplementary Fig. S4). In conclusion, as TF and CNV values change during therapy, ctDNA analysis enables detection of the fluctuations in tDNA dynamics that occur under selective pressure of surgery and Pt-based chemotherapy.

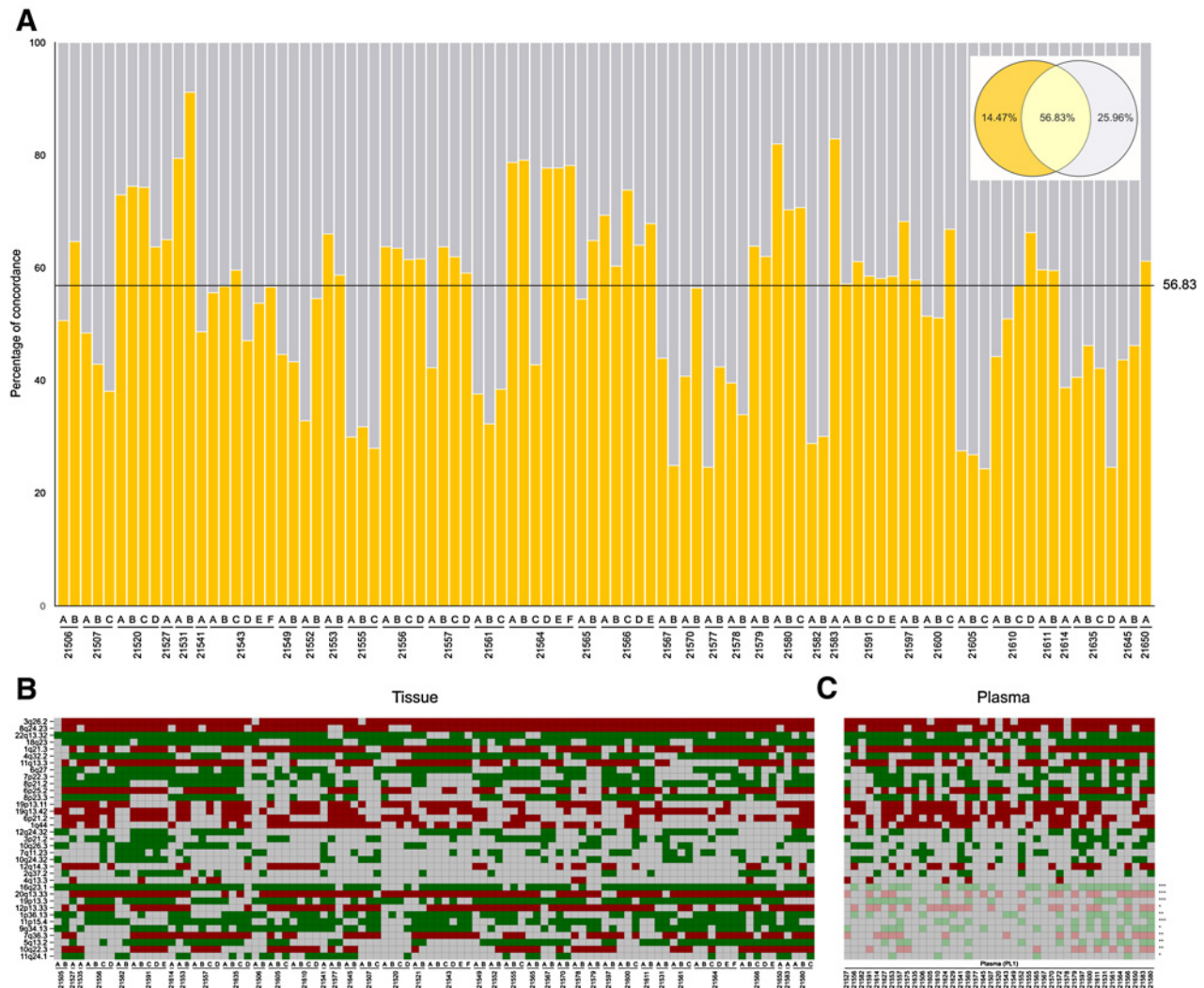


Figure 2.

SCNA pattern analysis between tDNA and ctDNA at T0. **A**, Bar chart showing the percentage of concordance of copy-number calls (y-axis) for each patient (x-axis) between each tissue biopsy (yellow bars) and matched ctDNA sample (gray bars). For each patient, the single tissue biopsy is named as reported in Supplementary Table S1. Solid line reports the median percentage of concordance across the entire cohort. Venn diagram showing the percentage of common, private to tissue, and private to plasma calls (insert). **B** and **C**, Heatmaps depicting the 35 cytobands found by GISTIC in gain (red square) or in loss (green square) in tissue and plasma samples at T0. Cytobands are reported on the y-axis and patient ID is reported on the x-axis. Dimmed colors in **C** refer to regions that were significantly (FDR < 0.1) less frequent in plasma samples than in tissue. Gray squares are neutral. For each cytoband, the difference in frequency between ctDNA and tDNA is reported as follows: *, FDR < 0.1; **, FDR < 0.05; ***, FDR < 0.001.

ctDNA levels at baseline reflect patients' survival

We next wished to find out whether TF and CNI values might be associated with Pt response and/or survival. To address the former issue, we compared the average levels of TF and CNI in 24 Pt-sensitive cases with those observed in 10 Pt-refractory/resistant and 10 Pt partially sensitive cases. Box plots (Supplementary Fig. S6) showed an overall comparable distribution of CNI and TF levels among the three groups. Next, we used univariate and multivariate survival models to assess the effects on PFS of demographic characteristics (age), clinical features (residual tumor), and the percentage of TF and CNI at T0. Overall survival was not analyzed as data were not available at the time of this study.

Kaplan–Meier curves show that the CNI parameter was not statistically associated with PFS ($P = 0.16$; Fig. 3A), whereas the

percentage of TF quantified in patients' plasma at T0 was associated with PFS (Fig. 3B). A TF level of 6.5% was identified as the best cutoff to stratify patients into cohorts with significantly different prognoses ($P = 0.02$). Patients with low TF (i.e., lower than 6.5, $n = 32$) demonstrated superior PFS (18.4 months) than patients with high TF (i.e., higher than 6.5, $n = 8$; 14.7 months). Multivariate analysis, including demographic characteristics and clinical features, confirmed a statistically significant association of TF amount measured in plasma samples with survival in terms of PFS (HR, 3.31; 95% confidence interval, 1.33–9.13; $P = 0.011$; Supplementary Table S5). Overall, although the lack of an independent validation set suggests that this data have to be considered with caution, at T0, patients with low TF in their plasma had significantly longer PFS compared with patients with high TF.

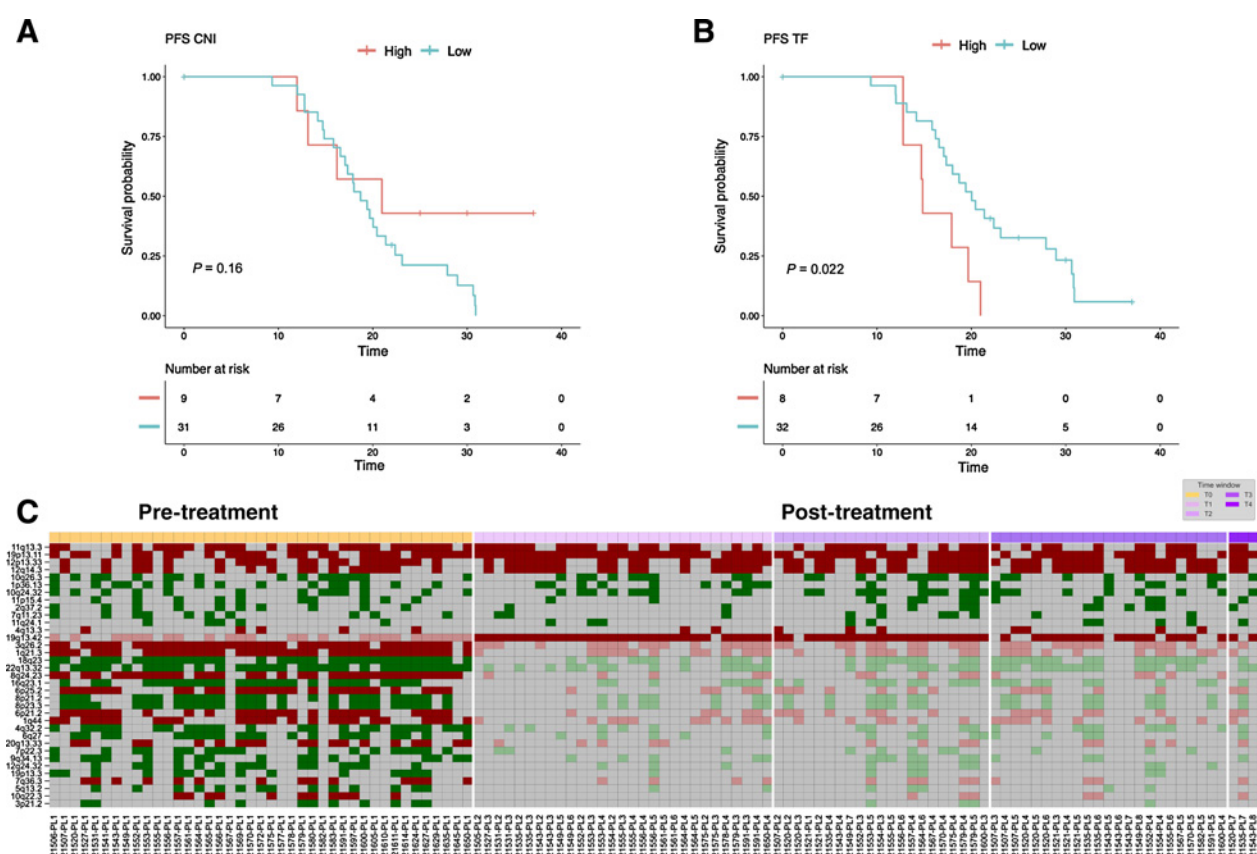


Figure 3.

Patients' survival analysis and dynamic changes in the tumor heterogeneity of ctDNA. Kaplan–Meier survival curves together with log-rank test P values describe the association between PFS and CNI (**A**) and TF (**B**) measures. The risk table below each curve shows the numbers of patients at risk at each timepoint. **C**, Heatmap describes the most recurrent 35 cytotbands identified by GISTIC analysis in the plasma ctDNA collected at T0 (“pre-treatment,” orange line, top left) or during follow-up after Pt-based chemotherapy of Pt-sensitive and Pt partially sensitive patients (“post-treatment,” purple line, top right). Red square indicates gain in copy-number variation for a specific locus/cytoband; green square depicts copy-number loss for a specific locus/cytoband; and gray square indicates no change (neutral). The names of plasma samples are shown on the x-axis. On the left side of the y-axis, the main cytotbands are reported. Dimmed colors refer to regions that were significantly less/more frequent in post-treatment samples than in pre-treatment samples or vice versa (*, FDR < 0.1; **, FDR < 0.05; ***, FDR < 0.005).

Clonal evolution after treatment shows heterogeneous pattern of genomic amplifications

Dynamic changes in the clonal heterogeneity composition of HGS-EOC is a critical therapeutic challenge confounding treatment of relapsed disease. We used sWGS data generated in serial plasma samples withdrawn before (PL-1) and after first-line Pt-based chemotherapy (PL-2 to PL-8; **Fig. 3C**) to monitor the evolution of the SCNA profile in Pt-sensitive and Pt partially sensitive patients, as the Pt-refractory/resistant cases ($n = 10$; see Supplementary Table S1) did not had plasma samples withdrawn in the post-treatment window. Using a 500-kbp bin resolution, we observed that the genomic landscape composition of post-treatment plasma samples was less heterogeneous than that reported in paired pre-treatment samples (**Fig. 3C**). Although we found the same 35 cytotbands identified previously at T0 (**Fig. 2C**), their frequency in the post-treatment plasma samples was different. In particular, 12 of 35 (34%) recurrent regions had comparable frequency levels between samples in the “pre-” and “post-treatment” time windows. Of these, cytotbands *11q13.3* and *19p13.11* were those more frequently identified (Supplementary Table S6). Twenty-two of 35 regions (62%) were significantly less frequently called in the “post-treatment” than in the “pre-treatment” samples (proportion test FDR < 0.1). Cytoband *19q13.42* was the only cytotband more frequently found in the “post-treatment”

(86.84%) than in the “pre-treatment” group (60.98%; Supplementary Table S6). Supplementary Fig. S7 shows the main genes in cytotbands *11q13.3*, *19p13.11*, and *19q13.42* that according to published work could play a key role in driving tumor relapse. The complete lists of genes are shown in Supplementary Tables S7–S9.

Serial TF analysis in ctDNA outperforms CA-125 in predicting patient relapse

Next, additional exploratory analyses were performed to assess the benefit of longitudinal monitoring of ctDNA by sWGS to anticipate the emergence of relapse after first-line Pt-based chemotherapy. In clinical practice, an increase in serum levels of CA-125 (>35 U/mL) is the current gold standard to trigger suspicion of disease progression. Here, we applied TF analysis to ctDNA samples collected after first-line treatment until the time of radiological progression (“post-treatment” time window) from 19 patients who had detectable ctDNA profiles at T0, and for whom post-treatment samples were available (**Figs. 4 and 5**; Supplementary Figs. S8–S12; Supplementary Table S1; “longitudinal data” section). **Figures 4 and 5 (A and B)** show four paradigmatic cases with detailed long-term clinical descriptions. In these patients, TF and CA-125 values measured longitudinally during treatment paralleled and anticipated tumor dynamics as assessed by radiological imaging.

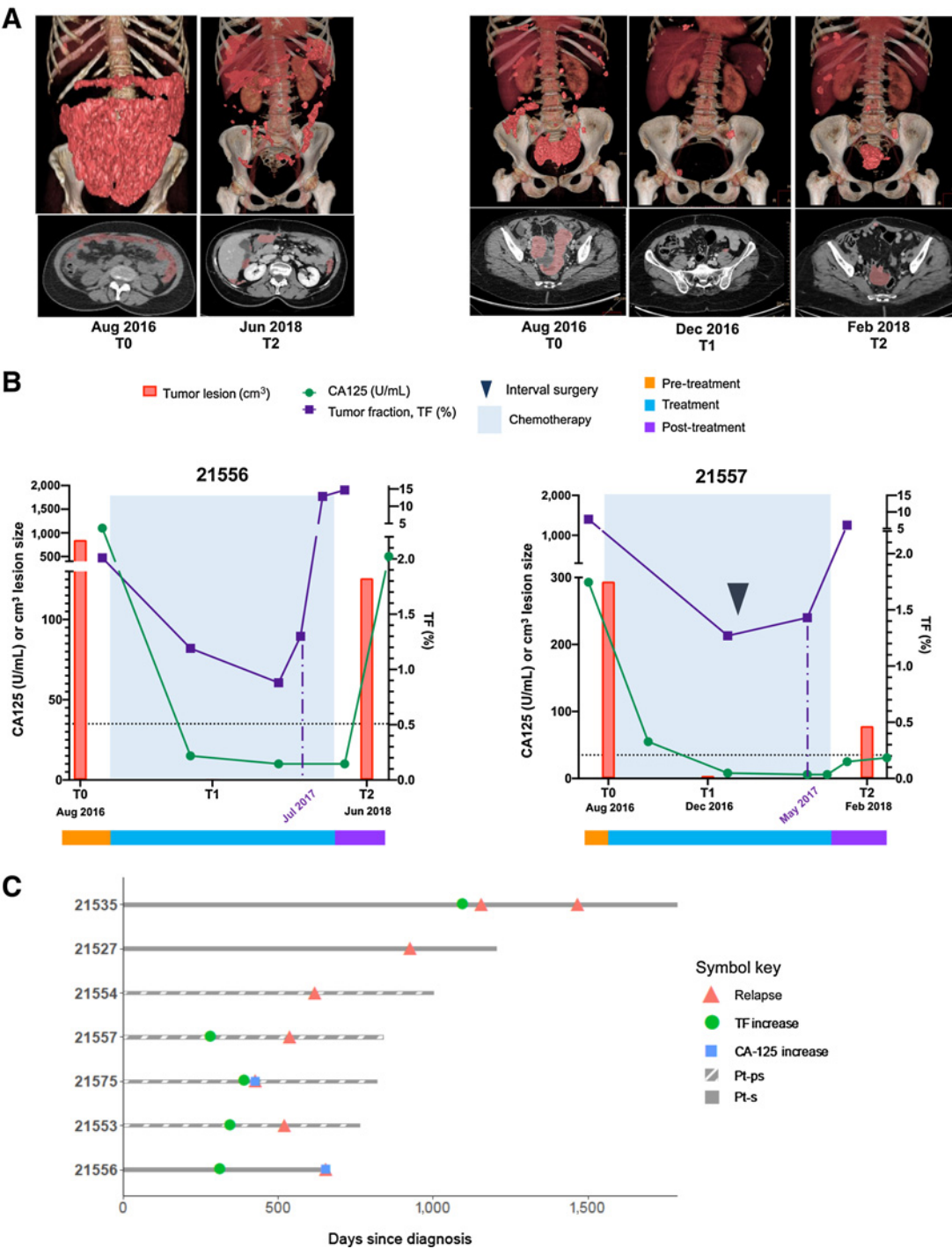
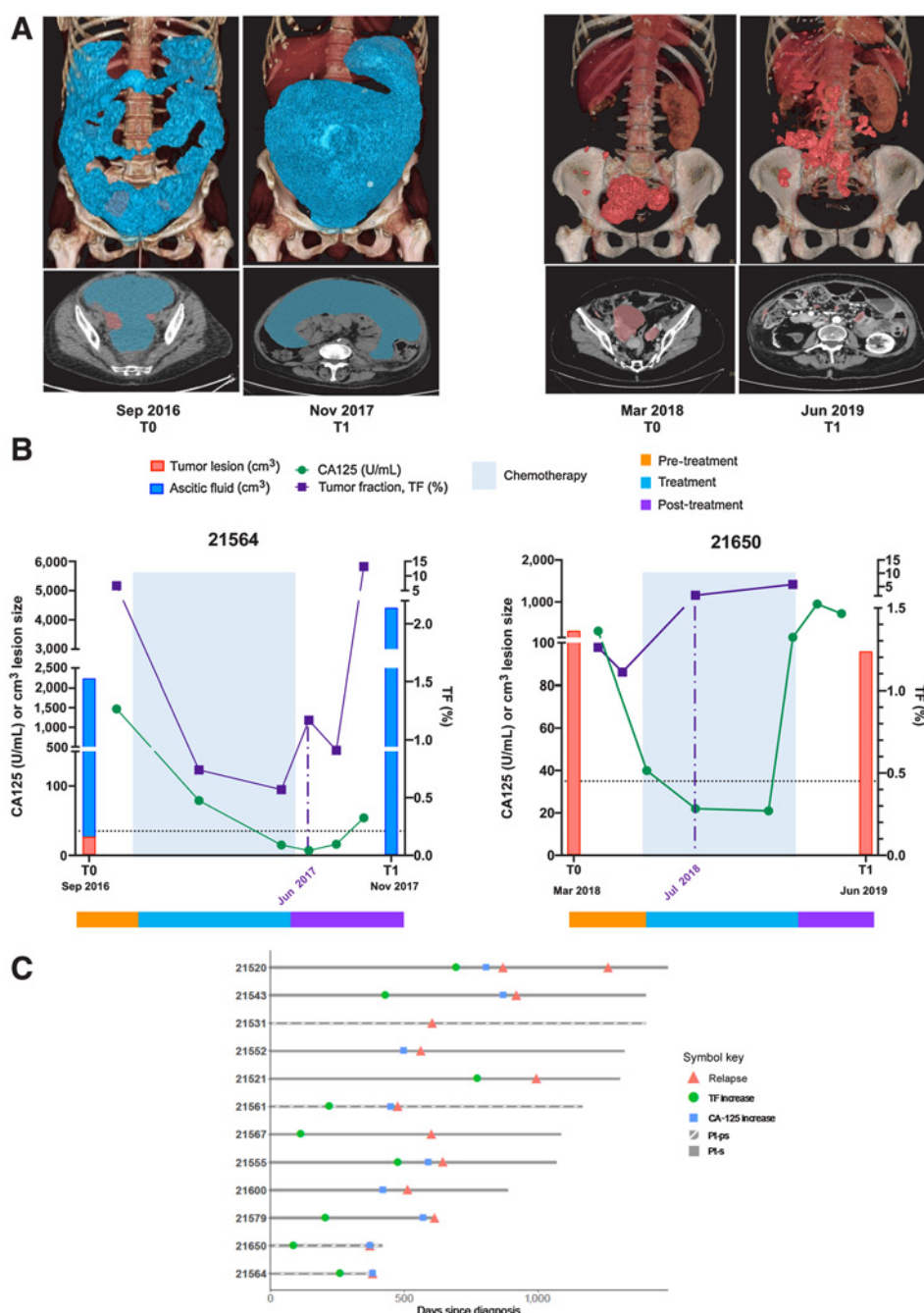


Figure 4. Longitudinal monitoring of ctDNA levels during clinical disease course in NACT patients. The levels of ctDNA for two patients with HGS-EOC receiving NACT protocol is shown in relation to the patient's clinical history, in months from primary diagnosis. **A**, Radiological images (CAT scan) with 3D reconstruction of the tumor mass spread in the abdominal cavity (red masses) at T0 and at different timepoints (T1 and T2). **B**, The TF% in cfDNA is reported for each plasma sample withdrawn against the CA-125 levels. Red bars refer to the tumor volume calculated from CAT analysis as described in Supplementary Materials and Methods. **C**, Swimmer plots. For each of the seven NACT patients analyzed, a horizontal line connects the T0 of HGS-EOC to the last follow-up (days). Solid or dashed lines denote the type of response as Pt sensitive (Pt-s) or Pt partially sensitive (Pt-ps), respectively. The symbols indicate the point in time of relapse, and the timepoints where an increase of the TF or the CA-125 might be an indication of recurrence, as shown in the legend.

**Figure 5.**

Longitudinal monitoring of ctDNA levels during clinical disease course in CT patients. The levels of ctDNA for two patients with HGS-EOC receiving CT protocol is shown in relation to the patients' clinical history, in months from primary diagnosis. **A**, Radiological images (CAT scan) with 3D reconstruction of the tumor mass (red, solid tumor, blue ascites) spread in the abdominal cavity at T0 and at relapse (T1). **B**, The TF% in cfDNA is reported for each withdrawn sample against the CA-125 levels. Red bars refer to the tumor volume calculated from CAT scan analysis as described in Supplementary Materials and Methods. **C**, Swimmer plots. For each of the twelve CT patients analyzed, a horizontal line connects the T0 to the last follow-up (days). Solid or dashed lines denote the type of response as Pt sensitive (Pt-s) or Pt partially sensitive (Pt-ps), respectively. The symbols indicate the point in time of relapse, and the timepoints where an increase of the TF or the CA-125 might be an indication of recurrence, as shown in the legend (data are reported in Supplementary Table S1, "longitudinal data" section).

Across all cases, we observed that while CA-125 levels dropped below the threshold of 35 U/mL after treatment, plasma ctDNA was persistently detectable over time, indicating the presence of resistant tumor lesions. Currently, there is no universally accepted parameter to establish a significant level of increase in TF. Therefore, in line with the RECIST 1.1 criteria, which consider an increase of 20% in the sum measurement of tumor target lesions as parameter to define the disease progression (24), we set an increase of 20% over baseline as cut-off point to determine a significant increase in TF. Our results show that an increase in TF predicts radiological recurrence more accurately than CA-125 values do, anticipating clinical and radiological progression as long as 8 months prior to its occurrence (range, 1–16; Figs. 4 and 5C).

Details of two cases (21556 and 21557; Fig. 4A and B) may be instructive. These patients received treatment according to the NACT protocol because of presence of unresectable disease. Both presented with complete responses to chemotherapy (assessed during the interval debulking surgery, after three or four cycles of chemotherapy) and relapsed after 14 and 8 months, respectively. In the first case, ctDNA levels anticipated the CA-125 increase by 11 months. In the second case, ctDNA was detectable throughout chemotherapy, while CA-125 decreased to normal levels without an increase at relapse. Therefore, in this case, the persistence of disease was anticipated by ctDNA analysis 8 months prior to disease manifestation. Cases 21564 and 21650 (Fig. 5A and B) underwent upfront cytoreductive surgery and received

six cycles of adjuvant chemotherapy. Levels of CA-125 were above the cutoff in both cases at T0 and decreased to below normal after three and two cycles, respectively. CAT scan at the end of adjuvant chemotherapy was performed for patient 21564, but not for patient 21650, where complete response was defined only by CA-125 levels. Both patients were classified as Pt resistant, as both relapsed 6 months after chemotherapy. Unlike CA-125 levels or radiological imaging, concentrations of ctDNA anticipated relapsed disease 4 and 9 months before it occurred. When cases were considered together, in almost 70% cases (five/seven treated with CT and nine/12 with NACT), a 20% increase in the ctDNA TF predicted clinical radiological progression 176 days (mean, range:18–272) prior to its manifestation, rendering this measure superior to conventional CA-125 levels (Figs. 4C and 5C for NACT and CT cases, respectively).

Discussion

In this study, we demonstrate for the first time that, TF amount in baseline plasma ctDNA is an independent prognostic marker of disease relapse in HGS-EOC, and that, unlike CA-125 levels, longitudinal monitoring of ctDNA can anticipate disease progression of a few months. Furthermore, we found that ctDNA profiling accurately represents the spatial and temporal heterogeneity of tumor biopsies. Our results provide proof of principle that sWGS analysis of ctDNA can potentially be employed in the clinic for the longitudinal monitoring of tumor evolution.

The use of blood-based ctDNA has recently provided a noninvasive tool to molecularly profile patients' tumors over time (25). A number of studies in metastatic breast cancer, colorectal cancer, and lung cancer (26–29) have shown the potential of ctDNA analysis to predict prognosis and treatment response to anticancer drugs, as well as to investigate the dynamic evolution of tumor heterogeneity. Next-generation sequencing technologies (e.g., targeted sequencing, whole-exome sequencing, and WGS) have so far allowed the precise and highly sensitive quantification of genomic variants in ctDNA (26, 30, 31).

In the HGS-EOC setting, only few studies have evaluated the potential of ctDNA analysis as a prognostic biomarker (19, 32, 33). From a biological standpoint, HGS-EOC is characterized by SCNAs that permeate the entire genome and by the presence of a clonal pathogenic variant in the *TP53* gene as an early event toward malignancy. As many studies have demonstrated, tracking changes in the frequency of ctDNA-mutant alleles of single pathogenic variants represent an ideal biomarker to monitor disease in real time (2, 19, 32–34).

However, tracking changes on a single hotspot may not be sufficient to capture and understand the complexity of the highly unstable genome of HGS-EOC. Furthermore, *TP53* alterations are almost always patient exclusive, and thus they cannot be determined without the use of upfront exome-wide analysis of tumor tissue. Assessing the SCNAs profile in ctDNA has long been challenging because yield and fraction of tumor-derived cfDNA vary substantially. However, recent studies using both qPCR-based protocols and sWGS demonstrate the feasibility of using somatic chromosomal rearrangements to identify plasma ctDNA, suggesting that SCNA in ctDNA samples might be a useful tool to discriminate between patients with benign adnexal masses and those with malignant ones (35, 36).

Moreover, sWGS has been used to detect and quantify TF in plasma ctDNA (23), offering an unbiased and unique high-throughput opportunity to study the evolution of genome-wide copy-number alterations in cfDNA of patients with HGS-EOC. Serial analysis of plasma samples from baseline to the end of follow-up allowed us to infer some biological features of ctDNA and to define the extent to which the

genomic landscape derived from ctDNA represents the spatial and temporal heterogeneity of tumor biopsies, especially when these alterations may be derived from unprofiled or not resected tumor lesions localized in the abdominal cavity.

In this study, using an *in silico* size selection strategy and dedicated algorithms, we estimated and monitored the percentage of TF in plasma ctDNA obtained from patients with HGS-EOC by sWGS. Overall, our results suggest that copy-number profiles of tumor biopsies at T0 are well represented by their liquid derivative and that chemotherapy might have influenced the clonal composition of relapsed tumors with the emergence of newly frequent cytobands, like *19q13.42*, while cytobands *11q13.3* and *19p13.11* were comparable between pre- and post-treatment samples. These data drive two important considerations: first, they are consistent with the hypothesis that an upfront therapy may suppress the outgrowth of tumor clones sensitive to Pt-based therapy, reducing the genomic heterogeneity of relapsed disease as compared with the primary tumor, naïve to chemotherapy and second, these findings clearly show ongoing clonal evolution in patients' tumor as therapy is being administered and suggest the emergence of new altered regions that can be used as future biomarkers for patient's monitoring after Pt-based drugs, even when matched solid biopsies are not available. Although a detailed description of the functional roles of the coding and noncoding genes (miRNA) mapped in these cytobands is beyond the scope of this work, some biological information could be gained from the ctDNA analysis of sWGS data. Of the three previously selected cytobands, gain on chromosome *11q13.3* is largely described in the literature as a genomic feature of various solid tumors, like breast (37, 38), esophageal squamous cell (39), prostate (40), and head and neck cancer (41). Many genes involved in the control of cell cycle (*CCND1*), apoptosis (*FADD* and *BAD*), DNA repair (*EMSY* and *XRR1*), folate receptors (*FOLR1*, *FOLR2*, and *FOLR3*), angiogenesis (*VEGFB*), as well as a novel miRNA with oncogenic function (*miR-548*; ref. 39), are located in this region and their overexpression is compatible with the common features of tumor cells that sustain growth and proliferation of both primary and relapsed disease. Among the different genes located on cytoband *19p13.11*, the *BRD4* gene is of particular interest for its potential therapeutic role. As a member of the BET family, *BRD4* has largely been studied in the past as a molecular target of a new class of small-molecule inhibitors (42). If confirmed, the finding that, *BRD4* overexpression is a feature of relapsed disease, could pave the way for novel therapeutic approach of relapsed disease on ovarian cancer. Differently, the emergence of cytoband *19q13.42* after Pt-based chemotherapy is a novel finding, with no previous data ever reported in the literature. Many zinc finger and long noncoding genes are located in this region, among which oncomiRNAs *miR-371*, *miR-372*, *miR-520*, and *miR-521*, and the *AURKC* gene are of potential interest to define the biology of relapsed disease (43–46).

All patients in our cohort had detectable baseline ctDNA levels and remained ctDNA positive during longitudinal plasma measurements, with no clearance of the ctDNA from their plasma. This finding indicates the presence of persisting micrometastases despite treatment, which is not adequately detected by a rise in CA-125 levels or imaging evaluations. In fact, all patients enrolled in this study experienced relapse after first-line Pt-based therapy, and inevitably died because of disease progression (data not shown). Remarkably, we found that an increase in the TF frequency during the continuous patient monitoring throughout and after therapy anticipated disease progression better than CA-125 levels. Moreover, as for patients with metastatic triple-negative breast cancer, we found that the amount of baseline TF (>6.5%) was an independent prognostic marker of short relapse (47).

The main weaknesses of this proof-of-concept study are its retrospective nature, the limited sample size, and the lack of a second, independent validation cohort. Nevertheless, its unique strength is that it might constitute a promising harbinger of new possibilities for disease monitoring and prognosis. Future clinical trials need to be designed with large sample size cohorts to estimate the sensitivity and accuracy of this approach, as well as to independently validate the prognostic significance of TF at baseline. In particular, monitoring ctDNA during treatment could allow a more objective evaluation of the response than CA-125 levels, thus optimizing the therapeutic strategy, as well as the duration of chemotherapy. This could be of outmost importance in the maintenance setting with PARP inhibitors or with bevacizumab. It seems noteworthy that the lower sensitivity of the sWGS analysis compared with other suitable methods is counterbalanced by its high-throughput nature, speed, and cost effectiveness. Furthermore, it is applicable to all patients with HGS-EOC without previous assessment of patients' specific probes, such as hotspot mutational analysis of *TP53* gene by droplet digital PCR. Although it is merely speculative at this moment, we expect that SCNA plasma analysis in ctDNA will become informative for longitudinal monitoring of the homologous recombination status, in those patients that are currently receiving PARP inhibitor treatment, and thus are at high risk of relapse. Studies are still ongoing, trying to overcome technical limitations to define plasma SCNA signature in ctDNA related to homologous recombination status.

In conclusion, this study demonstrates that the use of sWGS for serial monitoring of the SCNA landscape in plasma of patients with HGS-EOC provides a marker of relapse that anticipates disease progression several months earlier than the standard serum marker, CA-125. Furthermore, sWGS analysis provides information of new biological features characterizing disease relapse. Its relatively low cost, short turnaround time (2 weeks including library preparations, sequencing, and data analysis), and sensitivity render sWGS of plasma analysis a tantalizing, but realistic potential application in future clinical trials in patients with HGS-EOC, as it could allow the optimization and tailoring of adjuvant therapy and maintenance treatment for every patient.

Authors' Disclosures

L. Beltrame reports personal fees from Eli Lilly outside the submitted work. No disclosures were reported by the other authors.

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