Profiling cancer gene mutations in longitudinal epithelial ovarian cancer biopsies by targeted next-generation sequencing: a retrospective study

L. Beltrame¹,†, M. Di Marino¹,†, R. Fruscio³,‡, E. Calura⁵, B. Chapman⁶, L. Clivio¹, F. Sina³, C. Mele², P. Iatropoulos², T. Grassi³, V. Fotia⁷, C. Romualdi⁵, P. Martini⁵, M. Noris², L. Paracchini¹, I. Craparotta¹, M. Petrillo³, R. Milani³, P. Perego⁴, A. Ravaggi⁹, A. Zambelli¹⁰, E. Ronchetti¹¹, M. D’Incalci¹,‡,§* & S. Marchini¹,§

Departments of ¹Oncology; ²Molecular Medicine Laboratory, Immunology and Genetic of Rare Diseases and Organ Transplantation, Centro di Ricerche Cliniche per le Malattie Rare “ALDO e CELE DACCO”; ³IRCCS ‘Mario Negri’ Institute for Pharmacological Research, Milano; ⁴Clinic of Obstetrics and Gynecology; ⁵Department of Pathology, University of Milano-Bicocca, San Gerardo Hospital, Monza; ⁶Department of Biology, University of Padova, Padova, Italy; ⁷Bioinformatics Core, Harvard School of Public Health, Boston, USA; ⁸PhD Program in Experimental Medicine, University of Pavia, Pavia; ⁹Gynecologic Oncology Unit, Catholic University of the Sacred Heart, Rome; ¹⁰Division of Gynecologic Oncology, Angelo Nocivelli Institute of Molecular Medicine, University of Brescia, Brescia; ¹¹Unit of Medical Oncology, Azienda Ospedaliera Papa Giovanni XXIII, Bergamo; ¹²Laboratory of Experimental Oncology and Pharmacogenomics, IRCCS Salvatore Maugeri Foundation, Pavia, Italy.

Received 2 September 2014; revised 2 March 2015; accepted 17 March 2015

Background: The majority of patients with stage III–IV epithelial ovarian cancer (EOC) relapse after initially responding to platinum-based chemotherapy, and develop resistance. The genomic features involved in drug resistance are unknown. To unravel some of these features, we investigated the mutational profile of genes involved in pathways related to drug sensitivity in a cohort of matched tumors obtained at first surgery (Ft-S) and second surgery (Sd-S).

Patients and methods: Matched biopsies (33) taken at Ft-S and Sd-S were selected from the ‘Pandora’ tumor tissue collection. DNA libraries for 65 genes were generated using the TruSeq Custom Amplicon kit and sequenced on MiSeq (Illumina). Data were analyzed using a high-performance cluster computing platform (Cloud4CARE project) and independently validated.

Results: A total of 2270 somatic mutations were identified (89.85% base substitutions 8.19% indels, and 1.92% unknown). Homologous recombination (HR) genes and ATM, ATR, TOP2A and TOP2B were mutated in the majority of Ft-S, while TP53 and PTEN were mutated in the entire dataset. Only 2% of mutations were conserved between matched Ft-S and Sd-S. Mutations detected at second surgery clustered patients in two groups characterized by different mutational profiles in genes associated with HR, PI3K, miRNA biogenesis and signal transduction.

Conclusions: There was a low level of concordance between Ft-S and Sd-S in terms of mutations in genes involved in key processes of tumor growth and drug resistance. This result suggests the importance of future longitudinal analyses to improve the clinical management of relapsed EOC.

Key words: NGS, epithelial ovarian cancer, matched tumor biopsies

introduction

Epithelial ovarian cancer (EOC) is generally sensitive to first-line chemotherapy, and the vast majority of patients respond to platinum (Pt)-based therapy after debulking surgery. Unfortunately, the median progression-free survival (PFS) lasts only 18 months, and more than 80% of Pt-responsive patients relapse with a disease that progressively becomes Pt-resistant. They tend to die within 5 years from diagnosis [1]. Tumor stage, debulking status and Pt-free interval are the main prognostic parameters used in the clinical management of EOC, while the molecular characteristics correlated with patient outcome and response to therapy are unknown. Studies carried out so far in large cohorts of patients at both the genetic and epigenetic levels, failed to provide reliable predictive biomarkers to improve patient stratification, or genomic alterations critical for the development of a targeted approach against EOC [2]. With the exception of bevacizumab,
there are currently no targeted biological therapies approved for treatment of ovarian carcinomas. Recently, treatment of EOC with PARP inhibitors (PARPi) has been considered based on homologous recombination (HR) status [3–5]. One reason for the scarcity of therapeutic options is the complex and heterogeneous molecular nature of EOC [6]. In addition, there is no molecular information available on tumor biology at relapse, as second debulking surgery for EOC is carried out in only a small fraction of patients who relapse. Thus, we currently do not know whether the molecular scenario connected with tumor relapse mirrors that in tissues obtained at primary surgery. Furthermore, the molecular drivers associated with relapse and resistance are unknown. This lack of knowledge intimates the urgent need to evaluate time-dependent proximal tumor markers for second-line chemotherapy in EOC, in analogy to the situation in breast and lung cancer [7–10].

To this end, 33 EOC patients from whom matched longitudinal biopsies were available were selected for the study, and the genomic evolution of 65 genes known to be involved in tumor progression and therapy response were investigated by next-generation sequencing (NGS) approach.

materials and methods

patient cohort

A cohort of 33 EOC patients with matched primary surgery (Ft-S) and second surgery biopsies (Sd-S) was selected from the 'Pandora' tumor tissue collection, settled at 'Mario Negri' institute. The study has been carried out following the principles of the Declaration of Helsinki; the local scientific ethical committees approved the collection and usage of tumor samples. Written informed consent was obtained from all patients.

target re-sequencing libraries and massively parallel sequencing

Sequences for selected 65 genes were generated using TrueSeq amplicon panel (TSCA, Illumina, Palo Alto, CA) as described in supplementary Section 1, available at Annals of Oncology online. Quantified libraries were sequenced on the MiSeq platform (Illumina) using the 2 × 150 bp configuration (300 cycles) and run on V2 sequencing flow cell. Details are reported in supplementary Section 2, available at Annals of Oncology online.

results

patient cohort

Target sequencing analysis was carried out on a cohort of 33 patients with matched Fs-S and Sd-S biopsies. Histological and clinical parameters at diagnosis (Table 1) show that all cases, except one, presented with advanced stage EOC, of whom 75% were high grade (HG, 25 of 33) and 25% low grade (LG, 8 of 33). The most frequent histotype was serous (n = 24, 72.7%), although endometrioid (n = 4, 12.1%), mucinous (n = 2, 6.1%), mixed (n = 2, 6.1%) and clear-cell histotypes (n = 1, 3%) were included. The mean age at diagnosis was 56 years with a mean follow-up of 4.5 years. Table 2 summarizes the clinical information including therapeutic strategies and response to therapy during the follow-up, according to response evaluation criteria in solid Tumors (RECIST) criteria [11]. Specimens were removed from ovaries at Ft-S, and from the peritoneal cavity (omentum or peritoneum) at Sd-S. While Pt-based therapy used to be the mainstay for EOC until recently, second line therapy options have been more varied. Therefore, our Sd-S collection is less homogeneous than the Ft-S collection (Table 2). Detailed information is reported in supplementary Table S3.1, available at Annals of Oncology online.

The workflow depicted in Figure 1 shows the details of our targeted re-sequencing approach and the two objectives of the study: (i) defining the evolution of the genomic architecture from Ft-S to Sd-S and (ii) defining the level of concordance in matched biopsies.

somatic variant call database

Our pipeline of analysis (supplementary Section 2, available at Annals of Oncology online) identified 2270 somatic variants functionally classified as single nucleotide polymorphism, both synonymous and nonsynonymous, indel or variant of unknown significance (VUS) for noncoding mutations mapping in intergenic regions, UTR or canonical splicing sites (Table 3). Variants were with a wide inter/intraindividual variation: the average of somatic mutations per patient is 62.03 ± 40.6 at Ft-S and 37.72 ± 51.16 at Sd-S, with individual variation ranging from 25 to 178 at Ft-S and 4–253 at Sd-S (Table 3). Seven hundred fifteen were nonsynonymous, 245 synonymous, 100 indels and 1210 as VUS (Table 3). Synonymous mutations were then excluded from the analyses. Supplementary Figure S3.1, available at Annals of Oncology online, shows that the number of transitions and transversions at Ft-S and Sd-S are comparable, while indels were more frequent in Ft-S than in Sd-S (Table 3, supplementary Figure S3.1, available at Annals of Oncology online).

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Patient characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical annotations</strong></td>
<td>No. of patients, N (%)</td>
</tr>
<tr>
<td><strong>Histotypes</strong></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>24 (72.72)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>4 (12.12)</td>
</tr>
<tr>
<td>Mixed</td>
<td>2 (6.06)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>2 (6.06)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>1 (3.04)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>II A</td>
<td>1 (3.03)</td>
</tr>
<tr>
<td>III A</td>
<td>1 (3.03)</td>
</tr>
<tr>
<td>III B</td>
<td>4 (12.12)</td>
</tr>
<tr>
<td>III C</td>
<td>21 (63.64)</td>
</tr>
<tr>
<td>IV</td>
<td>6 (18.18)</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>25 (75.75)</td>
</tr>
<tr>
<td>Low</td>
<td>8 (24.25)</td>
</tr>
<tr>
<td><strong>Relapsing</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>33 (100)</td>
</tr>
<tr>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td><strong>Mean age [min–max] (years)</strong></td>
<td>56 [28–79]</td>
</tr>
<tr>
<td><strong>Mean follow-up [min–max] (years)</strong></td>
<td>4.5 [1–20]</td>
</tr>
<tr>
<td><strong>Total number of patients</strong></td>
<td>33</td>
</tr>
</tbody>
</table>

Table summarises the main clinical and histological data of patients enrolled in the study. NA, not available.
neutrally selected (<1) [12]. The NS/S ratio was >1 suggesting genes have been under positive selection pressure (>1) or were pressure on amino acid replacement mutations for protein-versus synonymous mutations (NS/S) as a measure of selective pressure (online). We next calculated the ratio between nonsynonymous versus synonymous mutations (NS/S) as a measure of selective pressure on amino acid replacement mutations for protein-coding genes. NS/S ratio defines whether mutations affecting genes have been under positive selection pressure (>1) or were neutrally selected (<1) [12]. The NS/S ratio was >1 suggesting that mutations were selected under positive pressure, probably by chemotherapy at Sd-S or by tumor outgrowth at Ft-S. Unsupervised cluster analysis revealed a complex and heterogeneous architecture with poor association between matched Ft-S and Sd-S (supplementary Figure S3.2, available at Annals of Oncology online).

**mutation profile at Ft-S**

The complex and heterogeneous results reported in supplementary Figure S3.3, available at Annals of Oncology online, confounded the strategy to use unsupervised cluster analysis to stratify Ft-S biopsies based on genomic mutation profiles. The heatmap in Figure 2A shows supervised stratification based on the morphological and clinical features. In line with the TGCA data [4], high-grade serous ovarian cancer (HGS-EOC) are characterized by mutations in the HR pathway (13 of 19 cases, 68%) and in the TP53 gene (11 of 19 cases, 58%). Considering the other histological parameters, mutations in TP53 were not counted in low grade serous epithelial ovarian cancer (LGS-EOC), while HR mutations were observed in both HG-EOC (two mixed, two endometroid and one clear cell) and LG-EOC (four serous and one endometroid). KRAS mutations were called in eight of 33 cases (24.2%), independently of tumor grade and histology, while BRAF mutations were restricted to HG-EOC. Bars on the left side of the picture describe the total number of mutations counted for each gene. Genes associated with DNA damage signaling (ATM and ATR), drug resistance (TOP2A and TOP2B), ECM interactions (FN1, COL3A1 and VIM), EMT transcription factor (ZEB1) and signal transduction (FGFR2 and IGFR1) were those with the highest number of mutations (Figure 2A). Almost 70% of Ft-S tumors (23 of 33) harbored somatic inactivating mutations in at least one gene belonging to the HR pathway. Compared with patients with no mutations in the HR pathway (Figure 2A), these biopsies harbor a great number of mutations in all the other pathways investigated. To allow deeper insight into whether the clonal architecture organization of somatic variants affects genes directly or indirectly involved in the HR pathway, the complete list of variants called in genes belonging to the HR core and DNA damage signaling pathways are shown (supplementary Table S3.2, available at Annals of Oncology online). Signature validation confirmed the robustness of our analysis although droplet digital PCR data had a different sensitivity for mutations with allelic fraction (AF) ranging from 1% to 4% (supplementary Table S4.3, available at Annals of Oncology online). Closer inspection revealed that ATM/ATR variants were very common in all patients, with median AF above 30% (Figure 2B), while the median AF for the BRCA1/2 gene ranges from 0.1% to 4% (Figure 2B). These results suggest that tumor biopsies comprise populations of cells containing largely identical somatic alterations in ATM/ATR genes, underlining the importance of moving beyond the classical BRCA1/2 analysis to correlate HR defects to treatment response.

**matched Ft-S and Sd-S mutations**

The second aim of our study was to identify concordant mutations. Figure 3A shows that clonal structure at relapse is more homogeneous than at Ft-S, as the number of somatic events,
mainly indels (red bars), largely exceeded those counted at Sd-S (paired t-test, \( P < 0.0001 \)). In Table 3, the term ‘shared’ defines those somatic loci found mutated in both Ft-S and in Sd-S, but not necessarily in matched biopsies. Somatic loci found mutated in matched biopsies have been defined as ‘concordant’ (Table 3). Fifty-five loci across 21 genes are shared. Of these, 36 loci (65.4%) were concordant. At 200× of coverage, the concordance rate was 2.2% (supplementary Table S3.3, available at Annals of Oncology online). To exclude that sequencing depth could bias this result, the concordance rate for variants with AF \( \geq 1\% \) was analyzed at 1000×, with comparable results (supplementary Table S3.3, available at Annals of Oncology online). By increasing the AF

---

**Table 3. Somatic mutations in Ft-S and Sd-S biopsies**

<table>
<thead>
<tr>
<th>Tumor biopsy</th>
<th>Somatic mutations per patient</th>
<th>Interindividual range</th>
<th>Nonsynonymous mutations</th>
<th>Synonymous mutations</th>
<th>Insertions/deletions</th>
<th>VUS</th>
<th>Total</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ft-S</td>
<td>62.03 ± 40.60</td>
<td>25–178</td>
<td>350</td>
<td>121</td>
<td>8</td>
<td>556</td>
<td>1107</td>
<td>≥200×</td>
</tr>
<tr>
<td>Sd-S</td>
<td>37.72 ± 51.16</td>
<td>4–253</td>
<td>345</td>
<td>122</td>
<td>8</td>
<td>633</td>
<td>1108</td>
<td></td>
</tr>
<tr>
<td>Shared (concordant)</td>
<td>–</td>
<td>–</td>
<td>20 (11)</td>
<td>2 (2)</td>
<td>12 (11)</td>
<td>21 (12)</td>
<td>55 (36)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>–</td>
<td>–</td>
<td>715</td>
<td>245</td>
<td>100</td>
<td>1210</td>
<td>2270</td>
<td></td>
</tr>
</tbody>
</table>

Table summarizes the number of somatic mutations. Somatic mutations are functionally classified into nonsynonymous, synonymous, insertions/deletions; alterations in intergenic regions, splicing sites and UTRs are classified as VUS. Somatic mutations present in unmatched Ft-S and Sd-S samples are defined as ‘shared’. Concordant mutations are those called in at the same genetic locus, in matched Ft-S and Sd-S biopsies.
threshold, we observed a slight increase in concordance rate (AF $\geq 3\%$, 13.04%; AF $\geq 10\%$, 25%–27%, $P < 0.01$, supplementary Table S3.3, available at *Annals of Oncology* online), independently of the coverage ($P = 0.48$, supplementary Section 3, available at *Annals of Oncology* online). These data suggest that the low concordance rate between matched Ft-S and Sd-S is due to the high number of tumor clones with less representative variants. Supplementary Table S3.4, available at *Annals of Oncology* online, lists all concordant point mutations and indels, and their predicted effects on protein function, while supplementary Tables S4.1 and S4.3, available at *Annals of Oncology* online, show the results of independent validation assays. The heatmap reported in Figure 3B
Figure 3. (A) Mutational load. The diagram describes for each patient, the mutational load measured at Ft-S and Sd-S. Mutations are categorized according to their predicted effect. Blue bars are nonsynonymous and VUS. Red bars indels. Green bars, synonymous mutations; (B) Concordant somatic mutations. Heat map showing the distribution of concordant somatic mutations. Mutations are reported for each patient in a false color scale; gray boxes indicate wt sequence. Color bars in the upper part show information as reported in Table 1: histotype (orange, serous; green, endometroid; gray, mixed; yellow, clear cell; blue, mucinous) and grade (red, high grade; green, low grade). Genes are grouped into pathways, depicted by color palette, as described in supplementary Table S1.2, available at Annals of Oncology online.
shows the distribution of the 36 concordant mutations across the 33 patients. As far as early events in HGS-EOC carcinogenesis are concerned, TP53 mutations were the most frequently observed (7 of 19 patients). A cluster of three LG-EOC (20 738, 20 783, 20 821) and one HGS-EOC (20 788) are characterized by concordant mutations in genes BRCA1, BRIP1, PIK3CA, PTEN, AKT2, mTOR, and DROSHA. Pathways concerning HR, and cell signaling through PI3K-AKT-mTOR were the main ones harboring concordant mutations.

**mutation profile at Sd-S**

Considering the follow-up, analysis of the cohort of patients resistant to primary Pt-therapy showed that their Sd-S biopsies had drug resistance traits, although the data for one patient was unavailable (Table 2). Ten out of 25 patients initially sensitive to first-line chemotherapy presented Sd-S biopsies with resistance attributes, while seven were sensitive. Data for eight patients were unavailable (Table 2 and supplementary Table S3.1, available at Annals of Oncology online). It is difficult to draw any conclusion from these data as to the impact of genetic variants on survival or on the development of resistance, although some correlations were observed (supplementary Section 5, available at Annals of Oncology online). Different from supplementary Figure S3.3, available at Annals of Oncology online, unsupervised cluster analysis at Sd-S revealed two main clusters, MI (14 patients, 42.4%) and MII (19 patients, 57.6%), with intermingled histological and clinical parameters, but with a different mutational landscape (Figure 4A). Statistical analysis confirmed that cluster MII is enriched in patients mutated in genes belonging to the HR (P = 0.057, supplementary Table S3.5, available at Annals of Oncology online), the PI3K signaling (adjusted P = 0.057, supplementary Table S3.5, available at Annals of Oncology online) and miRNA biogenesis pathways (adjusted P = 0.063, supplementary Table S3.5, available at Annals of Oncology online). Group MI was mainly enriched in patients mutated in signal transduction pathway genes (adjusted P = 0.057, supplementary Table S3.5, available at Annals of Oncology online). Box plots shown in Figure 4B depict these differences. Stacked plots in supplementary Figure S3.4, available at Annals of Oncology online, depict the number of mutations in each gene in cluster MI and MII, respectively, for selected pathways. The low AF ratio for variants in genes belonging to the HR and DNA signaling pathways measured at Sd-S, suggests that, at relapse, tumor biopsies comprise few populations of cells sharing the same alterations (supplementary Figure S3.5 and supplementary Table S3.2, available at Annals of Oncology online). It is not known whether these are new mutations acquired after initial therapy or were present at very low levels already at diagnosis and then selected for recurrence.

**discussion**

The two main findings of our target re-sequencing study are that somatic mutations show a low rate of concordance between primary and recurrent disease, and that the genomic architecture of relapsed disease is less heterogeneous than that of the primary disease. For the clinical management of HGS-EOC, assessing the temporal stability of somatic mutations emerges as a relevant issue. This is in particular the case for genes belonging to the HR pathway, which is now recognized as companion diagnostic tool for the treatment using PARPi [3, 4]. After one or more courses of Pt-based therapy, the decision to treat relapsed disease with PARPi is based on the evaluation of BRCA1/2 status made at primary surgery, when patients are naïve to therapy. What was not yet known is whether these somatic mutations are stable over time, and whether other genes beyond BRCA1/2 could be relevant for therapeutic decisions related to PARPi. The few studies reported so far are inconsistent as far as this issue is concerned. Discrepancies are related to the different technologies used, genes selection criteria, the small size of patients enrolled in the study, as well as the non-uniform time points in the treatment settings.

As to the finding that there is low concordance between primary and recurrent disease, our data are consistent with those obtained by Pennington et al. They analyzed a panel of 30 genes (i.e. BROCA test) in a cohort of 23 matched primary and recurrent pairs of EOC patients [13, 14]. As an early driver event in tumor carcinogenesis, TP53 mutations were found to be more stable over time compared with somatic mutations affecting the HR pathway. In general, they observed a relatively low rate of concordance.

In contrast, Castellarin et al. found that 89% of mutations found by exome sequencing of EOC tumor cells harvested from the ascites of three relapsed patients were already present in matched primary tumors. The authors speculated that recurrent tumors arise from selective persistence or outgrowth of pre-existing tumor clones with accumulated new mutations [15]. The reason for this discrepancy is unclear but it might be that cancer cells present in the ascites are not necessarily representative of the tumor cells present in metastatic nodules. The therapies received by the three patients reported by Castellarin were similar to those given to our patients, as they all included carboplatin and taxol.

A third study reported in a case a high level of concordance between primary and relapsed disease, taken after 42 months, suggesting that populations with stable clonal structure are a feature of long survivorship patients [16].

As to the second result- the genomic architecture of relapsed disease is less heterogeneous than that of the primary disease-the reduced mutational load observed at Sd-S (Figure 3A) means that most of the somatic mutations called at Ft-S have been under negative selection during multiple rounds of chemotherapy. In other words, Pt-based regimes have killed a large proportion of clones present in the tumor cell population, independent of variance ratios. This issue is exemplified by the ATM/ATR genes. In all Ft-S cases of our series, we found loss of functional mutations in the ATM/ATR genes, with variance ratio above 30%, and very seldom at recurrence. This result suggests that mutations in these genes confer high sensitivity to Pt-based therapy, as cells that expressed the mutated genes were killed by treatment. From a therapeutic perspective, specific inhibitors of these two kinases, currently under development, could be used as potentially useful drugs [17].

Secondary results worthy of discussion are: (i) patients with LG-EOC could also benefit from treatment with PARPi; (ii) other genes than BRCA1/2 could be used for therapeutic decisions as to treatment with PARPi. It has been assumed up to now that HGS-EOC with germline and somatic BRCA1/2
Figure 4. (A) Mutation profile at Sd-S. Hierarchical clustering of somatic mutations called at Sd-S groups patients into two distinct clusters, named MI and MII. Mutations are reported for each patient in a false color scale; gray boxes indicate wt sequence. Genes are grouped into pathways, depicted by color palette, as described in supplementary Table S1,2, available at Annals of Oncology online. Color bars in the upper part show information at diagnosis as reported in Tables 1: grade (red, high grade; green, low grade) and histotype (orange, serous; green, endometroid; gray, mixed; yellow, clear cell; blue, mucinous). (B) Pathway analysis at Sd-S. Bar plots show the % of patients found mutated at Sd-S, in at least one gene of the selected pathways. MI and MII correspond to clusters reported above.
gene alterations in the primary EOC tumor fails to re-therapy. Better chance to be effective in second-line than in directly against topoisomerase II such as anthracyclines have a mutated at Sd-S than at Ft-S. This result suggests that drugs damage signaling, such as pan in every HR gene, as well as in genes belonging to DNA function of the HR core pathway [18]. In contrast to the current management of EOC.

In our study, we identified loss of function mutations in our panel in every HR gene, as well as in genes belonging to DNA damage signaling, such as ATM/ATR, that indirectly affect the function of the HR core pathway [18]. In contrast to the current dogma that loss of function mutations in BRCA1/2 increases the response rate to PARPi, our data intimate that other genes can phenocopy the effects of BRCA1/2 mutations. This conclusion suggest that biomarkers of responsiveness to PARPi should not only include analysis of BRCA1/2.

Finally, the TOP2A and TOP2B genes were less frequently mutated at Sd-S than at Ft-S. This result suggests that drugs directed against topoisomerase II such as anthracyclines have a better chance to be effective in second-line than in first-line therapy.

In conclusion, our results suggest that the status of major gene alterations in the primary EOC tumor fails to reflect adequately the status at relapse. Albeit the results are preliminary, they represent proof of principle for the use of longitudinal bi-opsies in addition to companion diagnostic tools in the clinical management of EOC.

acknowledgements
Nerina and Mario Mattioli Foundation, ACTO Foundation, Cloud4CARE program, Italian Association of Medical Oncology (AIOM) foundation. We thank Loris Bernard, IE0-Cogentech, Milan, Italy, for technological advices and methodological discussion and Prof. Andreas Gescher (Leicester, UK) for editing the manuscript.

funding
This work was supported by Italian Association for Cancer Research (grant number IG15177 to SM; AIRC fellowship no. 14982 to EC), and Cariplo Foundation (grant number 2013-0815 to SM and CR).

disclosure
The authors have declared no conflicts of interest.

references