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# Feasibility of tumor testing for BRCA status in high-grade serous ovarian cancer using fresh-frozen tissue based approach

Claudia Marchetti [a], Angelo Minucci [c], Marco D'Indinosante [a,b], Raffaella Ergasti [a,b], Martina Arcieri [a,b], Ettore Domenico Capoluongo [d], Antonella Pietragalla [a], Chiara Caricato [b], Giovanni Scambia [a,b,*], Anna Fagotti [a,b]

[a] Department of Woman, Child and Public Health, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy
[b] Catholic University of the Sacred Heart, Rome, Italy
[c] Molecular and Genomic Diagnostics Laboratory, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy
[d] Università Federico II-CEINGE, Biotecnologie Avanzate, Napoli, Italy.

## HIGHLIGHTS

- Fresh-frozen-tissue (FFT) based BRCA screening workflow is feasible and reliable
- The concordance of tumor test *versus* germline BRCA test was 86.3%.
- FFT-screening led us to identify 6% of patients with tumor BRCA mutation only, who might be eligible for PARPi treatment

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## ABSTRACT

*Objective.* For many years, BRCA mutational status has only been considered as a predictor of ovarian cancer susceptibility and as a prognostic factor. Nonetheless, in the era of precision medicine, it has also become a predictive biomarker of response to platinum-based-chemotherapy and, more recently, to PARP-inhibitors, also in the frontline setting.
We assessed the feasibility of a fresh frozen tissue-based-BRCA-screening workflow in a tertiary referral center.

*Methods.* We consecutively enrolled a series of 456 newly diagnosed FIGO-Stage IIIC-IV, high grade serous-ovarian cancer patients. All patients receiving tumor-biopsy underwent tBRCA-testing.

*Results.* Clinically relevant tissue-BRCA (tBRCA) variants were observed in 145 women (31.8%), particularly we recognized 89 (61.4%) patients with BRCA1-pathogenetic variants (PVs) and 56 women (38.6%) with BRCA2-PVs. Among 292 tBRCA wild-type (wt) patients, 88 cases were germline BRCA tested (gBRCA) and 86 (97.8%) were confirmed as gBRCAwt, while 1 (1.1%) had gBRCA variant of uncertain significance and 1 had gBRCA mutation (1.1%).
The concordance of tumor test *versus* germline BRCA test was 86.3% (209/242).
Large genomic rearrangements (LGRs) were suspected in 13/292 tBRCAwt patients (4.5%) by using bioinformatic algorithm and multiplex ligation-dependent probe amplification (MLPA) was performed, with evidence of PVs in only 1 case.

*Conclusions.* Fresh-frozen tissue-based BRCA screening workflow is feasible and reliable. It allows to enlarge the BRCA mutated population that might receive PARPi with the greatest benefit, without missing cascade testing for family members and therefore, maintaining its preventive role.



## 1. Introduction

Ovarian cancer (OC) is the third most common gynecological malignancy and the leading cause of mortality from cancer among women [1]. Women who carry a *BRCA* pathogenic variant (PV) are at highest risk of developing OC, with a lifetime risk up to 44% by 80 years of age [2].

For many years, *BRCA* mutational status has only been considered as a predictor of ovarian cancer susceptibility and as a prognostic factor. Nonetheless, in the era of precision medicine, it has also become a predictive biomarker of response to platinum-based-chemotherapy and, more recently, to PARP inhibitors, also in the frontline setting [3]. For these reasons, prompt identification of *BRCA* carriers is mandatory.

* Corresponding author at: Department of Women's and Children's Health, Fondazione "Policlinico Universitario A.Gemelli"-IRCCS, Largo Agostino Gemelli 8, 00168 Rome, Italy.
*E-mail address:* giovanni.scambia@policlinicogemelli.it (G. Scambia).

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Traditionally, the *BRCA* testing has been performed on peripheral blood for the detection of constitutional PVs, showing that approximately 20–25% of patients with high grade serous ovarian cancers (HGSOC) have a chance of carrying a germline PV (gPV) in *BRCA* genes [4,5]. Recently, an ASCO statement clearly established [6] that each woman with epithelial ovarian cancer should have germline *BRCA (gBRCA)* testing.

On the other side, tissue PVs (tPVs) in *BRCA1* and *BRCA2* have recently been found in approximately 3.5%–8.5% HGSOC tissue samples, without an underlying germline PV [7–10]. Importantly, also patients with tumor *BRCA (tBRCA)* PVs show to benefit from treatment with PARPi [11].

Therefore, *BRCA* testing on formalin-fixed paraffin-embedded (FFPE), which permits the simultaneous assessment of both somatic and germline PVs, is becoming of increasing diagnostic and clinical importance in HGSOC patients. Many laboratories, which have adopted Next Generation Sequencing (NGS) technologies for *gBRCA* testing, are now applying this technology for *tBRCA* testing [12,13], thanks to its ability to detect a somatic variant, even if present at low percentage [14]. However, FFPE tissues may undergo extensive degradation and chemical modification of DNA resulting from formalin fixation and paraffin embedding [14]. Indeed, up to 3–5% of gPVs can be missed when *tBRCA* testing on FFPE samples is performed [15,16]. More recently, fresh frozen tissue (FFT) has been proposed as an alternative procedure [17,18] for *tBRCA* testing thanks to the reduction in the damage to nucleotides, thus allowing extraction of high-quality DNA, comparable to DNA extracted from blood [19].

Here we present a prospective study aiming at investigating feasibility and reliability of an FFT-based BRCA screening workflow in a consecutive HGSOC population in a tertiary referral center. Moreover, *tBRCA* status was correlated with clinico-pathological characteristics and survival outcomes.

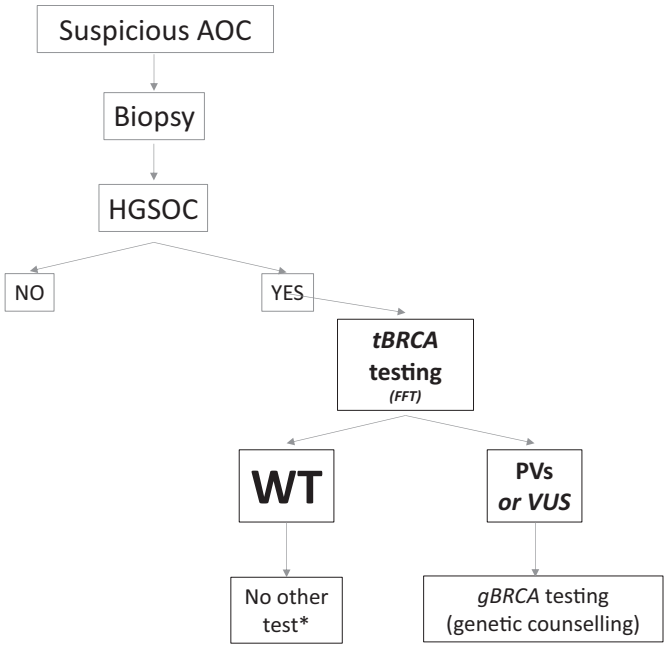


**Fig. 1.** Gynecologic Oncology algorithm. AOC: advanced ovarian cancer; HGSOC: high grade serous ovarian cancer.; tBRCA: tumor-tissue BRCA testing; PV: pathogenetic variant; VUS: variant of unknown significance; gBRCA-wt: germline BRCA wild type. *Genetic counselling is recommended in presence of personal or family history for ovarian cancers.

## 2. Materials & methods

### 2.1. Patients

We consecutively enrolled a series of newly diagnosed FIGO Stage IIIC-IV, HGSOC patients, admitted at the Gynecologic Oncology Unit of the Catholic University of the Sacred Heart tested for *tBRCA*, between January 2017 and June 2019.

In this time frame, all HGSOC patients receiving tumor biopsy at our Department underwent *tBRCA* testing, after gynecologic oncology counseling. According to our internal protocol, *tBRCA* mutated (*tBRCAm)* patients or those with a variant of uncertain significance (VUS) received blood sample analysis to define if the *BRCA* variant had germline or tumor origin. In *gBRCA* mutated *(gBRCAm)* women, genetic counseling was recommended (Fig. 1).

According to our Institutional model, patients were initially submitted to clinical evaluation, CT-scan and staging laparoscopy (S-LPS) [19] to be triaged to primary debulking surgery (PDS) or neoadjuvant chemotherapy (NACT). Regardless upfront treatment strategy, all women received six cycles of carboplatin 5–6 AUC/ paclitaxel 175 mg per square meter of body-surface area and after chemotherapy administration were entered into routine follow-up program including gynecological examination, CA125 assessment and CT-scan every 6 months. All women gave a written informed consent for their data to be collected and analyzed for scientific purpose. The Institutional Review Board approved the study (CICOG-01-07-19/35).

Medical records were reviewed for data relevant to medical history, surgery results, treatment approach and genetic counseling. Intraperitoneal tumor burden was evaluated using a laparoscopic predictive-index value (LPS-PIV) [20], classifying women as having: *low tumor load* in presence of LPS-PIV < 8, and *high tumor load* when a LPS-PIV ≥ 8 was observed. In all patients selected for PDS, maximal surgical effort was attempted, and residual tumor recorded. Complexity of surgical procedures in patients receiving PDS was graded according to the surgical complexity score (SCS) by Aletti et al. [21].

### 2.2. DNA extraction and evaluation

Tissue samples underwent hematoxylin/eosin expert pathology review to confirm HGSOC and estimate the tumor tissue content (TTC%). After biopsy or surgery, FFT samples were immediately stored in liquid nitrogen until the amplification step to achieve the highest efficiency in multiplex PCR. DNA was isolated from HGSOC FFT (all samples) in areas with a minimum TTC of 30–40% using an automated device (MagCore HF16 Plus, Diatech Lab Line, Jesi, Italy).

According to standard protocol, the tissues were cut in small pieces (up to 30 mg) and put into a micro centrifuge tube adding 400ul of GT Buffer and 20ul of Proteinase k and incubated at 55 °C for 90 minutes, until the samples were completely lysed. Finally, the samples were transferred to the MagCore sample tube and carried out according to manufacturer's instructions.

DNA concentration and quality were determined using Qubit dsDNA HS assay (Life Technologies, Gaithersburg, USA). Spectral analysis was performed by NanoPhotometer (Implen, München, Germany) to determine DNA concentration and purity, while DNA integrity was verified by 0.8% agarose gel electrophoresis. Finally, DNA samples showing concentration < 15 ng/mL, low quality (A260/A280 < 1.7), or a smeared profile were re-extracted.

### 2.3. BRCA testing

All patients received gynecologic oncologist counseling before tissue BRCA testing and a signed written informed consent. The genetic/molecular biology algorithm is described in Fig. 2.

BRCA Devyser Kit (Devyser, Svezia) [22] was used to analyze the entire coding sequences of BRCA1/2 genes, including 20–60 bases of adjacent intronic sequence of each exons. The sequencing process was carried out according to Illumina's protocols (Illumina, San Diego, CA,

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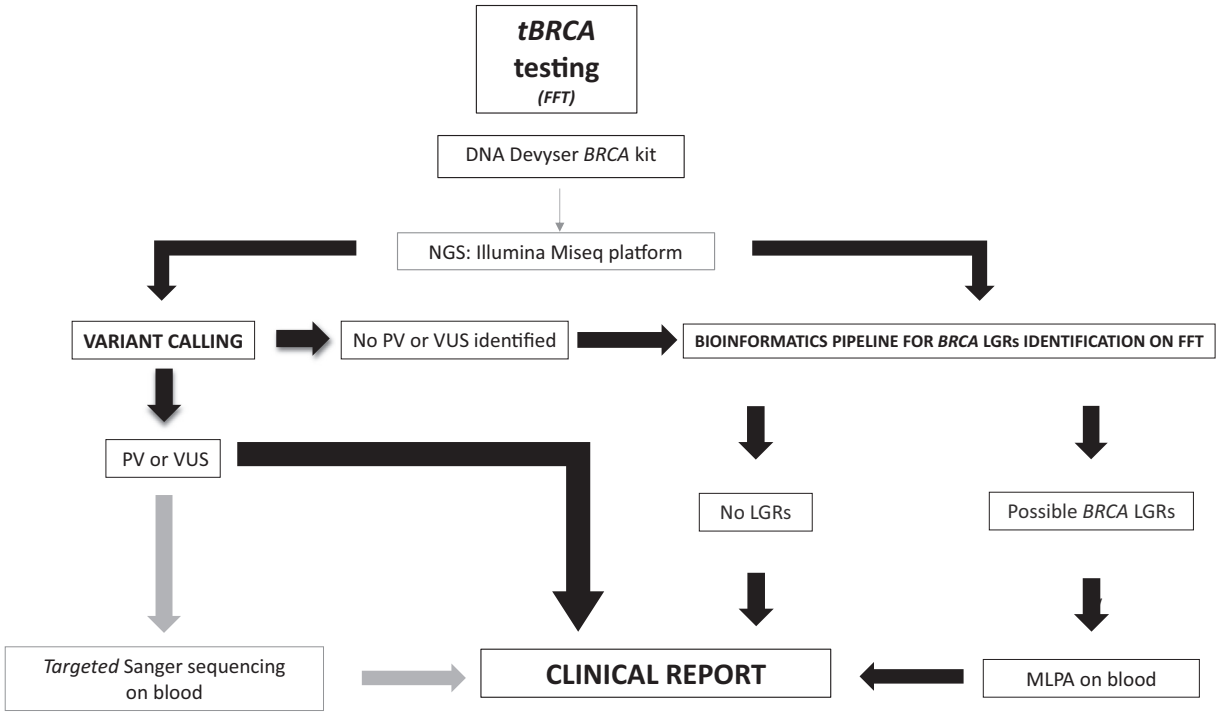


**Fig. 2.** The genetic/molecular biology algorithm. NGS: next generation sequencing; PV: pathogenetic variant; VUS: variant of unknown significance; LGRs: large genomic rearrangements; MLPA: Multiplex ligation-dependent probe amplification.

USA). Sequence data were processed using CE-IVD Amplicon Suite Software v.1.0 (SmartSeq s.r.l, Novara, Italy, www.smartseq.it/) to parse barcode reads, to align reads to the HG19 reference genome and to generate run metrics, including depth sequencing, total read counts and quality. The read length was pair-end and a cut-off of 5% for the Variant Allele Frequency (VAF) was applied. The median coverage for all samples was 2000×. In addition, *BRCA* large genomic rearrangements (LGRs) were also investigated, as previously reported [23,24].

### 2.4. In silico analysis and variant annotation

We excluded variants with minor allele frequencies (MAF) >1% in the following databases: *1000 Genomes* (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/), *dbSNP* (http://www.ncbi.nlm.nih.gov/projects/SNP), the *Exome Aggregation Consortium* (ExAC, http://exac.broadinstitute.org) and *Kaviar* (http://db.systemsbiology.net/kaviar/). The nomenclature of *BRCA* variants was based on the *BRCA1* and *BRCA2* cDNA sequence (NCBI Reference Sequences: NM_007294.4 and NM_000059.4). Annotations of putative functional variants were defined using *ClinVar* (https://www.ncbi.nlm.nih.gov/clinvar/) and *BRCA Exchange* (https://brcaexchange.org/), according to the recommendations of the Human Genome Variation Society (HGVS, http://www.hgvs.org/). Additionally, if the variants were not reported in any of the databases including the *1000 Genome*, *dbSNP*, *ClinVar*, or *BRCA exchange*, we defined them as *novel*.

### 2.5. Variants of uncertain significance (VUS)

*BRCA* variants reported in the above-mentioned databases associated to conflictual data of pathogenicity were considered VUS. To predict the functional effects of these VUSs, *in silico* analyses were performed using *SIFT* (https://sift.bii.a-star.edu.sg/), *Priors* (http://priors.hci.utah.edu/PRIORS/), *Provean* (http://provean.jcvi.org/index.php) and *Mutation taster* (http://www.mutationtaster.org/) tools.

### 2.6. Sanger sequencing

Tumor *BRCA* variants (*pathogenic* or *likely pathogenic* and VUS) detected by NGS were confirmed on blood by bi-directional Sanger Sequencing (ABI PRISM 3500, Life Technologies, Inc., Carlsbad, CA, USA), in order to determine the tumor or germline status. Genomic DNA was isolated and extracted from peripheral blood. Sanger sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Thermo Fisher Scientific, Inc.) and sequencing results were analyzed using SeqScape software version 2.5 (Applied Biosystems).

### 2.7. Sample size and statistical analysis

Based on previous experience, the proportion of *tBRCAm* patients is expected to be about 30% [25]. To calculate a 95% confidence interval with a margin of error no >0.05, we estimated to enroll at least 323 patients (±10%) in the timeframe (30 months).

Differences according to *BRCA* mutational status in terms of clinicopathological features at diagnosis and treatment details were analyzed using the Pearson c2 exact test and Kruskal-Wallis test, as appropriate. Medians and life tables were computed using the product limit estimate by Kaplan-Meier method [26,27] and the log-rank test was used to assess the statistical significance [28] PFS was defined as the time elapsed between the date of diagnosis and recurrence.

All statistical calculations were performed using the software SPSS, Version 25.

## 3. Results

Overall, 456 women with advanced HGSOC, admitted at our Institution, received *tBRCA* testing from January 2017 through June 2019.

All tests were performed at time of primary diagnosis. Adequate DNA quality and concentration was extracted from frozen tissue in all

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cases (100%), due to few areas of necrosis, inflammation or immune infiltrate, according to laboratory's standard.

Clinically relevant *tBRCA* variants were observed in 145 women (31.8%). In particular, we documented *BRCA1* PVs in 89 cases (61.4%), and *BRCA2* PVs in the remaining 56 women (38.6%). In detail, among women with tPVs, 138 patients (95.1%) underwent *gBRCA* testing and we discovered that PVs were confirmed on the blood in 117 patients (80.6%) while 20 patients (13.8%) presented a wild-type (*wt*) genotype and 1 a gBRCA VUS (0.7), which was confirmed to be the same mutation found on the tissue.

Among 292 t*BRCAwt* patients, in 88 cases gBRCA test was available and 86 (86/88, 97.7%) were confirmed gBRCAwt, while 1 (1/88, 1.1%) had gBRCA-VUS and 1 had gBRCA mutation (1/88, 1.1%), discovered by the identification of LGRs, which overall were suspected in 13/292 patients (4.5%) by using bioinformatic algorithm. In these women, multiplex ligation-dependent probe amplification (MLPA) was required and data were collected, with evidence of PVs in only 1 case. In this tBRCAwt/gBRCAmut patient, the algorithm had shown a doubtful signal of the copy number of BRCA-exon 2; following MLPA on blood, this doubtful signal of the somatic test was confirmed to be a structural rearrangement in BRCA1.

Lastly, 19 patients (4.2%) were found with a tBRCA-VUS; in 16 cases gBRCA test was performed and 6 (6/16, 31.6%) were confirmed as gBRCA-VUS while 10 (10/16, 52.6%) were a gBRCAwt.

Overall, 118 of 456 (25.8%) women were both gBRCAmut/tBRCAmut (117 patients) or gBRCAmut/tBRCAwt (1 patient) compared with 31.8% of tBRCAmut patients in the whole group, defining a 6% difference between the two procedure in identifying BRCA mutation.

In this population, the *concordance* of tumor test *versus* germline *BRCA* test was 86.3% (209/242), expressed as the ratio between the patients with concordance of the two tests over the total number of patients who performed the germinal BRCA (Fig. 3).

A detailed description of *tBRCA* PVs or VUS identified is reported in in Fig. 3 and Supplemental Table S1.

Patients in the *tBRCAm* group were approximately 5 years younger compared with *tBRCAwt* genotype women (57 *vs* 62 years old; *P* value 0.001) [Table 1]; the median age of tBRCAmut only patients, was 60 years (range 30–82). No differences were documented in terms of distribution of clinicopathological features at diagnosis according to both *tBRCA* status and type of PVs.

Regarding treatment strategy, there were no differences according to *BRCA* mutational status between rate of PDS and NACT, with no statistically significant difference of complete/optimal cytoreduction in the PDS population [Table 1].

With regard to maintenance treatment, 131 patients of the overall population (28.7%) received bevacizumab, with no differences between the Groups (85 *tBRCAwt* patients *vs* 46 *tBRCAm* patients, *p*-value = 0.19). Eleven (7.5%) patients received PARP-i, all in *tBRCAm* group.

With a median follow-up time of 18 months, recurrent disease was observed in 185 women (40.6%), with significant differences in terms of median PFS according to *BRCA* status (median *tBRCAwt* PFS 17.0 *vs* *tBRCAm* 25.0 months; log-rank p-value = 0.0001) [Fig. 4].

## 4. Discussion

To the best of our knowledge, this is the first prospective study analyzing feasibility and reliability of an FFT-based BRCA screening workflow in a large series of advanced HGSOC patients. Here we show that feasibility is 100%, meaning that no sample was considered inadequate for DNA extraction due to storage reasons or tissue content (necrosis or extreme inflammatory infiltration). This is in line with literature evidences, supporting that quality of extracted DNA on FFT is higher, due to reduction in damage to nucleotides, making it comparable with blood extracted DNA [23]. This result also underlines the importance of the entire "supply

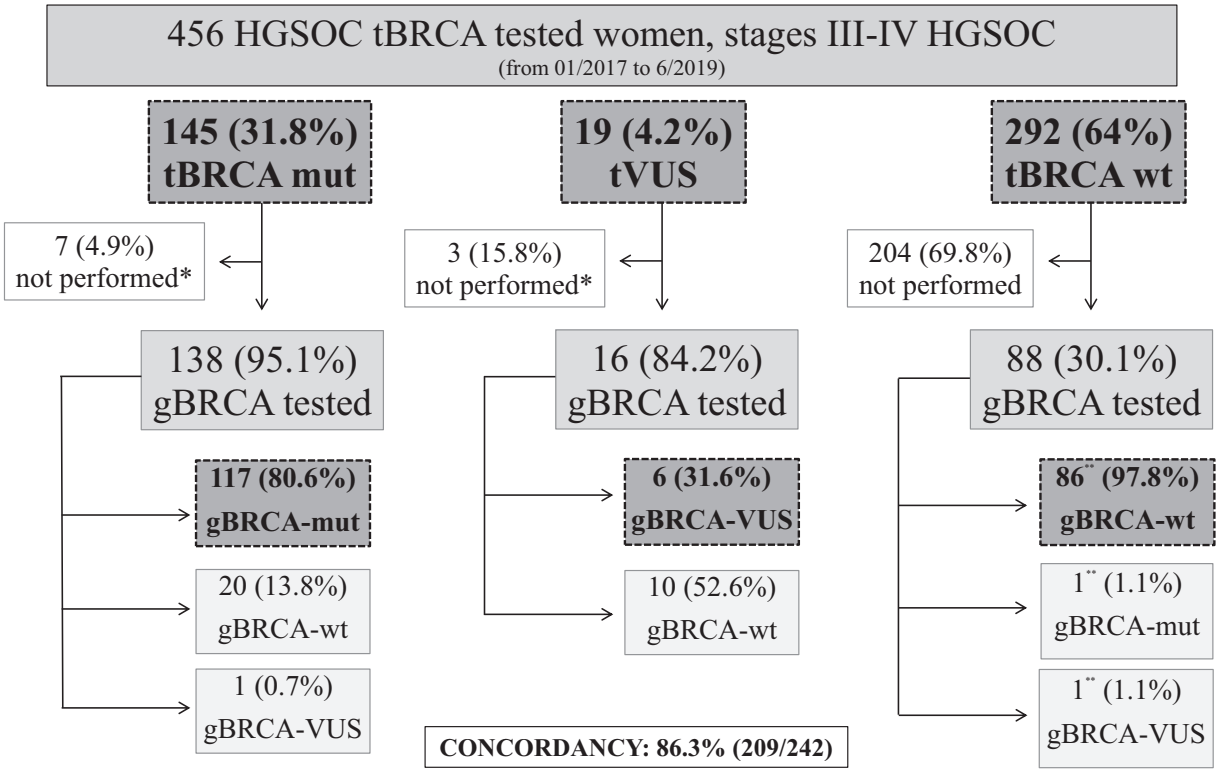


**Fig. 3.** Flowchart of patients and BRCA-status. HGSOC: high grade serous ovarian cancer; tBRCA: tumor-tissue BRCA testing; tBRCA-mut: tumor-tissue BRCA 1 or 2 mutated; tVUS: variant of unknown significance on tumor-tissue testing; tBRCA-wt: tumor-tissue BRCA wild-type; gBRCA: germline BRCA testing; gBRCA-mut: germline BRCA 1 or 2 mutated; gVUS: variant of unknown significance on germline testing; gBRCA-wt: germline BRCA wild-type *Patients' refusal or decision;§ LGRs were suspected according to the bioinformatic algorithm and gBRCA test was performed.

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**Table 1**
Distribution of patients' clinicopathological characteristics at diagnosis according to BRCA mutational status.

| | All patients n (%) | tBRCAwt n (%) | tBRCAmut n (%) | p-Value[a] |
|---|---|---|---|---|
| All cases | 456 | 311 (68.2) | 145 (31.8) | |
| Median age at diagnosis (range, years) | 60 (27–89) | 62 (27–89) | 57 (30–84) | *0.001* |
| Family history of cancer | | | | |
| No | 181 (39.7) | 141 (45.3) | 40 (27.6) | *0.0001* |
| Yes | 275 (60.3) | 170 (54.7) | 105 (72.4) | |
| Type of BRCA mutation | | | | |
| BRCA1 | NA | NA | 89 (61.4) | NA |
| BRCA2 | | | 56 (38.6) | |
| CA125, mean (SD), UI/mL | 4394 (9648) | 4308 (9436) | 4578 (10120) | 0.78 |
| FIGO stage at diagnosis | | | | |
| III | 335 (73.5) | 223 (71.7) | 112 (77.2) | 0.12 |
| IV | 121 (26.5) | 88 (28.3) | 33 (22.9) | |
| LPS-PIV | | | | |
| <8 | 193 (42.3) | 124 (39.9) | 69 (47.6) | 0.07 |
| ≥8 | 263 (57.7) | 187 (60.1) | 76 (52.4) | |
| Primary treatment strategy | | | | |
| PDS | 212 (46.5) | 143 (46) | 69 (47.6) | 0.41 |
| NACT | 244 (53.5%) | 168(54) | 76 (52.4) | |
| Surgical complexity[b] | | | | |
| 1–2 | 104 (49) | 67 (47) | 37 (54.4) | 0.19 |
| 3 | 107 (51) | 76 (53) | 31 (45.6) | |
| RT at primary surgery (PDS)[c] | | | | |
| 0 | 180 (85.7) | 117 (82.4) | 63 (92.6) | 0.13 |
| 1–10 mm | 23 (11) | 19 (13.4) | 4 (5.9) | |
| >1 cm | 7 (3.3) | 6 (4.2) | 1 (0.5) | |

tBRCAmut: tissue BRCA1/2 mutations; tBRCAwt: tissue wild-type BRCA genotype; FIGO: International Federation of Gynecology and Obstetrics; LPS-PIV: laparoscopic predictive index value; PDS: primary debulking surgery; NACT: neoadjuvant chemotherapy; IDS: interval debulking surgery; RT residual tumor.
[a] Calculated using the Chi square test.
[b] Calculated only in women treated with PDS, in 1 patient data not available.
[c] Calculated only in women treated with PDS, in 2 patients data are not available.

chain", starting from tissue selection made at time of surgery, either by laparoscopy or laparotomy, flowing through storage, pathological and molecular analysis.

The FFT-based BRCA screening approach has identified 32% tBRCAmut patients, confirming other and our previous experiences [29]. Nonetheless, the higher prevalence of gBRCA1/2 mutations in our population, which was roughly 26%, might be ascribed to the already known higher prevalence in some specific Italian subgroups [30].

Furthermore, the FFT-BRCA testing approach led us to identify 6% of patients with tumor-tissue BRCA mutation only, who might be eligible for PARPi maintenance treatment. One can argue that, with the recently presented data according to which PARPi increase the PFS expectations even in absence of a BRCA mutation [31,32], there is no meaning to further perform tumor-tissue test and we should rather move back to the only-blood test for preventive measures. Nonetheless, albeit this could be expected if drugs will be licensed without a molecular driver, there might also be the risk of losing important information, since BRCA mutational status still represents the strongest predictive factor of platinum sensitivity. Moreover, we are still facing with the identification of a "real" HRD population that benefit the most form PARPi therapies; with this regard, FFT has been proved to be more adequate to identify HRD genes, compared with FFPE [33].

On the other side, gBRCA testing was performed in 88 tBRCAwt patients (30%), either required by the bioinformatics algorithm or

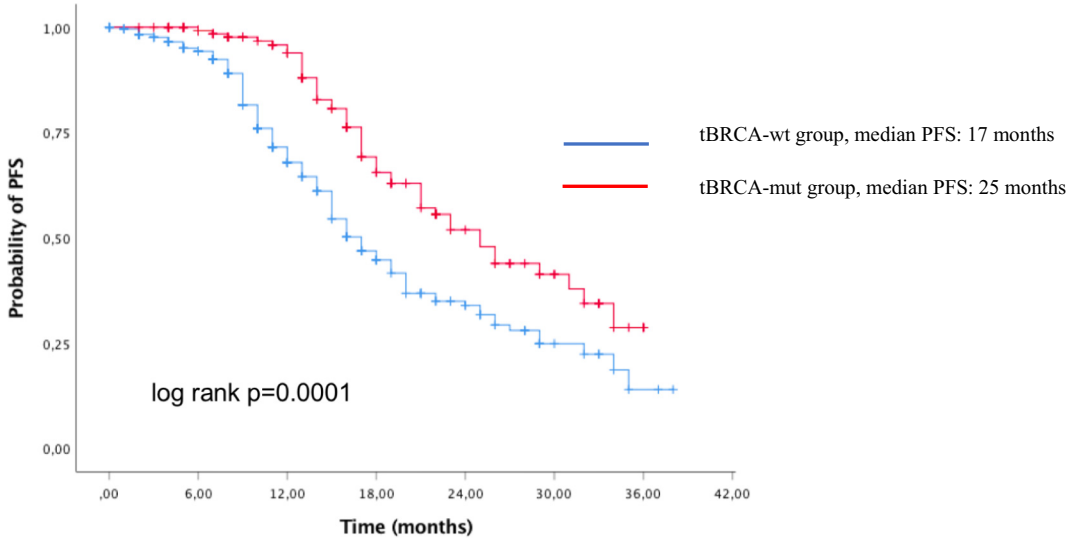


**Fig. 4.** Kaplan-meyer plots for progression-free survival (PFS) according to BRCA status. tBRCA-mut: tumor-tissue BRCA 1 or 2 mutated; tBRCA-wt: tumor-tissue BRCA wild-type; PFS: progression free-survival.

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spontaneously done. In 87 (98.9%) women, results on blood confirmed the absence of a germline BRCA mutation, suggesting that the FFT-BRCA testing approach is effective even in terms of "prevention". Moreover, we were able to identify a gPV in 1 tBRCAwt patient in which LGRs were suspected according to the bioinformatic algorithm; this further confirms that discordances previously ascribed to DNA extraction from FFPE are lowered by the high-quality DNA obtained from FFT, which allows an easier and accurate identification of LGRs. Of course, these data should be interpreted with caution as we did not perform concurrent blood and tissue testing, and further studies with universal testing might help in definitively clarify this issue.

Finally, with regard of outcomes, in this large population we confirmed that tumor clinical characteristic, surgical approach and survival expectations are similar to those commonly attributed to gBRCA positive HGSOC patients; as expected tBRCAmut patients progress later than the tBRCAwt counterpart [34].

We acknowledge there are some weakness in this approach, including higher expenses for collection, transferring and storage than paraffin embedded tissue samples, and the need for strict collaboration among different professionals. On the other hand, if we perform first gBRCA test to all HGSOC patients, according to the ASCO statement [4] we will find 75–80% of gBRCAwt cases. They will undergo tBRCA testing, with a tBRCA mutation found in no more than 15% of the cases. As a consequence, 65% of EOC patients will receive an unneeded and expensive double-test procedure. On the other hand, if we assume that around 30–35% of HGSOC harbor a tBRCA mutation, only they will undergo gBRCA testing, with a gBRCA mutation confirmed in 20–25% of the cases. Crudely, this second option seems to be more cost saving, but clearly further analysis is needed to confirm this hypothesis [35].

In conclusion, we present the largest series of a FFT-BRCA testing algorithm, showing that this approach is feasible and reliable. It allows to enlarge the BRCA mutated population that might receive PARPi with the greatest benefit, without missing cascade testing for family members and therefore, maintaining its preventive role. We should take the challenge of reducing the impact of unnecessary and expensive procedure by increasing our commitment toward an effective "in-house" BRCA testing approach.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2020.06.479.

## Author contributions

C.M. planned the study, conducted statistical analysis and drafted the manuscript. A.M. drafted the manuscript, advised on results interpretation. M.D. collected data and drafted the manuscript. R.E., M.A., C.C. collected data. E.D.C. and A.P. revised the manuscript. G.S. planned the study, advised on results interpretation, drafted and revised the manuscript A.F. planned the study, advised on results interpretation, drafted and revised the manuscript. All authors read and approved the final manuscript.

## Declaration of competing interest

All authors declare no competing interests.

## Acknowledgements

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