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Clinical impact of next-generation sequencing multi-gene panel highlighting the landscape of germline alterations in ovarian cancer patients

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Summary

1. Abstract

BRCA1 and BRCA2 are the most frequently mutated genes in ovarian cancer (OC), crucial both for the identification of cancer predisposition and therapeutic choices. However, germline variants in other genes could be involved in OC susceptibility. We characterized OC patients to detect mutations in genes other than BRCA1/2 that could be associated with a high risk to develop OC, and that could permit patients to enter the most appropriate treatment and surveillance program. Next-Generation Sequencing analysis with a 94-gene panel was performed on germline DNA of 219 OC patients. We identified 34 pathogenic/likely-pathogenic variants in BRCA1/2 and 38 in other 21 genes. Patients with pathogenic/likely-pathogenic variants in non-BRCA1/2 genes developed mainly OC alone compared to the other groups that developed also breast cancer or other tumors ($p=0.001$). Clinical correlation analysis showed that low-risk patients were significantly associated with platinum sensitivity ($p<0.001$). Regarding PARP inhibitors (PARPi) response, patients with pathogenic mutations in non-BRCA1/2 genes had significantly worse PFS and OS. Moreover, a statistically significant worse PFS was found for every increase of one thousand platelets before PARPi treatment. To conclude, knowledge about molecular alterations in genes beyond BRCA1/2 in OC could allow for more personalized diagnostic, predictive, prognostic, and therapeutic strategies for OC patients.

2. Introduction

Ovarian cancer is the second cause of death from gynecological malignancies, and the seventh most common cause of cancer death worldwide. [1] The median progression-free survival (PFS) and overall survival (OS) for advanced ovarian cancer range between 12 and 24 months and 29 and 65 months, respectively. [2,3] The most common ovarian neoplasm is a high-grade serous histological subtype, accounting for about 70% of cases and causing the majority (90%) of ovarian cancer deaths.

Other histologic subtypes include low-grade serous, endometrioid, clear-cell, and mucinous ovarian cancers. [4-6] Mucinous ovarian cancer is a rare tumor, probably accounting for 3% of all epithelial ovarian cancers. Stage III or IV mucinous ovarian cancer patients have a poorer prognosis than women with other, more common subtypes (particularly serous or endometrioid ovarian cancer), and may be related to a poorer response to chemotherapy. [7]

High-grade serous ovarian cancer is frequently associated with DNA repair deficiencies. [8] Alterations in DNA repair pathways represent a common feature of carcinogenesis, as they can drive malignant transformation with the accumulation of genomic alterations in cancer cells. [9] Conversely, the presence of multiple DNA repair systems allows cancer cells to have a compensating mechanism to avoid non-viable amounts of genotoxic stress that would ultimately lead to cell death. [10]

In around 18% of ovarian cancer patients, it is possible to identify germline mutations in *BRCA1* and *BRCA2*, especially in those with high-grade serous carcinoma. [11,12] When combined with *BRCA* deficiencies resulting from somatic mutations or epigenetic silencing, it appears that up to half of all high-grade serous ovarian cancers have a *BRCA* dysfunction. [13-16] About 10%–14% of ovarian endometrioid carcinomas present deficiencies in mismatch repair proteins by immunohistochemistry, accounting for the microsatellite instability phenotype. [17] Conversely, to high-grade serous ovarian cancer, mucinous ovarian cancers are not associated with *BRCA* mutations or defects in homologous recombination. The most frequent alterations are *KRAS* mutations (in 40% to 65% of cases), *c-MYC* amplifications (65% of cases), *HER2* amplifications (20% to 38% of cases), and *TP53* mutations (50% to 75% of cases). In addition, other alterations have been identified at lower frequencies, such as homozygous deletions in *CDKN2A/B* (in 25% of cases), mutations in *PI3KCA*(13%), and mutations in *PTEN*, *BRAF*, *FGFR*, *KIT*, or *STK11* (2% to 5% of cases). [18]

Although the tumor stage, residual disease after surgical debulking, response to chemotherapy, and *BRCA1/2*-mutation status all affect the outcome of ovarian cancer, the variability in PFS and OS among patients with similar clinical and pathological characteristics makes it difficult to reliably predict outcome. In 2003, Coukos and coworkers reported for the first time that the presence of tumor-

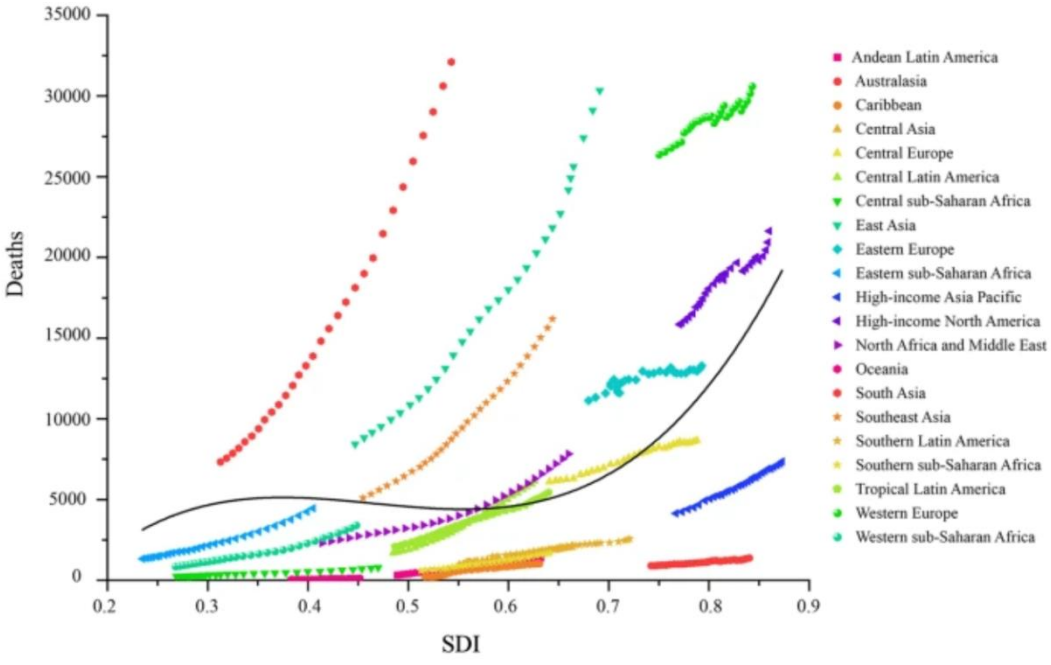
infiltrating lymphocytes (TIL) CD3+ correlated with improved clinical outcome in advanced ovarian carcinoma. [19]

1.1 Epidemiology – the global burden

Ovarian cancer has an insidious onset, and the prognosis is often poor because it is usually difficult to treat with conventional therapies due to recurrence and drug resistance. [20]

Today, in a global perspective, the number of people with the disease varies greatly from country to country. Many variabilities add to the fact that ovarian cancer is a complex disease that has emerged as major global public health concern. In 2019, the number of ovarian cancer incident cases was 294422 (260649 to 329727). The overall burden of ovarian cancer was on the rise, especially in the number of cases, with a percentage change of 107.8% (76.1 to 135.7%) compared to 1990. Nevertheless, the percentage changes of global age-standardized incidence rate kept stable during the same period. All three groups (15–49, 50–69, and 70 + age groups) showed an increasing trend in the number of cases between 1990 and 2019, with the highest cases number in 2019 in the 50–69 age group. The age group of largest percentage change in the number of incident cases was 70 + age group, were 119.9% (92.9 to 143.9%).

Fig.1 – The correlation of ovarian cancer deaths and social-demographic index (SDI), 1990-2019. The black line represents the average expected relationship between SDIs and deaths for ovarian cancer based on values from all countries from 1990 to 2019. [adapted from Zhang S, et al. BMC Public Health. 2022;22:1455. - 21]

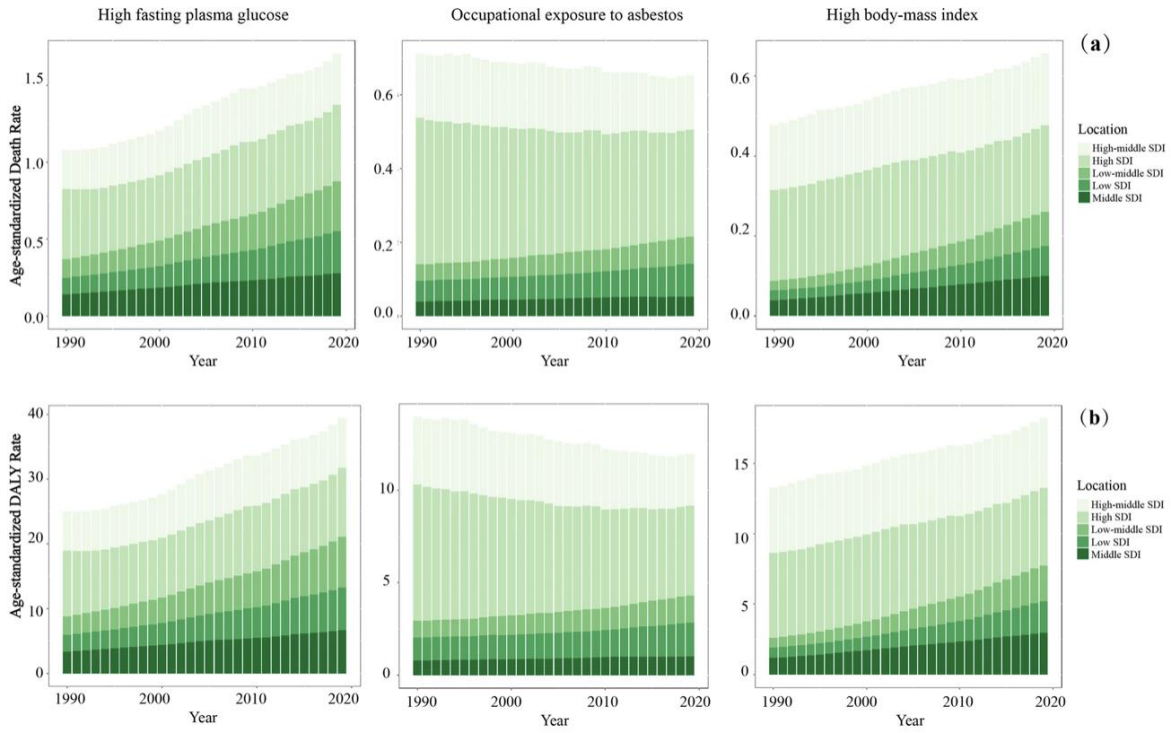


In 2019, the number of deaths for women due to ovarian cancer was 198412 (175357 to 217665). The age-standardized death rate due to ovarian cancer was 4.6 (4.0 to 5.0) per 100,000 in 2019. [21]

Among the most specific risk factors attributed to all deaths of ovarian cancer globally in 1990, the top three were high fasting plasma glucose, high body-mass index, and occupational exposure to asbestos, respectively. In 2019, the same pattern of risk factors for the number of ovarian cancer deaths worldwide did not change.

In 2019, the risk factor that led to the highest number of deaths was high fasting plasma glucose, accounting for 15736 (3023 to 36227) or age-standardized death rate of 0.4 (0.1 to 0.8) per 100000. The corresponding age-standardized death rate has shown an increase over the last 30 years (34.7% (18.6 to 51.4%)). [21]

Fig. 2 – The ovarian cancer age-standardized death rate (ASDR – **a**) and age-standardized disability-adjusted life year rate (ASDALYR – **b**) attributable to risk factors between 1990 and 2019 by social-demographic index (SDI) regions. [adapted from Zhang S, et al. BMC Public Health. 2022;22:1455. - 21]



Occupational exposure to asbestos was the second leading cause of ovarian cancer deaths globally, with an age-standardized death rate of 0.1 (0.1 to 0.2) per 100000. From 1990 to 2019, age-standardized death rate caused by this risk factor showed a decreasing trend year by year, with a decrease of 24.9% (-46.7 to -7.4%) in 2019. Among all social-demographic index quintiles, only high social-demographic index quintile showed a decreasing trend in age-standardized death rate,

decreasing by 26.8% (-47.9 to -7.1%), while the changes in other areas were not statistically significant in value. High body-mass index was the third leading cause of ovarian cancer deaths globally, with an age-standardized death rate of 0.1 (0.0 to 0.3) per 100000, while from 1990 to 2019, the value showed a slow upward trend with a 16.4% (2.7 to 32.0%) increase. [21]

1.2 DNA Repair Systems

Potentially harmful agents, comprising oxidative stress, ultraviolet light and ionizing radiation, and the use of alkylating and anti-tumor agents, continuously interact with human DNA. Five DNA repair mechanisms are exploited by cells: base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), and non-homologous end-joining (NHEJ).

BER protects against single-base DNA damage caused by spontaneous depurinations, methylating and oxidizing agents, or other genotoxicants. [22] BER consists of the removal of damaged bases by DNA glycosylases. There are 11 of these enzymes in humans, and each identifies specific lesions; they bind the altered deoxynucleoside in an extrahelical position and catalyze the cleavage of the base–sugar bond. APE-1 is a protein with an endonuclease activity that makes a 5' nick in the DNA structure and a 3' hydroxyl that is recognized by DNA repair polymerase β. Poly (ADP-ribose) polymerase-1 (PARP-1) binds to the 5' nick, acting as a nick surveillance protein. PARP-1 is one of the BER complex proteins involved in DNA interruption detection and DNA repair. [23] BER consists of different steps: excision of the base, incision, end processing, and repair synthesis (gap filling and ligation).

Slyskova et al. found that DNA repair capacity (DRC) linked to BER is similar in tumor tissues and adjacent healthy epithelium, suggesting that alterations of BER may be not the crucial events in malignant transformation; however, they could be involved in chemical sensitivity of tumor cells to drugs. [24]

The MMR system acts against DNA damaging agents in post-replication correction of extra-helical loops and nucleotide mispairs. MMR includes the *MLH1*, *PMS2*, *MSH2,* and *MSH6* genes. Alterations in MMR genes cause microsatellite instability, a mutator phenotype, and a predisposition to colorectal cancer. [25] Moreover, tumors with MMR deficiency show significantly more somatic alterations than MMR efficiency, resulting in an increased neoantigen burden and immunogenicity. Indeed, tumors with MMR deficiency are responsive to immune checkpoint inhibitors. [26]

NER consists of about 30 peptides and is involved in the repair of DNA with helix distorting damages, including that caused by UV light, environmental mutagens, and chemotherapeutic agents. [27] The main steps in NER are as follows: recognition of a DNA defect; recruitment of a repair

complex; involvement of helicases for DNA repair; incision of the damaged strand, which results in a single-strand fragment of 24–32 nucleotides; DNA synthesis to fill in the gap; and ligation to form the final phosphodiester bond. [28] Indeed, XPC-RAD23B recognize lesions, and interacts with TFIIH, a transcription initiation complex, prying the DNA open with an XPD subunit. XPB recruits XPA, RPA, and XPG, allowing the formation of a pre-incision complex. XPA interacts with ERCC1-XPF, making a 5' incision of the lesion. DNA ligase IIIa/XRCC1 or DNA ligase I concludes the NER process. [29]

Slyskova et al. identified alterations of DRC in sporadic colorectal cancer and hypothesized a role of NER in carcinogenesis. [30,31]

A double strand break (DSB) is the most lethal damage to the genome that can derive from anti-cancer treatments (e.g., ionizing radiation or the topoisomerase inhibitors) [32] or physiologic pathways (e.g., genetic recombination during meiosis). [33]

The HR system is an error-free mechanism that repairs DSBs using a homologous DNA template; during the S/G2 phase of the cell cycle, cyclin-dependent kinases stimulate DNA end resection and activate the HR pathway. The HR process initiates by the end resection generating a long stretch of single-strand DNA from DNA break ends. The HR pathway includes BRCA1 as part of BASC, a large complex linked to genome surveillance composed of MLH1, MSH2, and MSH6 (mismatch repair proteins), an MRN (Mre11–Rad50–Nbs1) complex, and ATM and Bloom (BLM) syndrome helicase. [34] The HR system also involves BRCA2, which forms a complex with Rad51, binding the exposed DNA and permitting Rad51 to load onto the break and assemble the presynaptic filament [35]. The main reactions in HR are catalyzed by the Rad51/RecA family DNA recombinases [36].

RAD51 mutations have been identified in ovarian cancer; specifically, deleterious variants were shown in *RAD51B*, *RAD51C*, and *RAD51D* (nonsense, frameshift, and splice), with a predominance for *RAD51C* and *RAD51D* mutations. [37] Literature data shows that tumors with *RAD51C* and *RAD51D* mutations presented sensitivity to PARP inhibitors, suggesting a novel therapeutic option for this setting of patients [38,39].

The NHEJ system is active during all phases of the cell cycle and ligates DSBs ends without a template. In NHEJ, the DSBs are first recognized by a heterodimer consisting of Ku70 and Ku80 (Ku). The degradation of short regions of the 5' or 3' ends by both exonuclease or endonuclease enzymes (e.g., Artemis) is included in the end resection that generates or exposes small regions of microhomology (≤4 nucleotides) between the strands, facilitating end joining. Artemis is recruited with DNA-dependent protein kinase catalytic subunits, which have a high affinity for DNA ends. Nucleotide addition can occur by the Pol X family polymerases. The DNA ligase IV complex, consisting of XRCC4, XLF, and perhaps PAXX, performs the ligation step for either strand of the

DSBs. Alternative joining pathways can be involved in DSBs: backup NHEJ (B-NHEJ) makes use of PARP1, PARP2, and ligase III; and microhomology-mediated end-joining (MMEJ) can be considered as a form of B-NHEJ other than alternative end joining (Alt-EJ) [40]. PARP1 could compete with RAD51 and BRCA2 for the further processing of resected ends at DSBs, after the initial phase of end resection. The activation of Alt-EJ is mediated by PARP1, and this determines the aligning of short homologous sequences (i.e., microhomology) in the broken ends of DSBs. The repair pathway mediated by microhomology translates into the generation of small deletions, surrounded by microhomologies. The activity of PARP inhibitors inducing synthetic lethality in BRCA1/2-null cells suggests that PARP1-mediated Alt-EJ compensates for HR in HR-deficient cells. [41]

Checkpoints in G1/S, intra-S, and G2/M phases control the progression of cell cycle. The type of DNA lesions activates different DNA damage response proteins. [42] NHEJ is prevalent throughout the cell cycle, whereas HR is dominant during the S and G2 phases, when factors promoting extensive end resection are more effective. [43] Cyclin-dependent kinases favor extensive resection during cell cycle, through enzymes and DNA damage response checkpoint proteins, such as ATM and ataxia telangiectasia and Rad-3 related. Moreover, ATM phosphorylation is involved in the pathway that allows HR or NHEJ activation. [44] In this context, end processing developing a long 3' single strand DNA depends on how long DSBs remain unrepaired, which leads to activation of single-strand annealing (SSA). SSA consists of a non-conservative, homology-directed repair pathway that necessitates >20 bp of homology and presents a loss of nucleotides. [45] RAD52 protein is required for the annealing of complementary single strand DNA in the SSA pathway.

Direct DNA repair of base alkylations lesions involves MGMT protein, which repairs the O^6-methylguanine (highly mutagenic) and human AlkB homologues (*ALKBH1*, *ALKBH2*, and *ALKBH3*). These kinds of lesions can occur during all phases of the cell cycle, so there is no cell cycle regulation for the genes involved in direct repair. [46]

Germline mutations in genes of repair cause a predisposition to cancer. In particular, germline mutations in *BRCA1* and *BRCA2* are associated mainly with ovarian and breast carcinoma, [47] but sporadic cancers show alterations in *BRCA* genes. Indeed, *BRCA1* interacts with *BRCA2*, and similar phenotypic effects result from *BRCA1* and *BRCA2* mutations. [48] Thus, *BRCA1* and *BRCA2* are two genes that are crucial for repairing DNA damage and for ensuring genomic stability, preventing the accumulation of gross chromosomal rearrangements that would ultimately lead to either cellular apoptosis or tumor formation. [49,50]

1.3 Consequences of DNA Repair Deficiencies in Ovarian Cancer Pathology

Based on epidemiologic studies, about 65% to 75% of all cases of hereditary ovarian cancer are caused by gene mutations in *BRCA1* or *BRCA2*. The third major cause of hereditary ovarian cancer is hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, which accounts for an additional 10% to 15% of all inherited, cases. [51] HNPCC is caused by mutations in genes involved in the MMR system.

It is widely acknowledged that HR-deficient ovarian cancers are enriched for high-grade serous histology (Table 1). *BRCA* abnormalities seldom occur in non-high-grade serous ovarian carcinoma subtypes. [52] HR deficiency endows ovarian cancers with a clinical phenotype that is characterized by visceral relapse, a slightly younger age at diagnosis, and a better response to platinum-based chemotherapy, PARP inhibitors, and anthracyclines. [41]

Usually, only one mutated allele results from inherited germline defects (typically mutations), and loss of the other allele occurs somatically, as in Lynch syndrome (or HNPCC), an autosomal dominant condition that predisposes the patient to cancer development (especially colorectal, ovarian, and endometrial cancer). [53] Alternatively, sporadic MMR deficient tumors are often due to hypermethylation of the *MLH1* promoter resulting in epigenetic silencing. [54]

The distribution of ovarian cancer histotypes in MMR-deficient patients differs considerably from that generally observed: non-serous histologies are more common, and often show endometrioid or a clear cell differentiation (Table 1). [55] Ovarian endometrioid cancers (~10%–25% of all ovarian carcinomas) are predominantly seen in perimenopausal women, and arise from endometriosis, which appears to act as a precursor. Endometrioid histology frequently harbors AT-rich interactive domain 1A (*ARID1A*) mutations, leading to loss of ARID1A protein expression, B-catenin (*CTNNB1*) somatic mutations, *PTEN* mutations, and microsatellite instability. [56,57] In particular, MMR-deficiency, accounting for the microsatellite instability phenotype, has been reported in 10%–14% of ovarian endometrioid carcinomas. Primarily, loss of *MSH2* and/or *MSH6* accounts for over 50% of MMR-deficient ovarian endometrioid carcinomas. [16,58]

Clear-cell ovarian cancer is a rare subtype characterized by a worse prognosis when diagnosed at an advanced stage, due to low chemosensitivity. Howitt and coworkers demonstrated that 10% of clear-cell ovarian cancer exhibited microsatellite instability and roughly 27% *ARID1A* loss. [59]

Table 1 - Characteristics of different ovarian cancer histological types. [50]

Clinical Characteristics	High-grade Serous	Low-grade Serous	Clear Cell	Endometrioid	Mucinous
Prevalence	65%–70%	3%	5%–10%	10%–15%	2%–8%
Hereditary risk	18%–20% present germline BRCA1/2 mutations	unknown	unknown	10%–14% endometrioid tumors are associated with HNPCC syndrome	unknown
Stage at diagnosis	Advanced	Early Advanced	Early	Early	Early
Genetic alterations	p53 p16 pRb pathway Homologous recombination defects (BRCA1/2, RAD51)	BRAF or KRAS	HNF-1β IL6/JAK2/STAT3 PI3K MSI ARID1A	PTEN β-Catenin KRAS MSI ARID1A	K-ras c-MYC HER2
Chemotherapy response	80%	26–28%	15%	unknown	15%
Immune infiltrate	High, more commonly associated with BRCA1 defects	Low	Generally low, higher when associated with MSI	Generally low, higher when associated with MSI	Low

1.4 Immune-Consequences of DNA Repair Defects

The first evidence of a relationship between ovarian cancer and the immune environment was reported by Zhang and coworkers in 2003. The authors demonstrated that the presence of intratumoral T cells correlated with the clinical outcome of advanced ovarian carcinoma. [18] Of note, TILs are more frequently present in serous carcinomas, compared to either endometrioid or clear-cell carcinomas (Table 1). Clarke and coworkers also performed an exploratory analysis in a small case series, observing a significant association between intraepithelial TIL and *BRCA1* mutations or promoter methylation causing loss of expression, mainly in high-grade serous ovarian cancer. [60] Since then, others authors have identified "prominent intraepithelial lymphocytes" as a distinguishing feature of *BRCA1/2*-mutated tumors, with a higher mutational load. [61]

Although both genes encode proteins that participate in the HR pathway, the reason why germline *BRCA1* mutations seem to confer a higher risk of developing ovarian cancer than germline *BRCA2* mutations is probably related to their earlier and more substantial role in DNA damage response and cell-cycle regulation. Indeed, *BRCA1*-mutant, high-grade serous ovarian cancers present a specific molecular subtype with a distinct gene expression signature, which seems related to specific amplification events at 8q24 and on the X chromosome. Conversely, *BRCA2*-mutant tumors more closely resemble "wild-type" high-grade serous ovarian cancer. [62] Consequently, it seems that *BRCA2*-disrupted tumors, although harboring similar numbers of point mutations, are less immunogenic than *BRCA1*-disrupted tumors. [63]

Strickland and coworkers demonstrated that a higher neoantigen load in the *BRCA1/2*-mutated ovarian cancers compared to HR-proficient tumors translates to a significantly higher number of CD3+ TILs compared to HR-proficient tumors. Moreover, HR-proficient tumors showed a lower PD-L1 expression on the surface of intraepithelial and peritumoral immune cells compared to the *BRCA1/2*-mutated tumors, supporting a link between *BRCA1/2*-mutation status, immunogenicity, and improved survival in high grade serous ovarian cancer. [64]

With regard to defects in the MMR system, it has been demonstrated that mismatch repair–deficient cancers are associated with 10- to 100-fold more somatic mutations as MMR–proficient cancers, and contain prominent lymphocyte infiltrates, a finding consistent with an immune response. In an unselected series of ovarian clear cell carcinoma, with around 6% of MMR deficiency, peritumoral lymphocytes were more frequent in MMR-deficient tumors. [65] Indeed, it has been seen that an MMR–deficient tumor microenvironment strongly expresses several immune checkpoint ligands, (e.g., PD-1, PD-L1, CTLA-4, LAG-3, and IDO), indicating an immune escape process where their active immune microenvironment is counterbalanced by immune inhibitory signals. [66]

MMR-deficient tumors were shown to be more frequently resistant to chemotherapy and in particular to methylating agents and platinum compounds. [67] A possible explanation may be related to the incapability of MMR proteins involved in DNA damage response to recruit ATM/ATR, which in turn leads to cell cycle arrest, DNA repair, or apoptosis. [68]

Alterations in DNA repair pathways are not the only events that may have immune consequences. In fact, inflammation is the process where reactive oxygen and nitrogen species (RONS) and other mediators, including cytokines, metalloproteinases (MMPs), and PGE2, are produced by inflammatory cells. The same inflammatory signals may, in turn, amplify and perpetuate the inflammatory cascade—e.g., MMPs induce reactive oxygen intermediates, whereas cytokines induce PGE2. The cGAS/STING pathway consists of the activation of a cGAS enzyme by aberrant cytosolic

DNA that produces cGAMP, activating the STING protein, leading to the production of pro-inflammatory cytokines, such as type I interferon (IFN), that boost the immune response. [69]

Inflammation has the capability to induce the production of HIF-1α in cancer cells, because of inflammatory cytokines (TNF and IL-1β), prostaglandin (PGE2), and RONS. HIF-1α in turn downregulates MMR proteins, such as MSH2 and MSH6, by displacing c-Myc from *MSH2*/*MSH6* promoters. A potent RONS, hydrogen peroxide, may damage several proteins and enzymes, including MMR members, disrupting their function and ultimately inactivating this DNA-repair pathway. The BER pathway, which serves to repair DNA damage caused by UV exposure and chemotherapeutic agents, appears to be affected by IL-6, which induces hypermethylation in multiple myeloma cells, leading to dysfunction of the key nucleotide excision repair component hHR23B. [70] Moreover, HIF-1α induces microRNA-373, which downregulates the expression of the NER component RAD23B. [71]

1.5 Inflammatory indexes and ovarian cancer

Several prognostic factors have been proposed to reliably predict ovarian cancer outcome, including histology, tumor stage, and residual disease after surgical debulking, response to chemotherapy and BRCA1/2-mutation status. [50] Indeed, HGSOC is frequently associated with homologous repair (HR) deficiencies whereas microsatellite instability (MSI) phenotype has been reported in up to 14% of ovarian endometrioid carcinomas and in about 10% of clear cell ovarian cancer.

A gene expression analysis of endometrioid ovarian cancer and HGSOC identified four distinct molecular subtypes (“immunoreactive,” “differentiated,” “proliferative” and “mesenchymal”) that did not have, however, a survival time significantly different. [72] A reanalysis of the TCGA classification on a Mayo Clinic cohort of HGSOC indicated the longest survival for the immunoreactive subtype. [73] However, this classification needs to be validated and the authors were not able to define the predictive role of each subtype. In this context, it was previously reported that a high presence of tumor infiltrating lymphocytes (TILs), especially intraepithelial CD4+ and CD8+, correlates significantly with improved outcome. [19,64,74]

As opposed to patients that exhibit a robust immune response in terms of TILs presence and display a better prognosis, tumor immune-escape (a mechanism by which antitumor immunity is effectively neutralized) is one of the main reasons for disease progression and treatment failure. Tumor cells, immune-suppressive T regulatory cells (FOXP3+ CD4+), tumor-associated macrophages (TAMs) are responsible of the inhibition of the activity of immune effectors cells within the tumor microenvironment, including CD4+ T cells, CD8+ T cells, and NK cells, through

the concerted action of a plethora of mediators comprising cytokines (such as IL-10, TGF-β, PGE2) and membrane-bound ligands including B7-H1 and programmed cells death protein 1 (PD-1). [75] In this context, neutrophils can exert effects that might be either tumor promoting or tumor suppressive, depending on the context. Early-stage EOCs secrete factors that stimulate influx of neutrophils into the premetastatic omental niche and induce these neutrophils to form neutrophil extracellular traps (NETs). Cancer cells that are shed by tumors into the circulating peritoneal fluid become trapped by NETs and then form implants on the omentum. [76]

The neutrophil-to-lymphocyte ratio (NLR) (defined as the ratio of neutrophil to lymphocyte count) is the most widely used inflammatory marker to evaluate the systemic potential balance between neutrophil-dependent pro-tumor inflammation and lymphocyte-associated anti-tumor immune response. Elevated NLR in EOC patients has been found to be associated with poor prognosis. [77,78] Again, more recent studies confirmed that a high NLR is correlated with an immunosuppressive profile and is associated to poorer overall survival and could be a predictive marker for treatment efficacy. [79,80]

Regardless all the prognostic factors evaluated till today, platinum-sensitivity, defined as patients who experience recurrence after 6 months from the end of primary platinum-based chemotherapy, is considered the main issue in treatment decision and a factor for predicting survival outcomes. [81] Since inflammatory indexes suggested being associated with treatment efficacy also in this setting, [82] we conducted further analyses within our project in order to evaluate if inflammatory indexes may be associated with germline mutational status and/or with response to PARP inhibitors.

2. Project objectives

Given the strict interplay between DNA repair dysfunction and ovarian cancer development and progression, with about 18% of ovarian cancer patients with germline mutations in *BRCA1* or *BRCA2*, especially in those with high-grade serous carcinoma, we decided to evaluate the frequency of other germline mutations in genes involved in DNA repair pathways .

Thus, we conducted a study in order to evaluate the clinical impact of germline alterations in DNA repair genes (not only in *BRCA1* or in *BRCA2*) in ovarian cancer patients, with three specific aims:

- Aim 1: identify the risk of second malignancies in relation of germline mutations harbored;
- Aim 2: evaluate the clinical outcome of ovarian cancer patients in relation with the mutational status;
- Aim 3: correlate inflammatory indexes with the mutational status with regard of ovarian cancer patient prognosis.

3. Materials and Methods

3.1 Ethics statement

The study was performed in accordance with the Good Clinical Practice and the Declaration of Helsinki and approved by the AVR Ethics Committee (protocol 6326/2020). All the patients enrolled in the study have signed informed consent for the genetic analyses and for the use of the results for research purposes.

3.2 Patients and samples

Patients with a diagnosis of ovarian cancer referring to the IRST Genetic Counseling service or to the Oncology units of the Area Vasta Romagna (AVR) catchment area in the years 2014-2018 were included in this study. To be considered eligible for this multicenter, retrospective study, patients must have a histological confirmed diagnosis of ovarian cancer; patient must have been treated with a first line chemotherapy; patients must have a clinical history available and patients must have performed a blood sample withdrawal for *BRCA1/2* germline alteration. Patients were excluded if information on BRCA status was not available or if clinical history was not complete.
All the consecutive ovarian cancer patients referred from 1st January 2014 to 31th December 2018 were considered eligible for this multicenter, retrospective study. The following data were collected from all consenting patients after registration:

- demographic data: birthday, weight and height at the time of treatment initiation, ECOG performance status;
- Tumor information: date of diagnosis, ovarian cancer histology, grade and stage, date of second malignancy onset, type of tumor and its main characteristics;
- Treatment information: use of neoadjuvant treatment, date of start and end of chemotherapy, chemotherapeutic regimen with doses, number of cycles administered, type of surgery, date of surgery, residual disease at surgery, date of progression/relapse (if any), number and types of further therapeutic regimens;
- Maintenance treatment: type of PARP inhibitor used (if any), start and stop date, after with line, reason for discontinuation, type of treatment use thereafter and date of post progression with the subsequent regimen;
- Date of death or last follow-up (if still alive).

Blood sample was collected at the time of genetic counseling referral, mainly at the time of diagnosis. Some patients may have been referred later on at the time of relapse. Peripheral blood of

patients was collected and stored at -80°C for the subsequent molecular analyses. Genomic DNA was extracted with QIAamp DNA mini kit (Qiagen) and quantified using Qubit dsDNA BR Assay kit (Thermo Fisher Scientific).

Information on neutrophil, lymphocyte and platelet counts from blood tests carried out at baseline (immediately before the 1st cycle of the first line) and before maintenance therapy, initiation was collected. SII was calculated as (platelet count × neutrophil count)/lymphocyte count, and NLR was obtained by dividing the absolute neutrophil count by the absolute lymphocyte count. NLR ≥ 3 and SII ≥ 730 were considered as high values.

After treatment completion, patients were followed up with physical examination, radiographic evaluation (CT scan of the chest and abdomen) and CA125 blood test every 3–4 months in the first two years, and every 6 months thereafter. After the 5th years, patients were visited annually. Progression was defined as the appearance of a new lesion or the increase in dimension of a known metastasis according to the Response Evaluation Criteria in Solid Tumors. Increase in tumor marker alone was not considered a progressive disease (PD).

3.3 Next-generation sequencing (NGS) analysis on blood samples

Sequencing libraries were generated using 50 ng of genomic DNA. Libraries were enriched for the regions of interest with the Trusight Cancer panel (Illumina), including the coding regions and flanking introns of 94 genes involved in hereditary cancer (Table 2). The sequencing was performed using the MiSeq platform (Illumina) with MiSeq Reagent Kit v2 configured 2x150 cycles, according to manufacturer's instructions, as previously described. [83,84]

Table 2 - List of the 94 genes included in the Trusight Cancer panel

Genes									
AIP	*ALK*	*APC*	*ATM*	*BAP1*	*BLM*	*BMPR1A*	*BRCA1*	*BRCA2*	*BRIP1*
BUB1B	*CDC73*	*CDH1*	*CDK4*	*CDKN1C*	*CDKN2A*	*CEBPA*	*CEP57*	*CHEK2*	*CYLD*
DDB2	*DICER1*	*DIS3L2*	*EGFR*	*EPCAM*	*ERCC2*	*ERCC3*	*ERCC4*	*ERCC5*	*EXT1*
EXT2	*EZH2*	*FANCA*	*FANCB*	*FANCC*	*FANCD2*	*FANCE*	*FANCF*	*FANCG*	*FANCI*
FANCL	*FANCM*	*FH*	*FLCN*	*GATA2*	*GPC3*	*HNF1A*	*HRAS*	*KIT*	*MAX*
MEN1	*MET*	*MLH1*	*MSH2*	*MSH6*	*MUTYH*	*NBN*	*NF1*	*NF2*	*NSD1*
PALB2	*PHOX2B*	*PMS1*	*PMS2*	*PRF1*	*PRKAR1A*	*PTCH1*	*PTEN*	*RAD51C*	*RAD51D*
RB1	*RECQL4*	*RET*	*RHBDF2*	*RUNX1*	*SBDS*	*SDHAF2*	*SDHB*	*SDHC*	*SDHD*
SLX4	*SMAD4*	*SMARCB1*	*STK11*	*SUFU*	*TMEM127*	*TP53*	*TSC1*	*TSC2*	*VHL*
WRN	*WT1*	*XPA*	*XPC*						

3.4 Data analysis and variant calling

Paired-end sequencing reads were aligned to the reference human genome (UCSC hg19) with the Burrows-Wheeler algorithm v0.7.15-r1140 [85]. Sequences around insertions and deletions (indels) were realigned locally with GATK v3.6-0 [86]. Then picard MarkDuplicates v2.6.0 (http://broadinstitute.github.io/picard/) was used to remove duplicate read-pairs artifacts arising during PCR amplification or sequencing. Data then underwent Base Quality Score Recalibration (BQSR) to ensure good call quality and to reduce the number of false positives (again with GATK). Variant calling was separately performed with GATK UnifiedGenotyper and freebayes v1.0.2-58 [87], then the resulting VCF files were merged with GATK CombineVariants. ANNOVAR v2016-02-01 was used for genomic and functional annotations of detected variants [88], while coverage statistics were computed with DepthOfCoverage utility of GATK and downstream custom bash/R scripts. The resulting annotated list of variants was filtered for variants present in exonic regions or in the 20 bases flanking each exon.

3.5 Additional BRCA1/2 analyses

BRCA1/2 regions covered <50X were amplified by standard PCR and sequenced using the Big Dye Terminator v.3.1 cycle sequencing kit (Thermo Fisher Scientific) on ABI-3130 Genetic analyzer (Applied Biosystems). Multiplex Ligation-dependent Probe Amplification (MLPA) analysis with BRCA1-P002 and BRCA2-P045 kits (MRC Holland) was performed to identify gross deletions/insertions not detectable by sequencing. MLPA results were analyzed by Coffalyser software (MRC Holland).

3.6 Variant classification

Genetic variants were classified into five classes, according to IARC recommendations. [89] *BRCA1/2* variants classification was performed consulting the main *BRCA* mutation databases, such as BRCA Exchange, BRCA Share, LOVD. [90-92] Sequence variants in the other 92 genes were classified using ClinVar [93] and dbSNP. [94] The variants absent in any of these databases were classified using VarSome [95] according with the guidelines of the American College of Medical Genetics. [96]

3.7 Inflammatory indexes

Information on neutrophil, lymphocyte and platelet counts from blood tests carried out at baseline (immediately before the 1st cycle of the first line) and maintenance treatment initiation was collected. SII was calculated as (platelet count × neutrophil count)/lymphocyte count, NLR was obtained by dividing the absolute neutrophil count by the absolute lymphocyte count and PLR resulted by the ratio between platelets and lymphocyte count. NLR, PLR and SII were dichotomized according with their median values.

3.8 Statistical analysis

Data were summarized by mean ± standard deviation (SD) or median, interquartile (IQ) range and minimum and maximum value, as appropriate, for continuous variables and through natural frequencies and percentages for categorical ones. The association between categorical variables was tested by the Pearson's χ^2 test or the Fisher's exact test, as appropriate, whereas those between a continuous variable and a categorical one was tested by means of the Student *t*-test or the F test or the analogous non-parametric tests, when appropriate.

Platinum sensitivity was defined as the time in months from the date of end of platinum-based chemotherapy until the date of relapse or death from any cause, whichever occurred first. Alive patients without relapse were censored at the time of last follow-up.

The prognosis of patients treated with PARP inhibitors was investigated in terms of progression-free survival (PFS) defined as the time in months from the date of inhibitor initiation until disease progression or death from any cause, whichever occurred first and overall survival (OS) define as the time in months from the date of inhibitor initiation until death from any cause. Patients were censored at the date of last follow-up update.

Time-to-event outcomes were analyzed by means of the Cox proportional hazards model; the effect of biological and clinical covariates was reported in terms of hazard ratios (HRs) and corresponding 95% confidence intervals (CIs).

All statistical analyses were performed using STATA 15.0 software (College Station, TX, USA).

4. Results

4.1 Study population

Between January 2014 and December 2018, 219 patients were recruited in this study, with a mean age of 69 years (standard deviation ± 11.3). Principal clinical characteristics of our study population are presented in Table 3. Patient characteristics were in line with the ones commonly seen in routine clinical practice: the majority (74%) were high grade serous ovarian cancer. At diagnosis, 164 patients (74.9%) presented a disease with an advanced stage (FIGO III/IV) and 79 patients (36.1% of our study population) had ascites at clinical presentation. Of note, only 115 (52,5%) patients of our casuistry were defined as low risk patients. This category was defined if patients underwent to primary debulking surgery without residual disease.

Table 3 - Patient characteristics

Patient characteristics	**No. 219 pts**	(%)
Ovarian cancer histology		
High grade serous	162	(74)
Endometrioid	16	(7,3)
Clear cell	12	(5,5)
Others	29	(13,2)
Grade		
G1	12	(5,8)
G2	4	(1,8)
G3	188	(85,8)
Missing	15	(6,8)
Stage		
I/II	43	(19,6)
III/IV	164	(74,9)
Missing	12	(5,5)
Ascites		
No	126	(57,5)
Yes	79	(36,1)
Unknown	14	(6,4)
Risk category		
Low risk	115	(52,5)
High risk	93	(42,5)
Missing	11	(5,0)

4.2 Mutational status

The molecular analysis of the 219 patients showed a mean target coverage of 404X and a 95.3% mean percentage of target covered >50X. We observed 42583 variants in the exonic and splicing regions of 94 genes. Going in major details, we observed 2501 variants in *BRCA1* or *BRCA2* genes, classified according to IARC guidelines and online databases in:

- 14 pathogenic/likely pathogenic variants in *BRCA1*;
- 20 pathogenic/likely pathogenic in *BRCA2*;
- 17 variants of uncertain significance (VUS) of which 4 in *BRCA1* and 13 in *BRCA2* gene;
- 2450 benign variants.

All together, the 34 *BRCA1/2* pathogenic/likely-pathogenic variants were present in 34/219 patients (15.5%), specifically 14/219 (6.4%) had a *BRCA1* mutation (mean age 55.93 years ± 6.40) and 20/219 (9.1%) had a *BRCA2* mutation (mean age 64 years ± 7.47). Mutations details are shown in Table 4. We also found that 17/219 patients (7.8%) harbored a VUS in *BRCA1/2* genes, of whom 2 had also a pathogenic variant in *BRCA1*.

Considering the other 92 genes of the panel, we observed 40082 variants that were classified according to ACMG guidelines in:

- ✓ 38 pathogenic/likely-pathogenic variants;
- ✓ 4710 variants of uncertain significance (VUS);
- ✓ 35334 benign variants.

The 38 pathogenic/likely-pathogenic variants were present in 21 genes in 36/219 patients (16.4%) with mean age of 62.52 years ± 14.26: *PPM1D* (8 variants), *MUTYH* (4 variants), *MITF* (3 variants), *RAD51C* (3 variants), *BRIP1* (2 variants), *ALK* (2 variants), *CHEK2* (2 variants), *PRF1* (1 variant), *PALB2* (1 variant), *FANCD2* (1 variant), *ERCC5* (1 variant), *MLH1* (1 variant), *SBDS* (1 variant), *TP53* (1 variant), *EGFR* (1 variant), *RECQL4* (1 variant), *ERCC2* (1 variant), *MSH2* (1 variant), *ERCC3* (1 variant), *FANCL* (1 variant), *HOXB13* (1 variant).

Table 4 - List of the 34 BRCA1/2 pathogenic/likely pathogenic variants identified in the case series.

Patient ID	1st cancer	Age at onset	2nd cancer	Age at onset	Gene	Exon	DNA (HGVS)	Protein (HGVS)	Variant type	IARC class	dbSNP	ClinVar
A125	BC	48	OC	50	*BRCA2*	23	c.9097dupA	p.Thr3033AsnfsTer11	frameshift insertion	5	rs397507419	pathogenic
A284	OC	49	-	-	*BRCA1*	20	c.5266dupC	p.Gln1756ProfsTer74	frameshift insertion	5	rs80357906	pathogenic
A643	BC	66	OC	70	*BRCA2*	11	c.3897_3901del	p.Glu1299AspfsTer7	frameshift deletion	4	-	-
A793	BC	54	OC	67	*BRCA2*	11	c.3847_3848del	p.Val1283LysfsTer2	frameshift deletion	5	rs80359405	pathogenic
A835	BC	40	OC	61	*BRCA1*	14	c.4484G>T	p.Arg1495Met	missense variant	5	rs80357389	pathogenic
A882	OC	55	-	-	*BRCA1*	11	c.4035delC	p.Glu1346LysfsTer20	frameshift deletion	5	rs80357711	pathogenic
A884	BC	52	OC	60	*BRCA2*	11	c.3743_3746del	p.Ser1248ArgfsTer10	frameshift deletion	5	rs80359403	pathogenic
A891	OC	64	-	-	*BRCA1*	11	c.1513A>T	p.Lys505Ter	nonsense variant	5	rs397508877	pathogenic
A899	OC	61	BC	66	*BRCA2*	16	c.7618-2A>G	p.?	splicing variant	5	rs886040940	pathogenic
A922	BC	42	OC	66	*BRCA2*	11	c.6468_6469del	p.Gln2157IlefsTer18	frameshift deletion	5	rs80359596	pathogenic
A938	BC	46	OC	52	*BRCA1*	8	c.529delT	p.Ser177LeufsTer57	frameshift deletion	5	rs80357758	pathogenic
B160	OC	66	-	-	*BRCA2*	11	c.4889C>G	p.Ser1630Ter	nonsense variant	5	rs80358711	pathogenic
B165	OC	74	-	-	*BRCA2*	14	c.7180A>T	p.Arg2394Ter	nonsense variant	5	rs80358946	pathogenic
B166	BC	71	OC	71	*BRCA1*	11	c.3748G>T	p.Glu1250Ter	nonsense variant	5	rs28897686	pathogenic
B215	OC	66	RCC	65	*BRCA2*	11	c.5868dupT	p.Ile1957TyrfsTer3	frameshift insertion	4	-	-
B220	OC	51	-	-	*BRCA2*	13	c.6998dupT	p.Pro2334ThrfsTer6	frameshift insertion	5	rs754611265	pathogenic
B245	OC	57	-	-	*BRCA1*	11	c.3700_3704del	p.Val1234GlnfsTer8	frameshift deletion	5	rs80357609	pathogenic
B270	OC	56	-	-	*BRCA1*	10	c.615dupA	p.Gln206ThrfsTer10	frameshift insertion	4	rs1567803215	uncertain significance
B294	OC	64	-	-	*BRCA2*	11	c.3046G>T	p.Glu1016Ter	nonsense variant	5	rs748508287	pathogenic
B295	OC	57	-	-	*BRCA2*	10	c.1813delA	p.Ile605TyrfsTer9	frameshift deletion	5	rs80359306	pathogenic
B319	OC	53	-	-	*BRCA1*	24	c.5503C>T	p.Arg1835Ter	nonsense variant	5	rs41293465	pathogenic
B336	OC	63	-	-	*BRCA2*	11	c.6037A>T	p.Lys2013Ter	nonsense variant	5	rs80358840	pathogenic
B351	OC	65	-	-	*BRCA2*	11	c.4284dupT	p.Gln1429SerfsTer9	frameshift insertion	5	rs80359439	pathogenic
B359	OC	49	-	-	*BRCA1*	14	c.4484G>T	p.Arg1495Met	missense variant	5	rs80357389	pathogenic

B363	OC	49	-	-	*BRCA1*	24	c.5468-1G>A	p.?	splicing variant	5	rs80358048	pathogenic
B365	OC	54	-	-	*BRCA1*	11	c.850C>T	p.Gln284Ter	nonsense variant	5	rs397509330	pathogenic
B372	OC	52	-	-	*BRCA1*	11	c.850C>T	p.Gln284Ter	nonsense variant	5	rs397509330	pathogenic
B404	OC	76	-	-	*BRCA2*	11	c.2905C>T	p.Gln969Ter	nonsense variant	5	rs886038080	pathogenic
B409	BC	35	OC	58	*BRCA2*	11	c.2684delC	p.Ala895ValfsTer9	frameshift deletion	5	rs80359342	pathogenic
B413	OC	58	-	-	*BRCA2*	11	c.4284dupT	p.Gln1429SerfsTer9	frameshift insertion	5	rs80359439	pathogenic
B418	OC	61	-	-	*BRCA1*	14	c.4484G>T	p.Arg1495Met	missense variant	5	rs80357389	pathogenic
B465	OC	69	-	-	*BRCA2*	11	c.2684delC	p.Ala895ValfsTer9	frameshift deletion	5	rs80359342	pathogenic
B519	OC	77	-	-	*BRCA2*	27	c.9871del	p.Ser3291LeufsTer22	frameshift deletion	5	rs886040854	pathogenic
B682	OC	64	-	-	*BRCA2*	11	c.3847_3848del	p.Val1283LysfsTer2	frameshift deletion	5	rs80359405	pathogenic

OC: ovarian cancer
BC: breast cancer
RCC: renal cell carcinoma

Mutation details are shown in Table 5. Out of these 36 patients, two had pathogenic/likely-pathogenic mutations in two different genes (B184 in *CHEK2* and *EGFR*, and B421 in *ALK* and *FANCD2*) whereas four had also a pathogenic mutation in *BRCA1/2* genes (1 in *BRCA1* and 3 in *BRCA2*), so these four patients were considered in *BRCA1/2* mutated group (Fig. 3).

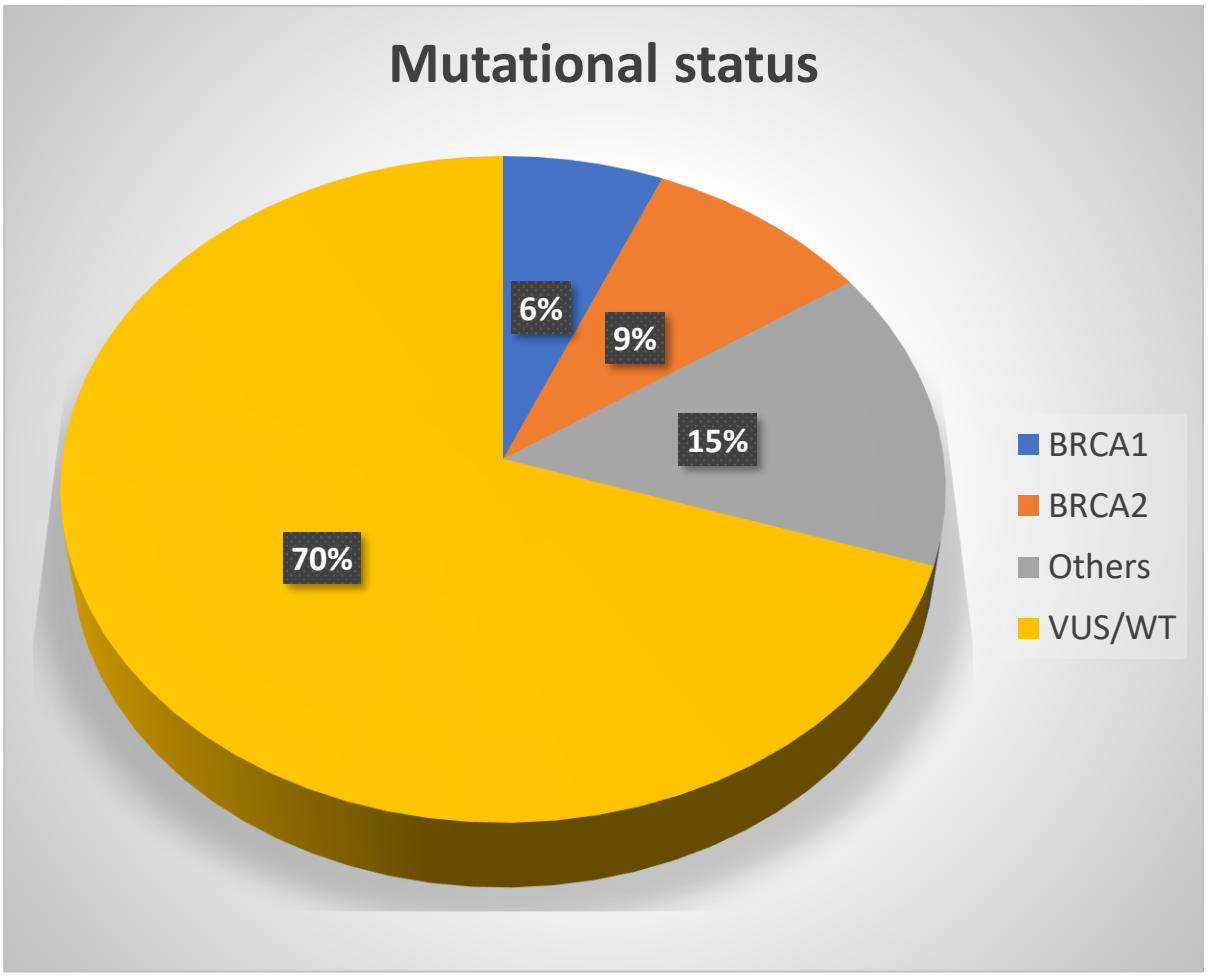
Table 5 - List of the 38 pathogenic/likely pathogenic variants in genes other than *BRCA1/2* identified in the case series.

Patient ID	1st cancer	Age at onset	2nd cancer	Age at onset	*BRCA1/2* status	Gene	Transcript	Exon	DNA (HGVS)	Protein (HGVS)	Variant type	IARC class	dbSNP	ClinVar
A284	OC	49	-	-	*BRCA1+*	*ERCC3*	NM_000122	11	c.1757delA	p.Gln586ArgfsTer25	frameshift deletion	5	rs753182861	pathogenic
A893	OC	52	-	-	wt	*PPM1D*	NM_003620	6	c.1535dupA	p.Asn512LysfsTer16	frameshift insertion	4	rs763475304	-
A906	OC	50	-	-	wt	*SBDS*	NM_016038	2	c.258+2T>C	p.?	splicing variant	5	rs113993993	pathogenic
A912	OC	74	-	-	wt	*PPM1D*	NM_003620	6	c.1426G>T	p.Glu476Ter	nonsense variant	4	rs1296018768	-
A913	OC	64	-	-	wt	*PPM1D*	NM_003620	6	c.1654C>T	p.Arg552Ter	nonsense variant	5	rs779070661	pathogenic
A916	NET	69	OC	73	wt	*BRIP1*	NM_032043	9	c.1201_1204dup	p.Ala402ValfsTer21	frameshift insertion	5	rs730881647	pathogenic
A917	OC	59	-	-	wt	*PPM1D*	NM_003620	6	c.1273delG	p.Asp425IlefsTer6	frameshift deletion	4	-	-
A918	OC	54	-	-	wt	*BRIP1*	NM_032043	8	c.1018_1019insCT	p.Leu340ProfsTer9	frameshift insertion	5	rs878855134	pathogenic
A939	OC	75	-	-	wt	*TP53*	NM_000546	8	c.817C>T	p.Arg273Cys	missense variant	5	rs121913343	pathogenic
A944	OC	65	-	-	wt	*PPM1D*	NM_003620	6	c.1654C>T	p.Arg552Ter	nonsense variant	5	rs779070661	pathogenic
B144	OC	47	-	-	wt	*MUTYH*	NM_012222	12	c.1162C>T	p.Gln388Ter	nonsense variant	5	rs587783057	pathogenic
B167	OC	54	-	-	wt	*MUTYH*	NM_012222	13	c.1178G>A	p.Gly393Asp	missense variant	5	rs36053993	pathogenic
B184	OC	77	-	-	wt	*EGFR*	NM_005228	7	c.844G>T	p.Glu282Ter	nonsense variant	4	-	-
						CHEK2	NM_007194	11	c.1232G>A	p.Trp411Ter	nonsense variant	5	rs371418985	pathogenic
B204	OC	79	-	-	wt	*PPM1D*	NM_003620	6	c.1281G>A	p.Trp427Ter	nonsense variant	5	rs1064797099	pathogenic
B205	OC	39	-	-	wt	*ALK*	NM_004304	16	c.2782dupT	p.Cys928LeufsTer20	frameshift insertion	4	rs1218092221	-
B220	OC	51	-	-	*BRCA2+*	*HOXB13*	NM_006361	1	c.251G>A	p.Gly84Glu	missense variant	4	rs138213197	conflicting
B243	OC	75	-	-	wt	*RECQL4*	NM_004260	15	c.2300delT	p.Val767GlyfsTer76	frameshift deletion	4	rs752895803	-
B303	OC	56	-	-	wt	*RAD51C*	NM_058216	7	c.905-2_905-1del	p.?	splicing variant	5	rs587781995	pathogenic
B330	OC	70	-	-	wt	*MITF*	NM_000248	9	c.952G>A	p.Glu318Lys	missense variant	5	rs149617956	pathogenic
B336	OC	63	-	-	*BRCA2+*	*PPM1D*	NM_003620	6	c.1465delT	p.Ser489LeufsTer2	frameshift deletion	4	-	-
B357	OC	85	-	-	wt	*PRF1*	NM_005041	2	c.160C>T	p.Arg54Cys	missense variant	5	rs200430442	pathogenic
B391	OC	46	-	-	wt	*MITF*	NM_000248	9	c.952G>A	p.Glu318Lys	missense variant	5	rs149617956	pathogenic
B406	OC	69	-	-	wt	*PALB2*	NM_024675	4	c.1140_1143del	p.Ser380ArgfsTer43	frameshift deletion	5	rs1257545151	pathogenic
B419	OC	73	-	-	wt	*ERCC2*	NM_000400	21	c.2005_2006del	p.Arg669GlyfsTer104	frameshift deletion	4	rs757535186	-
B421	OC	40	-	-	wt	*ALK*	NM_004304	16	c.2782dupT	p.Cys928LeufsTer20	frameshift insertion	4	rs1218092221	-

						FANCD2	NM_033084	35	c.3541C>T	p.Gln1181Ter	nonsense variant	5	-	pathogenic
B426	OC	78	-	-	wt	*RAD51C*	NM_058216	1	c.93delG	p.Phe32SerfsTer8	frameshift deletion	5	rs730881942	pathogenic
B458	OC	76	-	-	wt	*ERCC5*	NM_000123	15	c.3285_3294del	p.Ser1096AspfsTer12	frameshift deletion	4	-	-
B476	TC	26	OC	45	wt	*MUTYH*	NM_012222	7	c.527A>G	p.Tyr176Cys	missense variant	5	rs34612342	pathogenic
B513	OC	54	-	-	wt	*RAD51C*	NM_058216	7	c.905-2_905-1del	p.?	splicing variant	5	rs587781995	pathogenic
B519	OC	77	-	-	*BRCA2*+	*MSH2*	NM_000251	5	c.942+2delT	p.?	splicing variant	4	rs587779194	likely pathogenic
B542	OC	82	-	-	wt	*PPM1D*	NM_003620	6	c.1654C>T	p.Arg552Ter	nonsense variant	5	rs779070661	pathogenic
B571	OC	51	-	-	wt	*FANCL*	NM_018062	14	c.1096_1099dup	p.Thr367AsnfsTer13	frameshift insertion	4	rs759217526	conflicting
B612	OC	78	-	-	wt	*CHEK2*	NM_007194	4	c.514dupA	p.Thr172AsnfsTer14	frameshift insertion	5	rs1601823546	pathogenic
B618	OC	68	-	-	wt	*MUTYH*	NM_012222	13	c.1178G>A	p.Gly393Asp	missense variant	5	rs36053993	pathogenic
B693	OC	43	-	-	wt	*MLH1*	NM_000249	12	c.1039-1G>C	p.?	splicing variant	4	rs267607819	likely pathogenic
B697	OC	54	-	-	wt	*MITF*	NM_000248	9	c.952G>A	p.Glu318Lys	missense variant	5	rs149617956	pathogenic

OC: ovarian cancer
NET: neuroendocrine tumor
TC: thyroid carcinoma

Figure 3 – Frequency of germline mutation in our patient population



4.3 Mutational status subgroups

Our study population was grouped according to the germline mutational status in four groups:

a) *BRCA1* mutated (14 patients)
b) *BRCA2* mutated (20 patients)
c) Other genes (32 patients)
d) Wild type population (153 patients).

Since VUS in *BRCA1/2* demonstrated a similar clinical outcome compared to wild type patients, also as regard to PARP inhibitors efficacy [97], we categorized these patients in a single group. As reported in table 6 no significant differences in patients' characteristics were seen among the different subgroups: not for age at diagnosis (even if *BRCA2* patients were more frequently older), nor for histology, nor for stage nor for clinical presentation.

Table 6 – Patient characteristics according to mutational status

	BRCA1 (n=14)	*BRCA2* (n=20)	OTHERS (n=32)	VUS + WT (n=153)	TOTAL (n=219)	p
Mean age ± SD	55.9 ± 6.4	64 ± 7.5	62.5 ± 14.3	59.6 ± 11.0	60.1 ± 11.3	0,083
Histology						0.497
High grade serous	11 (78.57)	19 (95.00)	23 (71.88)	109 (71.24)	162 (73.97)	
Endometrioid	1 (7.14)	0	2 (6.25)	13 (8.50)	16 (7.31)	
Other	2 (14.29)	1 (5.00)	7 (21.88)	31 (20.26)	41 (18.72)	
Grade						0.721
G1	1 (7.14)	0	3 (11.11)	8 (5.56)	12 (5.88)	
G2	0	0	0	4 (2.78)	4 (1.96)	
G3	13 (92.86)	19 (100.00)	24 (88.89)	132 (91.67)	188 (92.16)	
missing	-	1	5	9	15	
Stage						0.760
I/II	3 (21.43)	2 (10.53)	6 (22.22)	32 (21.77)	43 (20.77)	
III/IV	11 (78.57)	17 (89.47)	21 (77.78)	115 (78.23)	164 (79.23)	
missing	-	1	5	6	12	
Ascites						0.766
No	8 (61.54)	12 (63.16)	19 (70.37)	87 (59.59)	126 (61.46)	
Yes	5 (38.46)	7 (36.84)	8 (29.63)	59 (40.41)	79 (38.54)	
missing	1	1	5	7	14	
Visceral metastasis						0.491
No	5 (83.33)	11 (84.62)	12 (93.31)	61 (92.42)	89 (90.82)	
Yes	1 (16.67)	2 (15.38)	1 (7.69)	5 (7.58)	9 (7.18)	
missing	8	7	19	87	121	
Risk						0.925
Low	7 (50.00)	10 (52.63)	14 (51.85)	84 (56.76)	115 (55.29)	
High	7 (50.00)	9 (47.37)	13 (48.15)	64 (43.24)	93 (44.71)	
missing	-	1	5	5	11	

*4.4 **Aim 1** – Germline mutational status and second malignancy*

Among our 219 ovarian cancer patients, 182 patients developed ovarian cancer alone, 16 ovarian cancer and breast cancer and 21 patients developed ovarian cancer and another tumor. Table 7 reports the other tumors identified with the specific mutation. We observed an association between the types of tumor and mutational status ($p<0.001$). In particular, patients with pathogenic mutations in genes other than *BRCA1/2* developed ovarian cancer alone in 30 (93.8%) patients. Two patients (6.2%) developed ovarian cancer and a neuroendocrine tumor (1 patient – mutation in *BRIP1*) and a

thyroid cancer (1 patient – mutation in *MUTYH*). Only 6 out of 153 (3.9%) patients developed ovarian and breast cancer if did not harbor pathogenic variants in any of the genes analyzed, in contrast to 10 out of 34 (29.4%) patients with *BRCA1/2* pathogenic mutations.

All the 3 patients who developed ovarian and breast cancer with a germline BRCA1 mutation had a breast cancer diagnosis (median age 42 years) before the onset of ovarian cancer (median age 62 years). Again, in the 7 patients with a germline BRCA2 mutation, the onset of breast cancer preceded always ovarian cancer diagnosis, with a median age of 52 years and 66 years, respectively.

Table 7 – Mutational status group and second malignancy

	BRCA1 pathogenic (n=14)	*BRCA2* pathogenic (n=20)	Pathogenic in OTHER GENES (n=32)	WT + *BRCA1/2* VUS (n=153)	TOTAL (n=219)	p value
Ovarian cancer	11 (78.6)	12 (60)	30 (93.8)	129 (84.3)	182	
Ovarian and breast cancer	3 (21.4)	7 (35)	0	6 (3.9)	16	<0.001
Ovarian cancer and another tumor	0	1 (5)	2 (6.2)	18 (11.8)	21	

*4.5 **Aim 2** – Mutational status and clinical outcome*

We then analyzed the response to platinum-based chemotherapy of 208 patients (11 patients were undetermined). Ovarian cancer patients were classified as low risk if residual disease did not occur during primary debulking surgery. Patients with residual disease and/or patients who underwent interval surgery were classified as high risk. Median time from the last administration of a platinum-based chemotherapy and the relapse or death was 24.2 months (IQ range 10.61 – 43.50) in our study population. Among the 115 patients in the low risk group, the median platinum-free interval was 35.8 months (IQ range 14.98-58.11). In the high risk group (93 patients), the median platinum-free interval was 15.2 months (IQ range 6.50 – 26.41), significant lower than the other group (p<0.001). Table 8 shows the association between platinum sensitivity and histology.

Table 8 - Association between platinum sensitivity and histology in our case series.

	Serous high-grade (n=162)	Endometrioid (n=16)	Other (n=41)	Total (n=219)	*P*
Platinum sensitivity					0.210
Median [IQ range]	22.39 [10.40 – 42.82]	19.09 [8.71 – 38.3]	37.73 [14.85 – 48.52]	24.15 [10.61 – 43.50]	
Min - max	0 – 124.74	0.16 – 128.81	0.62 – 329.4	0 – 329.4	
missing	10	1	15	26	

No statistically significant association between median platinum-free interval (PFI) and mutational status subgroup was observed (Table 9). However, whereas there were similar median PFI between the wild type group and the group with germline mutation in other genes (about 21 months), a clinical relevant, although not statistical proven, difference was seen between *BRCA1* group and *BRCA2* group: 15.8 months vs 42.5 months, respectively.

Table 9 – Median time of platinum-free interval in relation to mutational status

Platinum sensitivity	***BRCA1* pathogenic (n=14)**	***BRCA2* pathogenic (n=20)**	**Pathogenic in Other Genes (n=32)**	**VUS + WT (n=153)**	**TOTAL (n=219)**	**P**
Median [IQ range]	15.8 [7.8 – 37.5]	42.5 [15.4 – 52.1]	21.8 [8.8 – 33.8]	23.9 [9.7 – 43.0]	23.7 [10.1 – 43.3]	0.167
Minimum - maximum	5.4 – 108.3	4.3 – 124.7	0 – 118.9	0.2 - 329.4	0 – 329.4	
missing	2	3	8	13	26	

In our case series, 43 patients were treated with a maintenance treatment in subsequent lines than the first one. Out of 43:

- 5 presented a pathogenic germline mutation in *BRCA1*
- 7 presented a pathogenic germline mutation in *BRCA2*

- 4 patients a pathogenic germline mutation in 4 other genes (*PALB2*, *ERCC2*, *ALK* and *MITF*)
- 27 patients had no pathogenic mutations in any of the 94 genes.

Due the low number of the patients treated with maintenance PARP inhibitor, we decided to group patients with germline mutation in *BRCA1* or in *BRCA2* in the same group. We observed that patients with *BRCA1/2* VUS or without germline mutations had a similar outcome compared to patients with a pathogenic mutation in *BRCA1/2* genes (HR=1.15, 95% CI 0.53 – 2.48, *p*=0.715; HR=0.72, 95% CI 0.27 – 1.91, *p*=0.511, for PFS and OS respectively). Differently, patients with pathogenic mutations in genes other than *BRCA1/2* had a significantly worse PFS (HR=3.56, 95% CI 1.05 – 12.04, *p*=0.042) and a worse OS (HR=1.38, 95% CI 0.15 – 12.13, *p*=0.772), as shown in Table 10.

Table 10 - Clinical outcome of patient in PARP inhibitor maintenance treatment in relation with mutational status

Mutational status	**PFS**		**OS**	
	HR (95% CI)	**p Cox**	**HR (95% CI)**	**p Cox**
BRCA1/2 pathogenic	1		1	
Pathogenic in OTHER GENES	3.56 (1.05 – 12.04)	0.042	1.38 (0.15 – 12.13)	0.772
WT + BRCA1/2 VUS	1.15 (0.53 – 2.48)	0.715	0.72 (0.27 – 1.91)	0.511

*4.6 **Aim 3**: Inflammatory index, mutational status and ovarian cancer specific outcome*

Data on pre-treatment inflammatory index (NLR, PLR, and SII) levels was available for 118 patients enrolled. Median NLR value was 246 (IQ range 171 - 339), median PLR value was 200 (IQ range 140 - 280) and median SII was 739 (IQ range 432 - 1349). We did not observe any significant correlation between inflammatory indexes and mutational status (Table 11).

Table 11 *- Levels of neutrophils, lymphocytes, platelets, NLR, PLR, SII before treatment initiation and mutational status.*

	BRCA1+ (n=14)	*BRCA2*+ (n=20)	Other genes (n=32)	WT (n=153)	Total (n=219)	*P*
Neutrophils						0.347
Median [IQ range]	3310 [2230 - 5010]	4200 [2950 - 5730]	3510 [2390 - 4945]	4150 [2880 - 5620]	4115 [2770 - 5440]	
Min - max	1430 - 11300	2560 – 12970	1600 – 14300	1960 - 11450	1430 - 14300	
missing	4	9	12	64	89	
Lymphocytes						0.261
Median [IQ range]	1350 [945 - 1855]	1480 [1280 - 1660]	1530 [1320 - 1950]	1645 [1365 - 1995]	1585 [1330 - 1920]	
Min - max	178 - 2120	690 – 2150	1010 - 3760	260 - 3200	178 - 3760	
missing	6	9	13	73	101	
Platelets						0.986
Median [IQ range]	339 [172 - 403]	331 [231 - 488]	325.5 [276 – 391.5]	306 [255 - 404]	319 [256 - 410]	
Min - max	133 - 927	199 - 569	96 - 693	176 - 853	96 - 927	
missing	4	9	12	64	89	
NLR						0.156
Median [IQ range]	322 [212 – 520]	282 [187 – 450]	203 [151 – 328]	252 [166 – 342]	246 [171 – 339]	
Min - max	172 – 803	163 – 1323	750 – 1051	920 – 1516	750 – 1516	
missing	6	9	13	73	101	
PLR						0.360
Median [IQ range]	300 [160 – 430]	260 [160 – 310]	190 [140 – 280]	190 [140 – 240]	200 [140 – 280]	
Min - max	100 – 740	130 – 700	50 – 580	70 – 990	50 – 990	
missing	6	9	13	73	101	
SII						0.437
Median [IQ range]	970 [641 – 1109]	1029 [442 – 2078]	541 [357 – 1432]	775 [429 – 1316]	739 [432 – 1349]	
Min - max	133 - 927	409 – 4381	216 – 3701	198 – 7488	198 – 7488	
missing	6	9	13	73	101	
NLR dichot, n (%)						0.067
< 246	2 (25.00)	4 (36.36)	14 (73.68)	39 (48.75)	59 (50.00)	
≥ 246	6 (75.00)	7 (63.64)	5 (26.32)	41 (51.25)	59 (50.00)	
missing	6	9	13	73	101	
PLR dichot, n (%)						0.864
< 200	3 (37.50)	5 (45.45)	10 (52.63)	42 (52.50)	60 (50.85)	
≥ 200	5 (62.50)	6 (54.55)	9 (47.37)	38 (47.50)	58 (49.15)	
missing	6	9	13	73	101	
SII dichot, n (%)						0.268
< 739	3 (37.50)	4 (36.36)	13 (68.42)	39 (48.75)	59 (50.00)	
≥ 739	5 (62.50)	7 (63.64)	6 (31.58)	41 (51.25)	59 (50.00)	
missing	6	9	13	73	101	
Platelets, n (%)						0.361
≤ 450	8 (80.00)	7 (63.64)	18 (90.00)	73 (82.02)	106 (81.54)	
> 450	2 (20.00)	4 (36.36)	2 (10.00)	16 (17.98)	24 (18.46)	
missing	4	9	12	63	89	

Information on inflammatory indexes before PARP inhibitor treatment initiation was available for 29 patients. Considering NLR, PLR and SII as continuous variables, no statistically significant association was found for both PFS and OS. However, for every one standard deviation increase in platelets, a statistically significant higher risk of disease progression in terms of PFS was found (HR=1.52, 95% CI 1.03 – 2.26, p=0.037), as shown in Table 12. No association was found for OS (HR=1.18, 95% CI 0.62 – 2.27, p=0.614).

Table 12 - Results from univariate Cox analysis between inflammatory indexes and PFS

PFS	**HR (95% CI)**	**p value**
neutrophils	0.99 (0.66 – 1.50)	0.981
lymphocytes	1.02 (0.69 – 1.50)	0.922
platelets	1.52 (1.03 – 2.26)	0.037
NLR	0.93 (0.64 – 1.36)	0.702
PLR	1.51 (0.97 – 2.34)	0.067
SII	1.31 (0.88 – 1.96)	0.184

HRs refers to a one standard deviation increase in the continuous variable

5. Discussion

5.1 General considerations

In about 18% of ovarian cancer patients, it is possible to identify germline mutations in *BRCA1* and *BRCA2*, especially in those with high-grade serous carcinoma [11,12]. HR deficiency endows ovarian cancers with a clinical phenotype characterized by visceral relapse, a slightly younger age at diagnosis, and a better response to platinum-based chemotherapy, PARP inhibitors, and anthracyclines [41]. However, alterations in *BRCA1* or in *BRCA2* translate also in higher risk of second malignancies. Considering the results obtained in the last years in better outcome of ovarian cancer patients, information on possible strategies of follow up of these cases are needed.

In this context, since ovarian cancer is a neoplasm strictly linked to alteration in DNA repair genes, we decided to characterize a consecutive series of ovarian cancer patients in order to other germline alterations that might affect patient outcome. For this reason, we used a panel of 94 genes including almost all the genes involved in the main hereditary cancer syndromes with the aims of:

- Aim 1: identify the risk of second malignancies in relation of germline mutations harbored;
- Aim 2: evaluate the clinical outcome of ovarian cancer patients in relation with the mutational status;
- Aim 3: correlate inflammatory indexes with the mutational status with regard of ovarian cancer patient prognosis.

5.2 Discussion on the Aim 1

Current clinical genetic tests for ovarian cancer have been based only on *BRCA1* and *BRCA2* analysis, despite new evidence of a higher number of genes eligible for testing. [98] In our study, performed on 219 ovarian cancer patients referring to the IRST Genetic Counseling service or to the Oncology units of the AVR catchment area, we observed a total of 72 pathogenic/likely-pathogenic variants in 70/219 (32%) patients. In particular, 14 variants were found in *BRCA1* gene, 20 in *BRCA2* and 38 pathogenetic/likely-pathogenic variants were found in other 21 genes. The 38 pathogenic/likely-pathogenic variants in genes other than *BRCA1/2* were observed in 36 patients, 32 of whom did not present any pathogenic/likely-pathogenic variants in *BRCA1/2* genes. The most frequently mutated genes in our case series were: *PPM1D*, *MUTYH*, *MITF*, *RAD51C*, *BRIP1*, *ALK*, *CHEK2*.

BRCA1 and *BRCA2* are part of the BRCA-Fanconi anemia pathway and additional Fanconi genes *BRIP1* (*FANCJ*) and *RAD51C* (*FANCO*) have each been associated with inherited risk of

ovarian cancer. [99-101] *PPM1D* variants are associated with predisposition to breast and ovarian cancer [102,103] along with *MUTYH* [104,105] and *CHEK2*. [105] Moreover, mutations in the mismatch repair genes that cause Lynch syndrome (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) predict cancer risks of ovarian cancer. [106,107] In our case series, we detected a pathogenic/likely-pathogenic mutation in *MLH1* gene in 1 patient who did not present any other mutations in *BRCA1/2* genes and a pathogenic/likely-pathogenic mutation in *MSH2* in 1 patient who present a pathogenic/likely-pathogenic mutation in *BRCA2* gene. Panagiotis et al. underlined that germline sequencing of *BRCA1* and *BRCA2* should be performed in the context of a multigene panel that includes also *RAD51C*, *RAD51D*, *BRIP1*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *PALB2*. [108] These data highlighted that these mutations are associated with higher risk of ovarian cancer development, so it is noteworthy to introduce a multigene panel in standard genetic analysis protocols for patients with suspected hereditary ovarian cancer. Fortunately, we did not find a higher rate of second malignancies, maybe because the short median follow-up on time of our study population, but we cannot exclude that a longer observation period could demonstrate an increased risk.

Interestingly, all the patients with a germline *BRCA1* ore *BRCA2* mutation and a second malignancy, developed first a breast cancer and thereafter an ovarian cancer. This observation highlights the need of a strict follow up in order to identify ovarian cancer early and/or to dis-cuss with the patient about a risk-reducing salpingo-oophorectomy. This procedure demonstrated also to reduce the risk of breast cancer in the immediate 5 years after surgery and in the longer-term, especially in younger women. [109,110]

5.3 Discussion on the Aim 2

Although most patients with ovarian cancer initially respond to platinum-based chemotherapy, about 20% of women will experience disease progression ≤6 months after the last cycle of a platinum-based regimen (platinum-resistant or platinum-refractory). [111] Many efforts have been made over the years to develop predictive biomarkers of platinum-sensitivity. [82]

We found that patients without residual disease after primary debulking surgery (low-risk patients) had a median time from the last administration of platinum-based chemotherapy and the relapse or death significantly higher (35.8 months) than high-risk patients (15.2 months) ($p<0.001$). We also hypothesized a better clinical outcome in patients with the DNA damage response genes altered, because of a worse platinum-induced DNA interstrand cross-links repair capability. [112] However, we failed to demonstrate such association, maybe because the small number of patients in this subgroup.

A subset of 43 patients were treated with PARPi as maintenance treatment in patients with platinum-sensitive ovarian cancer. Patients with pathogenic mutations in genes other than *BRCA1/2* had a significantly worse PFS and OS compared to patients with a pathogenic mutation in *BRCA1/2* genes, suggesting that this may be associated to specific biological mechanisms. However, due to the small number of cases, we could not speculate about it. On the other hand, patients with *BRCA1/2* VUS or without germline mutations had a similar outcome of patients with a pathogenic mutation in *BRCA1/2* genes, confirming literature data. [113]

5.4 Discussion on the Aim 3

There is increasing evidence that inflammation plays an essential role in the development and progression of cancer through the secretion of cytokines and chemokines, facilitating angiogenesis and proliferation and preventing apoptosis. [114] In the tumor microenvironment, neutrophils are capable of favoring cancer progression through the production of tumor necrosis factor, interleukin (IL)-1, and IL-6. These cells also promote adhesion and seeding of distant organ sites through the secretion of circulating VEGF and proteases. [115,116] Platelets induce circulating tumor cell epithelial–mesenchymal transition and promote extravasation to metastatic sites. [117] Lymphocytes exert a critical role in the cancer-specific immune response by inducing cytotoxic cell death and inhibiting tumor cell proliferation and migration. This tumor microenvironment explains why increased tumor-infiltrating lymphocytes are associated with good prognosis. [118,119]

We previously demonstrated that inflammatory indexes (NLR and SII) were independent prognostic factors in recurrent platinum-sensitive ovarian cancer patients. [82] In this analyses, only platelets were correlated with PFS, demonstrating their important role in ovarian cancer not only as poor prognostic factor, [120] but also as possible predictive factor of response to PARP inhibitors. However, a validation of these easy biomarkers in a larger case series is warranted.

5.5 Limitations

It is important to underline some limitations of our study, including its retrospective nature, which may lead to bias in the data analysis. In order to identify the risk of second malignancy, maybe a longer follow up and larger casuistry would be needed. However, interesting observations were made also in our study, especially if we consider that all the patient with ovarian and breast cancer due to a germline pathogenic mutation in *BRCA1* or *BRCA2* developed a breast cancer before the onset of ovarian cancer.

Another important limitation regards the analyses of germline data only. Thus, we could have missed all the possible somatic mutations that might have an impact on patient clinical outcome, but it is our objective to implement this evaluation in the near future. Moreover, when we analyzed the impact of inflammatory indexes as a function of treatment, the number of patients per treatment group was fairly low, especially for patients treated with PARP inhibitors. Thus, the results in patients with pathogenic mutations in genes other than *BRCA1/2* have to be considered only exploratory.

6. Conclusions and future perspectives

Knowledge about molecular alterations in genes beyond *BRCA1/2* in ovarian cancer could allow for more personalized diagnostic, predictive, prognostic, and therapeutic strategies for the patient without forgetting the clinical implications for her own family members. Ovarian cancer frequently harbors germline mutation in DNA repair genes and our casuistry confirms previous literature data (the rate of *BRCA1/2* germline mutation is 15,5%). This not negligible rate of germline mutations in other DNA repair genes (16,4%) does not translate in an increased risk of a second malignancy in our study, but this could have been limited by the short follow up.

However, the study is only at its beginning. Our aim is to implement our analyses by evaluating the somatic mutational status of our casuistry in order to correlate this data with patient outcome. Moreover, we want to look in deep in the correlation between type of surgery ant time to treatment initiation in relation with mutational status.

7. References

1. GLOBOCAN. Available online: **http://gco.iarc.fr/** (accessed on 18 April 2019).
2. Vergote I, Tropé CG, Amant F, et al. Neoadjuvant chemotherapy or primary surgery in stage IIIC or IV ovarian cancer. *N Engl J Med*. 2010;363:943-953.
3. Armstrong DK, Bundy B, Wenzel L, et al. Intraperitoneal cisplatin and paclitaxel in ovarian cancer. *N Engl J Med*. 2006;354:34-43
4. Prat J, D'Angelo E, Espinosa I. Ovarian carcinomas: at least five different diseases with distinct histological features and molecular genetics. *Hum Pathol*. 2018;80:11-27.
5. McCluggage WG. Morphological subtypes of ovarian carcinoma: a review with emphasis on new developments and pathogenesis. *Pathology*. 2011;43:420-432.
6. Kurman RJ, Carcangiu ML, Herrington CS, Young RH. *WHO classification of tumours of female reproductive organs, 4th ed*; International Agency for Research on Cancer: Lyon, France, 2014; pp. 10–40.
7. Karabuk E, Kose MF, Hizli D, et al. Comparison of advanced stage mucinous epithelial ovarian cancer and serous epithelial ovarian cancer with regard to chemosensitivity and survival outcome: a matched case-control study. *J Gynecol Oncol*. 2013;24:160-166.
8. Risch HA, McLaughlin JR, Cole DE, et al. Population *BRCA1* and *BRCA2* mutation frequencies and cancer penetrances: a kin-cohort study in Ontario, Canada. *J Natl Cancer Inst*. 2006;98:1694-1706.
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
10. Dietlein F, Thelen L, Reinhardt HC. Cancer-specific defects in DNA repair pathways as targets for personalized therapeutic approaches. *Trends Genet*. 2014;30:326-339.
11. Press JZ, De Luca A, Boyd N, et al. Ovarian carcinomas with genetic and epigenetic BRCA1 loss have distinct molecular abnormalities. *BMC Cancer*. 2008;8:17.
12. Hennessy BT, Timms KM, Carey MS, et al. Somatic mutations in BRCA1 and BRCA2 could expand the number of patients that benefit from poly (ADP ribose) polymerase inhibitors in ovarian cancer. *J Clin Oncol*. 2010;28(22):3570-3576.
13. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma [published correction appears in *Nature*. 2012 Oct 11;490:298]. *Nature*. 2011;474:609-615.
14. Hilton JL, Geisler JP, Rathe JA, Hattermann-Zogg MA, DeYoung B, Buller RE. Inactivation of BRCA1 and BRCA2 in ovarian cancer. *J Natl Cancer Inst*. 2002;94:1396-1406.
15. Geisler JP, Hatterman-Zogg MA, Rathe JA, Buller RE. Frequency of *BRCA1* dysfunction in ovarian cancer. *J Natl Cancer Inst*. 2002;94:61-67.

16. Mukhopadhyay A, Curtin N, Plummer R, Edmondson RJ. PARP inhibitors and epithelial ovarian cancer: an approach to targeted chemotherapy and personalised medicine. *BJOG*. 2011;118:429-432.
17. Aysal A, Karnezis A, Medhi I, Grenert JP, Zaloudek CJ, Rabban JT. Ovarian endometrioid adenocarcinoma: incidence and clinical significance of the morphologic and immunohistochemical markers of mismatch repair protein defects and tumor microsatellite instability. *Am J Surg Pathol*. 2012;36(2):163-172.
18. Morice P, Gouy S, Leary A. Mucinous Ovarian Carcinoma. *N Engl J Med*. 2019;380:1256-1266.
19. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med*. 2003;348(3):203-213.
20. Jayson GC, Kohn EC, Kitchener HC, Ledermann JA. Ovarian cancer. *Lancet*. 2014;384:1376–1388.
21. Zhang S, Cheng C, Lin Z, et al. The global burden and associated factors of ovarian cancer in 1990-2019: findings from the Global Burden of Disease Study 2019. *BMC Public Health*. 2022;22:1455.
22. Evans AR, Limp-Foster M, Kelley MR. Going APE over ref-1. *Mutat Res*. 2000;461:83-108.
23. Carter RJ, Parsons JL. Base Excision Repair, a Pathway Regulated by Posttranslational Modifications. *Mol Cell Biol*. 2016;36(10):1426-1437.
24. Slyskova J, Korenkova V, Collins AR, et al. Functional, genetic, and epigenetic aspects of base and nucleotide excision repair in colorectal carcinomas. *Clin Cancer Res*. 2012;18(21):5878-5887.
25. Bernstein C, Bernstein H, Payne CM, Garewal H. DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. *Mutat Res*. 2002;511:145-178.
26. Le DT, Uram JN, Wang H, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med*. 2015;372:2509-2520.
27. Geske FJ, Nelson AC, Lieberman R, Strange R, Sun T, Gerschenson LE. DNA repair is activated in early stages of p53-induced apoptosis. *Cell Death Differ*. 2000;7:393-401.
28. Spivak, G. Nucleotide excision repair in humans. *DNA Repair*. 2015;36:13–18.
29. Schärer OD. Nucleotide excision repair in eukaryotes. *Cold Spring Harb Perspect Biol*. 2013;5:a012609.

30. Slyskova J, Naccarati A, Pardini B, et al. Differences in nucleotide excision repair capacity between newly diagnosed colorectal cancer patients and healthy controls. *Mutagenesis*. 2012;27:519-522.
31. Slyskova J, Cordero F, Pardini B, et al. Post-treatment recovery of suboptimal DNA repair capacity and gene expression levels in colorectal cancer patients. *Mol Carcinog*. 2015;54:769-778.
32. Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer*. 2008;8:193-204.
33. Neale MJ, Keeney S. Clarifying the mechanics of DNA strand exchange in meiotic recombination. *Nature*. 2006;442:153-158.
34. Gudmundsdottir K, Ashworth A. The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene*. 2006;25:5864-5874.
35. Yang H, Jeffrey PD, Miller J, et al. BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure. *Science*. 2002;297(5588):1837-1848.
36. Kowalczykowski SC. An Overview of the Molecular Mechanisms of Recombinational DNA Repair. *Cold Spring Harb Perspect Biol*. 2015;7:a016410.
37. Golmard L, Castéra L, Krieger S, et al. Contribution of germline deleterious variants in the RAD51 paralogs to breast and ovarian cancers. *Eur J Hum Genet*. 2017;25(12):1345-1353.
38. Loveday C, Turnbull C, Ramsay E, et al. Germline mutations in RAD51D confer susceptibility to ovarian cancer. *Nat Genet*. 2011;43:879-882.
39. Min A, Im SA, Yoon YK, et al. RAD51C-deficient cancer cells are highly sensitive to the PARP inhibitor olaparib. *Mol Cancer Ther*. 2013;12(6):865-877.
40. Durante M, Bedford JS, Chen DJ, et al. From DNA damage to chromosome aberrations: joining the break. *Mutat Res*. 2013;756(1-2):5-13.
41. Vanderstichele A, Busschaert P, Olbrecht S, Lambrechts D, Vergote I. Genomic signatures as predictive biomarkers of homologous recombination deficiency in ovarian cancer. *Eur J Cancer*. 2017;86:5-14.
42. Cimprich KA, Cortez D. ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol*. 2008;9:616-627.
43. Chang HHY, Pannunzio NR, Adachi N, Lieber MR. Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nat Rev Mol Cell Biol*. 2017;18:495-506.
44. Zhou Y, Lee JH, Jiang W, Crowe JL, Zha S, Paull TT. Regulation of the DNA Damage Response by DNA-PKcs Inhibitory Phosphorylation of ATM. *Mol Cell*. 2017;65:91-104.

45. Bhargava R, Onyango DO, Stark JM. Regulation of Single-Strand Annealing and its Role in Genome Maintenance. *Trends Genet*. 2016;32:566-575.
46. Mjelle R, Hegre SA, Aas PA, et al. Cell cycle regulation of human DNA repair and chromatin remodeling genes. *DNA Repair (Amst)*. 2015;30:53-67.
47. Goyal G, Fan T, Silberstein PT. Hereditary cancer syndromes: utilizing DNA repair deficiency as therapeutic target. *Fam Cancer*. 2016;15:359-366.
48. Welcsh PL, Owens KN, King MC. Insights into the functions of BRCA1 and BRCA2. *Trends Genet*. 2000;16:69-74.
49. Yu VP, Koehler M, Steinlein C, et al. Gross chromosomal rearrangements and genetic exchange between nonhomologous chromosomes following BRCA2 inactivation. *Genes Dev*. 2000;14:1400-1406.
50. Farolfi A, Gurioli G, Fugazzola P, et al. Immune System and DNA Repair Defects in Ovarian Cancer: Implications for Locoregional Approaches. *Int J Mol Sci*. 2019;20:2569.
51. Bewtra C, Watson P, Conway T, Read-Hippee C, Lynch HT. Hereditary ovarian cancer: a clinicopathological study. *Int J Gynecol Pathol*. 1992;11:180-187.
52. McAlpine JN, Porter H, Köbel M, et al. BRCA1 and BRCA2 mutations correlate with TP53 abnormalities and presence of immune cell infiltrates in ovarian high-grade serous carcinoma. *Mod Pathol*. 2012;25:740-750.
53. Peltomäki P. Lynch syndrome genes. *Fam Cancer*. 2005;4(3):227-232.
54. Beggs AD, Domingo E, Abulafi M, Hodgson SV, Tomlinson IP. A study of genomic instability in early preneoplastic colonic lesions. *Oncogene*. 2013;32:5333-5337.
55. Ketabi Z, Bartuma K, Bernstein I, et al. Ovarian cancer linked to Lynch syndrome typically presents as early-onset, non-serous epithelial tumors. *Gynecol Oncol*. 2011;121:462-465.
56. Wiegand KC, Shah SP, Al-Agha OM, et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med*. 2010;363:1532-1543.
57. Parra-Herran C, Lerner-Ellis J, Xu B, et al. Molecular-based classification algorithm for endometrial carcinoma categorizes ovarian endometrioid carcinoma into prognostically significant groups. *Mod Pathol*. 2017;30:1748-1759.
58. Rambau PF, Duggan MA, Ghatage P, et al. Significant frequency of MSH2/MSH6 abnormality in ovarian endometrioid carcinoma supports histotype-specific Lynch syndrome screening in ovarian carcinomas. *Histopathology*. 2016;69:288-297.
59. Howitt BE, Strickland KC, Sholl LM, et al. Clear cell ovarian cancers with microsatellite instability: A unique subset of ovarian cancers with increased tumor-infiltrating lymphocytes and PD-1/PD-L1 expression. *Oncoimmunology*. 2017;6:e1277308.

60. Clarke B, Tinker AV, Lee CH, et al. Intraepithelial T cells and prognosis in ovarian carcinoma: novel associations with stage, tumor type, and BRCA1 loss. *Mod Pathol*. 2009;22:393-402.
61. Birkbak NJ, Kochupurakkal B, Izarzugaza JM, et al. Tumor mutation burden forecasts outcome in ovarian cancer with BRCA1 or BRCA2 mutations. *PLoS One*. 2013;8:e80023.
62. George J, Alsop K, Etemadmoghadam D, et al. Nonequivalent gene expression and copy number alterations in high-grade serous ovarian cancers with BRCA1 and BRCA2 mutations. *Clin Cancer Res*. 2013;19:3474-3484.
63. Nelson BH. New insights into tumor immunity revealed by the unique genetic and genomic aspects of ovarian cancer. *Curr Opin Immunol*. 2015;33:93-100.
64. Strickland KC, Howitt BE, Shukla SA, et al. Association and prognostic significance of BRCA1/2-mutation status with neoantigen load, number of tumor-infiltrating lymphocytes and expression of PD-1/PD-L1 in high grade serous ovarian cancer. *Oncotarget*. 2016;7:13587-13598.
65. Bennett JA, Morales-Oyarvide V, Campbell S, Longacre TA, Oliva E. Mismatch Repair Protein Expression in Clear Cell Carcinoma of the Ovary: Incidence and Morphologic Associations in 109 Cases. *Am J Surg Pathol*. 2016;40:656-663.
66. Llosa NJ, Cruise M, Tam A, et al. The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov*. 2015;5:43-51.
67. Hewish M, Lord CJ, Martin SA, Cunningham D, Ashworth A. Mismatch repair deficient colorectal cancer in the era of personalized treatment. *Nat Rev Clin Oncol*. 2010;7:197-208.
68. Viale G, Trapani D, Curigliano G. Mismatch Repair Deficiency as a Predictive Biomarker for Immunotherapy Efficacy. *Biomed Res Int*. 2017;2017:4719194.
69. Bose D. cGAS/STING Pathway in Cancer: Jekyll and Hyde Story of Cancer Immune Response. *Int J Mol Sci*. 2017;18:2456.
70. Peng B, Hodge DR, Thomas SB, et al. Epigenetic silencing of the human nucleotide excision repair gene, hHR23B, in interleukin-6-responsive multiple myeloma KAS-6/1 cells. *J Biol Chem*. 2005;280:4182-4187.
71. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis*. 2009;30(7):1073-1081.
72. Cancer Genome Atlas Research Network Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011;474:609–615.

73. Konecny GE, Wang C, Hamidi H, Winterhoff B, Kalli KR, Dering J, et al. Prognostic and therapeutic relevance of molecular subtypes in high-grade serous ovarian cancer. *J. Natl. Cancer Inst.* 2014;106:dju249.
74. Pinto MP, Balmaceda C, Bravo ML, Kato S, Villarroel A, Owen GI, et al. Patient inflammatory status and CD4+/CD8+ intraepithelial tumor lymphocyte infiltration are predictors of outcomes in high-grade serous ovarian cancer. *Gynecol Oncol*. 2018;151:10–17.
75. Worzfeld T, Pogge von Strandmann E, Huber M, Adhikary T, Wagner U, Reinartz S, Müller R. The unique molecular and cellular microenvironment of ovarian cancer. *Front. Oncol*. 2017;7:24.
76. Lee W, Ko SY, Mohamed MS, Kenny HA, Lengyel E, Naora H. Neutrophils facilitate ovarian cancer premetastatic niche formation in the omentum. *J. Exp. Med.* 2019;216:176–194.
77. Templeton AJ, McNamara MG, Šeruga B, Vera-Badillo FE, Aneja P, Ocaña A, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: A systematic review and meta-analysis. *J. Natl. Cancer Inst.* 2014;106:dju124.
78. Ethier JL, Desautels DN, Templeton AJ, Oza A, Amir E, Lheureux S. Is the neutrophil-to-lymphocyte ratio prognostic of survival outcomes in gynecologic cancers? A systematic review and meta-analysis. *Gynecol Oncol.* 2017;145:584–594.
79. Baert T, Van Camp J, Vanbrabant L, Busschaert P, Laenen A, Han S, et al. Influence of CA125, platelet count and neutrophil to lymphocyte ratio on the immune system of ovarian cancer patients. *Gynecol. Oncol.* 2018;150:31–37.
80. Farolfi A, Petrone M, Scarpi E, Gallà V, Greco F, Casanova C, et al. Inflammatory indexes as prognostic and predictive factors in ovarian cancer treated with chemotherapy alone or together with bevacizumab. A multicenter, retrospective analysis by the MITO group (MITO 24) *Target. Oncol.* 2018;13:469–479.
81. Paik ES, Sohn I, Baek SY, Shim M, Choi HJ, Kim TJ, et al. Nomograms predicting platinum sensitivity, progression-free survival, and overall survival using pretreatment complete blood cell counts in epithelial ovarian cancer. *Cancer Res. Treat*. 2017;49:635–642.
82. Farolfi A, Scarpi E, Greco F, Bergamini A, Longo L, Pignata S, et al. Inflammatory indexes as predictive factors for platinum sensitivity and as prognostic factors in recurrent epithelial ovarian cancer patients: a MITO24 retrospective study. *Sci Rep*. 2020;10:18190.
83. Tedaldi G, Tebaldi M, Zampiga V, et al. Multiple-gene panel analysis in a case series of 255 women with hereditary breast and ovarian cancer. *Oncotarget*. 2017;8:47064-47075.
84. Tedaldi G, Tebaldi M, Zampiga V, et al. Male Breast Cancer: Results of the Application of Multigene Panel Testing to an Italian Cohort of Patients. *Diagnostics (Basel)*. 2020;10:269.

85. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25:1754-1760.
86. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res*. 2010;20:1297-1303.
87. Garrison, E.; Marth, G. Haplotype-based variant detection from short-read sequencing. 2012, 1–9.
88. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res*. 2010;38:e164.
89. Plon SE, Eccles DM, Easton D, et al. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. *Hum Mutat*. 2008;29:1282-1291.
90. Cline MS, Liao RG, Parsons MT, et al. BRCA Challenge: BRCA Exchange as a global resource for variants in BRCA1 and BRCA2. *PLoS Genet*. 2018;14:e1007752.
91. Béroud C, Letovsky SI, Braastad CD, et al. BRCA Share: A Collection of Clinical BRCA Gene Variants. *Hum Mutat*. 2016;37:1318-1328.
92. Fokkema IF, Taschner PE, Schaafsma GC, Celli J, Laros JF, den Dunnen JT. LOVD v.2.0: the next generation in gene variant databases. *Hum Mutat*. 2011;32:557-563.
93. andrum MJ, Lee JM, Riley GR, et al. ClinVar: public archive of relationships among sequence variation and human phenotype. *Nucleic Acids Res*. 2014;42(Database issue):D980-D985.
94. Sherry ST, Ward MH, Kholodov M, et al. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res*. 2001;29:308-311.
95. Kopanos C, Tsiolkas V, Kouris A, et al. VarSome: the human genomic variant search engine. *Bioinformatics*. 2019;35:1978-1980.
96. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17:405-424.
97. Musacchio L, Marchetti C, Boccia SM, et al. 31P - Survival outcomes in BRCA pathogenetic mutated, variant of unknown significance, and wild type ovarian cancer patients treated with PARP inhibitors. *Ann of Oncol*. 2022;33:S395-S401.
98. Amin, N.; Chaabouni, N.; George, A. Genetic testing for epithelial ovarian cancer. Best Pract. Res. Clin. Obstet. Gynaecol. 2020, 65, 125–138, doi:10.1016/j.bpobgyn.2020.01.005.
99. Norquist BM, Harrell MI, Brady MF, et al. Inherited Mutations in Women With Ovarian Carcinoma. *JAMA Oncol*. 2016;2:482-490.

100. Ramus SJ, Song H, Dicks E, et al. Germline Mutations in the BRIP1, BARD1, PALB2, and NBN Genes in Women With Ovarian Cancer. *J Natl Cancer Inst*. 2015;107:djv214.

101. Song H, Dicks E, Ramus SJ, et al. Contribution of Germline Mutations in the RAD51B, RAD51C, and RAD51D Genes to Ovarian Cancer in the Population. *J Clin Oncol*. 2015;33:2901-2907.

102. Ruark E, Snape K, Humburg P, et al. Mosaic PPM1D mutations are associated with predisposition to breast and ovarian cancer. *Nature*. 2013;493:406-410.

103. Bonache S, Esteban I, Moles-Fernández A, et al. Multigene panel testing beyond BRCA1/2 in breast/ovarian cancer Spanish families and clinical actionability of findings. J *Cancer Res Clin Oncol*. 2018;144:2495-2513.

104. Hutchcraft ML, Gallion HH, Kolesar JM. MUTYH as an Emerging Predictive Biomarker in Ovarian Cancer. *Diagnostics (Basel)*. 2021;11:84.

105. Kurian AW, Ward KC, Abrahamse P, et al. Time Trends in Receipt of Germline Genetic Testing and Results for Women Diagnosed With Breast Cancer or Ovarian Cancer, 2012-2019. *J Clin Oncol*. 2021;39:1631-1640.

106. Bonadona V, Bonaïti B, Olschwang S, et al. Cancer risks associated with germline mutations in MLH1, MSH2, and MSH6 genes in Lynch syndrome. *JAMA*. 2011;305:2304-2310.

107. Ten Broeke SW, van der Klift HM, Tops CMJ, et al. Cancer Risks for PMS2-Associated Lynch Syndrome [published correction appears in *J Clin Oncol*. 2019 Mar 20;37(9):761]. *J Clin Oncol*. 2018;36:2961-2968.

108. Konstantinopoulos PA, Norquist B, Lacchetti C, et al. Germline and Somatic Tumor Testing in Epithelial Ovarian Cancer: ASCO Guideline. *J Clin Oncol*. 2020;38:1222-1245.

109. Choi YH, Terry MB, Daly MB, et al. Association of Risk-Reducing Salpingo-Oophorectomy With Breast Cancer Risk in Women With BRCA1 and BRCA2 Pathogenic Variants. *JAMA Oncol*. 2021;7:585-592.

110. Wang Y, Song Z, Zhang S, Wang X, Li P. Risk-reducing salpingo-oophorectomy and breast cancer risk in BRCA1 or BRCA2 mutation carriers: A systematic review and meta-analysis. *Eur J Surg Oncol*. 2022;48:1209-1216.

111. Friedlander M, Trimble E, Tinker A, et al. Clinical trials in recurrent ovarian cancer. *Int J Gynecol Cancer*. 2011;21:771-775.

112. Blazek D, Kohoutek J, Bartholomeeusen K, et al. The Cyclin K/Cdk12 complex maintains genomic stability via regulation of expression of DNA damage response genes. *Genes Dev*. 2011;25:2158-2172.

113. Eoh KJ, Park HS, Park JS, et al. Comparison of Clinical Outcomes of BRCA1/2 Pathologic Mutation, Variants of Unknown Significance, or Wild Type Epithelial Ovarian Cancer Patients. *Cancer Res Treat*. 2017;49:408-415.

114. Moses K, Brandau S. Human neutrophils: their role in cancer and relation to myeloid-derived suppressor cells. *Semin Immunol*. 2016;28:187–96.

115. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell*. 2010;140:883–99.

116. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet*. 2001;357:539–45.

117. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces epithelial-mesenchymal-like transition and promote metastasis. *Cancer Cell*. 2011;20:576–90.

118. Zhang L, Conejo-Garcia JR, Katsaros D, et al. Intratumoral T cells,recurrence, and survival in epithelial ovarian cancer. *N Engl J Med*. 2003;348:203–13.

119. Leffers N, Gooden MJ, de Jong RA, et al. Prognostic significance of tumor-infiltrating T-lymphocytes in primary and metastatic lesions of advanced stage ovarian cancer. *Cancer Immunol Immunother*. 2009;58:449–59.

120. Stone RL, Nick AM, McNeish IA, et al. Paraneoplastic thrombocytosis in ovarian cancer. *N Engl J Med*. 2012;366:610-618. Erratum in: *N Engl J Med*. 2012;367:1768.