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Multigene panel testing in Hereditary Breast and Ovarian Cancer: an effective liquid biopsy approach to identify mutations in genes involved in the Homologous Recombination pathway

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Abstract

Background: Hereditary breast and ovarian cancer (HBOC) syndrome is an autosomal dominant inherited disorder that include 5–7% of all breast cancer (BC) cases and 10-15% of all ovarian cancer (OC) cases. *BRCA1* and *BRCA2* are the most common genes associated to HBOC syndrome. However, hereditary syndrome could be associated with germline PVs in several high- and moderate-risk genes. In recent years, Next-Generation Sequencing (NGS) has allowed to study multiple genes simultaneously, to reduce analysis costs, to led to an explosion of genetic data, and to offer more information to patients.

Methods: We retrospectively collected and analyzed to *BRCA1/2* test 876 patients affected by BC and OC (531 of BC, 345 of OC) among January 2016 to August 2020. Successively, we analyzed 192 patients resulted *BRCA1/2* negative with a strong personal and/or family history to BC and/or OC by using Multi-gene panel testing. We evaluated 22 genes involved in risk of hereditary breast, ovarian and colorectal cancer, and other inherited tumor syndromes.

Results: Analysis conducted with multi-gene panel testing revealed that 28 (14.6%) BC and OC patients showed PVs/LPVs in genes no-*BRCA*. In particular, we analyzed 165 BC patients and 27 OC patients, and we obtained 27 and 4 patients with PVs/LPVs in genes no-*BRCA* respectively. BC patients with alteration in gene over *BRCA* hardly showed TNBC than patients with *BRCA1/2* or all wt. Moreover, among BC patients with genes altered beyond *BRCA* the 45.8% showed a Bilateral Breast Cancer. In OC group we observed that 75% of patients with PVs/LPVs in genes over *BRCA* showed a previously personal history of BC or other cancer.

Conclusion: Our analysis showed that the 14.6% of patients *BRCA*-negative with a strong personal and/or family history to BC and/or OC presented alteration in genes beyond *BRCA1/2*. This result highlighted the importance of multi-gene panel testing which should be extended at all these patients and be included in clinical practice.

Summary

Many patients affected by Hereditary Breast and Ovarian Cancer (HBOC) syndrome with a strong personal and/or family history to BC and/or OC that result negative for *BRCA1* or *BRCA2* pathogenic variants, need a further genetic testing through a multi-gene panel. Some patients with negative test result for *BRCA1/2* pathogenic variants may harbour pathogenic variants or likely pathogenic variants in several high- and moderate-risk genes, including *ATM, CHEK2, PALB2, PTEN, TP53, STK11*. Of course, the use of multi-gene panel provides clinicians more information compared to single testing and is recommended to reduce time and costs of analysis. Therefore, we focused on the potential clinical impact of NGS-based multi-gene panel testing in HBOC patients with a strong personal and/or family history to BC and/or OC, in order to evaluate the utility of perform a most comprehensive genetic analysis in these subjects. Our study revealed that the use of NGS-based multiple-gene panel testing could increase the detection rates of germline alterations in these patients. Indeed, a significant proportion (14.6%) of PVs/LPVs was found with the aim to offer specific risk-reducing measures and free prevention programs for patients and their families.

CHAPTER 1

Background Rationale and Objectives

1.1 Background

During the 19s century, many studies allowed to understand women from specific families were prone to develop breast and ovarian cancer [1]. Peoples that shown this predisposition ware identify with Hereditary breast and ovarian cancer (HBOC) syndrome, an autosomal dominant inherited disorder that include 5–7% of all breast cancer (BC) cases and 10-15% of all ovarian cancer (OC) cases [2]. Patients with HBOC syndrome may have an increased risk of developing other types of cancer such as pancreatic cancer, prostate cancer, and melanoma. Until date National Comprehensive Cancer Network (NCCN) established clinical guidelines to distinguish HBOC patients [3].

In worldwide, lung cancer represent the most commonly diagnosed cancer and the main cause of cancer death in both sex [4]. However BC and OC are in the top ten of the most common cancer for incidence and mortality in females [5]. BC represents the most diagnosed tumours in females [6]. In general, different environmental and genetic risk factors influenced BC and OC. Furthermore, the majority of BC and OC showed sporadic nature (75-80%), about 15-20% was considered familial-type and 5-10% was hereditary [7]. In this contest, *BRCA1* and *BRCA2* are the most common genes associated to HBOC syndrome showing a germline pathogenic variant (PV) or likely-pathogenic variant (LPV). In fact, the risk to develop breast cancer was observed into 50–85% *BRCA1* and *BRCA2* carriers, while to ovarian cancer *BRCA1* and *BRCA2* carriers were characterised by a risk of 15–45% and 10–20% respectively [7]. However, hereditary syndrome could be associated with germline PVs in several high-risk genes such as *CDH1*, *PALB2, PTEN*, *STK11*, *TP53*,

or moderate-risk genes like *ATM*, *CHEK2*, *BARD1*, *BRIP1*, *RAD51C*, *RAD51D* [8].
In recent years, Next-Generation Sequencing (NGS) has allowed to study multiple genes simultaneously, to reduce analysis costs, to led to an explosion of genetic data and to offer more information to patients.

1.1.1 Breast cancer

The most common cancer's type in female was the breast cancer, with one out of eight women developing a BC during lifetime [9].
BC represent about 30% of cancers in women worldwide at any age, with a higher incidence in the most economically advanced States. BC is the most frequently diagnosed and also the leading cause of cancer death at any age. However, mortality rate is constantly decreasing probably to major diffusion of screening and prevention programs associated at analyzes of *BRCA1/2* genes.
BC is a multifactorial disease involving lifestyle, hormonal balance and inherited predisposition [10] Different factors are implicated in the development of BC, like as sex, age and family history. Females show a 100 times greater risk than men; in fact, male breast cancer represent <1% of all breast cancer diagnosed. The most frequent new diagnoses were revealed at an age >55 years old. This correlation is probably related to steroid hormones that act in continuous during lifetime, progressive DNA damage and epigenetic modification [11]. The 5-10% of all BC are considered as hereditary and they are related to *BRCA1* and *BRCA2* genes. Over the past decades, an increase of risk to develop BC is associated with a confirmed family history of BC cases. Moreover, hereditary tumor are often bilateral and an early age onset.
Based on molecular and histological features, BC could be divided into five major subtypes: luminal A, luminal B, luminal/human epidermal growth factor receptor 2 (HER2), HER2 enriched, and triple negative (TNBC) which includes, only in part, the basal like subtype [12]. Estrogen Receptor (ER), Progesterone Receptor (PR) and HER2 with Ki67 represent most valid and widely used biomarker in BC and they are evaluated directly in tumor tissue. Seeing as tumor tissue employment is difficult, validated circulating biomarkers as TPS, CEA, and CA 15-3 are necessary to use. However, to date, these biomarkers cannot serve as prognostic biomarker but have a potential negative predictive role in BC patients.
The most relevant predictive biomarker in BC patients is ER, its used as marker for endocrine therapy and its theorized that ER level are correlated to the positive effect of antiestrogenic therapy [13].

Another frequently assessed biomarker in BC is PR that is related to ER because PR could be induced by estrogen. Moreover, ER and PR evaluation are strongly recommended for BC patients management [14].

In addition to ER and PR, another predictive biomarker is HER2. High expression of HER2 is correlated to metastasis, invasion, and tumor cells proliferation [15].

About 60–70% of BC cases in premenopausal women was BC-hormone receptors positive (HR+). Therapeutic approach chosen to these patients was hormonotherapy employing tamoxifene to estrogen blockade and aromatase inhibitors (anastrozole or exemestane) to prevent hormone production from ovary [16]. Endocrine therapy was the best treatment to patients with BC HR+ and HER2-negative regardless the use of cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors [17] and independently of *BRCA* status.

HER2+ represents approximatively the 20% of all BC. These patients were treated with a combination of chemotherapy and anti-HER2 monoclonal antibody therapy [18].

TNBC represent about 10-20 % of all BC, it is especially proliferative and aggressive characterized by poor prognosis than other BC type for patients. It is defined immunohistochemically as breast cancer that does not excessively express HER2 and is negative for ER and PR [19]. TNBC is classified in six categories: basal-like 1 (BL-1), basal-like 2 (BL-2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem cell-like (MSL), luminal androgen receptor (LAR). TNBCs treatment was most difficult than other BC types, yet the chemotherapy was the gold standard nowadays. However, the combination of chemotherapy with recombinant humanized monoclonal antibody against Vascular Endothelial Growth Factor (VEGF) represented a valid treatment alternative [20] [21].

TNBC are often characterized by PVs/LPVs in *BRCA1* gene and pathological features determine frequently a infiltrating ductal carcinomas subtype with high grade and geographic necrosis [10]. While BC exhibiting PVs/LPVs in *BRCA2* gene showed features like sporadic BC with 2/3 grade carcinomas, ER+, PR+ and HER2- [22].

Besides standard anthracycline- and taxane-based chemotherapy, recent studies have better elucidated the potential role of platinum agents in patients with *BRCA*-mutated breast cancer.

Recently, the Poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi) discovery has offered new therapeutic choices to patients affected by *BRCA*-mutated ovarian, breast and pancreatic cancer. Robson and colleagues in OlympiAD study demonstrated that in patients with HER2-negative metastatic breast cancer and a germline *BRCA* mutation have a significant increase in progression-free survival (PFS) when treated with Olaparib, a poly

adenosine diphosphate-ribose polymerase inhibitor (PARPi), monotherapy rather than with standard therapy. However, death was 42% lower than patients treated with standard therapy in this setting of patients [23].

In 2019, Robson et al. reported the final Overall Survival (OS) in the OlympiAD study. A statistically significant improvement in patients treated with Olaparib was not observed compared to chemotherapy standard. A greater increase in OS were presents when patients not received chemotherapy before Olaparib treatment [24].

In BrighTNess study patients are randomized at adjuvant treatment with carboplatin versus carboplatin and Veliparib versus chemotherapy standard. The veliparib/carboplatin combination showed a higher pathological complete response (pCR) rate than standard treatment also in patients with PVs/LPVs in *BRCA1/2* genes [25].

In 2017, EMBRACA study led to the registration of Talazoparib, another PARPi, by FDA. The authors compare Talazoparib with standard therapy in women affected by BC HER2- and PVs/LPVs in *BRCA1/2*. The primary endpoint was the PFS, that was statistically significant in women treated with Talazoparib (8.6 months vs 5.6 months) [26]. In 2020 the authors evaluate the final OS that not result significantly improve in Talazoparib arm compered to chemotherapy [27].

Andrè and colleagues shown that patients HR-positive HER2-negative advanced breast cancer with *PIK3CA*-mutation have a significant improvement in progression-free survival and better overall response by adding an α-specific PI3K inhibitor to standard treatment [28].

1.1.2 Ovarian cancer

Ovarian cancer is the third gynaecological cancers diagnosed after cervical and uterine and the fifth cause death in women [29][30].

Considering the morphology, OC are divided in epithelial and non-epithelial origin; the epithelial represented about 90% of all OC [31][32]. Epithelial types are histologically classified in serous (52%), endometrioid (10%), mucinous (6%), or clear cell (6%), and the remaining 25% are rare or unspecified. Among them, clear cells and mucinous are less frequent but have a higher mortality, compared to other types [33].

Moreover, epithelial cancers are divided in I type and II type by clinicopathologic features [34]. Type I are low-grade, unilateral and cystic tumours. Usually, these OC originate from extraovarian benign lesions that acquire a phenotype malignant after mutations [35].

Type II includes high-grade serous carcinomas; the most common OC subtypes are high-grade, bilateral and particularly aggressive. Women with high-grade OC often present

extraovarian disease and ascites [34].

Epithelial ovarian cancer (EOC) is known to have a poor prognosis because it's diagnosed at advanced stage, in most cases is asymptomatic and usual symptoms are often unspecific. Today, the primary prognostic factor remains to anticipate the stage at diagnosis. A study estimating a combination of gynaecological ultrasonography and Ca125 testing has not led to a strong mortality decrease but additional follow-up was required [36]. Currently, the strategy for advanced stage OC is primary debulking surgery followed by standard therapy. Treatment standard is platinum-based and provides six cycles of carboplatin plus paclitaxel every 3 weeks [37]. In the past few years, different studies led to approval of bevacizumab adding to standard therapy and in maintenance in EOC patients with improves PFS [38], [39].

The most common risk factor to OC is represented by family history of breast and/or ovarian cancer with a genetic predisposition determined by *BRCA1* and *BRCA2* in 25% of OC affected women [30], [40]. *BRCA* test is fundamental at moment of OC diagnosed, indeed *BRCA* have a role to estimate the cancer treatment efficacy. In the past years three different groups showed that OC patients with a germline *BRCA1/2* PV exhibited an improved sensitivity to platinum derivatives therapies [41]–[43].

Recently, Moore et al. in SOLO-1 trial demonstrated that women with OC and germline or somatic PV in *BRCA1/2* have an improvement in terms of PFS when treated with Olaparib as maintenance after response to first-line platinum-based chemotherapy [44]. Besides SOLO-1 trial, other studies have shown statistically significant results regarding treatment with PARPi in OC patients.

PAOLA-1 trial demonstrated that treatment with Olaparib and bevacizumab as first-line maintenance therapy have shown an improvement in PFS regardless *BRCA* status [45]. In VELIA trial women affected by OC with *BRCA* mutations showed a largest benefit in term of PFS when treated with veliparib and chemotherapy combination and subsequently veliparib as maintenance [46].

Females with OC presents germline PVs/LPVs in genes beyond *BRCA1/2* belonging to the Homologous Recombination (HR) pathway or mismatch repair (MMR) pathway. HR status was used as stratification factor in PRIMA trial. This trial proves that OC patients with Homologous Recombination Deficiency (HRD) treated with Niraparib have significant clinical benefit over placebo [47]. The presence of PVs/LPVs in MMR genes were reported in 10-12% of all histological subtypes; these patients could be treated with pembrolizumab [30]. In this contest, a multigene panel testing with genes involved in HR and MMR pathways

is recommended to evaluate germline PVs/LPVs and to offer the best therapeutic choice for HBOC patients.

1.1.3 Genetic counselling

Genetic counselling consists of multidisciplinary team composed by oncologist, geneticist biologist or medical, psychologist and tumor patients affected named proband. During counselling, geneticist and proband trace patient personal and family history through the family tree reconstruction. In families where is the geneticist highlighted a suspected hereditary transmission, the identification of PVs/LPVs carriers is necessary. Once identified a PVs/LPVs in proband, the variant research is extended to family members.

Proband will be directed to genetic test in the case of personal history responds to specific criteria[48]:

Table 1. Criteria to Genetic Counselling in patients affected by Breast and Ovarian Cancer

Personal History of
Male Breast Cancer
Women with Breast and Ovarian Cancer
Women with Breast Cancer <36 years
Women with TNBC <60 years
Women with Bilateral Breast Cancer <60 years
Personal History of Breast Cancer <50 years and first-degree family members affected by
Breast Cancer <50 years
Non-Mucinous And Non Borderline Ovarian Epithelial Carcinoma At Any Age
Bilateral Breast Cancer
Male Breast Cancer
Personal History of Breast Cancer >50 and almost 2 first-degree family members with Breast or Ovarian Cancer
Personal History of PVs/LPVs in Breast and Ovarian Cancer Susceptibility Genes

Genetic test results are:

-informative: PVs/LPVs is identified;

-non informative: PVs/LPVs is not identified but it cannot be excluded that it is present, or in genetic test is identified a Variant of Unknown Significance (VUS);

-negative: only when a PVs/LPVs identified in a proband but it is not present in family member.

Variant classification is based on different criteria developed by the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) consortium (https://enigmaconsortium.org/) and according to IARC recommendations [48]. Therefore,

detected genetic variants were divided into five classes:

-class I (benign);

-class II (likely benign);

-class III (VUS);

-class IV (likely pathogenic);

-class V (pathogenic).

1.1.4 Homologous Recombination Pathway

It has been estimated that cells develop thousands of DNA damage every day [49], because the DNA is frequently exposed at different agents exogenous and/or endogenous. Therefore, normal cells present several DNA repair mechanisms including nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombination repair (HRR), and non-homologous end joining (NHEJ) [50]. HRR and NHEJ are the principal pathway implicated in repair of double stranded DNA breaks (DSBs).

NHEJ which happens during G0/G1 cell cycle phase is an error prone pathway that does not use intact sister chromatid as a template. Therefore DNA break ends are directly ligated among them causing small insertions or deletions [51], [52].

HRR occurs in proliferating cells during late S and G2 phases of cell cycle; it is a highly conserved pathway that allows DNA DSBs repair in an accurate manner based on sister chromatid as template [53]. BRCA1/2 are the main proteins involved in HRR pathway. When cells are BRCA1/2 deficient, the repair of DNA is based on Non-Homologous End Joining which determines an increase of DNA errors and genomic instability [54].

In this contest, polyADP-ribosylation (PARylation) represent a crucial post-translational protein modification involved in DNA damage repair [55]. In human cell, PARylation is catalysed by poly(ADP-ribose) polymerases (PARPs) which is composed by 17 members [56]. Among 17 members, PARP-1, PARP-2 and PARP-3 are implicated in DNA damage response, while PARP-5a and PARP-5b, also called tankyrase-1 and tankyrase-2, are involved to maintain genomic stability.

PARP-1 is the most famous and most common studied PARP members implicated in PAR chains synthesis. PARP-1 use NAD+ as substrate to catalyse the unit transfer of ADP-ribose (ADPR) moieties onto different nuclear proteins [57]. In normal cells, PAR chains are degraded by PAR glycohydrolase (PARG) with exoglycosidic and endoglycosidic activities, or through ADP-ribosylhydrolase 3 (ARH3) with only exoglycosidase activity. When PAR chains cannot be hydrolyzed, the excessive presence of protein-free PAR chains induce cell

death through a form of programmed cell death named parthanatos [58].

PARP-1 contains 6 domains. Only two domains do not have known function (C and E domains). Domain A is DNA-binding domain with two zinc-finger necessary to interact with DNA breaks. Domain B contain a nuclear localization signal (NLS) essential to PARP-1 nuclear transport [59]. Domain D is needful to interface to different nuclear partners through BRCT motif. Domain F show a catalytic activity [60].

PARP-2 is a nuclear protein that interacts with PARP-1 sharing proteins involved in Single Strand Break Repair (SSBR) and Base Excision Repair (BER). PARP-2 catalytic domain shown a 69% similarity with PARP-1, while DNA-binding domain is different.

PARP-3 is a centrosome component able to interact with PARP-1, with which shares two domains with 61% of similarity [61].

PARylation process intervenes in DNA repair pathway in both SSBs and DSBs. In presence of SSBs, PARP-1 cooperate with SSBR factor X-ray repair cross-complementing protein 1 (XRCC1) trough BRCT domain assembling SSBR machinery [62].

Schultz et al. in 2003 shown that PARP-1 is not indispensable for HR; in addition, PARylation seems to have a small effect on HR in PARP-depleted cells [63].

Nevertheless, PARP-1 has been associated with HR-repair, the reactivation of replication fork trough recruitment of MRE11, and RAD51 promoting the correct DNA replication [64], [65].

PARPi act by synthetic lethality mechanism against cancer with HRD. Synthetic lethality happens when cell death is caused by a simultaneous disruption of two genes [66]. In HRD situation (for example in *BRCA*-mutated cells), PARP inhibition led to replication fork collapse that cannot be repaired because the HR activity is disrupted and so cell death occurs [67] (Figure 1).

Homologous recombination pathway is composed, over BRCA1/2, by a multitude of other DNA repair proteins such as ATM, CHEK2, BARD1, BRIP1, MRE11, RAD50, NBN, RAD51C, RAD51D, and PALB2 (Figure 2). In presence of DNA damage, BARD1 identifies PAR chain and recruits BRCA1/BARD1 heterodimer to promote efficient HR [54]. Another complex included in HR is MRN complex composed by MRE11, RAD50 and NBN proteins, which serves to cut DSBs ends. This complex is essential to signal DNA damage and to stimulate Ataxia Telangiectasia Mutated (ATM) protein that provide to process DSBs in SSBs [53]. BRCA2 regulates RAD51 and its paralogues (RAD51B, RAD51C and RAD51D) that searching sequence homology on the sister chromatid to use as a reference. PALB2 interact with BRCA2 and BRCA1. BRCA2 is regulated by PALB2 that enables nuclear

localization of BRCA2 in DNA damage sites [51], [68], [69]. Instead BRCA1 controls PALB2-dependent loading of BRCA2-RAD51 repair machinery at double stranded DNA breaks [70]. BRCA1-interacting protein carboxy-terminal helicase1 (BRIP1) is also suggested to be involved in HRR by recruiting BRCA1 to DSBs.

One of major problems in PARPi treatment is the develop of acquired resistance following an initial responsiveness to therapy. The principal mechanism of resistance to PARPi is restoring HR capacity, DSBs are repaired and tumor cell continues to survive. HR capacity is restored by two mechanisms: the suppression of NHEJ activity, and BRCA1/2 reactivation through reverse mutation [66].

NHEJ can be suppressed by 53BP1 depletion that saves BRCA mutated cells and reduce hypersensitivity to PARPi [71]. Another mechanism to suppress NHEJ pathway is represented by the inhibition of REV7, that stimulate NHEJ through shRNA promoting HR activity [72].

The main known system to restore HR is the reactivation of BRCA1/2 by secondary mutations, that restored the Open Reading Frame (ORF) of *BRCA* genes. Several studies confirmed that reverse mutations are present in patients with germline and/or somatic *BRCA1/2* PVs/LPVs [73] and decrease of PFS during Rucaparib treatment from 9 to 1.8 months [74]

1.1.5 High Penetrance Genes

BRCA1 and BRCA2

The two major genes involved in HBOC syndrome are *BRCA1* and *BRCA2*, two tumour suppressors developed in 1994 [75] and 1995 [76] respectively. Pathogenic Variants/Likely Pathogenic Variants identified in *BRCA1/2* are inherited in autosomal dominant manner and lead to an increased risk to develop HBOC syndrome.

BRCA1 gene (17q21) is discovered in 1994 by Miki et al.; it is composed by 24 exons of which 23 codificant a protein involved to different cellular process such as DNA-repair, cell-cycle arrest and genomic stability [75]. This protein contains a RING domain at N-terminal, a central region composed for majority by exons 11-13, and BRCT domain at C-terminal. RING domain is composed by a RING finger and two alpha helices in exons 2-7. RING structure is stabilized by two Zn^{2+} atoms, organized by Zn^{2+}-binding loops through seven conserved cysteine residues and one conserved histidine residue [77]. BRCA1 interact with BARD1 (BRCA1 Associated RING Domain protein 1) via N- and C-terminal helices in RING

finger. This interaction increases BRCA1 ubiquitin ligase activity and hidden the nuclear export sequence (NES) of both BRCA1 and BARD1 to retain both proteins in nucleus [78]–[80].

Region between exons 11 and 13 cover more than 65% of all BRCA1 protein and codify for two nuclear localization sequences (NLS) and binding sites for different proteins such as retinoblastoma protein (RB), C-MYC, RAD50 and RAD51 involved in several cellular pathways. This portion includes a part of a coiled-coil domain which mediates interactions with PALB2, a protein involved in in DNA damage repair with *RAD50* and *RAD51* genes [81].

BRCA1 C-terminal (BRCT) domain is a conserved domain in several proteins including BRCA1. BRCT domain regulates the interaction between BRCA1 and proteins phosphorylated by ATM and ATR induced by DNA damage. Based on capacity to recognize phosphoproteins, BRCT domains are divided in two types: class I that identify phosphoserine (pSer) residues, and class II that recognize both pSer and phosphothreonine (pThr) residues [82].

BRCA2 gene (13q12-q13) is discovered in 1995 by Wooster et al. It consists of 27 exons of which 26 codificant a protein involved in repair of DNA double-strand break by Homologous Recombination pathway. Protein structure is composed by PALB2 bond site at N-terminal, eight BRC repeats that bind with different affinity RAD51, a DNA binding domain (DBD) that recognize single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). In the C-terminal are presents a nuclear localization sequence (NLS) and the bond site for RAD51 mediated by CDK activity [83].

TP53

TP53 gene located on 17p13.1 encodes a tumor suppressor that regulate several cellular processes such us cell cycle arrest, apoptosis, senescence and DNA repair. For all these reasons, tp53 is named “the guardian of the genome” and indeed the 50% of sporadic tumors showed a somatic mutation in *TP53* gene [84].

Germline PVs/LPVs detected in *TP53* gene are associated with autosomal dominant disorder, characterized by a high predisposition to different types of cancer, called Li-Fraumeni syndrome [85]. Patients that exhibiting this syndrome have a risk increase to develop BC of about 25-80% [86] and to acquire a OC during lifetime but is not well determined [87].

PTEN

PTEN gene is located on 10q23.31. The protein is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase that negatively regulates the MAPK (Mitogen-Activated Protein Kinase) pathway through its protein phosphatase activity [88]. In *PTEN* gene the presence of germline PVs/LPVs are related to Cowden syndrome, an autosomal dominant disorder characterized by benign hamartomas and an increased risk to develop breast, thyroid, uterine, and other cancers [89]. Indeed, in patients with PVs/LPVs in *PTEN* gene, the risk during lifetime to develop a BC is estimated in around 25–85%, while for OC the risk is low or none [90].

STK11

STK11 gene is located on chromosome 19p13.3 and encodes a protein with serine/threonine kinase activity. Germline PVs/LPVs are correlated with Peutz-Jeghers syndrome, an autosomal dominant disorder described by melanocytic macules, gastrointestinal hamartomatous and a predisposition to develop different cancer types. In particular, patients with PV/LPVs in *STK11* gene have an increased risk to develop BC and gynaecological cancers estimated in 32–54% and 13% respectively [91].

CDH1

CDH1 is positioned on chromosome 16q22.1 and encodes E-cadherin involved in different cellular process including prevents invasiveness and metastatization [92]. An autosomal dominant condition called Hereditary Diffuse Gastric Cancer syndrome is related to germline PVs/LPVs in the *CDH1* gene. This syndrome predisposes to diffuse-type gastric cancer and lobular breast cancer, with a risk of 39–52% to develop a BC [93].

1.1.6 Moderate and Low Penetrance Genes

PALB2

PALB2 gene is located on 16p12.2 and encodes protein that collaborates with BRCA2 in HR pathway, binding SSBs and directly interacts with RAD51 to stimulate strand invasion in HR process. *PALB2* PVs/LPVs are recently related to an increase in BC risk of about 50% for females and 1% for man, and a low risk of OC and pancreatic cancer respectively of 5% and 2-3% [94].

BRIP1

BRIP1 gene is positioned on chromosome 17q23.2 and encodes a protein with DNA helicase activity that binds directly BRCA1 at BRCT repeats. *BRIP1* PVs/LPVs are associated with a higher risk of OC assessed of about 6%, while BC risk is low or none [95].

ATM

ATM gene is located on 11q22.3 and is composed by 66 exon that codifies to 3056 amino acids [96]. ATM is a serine/threonine protein kinase, discovered in 1995 by Savitsky et al., implicated in developed of an autosomal recessive condition named Ataxia-Telangiectasia [97]. Few years later, in 1998 two different group showed that ATM is also involved in DNA damage repair and has been implicated in an increase of ~2-fold risks of breast cancer. Whereby is classified as Moderate-risk BC gene [98], [99].

CHEK2

CHEK2 gene located on 22q12.1, encodes a nuclear Serine/Threonine kinase involved in different cellular process [100]. In presence of DSBs, ATM catalyzes the phosphorylation of CHEK2 which in turn phosphorylates CDC25C blocking entry into mitosis [101], moreover CHEK2 phosphorylates p53 tumor suppressor protein prevents its degradation and stoppes cell cycle in G1 phase [102]. Germline PVs/LPVs in *CHEK2* gene are related with an increased risk of BC estimated to be 25–39% during a lifetime [103]. Risk is modulated by family history: in carriers without affected family member the risk is around 20%, and it grows up to 44% when both first- and second-degree families are affected [104]. In particular, an increase of BC risk of two- to three-fold in women and ten-fold in man was associated a variant named c.1100delC in *CHEK2* [105].

Besides BC risk, PVs/LPVs in *CHEK2* has been related to different cancers as well as prostate, gastric, colorectal and with much debate OC [5].

BARD1

BARD1 gene localized on chromosome 2q35 is composed of 11 exons. Produced protein consists of 777 amino acids that interacts with N-terminal region of BRCA1 creating a heterodimer with ubiquitin E3 ligase activity involved in DNA damage response pathways and cell cycle regulation [106]. *BARD1* PVs/LPVs has been associated with an increase of BC and OC risks, even if there is no clear evidence especially in OC cases [95]. However, considering the involvement of BARD1 in HR pathway, clinical trials have been developed

to evaluate PARP inhibitors treatment in patients with BC and PVs/LPVs in *BARD1* gene [85].

RAD51

RAD51 gene encodes a protein involved in Homologous Recombination pathway during DSBs; it presents seven different paralogs (RAD51, RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, and DMC1). Monoallelic PVs/LPVs in *RAD51* and its paralogs have been associated to cancer predisposition (in particular *RAD51B*, *RAD51C*) and *RAD51D* in OC, while *RAD51*, *RAD51B*, and *XRCC2* in BC [108]. In particular, *RAD51C* located on chromosome 17q22 is associated with around 7% OC risk, whereas BC risk is debatable [109]. Instead PVs/LPVs of *RAD51D*, located on 17q22, are associated to OC risk around 15% but BC risk is questionable [110].

MRN complex

The MRN complex is formed by three proteins encoded by *MRE11*, *RAD50*, and *NBN* genes implicated in DSBs repair and able to act as tumor suppressors by regulating genomic stability [5]. Germline PVs/LPVs in these genes have been associated to an increase in BC and/or OC risks [111]. In particular, *NBN* gene, positioned in 8q21.3, encoded a Nibrin protein essential to MRN complex localization, and its interaction with other proteins involved in DSBs signaling. PVs/LPVs in *NBN* gene influence cancer predisposition with an increase in BC, prostate cancer, medulloblastoma, and melanoma [112].

1.1.7 Mismatch repair

Mismatch repair system (MMR) is a type of DNA damage repair responsible to preserve genomic stability by correcting spontaneous base-base mispairs and INDELS developed during DNA replication. The presence of MMR alteration leads to mutational rate increase and high microsatellite instability (MSI) [113].

Mismatch repair pathway is composed by 5 proteins that acts as heterodimers: MLH1, MSH2, MSH6, PMS2 and EpCAM (a regulator of MSH2). *MSH2* gene on chromosome 2p21 encode to MSH2 protein that heterodimerize with two different partners, MSH6 and MSH3. However, MSH2/MSH6 heterodimer is more present than MSH2/MSH3 because MSH6 is expressed 10 more times than MSH3 [114]. MSH2/MSH6 function is to detect single base mismatches and dinucleotide insertion/deletion and successively to engage additional molecules as Proliferating Cell Nuclear Antigen (PCNA), Replication Factor C (RFC),

MLH1/PMS2 heterodimer and exonuclease 1 (Exo1) by leading to mismatch final dissociation [115].

MLH1 gene, located on chromosome 3p21, encode protein that form heterodimer with three different monomers, PMS2, PMS1 and MLH3 enrolled after mismatch identification by MSH2/MSH6 heterodimer starting the repair process. MLH1/PMS2 contain an endogenous endonuclease activity that nick unmethylated strand and provides an access point for EXO1 nuclease to degradation of DNA strand containing mispaired bases [116].

Germline PVs/LPVs in MMR genes determine an autosomal dominant syndrome called Lynch Syndrome, that is associated to an increased risk to develop colorectal cancer, endometrial cancer, epithelial ovarian cancer, stomach, pancreatic, brain ecc… [117]. This syndrome is responsible of about 10–15% of OC; germline/somatic MMR deficiency characterize about 1-8% of serous OC. [118]. In particular, OC risk is associated to *MLH1* PVs/LPVs in 10-20% of patients, to *MSH2* PVs/LPVs in 17–24%, to *MSH6* PVs/LPVs in 8–13% of patients, and to PVs/LPVs in *PMS2* gene the OC risk is analogous to generic population [119].

Considering all these conditions, in 2020 ASCO guideline has been approved treatment with pembrolizumab, a monoclonal anti-programmed cell death-1 antibody, in the setting of recurrent disease in patients with mismatch repair deficiency independently of primary site [30].

1.1.8 Multi-gene Panel Testing in HBOC patients

In patients with HBOC syndrome *BRCA1/2* testing is recommended. However, it is becoming necessary to study multiple genes in short-time and cost-effective manner. This scenario it has become possible with the advent of next-generation sequencing (NGS), which allow the simultaneous sequencing of multiple samples and genes, and in particular the multi-gene panel testing. To be accepted by scientific community, multi-gene panel approach should have a high rate of analytic concordance with more conventional sequencing methods as Sanger sequencing and Multiplex Ligation-Dependent Probe Amplification (MLPA). Another advantage, besides the cost reduction, is a possible therapeutic implementation in patients that showing a PVs/LPVs in genes included in multi-gene panel rather than single gene testing. Moreover, a germline mutation revealed could have an implication not only in therapeutic choice but also in the prevention of patient or in an unaffected relative. In 2016, NCCN recommended primary prevention and early detection in patients with PVs/LPVs [3].

However, the approach with multi-gene panel showing diverse disadvantages such as an increasing of VUS and PVs/LPVs in moderate/low-penetrance genes or with limited clinical relevance. These disadvantages can determine a risk overestimation and an increasing number of interventions without benefits.

To reduce this overestimation, the choice of multi-gene panel testing should be guided by personal and family history of patients and the multi-gene approach should be offer to patients at high risk.

In the last few years, different studies have focused on multi-gene panel testing use on HBOC patients.

In 2017 Kurian et al. showed an increased BC risk associated with 8 diverse genes *ATM*, *BARD1*, *BRCA1*, *BRCA2*, *CHEK2*, *PALB2*, *PTEN*, *TP53* while no mutations in MMR genes was associated at BC risk. Moreover, the researcher founded *ATM*, *BRCA1*, *BRCA2*, *BRIP1*, *MLH1*, *MSH6*, *MSH2*, *NBN*, *RAD51C*, *RAD51D*, and *STK11* genes strongly associated with OC risk. Interestingly, for NBN gene no association was revealed with breast and ovarian cancers [120].

Germani et colleagues, in their study on 733 patients with BC, OC and pancreatic cancer (PC) described a PVs/LPVs in 14% of BC/OC patients beyond *BRCA1/2* genes, in particular *CHEK2*, *RAD51C*, *ATM*, *MLH1*, *MSH2* and *RECQL* genes. However, the investigators showed an elevated presence of VUS in about 20% of patients that often causes problem in risk assessment and usually anxiety in patients [121].

Fanale et al in 2020, study 139 patients with bilateral breast cancer (BBC) and find that 37.4% have a PVs/LPVs in high- and moderate-penetrance BC susceptibility genes like *BRCA1*, *BRCA2*, *PTEN*, *PALB2*, *CHEK2*, *ATM*, *RAD51C*. In particular, is showed a strong correlation between BBC and LPVs/PVs in *PALB2* and *CHEK2* genes [8].

1.2 Rationale and objectives

Considering the extensive knowledge about preventive role of *BRCA1/2* genes, in the last few years *BRCA* genes hired an important predictive value to therapeutic response. In fact, recently FDA approved different PARP inhibitor to treatment of patients affected by BC or OC with PVs/LPVs in *BRCA1/2* achieving an improve in terms of PSF and OS. *BRCA1/2* genes are involved in pathway of DNA repair named Homologous Recombination together other genes like as *PALB2*, *CHEK2*, *ATM*, *STK11*, *TP53*.

Today patients with a strong personal and/or family history to BC and/or OC resulted BRCA1/2 negative do not receive new possible therapeutic approaches and a specific risk-

reducing measures and their family members are not followed with prevention programs. However, the advent of NGS before and, in the last few years, the multi-gene panel testing gave these families the opportunity to be monitored over time and the possibility of accessing new therapeutic frontiers.

The aim of study was assessed inherited pathogenic variants/likely pathogenic variants in both *BRCA1/2* genes and other genes involved in Homologous Recombination pathway in patients affected by Hereditary Breast and Ovarian Cancer syndrome with multi-gene panel approach. The objective of this study was to evaluate the contribution of these alterations, showed in high- and moderate-penetrance genes, to identify probands who could benefit from PARP inhibitor treatment. Moreover, the identification of variants in genes beyond *BRCA1/2* suggest family members a specific risk-reducing measures by offering free prevention programs.

CHAPTER 2

Patients and Methods

2.1 Study population

We conducted a retrospective study at the “Sicilian Regional Center for the Prevention, Diagnosis and Treatment of Rare and Heredo-Familial Tumors” of the Section of Medical Oncology of University Hospital Policlinico “P. Giaccone” of Palermo. We collected and analyzed all medical information regarding 876 patients divided in 531 women and men with primary breast cancer and 345 women with ovarian cancer which were underwent to germline and somatic *BRCA1/2* test among January 2016 to August 2020. Successively, we analyzed 192 patients with a strong personal and/or family history to BC and/or OC resulted *BRCA1/2* negative with a multi-gene panel testing.

The medical personal history of patients was retraced during genetic counselling in presence of a multidisciplinary team constituted by an oncologist, a geneticist and a psychologist. All patients provided an informed consent and information regarding personal and familial history of cancer, family geographical origin, age of cancer diagnosis, histological tumor subtype, molecular phenotype and disease stages (I–IV), were anonymously recorded. Data concerning histological type and tumor diagnosis were reported by medical pathology in diagnostic core biopsies or tumor resections.

To breast cancers, relying on histological grade and biomarker expression, tumors were divided as luminal A-like (LA= ER/PR+ and HER2-, histological grade 1 or 2), luminal B-like (LB= ER/PR+ and HER2+, or ER/PR+, HER2-, and grade 3), HER2 enriched and triple negative (ER-, PR- and HER2-) [122].

After genetic counselling and Hereditary Breast and Ovarian Cancer syndrome (HBOC) risk estimation, the patients were evaluated for both germline and somatic *BRCA1/2* genetic screening in OC patients and germline test in BC patients based on criteria established by the Italian Association of Medical Oncology (AIOM) also reported in section 1.1.3 [123].

2.2 Sample Collection and Next-Generation Sequencing Analysis

Peripheral blood was collected from HBOC patients. In addition, to OC patients, biopsy Formalin-Fixed Paraffin-Embedded (FFPE) ovarian neoplastic tissue samples had been available as exploratory biopsies or neoplastic tissue removed with surgery. Tissues samples were sectioned at 4 μm with >20% of malignant origin by the laboratory of pathological anatomy section of the same hospital agency. Genomic DNA was isolated from the peripheral blood using the DNeasy® Blood Kit (QIAGEN), while the isolation of DNA from FFPE tissue had been performed by QIAamp® DNA FFPE Tissue Kit (QIAGEN). Genomic DNA was quantified by Qubit®3.0 fluorometer (Thermofisher Scientific) and its quality was provided by use of 2100 Bioanalyzer (Agilent Technologies). To conduct the *BRCA1/2* analysis in germline/somatic samples, we used 4 ng of DNA to prepare the barcoded library using BRCA Screen kit (4bases SA) that has allowed to investigate all the exons of *BRCA1* (NM_007300.3) and *BRCA2* (NM_000059.3) genes. This kit consists of three multiplex PCR primer pools and we employed 20 ng of DNA for each primer pool to multiplex PCR amplification, followed by barcode ligation and purification with Agentcourt AMPureXP reagent (Beckman Coulter). Quantity and quality of libraries was assessed by Qubit®3.0 fluorometer (Thermofisher Scientific) and Agilent 2100 Bioanalyzer on-chip electrophoresis (Agilent Technologies), respectively, as previously described [124]. Subsequently, libraries were mixed in an equal concentration and emulsion PCR was performed using the Ion OneTouch OT2 System (Thermofisher Scientific) with Ion 520 & Ion 530 Kit-OT2 (Thermofisher Scientific). At the end, sequencing was performed with Ion 520 Chip (Thermofisher Scientific) using Ion Torrent S5 (Thermofisher Scientific) instrument. The sequencing data was analyzed with two different software Amplicon Suite (SmartSeq s.r.l.) and Ion Reporter Software v.5.14 (Thermofisher Scientific).

To analyze multi-gene panel testing, we used a HEVA SCREEN kit (4bases SA) that has allowed to evaluate 22 genes involved in risk of hereditary breast, ovarian and colorectal cancer, and other inherited tumor syndromes (*ATM*, *APC*, *BARD1*, *BRCA1*, *BRCA2*, *BRIP1*,

CDH1, *CHEK2*, *EPCAM*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *NBN*, *PALB2*, *PMS2*, *PTEN*, *RAD50*, *RAD51C*, *RAD51D*, *STK11*, and *TP53*) with the same method above mentioned. The data analysis has been performed with the standardization of sequencing coverage depth to minimize the probability of false positive and false negative results. There is currently no consensus on the minimum coverage depth and each laboratory must set its own parameters. A minimum coverage of 5000x and 500x to sample coverage has been considered as somatic and germline cut off analysis respectively.

2.3 Sanger sequencing

Pathogenic variants and likely pathogenic variants identified with NGS were confirmed with Sanger sequencing using SeqStudio (Thermofisher Scientific) with BigDye Therminator 3.1 Cycle Sequencing Kit (Life Technologies)

2.3 CNV Analysis by Multiplex Ligation-Dependent Probe Amplification Analysis (MLPA)

The presence of Large Genomic Rearrangements (LGR) was furthermore tested by Multiplex ligation-dependent probe amplification (MLPA), by SALSA MLPA probemix P002-C2 for *BRCA1* gene and SALSA MLPA probemix P090 for *BRCA2* gene according to the manufacturer's instructions (MRC–Holland, Amsterdam, the Netherlands). Probe amplification products were investigated by capillary electrophoresis using ABI 3130 Genetic Analyzer (Applied Biosystems). Results were evaluated by GeneMapper™ Software Version 3.5 (Applied Biosystems) to determine peak heights and areas and fragment sizes in base pairs (bp), as described previously [125]. Positive results were validated with a second analysis using the same kit on another blood sample.

2.3 Genetic Variant Classification

The detected genetic variants were classified based on criteria developed by Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) consortium (https://enigmaconsortium.org/) and divided into five classes: benign (class I), likely benign (class II), variant of uncertain significance (VUS, class III), likely pathogenic (class IV), and pathogenic (class V) as previously reported in section 1.1.3. Several databases were used to identification and classification of genetic variants, such as ClinVar, BRCA Exchange, LOVD and Varsome.

The variant detected were named according to the recommendations for the description of sequence variants offered by the Human Genome Variation Society (HGVS) that is approved by, Human Variome Project (HVP), and the Human Genome Organization (HUGO) [126].

2.4 Statistical analysis

Clinico-pathological variables and prevalence of PVs/LPVs were evaluated for each subgroup of patients. The comparison between subgroups was made with Fisher's Exact test. P-values < 0.05 were considered significant. Statistical analyzes were conducted using IBM SPSS Statistics for Windows Version 23.0 (IBM Corporation, Armonk, NY, USA).

CHAPTER 3

Results

3.1 *BRCA1/2* analysis in BC patients

Between January 1, 2016, and February 28, 2020, 531 BC patients who met eligibility criteria for *BRCA1/2* gene testing, were included in the retrospective study. According to national guidelines, the genetic counselling and the *BRCA1/2* mutational screening were offered at the "Regional Center for the prevention, diagnosis and treatment of rare and heredo-familial tumors of adults" of the Section of Medical Oncology of the University Hospital Policlinico "P. Giaccone" of Palermo.

Analysis was conducted after an appropriate informed consent signed.

Molecular subtypes identified were 125 (23.5%) Luminal A (LA), 223 (42%) Luminal B (LB), 29 (5.5%) HER2-enriched (HER2E), and 154 (29%) TNBC.

Among the 531 BC, 83 (15.6%) were positive for *BRCA1/2* PV. In particular, 39 (47%) showed a PVs in *BRCA1* gene, 43 (51.8%) were *BRCA2*-positive, and 1 patient (1.2%) revealed a double heterozygosity for *BRCA1* and *BRCA2* genes. Between BC with PVs in *BRCA1* gene, 28 (71.9%) showed TNBC, 10 (25.6%) a LB tumor, 1 (2.5%) HER2E and none LA. Between BC *BRCA2*-positive, 29 (67.5%) had LB, 6 (13.9%) TNBC, 6 (13.9%) LA and 2 (4.7%) HER2E. Tumors of the *BRCA1* carriers were most frequently TNBCs (p=0.0001), while in *BRCA2* carriers were predominantly LB/HER2-negative (p=0.0014) (Table 2).

In this study, the typology and gene location of germline *BRCA1/2* PVs in triple-negative vs. luminal-like breast cancers to identify potential association between specific PVs and tumor phenotype was also evaluated. From data analysis, it was seen that 83 patients showed 45 PVs in *BRCA1/2* genes; 23 were in TNBC patients, of which 18 in *BRCA1* gene and 5 in

BRCA2 gene. Regarding the other molecular subtypes, 33 PVs in *BRCA1/2* genes in luminal-like BCs were observed, 8 in *BRCA1* and 25 in *BRCA2*, whereas 3 PVs was observed in HER2E BCs divided in 1 in *BRCA1* and 2 in *BRCA2* (Table 3 and 4).

In TNBC patients the most frequent PV was c.514del in *BRCA1* gene, showed in 5 probands. This PV determine the deletion of one Cytosine causing a frameshift and a creation of premature stop codon at position 62 of new reading frame producing a BRCA1 protein truncated or absent [127]. The second most common PV in *BRCA1*-positive TNBC patients was the c.3904G>T, showed in 3 families. This PV cause a substitution of Guanine in Thymine in exon 11 at codon 1302 determining a premature stop codon and a BRCA1 protein truncated. Based on this data, no association between PVs in *BRCA1/2* genes and TNBC was observed in Sicilian population because the PVs detected in both genes showed a low prevalence.

Regarding the gene localization of *BRCA1* PVs identified in TNBC patients, three hypothetical cluster regions were recognized in *BRCA1* gene that include RING domain, exon11 region and BRCT domain. Specifically, 10 (55.5%) out of 18 *BRCA1* PVs were reported in region of exon 11, 4 in BRCT domain and 2 in RING domain. About type mutation, 11/18 were frameshift mutation, 3 nonsense, 3 missense and 1 was a Large Genomic Rearrangement (LGR) involving exons 1 to 15 (figure 3).

In luminal-like BC patients, the most common founded PVs in *BRCA1/2* genes was c.1238del in *BRCA2* gene in 8 probands with LB breast cancer. This PV cause a deletion of a Thymine and a premature stop codon with a loss of protein function. Furthermore, most variants present in luminal-like BC patients arise in *BRCA2* gene, for example LA patients not showed PVs in *BRCA1* gene. Like as TNBC patients, also in luminal-like BC patients none PV in *BRCA1/2* genes showed a significant prevalence without significant association between *BRCA1/2* variants and luminal-like BC patients.

The gene localization of *BRCA1* PVs in luminal-like BC patients follows the equal distribution in the same three cluster regions. To *BRCA2* gene, PVs were localized in three supposed cluster region that include at N-terminus the PALB2 binding, BRC repeats (in the exon 11), and DNA binding helical domain. Regarding the mutation type, the majority (9) were frameshift mutations, 7 were intronic origin (IVS), 5 nonsense and 4 missenses (figure 4).

3.1.1 Association among BRCA1/2 PVs and clinical factors in BC patients

In TNBC group, *BRCA*-positive patients were mainly in premenopausal at BC diagnosis with mean age of 43.7 (median 43). In terms of mean age between *BRCA1* and *BRCA2* PV carriers, a statistically significant difference was noticed. The mean age for *BRCA1* carriers was 41.7 years (median: 42; range: 28–58 years) versus 52.8 years (median 52.5; range: 42–62 years) for *BRCA2* PV carriers, and versus 48.2 years (median 48; range: 30-70 years) in subjects with no *BRCA1/2* PVs. Based on this data, patients with TNBC *BRCA1*-positive developed a BC 6.45 years before non-carrier ($p<0.001$) and 11.1 years previous than *BRCA2* PV carriers ($p<0.001$) (Figure 5A). According to the age group, the prevalence of PVs was 35.3% (12/34) in age group ≤ 40 years, 47.1% (16/34) in age range of 41-50 years, 14.7% (5/34) in range 51-60 years and 2.9% (1/34) in patients with age >60 years (Table 5).

Patients *BRCA*-positive with luminal-like tumors had a mean age at BC diagnosis of 43.75 years (median 40), specifically 39.1 years (median: 36.5; range: 31-55 years) for *BRCA1*-carriers and 45.1 years (median: 41; range: 26-82 years) for *BRCA2*-carriers. Instead, patients without PVs in *BRCA1/2* genes had mean age at BC diagnosis of 45.7 years (21-84 years; median: 44.5). Patients *BRCA1*-positive with luminal-like developed BC 6.6 years before non-carrier ($p= 0.0538$), and 6 years earlier than *BRCA2* PVs carriers ($p=0.78$) (Figure 5B). By dividing luminal-like BC patient in age group, prevalence of PVs was 58% (27/46) in age group ≤ 40 years, 20% (9/46) in age range of 41-50 years, 13% (6/46) in range 51-60 years, and 9% (4/46) in patients with age >60 years (Table 5).

Clinico-pathological differences between *BRCA* PVs carriers and non-carriers BC patients were observed. In luminal-like BC patients, *BRCA*-carriers were frequently associated to lower ER ($p=0.001$) and PR expression ($p = 0.007$) and were more often HER2-negative ($p=0.048$). Moreover, in this subgroup, *BRCA* carriers showed a high proliferation rate (Ki67%; $p=<0.001$) and upper histological grade (Grade III vs. I/II) than non-carriers ($p=<0.001$). In both analyzed subgroups, *BRCA* carriers seem to have an axillary nodal involvement than non-carriers ($p=0.002$ and $p=0.016$, respectively), while no statistically significant difference was showed in tumor size (T) ($p=0.802$ and $p=0.92$, respectively).

Ductal histotype was the principal histotype in both subgroups, without substantial differences among *BRCA*-carriers and non-carriers ($p=0.337$ and $p=0.7$, respectively).

Contralateral BC was present in 96 (19.1%) patients; in particular 5/34 (14.7%) were TNBC *BRCA1/2* positive, 13/46 (28.2%) were luminal-like *BRCA1/2* positive and 78/422 (18.4%)

were *BRCA1/2* negative. In 78 contralateral BC *BRCA1/2*-negative, 12/120 (10%) were TNBC and 66 (21.8%) out of 302 were Luminal-like. In *BRCA1/2*-positive TNBC patients, contralateral BC were diagnosed at earlier age than *BRCA1/2*-negative (50vs56 years; p=0.033). Whereas in Luminal-like the difference was lower (52 vs 53 years).

Median time between the first and second tumor was 10 years in patients with TNBC *BRCA*-positive and 6.5 years in *BRCA*-negative (p=0.389). In Luminal-like patients, the median time of contralateral BC was shorter in *BRCA*-positive (4 years) and *BRCA*-negative patients (3 years) (p=0.465). In general, the median time of onset of bilateral tumors was lower in luminal-like than TNBC patients.

3.2 *BRCA1/2* analysis in OC patients

A total of 345 patients affected by OC was retrospectively analyzed; 85 of which (24.6%) were resulted altered in *BRCA1/2* genes. In particular, 56 (65.9%) were resulted *BRCA1*-positive and 29 (34.1%) showed a PVs/LPVs in *BRCA2* gene. The distribution of these variants was reported in Table 6.

The most frequent PV identified in 8 probands (9.2%) was c.4964_4982del in *BRCA1* gene. This variant is characterized by a deletion of 19 nucleotides that produces a frameshift with a premature stop codon and the loss of normal protein function. In our recently published work, this variant resulted the most widespread PV in the Sicilian population [7]. Indeed, today is considered a potential Sicilian founder mutation [128]. The second most recurrent variant detected was c.514del in *BRCA1* gene highlighted in 5 families (5.7%). Interestingly, a variant named c.4963T>G in *BRCA1* gene was noted in 3 (3.5%) different patients. Protein change causes a substitution of Serine with Alanine. Since Serine in position 1655 is a highly conserved residue, this variant is probably considered as Pathogenic.

In *BRCA2* gene, we have not noted a most recurrent variant. In fact, only 6 different variants were detected in no more than 2 (2.3%) patients.

A variant type in *BRCA1/2* genes was also evaluated. In fact, it was possible to identify 48 (56.4%) frameshift mutations (fs), 19 (22.3%) NonSense (NS), 11 (12.9%) Missense (M), 6 (7%) Intronic Variants Sequencing (IVS) and 1 (1.2%) Synonymous. In details, in *BRCA1* gene, we highlighted 33 (58.9%) variants of frameshift nature, 11 (19.6%) NonSense, 9 (16%) Missense and 3 (5.3%) IVS. Instead, in BRCA2 gene was identified 15 (51.7%) frameshift mutations, 8 (27.7%) NonSense, 3 (10.3%) IVS, 2 (6.9%) Missense and 1 (3.4%) synonymous PV (Figure 6).

Among 116 performed somatic tests, 15 (12.9%) were resulted *BRCA1/2* positive. In

particular, 10 PVs were revealed in *BRCA1* and 5 in *BRCA2* genes. However, any correlation noteworthy was not found about frequency distribution.

3.2.1 Association among patients BRCA1/2- positive and clinical factors in OC patients

Between OC patients *BRCA1/2*-carriers and no-carriers, the analysis reveal a statistically significant difference in age at diagnosis (median: 56vs58; mean: 57.9vs58.6; range: 24-86vs37-81; p=0.02). According to the age group, the prevalence of patients in both analyzed groups was in the 51-60 range, with 36.5% and 38.5% respectively, and in the 61-70 range (25.8%vs25.4%). However, no statistically significant difference was noted.

As expected, the most recurrent cancer site either in *BRCA1/2* wt and *BRCA1/2*-carriers was ovarian carcinoma with 82.3% and 63.5% of patients respectively. Noteworthy, the 34.1% of patients with bilateral ovarian carcinoma showed a PVs/LPVs in *BRCA1/2* genes versus only 8% of patients without variants (p<0.00001). Moreover, a little difference was noted in patients with primary peritoneal carcinoma of which 9.3% resulted *BRCA1/2* wt and only 2.4% were *BRCA1/2*-carriers.

Another important and statistically significant difference in patients with alteration in *BRCA* genes was highlighted. The 57.7% of these subjects showed a FIGO stage III versus 13.8% of patients without alterations (p=0.02), although 73.8% of these last patients had the FIGO stage unknown.

Regarding the histological subtypes, most patients presented a high-grade serous carcinoma (HGSC) either in *BRCA1/2*-carriers subgroup (80%) and in group of *BRCA*-no carriers (68.5%), as reported in literature. The second most frequent subtype in both analyzed group was the Endometrioid carcinoma, represented in 7.1% of *BRCA*-carriers and in 9.2% of no-carriers.

An important obtained result was that the 17.6% of *BRCA1/2* carriers showed a personal BC history before OC, while only 6.5% of patients *BRCA1/2* no-carriers showed a previous personal BC history (p=0.007). In particular, 9/29 (31%) patients with *BRCA2* PVs/LPVs had a prior BC history while only 6/56 (10.7%) *BRCA1*-carriers had a BC history before the OC. The clinicopathological characteristics of OC patients were reported in Table 7

3.3 Detection of Germline Pathogenic Variants in Cancer Susceptibility Genes by Multi-Gene Panel Testing

After *BRCA1/2* testing, 192 patients (165 of whom affected by BC and 27 affected by OC) were resulted *BRCA1/2* negative with a strong personal and/or family history of breast and ovarian cancer. These subjects were selected to multi-gene panel testing analysis, based on indication of the multidisciplinary team. Obtained data revealed that 28 (14.6%) BC and OC patients showed PVs/LPVs in genes no-*BRCA* (Figure 7).

In the165 BC patients (160 women and 5 men) analyzed with multi-gene panel approach, 24 (14.5%) showed PVs/LPVs in other BC susceptibility gene (no-*BRCA*) (Table 8). Specifically, 6 (25%) probands have been shown to harbour PVs in *MUTYH* (NM_001048171) gene, 4 (16.6%) probands in *CHEK2* (NM_001005735) gene, 3 (12.5%) probands in *RAD51C* (NM_002876) and in *PMS2* (NM_000535) genes respectively, 2 (8.3%) patients presented PVs in *PALB2* (NM_024675) and ATM (NM_000051) genes respectively. In addition, 1 (4.2%) PVs in *RAD50* (NM_005732), *PTEN* (NM_000314), *MSH2* (NM_000251) and *CDH1* (NM_004360) genes were presents in singular patients (Figure 8). Conducted analysis revealed that among 24 patients (no-*BRCA*), 11 (45.8%) were affected by Bilateral Breast Cancer (BBC). In detail, these 11 PVs were divided in this manner: 2 in *CHEK2*, 2 in *PALB2*, 2 in *ATM*, 2 in *RAD51C*, 2 in *MUTYH* and 1 in *MSH2* genes.

The most frequent PV in the retrospective analysis was a missense mutation in *MUTYH* gene in heterozygous condition named c. 1145G>A in 5 probands (20.6%), that is normally linked to MUTYH-associated colon polyposis (MAP) syndrome and colorectal cancer [129]. In our cohort, this PV was strongly associated with Luminal B and HER2 negative BC.

The second most recurrent PV was c.1229del in *CHEK2* present in 2 (8.3%) individuals affected by BBC. This result was according to published data in our precedent work yet [8]. In fact, this variant was correlated with BBC and in particular to BC with Luminal A/B phenotype, estrogen receptor positivity >60%, and progesterone receptor positivity between 20% and 60% [8].

Another frequent variant was a IVS in *CHEK2* gene called c.721+3 A>T, discovered in 2 (8.3%) patients. The nature of this variant is still to define. In fact, database as ClinVar reports it as Conflicting Interpretations of Pathogenicity (CIP), although could be probably considered a PV being a splice site mutation.

Despite *PALB2* has been shown to be one of the most frequently altered gene in BC patients, in our analysis only two patients (8.3%) exhibited an alteration in this gene.

However, both of these patients showed a BBC whereby a correlation between this PV and BBC could be hypothesize.

Among 27 OC patients, 4 (14.9%) were showed a PVs/LPVs in other genes no-*BRCA* (Table 8). In particular, 2 patients (50%) showed a PV in *MUTYH* gene, and 1 PV in *ATM* and *PMS2* genes were present in two patients (25%) singularly.

Patients with *MUTHY* PV presented variant named c.1145G>A in heterozygous condition as patients with BC. One patient showed a variant in *ATM* gene called c.4776+1G>T capable of acting as a donor splice site in intron 31 of the *ATM* gene. Considering *in silico* analysis, this variant has been classified as Likely Pathogenic because it is expected to disrupt RNA splicing and probably to delineate an absent protein product.

Last patient harbouring a PV had a c.2249G>A in *PMS2* gene in homozygous condition. This variant involves a substitution of glycine, highly conserved residue, with aspartic acid at codon 750 and has been noted in patients with constitutional mismatch repair deficiency syndrome.

3.3.1 Comparison between BRCA1/2 PVs/LPVs, Multi-Gene Panel Testing, all *wild-type* and clinical factors

The median age at diagnosis of BC patients with PVs/LPVs in gene over *BRCA1/2* was lower than *BRCA1/2* positive and all *wild-type* (wt) (median age: 32vs41 p=0.8; 32vs44 p=0.9;) with a mean age that conversely was higher than other groups (mean age: 45.6vs42.6vs44.5 respectively). Most patients with PVs/LPVs either on *BRCA1/2* and in other genes had an age at diagnosis ≤40 years.

Significant clinico-pathological differences among three subgroups of BC were observed (Table 9). Patients with alterations in *BRCA1/2* (86.8%) and without any alterations (77.4%) often presented a ductal BC rather than patients with alteration in genes over *BRCA1/2* (54.2%).

Interestingly, only 3 patients (12.5%) with PVs/LPVs in the panel were TNBC compared to 34 patients (40.9%; p=0.12) with *BRCA1/2* and 149 (25.3%; p<0.00001) without alterations in genes analyzed.

Another important observation was that PVs carriers in other genes did not have a high proliferation rate and a higher histological grade than *BRCA1/2* carrier (p=0.003) and-non carrier (p=0.24). Furthermore, an involvement of axillary nodal did not presented than other two groups (p=0.07; p=0.002).

As mentioned above, 11 patients resulted altered with multi-gene panel analysis had a BBC. This result was statistically significant either compared to *BRCA1/2* carriers (p=0.04) and to all wt (p=0.008).

Comparing only patients analyzed with multi-gene panel, no correlation statistically significant was showed between mutated and *wt*. However, noteworthy result was that patients mutated had a median age at diagnosis considerably lower than *wt* (median: 32 vs 43.5 years; p=0.24). Regarding patients with BBC, the median time between 1st and 2nd tumor was longer in mutated than patients without alteration.

Considering patients affected by OC, the median age at diagnosis was similar between subjects with PVs/LPVs in other genes over *BRCA1/2* and *wt* for multi-gene panel (52.5vs52 years, respectively). A comparison among OC patients with PVs/LPVs in *BRCA1/2* genes, multi-gene panel mutated and all wt was conducted. We observed that women with PVs/LPVs in genes over *BRCA1/2* tends to develop OC before than other two groups (median: 52.5vs56vs58 years). Dividing in age groups, women characterised by an alteration in genes over *BRCA1/2* genes were distributed in all different groups. In other two analyzed groups, the most represented zones were the patients over the sixth decade of life.

Based on tumors characteristics, patients mutated in multi-gene panel showed an ovarian carcinoma with high-grade serous carcinoma as histological subtype, and in the 50% of cases a FIGO stage III. Same results were observed in other two groups, though in all wt subgroup the majority of patients had a FIGO stage unknown.

At the end, *BRCA1/2*-carriers group and also the group of patients with PVs/LPVs in genes over *BRCA1/2* the 75% of patients showed a personal history of BC or other cancer. Clinico-pathological characteristic were reported in Table 10.

Obviously, these results are not considered statistically significant due to the low number of patients analyzed with multi-gene panel.

CHAPTER 4

Discussion

HBOC syndrome is an autosomal dominant inherited disorder that represent about 5–7% of all breast cancer (BC) cases and 10-15% of all ovarian cancer (OC) cases [2]. NCCN established clinical guidelines to distinguish HBOC patients [3] that had an increased risk to develop additional cancer like as pancreatic cancer, prostate cancer, and melanoma.

BC and OC are heterogeneous diseases with the involvement of different environmental and genetic risk factors. This heterogeneous nature is underlined from recent advances in genetic and genomic fields. Indeed, in the last few years genetic test request to integrate the information about prevention, surveillance and treatment decision making.

BRCA1/2 are main genes involved in inherited predisposition to HBOC syndrome. In particular, the presence of PVs/LPVs in these genes increase the risk to develop breast cancer for 50–85% *BRCA1/2* carriers and to develop ovarian cancer for 15–45% and 10–20% to *BRCA1* and *BRCA2* carriers respectively. However, several patients result negative for *BRCA1/2* analysis and further genetic testing are need using panel with many other genes. In fact, HBOC syndrome could be associated with germline PVs/LPVs in several high-risk genes such as *CDH1*, *PALB2*, *PTEN*, *STK11*, *TP53*, or moderate-risk genes like *ATM*, *CHEK2*, *BARD1*, *BRIP1*, *RAD51C*, *RAD51D*. For these reasons, to date the use of multi-gene panel testing containing several susceptibility genes is becoming progressively regular in clinical practice [8]. This is possible also thanks to the progress obtained by NGS technology which revolutionized the clinical approach to genetic testing. Recently, several studies showed that a considerable number of PVs/LPVs can be missed if syndromes like as HBOC, Cowden syndrome, Li–Fraumeni syndrome, Lynch syndrome are tested independently. Therefore, a multi-gene panel testing that covering all these syndromes were applied [130].

In this work, we have retrospectively analyzed 531 BC patients for germline PVs in *BRCA1/2* genes according to national guidelines. We revealed 83 patients with *BRCA1/2* PVs. These variants resulted more frequent in TNBC subgroup (22.1%), followed by Luminal B (18%), HER2E (10.2%) and Luminal A (4.8%). Analysis allowed to reinforce the strong association between TNBC and *BRCA1* PVs and between Luminal B BC and *BRCA2* PVs. In our cohort, BC *BRCA*-positive arise in younger women. Patients *BRCA1*-positive developed BC before than *BRCA2* carriers and non-carriers, moreover TNBC *BRCA1*-carriers had a greater difference in age at diagnosis than TNBC *BRCA2*-carriers.

Based on prognostic factors, patients *BRCA1/2* carriers showed a greater percentage of Ki67-positive cells, had a high histological grade and an axillary nodal involvement. Patients with Luminal-like BC and a PVs in *BRCA1/2* showed a lower expression of ER, PR and HER2.

Furthermore, differences in presence of bilateral tumors in BRCA1/2 carriers than no-carriers were observed.

In Luminal-like subgroup with *BRCA1/2* PVs, contralateral BC was more common and characterised by lower median time to second tumor development than TNBC and *BRCA1/2* no-carriers.

Regarding variant type, most *BRCA1* PVs in TNBC and PVs in *BRCA2* were frameshift mutations. In Luminal-like subtype we revealed an elevated percentage of intronic pathogenic variants in *BRCA2* gene. Based on distribution of PVs in *BRCA1/2* genes, we have noticed that two variants were probably associated with TNBC and Luminal-like respectively. In particular, *BRCA1*-c.514del was presented with high frequency in 5 families with TNBC and only 2 families with LB tumors. Specifically, this variant was associated at TNBC diagnosed at younger age characterized by poor prognostic factors as Ki67 percentage and nuclear grade. Furthermore, considering that this PVs is infrequently presented in Italy or in World, could be further studied for a possible founder effect for the Sicilian population.

In *BRCA2* gene, variant named c.1238del was noted in 8 families with LB tumors and in none TNBC families. Specifically, this PVs was associated at patients who carried a high proportion of bilateral breast tumors [8] that were HER2-negative with higher ER expression (range 70-95%).

Moreover, we tried to identify a possible genotype/phenotype correlation based on PVs site correlated to TNBC or Luminal-like risk in our cohort. We have noted that most PVs revealed in TNBC and Luminal-like BCs were located at exon 11 of both genes, that correspond to

majority of coding sequence in both genes and is considered a “coldspot” to missense PVs. In addition, we showed that other two regions as RING domain and BRCT domain could be involved in TNBC risk. However, our data do not allow to describe new regions other than OOCRs and BCCRs already known in literature. The heterogeneous distribution of PVs and their low frequency in TNBC patients could reflect the genetic heterogeneity of Sicilian population, maybe determined by the colonization by many and different peoples during history.

Knowledge about mutational background underlying the phenotype of each tumor can have not only prognostic but also therapeutic implications.

Systemic therapies are usually chosen through few well-established biomarkers of therapeutic response as ER, PR expression and HER2 overexpression.

In TNBC, that do not present these biomarkers, chemotherapy is the standard treatment and typically involves the use of anthracycline and taxane, thought in *BRCA*-positive platinum-based agents and PARP inhibitors showed a peculiar efficacy.

We hypothesized that the genetic heterogeneity could be present also in TNBC *BRCA1/2*-related, defining the phenotype of BCs associated with PVs. This could make them possible candidates to different treatment choices.

In our analysis group, patients with c.514del in *BRCA1* gene exhibited a less chemosensitivity than those harbouring c.3904G>T and c.5266dup both in *BRCA1* gene, this analysis, however, shows some potential limitations. Our research suggests that chemosensitivity in TNBC patients may largely vary in the same molecular phenotype of the tumor and should be interpreted carefully due to the limited number of patients analyzed.

Analysis on 345 patients with OC revealed that 85 (24.6%) presented alterations in *BRCA1/2* genes. In detail, we showed that the majority were *BRCA1*-positive (65.9%) and 34.1% showed a PVs/LPVs in *BRCA2* gene. About of distribution on both genes, we did not note a specific cluster region. However, in *BRCA1* gene, we registered a high prevalence of two possible Sicilian founder mutation named c.4964_4982del and c.514del described in 8 and 5 probands respectively. An important observation was the presence of variant called c.4963T>G in *BRCA1* gene revealed in 3 probands, and that its possible considered as pathogenic because cause a substitution of one highly conserved Serine residue in position 1655 with an Alanine.

In *BRCA2* gene, we have not observed a prominent variant among OC patients. By focusing on *BRCA1/2* variant type, we noted a predominance of frameshift mutations either in *BRCA1* and in *BRCA2* gene which remain the most common type of variants noted.

About of clinicopathological characteristics of OC patients, we observe a statistically significant difference based on median age at diagnosis that was 56 versus 58 years in *BRCA1/2* carriers and no-carriers respectively (p=0.02). In our analysis group, like as in literature, OC develops in the sixth decade of life indeed the most prevalence of patients arises in 51-60 years and 61-70 years zones.

Regarding cancer site, the most prevalent was ovarian carcinoma in 82.3% and 63.5% of *BRCA1/2* carriers and no-carriers respectively. An important result observed in this study was that the 34.1% of patients with bilateral ovarian carcinoma showed a PVs/LPVs in *BRCA1/2* genes, while only 8% of patients without variants showed a bilateral ovarian carcinoma (p<0.00001). Moreover, the 57.8% of *BRCA1/2* patients showed a FIGO stage III in comparison of 13.8% without alteration (p=0.02). At the end, the 17.6% of *BRCA1/2* carriers showed a personal BC history before OC, while only 6.5% of patients *BRCA1/2* no-carriers showed a previous personal BC history (p=0.007).

Successively, we investigated 192 HBOC patients with strong personal and/or family history resulted *BRCA1/2* negative selected by the consulting of multidisciplinary team. Analysis were conducted by multi-gene panel testing to understand if these patients harboured a PVs/LPVs in high- and moderate-risk genes involved in hereditary cancer syndrome. We revealed 28 (14.6%) patients with a PVs/LPVs in high- and moderate-risk genes. In particular, we analyzed 165 BC patients of which 24 (14.5%) with a PVs/LPVs and 27 OC patients of which 4 (14.9%) presented a PVs/LPVs in genes beyond *BRCA1/2*. These results are consistent with the literature review.

Interestingly, patients that presented PVs/LPVs in genes over *BRCA1/2* develop a BC or OC before patients with *BRCA1/2* PVs/LPVs (median: 50 vs 43 years)

In particular, we observed that variant named c.1145G>A in *MUTYH* gene were presented in 7 patients analyzed, 5 BC and 2 OC. In our study, this variant was reported in heterozygous condition and it is located in the 8-oxo-G binding site within the Nudix domain [131]. *In silico* studies supports a damaging impact on protein product, therefore it is classified as pathogenic. In literature has been noted that in homozygous and compound heterozygous state, it is linked to MUTYH-associated colon polyposis (MAP) syndrome and colorectal cancer.

In BC patients, a possible correlation between BBC and variant c.1229del, also known c.1100del, in *CHEK2* gene was noted. This variant was present in two patients, and both had a BBC and were Luminal B and HER2 negative. This correlation was also observed in our recently published work, where considered PV was showed in 5 patients with BBC [8].

Furthermore, 11 out of 24 (45.8%) BBC patients analyzed in this study revealed a PVs/LPVs in genes beyond *BRCA1/2*. Besides c.1229del in *CHEK2* gene, we noted alterations in 6 different genes as *PALB2*, *ATM*, *RAD51C*, *MUTYH* and *MSH2*; therefore, it is possible hypothesize a strongly association between BBC and genes involved in HR pathway.

By focusing on 27 OC patients analyzed with multi-gene panel approach, we have noted that 4 (14.8%) patients have PVs/LPVs in genes over *BRCA1/2* distributed in three different genes as *MUTYH*, *ATM* and *PMS2*. Comparing baseline and clinical-pathological characteristic, we did not note an important and statistically significant difference among three analyzed group probably because the number of patients examined was low. Therefore, a future prospective will be to implement this study by increasing collected data.

Investigating baseline and clinical-pathological characteristic of BC patients studied by multi-gene panel, our analysis revealed that patients with PVs/LPVs in genes no-*BRCA* develop a BC before than *BRCA*-carriers and all *wt* with a median age at diagnosis of 32 years vs 41 and 44 years respectively. Carriers of PVs/LPVs in genes over *BRCA1/2* had a reduced probability of developing a TNBC in comparison to *BRCA*-carriers and all *wt* which instead were often luminal-like. In relation at these results, subjects with PVs/LPVs in other genes over *BRCA1/2* had a tumor with a lower proliferation rate, a lower nuclear grade and did not had an involvement of axillary nodal assuming that these patients could be develop a BC less aggressive. In group of no-*BRCA* carriers, we noted a prevalence of BBC (45.8%) than *BRCA1/2* (22.9%) carrier and all wt patients (20.5%), tending to develop a 1st and 2nd BC later than other groups with a median time between two tumors much longer than all wt and similar than *BRCA1/2*-carriers.

In conclusion, in this study we highlight that *BRCA1*-related tumors have frequently a profile which seem like the TNBC subtype, while *BRCA2*-related tumors have a profile similar to luminal-like breast cancers, specifically the luminal B tumor subtypes. Moreover, the pathogenic variants discovered in TNBC patients were not widely overlapping with those detected in Luminal-like tumors, although no evident association between specific *BRCA1/2* PVs and TNBC or luminal-like tumors was noted.

On 85 OC patients with PVs/LPVs in *BRCA1/2* genes, we have found a high prevalence of two probably Sicilian founder mutations both in *BRCA1* gene, c.4964_4982del and c.514del in 8 probands and in 5 probands respectively. By focusing on baseline clinical characteristic, we observe that the 34.1% of patients with bilateral ovarian carcinoma showed a PVs/LPVs in *BRCA1/2* genes and that the 17.6% of *BRCA1/2* carriers showed a personal BC history before OC.

Regarding the 192 patients with a strong personal and/or family history of BC and/or OC resulted *BRCA1/2* negative analyzed with multi-gene panel testing, the data showed that 14.6% of these patients presented PVs/LPVs in genes over *BRCA1/2*.

These findings highlighted the importance of multi-gene panel testing, which should be extended to all patients with a strong personal and/or family history of BC and/or OC who are resulted *BRCA1/2*-negative, with the aim to offer specific risk-reducing measures and free prevention programs for patients and their families.

CHAPTER 5

Tables and Figures

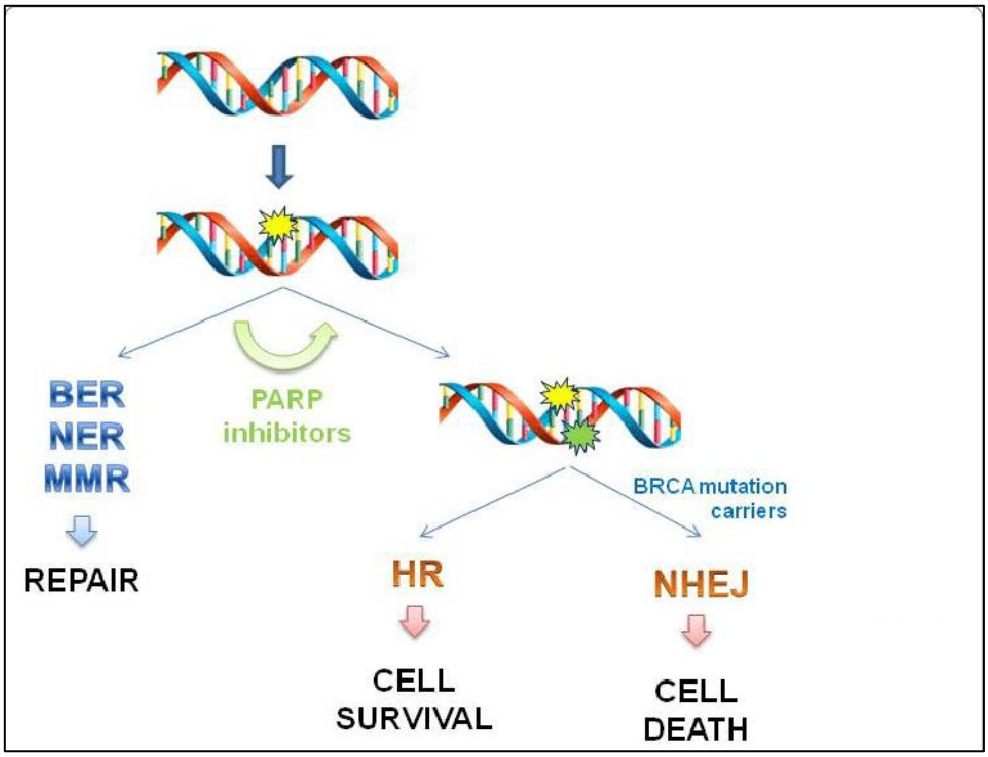

Figure 1. ***Synthetic lethality mechanisms****. When a single-strand breaks occurs, the repair is completed by BER, NER and MMR. If BER is compromised, through the inhibition of PARP, single strand breaks become double strand breaks. In patients with HRD, such as a BRCA-carriers, this damage causes the cancer cell death by activation of NHEJ. Toss A, Cortesi L (2013) Molecular Mechanisms of PARP Inhibitors in BRCA-related Ovarian Cancer. J Cancer Sci Ther 5: 409-416. doi:10.4172/1948-5956.1000234*

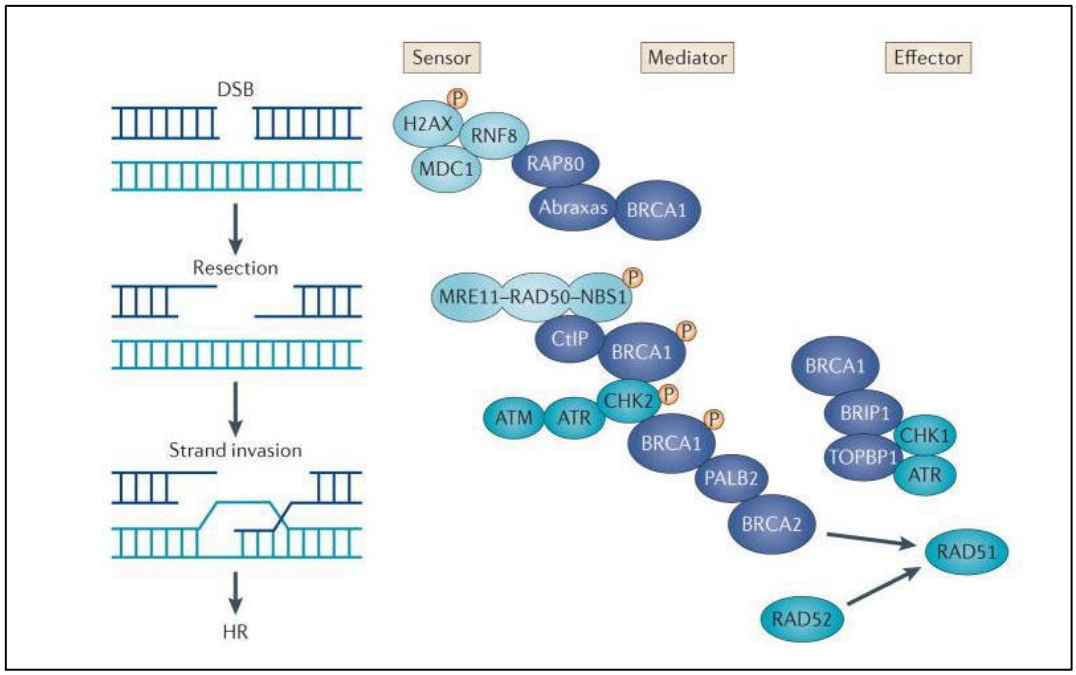

Figure 2. ***Molecular mechanisms of the DNA damage response*** *Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer. 2011 Dec 23;12(1):68-78. doi: 10.1038/nrc3181. PMID: 22193408; PMCID: PMC4972490*

Table 2. BRCA1/2 PV detection rate in Luminal A, Luminal B, HER2-enriched and TNBC patients.

	Total	***BRCA1***	***BRCA2***	**DH *BRCA1-BRCA2***	**No PVs**	**p value ***
Luminal A	125	0 (0%)	6 (4.8%)	0 (0%)	119 (95.2%)	p=0.213
Luminal B	223	10 (4.5%)	29 (13%)	1 (0.5%)	183 (82%)	p= 0.0014
HER2E	29	1 (3.4%)	2 (6.8%)	0 (0%)	26 (89.8%)	p=1.00
TNBC	154	28 (18.2%)	6 (3.9%)	0 (0%)	120 (77.9%)	p= 0.0001

*Comparison BRCA1 PVs vs BRCA2 PVs vs BRCA1/2 wt. DH= Double Heterozygosity; HER2E=Her2-enriched; TNBC=Triple Negative Breast Cancer

Table 3. BRCA1/2 pathogenic variants in TNBCs

Gene	Variant Type	HGVS Nomenclature	Protein change	No. Probands
BRCA1	Deletion	c.514del	p.Gln172fs	5 (14.5%)
BRCA1	SNV	c.3904G>T	p.Glu1302Ter	3 (9.1%)
BRCA1	Duplication	c.5266dupC	p.Gln1756Profs	3 (9.1%)
BRCA1	Deletion	c.4964_4982del	p.Ser1655fs	2 (6%)
BRCA1	SNV	c.3400G>T	p.Glu1134Ter	2 (6%)
BRCA1	Deletion	c.798_799del	p.Ser267fs	1 (2.9%)
BRCA1	Deletion	c.1360_1361del	p.Glu453_Ser454insTer	1 (2.9%)
BRCA1	Deletion	c.3228_3229del	p.Gly1077fs	1 (2.9%)
BRCA1	Deletion	c.1531del	/	1 (2.9%)
BRCA1	Deletion	c.5030_5033del	p.Thr1677fs	1 (2.9%)
BRCA1	Duplication	c.66dupA	p.Glu23Argfs	1 (2.9%)
BRCA1	SNV	c.5123C>A	p.Ala1708Glu	1 (2.9%)
BRCA1	Deletion	c.3266del	p.Leu1089fs	1 (2.9%)
BRCA1	Deletion	c.3599_3600del	p.Gln1200Argfs	1 (2.9%)
BRCA1	Deletion	c.882del	p.Asp295fs	1 (2.9%)
BRCA1	SNV	c.2722G>T	p.Glu908Ter	1 (2.9%)
BRCA1	Deletion	c.66_67del	p.Glu23fs	1 (2.9%)
BRCA1	LGR	c.-232_4675del	/	1 (2.9%)
BRCA2	Deletion	c.5851_5854del	p.Ser1951fs	2 (6%)
BRCA2	SNV	c.8954-15T>G	/	1 (2.9%)
BRCA2	Deletion	c.1238del	p.Leu413fs	1 (2.9%)
BRCA2	Deletion	c.9455_9456del	p.Glu3152fs	1 (2.9%)
BRCA2	Deletion	c.6082_6086del	p.Glu2028fs	1 (2.9%)

Table 4. BRCA1/2 pathogenic variants in Luminal-like and HER2E patients

Gene	Variant Type	HGVS Nomenclature	Protein change	No. probands
Luminal B				
BRCA1	Deletion	c.4964_4982del	p.Ser1655fs	3 (7.5%)
BRCA1	Deletion	c.514del	p.Gln172fs	2 (5%)
BRCA1	SNV	c.181T>G*	p.Cys61Gly	1 (2.4%)
BRCA1	SNV	c.2722G>T	p.Glu908Ter	1 (2.4%)
BRCA1	SNV	c.5096G>A	p.Arg1699Gln	1 (2.4%)
BRCA1	Deletion	c.3228_3229del	p.Gly1077fs	1 (2.4%)
BRCA1	Deletion	c.66_67del	p.Glu23fs	1 (2.4%)
BRCA1	SNV	c.3904G>T	p.Glu1302Ter	1 (2.4%)
BRCA2	Deletion	c.1238del	p.Leu413fs	8 (19.5%)
BRCA2	Deletion	c.9026_9030del	p.Tyr3009fs	2 (5%)
BRCA2	Deletion	c.6082_6086del	p.Glu2028fs	2 (5%)
BRCA2	SNV	c.476-2A>G	/	2 (5%)
BRCA2	Duplication	c.9253dup	p.Thr3085Asnfs	2 (5%)
BRCA2	SNV	c.8331+2T>C*	/	1 (2.4%)
BRCA2	SNV	c.631G>A	p.Val211Ile	1 (2.4%)
BRCA2	Deletion	c.5851_5854del	p.Ser1951fs	1 (2.4%)
BRCA2	SNV	c.8754+4A>G	/	1 (2.4%)
BRCA2	SNV	c.8632+2T>C	/	1 (2.4%)
BRCA2	SNV	c.6124C>T	p.Gln2042Ter	1 (2.4%)
BRCA2	SNV	c.7681C>T	p.Gln2561Ter	1 (2.4%)
BRCA2	Deletion	c.2808_2811del	p.Ala938Profs	1 (2.4%)
BRCA2	Duplication	c.1842dup	p.Asn615Terfs	1 (2.4%)
BRCA2	SNV	c.7007G>A	p.Arg2336His	1 (2.4%)
BRCA2	Deletion	c.1472del	p.Thr491Ilefs18	1 (2.4%)
BRCA2	SNV	c.396T>A	p.Cys132Ter	1 (2.4%)
BRCA2	Deletion	c.5595_5596del	p.Phe1866fs	1 (2.4%)
BRCA2	SNV	c.8487+1G>A	/	1 (2.4%)
Luminal A				
BRCA2	SNV	c.631G>A	p.Val211Ile	1 (16.67%)

BRCA2	SNV	c.8487+1G>A	/	1 (16.67%)
BRCA2	SNV	c.93G>A	p.Trp31Ter	1 (16.67%)
BRCA2	SNV	c.7007G>A	p.Arg2336His	1 (16.67%)
BRCA2	Duplication	c.5073dup	p.Trp1692Metfs	1 (16.67%)
BRCA2	SNV	c.8754+4A>G	/	1 (16.67%)
HER2E				
BRCA1	Duplication	c.5266dupC	p.Gln1756Profs	1 (33.3%)
BRCA2	Deletion	c.5073del	p.Lys1691fs	1 (33.3%)
BRCA2	Deletion	c.7679-7680del	p.Phe2560fs	1 (33.3%)

*These PVs are present in one proband showing double heterozygosity for *BRCA1* and *BRCA2* PVs.

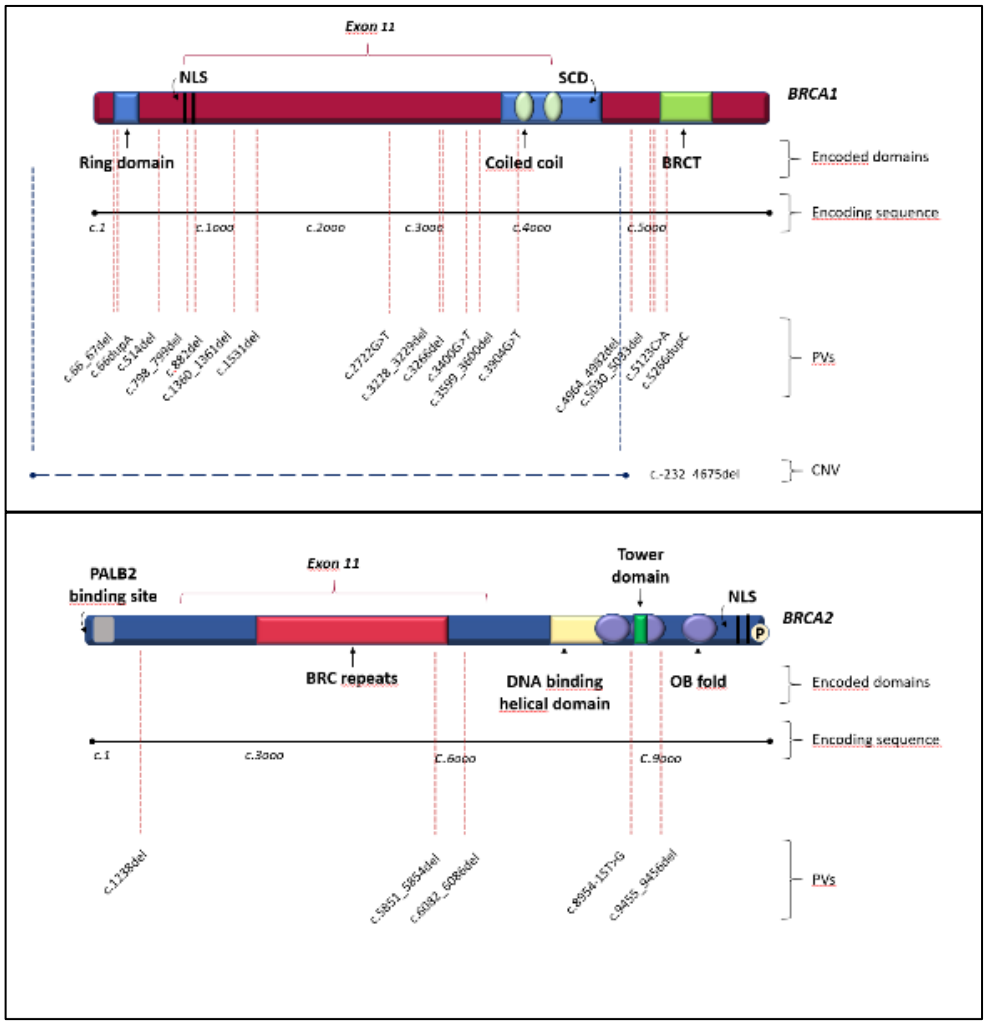

Figure 3. ***Gene location of BRCA1/2 PVs in TNBC patients****. Abbreviations: BRCT, BRCA1 C-terminus domain; CNV, Copy Number Variant; NLS, Nuclear Localization Sequence; OB, Oligonucleotide Binding; PV, Pathogenic Variant; SCD, Serine Cluster Domain*

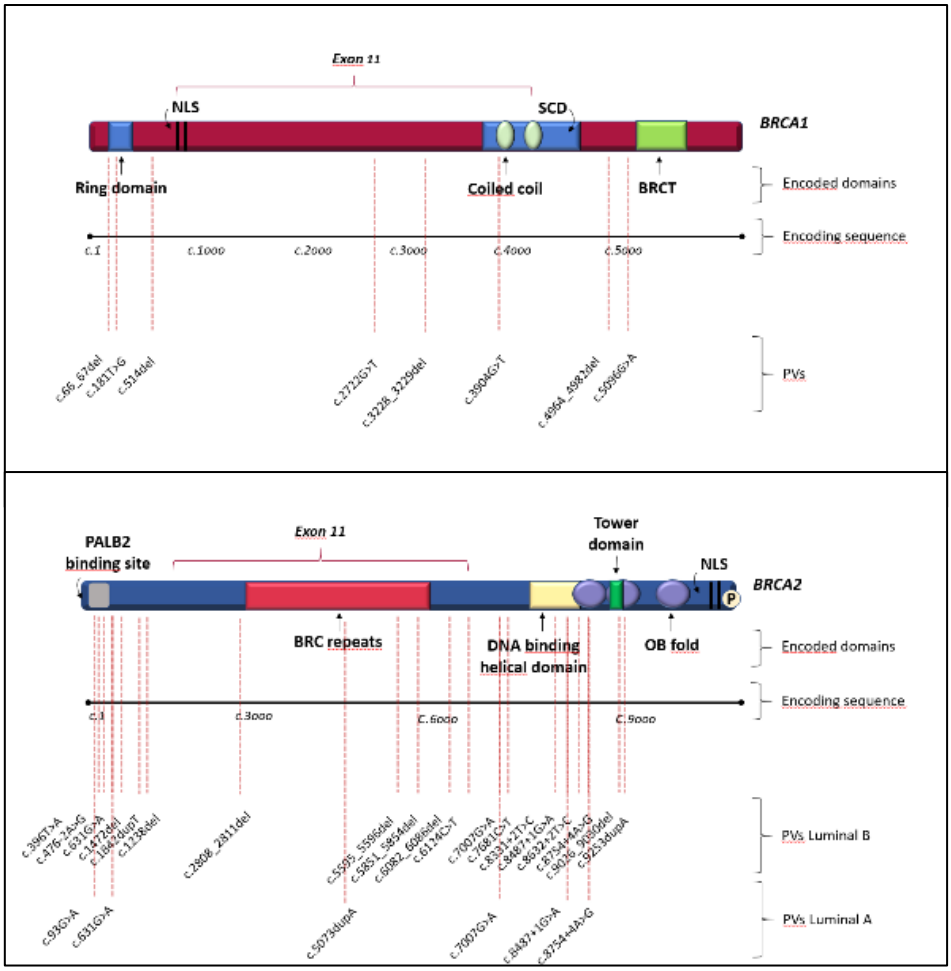

Figure 4. ***Gene location of BRCA1/2 PVs in luminal-like BC patients****. Abbreviations: BRCT, BRCA1 C-terminus domain; NLS, Nuclear Localization Sequence; OB, Oligonucleotide Binding; PV, Pathogenic Variant; SCD, Serine Cluster Domain*

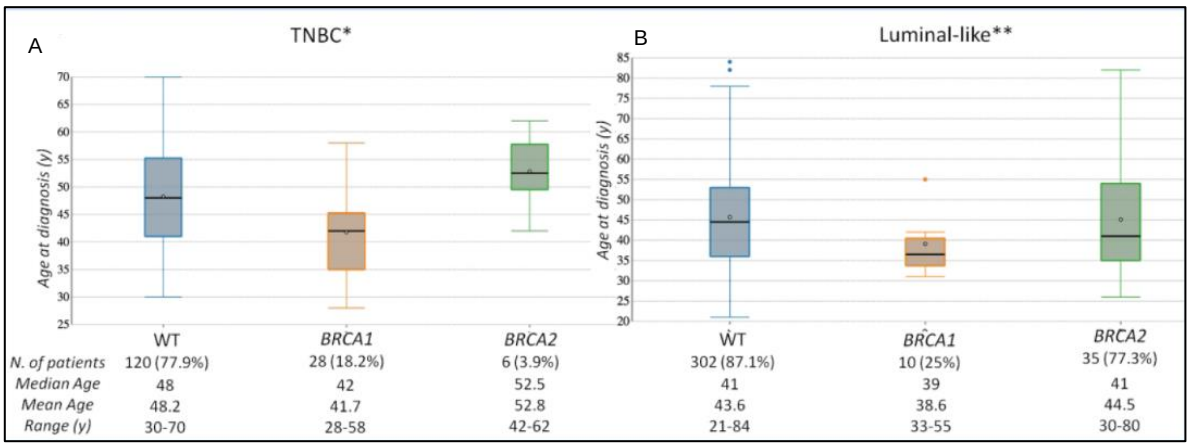

Figure 5. ***Boxplots showing difference in age at diagnosis among women without BRCA1/2 PVs versus women with BRCA1 or BRCA2 PV****. (a) TNBC subgroup; (b) Luminal-like BC subgroup.*WT vs BRCA1 p<0.001; WT vs BRCA2 p=0.26; BRCA1 vs BRCA2 p<0.001; **WT vs BRCA1 p=0.053; WT vs BRCA2 p=0.94; BRCA1Vs BRCA2 p=0.78*

Table 5. Baseline characteristics and clinico-pathological information for of Triple-Negative and Luminal-like BC patients

	TNBC*		Luminal-like**		*P value	**P value
	WT	***BRCA1/2***	**WT**	***BRCA1/2***		
Number of patients (502)	120 (77.9%)	34 (22.1%)	302 (86.8%)	46 (13.2%)	-	-
Age at diagnosis (y): Median Mean Range	 48 48.25 30-70	 43 43.7 28-62	 41 43.6 21-84	 40 42.8 30-80	0.013	0.308
Age groups (y) ≤40 41-50 51-60 >60	 30 (25%) 43 (35.8%) 36 (30%) 11 (9.2%)	 12 (35.3%) 16 (47.1%) 5 (14.7%) 1 (2.9%)	 140 (46.3%) 86 (28.4%) 49 (16.2%) 27 (9.1%)	 27 (58%) 9 (20%) 6 (13%) 4 (9%)	0.135	0.580
Histological Subtype Ductal Lobular Others unknown	 119 (99.2%) 0 (%) 1 (0.8%) \	 33 (97%) 0 (%) 1 (3%) \	 237 (78.5%) 32 (10.6%) 30 (9.9%) 3 (1%)	 36 (78.2 %) 7 (15.2%) 3 (6.6%) \	0.337	0.700
ER (%) ≤20 >20 unknown	\	\	 14 (4.6%) 267 (88.5%) 21 (6.9%)	 8 (17.4%) 34 (73.9%) 4 (8.7%)	-	0.001
PR (%) ≤20 >20 unknown	\	\	 60 (19.9%) 211 (69.9%) 31 (10.2%)	 19 (41.3%) 23 (50%) 4 (8.7%)	-	0.007
HER2 (%) pos neg unknown	\	\	 63 (20.9%) 220 (72.8%) 19 (6.3%)	 4 (8.7%) 42 (91.3%) /	-	0.048
Ki-67 (%) <20 20-50 >50 unknown	 10 (8.3%) 34 (28.4%) 76 (63.3%) \	 2 (5.9%) 9 (26.5%) 23 (67.6) \	 105 (34.8%) 120 (39.7%) 39 (12.9%) 38 (12.6%)	 6 (13%) 21 (45.7%) 15 (32.6%) 4 (8.7%)	0.854	<0.001
Histological grade G1 G2 G3 unknown	 4 (3.3) 18 (15%) 98 (81.7%) \	 1 (2.9%) 4 (11.8%) 29 (85.3%) \	 43 (14.3%) 141 (46.7%) 84 (27.8%) 34 (11.2%)	 1 (2.2%) 14 (30.4%) 26 (56.5%) 5 (10.9%)	0.882	<0.001
Tumor size (T) T1 T2 T3 T4 unknown	 74 (61.7%) 34 (28.3%) 10 (8.3%) 2 (1.7%) \	 18 (53%) 12 (35.3%) 3 (8.8%) 1 (2.9%) \	 142 (47.1%) 77 (25.6%) 5 (1.7%) 4 (1.4%) 73 (24.2%)	 19 (41.3%) 10 (21.7%) / 1 (2.2%) 16 (34.8%)	0.802	0.920
Axillary nodal Involvement (N) N0 N1 N2 N3 unknown	 88 (73.3%) 22 (18.3%) 8 (6.7%) 2 (1.7%) \	 13 (38.2%) 14 (41.3%) 6 (17.6%) 1 (2.9%) \	 126 (41.7%) 66 (21.9%) 13 (4.3%) 4 (1.3%) 93 (30.8%)	 16 (34.8%) 10 (21.7%) 2 (4.4%) 4 (8.7%) 14 (30.4%)	0.002	0.016
Bilateral Yes No	 12 (10%) 108 (90%)	 5 (14.7%) 29 (85.3%)	 66 (21.8%) 236 (78.2%)	 13 (28.2%) 33 (71.8%)	0.439	0.425
Median Age at diagnosis (y) Primary tumor Secondary tumor	 48 56	 40 50	 48 53	 41 52	0.033	0.0474
Time between 1st and 2nd Tumors (y) Median	 6.5	 10	 3	 4	0.389	0.465

*Comparison TNBC WT versus BRCA1/2; **Comparison Luminal-like WT versus BRCA1/2

Table 6. BRCA1/2 pathogenic variants in OC patients

Gene	Variant Type	HGVS Nomenclature	Protein change	No patients	Variant Nature
BRCA1	fs	c.4964_4982del	p.Ser1655fs	8 (9.4%)	G
BRCA1	fs	c.514del	p.Gln172fs	5 (5.9%)	G
BRCA1	M	c.181T>G	p.Cys61Gly	4 (4.8%)	G
BRCA1	M	c.4963T>G	p.Ser1655Ala	3 (3.6%)	G
BRCA1	fs	c.1356_1357AG[2]	p.Glu453_Ser454insTer	3 (3.6%)	G
BRCA1	fs	c.5266dupC	p.Gln1756Profs	3 (3.6%)	G
BRCA1	fs	c.3226_3227AG	p.Gly1077fs	2 (2.3%)	G
BRCA1	fs	c.3253dupA	p.Arg1085Lysfs	2 (2.3%)	G
BRCA1	NS	c.3904G>T	p.Glu1302Ter	2 (2.3%)	G
BRCA1	fs	c.115_116TG[1]	p.Cys39_Asp40delinsTer	2 (2.3%)	G
BRCA1	IVS	c.547+2T>A		2 (2.3%)	G
BRCA1	NS	c.3544C>T	p.Gln1182Ter	1 (1.2%)	G
BRCA1	fs	c.3695_3699GTAAA[1]	p.Val1234fs	1 (1.2%)	G
BRCA1	NS	c.4117G>T	p.Glu1373Ter	1 (1.2%)	G
BRCA1	NS	c.4327C>T	p.Arg1443Ter	2 (2.3%)	G
BRCA1	IVS	c.5406+5G>C		1 (1.2%)	G
BRCA1	fs	c.1029_1030insT	p.Ala344Ter	1 (1.2%)	G
BRCA1	NS	c.5297T>G	p.Ile1766Ter	1 (1.2%)	G
BRCA1	NS	c.3400G>T	p.Glu1134Ter	1 (1.2%)	G
BRCA1	M	c.65T>C	p.Leu22Ser	1 (1.2%)	G
BRCA1	fs	c.984_985insC	p.Asn329fs	1 (1.2%)	G
BRCA1	fs	c.2269delG	p.Val757Phefs	2 (2.3%)	S
BRCA1	NS	c.4576G>T	p.Glu1526Ter	2 (2.3%)	S
BRCA1	NS	c.2059C>T	p.Gln687Ter	1 (1.2%)	S
BRCA1	M	c.5252G>C	p.Arg1751Gln	1 (1.2%)	S
BRCA1	fs	c.1674del	p.Gly559fs	1 (1.2%)	S
BRCA1	fs	c.4891dupA	p.Ser1631fs	1 (1.2%)	S
BRCA1	fs	c.2292_2293AG	p.Glu765_Ser766insTer	1 (1.2%)	S
BRCA2	M	c.631G>A	p.Val211Ile	2 (2.3%)	G
BRCA2	fs	c.6082_6086del	p.Glu2028fs	2 (2.3%)	G
BRCA2	IVS	c.1909+1G>A		2 (2.3%)	G
BRCA2	fs	c.2808_2811del	p.Ala938Profs	2 (2.3%)	G
BRCA2	NS	c.3158T>G	p.Leu1053Ter	2 (2.3%)	G
BRCA2	fs	c.5851_5854del	p.Ser1951fs	2 (2.3%)	G
BRCA2	fs	c.6323_6324GT	p.Arg2108_Val2109insTer	1 (1.2%)	G

BRCA2	NS	c.5959C>T	p.Gln1987Ter	1 (1.2%)	G
BRCA2	fs	c.4284dup	p.Gln1429fs	1 (1.2%)	G
BRCA2	fs	c.5073dupA	p.Trp1692Metfs	1 (1.2%)	G
BRCA2	fs	c.5158dupT	p.Ser1720Phefs	1 (1.2%)	G
BRCA2	fs	c.5701_5714del	p.Ser1900_Glu1901insTer	1 (1.2%)	G
BRCA2	fs	c.6482_6485ACAA[1]	p.Lys2162fs	1 (1.2%)	G
BRCA2	NS	c.7480C>T	p.Arg2494Ter	1 (1.2%)	G
BRCA2	IVS	c.8331+2T>C		1 (1.2%)	G
BRCA2	fs	c.9026_9030del	p.Tyr3009fs	1 (1.2%)	G
BRCA2	fs	c.9253dupA	p.Thr3085Asnfs	1 (1.2%)	G
BRCA2	NS	c.6037A>T	p.Lys2013Ter	1 (1.2%)	G
BRCA2	NS	c.3883C>T	p.Gln1295Ter	1 (1.2%)	S
BRCA2	Syn	c.9117G>A	p.Pro3039=	1 (1.2%)	S
BRCA2	fs	c.3264delT	p.Gln1089Argfs	1 (1.2%)	S
BRCA2	NS	c.7366C>T	p.Gln2456Ter	1 (1.2%)	S
BRCA2	NS	c.7297C>T	p.Gln2433Ter	1 (1.2%)	S

G= Germline, S=Somatic

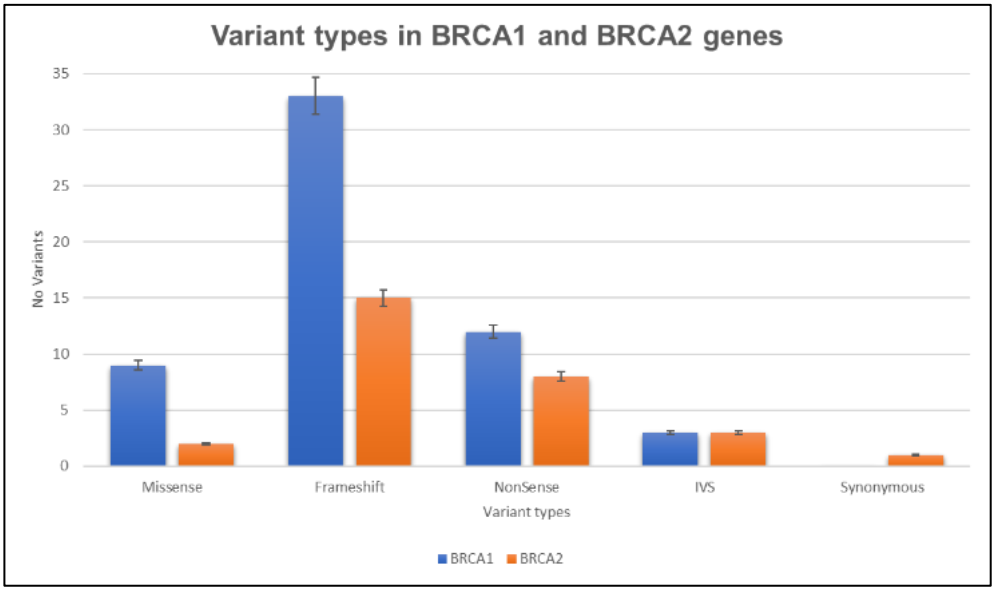

Figure 6. ***Distribution in variant types of PVs/LPVs in BRCA1 and BRCA2 genes****. IVS (Intonic Variant Sequences)*

Table 7. Baseline characteristics and clinico-pathological information of OC patients

	BRCA1/2 wt	BRCA1/2	*p value*
Number of patients: 345	260 (75.4%)	85 (24.6%)	***/***
Age at diagnosis (y): Median Mean Range	 58 58.6 24-86	 56 57.9 37-81	0.02
Age groups (y) ≤40 41-50 51-60 61-70 >70	 13 (5%) 37 (14.2%) 100 (38.5%) 66 (25.4%) 44 (16.9%)	 3 (3.5%) 19 (22.4%) 31 (36.5%) 22 (25.8%) 10 (11.8%)	0.39
Cancer site Ovarian carcinoma Bilateral Ovarian carcinoma Fallopian tube carcinoma Primary peritoneal carcinoma	 214 (82.3%) 21 (8%) 1 (0.4%) 24 (9.3%)	 54 (63.5%) 29 (34.1%) 0 (0%) 2 (2.4%)	< 0.00001
FIGO stage I II III IV unknown	 11 (4.3%) 10 (3.8%) 36 (13.8%) 11 (4.3%) 192 (73.8%)	 3 (3.5%) 4 (4.7%) 49 (57.7%) 9 (10.6%) 20 (23.5%)	0.02
Histological Subtype HGSC Clear cell Endometrioid LGSC Papillary Unknown	 178 (68.5%) 5 (1.9%) 24 (9.2%) 1 (0.4%) / 52 (20%)	 68 (80%) 2 (2.4%) 6 (7,1%) 3 (3.5%) / 6 (7%)	0.14
Personal cancer history before EOC Personal breast cancer history Personal others cancer history No cancer history	 17 (6.5%) 7 (2.7%) 236 (90.8%)	 15 (17.6%) 1 (1.2%) 69 (81.2%)	0.007
Surgery Staging Primary cytoreductive Unknown	 31 (11.9%) 114 (43.9%) 115 (44.2%)	 11 (12.9%) 32 (37.6%) 42 (49.4%)	0.5

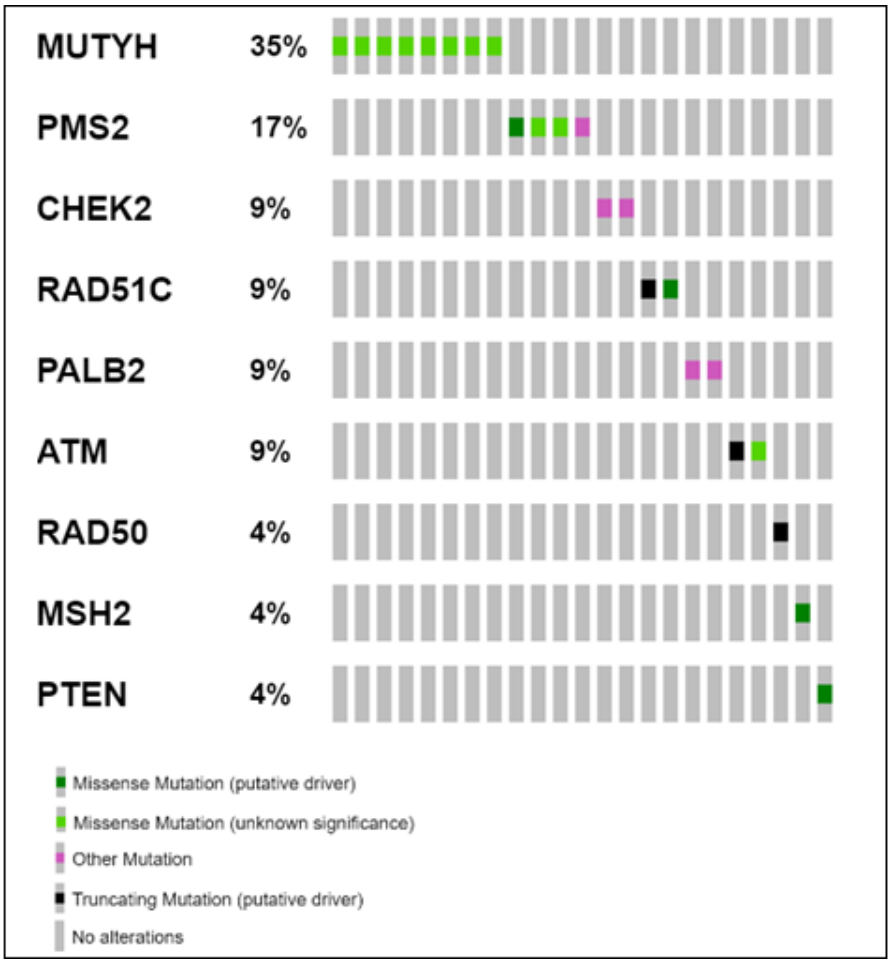

Figure 7. ***Distribution of PVs/LPVs revealed in 28 BC and OC patients analyzed with multi-gene panel testing. cancer patients.*** *The OncoPrint, showing the identified PVs/LPVs by heatmap, was obtained by the informatic tool Mutation Mapper-cBioPortal for Cancer Genomics. The Intronic Variant Sequences (IVS) are not shown*

Table 8.PVs/LPVs showed in patients affected by BC and OC analyzed by Multi-gene panel testing

Gene	Variant Type	HGVS Nomenclature	Protein change	Variant Interpretation	No. patients
Breast Cancer patients					
MUTYH	M	c.1145G>A	p.Gly382Asp	PV	5 (20.6%)
CHEK2	fs	c.1229del	p.Thr410fs	PV	2 (8.2%)
CHEK2	IVS	c.721+3A>T	/	CIP/PV	2 (8.2%)
CDH1	IVS	c.2164+2T>C	/	PV	1 (4.2%)
MSH2	M	c.1045C>G	p.Pro349Ala	CIP/PV	1 (4.2%)
PMS2	fs	c.2182_2184delinsG	p.Thr728Alafs	CIP/PV	1 (4.2%)
PMS2	M	c.137G>T	p.Ser46Ile	LPV	1 (4.2%)
PMS2	M	C.2T>C	p.Met1Thr	PV	1 (4.2%)
RAD51C	IVS	c.1026+5_1026+7del	/	LPV	1 (4.2%)
RAD51C	NS	c.224dup	p.Tyr75Ter	PV	1 (4.2%)
RAD51C	M	c.773G>A	p.Arg258His	LPV	1 (4.2%)
PTEN	M	c.284C>A	p.Pro95Gln	PV	1 (4.2%)
RAD50	NS	c.3598C>T	p.Arg1200Ter	PV	1 (4.2%)
MUTYH	M	c.494A>G	p.Tyr165Cys	PV	1 (4.2%)
PALB2	fs	c.758dup	p.Ser254fs	PV	1 (4.2%)
PALB2	fs	c.1050_1053del	p.Thr351fs	PV	1 (4.2%)
ATM	M	c.8147T>C	p.Val2716Ala	LPV	1 (4.2%)
ATM	NS	c.8818_8821dup	p.Ser2941Ter	PV	1 (4.2%)
Ovarian Cancer patients					
MUTYH	M	c.1145G>A	p.Gly382Asp	PV	2 (50%)
ATM	IVS	c.4776+1G>T	/	LPV	1 (25%)
PMS2	M	c.2249G>A	p.Gly750Asp	LPV	1 (25%)

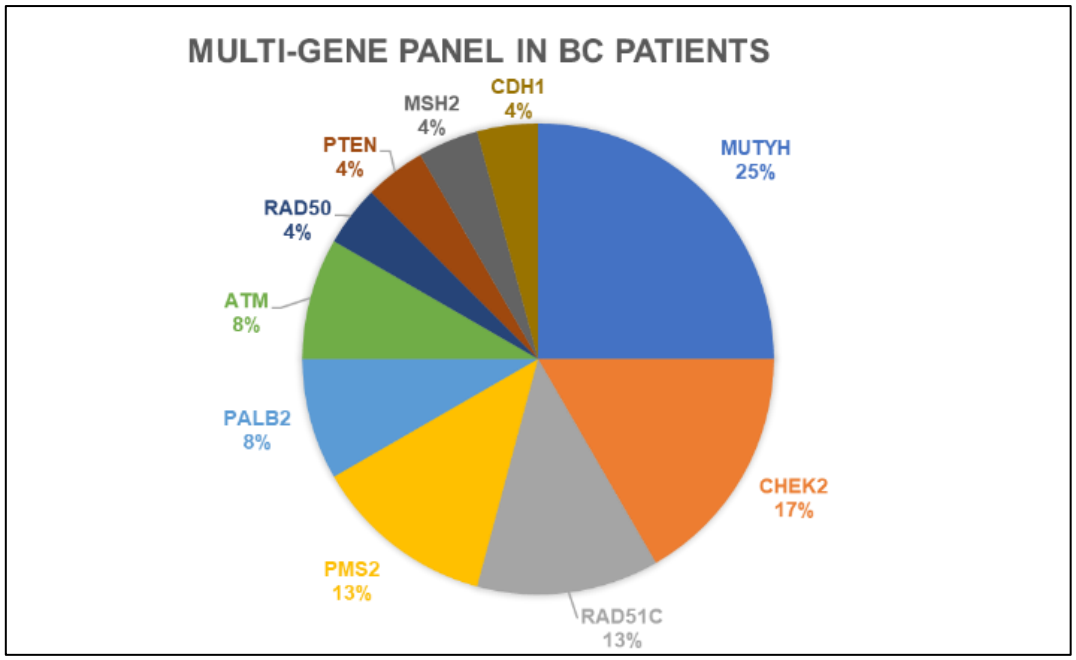

Figure 8. Distribution of genes altered in BC patients evaluated with Multi-gene panel testing

Table 9. Comparison of baseline characteristics and clinico-pathological information of BC patients analyzed to BRCA1/2 and Multi-gene panel testing

	BRCA1/2	Multi-gene Panel MUT	All WT	*p-value**	*p-value***
Number of patients	83	24	589	/	/
Age at diagnosis (y):					
Median	41	32	44	0.8	0.9
Mean	42.6	45.6	44.5		
Range	28-80	27-71	21-84		
Age groups (y)					
≤ 40	40 (48.2%)	9 (37.5%)	241 (40.9%)		
41-50	26 (31.3%)	7 (29.2%)	194 (32.9%)	0.48	0.86
51-60	13 (15.7%)	5 (20.8%)	105 (17.9%)		
> 60	4 (4.8%)	3 (12.5%)	49 (8.3%)		
Histological Subtype					
Ductal	72 (86.8%)	13 (54.2%)	456 (77.4%)		
Lobular	7 (8.4%)	2 (8.3%)	47 (8%)	0.47	0.8
Others	4 (4.8%)	2 (8.3%)	49 (8.3%)		
Unknown	/	7 (29.2%)	37 (6.3%)		
Molecular Subtype					
Luminal	46 (55.4%)	15 (62.5%)	397 (67.4%)		
HER2E	3 (3.7%)	1 (4.2%)	33 (5.6%)	0.12	<0.00001
TNBC	34 (40.9%)	3 (12.5%)	149 (25.3%)		
Unknown	/	5 (20.8%)	10 (1.7%)		
ER (%)					
≤ 20	8 (9.7%)	/	19 (3.2%)		
> 20	34 (40.9%)	16 (66.6%)	355 (60.3%)	0.02	0.5
Negative	37 (44.6%)	4 (16.7%)	165 (28%)		
Unknown	4 (4.8%)	4 (16.7%)	50 (8.5%)		
PR (%)					
≤ 20	19 (22.9%)	2 (8.4%)	76 (12.9%)		
> 20	23 (27.7%)	12 (50%)	286 (48.6%)	0.02	0.6
Negative	37 (44.6%)	5 (20.8%)	165 (28%)		
Unknown	4 (4.8%)	5 (20.8%)	62 (10.5%)		
Ki-67 (%)					
< 20	8 (9.7%)	5 (20.8%)	158 (26.9%)		
20-50	31 (37.3%)	8 (33.3%)	211 (35.8%)	0.003	0.24
> 50	39 (47%)	/	134 (22.7%)		
Unknown	5 (6%)	11 (45.9%)	86 (14.6%)		
Histological grade					
G1	2 (2.4%)	2 (8.4%)	62 (10.5%)		
G2	18 (21.7%)	8 (33.3%)	212 (36%)	0.0008	0.27
G3	58 (69.9%)	3 (12.5%)	226 (38.4%)		
Unknown	5 (6%)	11 (45.8%)	89 (15.1%)		
Tumor size (T)					
T1	38 (45.8%)	8 (33.3%)	261 (44.4%)		
T2	23 (27.7%)	4 (16.7%)	145 (24.6%)	0.9	0.44
T3	3 (3.6%)	/	18 (3%)		
T4	2 (2.4%)	/	6 (1%)		
unknown	17 (20.5%)	12 (50%)	159 (27%)		
Axillary nodal Involvement (N)					
N0	30 (36.1%)	2 (8.3%)	257 (43.7%)		
N1	24 (28.9%)	6 (25%)	108 (18.3%)	0.07	0.002
N2	8 (9.6%)	3 (12.5%)	23 (3.9%)		
N3	5 (6%)	/	9 (1.5%)		
unknown	16 (19.4%)	13 (54.2%)	192 (32.6%)		
Bilateral					
Yes	19 (22.9%)	11 (45.8%)	121 (20.5%)	0.04	0.008
No	64 (77.1%)	13 (54.2%)	468 (79.5%)		
Median Age at diagnosis (y)					
Primary tumor	40	53	46	0.01	0.75
Secondary tumor	50	63	52		
Time between 1st and 2nd Tumors (y)					
Median	8	7	3	0.87	0.09

*comparison BRCA1/2 vs Multi-gene panel MUT; ** comparison Multi-gene panel MUT vs All WT

Table 10.Comparison of baseline characteristics and clinico-pathological information of OC patients analyzed to BRCA1/2 and Multi-gene panel testing

	BRCA1/2	Multi-gene Panel MUT	*All WT*	*p value**	*p value***
Number of patients	85	4	283	**/**	**/**
Age at diagnosis (y):					
Median	56	52.5	58	0.9	0.9
Mean	57.9	55.5	58		
Range	37-81	38-79	28-84		
Age groups (y)					
≤40	3 (3.5%)	1 (25%)	22 (7.8%)	0.45	0.77
41-50	19 (22.4%)	1 (25%)	33 (11.6%)		
51-60	31 (36.5%)	/	112 (39.6%)		
61-70	22 (25.8%)	1 (25%)	72 (25.4%)		
>70	10 (11.8%)	1 (25%)	44 (15.6%)		
Cancer site					
Ovarian carcinoma	54 (63.5%)	4 (100%)	236 (83.4%)	0.24	0.01
Bilateral Ovarian carcinoma	29 (34.1%)	/	21 (7.4%)		
Fallopian tube carcinoma	0 (0%)	/	2 (0.7%)		
Primary peritoneal carcinoma	2 (2.4%)	/	24 (8.5%)		
FIGO stage					
I	3 (3.5%)	/	12 (4.2%)	0.3	0.9
II	4 (4.7%)	1 (25%)	10 (3.5%)		
III	49 (57.7%)	2 (50%)	39 (13.8%)		
IV	9 (10.6%)	/	11 (3.9%)		
unknown	20 (23.5%)	1 (25%)	211 (74.6%)		
Histological Subtype					
HGSC	68 (80%)	3 (75%)	194 (68.6%)	0.2	0.0004
Clear cell	2 (2.4%)	/	6 (2.1%)		
Endometrioid	6 (7,1%)	/	25 (8.8%)		
LGSC	3 (3.5%)	/	2 (0.7%)		
Papillary	3 (3.5%)	1 (25%)	2 (0.7%)		
Unknown	3 (3.5%)	/	54 (19.1%)		
Personal cancer history before EOC					
Personal breast cancer history	15 (17.6%)	2 (50%)	20 (7%)	0.001	0.001
Personal others cancer history	1 (1.2%)	1 (25%)	21 (7.4%)		
No cancer history	69 (81.2%)	1 (25%)	242 (85.6%)		
Surgery					
Staging	11 (12.9%)	2 (50%)	34 (12%)	0.1	0.07
Primary cytoreductive	32 (37.6%)	1 (25%)	126 (44.5%)		
Unknown	42 (49.4%)	1 (25%)	123 (43.5%)		

*Comparison between Multi-gene panel MUT and BRCA1/2; ** Comparison between Multi-gene panel MUT and All WT

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CHAPTER 9

Scientific Products

Scientific products congruent on research project

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