UNIVERSITÀ DEGLI STUDI DI PALERMO
Dottorato di ricerca in Oncologia e Chirurgia Sperimentali
Dipartimento di Discipline Chirurgiche Oncologiche e Stomatologiche (Di.Chir.On.S.)

Agnostic application of a Multigene Panel Testing including tumor susceptibility genes in Breast, Ovarian, Pancreatic and Prostate cancer patients

Doctoral Dissertation of:
Alessia Fiorino

Supervisor:
Prof. B. Vincenzi
Prof. N. Silvestris

Tutor:
Prof.ssa V. Bazan

Cotutor:
Prof.ssa L. Incorvaia

The Chair of the Doctoral Program:
Prof. A. Russo

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Abstract

Background: The pathogenic/likely pathogenic variants (PV/LPVs) into the high susceptibility *BRCA1* and *BRCA2* genes have been identified as conferring predisposition to the hereditary breast cancer (BC) and ovarian cancer (OC), such as pancreatic (PaC) and prostate cancer (PrC). However, several patients with significant personal and/or family history of cancer result negative to genetic testing of *BRCA1/2* genes. Next generation-based (NGS) multi-gene panel testing allows to analyze the molecular bases of hereditary disease investigating also other predisposing genes involved in homologous recombination and/or other pathways crucial in cancer development. Nowadays, the PV/LPVs in *BRCA1/2* genes and in homologous recombination genes assume not only a preventive meaning, but also a predictive significance of PARP inhibitors sensitivity.

Methods: In this observational study, 1878 patients have been selected for genetic testing of the *BRCA1/2* genes. The cohort was composed as follows: 1170 BC, 540 OC, 144 PaC and 24 PrC patients. Moreover, 527 patients were selected to the analysis with the NGS-based multi-gene cancer panel investigating 22 susceptibility genes involved in hereditary cancer syndromes. The type and gene location of each variant have been recorded in order to identify a potential genotype-phenotype correlation.

Results: Overall, 144 (12.3%) BCs harboured germline PV/LPVs in *BRCA1/2* genes; 109 (20.2%) OCs were carriers of germline PV/LPVs, while 19 (14.8%) showed somatic deleterious variants in *BRCA1/2* genes; 3 (2.1%) PaC and one (4.2%) PrC patients harboured germline PV/LPVs in *BRCA1/2* genes. Subsequently, the multi-gene panel analysis score revealed the carriers of PV/LPVs in other susceptibility genes: 65 (15.4%) of the 422 analyzed BC patients, 11 (15.7%) of the 70 analyzed OC patients, 7 (23.3%) of the 30 analyzed PaC patients and none PrC patients. Overall, this study emphasized the involvement of PV/LPVs in the homologous recombination genes in 59.1% of BCs, 54.5% of OCs and 57.1% of PaCs among all the carriers of PV/LPVs in other genes.

Conclusion: Further analysis are needed in specific setting of BC, OC, PaC, and PrC patients resulting negative to *BRCA1/2* genes genetic testing and with significant personal and/or family history of cancer. The molecular scores highlighted the important role of multi-gene panel testing that results crucial to better understand the molecular background of cancers. In future, molecular and clinical data together will lead to a more accurate risk assessment, to the develop of univocal and personalized intensive surveillance programs and/or risk reduction strategies and to develop novel treatment strategies.

Summary

Deep is the knowledge about the effect of pathogenic/likely pathogenic variants into the high susceptibility *BRCA1* and *BRCA2* genes conferring predisposition to the hereditary breast and ovarian cancer, such as pancreatic and prostate cancer. However, there are several affected subjects in families with multiple cases of cancer remain still unclear. Next generation-based multi-gene panel testing should be performed in selected settings of breast, ovarian, pancreatic, and prostate cancer patients resulting negative to genetic testing of *BRCA1/2* genes, and with significant personal and/or family history of cancers. The investigation of other susceptibility genes beyond *BRCA1/2* genes, with high or moderate penetrance, including *TP53, PTEN, STK11, CDH1, ATM, CHEK2, PALB2* genes, involved in homologous recombination and/or in several pathways crucial in hereditary cancer development is the main goal of this research, which assumes a preventive meaning. Moreover, the recent Food and Drugs Administration's and European Medicines Agency's approval of Poly (ADP) Ribose Polymerase inhibitors (PARPi)-based maintenance therapy for specific setting of breast, ovarian, pancreatic, and prostate cancers in carriers of an aberrant alteration of *BRCA1/2* genes and the literature evidence of PARPi sensitivity in patients with homologous recombination deficiency, make this study aimed to the identification of predictive novel biomarker of treatment choice. Furthermore, this study could contribute to define the potential prognostic aspects considering the debated improving on progression free survival and overall survival in patients treated with PARPi. For these goals and the need to develop a more accurate risk assessment and intensive surveillance programs has been conducted this research. This study reveals deleterious variants of genes involved in homologous recombination repair pathway identified in 59.1% of breast, 54.5% of ovarian and 57.1% of pancreatic cancers among the carriers of variants in other genes beyond *BRCA1/2* genes.

CHAPTER 1

Background, Rationale and Objectives

1.1 Background

The pathogenic/likely pathogenic variants (PV/LPVs) into the high susceptibility genes, *BRCA1* and *BRCA2*, have been widely studied and associated to an increased lifetime risk of Hereditary Breast and Ovarian Cancer (HBOC) syndrome, an autosomal dominant inherited disorder including 5–7% and 10–15% of all cases of breast cancer (BC) and ovarian cancer (OC), respectively[1-6]. The *BRCA1/2* genes are involved in the homologous recombination repair (HRR) pathway, and the loss of heterozygosity (LOH) of one of these genes leads to the homologous recombination deficiency (HRD), conferring genomic instability and predisposing to neoplastic disease[7,8]. Over the years, PV/LPVs in *BRCA1/2* genes have been identified also as risk factors to pancreatic cancer (PaC), prostate cancer (PrC) and other neoplastic diseases[9,10], leading the National Comprehensive Cancer Network (NCCN), the American Society of Clinical Oncology (ASCO) and the European Society for Medical Oncology (ESMO) to define clinical guidelines aimed at the management of high-risk patients[11-15]. Moreover, family history-based testing does not consider about a half of carriers harbouring PV/LPVs in HBOC syndrome predisposing genes without suggestive family history[16,17], and strategies to identify these high-risk subjects are under development[11]. To date, clinically validated multi-gene panel testing based on HBOC genes analysis have been offered to subjects with a relevant family history of cancer[11]. Nowadays, genetic test investigating the *BRCA1/2* genes represents a crucial step for patients who meet the eligibility criteria to test and assumes not only a preventive meaning but also a predictive and a potential prognostic value for the clinical management of patients[18-22]. Both germinal and somatic PV/LPVs in *BRCA1/2* genes have been defined as conferring sensitivity to Poly (ADP) Ribose Polymerase inhibitors

(PARPi)-based maintenance treatment, with improving progression free survival (PFS) and overall survival (OS) in several cancer patients[23-25]. The efficacy of PARPi as therapeutic choice for HRD tumors occurs through the “synthetic lethality”[26,27]. Recently, the Food and Drugs Administration (FDA) and the European Medicines Agency (EMA) approved PARPi treatment in the management of specific settings of BC, OC, PaC and PrC patients having a PV in *BRCA1/2* genes, enhancing the request for genetic testing[9,28-32]. Furthermore, HRD tumors have been deeply investigated and aberrant alterations of genes involved in the HRR and/or other pathways involved in differentiation, growth, proliferation, and survival of cancer cells turn out to be preventive for an early diagnosis and risk-reducing strategies, but also predictive of a possible targeted therapy and/or a potential prognostic factor in the clinical management of patients[21,22,33,34]. The cancer’s genetic scenario is increasingly growing, and this emerges from the several families with multiple neoplastic diseases, remain still without a clear genetic background[35]. The PV/LPVs in *BRCA1*, *BRCA2*, *TP53*, *PTEN*, *STK11* and *CDH1* genes lead to a high life-time risk of every single carrier of PV/LPVs compared to a subject belonging to general population, defined relative risk (RR), to develop several cancers[36-41]. Nowadays, the advent of the cost-effective and short-time Next Generation Sequence (NGS) analysis suggests that genetic alterations in other cancer susceptibility genes with different penetrance, high and/or moderate, such as *PALB2*, *BRIP1*, *ATM, CHEK2, BARD1, NBN, NF1, RAD51C, RAD51D* and other genes, confer a different RR to several neoplastic diseases[36,37,42,43]. Recently, findings have shown that the prevalence of inherited PV/LPVs and gene-specific RR estimates could depend on the ethnicity and different geographic location[44,45]. In this context, deeper molecular investigations are needed in BC, OC, PaC, and PrC patients resulting negative to *BRCA1/2* genes genetic testing and with relevant personal and/or family history of cancers[46,47] to make light on the molecular bases of the hereditary cancers and to observe the prevalence and the geographic distribution of inherited PV/LPVs with a multi-gene approach. The NGS molecular data and the clinical information together would result in a more accurate management for each cancer patient and result fundamental for risk assessment to develop univocal and tailored intensive surveillance programs and/or risk reduction strategies[48].

1.2 Breast cancer

BC is the most diagnosed cancer and the leading cause of death among Italian female population, with an increasing incidence trend of +0.3% per year. Thanks to the spread of screening programs and therapeutic advances, mortality is declining sharply (-6% from 2015 to 2020). Nationwide, more than 834.000 women received a diagnosis of BC, amounting to 43% of women with a prior diagnosis of BC and 23% of all prevalent cases. The 5-year survival rates for women with BC is about 87%. The RR of BC is increasing with the age. The incidence curve increases until menopause slowing down and rises again after 60 years old. The RR is multifactorial and the 5-10% of all BCs are hereditary, with a quarter of them associated to the PV/LPVs in the major susceptibility *BRCA1/2* genes and characterized often by contralateral and an early onset[49]. Woman affected by unilateral BC have an increased RR to develop a bilateral breast cancer (BBC)[50,51].

Based on histological features, BCs could be classified into five main molecular subtypes:

- "Luminal A" (LA) (about the 80% of luminal BCs)[52], with marked expression of estrogen receptor (ER) and progesterone receptor (PR) (>20%), lack of expression of the human epidermal growth factor receptor 2 (HER2) and low proliferative activity (Ki-67) (<20%);
- "Luminal B"/HER2 negative (LB/HER2-), with marked expression of ER and PR, lack of expression of HER2, but with high recurrence's risk showing a higher Ki-67 (≥20%) due to high expression of proliferation genes;
- "Luminal B"/HER2 positive (LB/HER2+), with marker expression of ER and PR, overexpression of HER2 or expression, and any value of Ki-67;
- "HER2-enriched" (HER2E) (about 10-15% of all BCs)[52], characterized by the expression or overexpression only of HER2[53];
- "Triple-Negative Breast Cancer" (TNBC) (about 20% of all BCs)[52], disease defined by the lack of expression of hormonal receptors and HER2.

Each type of BC is different in clinical features, with the LA characterized by a better prognosis and the HER2 and TNBC having the worst[49]. About 60–70% of BCs cases occurring in premenopausal women was hormone receptors positive. TNBCs are often related to PV/LPVs in the *BRCA1* gene and a poorly differentiated infiltrating ductal carcinomas (CDI) histological subtype, high Ki-67, high grade, and geographic necrosis, associated with higher mortality and morbidity and treated only with conventional chemotherapy[54,55]. On the other hand, BCs are related to PV/LPVs in the *BRCA2* gene

show a LA or LB subtype, a lobular histological subtype (CLI), with 2/3 grade, ER and PR expression and HER2 absence, similarly to sporadic BCs[56].

Looking beyond therapies, the potential role of platinum-based agents, standard anthracycline- and taxane-based chemotherapy, has emerged in patients with *BRCA1/2* genes deficient BC[57]. In 2018, recent findings lead to the FDA and EMA approval of PARPi-based therapy, with optimal improving in mortality rate, PFS and OS[58-61]. Olaparib was approved for the treatment of HER2- metastatic BC with germline PVs in the *BRCA1/2* genes (OlympiAD study)[23], while Talazoparib was approved for the HER2-advanced or metastatic BC with germline PVs in the *BRCA1/2* genes (EMBRACA study)[62].

1.3 Ovarian cancer

OC represents one of the most common gynaecological tumours, with the highest mortality rate[63,64]. In Italy, the Italian Association of Medical Oncology (AIOM) and Italian Association of Cancer Registries (AIRTUM) have estimated 5.200 new cases in 2020[65]. The most significant risk factor of OC is represented by family history of BC and/or OC due to the *BRCA1/2* genes aberrant alterations identified in almost 25% of all OCs[66]. Population-based studies have shown that OC patients have a prevalence of PV/LPVs in the *BRCA1/2* genes in more than 15% of cases, regardless of age at diagnosis and the presence of family history of BC or OC[6,67]. According to the histological features, OCs are classified in epithelial, representing 90% of all, and non-epithelial origin[68-70]. Among epithelial, 3% of OCs are mucinous and the other are non-mucinous[71]. Non-mucinous are divided in high-grade serous (70%); endometrioid (10%); clear cell (10%); low-grade serous (<5%)[71,72]. Moreover, epithelial cancers are divided in low-grade serous carcinoma (LGSC), unilateral, and cystic cancer[73], and high-grade serous carcinoma (HGSC); the most common OC subtypes are HGSCs, bilateral, and aggressive[74,75]. Epithelial OC is the most frequently diagnosed in advanced stage and with a poor prognosis, often asymptomatic or with unspecific symptoms. The prevalence of the PV/LPVs in the *BRCA1/2* genes is increasing in patients with serous OC (17-20%)[76] and increases up to 23–25% in HGSCs[77]. According to national AIOM guidelines, the *BRCA1/2* genes genetic testing for OC patients must be conducted in all non-mucinous and non-borderline ovarian epithelial carcinoma, fallopian tube carcinoma and primary peritoneal carcinoma[76]. The germline and/or somatic PV/LPVs of the *BRCA1/2* genes have a role as predictive factor of platinum improved sensitivity[78,79]. Over time, researchers demonstrate the improved response to platinum derivates for OC patients carrying a germline alteration in the

BRCA1/2 genes[80]. Several recent studies have reported an improvement in terms of PFS in case of OC treatment with PARPi-based therapy, alone or in combination with other drugs, leading in 2014 to the FDA's and EMA's approval for the treatment of OC[81-83].

Olaparib has been approved for the first time in 2014 for the treatment of advanced OC with germline PVs in the *BRCA1/2* genes (NCT0107662 study)[84]. Since 2014, several studies tested Olaparib alone or in combination with other drugs and in several settings of OC, leading to an increasing and evolving use to PARPi-based therapy in clinical practice, such as the SOLO-2, Study 19, SOLO-1, PAOLA-1, ARIEL2 and Study 10. In 2020, all these findings lead to the Olaparib approval in 2020 by the FDA (PAOLA-1 study) as first-line maintenance therapy of advanced HRD carcinomas in combination with bevacizumab with a complete or partial response to chemotherapy[85]. Rucaparib has been approved for the first time in 2016 by FDA and two years after by EMA for the treatment of advanced OC with germline/somatic PVs in *BRCA1/2* genes, after several chemotherapy treatments (ARIEL2 study and Study 10)[86]. To date, the FDA in 2019 and EMA in 2020, approved the same PARPi as maintenance therapy in recurrent OC, regardless of mutational status, but after response to platinum-based chemotherapy (ARIEL3 study)[87]. Niraparib has been approved for the first time in 2017 by the FDA and EMA for the treatment of recurrent OC, regardless of mutational status, after complete or partial chemotherapy response (ENGOT-OV16/NOVA study)[88]. In 2019, the FDA expanded Niraparib-based treatment to all OCs with HRD (QUADRA study)[89]. In 2020, the FDA approved the Niraparib for the treatment of recurrent OC, independently of mutational status and after complete or partial chemotherapy response (PRIMA study)[90]. Moreover, the major involved genes in OC are considered the HRR genes with a potential as predictive biomarkers of HRD and PARPi-sensitivity, but also the PV/LPVs in MMR genes have been identified in OCs[64,66], suggesting the crucial role of multi-gene panel testing.

1.4 Pancreatic cancer

PaC represents the fourth leading cause of death in women (7%) and the sixth in men (5%). Exocrine PaC is one of the most inauspicious diseases with a 5-year survival of 8.1%[91]. In Italy, 14.300 new diagnosis of PaC have been estimated in 2020 (6.900 men and 7.400 women), according to AIRTUM, and leading to an increasing incidence trend for men. In particular, the incidence trend is growing from the North to the Center of the country (20-29% in males and 12-24% in females). The 10-year survival rate is about 3%. The risk is multifactorial, but up to 10% of PaC patients show familiarity caused by Peutz-

Jeghers syndrome (more than 100-fold increased risk), or the Familial Atypical Multiple Mole Melanoma syndrome (20-30 times), or germline PV/LPVs in the *BRCA2* gene (3-10 times), or hereditary pancreatitis (10 times) and Lynch syndrome[92,93]. Overall, familial PaC affects 5-15% of individuals who develop PaC with early diagnosis, and who often have family history of cancer suggesting a hereditary syndrome[94,95]. Germline PV/LPVs have been identified in about 10-20% of all PaCs. Literature findings showed the predisposition to PaC conferred by *BRCA1*, *BRCA2*, *ATM*, *PALB2* and the debated *CDKN2A* gene. Overall, these and other genes conferring predisposition to BC and/or OC, have been identified as altered in the 3.5% of PaC cases[40,96,97]. The 17-25% of PaC present somatic variants in DNA damage response (DDR) genes, especially if involved in HRR[40,98,99]. Based on histological features, the pancreatic ductal adenocarcinoma (PDAC), is the most diffuse amounting to more than 90% of all cases[100]. Classical treatment strategies, such as chemotherapy and radiotherapy, cannot always, and in all patients, contrast the aggressive progression of PaC. To date, it is known the sensitivity to platinum derivates for PaC with PV/LPVs in the *BRCA1/2* genes. However, researchers are focused on the development of more efficacy targeted therapies and, in the recent years recent findings lead to the FDA's, in 2019, and EMA's, in 2020, approval of Olaparib as a maintenance treatment for germline *BRCA1/2* genes deficient metastatic PDAC that has not progressed on platinum-based chemotherapy (POLO study)[30,101,102].

1.5 Prostate cancer

PrC is the most frequent tumor and the third cause of death among men population in many Western countries. In Italy, PrC is the most frequent malignancy among males and accounts for more than 20% of all cancers diagnosed at the age of 50 and over. In 2020, about 36.074 new cases were expected. According to AIRTUM data, between 2008 and 2016, the incidence increased slightly (+3.4%) in men under 49 years old but remained unchanged in subsequent decades. The incidence shows a North-South gradient. These differences, in addition to the different frequency of PSA as screening test are likely to be explained by the different incidence of possible susceptibility factors and with the different lifestyle. The 5-years survival rate of patients with PrC is currently at 92% after diagnosis, which is significantly increasing because of the progressive spread screening by PSA administration. The etiology of PrC is multifactorial, and only a small subgroup of PrC patients (less than 15%) have a hereditary disease. PrC is associated with HBOC and Lynch syndrome[103]. Fortunately, PrC is often low-risk and/or diagnosed at an early stage,

even if a subset of PrC is characterized by an aggressive course. The first-line treatment used for PrC is based on the androgen-blocking agents, but most PrC develop resistance to these. The androgen-resistant metastatic PrC (mCPRC) has the worst prognosis, with a survival-rate of less than 2 years[40,104]. Moreover, mCRPCs could have somatic alterations in genes involved in DDR, about 23% of cases, while 10% have germline PV/LPVs and more than 50% had a LOH[105-107]. In 2019, FDA approved the use of Rucaparib for mCPRC treatment having *BRCA1/2* genetic variants after androgen receptor-directed therapy and a taxane-based chemotherapy, and lately for all HRD tumors (PROfound study)[108,109]. However, in 2020, the EMA's Olaparib approval is limited to *BRCA1/2* genes deficient patients affected by mCPRC[110].

1.6 Onco-genetic counselling

Patients who meet the eligibility criteria for *BRCA1/2* genes genetic testing (**Table 1**) are selected through the onco-genetic counselling based on the evaluation of patient's personal and family history, according to the guidelines of the Italian Association of Medical Oncology (AIOM). The evaluation is performed by a multidisciplinary group of experts consisting of medical oncologists, geneticists, molecular biologists, and onco-psychologists. The criteria are as follows: number of affected relatives, type of neoplasm, multiple primary tumors, age at diagnosis, sex, histological, immunohistochemical and molecular characteristics of the neoplastic disease. The variables are useful to identify an increased chance of finding a PV/LPV for >10-fold compared with the estimated prevalence in the general population. The identification of a PV/LPV in *BRCA1/2* genes assumes a preventive role, and a potential predictive meaning for the treatment choice for patients, but also for the first-degree relatives who access genetic "cascade" counselling for a surveillance program, such as screening for an early diagnosis and risk-reducing strategies. Recently, the *BRCA1/2* genes genetic testing has been extended to metastatic settings of PDAC and mCPRC[93].

The outcomes of the test will be:

-informative: the PV/LPV is identified;

-non-informative: the PV/LPV is not identified but the presence of an aberrant variants of other susceptibility genes cannot be excluded, or a Variant of Unknown Significance (VUS) is identified;

-negative: in case of lack of identification of a specific alteration investigated in a family member previously identified in a related proband.

1.7 High penetrance genes

1.7.1 *BRCA1* and *BRCA2*

The onco-suppressor *BRCA1* and *BRCA2* genes have been discovered by Hall et al. in 1994 and Wooster et al. in 1995, respectively, as maintaining chromosomal stability[2,3]. The BRCA1/2 proteins play a crucial role in DNA double-strand breaks (DSBs) repairing in the HRR pathway[4,111-113]. Inherited biallelic PV/PVs in *BRCA1/2* genes have been identified in several forms of Fanconi Anemia (FA). Germline single allele PV/LPVs in *BRCA1/2* genes are considered the main cause of HBOC. Moreover, PV/LPVs in these genes have been defined as conferring predisposition also to PaC and PrC and other tumors[114-118]. A quarter of all the hereditary BCs are due to PV/LPVs in the *BRCA1/2* genes, while more than 15% of all OCs had a *BRCA1/2* genes alteration[49,76,93].

According to AIOM, women carriers of a PV/LPV in *BRCA1* gene has a RR = 65% of developing BC, with a cumulative risk by age of 80 years of 72%. While women carriers of a PV/LPV in *BRCA2* gene show a RR = 40% of BC, with a cumulative risk by age of 80 years of 69%[5,41,119]. In male BCs, the most involved gene is *BRCA2* gene and the PV/LPVs in this gene lead to a RR = 4-16%, versus a RR = 0-4% for carriers of PV/LPVs in *BRCA1* gene[49]. The RR to develop an OC for carriers of aberrant variants in *BRCA1/2* genes has been estimated to be about 30-70%, with a cumulative risk of OC by the age of 80 years of 44% for carriers of variants in *BRCA1* gene and 17% for subjects with variants in *BRCA2* gene[76]. The *BRCA1/2* genes alterations can be identified in until almost 5-10% of primary PaC[92,120-122]. The *BRCA2* gene harbors the majority of PV/LPVs identified in PrCs, especially in the most advanced. 12% of all PrCs is due to a PV/LPV in DDR genes, mainly in *BRCA2* gene[103,116,123]. Moreover, the PV/LPVs of the *BRCA1/2* genes have been reported by both germline and somatic analysis from PaC patients at similar rates, but on the other hand, these were found more often in PrC tissue samples[40].

Recent evidence reported that the carrier patients harboring PV/LPVs in *BRCA1*/2 gene have a RR >60% of BC and a RR = 40-60% of OC for carriers of PV/LPVs in *BRCA1* gene, whereas a RR = 15-30% for carriers of PV/LPVs in *BRCA2* gene. Moreover, a RR <5% to develop PaC exist for carriers of a PV/LPV in *BRCA1* or in *BRCA2* gene and a RR = 30% of PrC is present for *BRCA2* gene deficient patients[11].

Breast cancer 1, BRCA1

The *BRCA1* gene (17q21.31) is composed by 24 exons (23 coding)[4,124]. The largest is the exon 11 encoding for more than 60% of the enzyme[125,126]. The BRCA1 protein (1863 aa) is

a nuclear effector playing a role in DNA repair, cell cycle control, and genomic stability[4]. BRCA1 is composed by several domains absolving multiple functions, such as a RING (Really Interesting New Gene) domain at the N-terminal end and a C-terminal BRCT domain[125,127] (**Figure 1**). The N-terminal end carries zinc-binding finger RING domain, and it is essential for the interaction of BRCA1 with BARD1 (BRCA1 Associated RING Domain protein 1)[128]. The BRCA1 protein's RING finger is responsible for the ubiquitin E3-ligase activity[129], resulting increased after BARD1 binding[125]. The interaction BRCA1/BARD1 causes the hiding of the Nuclear Export Sequence (NES) of the RING domain, resulting in the nuclear catching of both[125,130-132]. At the C-terminal end, two phosphopeptide-binding domains in the BRCT allow the interaction of BRCA1 with the effector CtIP (C-terminal binding protein 1 (CtBP1) interacting protein), the BRCA1 A Complex Subunit (ABRAXAS), and the BRCA1 interacting protein C-terminal helicase 1 (*BRIP1*/FACJ)[127,133,134]. The central exons 11-13 has two Nuclear Localization Signals (NLSs) allowing to translocate from the cytoplasm to the nucleus, one coiled coil domain allowing the interaction between BRCA1-BRCA2 through PALB2 (partner and localizer of BRCA2) and binding sites for different effectors, such as Retinoblastoma protein (RB), C-MYC, RAD50 and RAD51 (**Figure 1**)[127,135,136]. Through PALB2, BRCA1 places BRCA2 at DSB sites for the repair. The Serine Cluster Domain (SCD) at the C-terminal region allows the phosphorylation by ATM[113]. By associating with proteins, such as ATM, MSH2, MSH6, MLH1, RAD50 or MRE11- NBS1, it forms the BRCA1-Associated Surveillance Complex (BASC)[137], acting as a sensor and regulate the repair process after replication. The sequence alterations across the *BRCA1* gene sequence have been often identified into the RING domain, the exons 11-13 and the BRCT domain[125,126,138]. In most cases the lack of BRCA1 is caused by epigenetic changes, such as promoter methylation inactivating both the alleles of the gene[139]. Furthermore, the Loss of Function (LOF) of *BRCA1* gene in many cancers follows the Knudson's double-hit theory[140], but it has been proposed that single-allele aberrant variants on *BRCA1* gene lead to a haploinsufficiency causing genomic instability[141].

Breast cancer 2, BRCA2

The *BRCA2* (13q13.1) gene consists of 27 exons (26 coding)[3,142]. The exon 11 is extended for more than 50% of the sequence. The BRCA2 nuclear protein (3418 aa) is ubiquitinated and play a key role in cell-cycle control, proliferation pathways, transcription and DSBs repairing in HRR[113,143,144]. The BRCA2 effector consists in a DNA-binding

helical domain, recognizing single-strand DNA (ssDNA) and double-strand DNA (dsDNA), and in eight BRC repeats between the amino acid residues 1009 and 2083 useful to bind RAD51[145,146]. The BRCA2 protein has also by three Oligonucleotide Binding (OB) domain and a tower domain allowing the BRCA2 binding to ssDNA and/or dsDNA[113]. Its N-terminal end has a binding site for PALB2 (amino acids 21-39), and at the C-terminal end are localized the NLSs. BRCA2 has a phosphorylation site for cyclin-dependent kinase 2 (CDK2) (S3291) by which it binds to RAD51 recombinase[113] (**Figure 2**). In fact, BRCA2 targets RAD51 protein to ssDNA, enabling RAD51 to displace Replication Protein-A (RPA) from ssDNA and stabilizing RAD51ss-DNA filaments by blocking ATP hydrolysis. BRCA2 is not able to anneal ssDNA complexed with RPA but mediates the recruitment of the recombinase RAD51 to the DSBs through the assembling of the BRCA1-PALB2-BRCA2 complex[147,148]. Sequence alterations on *BRCA2* gene are associated with FA, HBOC syndrome and several setting of PaC and PrC[149]. Findings suggest that epigenetic regulations are involved also in *BRCA2* gene expression level[143,150].

1.7.2 *TP53*

The *TP53* gene (17p13.1), "the guardian of the genome"[151], encodes for p53 onco-suppressor involved in cell cycle arrest, apoptosis, senescence, and DDR[152,153]. The *TP53* gene missense's PV/LPVs have been identified in about the 50% of sporadic cancers[154,155]. Inherited PV/LPVs in *TP53* gene have been associated with the Li-Fraumeni syndrome, a rare autosomal dominant disorder consisting in a higher risk of childhood-, young- and adult-onset neoplastic diseases, such as BC, leukemia, adrenocortical carcinomas, central nervous system tumors, osteosarcomas, and soft-tissue sarcomas[152,156-158]. The carrier patients harboring germline PV/LPVs in *TP53* gene have a RR = 40% of BC and the RR to develop OC is not yet known. While the RR to develop PaC, colon cancer and other, such as sarcoma, brain, leukemia, and adrenocortical carcinoma, is not well established[11].

1.7.3 *PTEN*

The *PTEN* gene (10q23.3) encodes a phosphatidylinositol-3,4,5- trisphosphate 3-phosphatase, which negative regulate the PI3K pathway and Mitogen-Activated Protein Kinase (MAPK) cascade[159-162]. The PTEN effector maintains genomic stability and it is responsible for cell cycle, apoptosis, and metabolism[163,164]. The PV/LPVs in *PTEN* gene have been identified in many sporadic cancers, such as thyroid, endometrial, breast, and

neural cancer[165,166]. Inherited PV/LPVs in *PTEN* gene have been found in families with the Cowden's syndrome (CS). The CS is an autosomal dominant disorder, described for the first time in 1963, as a hereditary syndrome characterized by individuals with multiple hamartomas and a predisposition to several neoplastic disease, such as uterine, endometrial, thyroid, and BC[162,167]. The carrier patients harboring germline PV/LPVs in *PTEN* gene have a RR = 40% of BC, and the RR to develop OC and PaC is not yet known. While the RR to develop other cancers has been reported, such as colon (10%), thyroid (20%) and endometrial (20%) cancer[11].

1.7.4 *STK11* or *LKB1*

The *STK11* or *LKB1* gene (19p13.3) encodes for a serine/threonine kinase playing a role in several proliferation, growth, and metabolism[168,169]. Inherited PV/LPVs in *STK11* or *LKB1* gene have been linked to Peutz-Jeghers syndrome, a rare autosomal dominant disorder[170], characterized by gastrointestinal (GI) polyposis, mucocutaneous freckling, hyperpigmented macules and predisposition to colorectal cancer (CRC), PaC, BC, and OC[171,172]. The carrier patients harboring germline PV/LPVs in *STK11* gene have a RR = 40% of BC, and the RR to develop OC is not yet known. While the RR to develop PaC is 10-30%, the RR of colon cancer is 30% and the RR to GI cancer is 30% and Sertoli-Lyedig account to 10-20%[11].

1.7.5 *CDH1*

The *CDH1* gene (16q22.1) encodes an E-cadherin acting in invasiveness and metastatic events[173,174]. Somatic, and epigenetic alterations in the *CDH1* gene occur frequently in sporadic tumors, such as gastric cancer and BC[175,176], conferring a poor prognosis[177]. Inherited inactivating PV/LPVs in *CDH1* gene coding and spicing sequence have been identified as responsible for Hereditary Diffuse Gastric Cancer, an autosomal dominant disorder[178]. This disorder is predisposing to the rare hereditary diffuse-type of GI cancer and hereditary lobular BC[176]. The carrier patients harboring germline PV/LPVs in *CDH1* gene have a RR to develop lobular BC accounting to 40%, and the RR to develop OC and PaC is not yet known. On the other hand, the RR to develop diffuse GI is 35-45%[11].

1.8 Moderate and low penetrance genes

1.8.1 Fanconi Anemia genes: *PALB2*, *BRIP1* and other

The FA is a rare inherited recessive disorder characterized by multiple congenital malformations, progressive pancytopenia, predisposition to hematological and solid tumors[179]. The FA genes are involved in several cellular processes in response to replication stress, in the repair of interstrand DNA crosslinks and mainly in HRR pathway[180,181]. The FA is due to homozygous PV/LPVs in one of 22 genes involved in the FA/BRCA pathway, except for the X chromosomal *FANCB* gene[182,183]. FA/BRCA pathway include *BRCA1*/*2* genes (*FANCS* and *FANCD1* respectively)[184-186]. Moreover, in BC and OC patients have been described monoallelic alterations in several FA genes[37].

The *PALB2* o *FANCN* gene (16p12.2) encodes an effector working with BRCA2 in HRR, leading to its nuclear localization and stimulating its activity in checkpoint and repairing[187,188]. PALB2 binds ssDNA and interacting with RAD51 enhances its strand invasion[189]. Germline monoallelic PV/LPVs in the *PALB2* gene conferred an increased RR = 40-60% of BC, a RR = 3-5% of OC and a RR = 2-3% of PaC[11]. Somatic PV/LPVs of *PALB2* gene have been identified in both PaCs and PrCs, whereas germline PV/LPVs have been found in PaC patients, with a higher RR[40,97].

The *BRIP1* (BRCA1 Interacting Helicase 1) or *FANCJ* gene (17q23.2) encodes for a protein with DNA helicase activity interacting to BRCA1 and playing a role in cell cycle checkpoint control and mitosis[190]. The carrier patients of PV/LPVs of *BRIP1* gene confer a RR = 5-10% of OC and the RR of BC is not yet known[11].

1.8.2 *ATM*

The *ATM* gene (11q22.3) encodes a serine/threonine PI3/Pi4 kinase playing a pleiotropic role in repairing DBSs and in cell cycle regulation[191,192]. ATM can activate BRCA1 and BRCA2 in HRR and it is involved also in NHEJ. Biallelic PV/LPVs identified in the Ataxia-telangiectasia-mutated (*ATM*) gene have been related to a rare autosomal recessive syndrome, the Ataxia-telangiectasia, consisting in early onset cancers with a progressive cerebellar ataxia occurring in early infancy characterized by telangiectasis and immune defects[193]. Lymphoma of the B-cell type is the most frequently observed neoplastic disease and numerous cases of leukemias[194].

Monoallelic PV/LPVs in *ATM* gene have been identified as conferring a RR = 25-30% of BC, a RR ≤ 5% of OC, a RR < 5% of PaC and a RR = 30% of PrC[11].

1.8.3 *CHEK2*

The *CHEK2* gene (22q12.1) encodes a nuclear serine/threonine kinase (Chk2) activated in response to DSBs by ATM in HRR[195]. The *CHEK2* gene effector is involved in mitotic function, cell cycle arrest and apoptosis[196,197]. Chk2 activates proteins, such as BRCA1 and p53, allowing to ensure chromosomal stability[198,199]. Germline PV/LPVs in *CHEK2* gene confer a RR of BC estimated to be 25–30%. The RR of OC and PaC is not yet known and, moreover, it seems to confer a RR = 15% of colon cancer[11]. Furthermore, somatic *CHEK2* gene alterations have been found in sporadic tumors[200].

1.8.4 *BARD1*

The *BARD1* gene (2q35) encodes a full-length onco-suppressor protein (BARD1-FL) interacting with the N-terminal region of BRCA1[201,202]. BARD1 plays a crucial role through the BRCA1/BARD1 heterodimer assembling with ubiquitin E3 ligase activity in DDR and cell cycle regulation[203,204]. The *BARD1* gene PV/LPVs have been defined as predisposing only to BC with a RR of about 20%[11].

1.8.5 *RAD51* family

The *RAD51* gene (17q22) encodes several enzymes playing a role in HRR. The RAD51 family is composed by seven different paralogs (RAD51, RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, and DMC1), combining in two complexes with different role at different levels of the same DDR system, the BCDX2 (RAD51B-RAD51C-RAD51D-XRCC2) and the CX3 (RAD51C-XRCC3) complex[205-207]. Biallelic PV/LPVs of the *RAD51*, *RAD51C*, and *XRCC2* genes (*FANCR*, *FANCO*, and *FANCU*, respectively) have been linked to FA and BC[208]. Monoallelic PV/LPVs in *RAD51* gene have been related to BC and OC, such as *RAD51B*, *RAD51C*, and *RAD51D* genes to OC and *RAD51*, *RAD51B*, and *XRCC2* genes to BC[209-211]. The major and well known RAD51 paralogs are *RAD51C* and *RAD51D* genes. The *RAD51C* gene (17q22) encodes an effector having its role in HRR and works with other paralogs[212]. The RR of BC for carriers of PV/LPVs in *RAD51C* gene is estimated to be 20% and the RR of OC is 10%[11].

The *RAD51D* gene (17q12) encodes a tumor suppressor protein creating complexes with the other paralogs of its RAD51 family in the HRR pathway[213]. The RR of developing BC for carriers of PV/LPVs in *RAD51D* gene is estimated to be 10%, such as the RR of OC is 10%[11].

1.8.6 MRE-RAD50-NBS1 complex

The complex composed by the *MRE11*, *RAD50*, and *NBN* genes products (MRN complex) is involved in DNA end resection in HRR, DSBs restoring through NHEJ, telomere preservation, DNA replication and cell cycle checkpoints[152,214]. In presence of DSBs, the MRN complex stimulate other effectors, including ATM, to start the DDR and cell cycle arrest against cancer[214]. Germline PV/LPVs in *MRE11*, *RAD50*, and *NBN* genes have been associated to an increased risk of BC, OC, CRC, GI and PrC, but also leukemia and melanoma[215-217].

The *NBN* gene (8q21.3) encodes a Nibrin protein with a crucial role in MRN complex localization and in the interaction with other proteins[218]. Germline homozygous PV/LPVs in *NBN* gene have been defined as responsible for Nijmegen breakage syndrome (NBS)[219], an autosomal recessive disorder associated to progressive microcephaly, intrauterine growth retardation and short stature, recurrent sinopulmonary infections, an increased risk for cancer, and premature ovarian failure in females. Germline PV/LPVs in NBN gene confer RR in life to develop in BC, OC, PrC, medulloblastoma, and melanoma [220,221]. Homozygous PV/LPVs in *RAD50* and *MRE11* genes have also been identified in NBS-Like Disorder a rare, genetic multiple congenital anomalies/dysmorphic syndrome[222,223]. At the same time, *NBN* gene alterations have been found in BC, PrC and melanoma cancer patients[224]. The PV/LPVs of NBN gene have been found in 0.21% of sporadic PaCs and 0.59% of familial PaCs[97], while in sporadic PrCs represent the 2%[107]. On the other hand, somatic PV/LPVs have been not identified for more than 2% in both PaCs and PrCs[40]. The RR of OC is yet not known[37,225,226].

1.8.7 Mismatch Repair genes

The mismatch repair (MMR) genes are the *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM* genes having different role in the same pathway[227]. The PV/LPVs in MMR genes alteration leads also to a high microsatellite instability[228]. Germline PV/LPVs of the MMR genes are liked to Lynch syndrome (LS), an autosomal dominant disease, including a higher RR of developing CRC, endometrial and other tumors, including PaC and PrC[229,230]. Findings reported the involvement of the *EPCAM* gene in LS as it is localized upstream of *MSH2* gene[231]. The LS consist in a predisposition also to endometrioid or clear cell OC with a RR = 4–12%[232]. Germline/somatic MMR deficiency has been found in 1-8% of serous OC[233]. The RR of OC is related to PV/LPVs in *MLH1* gene (10-20%), in *MSH2* gene (17–24%), in *MSH6* gene (8–13%), while the RR of OC related to *PMS2* gene is equal to generic

population[232]. To date, it is not clear the impact of the PV/LPVs in MMR genes in the RR of BC[46,234]. Germline PV/LPVs in MMR genes have been found in PaC and mCRPC regardless of family history of cancer[97,235]. The PV/LPVs in MMR genes have been found in 0.8% of PaC samples and in almost 3% of PrC samples[98,236].

1.9 Rationale

Deep is the knowledge about the impact of the PV/LPVs of *BRCA1/2* genes in hereditary cancers. However, numerous patients result negative to genetic testing of *BRCA1/2* genes, despite the relevant personal and/or family history of cancers. Furthermore, family history-based testing does not consider about a half of carriers harbouring PV/LPVs in HBOC syndrome predisposing genes[11]. Multi-gene panel testing could define a more detailed molecular profile of the HRR genes and of other genes involved in hereditary cancers to develop more accurate risk assessments, clinical interventions, and targeted therapies. In fact, in the last years has emerged the role of the PV/LPVs in the *BRCA1/2* genes as predictive biomarker of PARPi-treatment choice. The recent FDA and EMA approval of PARPi-based maintenance therapy for the treatment of specific settings of BC, OC, PDAC and PrC resulted crucial in improving PFS and OS for patients. The diffusion of the NGS-based multi-gene panel testing in several setting of BC, OC, PaC and PrC could be essential to understand their molecular basis, investigating beyond the *BRCA1/2* genes[46,47]. To date the NCCN, the ASCO and the ESMO guidelines developed strategies to identify high risk patients, but there are no univocal guidelines[11-15]. The advent of NGS has allowed to perform molecular investigations in a multiplexing approach, reducing time and costs of each analysis, obtaining more information of every single patient[35]. The aim of this study was to develop a more accurate hereditary cancer risk assessment to define specific and wider prevention paths for carriers of PV/LPVs in cancer susceptibility genes and to identify novel potential biomarker for therapeutic purposes.

1.10 Objectives

Based on a Breast Cancer BRCA System database retrospectively collected at University Hospital Policlinico "P. Giaccone" of Palermo, the main goal of this research was to collect and describe the typology and gene location of germline PV/LPVs of the main susceptibility *BRCA1/2* genes detected in BC, OC, Pac and PrC patients who met the eligibility criteria to genetic testing (see section Patients and Methods).

Moreover, the aim was to perform in a selected subset of patients (see section Patients and Methods) a broader multi-gene NGS-based analysis to investigate the prevalence of different inherited PV/LPVs of other genes involved in HRR and/or in other pathways crucial in cancer development and responsible for hereditary cancer syndromes. Overall, the possible genotype-phenotype and clinico-pathological correlations have been carried out. This study could be useful to define a more accurate hereditary cancer's risk assessment and to identify high risk patients for tailored preventive and therapeutic strategies.

CHAPTER 2

Patients and Methods

2.1 Study population

This observational study of Sicilian population has been carried out based on a Breast Cancer BRCA System database collected at the "*Sicilian Regional Center for the Prevention, Diagnosis and Treatment of Rare and Heredo-Familial Tumor of adults*" of the Section of Medical Oncology of University Hospital Policlinico "P. Giaccone" of Palermo, according to the eligibility criteria to genetic test defined by the AIOM guidelines (**Table 1**).

The cohort was composed by primary BC patients included from January, 1th 2016 to October, 20 2022; patients affected by OC included from January, 1th 2017 to October, 20 2022; PaC patients included from January, 1th 2019 to October, 20 2022; and PrC patients included from January, 1th 2021 to October, 20 2022.

On the other hand, a subset of patients resulting negative to *BRCA1/2* genes genetic testing have been selected to the broader analysis with the NGS-based multi-gene cancer panel investigating 22 susceptibility genes (*ATM*, NM_000051.4; *APC*, NM_000038.6; *BARD1*, NM_000465.4; *BRCA1*, NM_007294.4; *BRCA2*, NM_000059.4; *BRIP1*, NM_032043.3; *CDH1*, NM_004360.5; *CHEK2*, NM_001005735.2; *EPCAM,* NM_002354.3; *MLH1*, NM_000249.4; *MSH2*, NM_000251.3; *MSH6*, NM_000179.3; *MUTYH*, NM_001048171.1; *NBN*, NM_005732.4; *PALB2*, NM_024675.4; *PMS2*, NM_000535.7; *PTEN*, NM_000314.8; *RAD50*, NM_005732.4; *RAD51C*, NM_058216.3; *RAD51D*, NM_001142571.2; *STK11*, NM_000455.5 and *TP53,* NM_000546.6)[49,76,93].

The multi-gene testing has been performed on patients resulting negative to *BRCA1/2* genes genetic testing and showing at least one of the following criteria: (i) at least other two first-degree relatives affected by BC, OC, and/or PC; (ii) early onset of cancer (age at diagnosis ≤ 36 years); or (iii) presence of synchronous/ metachronous tumours (e.g. bilateral BC, BC and OC).

Clinical and molecular data have been collected and clinico-pathological correlation have been carried out. All patients provided and signed an informed consent, and the information regarding personal and family history of neoplastic disease, such as family geographical origin, age of cancer diagnosis, histological tumour subtype, molecular phenotype, disease stages (I–IV) and diagnosis have been recorded anonymously. Data about the ER, PR and HER2 receptor status, Ki67 status, and histological grade (Grades I, II and III) of the primary tumours were provided by medical pathology reports in diagnostic core biopsies or resections.

Overall, BCs were divided into LA (LA= ER/PR+ and HER2-, histological grade 1 or 2), LB (LB= ER/PR+ and HER2+, or ER/PR+, HER2-, and grade 3), HER2E (ER/PR- and HER2+) and TNBCs (ER-, PR- and HER2-)[237]. The OC patients were valued for both germline and somatic PV/LPVs in *BRCA1/2* genes[76]. The study was approved by ethical committee (*Comitato Etico Palermo 1*; approval number: 3/2020) of the University-affiliated Hospital A.O.U.P. "P. Giaccone" of Palermo. Each patient has been valued on probability score to be carrier of a PV/LPV in *BRCA1/2* genes through the use of available tools, such as BRCAPRO genetic risk prediction model, and according AIOM guidelines[93,238,239]. The criteria adopted by the AIOM guidelines are focused on personal and family history and age of cancer onset, with the aim to identify high risk subjects harbouring a PV/LPV in the HBOC susceptibility genes[5].

2.2 Sample selection and Next-Generation Sequencing analysis

Peripheral blood samples, from BC, OC, PaC and PrC patients, were collected at diagnosis through a vacutainer syringe containing EDTA. The OC's tissue samples were accessible as exploratory biopsies or neoplastic tissue obtained by surgery and available as Formalin-Fixed Paraffin-Embedded (FFPE). Tissues specimens were sectioned at 10 µm with >20% of malignant origin by the laboratory of pathological anatomy section of the same hospital agency. The extraction of DNA from both peripheral blood and FFPE tissue has been obtained using a commercially available extraction kit according to the manufacturer protocol. The obtained DNA has been quantified using a fluorometer and its quality has been evaluated. The *BRCA1/2* genes analysis in germline and/or somatic samples required 20 ng of DNA to the barcoded library preparation phase. Since 2016 to 2019, the kit used for *BRCA1/2* genes' analysis was performed using a manual workflow's protocol, while starting from 2020 it has been replaced by an automated workflow. Both the procedures have allowed to investigate all the coding and splicing sequences of

BRCA1 and *BRCA2* genes. According to the manufacturer protocol, in the manual procedure, the library has been obtained by using three multiplex PCR primer pools useful to amplify all the coding sequences of the examined genes. There have been employed 20 ng of DNA to each primer pool for PCR amplification in multiplexing condition. The next step has been the partial digestion of the amplicons' ends to allow the barcode ligation followed by a purification step. The quantity and quality of libraries have been valued using a fluorometer and an on-chip electrophoresis, respectively[240]. Later, an equimolar library has been generated and an emulsion PCR have been set up to obtain the target enrichment and the template ssDNA preparation. The final phase is represented by sequencing with the Ion Torrent S5 (Thermofisher Scientific) NGS platform. Molecular data have been analyzed with specific software. To perform multi-gene panel testing analysis have been used a manual workflow kit to evaluate 22 susceptibility genes, with high, moderate, and low penetrance, involved in risk of several inherited neoplastic syndromes, according to a similar protocol to the previously mentioned. The data analysis has been conducted through the standardization of sequencing coverage depth to minimize the probability of false positive and false negative scores. Among laboratories, it has not been established a consensus on the minimum coverage depth and each one must set its own parameters. In our laboratory, a minimum coverage depth of 5000x and 500x has been considered as somatic and germline samples' cut off analysis, respectively.

2.3 Sanger sequencing analysis

Sanger sequencing analysis have been used to confirm the identified PV/LPVs identified on *BRCA1/2* genes. The ABI 3130 Genetic Analyzer (Applied Biosystems) and, more recently, the SeqStudio (Thermofisher Scientific) have been used with a specific kit and according to manufacturer's protocols.

2.4 Copy Number Variation (CNV) Analysis by Multiplex Ligation-Dependent Probe Amplification Analysis

The identification of Large Genomic Rearrangements (LGR) through NGS analysis was confirmed by Multiplex ligation-dependent probe amplification (MLPA). Probe amplification products were analysed by capillary electrophoresis using the ABI 3130 Genetic Analyzer (Applied Biosystems) and the SeqStudio (Thermofisher Scientific). Final scores were analysed by a specific software to observe peak heights and areas and fragment sizes in

base pairs (bp)[241]. In case of positive scores, the results have been confirmed conducing a second analysis on another blood sample following the same protocol.

2.5 Genetic variants classification

According to AIOM guidelines, variants are classified adopting the five-class classification proposed by the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA)[242] and the International Agency for Research on Cancer (IARC) recommendations[243], into:

- Class 1: **Benign**,
- Class 2: **Likely Benign**,
- Class 3: **Variant of Uncertain Significance**,
- Class 4: **Likely Pathogenic**,
- Class 5: **Pathogenic**.

The *in-silico* analysis has been performed using online available databases, such as ClinVar, VarSome, BRCA Exchange, LOVD, PolyPhen-2, Sift, HCI Priors V2.0 Software, to investigate the molecular and clinical involvement variants. The gene and variants identified were characterized according to the systematic nomenclature of the recommendations for the description of sequence variants established by the Human Genome Variation Society (HGVS) authorized by the HGVS, Human Variome Project (HVP), and the Human Genome Organization (HUGO)[244].

2.6 Statistical analysis

Clinico-pathological variables and prevalence of each genetic variants were evaluated in the examined cohort. The comparison between each clinical feature was made with Fisher's Exact test. P-values <0.05 were considered significant. Statistical analyses have been performed through the IBM SPSS Statistics for Windows Version 23.0 (IBM Corporation, Armonk, NY, USA).

CHAPTER 3

Results

3.1 Patients selected and molecular subtypes

Overall, 1878 patients were included in this observational study and selected for genetic testing of the susceptibility *BRCA1/2* genes, according to the eligibility AIOM criteria (see section Patients and Methods).

The cohort were composed by 1170 BC patients (1116 women and 54 men); 540 OC patients; 144 PaC patients (65 women and 79 men) and 24 PrC patients.

The collection of the available clinical data covered a total of 1023 BC patients out of 1170 analyzed, bringing out BCs with the following molecular subtypes: 246 (24%) LA, 411 (40.2%) LB, 40 (3.9%) HER2E and 326 (31.9%) TNBCs. About the remaining 147 BC patients, the available clinical information was incomplete, but the molecular data have been reported.

The OC patients showed the following histological subtypes: 2 serous, low-grade (0.4%); 347 serous, high-grade (64.3%); 53 endometrioid (9.8%); 13 clear cells (2.4%); 32 papillary (5.9%); 93 with unknown histological subtypes (17.2%).

The PaC patients had previously a PDAC (63%). The PrC showed a 66% of cancers with Gleason 7-9.

3.2 Analysis of *BRCA1/2* genes in BC patients

Overall, 144 (12.3%) out of 1170 BC patients resulted carriers of PV/LPVs in *BRCA1/2* genes: 135 women (93.75%) e 9 men (6.25%). Among which, 72 (50%) had a PV/LPV in *BRCA1* gene, 70 (48.6%) in *BRCA2* gene, while 2 patients (1.4%) showed double heterozygosity for PV/LPVs in both *BRCA1* and *BRCA2* genes.

Among the 144 carrier patients, 67 (46.5%) had a TNBC, 53 (36.8%) had a LB, 9 (6.3%) had a LA, 3 (2.1%) had a HER2E and 12 (8.3%) had an unknown molecular subtype. Out of 72 carriers of variants in *BRCA1* gene, 48 (66.7 %) had a TNBC, 17 (23.6%) a LB, none had a LA, one (1.4%) had a HER2E tumor and 6 (8.3%) had an unknown molecular subtype. Out of 70 carriers of variants in *BRCA2* gene, 35 (50%) had a LB, 18 (25.7%) a TNBC, 9 (12.85%) a LA, 2 (2.85%) a HER2E tumor and 6 (8.6%) had an unknown molecular subtype. Moreover, both one TNBC patient and one patient with a LB tumor showed double heterozygosity for PV/LPVs in *BRCA1/2* genes. The PV/LPVs detection rate among the molecular subtypes and between women and men is shown in **Figure 3a, 3b** and **3c**.

Overall, BC patients harboring a PV/LPV in *BRCA1* gene developed mainly a TNBC and patients carrying a *BRCA2* gene aberrant alteration had more frequently a LB/HER2-negative BC (**Table 2**).

The typology and gene location of each PV/LPV have been recorded, especially with a TNBCs vs LA/LB/HER2E BCs comparison, with the aim to identify a potential association between a specific genetic alteration and a BC's molecular subtype.

The molecular data showed 70 different PV/LPVs in *BRCA1/2* genes identified in 144 BC patients, among which 4 in unknown molecular subtypes. In detail, 40 different PV/LPVs have been found among TNBC patients, of which 25 in *BRCA1* gene and 15 in *BRCA2* gene (**Table 3** and **Figure 4a** and **4b**); 36 PV/LPVs have been found in LB patients, 11 in *BRCA1* gene and 25 in *BRCA2* gene, whereas 9 PV/LPVs of *BRCA2* gene were observed in LA and 3 PV/LPVs have been identified in HER2E, one in *BRCA1* gene and 2 in *BRCA2* gene (**Table 4** and **Figure 5a** and **5b**).

Overall, 28 different PV/LPVs in *BRCA1* gene have been identified and 17 have been identified only in TNBCs and 3 only in the LB phenotype. On the other hand, 8 have been identified both in TNBCs and LB, including one LGR, while one PV has been identified in both TNBCs and HER2E. Overall, 38 different PV/LPVs of *BRCA2* gene have been identified and 16 have been identified only in LB, 7 only in TNBCs, 3 in LA and 2 in HERE. On the other hand, 7 were identified in both TNBC and LB subtype, 5 were both in LA and

LB, one was in TNBCs and LA and 3 in TNBCs, LA and LB. Based on available molecular data, the spectrum of the PV/LPVs identified in TNBC patients was not overlapping the variants harboured by LA/LB/HER2E patients (**Table 3** and **4**).

Among TNBC patients the most frequent identified PV were the c.4964_4982del, *BRCA1* (11.9%) and the c.514del, *BRCA1* (10.4%), according to HGVS nomenclature, identified in 8 and 7 probands respectively. The c.4964_4982del, *BRCA1* is a deletion of an extended fragment composed by 19 nucleotides of the coding sequence leading to a premature stop codon formation and it has been defined a founder variant of the Southern Italy[245-247]. The c.514del, *BRCA1* is a deletion of one cytosine causing a frameshift, resulting in the substitution of the amino acid glutamine with asparagine at codon 172, and in a premature stop signal creation leading to a BRCA1 truncated or absent protein[247,248]. Into *BRCA1* gene coding and splicing sequence, the PV/LPVs were located into three hypothetical cluster regions, such as the RING N-terminal domain (nucleotide: 185; codon: 23), the exon11 (nucleotides: 916-4023; codons: 267-1302) and the BRCT C-terminal domain (nucleotides: 5083-5382; codons: 1655-1756)[249]. In detail, one variant was in the the RING N-terminal domain, 10 PV/LPVs in the exon 11 and 5 in the BRCT C-terminal domain (**Figure 4a**). About the type of each alteration, 12 were frameshift (Fs), 7 nonsense (NS), 1 missense (M), 2 intronic variants (IVS) and 3 were LGR (**Table 3**). The PV/LPVs in *BRCA2* gene seemed to be a distributed along the whole sequence (**Figure 4b**). About the type of the variants, 2 PV/LPVs were in the N-terminal region, 7 were in exon 10-11, with 4 in the BRC repeats and 1 in the DNA binding domain[249]. Regarding the type of the alterations, 7 were Fs, 3 were NS, and 5 were IVS (**Table 3**). Among TNBCs, have been also identified three *Large Genomic Rearrangement* (LGR): the c.-232_4675del, *BRCA1* (exons 1-15), a big deletion of the exon 14 of *BRCA1* gene reported as 17q21.31 (41226308-41226571)x1, and a big deletion of the exons 15-16 of *BRCA1* gene reported as 17q21.31 (41219595-41223282)x1 (**Table 3**).

Among LA/LB/HER2E patients, the most frequent PV in *BRCA1/2* genes was the c.1238del, *BRCA2* identified in 7 (10.8%) LB and one LA (1.5%) patients. The c.1238del, *BRCA2* leads to the deletion of a thymine causing a frameshift resulting in the substitution of a leucine with a histidine at codon 413 and in the premature stop signals formation with a LOF of the protein[51,250]. In this study, it emerges how the *BRCA2* gene harbours the largest proportion of PV/LPVs in LA/LB patients. In addition, patients affected by a LA tumour show only aberrant genetic alterations of the *BRCA2* gene. The genetic localization of the PV/LPVs in both the *BRCA1* and *BRCA2* genes in this subset of BC patients has a

similar distribution into the three cluster regions. Into *BRCA2* gene coding and splicing sequence, the PV/LPVs were located into three hypothetical cluster regions, such as the BRC repeats into the exons 10-11 (nucleotides: 4088-6352; codons: 1287-2042) and the exons 10-11 (nucleotides: 1466-6819; codons: 413-2198), and DNA binding helical domain near the C-terminal region (nucleotides: 7909-9683; codons: 2561-3152)[249]. In detail, 6 PV/LPVs were in the N-terminal region, 9 variants were in the BRC repeats into the exon 11, 4 in the exon 10 and 7 DNA binding helical domain near the C-terminal region (**Figure 5b**). About the type of each variant, 19 were Fs, 8 were NS, 2 were M and 8 were IVS (**Table 4**). The PV/LPVs in the *BRCA1* gene seemed to be located into the three hypothetical cluster regions, but there were poor represented in LA/LB/HER2E BCs. Into *BRCA1* gene, 2 PV/LPVs were located into the RING N-terminal domain, 5 in the exon 11 and 3 in the BRCT C-terminal domain (**Figure 5a**). About the *BRCA1* gene variants type, 6 were Fs, 3 were NS, 2 were M and one LGR has been identified (**Table 4**). The results showed a potential association with the TNBC subgroup and the c.4964_4982del, *BRCA1* and the c.514del, *BRCA1*, although these have been identified also in LB patients with a relatively high frequency. Moreover, the c.4964_4982del, *BRCA1* and the c.514del, *BRCA1* have been identified in TNBC women with younger age than in LB and with higher Ki-67 and histological grade. Whereas the c.1238del, *BRCA2* has been found in LA/LB HER2- patients with a higher frequency than TNBC patients, with higher expression of ER and in 2 BBCs with onset <40 years old. The other variants showed a low prevalence suggesting the absence of a potential association with a specific molecular subtype (**Table 3** and **4**).

Furthermore, 64 (5.5%) out of 1170 patients were carriers of VUSs. The VUSs have been investigated *in silico* through online available tools (see section Patients and Methods). Overall, 19 VUSs have been identified in *BRCA1* gene: 7 in LA, 5 in LB, 4 in TNBCs, one in HER2E and one in an unknown molecular subtype. While 44 VUSs have been identified in *BRCA2* gene: 16 in TNBCs, 15 in LB, 9 in LA and 4 in unknown molecular subtypes. One patient showed double heterozygosity for VUSs in the involved genes. The *BRCA2* gene seemed to harbour the most variety of VUSs and almost exclusively of the M type.

A correlation between the localization of each variant and the Breast Cancer Cluster Regions (BCCRs) and the Ovarian Cancer Cluster Regions (OCCRs) has been observed (**Table 3** and **Table 4**; **Figure 4a** and **4b, Figure 5a** and **5b**). The TNBC subset showed a correlation with the so called BCCRs for 2 variants of *BRCA1* gene (5 patients) and 6 variants of *BRCA2* gene (7 patients), on the other hand, TNBC subset showed correlation

with the OCCRs in 7 variants of *BRCA1* gene (12 patients) and in 2 variants of *BRCA2* gene (2 patients). The LB subset showed a correlation with the BCCRs for one variant of *BRCA1* gene (one patient) and 11 variants of *BRCA2* gene (20 patients), on the other hand, LB showed correlation with the OCCRs in 4 variants of *BRCA1* gene (4 patients) and in 2 variants of *BRCA2* gene (3 patients). The LA subset showed a correlation with the BCCRs for 3 variants of *BRCA2* gene (4 patients) and a correlation with the OCCRs in 4 variants of *BRCA2* gene (3 patients). The HER2E subset showed a correlation with the BCCRs for one variant of *BRCA2* gene (one patient) and a correlation with the OCCRs in one variant of *BRCA1* gene (one patient) and one variant of *BRCA2* gene (one patients). The potential correspondence between variants and BCCRs seemed to be more represented in LB subset.

3.3 Association among PV/LPVs in *BRCA1/2* genes and clinical data in BC patients

The mean age at diagnosis among carriers of a *BRCA1/2* gene PV/LPV was 43,6 years (median: 42; range: 25-80), with a mean age of 42,7 years for women (median: 41; range: 25-80) and 57,4 years for men (median: 58; range: 36-80). An earlier tumor onset has been observed for patients with a *BRCA1* gene alteration having a mean age of 41,7 years (median: 41; range: 26-65), 41,75 years for women (median: 41; range: 26-65) and 41 years for men (median: 41; range 36-46). On the other hand, carrier patients of a *BRCA2* gene alteration have been characterized by a mean age at diagnosis of 45,7 years (median: 42; range: 25-80), in detail 43,8 years for women (median: 40; range: 25-80), while 62,1 years was the mean age among men (median: 60; range: 42-80). The two double heterozygous patients were women showing an age at diagnosis of 31 and 49 years old. The carrier patients of PV/LPVs in *BRCA1* gene had an earlier onset both for woman and men, while carriers of PV/LPVs in *BRCA2* gene showed a latest onset. The mean age among non-carrier patients was 47,2 years (median: 46; range: 21–93 years), with women having a mean age of 46,4 years (median: 46; range: 22-93) and men having a mean age of 62 years old (median: 64; range: 21-87). The non-carriers were aged more similarly to carriers of PV/LPVs in *BRCA2* gene.

Overall, in BC cohort the 63% of carrier women were premenopausal at diagnosis (age before 50 years; mean age: 39.9; median: 40) and a women developed a BC about 14 years before a man, except for carriers of PV/LPVs in *BRCA1* gene.

In the TNBC subgroup, the most of carriers of variants in *BRCA1/2* genes (73.1%) was premenopausal at diagnosis (before the age of 50 years; mean age of 41.3 years; median: 42). The TNBC carrier patients developed a BC at the mean age of 44,9 years (median: 49; range 21-93).

A statistically significant difference emerges in mean age at diagnosis between carriers of PV/LPVs in *BRCA1*/*2* genes and non-carriers, with the carriers showing an early age at diagnosis (p=0.0005). Moreover, the TNBC patients carrying PV/LPVs in *BRCA1* gene developed BC at a mean age of 42,9 (median: 42; range: 26-65), about 8 years earlier than carriers of variants in *BRCA2* gene (median age: 50; mean age: 50; range: 32-69) and non-carriers (median age: 50; mean age: 50.6; range: 21-93). The prevalence of PV/LPVs in the age groups of patients was 34.3% (23/67) in TNBC patients ≤40 years old, 41.8% (28/67) among 41-50 years, 13.4% (9/67) among 51-60 years, and 10.4% (7/67) in patients with >60 years (**Table 5**).

In LA/LB and HER2E subset, the vast majority (68.9%) was premenopausal at diagnosis, mean age 39.6 (median: 39). The LA/LB and HER2E carrier patients developed a BC at the mean age of 42.9 years (median: 40; range 25-80). A statistically significant difference emerges in mean age at diagnosis between carriers of PV/LPVs in *BRCA1*/*2* genes and non-carriers, with the carriers showing an early age at diagnosis (p=0.05). Moreover, LA/LB and HER2E patients carrying PV/LPVs in *BRCA1* gene developed BC at a mean age of 40.3 (median:39; range: 31-55), about 4 years earlier than *BRCA2* gene's PV/LPVs carrier patients (median age: 40; mean age: 44.2; range: 25-80) and 4 years earlier than non-carriers (median age: 44; mean age: 45.9; range: 24-87). The prevalence of PV/LPVs was 52.3% (34/65) in patients aged ≤40 years, 24.6% (16/65) among patients with 41-50 years, 16.9% (11/65) among patients of 51-60 years, and 6.2% (4/65) among patients having >60 years (**Table 5**).

Significant clinicopathological differences have been observed between carrier and non-carrier BC patients.

The LA/LB and HER2E carrier patients showed a higher Ki-67 compared with non-carrier patients (p<0.0001) and the tumor grading is more often grade III in carriers compared to non-carriers (p<0.0001). The entire cohort showed a most diffuse CDI histological type without statistically significant differences between carriers and noncarriers.

160 contralateral tumors were identified (15.6%): 32 (20%) out of 160 were TNBC among which 7 with PV/LPVs in *BRCA1* gene, 2 with PV/LPVs in *BRCA2* gene and one showed double heterozygosity for *BRCA1/2* genes; 117 (73.1%) were LA/LB/HER2E among which

2 with PV/LPVs in *BRCA1* gene, 8 with PV/LPVs in *BRCA2* gene and one showed double heterozygosity for *BRCA1/2* genes. Overall, 13.1% of all BBCs were carriers of PV/LPVs in *BRCA1* gene. About the other 11 BBCs, the clinical information was incomplete to determine the molecular subtype. The mean age at diagnosis of the BBCs of the TNBC subtype was 45.9 years (median: 44; range 32-62), while the mean age at diagnosis of LA/LB and HER2E with BBCs was 48.4 years (median: 48; range: 21-80). The BBCs with TNBC molecular subtype and with *BRCA1/2* genes aberrant alterations had an earlier onset than non-carriers (median: 39 vs 48 years), such as BBCs with a LA/LB/HER2E BCs (34 vs 48 years).

The time of onset between the first and the second tumor was about 10 years both for carriers and non-carriers in TNBC patients. On the other hand, the time between the first and the second tumor in LA/LB/HER2E patients was about 12 years in carrier patients and 5 years in non-carriers. It emerges that the BBCs diagnosis is earlier in LA/LB/HER2E subsets compared to TNBC, but without significant statistically results. The clinical and molecular data between TNBC vs LA/LB/HER2E BCs is reported in **Table 5**.

Moreover, 25 out of 64 (39.1%) patients with VUSs developed an early onset of BC (age <40 years old). The TNBC subset with VUSs received a diagnosis at 50.8 years (median: 52; range 27-74), compared with the LA/LB and HER2E subset that received a diagnosis at 42.4 (median: 39; range: 27-63).

3.4 Analysis of *BRCA1/2* genes in OC patients

Overall, 128 (23.7%) out of 540 OC patients resulted carriers of germline and somatic PV/LPVs in *BRCA1/2* genes. In detail, 72 (56.3%) had a germline PV/LPV in *BRCA1* gene and 37 (28.9%) had a germline PV/LPV in *BRCA2* gene. Moreover, 19 (14.8%) out of 128 patients were carriers of somatic variants in both *BRCA1* and *BRCA2* genes, 12 (63.2%) in *BRCA1* gene, 6 (31.6%) in *BRCA2* gene and one (5.3%) sample showed double heterozygosity for variants of *BRCA1/2* genes. Furthermore, a not well characterized LGR has been identified as a big duplication in the exon 12 of *BRCA1* gene and reported as 13q13.1 (32900212-32972932)x1 (**Table 6**). Overall, women harbouring PV/LPVs in *BRCA1* gene developed with almost twice frequency an OC than carriers of variants in *BRCA2* gene.

The typology and gene location of each PV/LPV in *BRCA1/2* gene have been recorded, with a BCs vs OCs comparison, aiming to identify a potential association between a

specific variant and a tumour. Overall, 71 different variants of *BRCA1/2* genes have been identified in OC patients: 40 in *BRCA1* gene and 31 in *BRCA2* gene. Among the variants, 45 different PV/LPVs have been exclusively identified among OC patients, while the other 26 were identified even in BC patients. Based on available information, no potential correlation between a specific PV/LPV in *BRCA1/2* genes and the disease has been observed (**Table 6**).

The most frequent PV/LPVs identified were the c.4964_4982del, *BRCA1* and the c.514del, *BRCA1*. Moreover, the c.9026_9030del, *BRCA2* and the c.6082_6086del, *BRCA2*, were the most prevalent among the variants of *BRCA2* gene, but with a lower frequency. The c.4964_4982del, *BRCA1* has been identified in 15 patients (11.7%) leading to a deletion of 19 nucleotides with a frameshift of the sequence, and a premature stop codon formation allowing LOF of the effector. This variant is considered a founder PV in Sicilian population[245-247]. The second most common PV characterized was the c.514del, *BRCA1* identified in 9 patients (7%) and leads to a deletion of one cytosine causing a frameshift alteration and a premature translational stop signal with a BRCA1 truncated or absent protein[248]. Furthermore, among the less frequent variants it has been identified the c.4963T>G, *BRCA1* in 3 (2.3%) patients that is considered “not provided” on ClinVar[251] and Likely Pathogenic on VarSome[252]. This variant leads to a protein change causing a substitution of a serine with an alanine in position 1655 and, since it is a highly conserved residue, this variant has probably a potential of pathogenicity[253]. On the other hand, the c.9026_9030del, *BRCA2* and the c.6082_6086del, *BRCA2* have been identified in 4 and 3 patients (3.1% and2.3% respectively) both leading to a premature stop codon formation causing a truncated or absence protein[248,254]. The genetic localization of the PV/LPVs identified in *BRCA1* gene in OC patients seem to be located into three hypothetical cluster regions, such as the RING N-terminal domain (nucleotide: 184-405; codon: 22-96), the exon 11 (nucleotides: 917-4023; codons: 267-1302) and the BRCT C-terminal domain (nucleotides: 5082-5680; codons: 1655-1855)[249]. In the *BRCA1* gene, 4 PV/LPVs were located into the RING N-terminal domain, 20 in the exon 11 and 6 in the BRCT C-terminal domain (**Figure 6a**). The genetic localization of the PV/LPVs identified in *BRCA2* gene in OC patients seem to be located into three hypothetical cluster regions, such as the BRC repeats into the exon 11 (nucleotides: 3386-6310; codons: 1053-2028) and the exons 10-11 (nucleotides: 1466-6714; codons: 413-2162), and DNA binding helical domain near the C-terminal region (nucleotides: 7909-9683; codons: 2494-3085)[255]. In the *BRCA2* gene, 14 PV/LPVs were in the BRC repeats, 22 in the exons 10-11 and 5 in the DNA binding helical

domain (**Figure 6b**). About the type of the variants in *BRCA1* gene, 17 were FS, 11 were NS, 7 were M, 4 were IVS and one was a LGR. About the type of variants in *BRCA2* gene, 14 FS variants, 12 NS variants, 3 IVS, one M and one Syn variant (**Table 6**).

Overall, 20 (3.7%) out of 540 patients had VUSs that have been investigated *in silico* (see section Patients and Methods). 6 VUSs have been identified in *BRCA1* gene and 14 VUSs have been identified in *BRCA2* gene. The *BRCA2* gene seemed to harbour the most variety of VUSs and almost exclusively of the M type.

Clinicopathological correlations and correlations between variant location and Ovarian Cancer Cluster Regions (OCCRs) has been observed (**Table 6** and **Figure 6a** and **6b**). The OC cohort showed a correlation with the so called BCCRs for 9 variants of *BRCA1* gene (14 patients) and 5 variants of *BRCA2* gene (5 patients), on the other hand, OC patients showed correlation with the OCCRs in 14 variants of *BRCA1* gene (18 patients) and in 8 variants of *BRCA2* gene (9 patients). The correspondence between variants and OCCRs seemed to be more represented in carriers of a PV/LPV in *BRCA1* gene.

3.5 Association among PV/LPVs in *BRCA1/2* genes and clinical data in OCs patients

Overall, the mean age at diagnosis among carriers of PV/LPVs in *BRCA1/2* genes was 56 years (median: 57; range: 27-81). An earlier OC onset has been observed for carrier patients of a germline alteration of the *BRCA1* gene having a mean age of 53 (median: 52; range: 27-74), while the mean age of a carrier of germline a PV/LPV in *BRCA2* gene was 59 years (median: 59; range: 28-81). The mean age of patients having somatic PV/LPVs was 62 years (median: 64; range: 47-78). The mean age for non-carrier patients was 59 years (median: 59; range: 23-84). Among the carrier patients the 28.4% were premenopausal at diagnosis, mean age 43 (median: 44). The prevalence of germline PV/LPVs was 10/109 (9.2%) in OC patients ≤40 years, 28/109 (25.6%) among 41-50 years, 40/109 (36.7%) among 51-60 years and 23/109 (21.1%) among 61-70 years and 8/109 (7.3%) among >70. The comparison between the age group distribution in carriers and non-carriers resulted statistically significant with an earlier onset in carrier women (p=0.02) (**Table 7**).

Overall, 191 (35.4%) bilateral OCs have been identified with 50 (26.2%) carriers of a germline PV/LPV in *BRCA1/2* genes, while 7 (3.7%) with somatic PV/LPVs in *BRCA1/2* genes. The bilateral OCs with a germline alteration in *BRCA1* gene had a mean age of 51 years (median: 55; range: 35-70), while the carriers of a germline PV/LPV in *BRCA2* gene

had a mean age of 54 years (median: 57; range: 37-67). The mean age of bilateral OCs patients having somatic PV/LPVs was 60 years (median: 60; range: 47-73). It emerges that the OCs with *BRCA1* genes germline alterations had an earlier onset compared with carriers of PV/LPVs in *BRCA2* gene and non-carriers (**Table 7**).

Statistically relevant correlation has been observed about the histological subtype, with the carriers showing a higher percentage of endometroid and papillary OCs (10.9% and 7%, respectively) compared to non-carriers (6.25% and 2.3%, respectively) (p=0.04). Among non-carrier of a PV/LPV in *BRCA1/2* genes, 92 (71.9%) had HGSC compared to 62% of carriers. Overall, 29.8% of bilateral OC cases had a PV/LPV in *BRCA1/2* genes, while 70.2% of bilateral OC patients was without germline variants. Overall, 12.8% of carrier patients of carriers with germline PV/LPVs showed a personal BC history before OC, and, on the other hand, 4.1% of non-carrier patients had a previous personal BC history. Whereas the 65% of carriers showed family history of cancer against 40% of non-carrier patients. The clinicopathological features of the OC cohort were reported in **Table 7**.

3.6 Analysis of *BRCA1/2* genes in PaC and PrC patients

Overall, 3 (2.1%) out of 144 PaC patients were men carriers of PV/LPVs in *BRCA2* gene and none resulted carrier of variants in *BRCA1* gene. The typology and gene location have been recorded, especially with a comparison with BCs and OCs previously examined. Among the 3 variants identified, one was exclusively identified in PaCs, while 2 were even in luminal-like tumors. Based on available information, no correlation between PV/LPVs in *BRCA1/2* genes and a specific tumour has been observed (**Table 8**).

The identified variants were the c.6990_6994del, *BRCA2*, identified only in PaCs, the c.7681C>T, *BRCA2*, and the c.8487+1G>A, *BRCA2*, both even identified in luminal-like tumours. The c.6990_6994del, *BRCA2*, is a deletion of 5 nucleotides causing a translational frameshift and leading to the formation of a premature stop codon, causing a truncation or the absence of the encoded protein[256,257]. The c.7681C>T, *BRCA2*, is a nonsense variant causing the formation of a premature translational stop signals[248,258]. The c.8487+1G>A, *BRCA2* is an IVS leading to an alteration of the splice-site and can interfere with mRNA splicing with the exon skipping, shortening, or involvement of intronic fragment[259,260] (**Table 8**). Moreover, the variants of *BRCA2* gene were all located in the *BRCA2* sequence encoding the DNA binding domain.

Overall, one (4.2%) out of 24 PrC patients was carrier of a PV in *BRCA2* gene. The variant was the nonsense c.3545_3546del, *BRCA2* leading to a truncated non-functional protein due to the formation of a truncated enzyme[248]. The identified variant was exclusively identified in PrC and was the c.3545_3546del, *BRCA2*[256]. The variant was located into the BRC repeats of the exon 11 of the enzyme. Based on available information, no correlation between PV/LPVs in *BRCA1/2* genes and a specific tumour has been observed (**Table 8**).
Overall, 7 (4.9%) out of 144 PaC patients and one (4.2%) out of 24 PrC patients were carriers of VUSs. The VUSs have been investigated *in silico* through online available tools (see section Patients and Methods). Among PaCs, One VUS have been identified in *BRCA1* gene, while 6 VUSs have been identified in *BRCA2* gene, while the PrC patient's VUSs was in *BRCA2* gene. The *BRCA2* gene seemed to harbour the most variety of VUSs and almost exclusively of the M type.

3.7 Association among PV/LPVs in *BRCA1/2* genes and clinical data in PaC and PrC patients

Overall, the mean age at diagnosis among PaC patients resulted carriers of PV/LPVs in *BRCA2* genes was 60.3 years (median: 63; range: 44-74), while the mean age for non-carrier patients was 64.5 years (median: 65; range: 34-86). The carriers were all men, two had a PDAC, while only one had a cribriform PaC. The prevalence of germline PV/LPVs was 0/3 (0%) in PaC patients ≤40 years, 1/3 (33.3%) among 41-50 years, 0/3 (0%) among 51-60 years and 1/3 (33.3%) among 61-70 years and 1/3 (33.3%) among >70. The PaCs with *BRCA1/2* genes germline alterations had an earlier onset compared with non-carriers.
Overall, none of carrier patients of alterations in *BRCA1/2* genes showed a personal history of cancer before PaC, and, on the other hand, 8.2% of non-carrier patients had a previous personal history of other cancers, such as BC, PrC, colon cancer, cholangiocancinoma, lymphoma, thyroid cancer.
The age at diagnosis of the only PrC patient resulted carrier of a PV in *BRCA2* gene was 34 years old.

3.8 Detection of germline variants through NGS-based multi-gene cancer panel analysis

Overall, 527 (28%) out of 1878 patients have been selected, including 422 BC, 70 OC, 30 PaC and 5 PrC patients (see section Patients and Methods), and analyzed through NGS-based multi-gene panel testing to identify the presence of PV/LPVs in other cancer susceptibility genes beyond *BRCA1/2* genes. The analysis revealed 83 (15.7%) out of 527 patients analyzed with PV/LPVs in susceptibility genes different from *BRCA1/2* genes. In detail, the analysis showed 65 BCs, 11 OCs, 7 PaCs and none PrCs harboring PV/LPVs (**Table 9**).

In BC's cohort, 422 BC patients have been tested (401 women and 21 men). Overall, 65 (15.4%) out of 422 resulted carriers of PV/LPVs, with the most involved genes as *MUTYH* gene in 16 (24.6%) patients and *CHEK2* gene in 14 (21.5%) patients (**Figure 7**). The most frequent variants were the c.1145G>A, *MUTYH*, identified mainly in heterozygous patients affected by LB/HER2- patients; the c.1395_1397del, *MUTYH* identified in LA/LB patients; the c.1229del, *CHEK2*, identified in LA/LB BCs among which two were LA BCCs with ER >60% and PR among 20% and 60%; the c.1165C>T, *CHEK2* identified in LA/LB patients; the IVS c.721+3A>T, *CHEK2* in LB with ER >90% and PR between 40% and 90% and the c.1441G>T, *CHEK2* in LA with ER >80% and PR >80% and the c.1065+5_1026+7del, *RAD51C* identified in one TNBC and two luminal-like BCs among which a BBCs. Although *PALB2* is known as one of the most frequently involved gene in BC predisposition, in our analysis 10.8% of all PV/LPVs have been identified in *PALB2* gene in patients with TNBC and LB tumours among which two BBCs[261]. Among carriers, 13 (20%) out of 65 were BBCs with the PV/LPVs distributed as follows: 3 *MUTYH*, 3 *ATM*, 2 *CHEK2*, 2 *RAD51C*, 2 *PALB2* and 1 *APC* genes. Overall, more than a half of carrier patients (59.1%) showed PV/LPVs in HRR genes. The detailed distribution rate of all variants is showed in **Table 9**.

In OC's cohort, 70 OC patients have been investigated. Overall, 11 (15.7%) out of 70 resulted carriers of PV/LPVs mainly in *MUTYH* gene (**Figure 7**). In detail, 5 (45.5%) out of 11 variants were in *MUTYH* gene and 4 of them were the monoallelic c.1145G>A, *MUTYH* identified in 3 HGSC (FIGO stage III and grading III), and one endometrioid carcinoma (FIGO stage II and grading II) having a strong family history for several cancers among whing colon cancer. Among carriers, 6 (54.5%) out of 11 were bilateral OCs with the PV/LPVs distributed as follows: 3 *MUTYH*, 1 *MLH1* and 1 *PMS2* genes. The monoallelic c.1145G>A, *MUTYH* has been identified in 3 HGSC, with FIGO stage III and grading III, and one endometrioid carcinoma, with FIGO stage II and grading II, having a strong family

history for several cancers among whing colon cancer. This missense variant, in a biallelic status, has been strongly associated with *MUTYH*-associated colon polyposis syndrome and colorectal cancer[262]. Overall, the half of carrier patients (54.5%) showed PV/LPVs in HRR genes, often identified simultaneously with the c.1145G>A, *MUTYH*, and 27.3% of carriers showed PV/LPVs MMR genes (**Table 9**).

In PaC's cohort, 30 PaC patients have been analyzed through multi-gene panel testing (12 women and 18 men) and 7 (23.3%) out of 30 showed PV/LPVs. The most involved gene resulted *ATM* gene (**Figure 7**) with 3 PV/LPVs identified in affected patients by a PDAC of the pancreatic head and were all women with personal and/or family history of BC and/or OC (42.9%). Overall, 57.1% of carrier patients showed PV/LPVs in HRR genes, 3 in *ATM* and one in *PALB2* gene (**Table 9**). In PrC's subset, 5 patients have been investigated through multi-gene panel testing, but no PV/LPVs have been identified.

Overall, the presence of VUSs have been investigated, even contemporary to PV/LPVs.

Overall, 30 (46.1%) out of 65 BC patients had PV/LPVs and VUSs simultaneously, while the carriers of only VUSs were 159 (37.7%) out of 422 BC patients. Among the carriers of VUSs, 82 were TNBC patients and 9 (11%) resulted carriers of PV/LPVs and VUSs contemporary, two of which in *PALB2* gene. Overall, 13 (20%) out of 65 of PV/LPVs carriers had a BBC. The PV/LPVs were identified as follows: 3 in *MUTYH*, 3 in *ATM*, 2 in *CHEK2*, 2 in *PALB2*, 2 in *RAD51C* and 1 in *APC* genes.

Overall, 3 (27.3%) out of 11 OC patients had a PV/LPV and a VUS simultaneously, while the carriers of only VUSs were 27 (38.6%) out of 70 OC patients. Among these, 8 (29.6%) out of 27 out of resulted affected by bilateral OC, as follows the PV/LPVs were identified: 1 *MUTYH*, 1 *PALB2*, 1 *CHEK2*, 2 *ATM*, 1 *PMS2*, 1 RAD50 and 1 *APC* genes.

Overall, 2 (28.6%) out of 7 PaC patients has PV/LPVs and VUSs simultaneously, while the carriers of only VUSs were 10 (33.3%) out of 30 OC patients.

None PrC patients resulted carriers of VUSs in other genes different from *BRCA1/2* genes.

3.9 Clinical factors comparison between carriers of PV/LPVs in *BRCA1/2* genes, carriers of PV/LPVs in other genes and non-carriers patients

Among BC patients, the median age at diagnosis of carrier patients of PV/LPVs in *BRCA1/2* genes was lower than patients with PV/LPVs in other gene, with a median age of 42 and 44 years respectively (mean age: 43.6 and 46; range: 25-80 and 25-71; p=0.04).

Significant clinico-pathological differences emerged among the three subsets of BCs

(**Table 10**). Moreover, the median age of non-carrier patients was 46 years (mean age: 47.2; range: 21-93). Overall, 53% of carrier patients of PV/LPVs in other genes patients was premenopausal at diagnosis, compared to 63% of carriers of PV/LPVs in *BRCA1/2* genes and 60% of non-carriers. Looking at molecular subtype among carrier patients of PV/LPVs in other genes, 19 (29.2%) had a LA, 19 (29.2%) a LB, 9 (13.8%) a TNBC, none a HER2E and 18 (27.7%) had an unknown molecular type. Moreover, the TNBC molecular subtype have been identified in the 46.5% of carriers of PV/LPVs in *BRCA1/2* genes vs 13.8% of carriers of variants in in other genes (p=0.0003) (**Table 10**) and 26% in non-carriers. As consequence, ER and PR were more frequently lower in carriers of variants in *BRCA1/2* genes compared to carriers of variants in other genes (p<0.00001 calculated for both ER and PR) and even in carriers of variants in other genes compared to non-carriers (p=0.03 calculated for ER and p=0.05 for PR) (**Table 10**). Moreover, carrier patients of PV/LPVs in *BRCA1/2* genes have more frequently a higher Ki-67% (p<0.00001) and histological grade (p<0.00001) than the carriers of PV/LPVs in other genes (**Table 10**). While the carriers of PV/LPVs in other genes showed higher histological grade (p=0.04) than non-carrier patients (**Table 10**). Among carriers of variant in gene beyond *BRCA1/2* genes, 20% was BBC against 14.6% and 13.2% of carriers of variants in *BRCA1/2* genes and non-carriers respectively. The median age of onset between the first and the second tumours was longer in *BRCA1/2* genes PV/LPVs carriers (10 years) and equal in carriers of other variants and in non-carriers (5 years).

Among OC patients, the median age at diagnosis was lower in patients with PV/LPVs in other genes different from *BRCA1/2* gene compared to carriers of variants in *BRCA1/2* genes, 46 and 54 years respectively (mean age: 55 and 52; range: 27-81 and 38-79), but with no statistically relevant scores. Moreover, the median age of non-carriers was 59 years (mean: 59; range: 28-84) and the comparison between non-carriers and carriers of PV/LPVs in other genes showed a younger age at diagnosis in carriers (p=0.02) (**Table 11**). As consequence, carrier patients of variants in genes different from *BRCA1/2* genes have an early onset of 8 years compared to carriers of PV/LPVs in *BRCA1/2* genes and 13 years before non-carriers. Furthermore, OC patients with PV/LPVs in other genes showed more frequently an endometrial histological subtype (45.5%), while the carriers of variants in *BRCA1/2* genes showed in 70.6% of cases a HGSC (p<0.001), while non-carriers present 60.7% of HGSC (p<0.0001) (**Table 11**). The personal history of BC was more relevant in carriers of PV/LPVs in *BRCA1/2* genes and in other susceptibility genes compared to non-carriers, but without statistically significant scores. Overall, 66% of carrier

patients of variants in genes different from *BRCA1/2* genes showed family history of cancers, while 40% of non-carriers OC present similar family history, against 76% in the subset with PV/LPVs in *BRCA1/2* genes.

Among PaC patients, the median age at diagnosis was lower in patients with PV/LPVs in other genes compared to carriers of variants in *BRCA1/2* gene, 57 and 63 years respectively (mean age: 58.2 and 60.3; range: 39-76 and 44-74). Furthermore, the median age of non-carriers is higher (median: 65; mean: 64.5; range: 34-86). As consequence, carrier patients of variants in genes different from *BRCA1/2* genes have an early onset of 6 years compared to carriers of variants in *BRCA1/2* genes and 8 years before non-carriers. Unfortunately, the information about clinical data was poor and further analysis are needed concerning PaC and PrC subsets.

CHAPTER 4

Discussion

Over the years, the PV/LPVs in *BRCA1/2* genes have been deeply investigated and mainly associated to an increased lifetime risk of HBOC and even to other cancers, such as PaC and PrC[1-6]. The genetic testing of the major susceptibility *BRCA1/2* genes turned out to be crucial for subjects who meet the eligibility criteria, assuming a preventive and a predictive meaning[18-20]. In fact, the PV/LPVs in the *BRCA1/2* genes are considered predictive biomarkers of PARPi treatment as maintenance therapy with improvements in PFS an OS[23-25]. The recent approval of FDA and EMA for PARPi treatment in the management of several settings of BC, OC, PaC and PrC patients having a deleterious variant in *BRCA1/2* genes has increased the request for genetic testing[28,29,31,32]. To date the NCCN, the ASCO and the ESMO defined clinical guidelines for the management of high-risk patients[11-15]. However, family history-based testing could not identify about a half of carriers of PV/LPVs in susceptibility genes involved in HBOC, and novel strategies to identify high-risk subjects are under development. Nowadays, clinically validated multi-gene testing is a valid molecular analysis for subjects with a relevant family history of cancer to deeper investigate HBOC related genes[11].

Recent discoveries and advancements in molecular biology techniques have made it possible to integrate the requests of genetic testing conferring a more accurate risk assessment[263]. To date, genetic testing aims to more detailed genetic information assuming a meaning in screening, diagnosis, prognosis and in the choice or development of personalized therapeutic treatment[264]. Moreover, other genes beyond the major susceptibility genes involved in pathways influencing pathogenesis could be disease-causing in several neoplastic inherited disease and such as the involvement of HRR effectors, and their genetic alterations could be representative of a PARPi-based therapy sensitivity[21,22,33,34]. To obtain a more accurate risk assessment, tailored intensive

surveillance programs and targeted therapies, the NGS-based multi-gene panel testing should be performed in specific settings of BC, OC, PaC, and PrC patients with a negative result to genetic testing of *BRCA1/2* genes and with personal and/or family history of cancers[11]. The HBOC syndrome is known to be related to germline PV/LPVs in *BRCA1/2* genes but also to other genes different from the major susceptibility genes, including the high penetrance *TP53, PTEN, STK11, CDH1, ATM, CHEK2, PALB2* genes and other with moderate penetrance, involved in HRR and/or in several pathways crucial in cancer development[36-41]. Due to the need of a more accurate risk assessment and the identification of novel biomarkers of treatment choice, the NGS-based multi-gene panel testing's request is increasingly growing. Moreover, multi-gene panels investigating a wide range of genes involved in different hereditary syndromes, could identify high-risk patients that might be missed[11].

In this observational study, 1878 cancer patients have been included and analysed through the genetic testing of the *BRCA1/2* genes. The cohort included as follows: 1170 BC patients (1116 women and 54 men); 540 OC patients; 144 PaC patients (65 women and 79 men) and 24 PrC patients.

The molecular scores showed: 144 (12.3%) BC patients harbouring PV/LPVs in *BRCA1/2* genes; 109 (20.2%) OC patients carrying germline PV/LPVs in *BRCA1/2* genes and 19 (14.8%) showing somatic deleterious variants in *BRCA1/2* genes; 3 (2.1%) PaC and one (4.2%) PrC patients harbouring germline PV/LPVs in *BRCA1/2* genes.

In BC subset, 144 (12.3%) were carriers of PV/LPVs in *BRCA1/2* genes and the results allowed to emphasize the strong association between carriers of PV/LPVs in *BRCA1* gene and the developing of a TNBC and carriers of PV/LPVs in *BRCA2* gene with LB/HER2-BC, with a different mutational spectrum of variants. The higher percentage of carriers was among TNBCs (46.5%). Our molecular scores agree with the literature, in fact, the TNBC molecular subtypes is known to be the most aggressive and it is associated to PV/LPVs in *BRCA1* gene[5,265]. Overall, 13.1% of all BBCs were carriers of PV/LPVs in *BRCA1/2* genes. Moreover, the median time of occurrence between the first and the second tumour was significantly lower among non-carriers luminal-like BCs than carriers and even than TNBCs[258].

Although no statistically relevant association is emerged between a specific PV/LPV and the molecular subtype, the molecular scores reported a higher frequency among TNBCs of the c.4964_4982del, *BRCA1* and the c.514del, *BRCA1* in 8 and 7 families respectively, against 5 and 4 families inheriting the variants in LB patients. The c.4964_4982del,

BRCA1 and the c.514del, *BRCA1* have been observed in TNBC younger women than in LB molecular subtype and with higher Ki-67 and histological grade. Both the PVs of the *BRCA1* gene have been observed with high frequency in Sicilian population[5]. Furthermore, the c.4964_4982del, *BRCA1* have been observed in several families with BCs and OCs and it was described as founder PV in Calabrian/Southern Italian population[266] but, nowadays, it is reported as a potential founder PV of Sicilian population[246]. The c.1238del, *BRCA2* has been identified in luminal-like tumours with a high frequency, 8 families vs only 2 among TNBCs, according to literature data[258]. In our cohort, the variant has been identified in LA/LB patients HER2- and with higher expression of ER and 2 BBCs with onset <40 years old. Overall, most variants were Fs in both *BRCA1* and *BRCA2* genes, regardless of molecular subtype, and a higher frequency of IVS were observed in *BRCA2* gene in luminal-like BCs, according to the literature[258].

Furthermore, patients harbouring PV/LPVs in *BRCA1* gene developed a disease before than carriers of PV/LPVs in *BRCA2* gene (mean age 41,7 vs 45,7 years) and non-carriers (mean age 47,2 years), with the TNBC patients showing an earlier age of onset than the other subsets. A statistically significant difference emerges in mean age at diagnosis between TNBC patients harbouring PV/LPVs in *BRCA1/2* genes and non-carrier patients, with the carriers showing an earlier age at diagnosis (p=0.0005). Moreover, LA/LB/HER2 patients harbouring PV/LPVs in *BRCA1/2* genes compared to non-carriers showed an earlier age at diagnosis (p=0.05). This study shows that carriers patients of variants in *BRCA1/2* genes involve frequently younger women, as reported in literature[267].

According to *Rebbeck et. al*[255], the type and genetic location of each variant have been recorded to identify a potential genotype/phenotype correlation. Molecular results showed the generation of clusters in both the sequences of the *BRCA1* and *BRCA2* genes, with TNBC showing 10 variants in exon 11 of *BRCA1* gene and luminal-like tumours with 9 variants in BRC repeats into the exon 11 of *BRCA2* gene, emphasizing the crucial role of these repeats in onco-suppressor's function[268-270]. In this study have been analysed also the putative BCCRs and OCCRs emerging in BC cohort the potential association between LB tumours and 11 variants of *BRCA2* gene (20 patients) into the BCCRs[255,271].

The mutational background assumes a preventive and prognostic value, but also a predictive meaning of treatment choice[264]. To date, ER, PR expression and HER2E overexpression are crucial for BC patients who could benefits from endocrine therapy[272]. However, the lack of these biomarkers in TNBCs determine chemotherapy as the standard treatment, involving anthracycline and taxane[273]. Furthermore, in carriers of PV/LPVs in

BRCA1/2 genes have been demonstrate the efficacy of platinum-based agents and PARPi[61].

In OC subset the molecular data emphasizes how women harbouring a variant in *BRCA1* gene developed with almost twice frequency the disease, than carriers of PV/LPVs in *BRCA2* gene (66.1% vs 33.9%), according to the literature[274].

The typology and gene location of each variant have been recorded, with a BCs vs OCs comparison, aiming to identify a potential association between a variant and a specific disease, but without a specific association.

The molecular scores reported 109 (20.2%) carriers of germline variants in *BRCA1/2* genes. The results showed a higher frequency of the c.4964_4982del, *BRCA1* and the c.514del, *BRCA1* observed respectively in 15 and 9 families. The c.4964_4982del, *BRCA1* has been observed in bilateral OC patients with a mean age of 51 years old (median: 50; range: 40-69) all with FIGO stage III and histological grade III, all with HGSCs. The c.514del, *BRCA1* has been observed in women with a mean age of 55 years (median: 61; range: 35-70), with HGSC and 50% of patients had a bilateral OCs with FIGO III and histological grade III. Moreover, it has been identified with low frequency the c.4963T>G, *BRCA1* in 3 patients with bilateral OC, histological grade III, in spite is reported as “not provided” on ClinVar[251] and as Likely Pathogenic on VarSome[252]. This type of M variant could be considered disease-causing[6]. Overall, most variants were Fs in both *BRCA1* and *BRCA2* genes. Moreover, the type and genetic location of variants have been recorded and it emerges in both *BRCA1* and *BRCA2* genes the distribution in cluster, with a more evidence of 20 variants in exon 11 of *BRCA1* gene and 14 variants in BRC repeats into the exon 11 of *BRCA2* gene, confirming the crucial role of these regions in cancer suppression[255].

Furthermore, the OC patients showing PV/LPVs in *BRCA1/2* gene had an earlier onset of disease with a mean age of 56 years, with a difference between carriers of variants in *BRCA1* and *BRCA2* gene, 53 vs 59 years respectively. On the other hand, non-carriers showed a mean age of 59 years. It emerges how OCs with *BRCA1* genes germline alterations had an earlier onset compared with carriers of PV/LPVs in *BRCA2* gene and non-carriers, with the exception for bilateral OCs. The age group with PV/LPVs was mainly involving women with 51-60 years old. The comparison between the age group distribution in carriers and non-carriers resulted statistically significant with an earlier onset in carrier women (p=0.02).

Moreover, the carrier of PV/LPVs in *BRCA1/2* genes showed a higher percentage of

endometroid, and papillary OCs (10.9% and 7%, respectively) compared to non-carriers (6.25% and 2.3%, respectively) (p=0.04), according to the literature[275]. The 71.9% of non-carrier patients showed HGSCs. Overall, 29.8% of bilateral OC cases had a PV/LPV in *BRCA1/2* genes. Furthermore, carriers of variants in *BRCA1* gene seemed to have an earlier onset compared to carriers of PV/LPVs in *BRCA2* gene (mean age of 51 and 54 years respectively) and non-carriers (mean age 57 years).

About the association with the putative BCCRs and OCCRs, in OC cohort emerges the association in 14 variants of *BRCA1* gene and 8 variants of *BRCA2* gene into the OCCRs[255].

In PaC subset the molecular scores reported 2.1% of all as carriers of variants in *BRCA2* gene. The carrier patients were all men with a mean age of 60.3 years, with two patients having a PDAC and one a cribriform PaC, according to the literature[276]. The identified PV/LPVs in the PaC subset were the c.6990_6994del, *BRCA2*, identified only in PaCs, the c.7681C>T, *BRCA2*, and the c.8487+1G>A, *BRCA2*, all located in the *BRCA2* sequence encoding the DNA binding domain, highlighting the role of the highly conserved DNA-binding domain (DBD) for the onco-suppressor function[146]. In PrC subset the molecular scores reported only one patient (4.2%) of 34 years as carriers of PV/LPVs in *BRCA1/2* genes. The variant was the nonsense c.3545_3546del, *BRCA2* located into the the BRC repeats of the exon 11. Unfortunately, due to the poor information available, more in-depth analyses are needed to make clinico-pathological correlations.

Subsequently, 527 (28%) patients have been selected for the NGS-based multi-gene panel testing, according to the multidisciplinary team, revealing 15.7% of carriers of PV/LPVs in other susceptibility genes. The multi-gene panel involved high- and moderate-penetrance genes predisposing to different hereditary cancer syndrome.

The analysis scores reported that 65 (15.4%) of the 422 analyzed BC patients, 11 (15.7%) of the 70 analyzed OC patients, 7 (23.3%) of the 30 analyzed PaC patients and none PrC patients harbored PV/LPVs in other genes.

Among BC patients, the median age at diagnosis of carrier patients of PV/LPVs in *BRCA1/2* genes resulted lower than patients with PV/LPVs in other gene, with a median age of 42 and 44 years respectively (p=0.04). Moreover, the median age of non-carrier patients was 46 years. Moreover, statistically relevant was the presence of TNBCs identified in the 46.5% of carriers of PV/LPVs in *BRCA1/2* genes vs 13.8% of carriers of variants in in other genes (p=0.0003), while about the 60% of BCs with genetic alterations in gene different from *BRCA1/2* genes were LA/LB. The most frequent variant was the

c.1145G>A, *MUTYH* identified in 7 BC and 4 OC patients. This M variants is reported in literature as associated to MUTYH-Associated Polyposis and able to damage the effector protein[262]. Among BC patients harboring variants in other genes, the most involved genes were *MUTYH* and *CHEK2* genes (24.6% and 21.5%, respectively). Moreover, the c.1229del, *CHEK2*, known as related to several cancer among which BC, OC and PrC, has been observed in two BBCs LB/HER2-[51,277,278]. Although in literature *PALB2* is known as highly predisposing to BC, in our analysis 10.8% of PV/LPVs were in *PALB2* gene in patients with TNBC and LB tumours among which two BBCs[261]. Overall, 20% were BBCs with a higher frequency compared to carriers of PV/LPVs in *BRCA1/2* genes and non-carriers, even with a time of occurrence between the first and the second tumor compared to non-carriers. BBCs presented mainly aberrant variants in *MUTYH* and *ATM* genes (both 23%) and with a lower frequency in *CHEK2*, *PALB2* and *RAD51C* genes (8.7% for each gene) emerging the potential association with HRR genes. Overall, more than a half of BC carrier patients (59.1%) showed PV/LPVs in HRR genes.

In OC subset, the earlier onset was in carrier patients of PV/LPVs in other genes compared to carriers of variants in *BRCA1/2* genes and non-carriers (median age: 46 vs 54 vs 59 respectively). Among OC patients harboring variants in other genes, the most involved genes were *MUTYH* gene accounting for 45.5% of identified variants and 4 of them were the monoallelic c.1145G>A, *MUTYH*. This variant has been identified in 3 HGSC, with FIGO stage III and grading III, and one endometrioid carcinoma, with FIGO stage II and grading II, having a strong family history for several cancers among whing colon cancer, according to the literature[279]. Among OC carrier patients of PV/LPVs in other genes, 6 (54.5%) out of 11 were bilateral OCs. Overall, a half of carrier patients of variants in other genes (54.5%) showed PV/LPVs in HRR genes, often identified simultaneously with the c.1145G>A, *MUTYH*, and 27.3% of carriers showed PV/LPVs in MMR genes, as reported in literature[64,66]. Moreover, OC patients with PV/LPVs in other genes showed with a higher frequency an endometrial histological subtype (45.5%), than the carriers of variants in *BRCA1/2* genes showed in 70.6% of cases a HGSC ($p<0.001$), and the non-carriers present 60.7% of HGSC ($p<0.0001$).

Overall, 66% of patient harbouring PV/LPVs in genes beyond *BRCA1/2* genes had family history of cancers, while 40% of non-carriers OCs had family history, in comparison to 76% of carriers of PV/LPVs in *BRCA1/2* genes.

In PaC emerges a younger age at diagnosis in carriers of PV/LPVs in other genes than carriers of PV/LPVs in *BRCA1/2* genes and non-carriers (median age: 57 vs 63 vs 65

respectively). Among PaC patients harboring variants in other genes have been identified 3 PV/LPVs in *ATM* gene, in affected women with PDAC of the pancreatic head and with personal and/or family history of BC and/or OC (42.9%). Overall, 57.1% of carrier patients showed PV/LPVs in HRR genes (3 in *ATM* gene and one in *PALB2* gene), according to literature[280].

In conclusion, this research emphasizes that the BC carriers of PV/LPVs in *BRCA1* gene developed mainly a TNBC and carriers of PV/LPVs in *BRCA2* gene a LB/HER2 BC, with a not overlapping mutational spectrum among molecular subtypes, with the carrier TNBCs showing the vast majority of PV/LPVs and the earlier onset. No statistically relevant association has emerged between specific PV/LPVs and the molecular subtype.

In OC subset has been observed how women harbouring a variant in *BRCA1* gene developed with almost twice frequency the disease, than carriers of PV/LPVs in *BRCA2* gene. No statistically relevant association has emerged between specific PV/LPVs and the disease. Overall, 144 (12.3%) BCs and 109 (20.2%) OCs resulted carriers of gemline variants in *BRCA1/2* genes and both the diseases showed the same more frequent variants, c.4964_4982del, *BRCA1* and the c.514del, *BRCA1* observed in a total of 28 and 20 families of our cohort.

Moreover, 15.4% of BC patients, 15.7% of OC patients, 23.3% of PaC patients showed PV/LPVs in genes beyond *BRCA1/2* genes. In total, the involvement of the PV/LPVs in HRR genes was 59.1% in BCs, 54.5% in OCs and 57.1% in PaCs of all the carriers of PV/LPVs in other genes. The molecular findings highlighted the crucial role of multi-gene panel testing that should be performed in BC, OC, PaC and PrC resulting negative to genetic testing of *BRCA1/2* genes, but with significant personal and/or family history of cancers. The molecular investigation through multi-gene panel allows to better understand the molecular background of hereditary cancers. However, further analysis are needed to obtain a more accurate risk assessment, to establish univocal and tailored intensive surveillance programs and/or risk reduction strategies and to develop novel strategies on therapeutic field.

CHAPTER 5

Tables and Figures

Table 1: *Eligibility criteria to BRCA1/2 genes genetic test in BC, OC, PrC and PrC patients.*

Personal history of:
1. Male BC
2. Woman with BC and OC
3. Woman with BC < 36 y.o.
4. Woman with Triple-Negative BC < 60 y.o.
5. Woman with bilateral BC at the age of 50 y.o.
6. Woman with OC not mucinous and not borderline at every age
7. Metastatic PDAC
8. Metastatic PrC
Personal history of BC at 50 y.o. e familiarity in fist degree relatives [a, b] for:
- BC < 50 y.o.
- OC not mucinous and not borderline at every age
- BBC
- Male BC
- Locally advanced or metastatic PaC
- Metastatic PrC
Personal history of BC > 50 y.o. and familiarity for BC, OC, metastatic PrC or locally advanced or metastatic PaC in 2 or more first degree relatives [a, b] between them (of which one of first degree with her [a, b])
Personal history of PrC and familiarity:
At least one first degree relatives with PrC non-Grade Group [1c] < 60 y.o.
At least 2 members of family with PrC non-Grade Group [1c] < 50 y.o.
Personal history of PaC and familiarity:
At least 2 first degree relatives [d] with PDAC
At least 3 family members with PDAC [e]
In presence of genetic testing eligibility criteria for hereditary syndromes with an increased risk of PaC
Family history of known inherited PV/LPV in a disease-causing gene

[a] *First-degree relatives= parents, brothers/sisters and son;* [b] *For BC and OC, in the paternal side of the family, it must include also second degree relatives (grandmother, aunts);* [c] *Grade Group 1 according to ISUP;* [d] *The condition not include both affected parents in present or past;* [e] *In the same blood line and with at* least *one first degree relatives. y.o.= years old* [93,281].

Table 2: *The detection rate of the PV/LPVs in BRCA1/2 genes in LA, LB, HER2E and TNBC patients.*

Molecular subtype	Total	*BRCA1*	*BRCA2*	No PV/LPVs	p value *
LA	246	0 (0%)	9 (3.7%)	237 (96.3%)	
LB	411	17 (4.1%)	35 (8.5%)	358 (87.1%)	
HER2E	40	1 (2.5%)	2 (5%)	37 (92.5%)	*p <0.00001*
TNBC	326	48 (14.7%)	18 (5.5%)	259 (79.4%)	
Unknown	147	6 (4%)	6 (4%)	135 (92%)	

One TNBC and one LB proband who showed double heterozygosity for BRCA1 and BRCA2 genes are not reported in Table 1.

**Comparison between carriers of PV/LPVs in BRCA1 gene vs carriers of PV/LPVs in BRCA2 gene vs BRCA1/2-wild type.*

Abbreviations: LA= Luminal A; LB= Luminal B; HER2E=Her2-enriched; TNBC=Triple Negative Breast Cancer; PV/LPVs= Pathogenic/ Likely Pathogenic Variants.

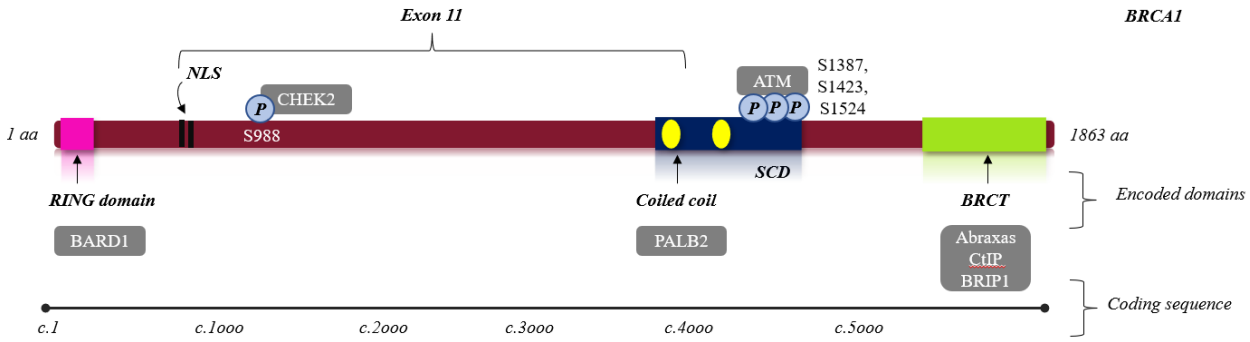


Figure 1: *Breast cancer 1, BRCA1.*

Exons: 24. Coding exons: 23. Transcript length: 7,094 bps. Translation length: 1,863 residues. BRCA1 protein structure is composed by a RING domain to interact with BARD1; the NLSs allowing the translocation from cytoplasm to nucleus; a serine residue phosphorylated by CHEK2; a coiled coil C-terminal domain to interact with PALB2; a BRCT domain to bind phosphorylated proteins; the SCD interacting with ATM (10.1038/nrc3181).

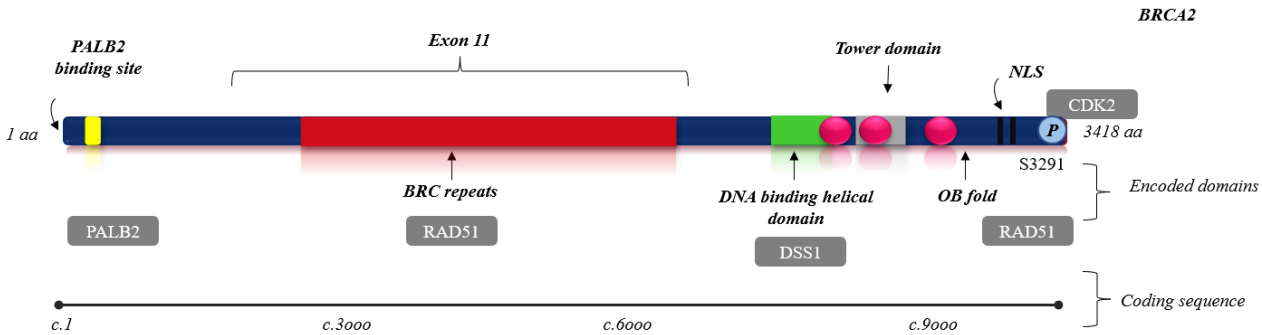


Figure 2: *Breast cancer 2, BRCA2.*

Exons: 27. Coding exons: 26. Transcript length: 10,930 bps. Translation length: 3,418 residues. BRCA2 protein structure is composed by the N-terminal domain containing the PALB2 binding site; eight BRC repeats in the central region of the protein useful to bind RAD51; a DNA binding domain; three Oligonucleotide Binding (OB) domain and a tower domain useful to stimulate the BRCA2 binding to ssDNA and/or dsDNA; the C-terminal NLS and a phosphorylation site for the cyclin-dependent kinase 2 (CDK2) able to bind RAD51 (10.1038/nrc3181).

Table 3: *The PV/LPVs of BRCA1/2 genes in TNBC patients.*

Gene	Type of variant	Molecular consequence	HGVS Nomenclature	Protein change	No. Probands	BCCR OCCR
BRCA1	Deletion	Fs	c.4964_4982del	p.Ser1655fs	8 (11.9%)	\
BRCA1	Deletion	Fs	c.514del	p.Gln172fs	7 (10.4%)	\
BRCA1	SNV	NS	c.3904G>T	p.Glu1302Ter	4 (6%)	OCCR
BRCA1	Deletion	Fs	c.798_799del	p.Ser267fs	3 (4.5%)	\
BRCA1	Duplication	Fs	*c.5266dup	p.Gln1756fs	3 (4.5%)	BCCR2'
BRCA1	Duplication	Fs	c.66dup	p.Glu23fs	2 (3%)	\
BRCA1	Deletion	NS	c.1360_1361del	p.Glu453_Ser454insTer	2 (3%)	\
BRCA1	Deletion	Fs	c.3228_3229del	p.Gly1077fs	2 (3%)	OCCR
BRCA1	SNV	NS	c.3400G>T	p.Glu1134Ter	2 (3%)	OCCR
BRCA1	IVS	/	c.134+2T>C	/	1 (1.5%)	\
BRCA1	SNV	NS	c.303T>G	p.Tyr101Ter	1 (1.5%)	BCCR1
BRCA1	Deletion	Fs	c.882del	p.Asp295fs	1 (1.5%)	\
BRCA1	Deletion	Fs	c.1531del	/	1 (1.5%)	OCCR
BRCA1	SNV	NS	c.2722G>T	p.Glu908Ter	1 (1.5%)	OCCR
BRCA1	Deletion	Fs	c.3266del	p.Leu1089fs	1 (1.5%)	OCCR
BRCA1	Deletion	Fs	c.3599_3600del	p.Gln1200fs	1 (1.5%)	OCCR
BRCA1	IVS	/	c.4096+3A>G	/	1 (1.5%)	\
BRCA1	Deletion	Fs	c.4137_4138del	p.Glu1380fs	1 (1.5%)	\
BRCA1	SNV	NS	c.4327C>T	p.Arg1443Ter	1 (1.5%)	\
BRCA1	Deletion	Fs	c.5030_5033del	p.Thr1677fs	1 (1.5%)	\
BRCA1	SNV	M	c.5123C>A	p.Ala1708Glu	1 (1.5%)	\
BRCA1	SNV	NS	c.5161C>T	p.Gln1721Ter	1 (1.5%)	\
BRCA1	LGR	/	c.-232_4675del	/	1 (1.5%)	-
BRCA1	LGR	/	17q21.31 (41226308-41226571)x1	/	1 (1.5%)	-
BRCA1	LGR	/	17q21.31 (41219595-41223282)x1	/	1 (1.5%)	-
BRCA2	Deletion	Fs	c.1238del	p.Leu413fs	2 (3%)	BCCR1'
BRCA2	Duplication	NS	c.1842dup	p.Asn615Ter	2 (3%)	\
BRCA2	IVS	/	*c.1909+1G>A	/	2 (3%)	\
BRCA2	Deletion	Fs	c.5851_5854del	p.Ser1951fs	2 (3%)	\
BRCA2	SNV	NS	c.396T>A	p.Cys132Ter	1 (1.5%)	BCCR1
BRCA2	IVS	/	c.476-2A>G	/	1 (1.5%)	BCCR1
BRCA2	IVS	/	c.517-2del	/	1 (1.5%)	BCCR1
BRCA2	SNV	NS	c.523C>T	p.Gln175Ter	1 (1.5%)	BCCR1
BRCA2	Deletion	Fs	c.1813del	p.Ile605fs	1 (1.5%)	\
BRCA2	Deletion	Fs	c.3847_3848del	p.Val1283fs	1 (1.5%)	OCCR1
BRCA2	Duplication	Fs	c.4284dup	p.Gln1429fs	1 (1.5%)	OCCR1
BRCA2	Deletion	Fs	c.6082_6086del	p.Glu2028fs	1 (1.5%)	\
BRCA2	IVS	/	c.8487+1G>A	/	1 (1.5%)	BCCR2
BRCA2	IVS	/	c.8954-15T>G	/	1 (1.5%)	\
BRCA2	Deletion	Fs	c.9455_9456del	p.Glu3152fs	1 (1.5%)	\

**These PV/LPVs are present in the same proband showing double heterozygosity for BRCA1 and BRCA2 genes.*

Abbreviations: TNBC=Triple Negative Breast Cancer; PV/LPVs= Pathogenic/ Likely Pathogenic Variants; SNV= Single Nucleotide Variant; IVS= Intronic Variant Sequence; Fs= Frameshift; M= Missense; NS= Nonsense; BCCR= Breast Cancer Cluster Region; OCCR= Ovarian Cancer Cluster Region.

Table 4: *The PV/LPVs of BRCA1/2 genes in LA/LB/HER2E patients.*

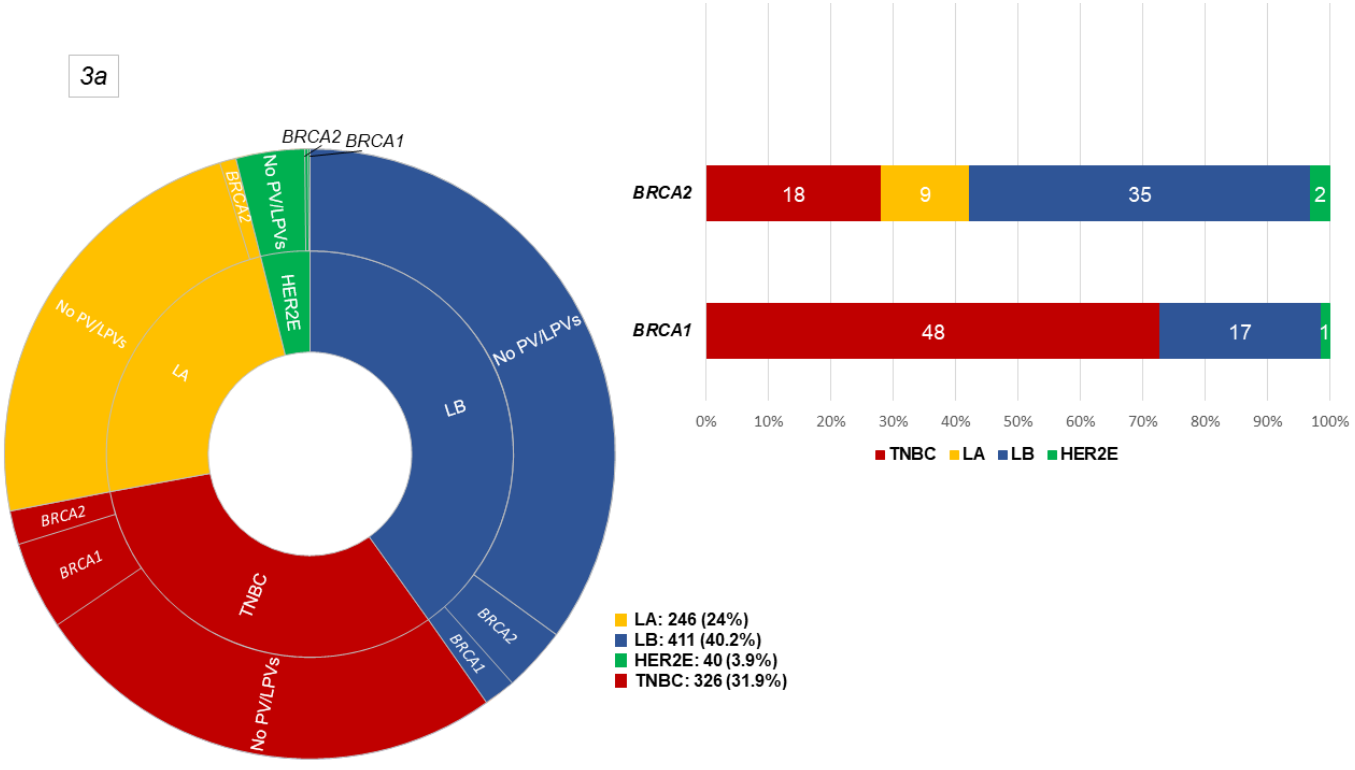
LB						
Gene	**Type of variant**	**Molecular consequence**	**HGVS Nomenclature**	**Protein change**	**No. Probands**	**BCCR OCCR**
BRCA1	Deletion	Fs	c.4964_4982del	p.Ser1655fs	5 (7.7%)	\
BRCA1	Deletion	Fs	c.514del	p.Gln172fs	4 (6.2%)	\
BRCA1	Deletion	Fs	c.66_67del	p.Glu23fs	1 (1.5%)	\
BRCA1	SNV	M	*c.181T>G	p.Cys61Gly	1 (1.5%)	BCCR1
BRCA1	Deletion	Fs	c.798_799del	p.Ser267fs	1 (1.5%)	\
BRCA1	SNV	NS	c.2722G>T	p.Glu908Ter	1 (1.5%)	OCCR
BRCA1	Deletion	Fs	c.3228_3229del	p.Gly1077fs	1 (1.5%)	OCCR
BRCA1	SNV	NS	c.3400G>T	p.Glu1134Ter	1 (1.5%)	OCCR
BRCA1	SNV	NS	c.3904G>T	p.Glu1302Ter	1 (1.5%)	OCCR
BRCA1	SNV	M	c.5096G>A	p.Arg1699Gln	1 (1.5%)	\
BRCA1	LGR	/	17q21.31 (41226308-41226571)x1	/	1 (1.5%)	\
BRCA2	Deletion	Fs	c.1238del	p.Leu413fs	7 (10.8%)	BCCR1'
BRCA2	SNV	NS	c.7681C>T	p.Gln2561Ter	3 (4.6%)	BCCR2
BRCA2	IVS	/	c.476-2A>G	/	2 (3.1%)	BCCR1
BRCA2	Deletion	Fs	c.5851_5854del	p.Ser1951fs	2 (3.1%)	\
BRCA2	SNV	M	c.7007G>A	p.Arg2336His	2 (3.1%)	OCCR2
BRCA2	Duplication	Fs	c.9253dup	p.Thr3085fs	2 (3.1%)	\
BRCA2	SNV	NS	c.93G>A	p.Trp31Ter	1 (1.5%)	BCCR1
BRCA2	SNV	NS	c.97G>T	p.Glu33Ter	1 (1.5%)	BCCR1
BRCA2	SNV	M	**c.631G>A	p.Val211Ile	1 (1.5%)	\
BRCA2	Duplication	NS	c.1842dup	p.Asn615Ter	1 (1.5%)	\
BRCA2	Deletion	Fs	c.1472del	p.Thr491IlefsTer18	1 (1.5%)	BCCR1'
BRCA2	Deletion	Fs	c.2808_2811del	p.Ala938fs	1 (1.5%)	\
BRCA2	Deletion	Fs	c.5595_5596del	p.Phe1866fs	1 (1.5%)	\
BRCA2	SNV	NS	c.5959C>T	p.Gln1987Ter	1 (1.5%)	\
BRCA2	Deletion	Fs	c.6082_6086del	p.Glu2028fs	1 (1.5%)	\
BRCA2	SNV	NS	c.6124C>T	p.Gln2042Ter	1 (1.5%)	\
BRCA2	Deletion	Fs	c.6591_6592del	p.Glu2198fs	1 (1.5%)	\
BRCA2	IVS	/	**c.7008-2A>T	/	1 (1.5%)	OCCR2
BRCA2	Deletion	NS	c.7910_7914del	p.Ala2637_Phe2638insTer	1 (1.5%)	BCCR2
BRCA2	IVS	/	*c.8331+2T>C	/	1 (1.5%)	BCCR2
BRCA2	IVS	/	c.8487+1G>A	/	1 (1.5%)	BCCR2
BRCA2	IVS	/	c.8632+2T>C	/	1 (1.5%)	BCCR2
BRCA2	IVS	/	c.8754+4A>G	/	1 (1.5%)	BCCR2
BRCA2	Deletion	Fs	c.9026_9030del	p.Tyr3009fs	1 (1.5%)	\
BRCA2	Deletion	Fs	c.9455_9456del	p.Glu3152fs	1 (1.5%)	\
LA						

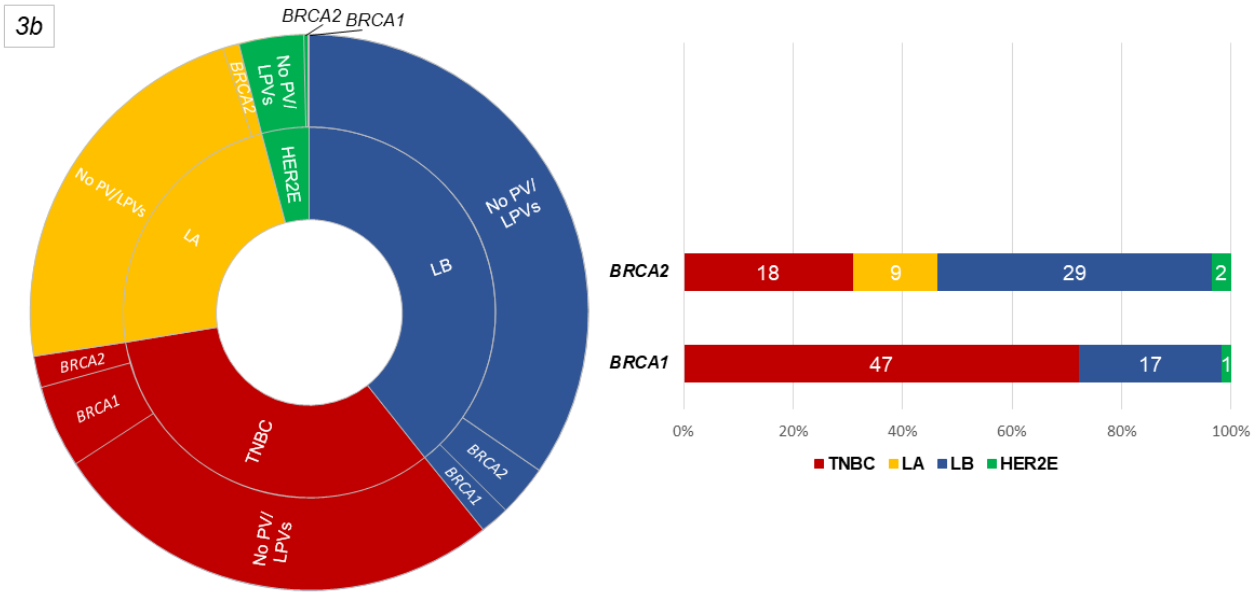
Gene	Type of variant	Molecular consequence	HGVS Nomenclature	Protein change	No. Probands	BCCR OCCR
BRCA2	Deletion	Fs	c.1238del	p.Leu413fs	1 (1.5%)	BCCR1'
BRCA2	Deletion	Fs	c.1813del	p.Ile605fs	1 (1.5%)	\
BRCA2	Duplication	NS	c.1842dup	p.Asn615Ter	1 (1.5%)	\
BRCA2	Deletion	Fs	c.3860del	p.Asn1287fs	1 (1.5%)	OCCR1
BRCA2	Duplication	Fs	c.5073dup	p.Trp1692fs	1 (1.5%)	OCCR1
BRCA2	Deletion	Fs	c.5722_5723del	p.Leu1908fs	1 (1.5%)	\
BRCA2	IVS	/	c.8487+1G>A	/	1 (1.5%)	BCCR2
BRCA2	IVS	/	c.8754+4A>G	/	1 (1.5%)	BCCR2
BRCA2	Deletion	Fs	c.9026_9030del	p.Tyr3009fs	1 (1.5%)	\
HER2E						
Gene	**Type of variant**	**Molecular consequence**	**HGVS Nomenclature**	**Protein change**	**No. Probands**	**BCCR OCCR**
BRCA1	Duplication	Fs	c.5266dup	p.Gln1756fs	1 (1.5%)	BCCR2'
BRCA2	Deletion	Fs	c.5073del	p.Lys1691fs	1 (1.5%)	OCCR1
BRCA2	Deletion	Fs	c.7679_7680del	p.Phe2560fs	1 (1.5%)	BCCR2

**These PV/LPVs are present in the same proband showing double heterozygosity for BRCA1 and BRCA2 genes.*

Abbreviations: LB= Luminal B; LA= Luminal A; HER2E=Her2-enriched; PV/LPVs= Pathogenic/ Likely Pathogenic Variants; SNV= Single Nucleotide Variant; IVS= Intronic Variant Sequence; Fs= Frameshift; M= Missense; NS= Nonsense; BCCR= Breast Cancer Cluster Region; OCCR= Ovarian Cancer Cluster Region.

3a





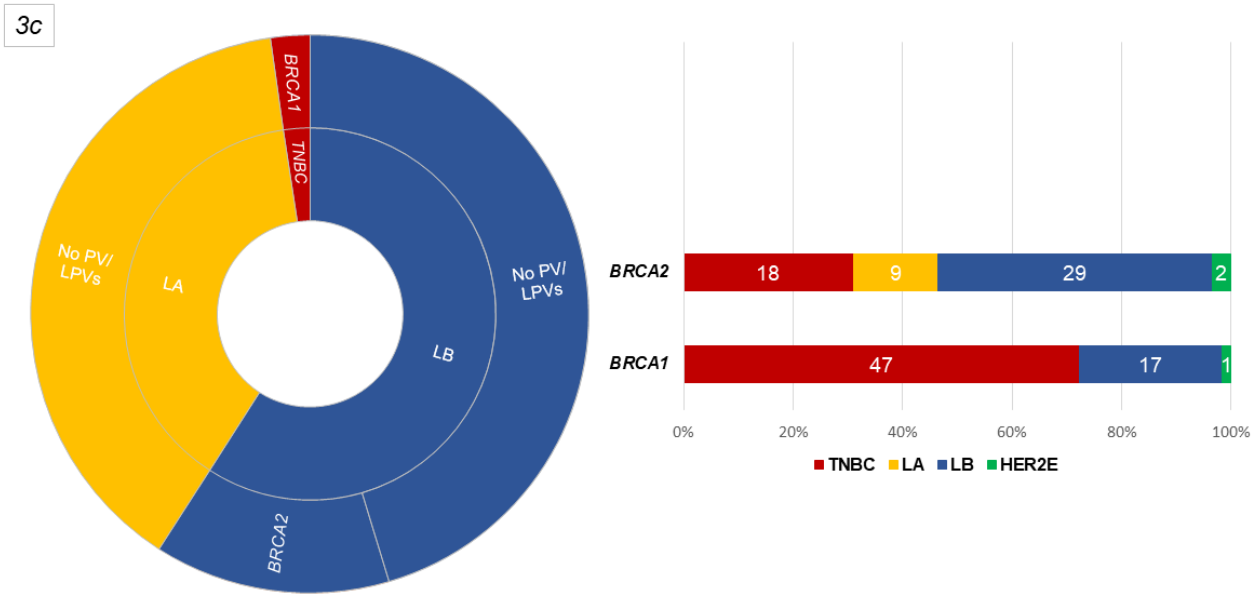


Figure 3: *a) The PV/LPVs of BRCA1/2 genes detection rate among the molecular subtypes. b) The PV/LPVs of BRCA1/2 genes detection rate among women. c) The PV/LPVs of BRCA1/2 genes detection rate among men.*

Abbreviations: LA= Luminal A; LB= Luminal B; HER2E=Her2-enriched; TNBC=Triple Negative Breast Cancer; PV/LPVs= Pathogenic/ Likely Pathogenic Variants.

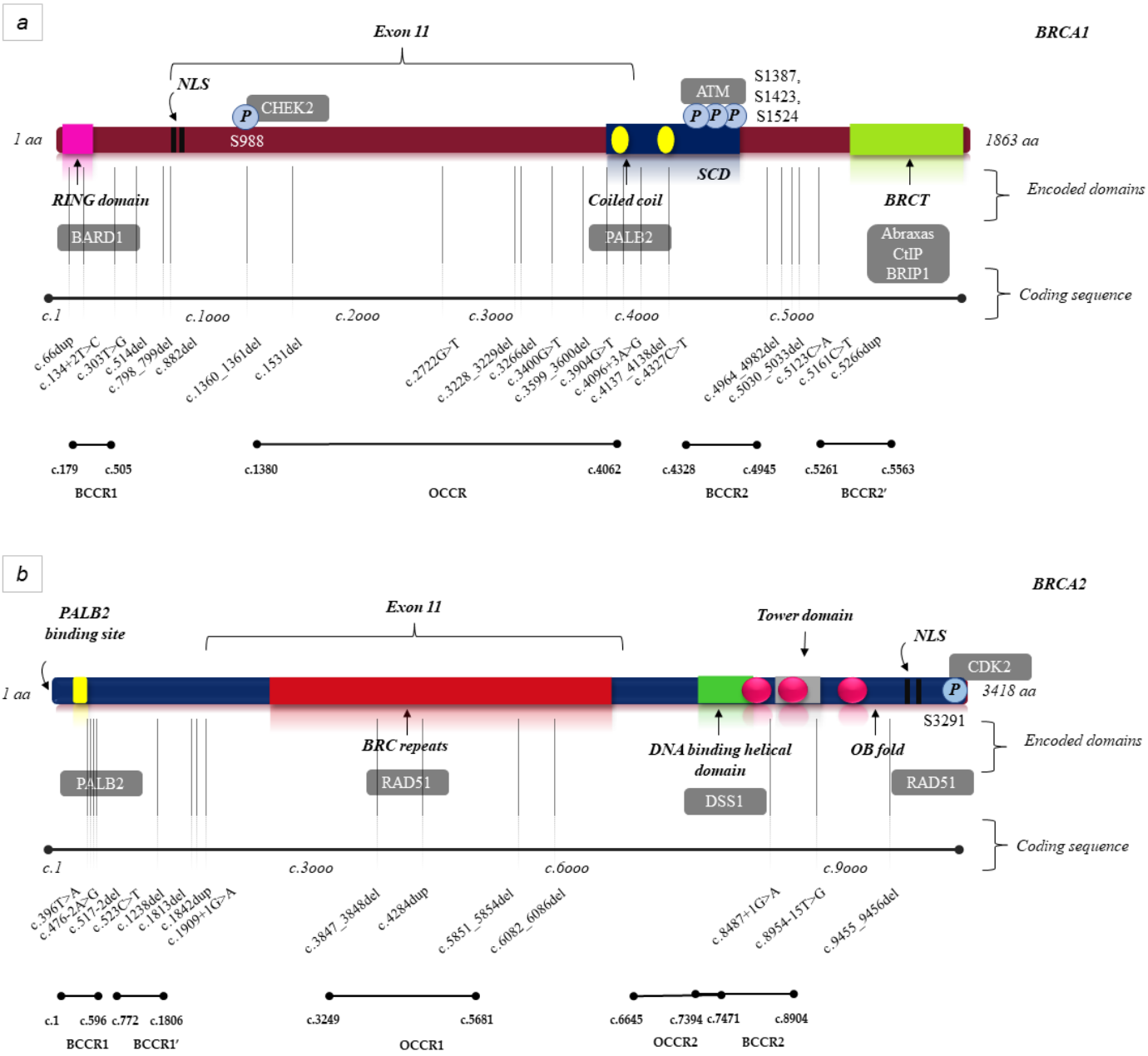


Figure 4: *Genetic location of PV/LPVs in BRCA1/2 genes among TNBC patients; a) PV/LPVs identified in BRCA1 gene sequence; b) PV/LPVs identified in BRCA2 gene sequence.*

Abbreviations: TNBC=Triple Negative Breast Cancer; PV/LPVs= Pathogenic/ Likely Pathogenic Variants; BCCR= Breast Cancer Cluster Region; OCCR= Ovarian Cancer Cluster Region; RING= Really Interesting New Gene; NLS= Nuclear Localization Sequence; BRCT= BRCA1 C-terminus domain; SCD= Serine Cluster Domain; OB= Oligonucleotide Binding.

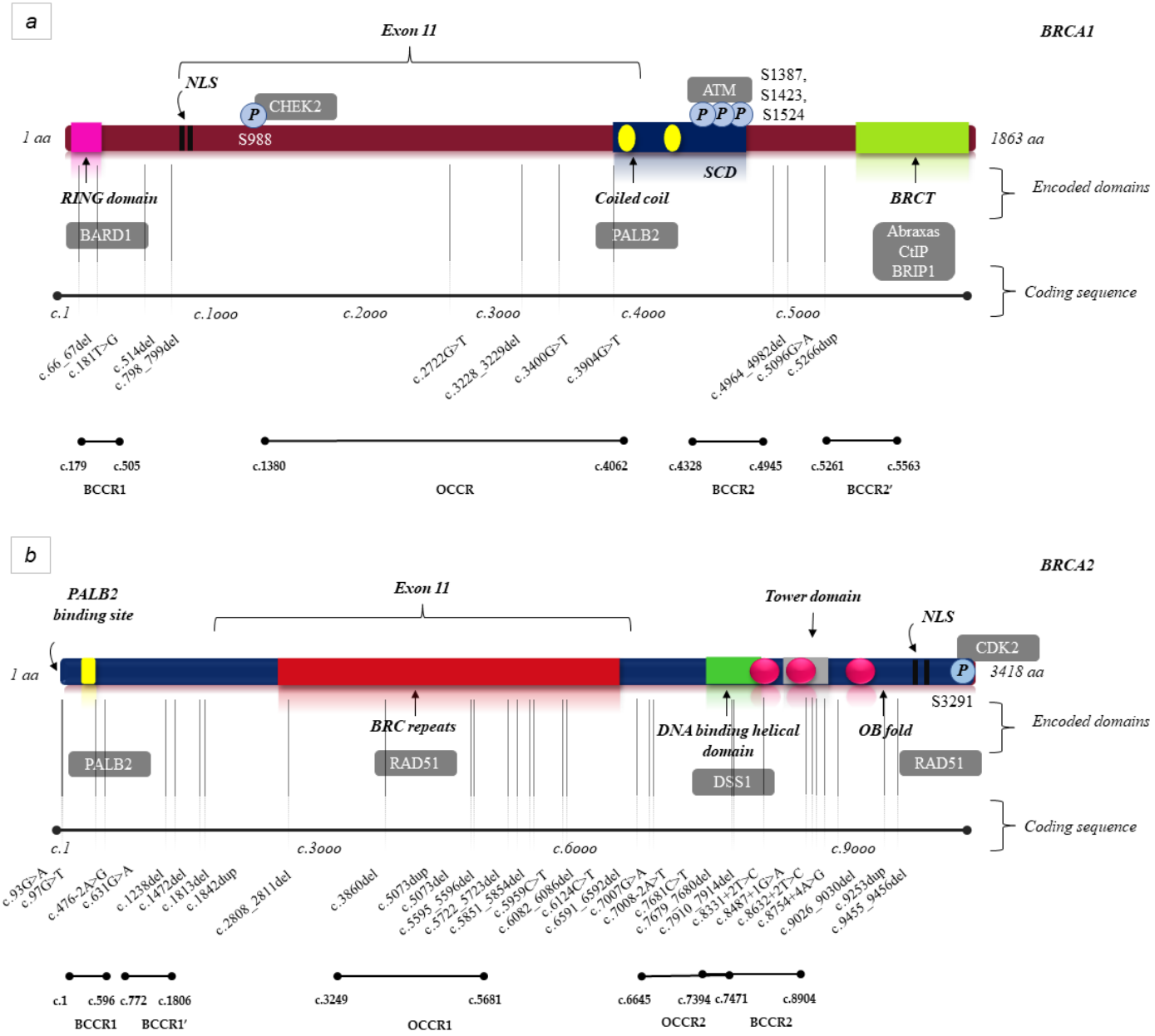


Figure 5: *Genetic location of PV/LPVs in BRCA1/2 genes among LA/LB/HER2E patients; a) PV/LPVs identified in BRCA1 gene sequence; b) PV/LPVs identified in BRCA2 gene sequence.*

Abbreviations: LA= Luminal A; LB= Luminal B; HER2E=Her2-enriched; PV/LPVs= Pathogenic/ Likely Pathogenic Variants; BCCR= Breast Cancer Cluster Region; OCCR= Ovarian Cancer Cluster Region; RING= Really Interesting New Gene; NLS= Nuclear Localization Sequence; BRCT= BRCA1 C-terminus domain; SCD= Serine Cluster Domain; OB= Oligonucleotide Binding.

Table 5: *Baseline features and clinico-pathological information of TNBC and LA/LB/HER2E BCs.*

	TNBC*		LA/LB/HER2E BCs**		*P value	**P value
	WT	***BRCA1/2***	**WT**	***BRCA1/2***		
Number of patients (1023)	259 (79.4%)	67 (20.6%)	632 (90.7%)	65 (9.3%)	-	-
Age groups (y) ≤40 41-50 51-60 >60	 42 (16.2%) 91 (35.1%) 88 (34%) 38 (14.7%)	 23 (34.3%) 28 (41.8%) 9 (13.4%) 7 (10.4%)	 230 (36.4%) 229 (36.2%) 102 (16.1%) 71 (11.2%)	 34 (52.3%) 16 (24.6%) 11 (16.9%) 4 (6.2%)	0.0005	0.05
Histological Subtype Ductal Lobular Others unknown	131 (50.6%) 3 (1.15%) 3 (1.15%) 122 (47.1)	29 (43.3%) 1 (1.5%) 1 (1.5%) 36 (53.7%)	496 (78.5%) 61 (9.7%) 32 (5.1%) 43 (6.8%)	54 (83.1%) 3 (4.6%) 3 (4.6%) 5 (7.7%)	-	-
ER (%) ≤20 >20 unknown	\	\	64 (10.1%) 554 (87.7%) 14 (2.2%)	9 (13.8%) 54 (83.1%) 2 (3.1%)		-
PR (%) ≤20 >20 unknown	\	\	162 (25.6%) 450 (71.2%) 20 (3.2%)	23 (35.4%) 40 (61.5%) 2 (3.1%)		-
HER2 (%) pos neg unknown	\	\	145 (22.9%) 450 (71.2%) 37 (5.9%)	9 (13.8%) 49 (75.4%) 7 (10.8%)		-
Ki-67 (%) <20 20-50 >50 unknown	23 (8.9%) 45 (17.4%) 86 (33.2%) 105 (40.5%)	2 (3%) 8 (11.9%) 27 (40.3%) 30 (44.8%)	231 (36.6%) 285 (45.1%) 75 (11.9%) 41 (6.5%)	9 (13.8%) 31 (47.7%) 20 (30.8%) 5 (7.7%)	-	<0.0001
Histological grade G1 G2 G3 unknown	2 (0.8) 18 (6.9%) 84 (32.4%) 155 (59.8%)	0 (0%) 3 (4.5%) 21 (31.3%) 43 (64.2%)	68 (10.8%) 276 (43.7%) 161 (25.5%) 127 (20.1%)	1 (1.5%) 21 (32.3%) 33 (50.8%) 10 (15.4%)	-	<0.0001
Bilateral Yes No	22 (8.5%) 237 (91.5%)	10 (14.9%) 57 (85.1%)	106 (16.8%) 526 (83.2%)	11 (16.9%) 54 (83.1%)	-	-
Median Age at diagnosis (y) Primary tumor Secondary tumor	 48 58	 39 49	 48 53	 34 46	-	-
Time between 1st and 2nd Tumors (y) Median	 10	 10	 5	 12		

**TNBC WT versus carriers of PV/LPVs in BRCA1/2 genes; **LA/LB/HER2E WT BCs versus carriers of PV/LPVs in BRCA1/2 genes*

Abbreviations: TNBC= Triple Negative Breast Cancer; LA= Luminal A; LB= Luminal B; HER2E=Her2-enriched; WT= Wild-Type; ER= Estrogen Receptor; PR= Progesterone Receptor; y= years old.

Table 6: *The PV/LPVs of BRCA1/2 genes in OC patients.*

Gene	Type of variant	Molecular consequence	HGVS Nomenclature	Protein change	No. Probands	Variant origin	BCCR OCCR
BRCA1	Deletion	Fs	c.4964_4982del	p.Ser1655fs	15 (11.7%)	G	\
BRCA1	Deletion	Fs	c.514del	p.Gln172fs	9 (7%)	G	\
BRCA1	SNV	M	c.181T>G	p.Cys61Gly	4 (3.1%)	G	BCCR1
BRCA1	SNV	NS	*c.4327C>T	p.Arg1443Ter	4 (3.1%)	G/S	\
BRCA1	Deletion	NS	c.1360_1361del	p.Glu453_Ser454insTer	3 (2.3%)	G	\
BRCA1	Duplication	Fs	c.3253dup	p.Arg1085fs	3 (2.3%)	G	OCCR
BRCA1	SNV	M	c.4963T>G	p.Ser1655Ala	3 (2.3%)	G	\
BRCA1	Duplication	Fs	c.5266dup	p.Gln1756fs	3 (2.3%)	G	BCCR2’
BRCA1	Deletion	NS	c.117_118del	p.Cys39_Asp40delinsTer	2 (1.6%)	G	\
BRCA1	Deletion	Fs	c.3228_3229del	p.Gly1077fs	2 (1.6%)	G	OCCR
BRCA1	SNV	NS	c.3904G>T	p.Glu1302Ter	2 (1.6%)	G	OCCR
BRCA1	IVS	/	c.4096+3A>G	/	2 (1.6%)	G	\
BRCA1	IVS	/	c.547+2T>A	/	2 (1.6%)	G	\
BRCA1	Insertion	Fs	c.5561_5562insC	p.Leu1854Profs26	2 (1.6%)	G	BCCR2’
BRCA1	SNV	M	c.65T>C	p.Leu22Ser	2 (1.6%)	G	\
BRCA1	SNV	M	c.286G>C	p.Asp96His	1 (0.8%)	G	BCCR1
BRCA1	Insertion	Fs	c.1029_1030ins	p.Ala344Ter	1 (0.8%)	G	\
BRCA1	Deletion	Fs	c.1854del	p.Arg618fs	1 (0.8%)	G	OCCR
BRCA1	SNV	NS	c.2722G>T	p.Glu908Ter	1 (0.8%)	G	OCCR
BRCA1	SNV	NS	c.3400G>T	p.Glu1134Ter	1 (0.8%)	G	OCCR
BRCA1	SNV	NS	c.3544C>T	p.Gln1182Ter	1 (0.8%)	G	OCCR
BRCA1	Deletion	Fs	c.3700_3704del	p.Val1234fs	1 (0.8%)	G	OCCR
BRCA1	Deletion	Fs	c.3700_3704del	p.Val1234fs	1 (0.8%)	G	OCCR
BRCA1	SNV	NS	c.4117G>T	p.Glu1373Ter	1 (0.8%)	G	\
BRCA1	SNV	M	c.4484G>T	p.Arg1495Met	1 (0.8%)	G	BCCR2
BRCA1	SNV	M	c.5297T>G	p.Ile1766Ser	1 (0.8%)	G	BCCR2’
BRCA1	IVS	/	c.5406+5G>C	/	1 (0.8%)	G	BCCR2’
BRCA1	Deletion	Fs	c.798_799del	p.Ser267fs	1 (0.8%)	G	\
BRCA1	Insertion	Fs	c.984_985ins	p.Asn329fs	1 (0.8%)	G	\
BRCA1	LGR	/	13q13.1 (32900212-32972932)x1	/	1 (0.8%)	G	-
BRCA1	Deletion	Fs	c.2269del	p.Val757fs	2 (1.6%)	S	OCCR
BRCA1	SNV	NS	c.4576G>T	p.Glu1526Ter	2 (1.6%)	S	BCCR2
BRCA1	Deletion	Fs	c.1258del	p.Asp420MetfsTer10	1 (0.8%)	S	\
BRCA1	Deletion	Fs	c.1674del	p.Gly559fs	1 (0.8%)	S	OCCR
BRCA1	SNV	NS	c.2059C>T	p.Gln687Ter	1 (0.8%)	S	OCCR
BRCA1	Deletion	NS	c.2296_2297del	p.Glu765_Ser766insTer	1 (0.8%)	S	OCCR
BRCA1	Deletion	Fs	c.3528_3585del	p.Phe1177HisfsTer14	1 (0.8%)	S	OCCR
BRCA1	IVS	/	c.4484+1G>A	/	1 (0.8%)	S	BCCR2
BRCA1	Duplication	Fs	c.4891dup	p.Ser1631fs	1 (0.8%)	S	BCCR2
BRCA1	SNV	M	c.5252G>C	p.Arg1751Pro	1 (0.8%)	S	\
BRCA2	Deletion	Fs	c.9026_9030del	p.Tyr3009fs	4 (3.1%)	G	\
BRCA2	Deletion	Fs	c.6082_6086del	p.Glu2028fs	3 (2.3%)	G	\
BRCA2	Duplication	NS	c.1842dup	p.Asn615Ter	2 (1.6%)	G	\
BRCA2	IVS	/	c.1909+1G>A	/	2 (1.6%)	G	\

BRCA2	Deletion	Fs	c.2808_2811del	p.Ala938fs	2 (1.6%)	G	\
BRCA2	SNV	NS	c.3158T>G	p.Leu1053Ter	2 (1.6%)	G	\
BRCA2	Duplication	Fs	c.4284dup	p.Gln1429fs	2 (1.6%)	G	OCCR1
BRCA2	Deletion	Fs	c.5851_5854del	p.Ser1951fs	2 (1.6%)	G	\
BRCA2	SNV	NS	c.5959C>T	p.Gln1987Ter	2 (1.6%)	G	\
BRCA2	SNV	M	c.631G>A	p.Val211Ile	2 (1.6%)	G	\
BRCA2	Deletion	Fs	c.1238del	p.Leu413fs	1 (0.8%)	G	BCCR1’
BRCA2	SNV	NS	c.2651C>G	p.Ser884Ter	1 (0.8%)	G	\
BRCA2	Duplication	Fs	c.5073dup	p.Trp1692fs	1 (0.8%)	G	OCCR1
BRCA2	Duplication	Fs	c.5158dup	p.Ser1720fs	1 (0.8%)	G	OCCR1
BRCA2	Duplication	NS	c.5681dup	p.Tyr1894Ter	1 (0.8%)	G	OCCR1
BRCA2	Deletion	NS	c.5701_5714del	p.Ser1900_Glu1901insTer	1 (0.8%)	G	\
BRCA2	SNV	NS	c.6037A>T	p.Lys2013Ter	1 (0.8%)	G	\
BRCA2	Deletion	Fs	c.6324del	p.Val2109LeufsTer10	1 (0.8%)	G	\
BRCA2	Deletion	Fs	c.6486_6489del	p.Lys2162fs	1 (0.8%)	G	\
BRCA2	SNV	NS	c.7480C>T	p.Arg2494Ter	1 (0.8%)	G	BCCR2
BRCA2	IVS	/	c.8331+2T>C	/	1 (0.8%)	G	BCCR2
BRCA2	IVS	/	c.8754+4A>G	/	1 (0.8%)	G	BCCR2
BRCA2	Duplication	Fs	c.9253dup	p.Thr3085fs	1 (0.8%)	G	\
BRCA2	Deletion	Fs	c.9253del	p.Thr3085fs	1 (0.8%)	G	\
BRCA2	SNV	NS	c.1528G>T	p.Glu510Ter	1 (0.8%)	S	BCCR1’
BRCA2	Deletion	Fs	c.3264del	p.Gln1089fs	1 (0.8%)	S	OCCR1
BRCA2	SNV	NS	c.3883C>T	p.Gln1295Ter	1 (0.8%)	S	OCCR1
BRCA2	Deletion	Fs	c.5794del	p.His1932IlefsTer31	1 (0.8%)	S	\
BRCA2	SNV	NS	*c.7297C>T	p.Gln2433Ter	1 (0.8%)	S	OCCR2
BRCA2	SNV	NS	c.7366C>T	p.Gln2456Ter	1 (0.8%)	S	OCCR2
BRCA2	SNV	Syn	c.9117G>A	p.Pro3039=	1 (0.8%)	S	\

**These PV/LPVs are present in the same proband showing double heterozygosity for somatic BRCA1 and BRCA2 genes.*

Abbreviations: OC= Ovarian Cancer; PV/LPVs= Pathogenic/ Likely Pathogenic Variants; SNV= Single Nucleotide Variant; IVS= Intronic Variant Sequence; Fs= Frameshift; M= Missense; NS= Nonsense; Syn= Synonymous; BCCR= Breast Cancer Cluster Region; OCCR= Ovarian Cancer Cluster Region; G= germline; S= somatic.

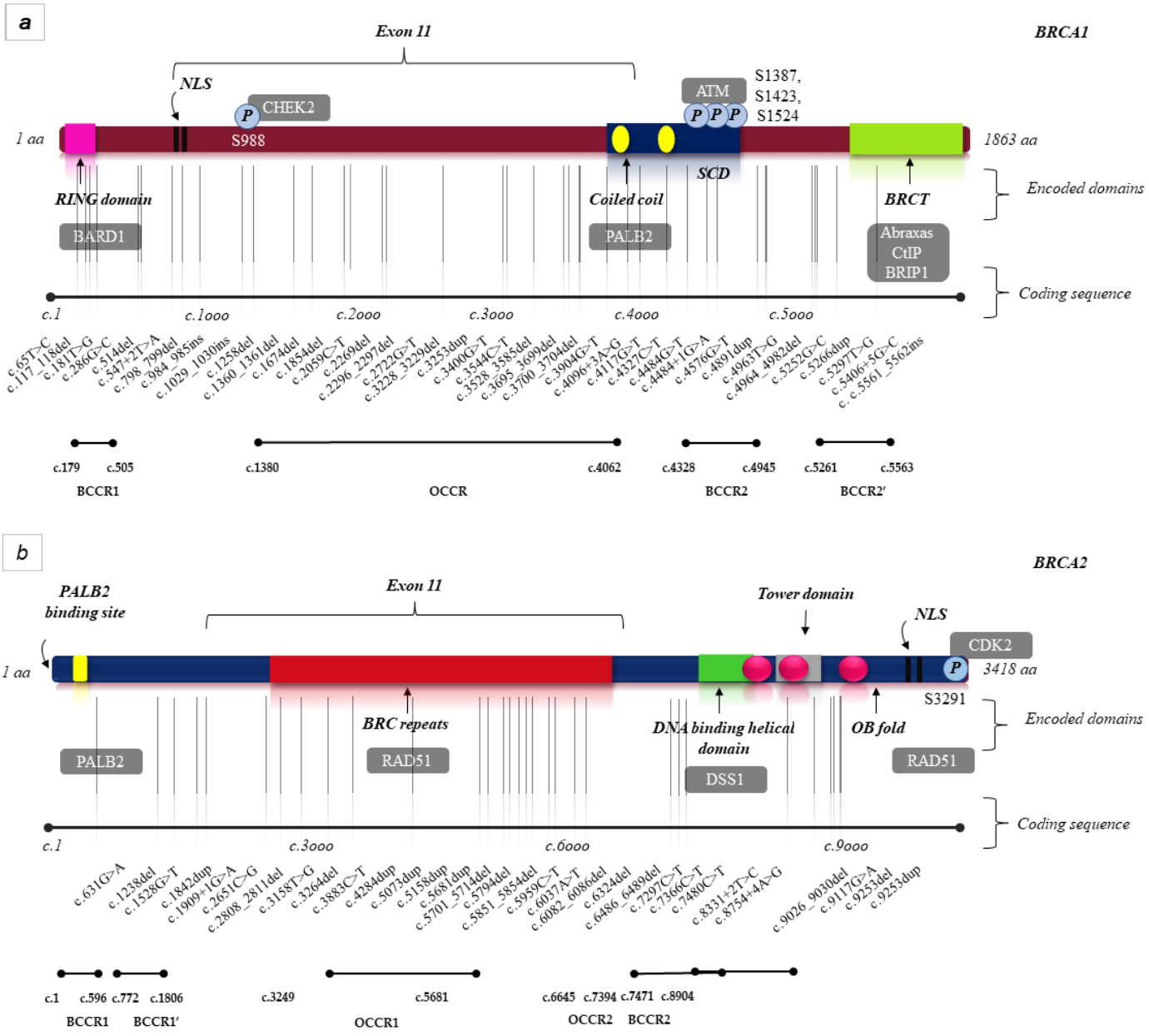


Figure 6: *Genetic location of PV/LPVs in BRCA1/2 genes among OC patients; a) PV/LPVs identified in BRCA1 gene sequence; b) PV/LPVs identified in BRCA2 gene sequence.*

Abbreviations: OC= Ovarian Cancer; PV/LPVs= Pathogenic/ Likely Pathogenic Variants; BCCR= Breast Cancer Cluster Region; OCCR= Ovarian Cancer Cluster Region; RING= Really Interesting New Gene; NLS= Nuclear Localization Sequence; BRCT= BRCA1 C-terminus domain; SCD= Serine Cluster Domain; OB= Oligonucleotide Binding.

Table 7: *Baseline features and clinico-pathological information of OC patients.*

	BRCA1/2 wt	*g/s BRCA1/2*	*p value*
Number of patients: 540	412 (76.3%)	128 (23.7%)	/
Age groups (y)			
≤40	22 (5.3%)	10 (7.8%)	
41-50	54 (13.1%)	30 (23.4%)	
51-60	123 (29.9%)	46 (35.9%)	0.02
61-70	90 (21.8%)	32 (25%)	
>70	65 (15.8%)	10 (7.8%)	
Unknown	58 (14.1%)		
Histological Subtype			
HGSC	255 (62%)	92 (71.9%)	
Clear cell	12 (2.9%)	1 (0.8%)	
Endometrioid	46 (10.9%)	8 (6.25%)	0.04
LGSC	2 (0.5%)	0 (0%)	
Papillary	29 (7%)	3 (2.3%)	
Unknown	68 (16.7%)	24 (18.75%)	
Personal cancer history before EOC			
Personal breast cancer history	17 (4.1%)	14 (11%)	0.04
Personal others cancer history	14 (3.4%)	3 (2.3%)	
No cancer history	381 (92.5%)	111 (86.7%)	

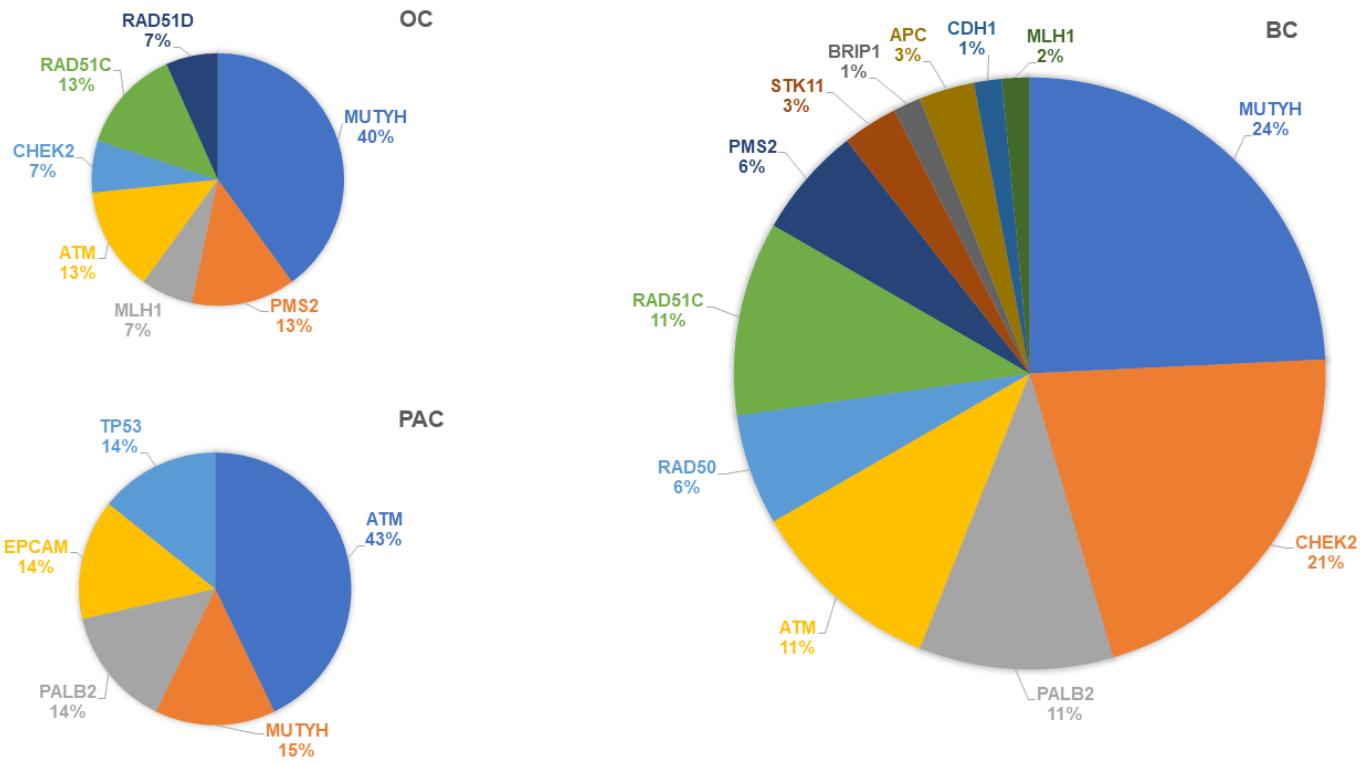
Abbreviations: HGSC= High Grade Serous Carcinoma; LGSC= Low Grade Serous Carcinoma; EOC= Epithelial Ovarian Cancer; g= germline; s= somatic; WT= wild-type; y= years old.

Table 8: *The PV/LPVs of BRCA1/2 genes in PaC and PrC patients.*

PaC							
Gene	**Type of variant**	**Molecular consequence**	**HGVS Nomenclature**	**Protein change**	**No. Probands**	**Variant origin**	**BCCR OCCR**
BRCA2	SNV	NS	c.7681C>T	p.Gln2561Ter	1	G	BCCR2
BRCA2	Deletion	Fs	c.6990_6994del	p.Ile2330fs	1	G	OCCR2
BRCA2	IVS	/	c.8487+1G>A	/	1	G	BCCR2
PrC							
Gene	**Type of variant**	**Molecular consequence**	**HGVS Nomenclature**	**Protein change**	**No. Probands**	**Variant origin**	
BRCA2	Deletion	NS	c.3545_3546del	p.Gln1181_Phe1182insTer	1	G	OCCR1

Abbreviations: PaC= Pancreatic Cancers; PrC= Prostate Cancer; PV/LPVs= Pathogenic/ Likely Pathogenic Variants; SNV= Single Nucleotide Variant; IVS= Intronic Variant Sequence; Fs= Frameshift; NS= Nonsense; BCCR= Breast Cancer Cluster Region; OCCR= Ovarian Cancer Cluster Region

Figure 7: *Distribution rate of the involved genes beyond BRCA1/2 genes in BC, OC, PaC and PrC patients*



evaluated with NGS-based multi-gene panel testing.

Abbreviations: BC= Breast Cancer; OC= Ovarian Cancer; PaC= Pancreatic Cancer; PrC= Prostate Cancer.

Table 9: *The PV/LPVs identified through multi-gene panel testing in BC, OC, Pac and PrC patients.*

BCs					
Gene	**Type of variant**	**Molecular consequence**	**HGVS Nomenclature**	**Protein change**	**No. Probands**
MUTYH	SNV	M	c.1145G>A	p.Gly382Asp	7
MUTYH	Deletion	Fs	c.1395_1397del	p.Glu466del	4
MUTYH	Insertion	Fs	c.1187_1188insGG	p.Glu396Glyfs43	2
MUTYH	SNV	M	c.494A>G	p.Tyr165Cys	1
MUTYH	SNV	M	c.1129C>T	p.Gln377Ter	1
MUTYH	SNV	M	c.1238G>T	p.Trp413Leu	1
CHEK2	IVS	/	c.721+3A>T	/	3
CHEK2	SNV	M	c.1165C>T	p.Arg389Cys	3
CHEK2	Deletion	Fs	c.1229del	p.Thr410fs	3
CHEK2	SNV	M	c.1441G>T	p.Asp481Tyr	3
CHEK2	SNV	M	c.844G>A	p.Glu282Lys	1
CHEK2	SNV	M	c.980G>A	p.Cys327Tyr	1
PALB2	DelIns	NS	c.661_662delinsTA	p.Val221Ter	2
PALB2	Deletion	NS	c.94del	p.Leu32Ter	1
PALB2	Duplication	Fs	c.758dup	p.Ser254fs	1
PALB2	Deletion	Fs	c.1050_1053del	p.Thr351fs	1

PALB2	SNV	NS	c.2566C>T	p.Gln856Ter	1
PALB2	SNV	NS	c.3549C>G	p.Tyr1183Ter	1
ATM	SNV	NS	c.2413C>T	p.Arg805Ter	2
ATM	Duplication	Fs	c.2502dup	p.Val835fs	1
ATM	SNV	Syn	c.3576G>A	p.Lys1192=	1
ATM	IVS	/	c.4776+1G>T	/	1
ATM	SNV	M	c.8147T>C	p.Val2716Ala	1
ATM	Duplication	NS	c.8818_8821dup	p.Ser2941Ter	1
RAD50	SNV	/	c.130-1G>T	/	1
RAD50	Deletion	Fs	c.326_329del	p.Trh109LysfsTer21	1
RAD50	IVS	/	c.551+1G>C	/	1
RAD50	SNV	NS	c.3598C>T	p.Arg1200Ter	1
RAD51C	IVS	/	c.1026+5_1026+7del	/	3
RAD51C	SNV	M	c.773G>A	p.Arg258His	2
RAD51C	Deletion	Fs	c.97_98del	p.Gln33fs	1
RAD51C	Insertion	Fs	c.226_227insAT	p.Ala76Metfs26	1
PMS2	SNV	M	c.2T>C	p.Met1Thr	1
PMS2	SNV	M	c.137G>T	p.Ser46Ile	1
PMS2	Deletion	Fs	c.2177del	p.Pro726fs	1
PMS2	DelIns	Fs	c.2182_2184delinsG	p.Thr728Alafs	1
STK11	SNV	M	c.368A>G	p.Gln123Arg	1
STK11	Deletion	Fs	c.1027del	p.Asp343ThrfsTer50	1
BRIP1	SNV	NS	c.1348G>T	p.Glu450Ter	1
APC	SNV	M	c.1121G>A	p.Arg374Gln	1
APC	SNV	M	c.3920T>A	p.Ile1307Lys	1
CDH1	IVS	/	c.2164+2T>C	/	1
OCs					
Gene	**Type of variant**	**Molecular consequence**	**HGVS Nomenclature**	**Protein change**	**No. Probands**
MUTYH	SNV	M	*** *c.1145G>A	p.Gly382Asp	**4**
MUTYH	SNV	NS	**c.479G>A	p.Trp160Ter	**1**
MUTYH	SNV	M	***c.1103G>A	p.Gly368Asp	1
PMS2	SNV	M	c.137G>T	p.Ser46Ile	1
PMS2	SNV	M	c.2249G>A	p.Gly750Asp	1
MLH1	Deletion	Fs	c.599_602delCAGT	p.Thr200LysfsTer28	**1**
ATM	Deletion	NS	*c.3802_3802delG	p.Glu1267_Val1268insTer	**1**
ATM	IVS	/	**c.4776+1G>T	/	**1**
CHEK2	SNV	M	c.1441G>T	p.Asp481Tyr	1
RAD51C	SNV	M	***c.680C>G	p.Pro227Arg	**1**
RAD51C	Deletion	**Fs**	c.622_623del	p.Ile208fs	**1**
RAD51D	SNV	NS	c.863G>A	p.Trp288Ter	**1**
PaC					
Gene	**Type of variant**	**Molecular consequence**	**HGVS Nomenclature**	**Protein change**	**No. Probands**
ATM	Insertion	Fs/NS	c.2503_2504insA	p.Val835AspfsTer7	1
ATM	SNV	M	c.8558C>T	**p.Thr2853Met**	1
ATM	SNV	NS	c.8977C>T	p.Arg2993Ter	1
MUTYH	SNV	M	c.1145G>A	p.Gly382Asp	1

PALB2	DelIns	NS	c.661_662delinsTA	p.Val221Ter	**1**
EPCAM	SNV	NS	c.227C>G	p.Ser76Ter	**1**
TP53	SNV	M	c.1016A>G	p.Glu339Gly	**1**

**These PV/LPVs are present in the same proband showing double heterozygosity.*

Table 10: *Comparison of clinico-pathological information between patients analysed in BRCA1/2 genes and Multi-gene panel testing*

	BRCA1/2	**Multi-gene Panel MUT**	**All WT**	***p-value****	***p-value******
Number of patients	144	65	961	/	/
Age groups (y) ≤ 40 41-50 51-60 > 60 Unknown	 58 (39.6%) 45 (30.6%) 20 (13.9%) 11 (7.6%) 10 (8.3%)	 22 (33.8%) 16 (24.6%) 20 (30.8%) 7 (10.8%)	 300 (31.2%) 326 (34%) 185 (19.3%) 118 (12.3%) 32 (3.3%)	0.04	-
Histological Subtype Ductal Lobular Others Unknown	 83 (57.6%) 4 (2.8%) 4 (2.8%) 53 (36.8%)	 36 (55.4%) 5 (7.7%) 5 (7.7%) 19 (29.2%)	 606 (63.1%) 58 (6%) 56 (5.8%) 241 (25.1%)	-	-
Molecular Subtype LA/LB HER2E TNBC Unknown	 62 (43.1%) 3 (2%) 67 (46.5%) 12 (8.3%)	 39 (60%) 0 (0%) 9 (13.8%) 17 (26.2%)	 556 (57.9%) 36 (3.7%) 250 (26%) 119 (12.4%)	0.0003	-
ER (%) ≤ 20 > 20 Negative Unknown	 9 (6.3%) 54 (37.5%) 68 (47.2%) 13 (9%)	 1 (1.5%) 40 (61.5%) 8 (12.3%) 16 (24.6%)	 22 (2.3%) 537 (55.9%) 286 (29.8%) 116 (12%)	<0.00001	0.03
PR (%) ≤ 20 > 20 Negative Unknown	 23 (16%) 40 (27.8%) 74 (51.4%) 7 (4.9%)	 3 (4.6%) 34 (52.3%) 12 (18.5%) 16 (24.6%)	 93 (9.7%) 430 (44.7%) 311 (32.4%) 127 (13.2%)	<0.00001	0.05
Ki-67 (%) < 20 20-50 > 50 Unknown	 11 (7.6%) 39 (27.1%) 47 (32.6%) 47 (32.6%)	 18 (27.7%) 17 (26.15%) 4 (6.15%) 26 (40%)	 237 (24.7%) 318 (33.1%) 145 (15.1%) 261 (27.1%)	<0.00001	-
Histological grade G1 G2 G3 Unknown	 1 (0.7%) 24 (16.7%) 54 (37.5%) 65 (45.1%)	 5 (7.7%) 27 (41.5%) 9 (13.8%) 24 (36.9%)	 69 (7.2%) 281 (29.2%) 247 (25.7%) 364 (37.9%)	<0.00001	0.04
Bilateral Yes No	 21 (14.6%) 123 (85.4%)	 13 (20%) 52 (80%)	 127 (13.2%) 834 (86.8%)	-	-
Median Age at diagnosis (y) Primary tumor Secondary tumor	 37 47	 47 52	 48 53	-	-
Time between 1st and 2nd Tumors (y) Median	 10	 5	 5		

**comparison carriers of PV/LPVs in BRCA1/2 vs Multi-gene panel MUT; ** comparison Multi-gene panel MUT vs All WT*

Abbreviations: TNBC= Triple Negative Breast Cancer; LA= Luminal A; LB= Luminal B; HER2E=Her2-enriched; WT= Wild-Type; ER= Estrogen Receptor; PR= Progesterone Receptor; y= years old.

Table 11: *Comparison between clinico-pathological information among OC patients analyzed to BRCA1/2 and Multi-gene panel testing*

	g *BRCA1/2*	Multi-gene Panel MUT	*All WT*	*p value**	*p value***
Number of patients	109	11	420	/	/
Age groups (y)					
≤40	10 (9.2%)	3 (27.3%)	22 (5.2%)	-	0.02
41-50	28 (25.7%)	2 (18.2%)	54 (12.9%)		
51-60	39 (35.8%)	1 (9.1%)	112 (26.6%)		
61-70	23 (21.1%)	2 (18.2%)	95 (22.6%)		
>70	9 (8.3%)	1 (9.1%)	67 (16%)		
Unknown		2 (18.2%)	70 (16.6%)		
Histological Subtype					
HGSC	77 (70.6%)	4 (36.4%)	255 (60.7%)	<0.001	<0.0001
Clear cell	2 (1.8%)	0 (0%)	13 (3.1%)		
Endometrioid	6 (5.5%)	5 (45.5%)	42 (10%)		
LGSC	3 (2.8%)	0 (0%)	2 (0.5%)		
Papillary	3 (2.8%)	1 (9.1%)	2 (0.5%)		
Unknown	18 (16.5%)	1 (9.1%)	106 (25.2%)		
Personal cancer history before EOC					
Personal breast cancer history	15 (13.8%)	2 (18.2%)	20 (4.8%)	-	0.05
Personal others cancer history	1 (1.8%)	1 (9.1%)	21 (5%)		
No cancer history	93 (85.3%)	8 (72.7%)	379 (90.2%)		

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

Abbreviations: HGSC= High Grade Serous Carcinoma; LGSC= Low Grade Serous Carcinoma; EOC= Epithelial Ovarian Cancer; g= germline; s= somatic; WT= wild-type; y= years old.

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Scientific Products

- *BRCA1/2 variants of unknown significance in hereditary breast and ovarian cancer (HBOC) syndrome: Looking for the hidden meaning.* Fanale D., Pivetti A., Cancelliere D., Spera A., Bono M., Fiorino A., Pedone E., Barraco N., Brando C., Perez A., Guarneri M.F., Russo T.D.B., Vieni S., Guarneri G., Russo A., Bazan V. Crit Rev Oncol Hematol. 2022 Apr;172:103626. doi: 10.1016/j.critrevonc.2022.103626. Epub 2022 Feb 10. PMID: 35150867.

- *Impact of deleterious variants in other genes beyond BRCA1/2 detected in breast/ovarian and pancreatic cancer patients by NGS-based multi-gene panel testing: looking over the hedge.* Bono M., Fanale D., Incorvaia L., Cancelliere D., Fiorino A., Calò V., Dimino A., Filorizzo C., Corsini L.R., Brando C., Madonia G., Cucinella A., Scalia R., Barraco N., Guadagni F., Pedone E., Badalamenti G., Russo A., Bazan V. ESMO Open. 2021 Aug;6(4):100235. doi: 10.1016/j.esmoop.2021.100235. Epub 2021 Aug 7. PMID: 34371384; PMCID: PMC8358413.

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ABSTRACT ASCO 2022

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ABSTRACT AIOM 2021

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ABSTRACT ASHG 2021

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