ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN
Oncologia, Ematologia e Patologia

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Ciclo XXXVI Ciclo

Settore Concorsuale: 06/D3

Settore Scientifico Disciplinare: MED/06

Exploring the role of rare germline variants in non-coding regions of cancer predisposition genes in triple-negative breast cancer patients”

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Esame finale anno 2024

SUMMARY

ABSTRACT:

BRCA1 and *BRCA2* are well-known genes that are associated with a significant increase in the risk of breast and ovarian cancers. However, current genetic screening is limited to *BRCA1/2* exons and intron/exon boundaries, and limited information exists about the impact of variants in *BRCA1/2* non-coding regions. As a result, the majority of variants identified in these regions remain unclassified, and about 80% of germline *BRCA1/2* tests result in a "negative" diagnosis. Introns and proximal untranslated regions remain relatively unexplored, but evidence of non-coding variants' impact on cancer risk and response to treatment is beginning to emerge. The aim of this project was to investigate the prevalence of non-*BRCA* pathogenic germline variants in patients with triple-negative breast cancer and risk factors, the authors used an NGS custom panel of promoter regions of 62 genes involved in cancer predisposition. We enrolled 144 consecutive triple-negative breast cancer patients who were wild type for germline *BRCA1/2* and identified 635 rare variants in non-coding regions of 28 genes, among the 144 patients. Clinical data were available for 75 patients, and these data were merged with the genomic dataset. Among these 75 patients, rare germline variants in *BRCA2* were statistically significantly related to worse overall survival (*p*-value=0.017 HR=4.76 (1.32-17.15)). No differences in Disease-free survival and overall survival were found for other genes. CDH1's rare variants were related to the highest percentage of non-pathological complete response after neoadjuvant chemotherapy (*p*-value=0.0273); *MLH1* and *PALB2* rare variants were found to be both related to bilateral breast cancer (*p-value*=0.0146 and p=0.0005, respectively). Rare variants of the *ATM* gene were associated with a positive family history (*p*-value 0.0408). However, due to the small sample size, these analyses should be considered only exploratory, and further studies are needed to confirm these findings.

INTRODUCTION: Breast Cancer

Etiology

Breast cancer is the most common cancer among women worldwide, despite the introduction of screening programs and advancements in therapeutic techniques contributing to increased survival rates over the past decades [1, figure 1]. The etiology of breast cancer is multifactorial, involving various aspects of a woman's lifestyle, hormonal status, and genetic predisposition to varying degrees. Although the exact cause of breast carcinoma development remains largely unknown, numerous risk factors capable of influencing the disease have been identified. Therefore, much like many other forms of cancer, breast cancer is the end result of the combined contributions of numerous environmental and hereditary factors, both positive and negative.

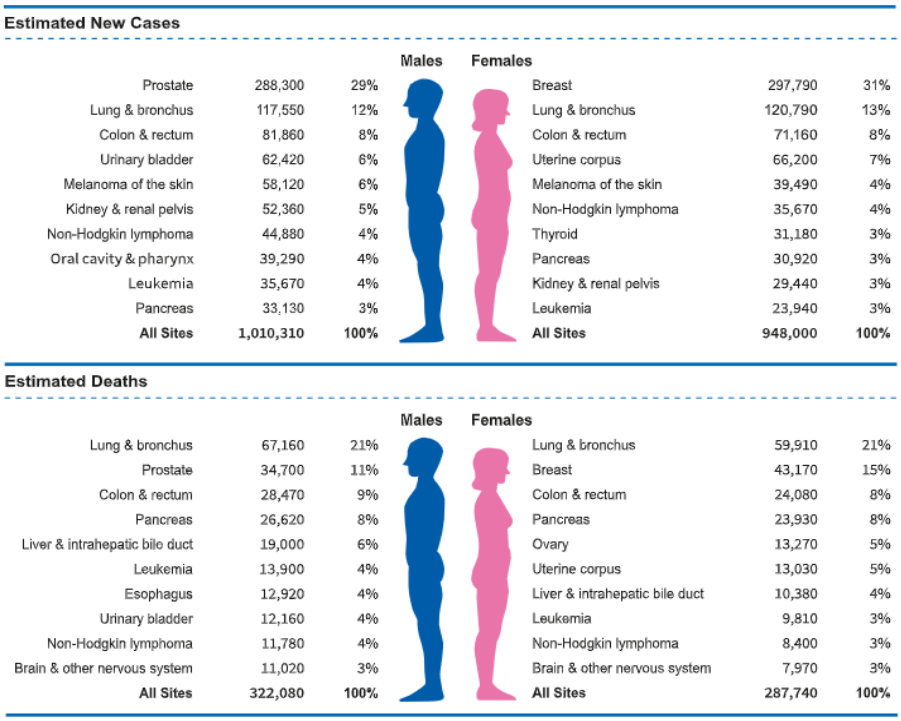

Figure 1. Ten leading cancer types for the estimated new cancer cases and deaths by sex, United States 2023. Estimates are rounded to the nearest 10, and cases exclude basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder. Ranking is based on modelled projections and may differ from the most recent observed data.

First and foremost, an individual's risk of developing breast cancer is strongly linked to age, with a direct proportional relationship. In this regard, breast cancer exhibits a distinctive age-specific incidence curve characterized by a bimodal pattern. While the incidence of this neoplasm is very low until the age of 30, it increases exponentially until the age of 50 and then stabilizes at a plateau around menopause (most likely due to hormonal changes). The majority of breast cancers are sporadic and caused by somatic mutations that are acquired during life. However, approximately 10-15% of breast cancers are familial and occur in multiple family members due to environmental risk factors or the presence of multiple gene polymorphisms. Finally, approximately 5-10% of breast cancers are hereditary and characterized by the presence of multiple cases in the same family and an earlier onset compared to sporadic tumors. Mutations in genes such as *BRCA1* and *BRCA2* are associated with a significant increase in the risk of developing breast cancer. In particular, mutations in these genes are responsible for approximately 50% of hereditary breast cancers. This pattern has led to the hypothesis of two forms of the disease, one premenopausal, primarily influenced by genetic, hormonal, and reproductive factors, and one postmenopausal, primarily influenced by dietary and endocrine factors [2]. Among dietary factors, particular importance has been given to high consumption of carbohydrates and saturated fats (fat intake is associated with an increase in plasma estrogen levels), as well as excessive alcohol consumption, following the demonstration by some studies of ethanol's ability to increase plasma levels of sex steroids [3-5]. Another condition closely related to an increased risk of breast cancer is obesity. In this circumstance, the increased concentration of insulin in circulation leads to increased liposynthetic activity by adipocytes, which in turn increases the production and circulation of leptin, a peptide recently associated with tumorigenesis in obese breast cancer patients [6, 7]. Among other aspects of a woman's lifestyle that can increase the risk of developing breast cancer are low physical activity and smoking. As mentioned earlier, estrogens play a central role in the etiology of breast carcinoma, being a group of steroid hormones involved in breast growth and development. Numerous studies have confirmed their carcinogenic role and the mechanisms by which these hormones are thought to exert it. In particular, it has been shown that estrogens have the ability to negatively interfere with DNA damage repair mechanisms, as well as to accelerate cell division, increasing the risk of developing the neoplasm [8, 9]. Therefore, reproductive factors that increase estrogen levels, including a longer fertile period due to early menarche and late menopause, low parity or nulliparity (reduced risk is observed, conversely, in women who have had children, with greater protection associated with more children and an earlier age at first pregnancy [10]), a first full-term pregnancy after the age of 30, and not breastfeeding, are all considered significant risk

factors. Oral contraceptives also fall into this category. By stimulating the ductal epithelium for a longer period (21 days) than the physiological cycle, they may increase the risk of developing the neoplasm. Similarly, Hormone Replacement Therapy (HRT), administered to menopausal women for the treatment of conditions like osteoporosis and cardiovascular diseases, is thought to increase the risk of breast cancer, especially in women with a *positive* family history. Introduced in the late 1960s to 1970s, the first generation of Hormone Replacement Therapy was based solely on estrogen (ERT), and as early as 1975, the first studies were published demonstrating its association with the onset of endometrial cancer among treated patients [11, 12]. These observations led to the need to develop alternative therapeutic strategies, leading to the introduction of a second generation of Hormone Replacement Therapy, based on the administration of estrogen in combination with progesterone (CHRT). It had long been known that the latter had a protective role against endometrial cancer [13], and soon the opinion spread that this effect could also be exerted at the breast tissue level [14]. Conversely, a few years ago, Ronald K. Ross *et al.* [15] stated that progesterone, in addition to not being able to protect the breast from the carcinogenic effect of estrogens, drastically increases the risk. The explanation for this phenomenon lies in the opposite biological effect that progesterone has in the two tissues, the endometrial and mammary glands. In the former case, progesterone exerts an inhibitory action on proliferation, while in the latter case, within the mammary gland, the influence of this hormone results in maximum mitotic activity stimulation, just like estrogens, promoting carcinogenesis [16]. Similar results have been obtained from a recent meta-analysis [17] and other assessments on the same topic [18-19]. Furthermore, an increased incidence of breast cancer has been reported following exposure to ionizing radiation. In this case, the risk is related to age (high for exposure at a young age, negligible for exposure after 40 years) and dose, with a latency period of about 10-15 years. Finally, all forms of benign cell proliferation, such as ductal or lobular hyperplasia, should be monitored, although the most important risk factor always remains to have a relative diagnosed with breast cancer [20]. To thoroughly understand the nature and histopathological characteristics of breast neoplasms, essential knowledge of the anatomy and histology of the mammary gland is required.

Molecular and immunophenotypic classification

Thanks to innovations in molecular biology, primarily the introduction of microarrays and Genome-wide approaches (GWAS), it has become possible to classify breast tumors based on their

molecular characteristics and genetic profiles, leading to the identification of at least molecular subtypes [21-26]:

- Luminal A: Neoplasms characterized by the expression of hormone receptors ER and PR, with a favorable prognosis.
- Luminal B: Neoplasms that, while expressing hormone receptors, have a high risk of recurrence due to a high proliferative index correlated with high expression of proliferation genes.
- HER2-positive: Neoplasms characterized by HER2 amplification.
- Basal-like: Neoplasms characterized by the absence of hormone receptor and HER2 expression, and increased expression of basal cytokeratins (CK5/6 and CK17).
- Claudin low: Neoplasms that are negative for hormone receptors and HER2, with a poor prognosis. They exhibit an expression pattern similar to stem cells, low expression of claudins (proteins involved in cell-cell junctions), and the presence of accompanying lymphocytic infiltration in tumor growth [27].

In addition to molecular classification based on gene profiles, clinical practice also identifies four immunophenotypic subgroups of breast cancer through immunohistochemical assessment, considering the status of hormone receptors, Ki-67 antigen, and HER2 [28]:

- Luminal A: Hormone receptor-positive, HER2-negative, and low proliferative activity.
- Luminal B/HER2-negative: Hormone receptor-positive, HER2-negative, with high proliferative activity.
- Luminal B/HER2-positive: Hormone receptor-positive, HER2 overexpressed or amplified, with any level of proliferative activity.
- HER2-positive (non-luminal): HER2 overexpressed or amplified, and both hormone receptors negative.
- Triple-negative (TNBC): Tumors characterized by the absence of hormone receptor and HER2 expression. This immunophenotypic group includes specific histotypes such as typical medullary and adenoid cystic carcinoma. From a genetic perspective, triple-negative

tumors have recently been associated with mutations in one of the two major breast cancer susceptibility genes, *BRCA1*. The cumulative risk of ER-negative breast cancer by age 70 for *BRCA1* mutation carriers was estimated to be 55% and the risk of ER-positive disease was 18%. The corresponding risks for *BRCA2* mutation carriers were 21% and 44% for ER-negative and ER-positive disease, respectively [29-30].

In most cases, the classification based on genetic profiles corresponds well with the classification based on the tumor's immunophenotype. For example, in approximately 80% of cases, there is a correspondence between the "basal-like" subgroup identified based on genetics and the "triple-negative" (TNBC) phenotype identified based on immunohistochemistry.

Table 1. Clinicopathological characteristics of patients with *BRCA1/2* pathogenic variants.

	BRCA1 pathogenic variant	BRCA2 pathogenic variant
Chromosome	17q21	13q12-13
Breast cancer		
Risk of cancer (by 70 years)	65% (44–78%)	45% (31–56%)
Histological type	Invasive ductal (~75%)	Invasive ductal (~75%)
	Atypical medullary (~10%)	Atypical medullary (<10%)
		Lobular or ductal with lobular feature type (up to 10%)
Histological grade	Mostly high (Grade III)	Mostly medium (Grade II) or high (Grade III)
TNBC	66–100%	14–35%
DCIS	Rare	Common
Ovarian cancer		
Risk of cancer (by 70 years)	39% (18–54%)	11% (2.4–19%)

Abbreviations: TNBC, triple-negative breast cancer; DCIS, ductal carcinoma *in* si*tu*

DNA REPAIR SYSTEMS

DNA repair systems are essential for maintaining the genomic integrity of every cell. Effective DNA damage repair requires the ability to detect damage, prevent the replication of damaged DNA, halt the cell cycle, and direct the cell toward apoptosis. During DNA replication, cells defend against nucleotide incorporation errors by utilizing the proofreading activity of the polymerase. In addition to this, cells must protect themselves from damage caused by spontaneous depurination catalyzed by water, ionizing radiation, reactive oxygen species that can originate endogenously from metabolism or exogenously. Even the body's own heat provides enough energy to detach adenine and guanine from DNA sugars. It is essential that DNA lesions are repaired before replication, as otherwise, these would be fixed as mutations in the daughter cells.

Homologous Recombination (HR)

Homologous recombination is an error-free mechanism that comes into play in repairing double-strand breaks (DSB) in DNA and is the primary mechanism for safeguarding genomic integrity in proliferating cells. A DSB can be caused by errors in DNA replication or exposure to ionizing radiation, genotoxic compounds, and oxidative stress. It is the most dangerous form of DNA damage as it damages the integrity of both DNA strands simultaneously. The DNA damage response (DDR) to DSBs involves numerous proteins, primarily grouped into three categories: damage-sensing proteins, effector proteins that carry out the repair, and mediator proteins that facilitate interactions between sensor and effector proteins. During DDR, the cell cycle checkpoint is activated, which slows down the cycle before or during replication (G1/S or intra-S checkpoint) or before cell division (G2/M checkpoint). In mammals, the absence of proper HR can lead to chromosomal rearrangements and genomic instability. One of the damage sensors is ATM, which undergoes autophosphorylation and monomerization (in the absence of DNA damage, it is in dimeric form) in the presence of double-strand damage, activating even in regions distant from the break because it can perceive chromatin conformational changes. ATM is then recruited to the break sites by the MRN complex, consisting of RAD50, MRE11, and NBS1, which quickly migrates to the damage site (it has exonuclease, endonuclease, and helicase activities). Once activated, ATM phosphorylates various substrates, including BRCA1, the MRN complex, and RPA. MRN remodels the ends, leaving single-stranded DNA (ssDNA) to which RAD51 is bound, localized by BRCA2. RAD51 forms a multimeric filamentous structure and mediates invasion of the homologous chromosome helix to search for a region of homology to repair the damage.

Holliday junctions are formed, characteristic of crossing-over; finally, with the help of a resolvase, the structure is resolved, and the damage is repaired [31].

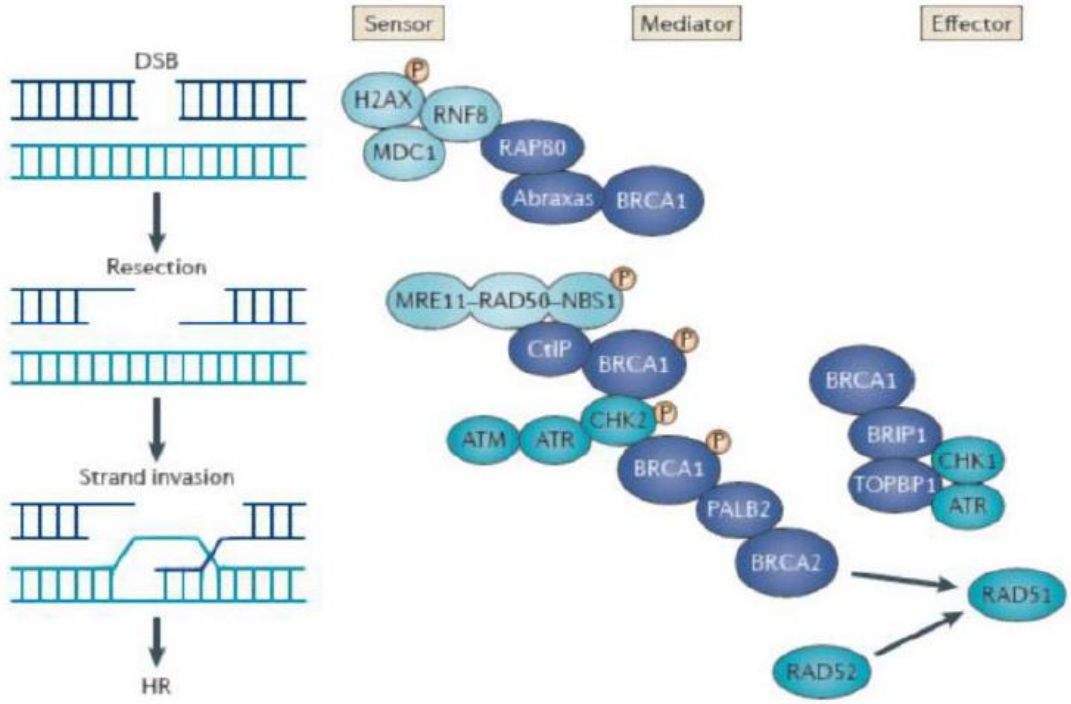

Figure 2. Main players in homologous recombination.

Non-Homologous End-Joining (NHEJ): Non-Homologous Recombination is another strategy for repairing double-strand DNA damage, and unlike homologous recombination, it can occur at any point in the cell cycle. NHEJ is error-prone and can lead to translocations due to imperfectly repaired junctions, which may result in the loss of genetic material. The molecular mechanism of NHEJ is distinct from that of HR, involving different sensors, mediators, and effectors. The damage sensors in NHEJ are Ku70 and Ku80, which bind to the free 3' and 5' ends of DNA to prevent degradation. They recruit the DNA-dependent protein kinase (DNA-PKCS) and ARTEMIS. The non-compatible DNA ends must be processed to create repairable terminal filaments. This processing is carried out by the MRN complex, which processes the 3' end, while the 5' end is processed by FEN1. Subsequently, XRCC4 and Ligase 4 come into play to seal the filaments [32-33].

Base Excision Repair (BER): BER is specialized in the removal of nucleotide alterations caused by chemicals present in the diet or generated by metabolism. BER is initiated by the action of a DNA glycosylase that recognizes the alteration and cleaves the glycosidic bond between the nitrogenous

base and the deoxyribose sugar. There are numerous glycosylases, and they can vary in specificity when recognizing a particular altered base. Altered bases are diverse; some examples include uracil, resulting from the hydrolytic deamination of cytosine, and 8-oxoguanine, caused by damage from oxygen's free radicals. First, the glycosylase removes the nitrogenous base, leaving behind an abasic site, which is recognized by the endonuclease APE1, which cleaves the DNA strand. Poly(ADP-ribose) polymerase-1 (PARP1) is a sensor that recognizes DNA breaks and ADP-ribosylates the ends of the cut strand, protecting it from degradation. Additionally, ADP-ribosylation serves as a recruitment signal for DNA ligase III, polymerase β, and scaffold proteins like XRCC1. The lyase activity of polymerase β removes the remaining sugar-phosphate group attached to the excised base. Subsequently, polymerase β fills the gap by inserting a nucleotide complementary to the undamaged DNA strand, and finally, DNA ligase III seals the repaired strand. PARP proteins belong to a family of multifunctional enzymes, with PARP1 being the most abundant. PARP1 and PARP2 are involved in BER and can also stimulate the early stages of repair at DNA replication fork stalls, repaired by HR [34].

Nucleotide Excision Repair (NER): NER primarily operates in the removal of bulky lesions, such as pyrimidine dimers and nucleotides with adducts. NER can be distinguished into two pathways: Transcription-Coupled Repair (TCR) and Global Genomic Repair (GGR). Despite this division, what changes is the mechanism of lesion recognition, while the removal of damage and repair remains the same. TCR is activated by a stall in RNA polymerase II, which recruits the CSA and CSB proteins. GGR is initiated by the Xeroderma Pigmentosum Complementation Group C (XPC) protein complex. Subsequently, XPB and XPD (two subunits with helicase activity in TFIIH) mediate the separation of DNA strands. Then, XPG at the 3' end and XPF-ERCC1 at the 5' end, with their endonuclease activity, cleave the damaged strand. Subsequently, the segment within the incision is removed. Finally, DNA polymerase δ or ε resynthesizes the missing strand portion, and a ligase seals the strand [35].

Mismatch Repair (MMR): In cases where the proofreading activity of DNA polymerase fails, resulting in the incorporation of an incorrect nucleotide, error correction must occur before the damage is fixed as a mutation through another round of replication. This task is handled by MMR. Mismatches can also occur following homologous recombination, cytosine deamination, and other sources of DNA damage. This system plays a crucial role in maintaining genomic stability. A mismatch causes distortion in the double helix's geometry, which can be recognized by certain repair enzymes. Interestingly, the cell can discriminate the newly synthesized strand, which contains the error, thus avoiding random mismatch removal. Random removal would result in a 50% chance of permanently fixing the error as a mutation. In E. coli, the newly synthesized strand is recognized by the absence of methylation, which is present in the parental strand. In E. coli, MutS can recognize and bind the mismatch, recruiting MutL, which acts as a bridge protein between MutS and MutH. MutH, in turn, binds to methyl groups and distinguishes the newly synthesized strand from the parental strand. The unmethylated newly synthesized strand is then cut by MutH,

and a helicase separates the two strands. The newly synthesized strand is excised and resynthesized. Eukaryotes do not use the methylation system, and the exact recognition criteria remain partially unclear. In eukaryotes, various homologs for MutS and MutL have been identified, known as MutS homolog (MSH) and MutL homolog (MLH), respectively. Homologs for MutH have not yet been identified. Different heterodimers of MSH isoforms have different functions; for example, MSH2-MSH3 heterodimers bind to mismatches caused by insertions or deletions, while MSH2-MSH6 heterodimers bind to single-base mismatches [36].

HEREDITARY BREAST TUMORS

Hereditary Breast Tumors: HBOC Syndrome

Cancer is a disease of cellular proliferation caused by the accumulation of a certain number of genetic alterations in a single cellular clone that undergoes neoplastic transformation first [37]. Based on the origin of the mutation responsible for this transformation, it is possible to distinguish two forms of breast cancer: sporadic and familial. Approximately 70% of breast cancer cases are represented by the sporadic form, meaning cancer that occurs in an individual who is the only one in the family to have developed this neoplasia. Therefore, it is a form of cancer primarily linked to environmental factors, which are responsible for the occurrence of "somatic" mutations within a single cell in the body. These mutations will not be passed on to subsequent generations but will lead, following the clonal expansion of the first transformed cell, to the appearance of a clinically evident tumor. In addition to the sporadic form, there is also familial breast cancer, which represents about 30% of breast carcinoma cases. It is defined as a form in which, within the same family, multiple neoplastic events of the same type occur due to exposure to the same environmental and dietary risk factors or the transmission of specific mutated genes that confer a predisposition to the development of cancer. In this latter case, breast cancer, in particular, is termed hereditary and is due to the transmission of a mutation present in the germ cells (germline mutation), which can be passed from one generation to the next according to Mendelian inheritance criteria. However, it is important to emphasize that inheriting a germline mutation does not mean inheriting cancer but rather inheriting a predisposition to develop that neoplasm more easily than the rest of the population. Environmental factors, as described earlier, and other genetic factors will then influence the development and onset of the disease. The hypothesis that breast cancer could have a familial or hereditary component was first proposed in 1757 by Le Dran. He described the case of a 19-year-old woman affected by breast cancer, just like her grandmother and maternal aunt, who had died from the same condition a few years earlier. In 1866, Broca analyzed a family consisting of ten women with breast cancer, spanning four generations. He was able to gather enough information to demonstrate the hereditary nature of this pathology, a characteristic further confirmed by other studies published in the 1980s. [38-41]. All these observations prompted the scientific community in those years to search for the possible genes involved in predisposition to breast cancer, leading to the identification, first in 1990 and later in 1994, of the two main susceptibility genes for this neoplasm: *BRCA1* and *BRCA2*, respectively [42,43]. These genes belong to the family of tumor suppressor genes, specifically categorized as "caretaker" genes, as they play a key role in DNA

damage repair and genomic stability regulation [44]. In particular, in the presence of one or more mutations in the *BRCA1* and *BRCA2* genes, women have a lifetime risk of developing breast cancer of approximately 70-80% and 50-60%, respectively. Furthermore, carrying pathogenic mutations in the *BRCA* genes also predisposes individuals to ovarian carcinoma, as mutations in *BRCA1* increase the risk of developing this neoplasm by about 50%, while mutations in *BRCA2* increase the risk by 30% [45]. To date, breast tumors that develop following the transmission of germline mutations in the two main high-penetrance susceptibility genes, *BRCA1* and *BRCA2*, constitute approximately 15% of all familial cases [46,47]. This suggests that numerous other genes are involved. Over the years, additional genes have been identified, and if mutated, they increase susceptibility to breast cancer. Examples include *PTEN*, *TP53*, *STK11*, *CHEK2*, *ATM*, and *PALB2*. These genes are part of a hereditary syndrome called Hereditary Breast and Ovarian Cancer (HBOC) [48]. Mutations in these latter genes predispose individuals to the disease with different penetrance compared to *BRCA1* and *BRCA2* genes. Considering the risk of breast cancer conferred by pathogenic mutations, predisposition genes can be mainly divided into three classes: high, moderate, and low penetrance. Figure 3 provides a simplified chart illustrating the main genes involved in familial breast cancer.

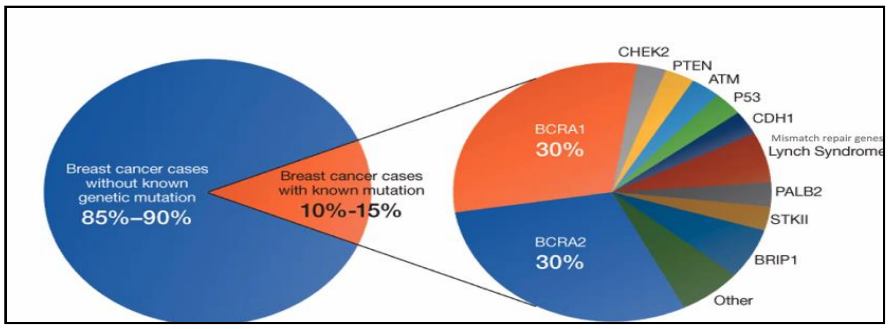

Figure 3. Susceptibility genes to BC and targetability.

In sporadic breast tumors, somatic mutations in *BRCA1* and *BRCA2* are extremely rare, although some tumors may not express the BRCA1 protein. In such cases, reduced protein expression can be achieved through hypermethylation of CpG islands in the promoter region [49]. This epigenetic alteration is strongly associated with gene silencing and, once established, can be transmitted to

offspring. It has also been observed that the presence of germline mutations in *BRCA1* and somatic promoter methylation are two mutually exclusive events.

Oncogenetic Counseling

The identification of alleles in the *BRCA1* and *BRCA2* genes capable of conferring a predisposition to breast and ovarian cancer has led to the introduction of genetic testing as the primary tool for early detection of "at-risk" individuals. In this context, since having a mutation in these genes does not guarantee the development of the disease but rather an increased susceptibility compared to the general population, genetic testing is part of a broader, multidisciplinary approach known as genetic counseling. During genetic counseling, patients have the opportunity to interact with various professionals, including oncologists, psychologists, and medical geneticists.

In general, after initially informing the patient and their family about the nature of the upcoming process, the main phases of genetic counseling include:

- Evaluation of personal and family medical history, including the construction of a family pedigree.
- Collection of clinical documentation from family members to determine the degree of familial and/or hereditary risk.
- Discussion of the potential benefits and limitations of genetic testing.

Genetic testing

Evaluation of the case based on the knowledge gained from the test and selection of the most appropriate prevention or early diagnosis option. Not all patients are directed to genetic testing, and the key role in determining eligibility is played by the first two phases of genetic counseling. Based on the data and information collected earlier, the oncologist estimates the percentage of the risk of developing breast cancer using statistical and probabilistic models. If the patient falls into the category of high familial risk, they will be offered the option of genetic testing. This category includes women who were diagnosed with breast cancer at a young age and women with a personal and/or family history of breast and ovarian cancer. According to the definition provided by the National Institutes of Health, genetic testing is defined as: "The analysis of DNA, RNA, chromosomes, proteins, metabolites, or other gene products for clinical purposes to detect genotypes, mutations, phenotypes, or karyotypes that cause or are likely to cause human heritable disease. This includes prenatal, neonatal, and carrier screening, as well as testing for families at risk. The results of such investigations may be applied to the diagnosis and prognosis of hereditary diseases, the prediction of disease risk, the identification of healthy carriers, and genotype-phenotype correlations" [50]. In our case, genetic testing is a predictive test, as it identifies individuals at high risk of developing the neoplasm through molecular analysis performed on DNA extracted from a peripheral blood sample. Therefore, all information obtained from genetic testing must be appropriately interpreted and integrated with the data obtained during genetic counseling and cannot be considered in isolation. Once individuals at risk are identified, the purpose of genetic testing is to initiate an oncological prevention pathway to reduce the risk of developing cancer. This pathway primarily involves intensive clinical and instrumental surveillance coupled with a heightened focus on lifestyle. For example, these patients are advised to prefer magnetic resonance imaging (MRI) over mammography due to its higher sensitivity [51]. Additionally, consideration can be given to prophylactic surgery or pharmacological prevention. In the former case, prophylactic mastectomy is the most effective intervention, reducing the risk by approximately 90%, although it is a highly invasive procedure with physical and psychological side effects [52]. In the latter case, tamoxifen treatment has been shown to reduce the risk of contralateral breast cancer in patients with breast cancer who test *positive* for *BRCA* mutations [53]. Furthermore, pathogenic mutations in *BRCA1* and *BRCA2* also serve as predictive factors for response to certain antitumor therapies used in ovarian cancer treatment, such as platinum-based therapy and PARP inhibitors [54,55]. Regarding the interpretation of results, genetic testing can produce three types of outcomes:

Positive result: when a mutation in the *BRCA1* and/or *BRCA2* genes associated with an increased risk of breast and/or ovarian cancer is identified. This mutation is termed "pathogenic." In this case, the previously described pathways should be initiated, and all other family members should also be considered for testing. Negative result: when no mutation in the two susceptibility genes is identified. In this situation, the risk of cancer is based on family history. However, the test's negativity could also be due to the presence of a false negative resulting from alterations in new genes possibly involved in breast cancer predisposition but not yet considered in the genetic test. Uncertain result: when a mutation is identified, but the risk of developing cancer is not yet estimated, referred to as a Variant of Uncertain Significance (VUS) [56]. Clinically, the presence of a VUS or Unclassified variant poses challenges for genetic counseling in families where these variants are identified, as they do not yet have clear biological relevance. Approximately 10-20% of *BRCA* genetic test results report the identification of a variant of uncertain significance. To date, approximately 1500 such variants have been identified, distributed in both *BRCA* genes. These variants are mainly missense mutations, silent mutations, intronic variants, frame deletions, and insertions. What complicates the classification of these variants is the lack of studies demonstrating whether these seemingly mild changes to the protein are sufficient to predispose to breast and/or ovarian cancer [57]. Therefore, the need to clarify the significance of all these variants is based on the fact that the difficulty in interpreting their biological role inevitably also affects the clinical aspect. The management of healthy individuals or those with a family history of cancer who receive an uncertain result from genetic testing is still very complex. At the same time, discovering that one carries a variant or polymorphism with unclear significance could lead to an underestimation or, conversely, an unnecessary overestimation of the risk, with potential psychological repercussions.

Multiple Genes Involved in Breast Cancer Predisposition

It is now clear, given the limited reliability and incompleteness of genetic tests for breast cancer predisposition, that the genes involved are many more than those currently known, and that Variants of Uncertain Significance (VUS) and polymorphisms related to these genes are yet to be discovered. Literature data and various databases, such as BIC and HGMD, demonstrate the involvement of additional genes beyond those examined through today's genetic test. In fact, as previously shown in Figure 3, 5% of hereditary susceptibility cases are linked to high-penetrance gene mutations such as *PALB2*, *TP53*, *PTEN*, *STK11*, and *CDH1*; another 5% are related to genes with low frequency, such as *CHEK2*, *ATM*, *NBN*, *MRE11A*, *RAD50*, and *BRIP1*; and a final 50% is associated with possible genes involved but not yet identified [58]. Variants of high and low-

penetrance genes are clearly related to breast cancer but can also be found in other neoplasms, including colorectal cancer, thyroid cancer, ovarian cancer, and lung cancer.

BRCA1/2 AND RELATED GENES IN BREAST CANCER CHEMOTHERAPY RESPONSE

The standard therapy for breast cancer treatment consists of surgical removal followed and/or preceded by chemotherapy. Therapy is defined as "Adjuvant" when administered after surgery and "Neoadjuvant" when given before surgery. Adjuvant therapies, performed after surgery, aim to target microscopic remnants that are not visible to the naked eye but might remain after the surgical procedure, which is focused on macroscopic removal. Neoadjuvant therapy, administered before surgery, aims to reduce the tumor mass's size to allow for a more conservative surgical approach. Additionally, it can guide long-term treatments based on the tumor's response to specific drug combinations [59]. The response to neoadjuvant therapy is assessed during the surgical procedure.

In breast cancer, the main chemotherapeutic agents used, which cause DNA damage, can be divided into four main groups:

- Alkylating agents
- Topoisomerase I and II inhibitors
- Platinum-based agents

Agents causing Double-strand breaks (DSB): Additionally, chemotherapy agents that inhibit cell growth are used. More recently, biotechnological-based pharmacological approaches have been adopted, allowing for greater therapy customization based on tumor characteristics. Alkylating Agents: These are agents like cyclophosphamide, which cause DNA damage by inducing inter-strand cross-linking. These cross-links lead to the arrest of DNA replication forks, resulting in the formation of double-strand DNA breaks (DSB) [60,61].

Topoisomerase I and II Inhibitors: Topoisomerases introduce temporary breaks in DNA strands to allow necessary unwinding before replication. Inhibiting topoisomerases stabilizes the topoisomerase-DNA complex, causing replication fork arrest and DSBs [62]. Drugs in this group include Anthracyclines (such as doxorubicin and epirubicin), which, in addition to topoisomerase inhibition, have the ability to induce inter-strand cross-links and generate reactive oxygen species (ROS) [63].

Platinum-Based Agents: Platinum compounds cause DNA adducts like intra- and inter-strand cross-links, which can lead to DNA replication arrest, S-phase arrest, replication fork collapse, DSBs, and consequently, apoptosis [64,65].

Agents Causing Double-Strand Breaks: These agents are capable of directly damaging DNA and causing DSBs. This group includes agents like bleomycin [66].

Mitotic Spindle Inhibitors: Both in breast and ovarian tumors, combinations of drugs are used, often with the addition of a taxane family drug like docetaxel or paclitaxel. These drugs stabilize the GDP-β-tubulin complex in microtubules, causing "microtubule freezing," inhibiting mitosis, and inducing apoptosis [67,68]. While taxanes like paclitaxel block microtubule depolymerization, vinca alkaloid derivatives like vinorelbine, also binding to β-tubulin, promote microtubule depolymerization [69,70].

PERSONALIZED THERAPIES:

With the advancement of molecular and biotechnological technologies, it has become possible to personalize treatment based on tumor characteristics. Personalized therapy is chosen after characterizing the tumor or the patient's genotype and aims to maximize benefits while minimizing unwanted effects.

Antibody and Hormone Therapies: Immunohistochemical analysis, specifically the analysis of ER, PR, and HER2 receptors, plays an essential role in choosing various personalized pharmacological approaches. Tumors positive for ER or PR receptors respond to hormone therapies like Tamoxifen, which, being a potent estrogen receptor antagonist, inhibits tumor growth. It has become the gold standard for endocrine treatment in pre- and post-menopausal women with estrogen-positive tumors [71]. In patients with overexpressed HER2 receptors, humanized monoclonal antibodies like Trastuzumab or Pertuzumab [72] can be used, targeting the extracellular domain IV and the dimerization arm of HER2, respectively. Another antibody is Lapatinib, which reversibly inhibits the intracellular tyrosine kinase activity of both HER2 and EGFR (also known as HER1). Bevacizumab is a humanized monoclonal antibody directed against Vascular Endothelial Growth Factor (VEGF), a critical cellular signal promoting angiogenesis. It can be used to counter metastatic breast cancer.

PARP Inhibitors: In recent years, a new class of molecules has been developed: poly(ADP-ribose) polymerase-1 (PARP1) inhibitors. This enzyme is involved in maintaining genomic integrity, primarily acting in base excision repair (BER) and repairing single-strand DNA lesions and breaks (figure 4). The inhibition of this enzyme, and consequently, the BER, results in the persistence of single-strand breaks (SSB). When involved in a replication fork, these SSBs can cause cell cycle arrest and may lead to double-strand breaks (DSB), which are repaired by homologous recombination (HR) or non-homologous end-joining (NHEJ). [73]. PARP inhibitors appear to be effective in BRCA1 and BRCA2 deficient tumors. This can be explained by synthetic lethality resulting from its inhibition, leading to the accumulation of DSBs in cells with defects in homologous recombination. Two genes are defined as synthetically lethal when a mutation in one of them is not lethal, but the inactivation of both leads to cell death [74]. Adding PARP inhibitors to conventional chemotherapy approaches could bring benefits in triple-negative breast cancer (TNBC) treatments. Monotherapy with PARP inhibitors could be effective in TNBC with *BRCA1/2* gene defects [75-77]. Even TNBC tumors without *BRCA* mutations could benefit from PARP inhibitors since many therapeutic agents used cause types of DNA damage normally countered by pathways involving PARP.

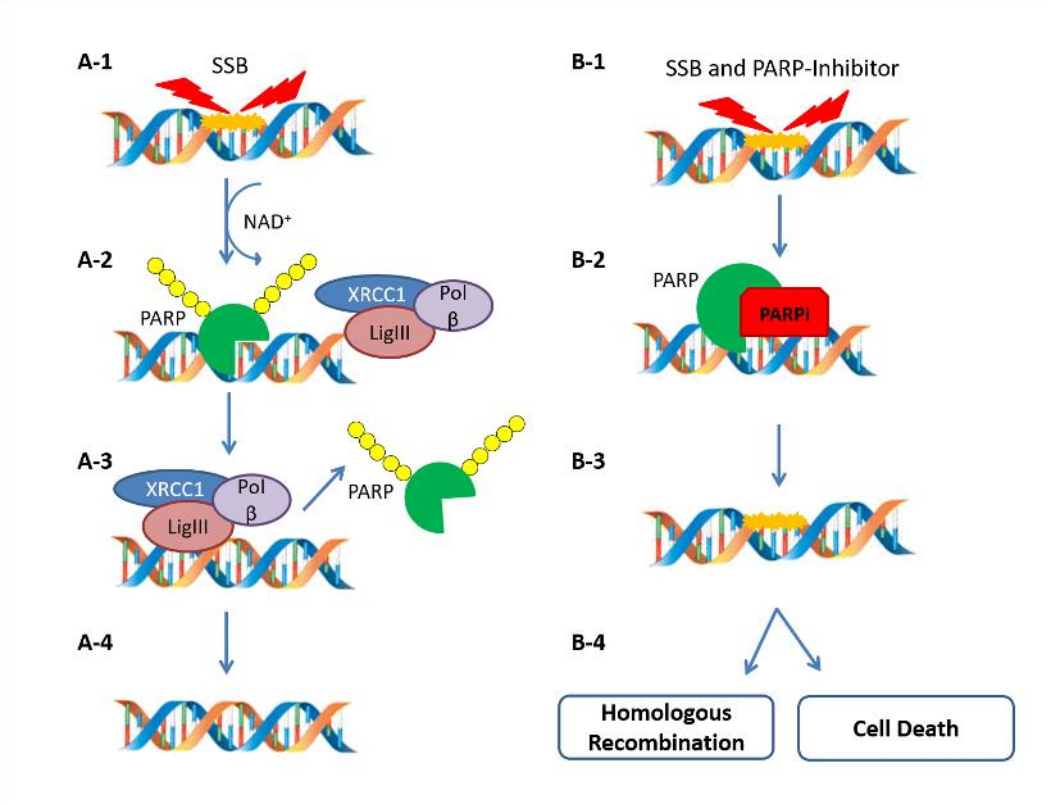

Figure 4. Molecular mechanism of PARP inhibition: When an SSB occurs (A-1), PARP-1 recruits scaffold proteins and forms an ADP-ribose chain on itself (PARylation) using NAD+ (A-2). The PARylation also promotes PARP-1 dissociation and leads scaffold proteins to repair the SSB (A-3 and A-4). When an SSB occurs in presence of a PARPi (B-

1), the PARPi binds the NAD+ binding site in a competitive way (B-2). Since the SSB cannot be repaired by PARP enzyme, the HRR system can repair the damage but the coexistence of HRD prevents the repair and induces cell death (B-3 and B-4).

Genetic Profile and Therapy Response

Most chemotherapeutic agents work by damaging DNA. Therefore, when the DNA repair systems are impaired, cells respond less effectively to chemotherapy-induced damage, influencing prognosis. Several studies have focused on the relationship between the BRCA1 protein and therapy response. BRCA1 is involved in various cellular processes, such as DNA response and repair, cell cycle checkpoints, apoptosis, transcription regulation, and ubiquitination. In DNA damage, its action is expressed both directly and indirectly, for example, through the large multiprotein complex called BRCA1-associated genome surveillance complex (BASC), which includes proteins that identify damage, such as ATM, and proteins directly involved in DNA repair, such as RAD50, MRE11A, NBN, and proteins from the Mismatch Repair (MMR) pathway, including MLH1, MSH2, and MSH6. While BRCA1 primarily acts, it might have a role in regulating mitotic control and could be involved in modulating the response to mitotic spindle-damaging agents. The mechanism is not yet entirely clear, but it could interact with γ-tubulin and participate in proper chromosome segregation during mitosis [78]. In a retrospective study where patients were treated with neoadjuvant therapy consisting of four cycles of anthracycline and cyclophosphamide, it was observed that mutations in *BRCA1* or *BRCA2* increased the response to chemotherapy [79]. In another retrospective study, it was highlighted that, in patients carrying mutations in the *BRCA1/2* genes, the best complete response was achieved in patients treated with neoadjuvant platinum-based therapy compared to neoadjuvant therapies based on CMF (cyclophosphamide, methotrexate, fluorouracil) and AT (doxorubicin and docetaxel), indicating that agents causing DSBs may be particularly effective when homologous recombination systems are less efficient. In one study, it was observed that tumors in patients with *BRCA1* mutations are more sensitive to DNA-damaging chemotherapy compared to tumors in patients with *BRCA2* mutations and sporadic tumors. However, a study on the expression of *BRCA1* in sporadic tumors contradicts what is observed in *BRCA*-mutated patients. This study reported that a decrease in *BRCA1* mRNA (defined as the mRNA expression level of *BRCA1* less than 55% compared to the expression levels of β-glucuronidase) in the tumor is associated with a less favorable response to anthracycline-based therapy [80]. The contradictory results observed in this study, compared to those describing

increased sensitivity to chemotherapeutic agents in tumors of *BRCA*-mutated patients, could be explained by the difference between reduced BRCA1 levels in sporadic tumors due to epigenetic mechanisms compared to the complete loss of BRCA1 function observed in *BRCA1*-mutated patients. Alternatively, these conflicting results may indicate that mRNA levels do not always reflect the presence of functional BRCA1 protein [81]. This effect is a consequence of the central role of BRCA1 in the DNA damage response: in the absence of a central component of the involved mechanisms, DNA-damaging chemotherapeutic agents can more easily induce apoptosis. This occurs because in the absence of BRCA1, the S-phase and G2/M transition checkpoints are not activated, and the cell, accumulating DSBs, is more easily directed towards apoptosis. It has been demonstrated, using siRNA technology, that the knockdown of *BRCA1* expression promotes apoptosis in response to platinum-based agents, confirming that these agents could be suitable for treating *BRCA1*-mutated tumors [82]. Platinum-based therapies are often adopted when there is resistance to anthracycline and taxane therapy and as palliative therapy. However, these therapies have an initial good response in TNBC patients, so clinical research on the use of cisplatin in TNBC patients, especially those carrying *BRCA1* mutations, has intensified. It is worth noting that despite the success of the initial treatment, the disease progresses rapidly, a phenomenon referred to as the "triple-negative paradox" [83]. On the other hand, *BRCA1*-mutated tumors exhibit resistance to mitotic spindle-damaging agents. BRCA1 participates in the mitotic spindle checkpoint during the metaphase-anaphase transition, necessary to ensure proper chromosome segregation in daughter cells. The disruption of the mitotic spindle caused by taxol derivatives leads to apoptotic cell death involving the SAPK/JNK (Stress Activated Protein Kinase/c-Jun N-terminal Kinase) pathway, which is also promoted by BRCA1. Indeed, it has been shown that BRCA1 can activate the transcription of GADD45, which, in turn, interacts with upstream regulators of SAPK/JNK, promoting its activation. Once activated, SAPK/JNK translocates to the nucleus and activates the pro-apoptotic protein BAD. However, the role of GADD45 in activating the SAPK/JNK pathway following DNA damage is not entirely clear [85]. Furthermore, BRCA1, acting as a "scaffold," can bring components of the stress response pathways into proximity, facilitating the MAPK cascade that leads to JNK activation. BRCA1, in collaboration with BARD1, is capable of ubiquitinating γ-tubulin, with which it co-immunoprecipitates during mitosis. This interaction appears to be responsible for BRCA1 ability to control centrosome fidelity, preventing hypertrophy and aneuploidies observed in breast tumor cells. Additionally, BRCA1 has been shown to regulate the transcription of MAD2, an essential component of the spindle checkpoint, which, by inhibiting the cdc20/APC complex (cdc20/Anaphase Promoting Complex), leads to mitotic arrest. It can be

observed that BRCA1 appears to act as a different mediator for apoptosis in breast tumor cells, depending on the nature of the chemotherapy agent used: under normal conditions, following DNA damage, it would promote repair, while following damage to microtubules, it promotes apoptosis. Therefore, in *BRCA1*-mutated patients, with the activation of these pathways missing, the efficacy of drugs that act on microtubules is reduced. In sporadic breast tumors, somatic mutations in *BRCA1*/*BRCA2* are extremely rare, but some tumors do not express the BRCA1 protein [86]. They are often associated with typical basal-like phenotype markers and are usually ER/PR-negative, thus presenting a "BRCAness" phenotype. The reduction in *BRCA1* expression can be achieved through hypermethylation of CpG islands in the promoter [87]. This epigenetic alteration is strongly associated with gene silencing, and once established, methylation is passed on to daughter cells. It has been observed that the presence of germline mutations in *BRCA1* and promoter methylation in the tumor are usually mutually exclusive events [88]. Investigating the expression status of *BRCA1* in sporadic tumors can be useful for both prognostic values and guiding therapeutic choices. For example, a reduction in *BRCA1* mRNA is associated with the acquisition of metastatic capacity, indicating that BRCA1 is required to maintain negative growth regulation in breast epithelial cells. Furthermore, it has been observed that promoter hypermethylation of *BRCA1* in BRCAness tumors can be directly used to estimate prognosis, which is unfavorable in such cases. In other studies, the loss of BRCA1 protein and methylation of CpG islands in the promoter have been specifically evaluated to identify BRCAness phenotypes in sporadic breast tumors, which can be targeted for therapy, such as with PARP inhibitors [89]. It has also been observed that tumors with a BRCAness phenotype are much more sensitive to high-dose platinum-based chemotherapy compared to conventional chemotherapy with 5-fluorouracil, epirubicin, and cyclophosphamide (FEC), further highlighting the sensitivity to agents causing DSBs in tumors with potential defects in BRCA1-related pathways [90]. It has been demonstrated that TNBC tumors treated with neoadjuvant anthracycline-based therapy show greater sensitivity to therapy, and patients with a complete response to therapy have a good prognosis; those associated with worse survival are those who did not achieve complete remission of the disease, which can be attributed to a higher propensity for recurrences [91]. A recent study examines how, in TNBC patients treated with neoadjuvant anthracycline-based therapy, the gene expression signature related to DNA repair defects is useful in distinguishing patients sensitive to this therapy. The hypothesis that patients with DNA repair defects might be more sensitive to agents like doxorubicin and have relative resistance to taxanes has been verified in several clinical studies. In one of these studies, patients were randomly assigned to receive neoadjuvant therapy with FEC (Fluorouracil, Epirubicin, Cyclophosphamide) or

a regimen mainly based on taxanes. It was observed that among patients treated with taxanes, those with defective DNA repair signatures were associated with therapy resistance. Among patients treated with FEC, those with DNA repair defect signatures had the highest complete response to the disease. Despite TNBC having a worse prognosis, they generally have a frequent complete response to neoadjuvant treatment. Additionally, carriers of *BRCA1* mutations have higher success in terms of a complete response to neoadjuvant therapy [92].

New Technologies in Biomedical Research

New technologies, such as microarrays and Next Generation Sequencing, have begun to change the way diagnostics and research are conducted. The ability to analyze entire transcriptomes, genomes, exomes, and gene groups promotes the development of personalized medicine and diagnostics by allowing for the acquisition of a substantial amount of data in a short time. The term "Next Generation Sequencing" refers to a series of new sequencing technologies no longer based on Sanger's dideoxy chain termination method but on platforms using different methods of base detection, which enable high-coverage sequencing (deep sequencing) and provide a very high yield of information over time (high-throughput). This allows, for example, the simultaneous sequencing of multiple samples. From a diagnostic perspective, it is interesting how Next Generation Sequencing platforms can be used to sequence multiple genes involved in a disease and multiple patients simultaneously, broadening the spectrum of detectable mutations in genes not routinely analyzed while maintaining comparable costs to Sanger sequencing. In the diagnostic routine, the *BRCA1* and *BRCA2* genes are mainly sequenced for HBOC syndrome in eligible individuals following genetic counseling, excluding all genes responsible for breast cancer with reduced penetrance [93]. In this context, a 2010 study describes the possibility of identifying hereditary mutations in breast and ovarian cancer using NGS techniques[94]. In Walsh and Lee's study, 20 patients with previously identified mutations were analyzed to verify the ability to identify different categories of mutations. By performing NGS analysis of all samples blindly (without knowing which mutation corresponded to each sample), they identified all variants without false *positive*s, demonstrating that these technologies are particularly useful for diagnostics.

GENES INVOLVED IN THE MOLECULAR PATHWAY OF DNA DAMAGE REPAIR

- Breast Cancer 1, Early Onset

  HGNC Symbol: *BRCA1*

  Chromosome: 17q21.31

Identified and cloned in 1994, *BRCA1* is a gene whose protein is involved in numerous cellular processes, including DNA repair, cell cycle control, and the maintenance of genomic stability. Initially, it was identified as a gene with 24 exons; however, exon 4 was subsequently removed as it was found to be an artifact of cloning, consisting of an Alu element. The exon numbering remained unchanged [95]. *BRCA1* has 32 splicing variants. The chromosomal region where *BRCA1* maps is rich in Alu repeats, accounting for approximately 41.5% of the region, while about 4.8% of the region consists of other repeated sequences [96]. The function of BRCA1 has been extensively studied. As early as 1995, it was observed that in sporadic tumors, *BRCA1* mRNA levels decreased during the transition from carcinoma in situ to invasive carcinoma. It was suggested that BRCA1 normally functions as a negative regulator of breast epithelium growth, and this function is compromised in carcinomas due to direct mutations or gene expression alterations [97]. In many tumors associated with BRCA1, numerous mutations have been identified in the RING and BRCT domains, indicating their involvement in tumor suppression. The E3 ubiquitin ligase activity of BRCA1 is emphasized when it binds to its partner, BARD1, through the RING domain. Through PALB2, BRCA1 localizes BRCA2 at double-strand break sites for repair through homologous recombination. By associating with many other proteins (such as ATM, MSH2, MSH6, MLH1, RAD50-MRE11-NBS1), it forms the BRCA1-associated genome surveillance complex (BASC), which can be localized associated with large nuclear foci. The BASC complex can function as a sensor of abnormal DNA structures and/or regulate the post-replicative repair process [98]. Although *BRCA1* and *BRCA2* mutations are rarely found in sporadic tumors, about 50% of these tumors exhibit reduced or absent BRCA1 expression. In most cases, this occurs through the combination of heterozygous loss with promoter methylation to inactivate both alleles [99]. It is hypothesized that *BRCA1*-mutated tumors follow the two-hit theory, which may explain accelerated carcinogenesis in familial cancer syndromes [100]. Furthermore, it has been proposed that single allele mutations in *BRCA1* have a haploinsufficiency effect, potentially accelerating hereditary carcinogenesis and facilitating genomic instability [101]. Mutations in *BRCA1*, with an autosomal dominant pattern of incomplete penetrance, result in the development of Hereditary Breast and

Ovarian Cancer (HBOC) syndrome, increasing the risk of developing breast tumors by up to 78% concerning age [102]. Mutations in *BRCA1* are also associated with pancreatic and prostate carcinoma.

- Breast cancer 2, early onset:

  HGNC Symbol: *BRCA2*

  Chromosome: 13q13.1

Identified in 1995 through positional cloning [104], *BRCA2* encodes a protein that shares the etiology of hereditary breast cancer with BRCA1 but has no structural homology with it. BRCA2 is directly involved in homologous recombination by binding to single-stranded DNA (ssDNA) and positioning RAD51 at DNA filament ends, allowing them to remove RPA from ssDNA. Thus, BRCA2 stabilizes the RAD51-ssDNA complexes, preventing ATP hydrolysis [105]. Biallelic mutations in *BRCA2* are associated with Fanconi anemia and monoallelic mutations, primarily, like *BRCA1*, with HBOC syndrome. In mutation carriers, they increase the risk of developing breast cancer by up to 56% in relation to age. Mutations in *BRCA2* are also associated with pancreatic cancer and glioma. Although associated with prostate cancer in only 0.1% of cases and pancreatic cancer in only 0.5% of cases, *BRCA2* mutations can increase the relative risk, compared to the general population, by up to 20 times for prostate cancer and up to 10 times for pancreatic cancer.

- BRCA1 Interacting Protein C-terminal Helicase 1:

  HGNC Symbol: *BRIP1*

  Chromosome: 17q22.2

Identified in 2001, *BRIP1* is a gene that encodes a DEAH helicase family protein. It is a DNA-dependent ATPase with 5'-3' helicase activity necessary for maintaining chromosomal stability. It possesses seven conserved helicase motifs and a nuclear localization signal. BRIP1 directly binds to BRCT repeats on BRCA1 and is directly involved in BRCA1 activity in repairing double-strand breaks (DSBs) [106]. Biallelic mutations in *BRIP1* are associated with Fanconi anemia, particularly in complementation group J, and monoallelic mutations mainly with breast and ovarian cancer.

- E-cadherin:

HGNC Symbol: *CDH1*

Chromosome: 16q22.1

CDH1 encodes the important cell adhesion protein E-cadherin. Identified in 1987 as uvomorulin, it shows an 80% nucleotide and amino acid sequence homology with its Mus musculus ortholog [107]. It is a calcium-dependent cell adhesion molecule, and its loss is usually associated with the epithelial-mesenchymal transition observed during the origin of metastatic cells. Germline mutations in *CDH1* are associated with gastric cancer and breast cancer, particularly the lobular type, with consequent loss of heterozygosity in tumor tissue [108].

- Checkpoint kinase 2:

  BHGNC Symbol: *CHEK2*

  Chromosome: 22q12.1

CHEK2, also known as *CHK2*, was identified in 1998 as the homolog of Saccharomyces cerevisiae *RAD53* and Schizosaccharomyces pombe cds1+. It encodes a serine-threonine kinase that is rapidly phosphorylated and activated in response to DNA replication blocks and DNA damage. It is involved in cell cycle arrest, DNA repair activation, and apoptosis in the presence of DSBs. It also interacts with ATM [109]. It has been shown that CHEK2 and BRCA1 interact and co-localize at foci. CHEK2 can regulate the function of BRCA1 following DNA damage by phosphorylating Ser-988, which is required for BRCA1 activation. The importance of Ser-988 phosphorylation by CHEK2 has been demonstrated in homozygous *BRCA1*-mutated cells (HCC1937), which are extremely sensitive to DNA damage. Resistance to DNA damage was restored when cells were transfected with the wild-type protein or a protein with a non-phosphorylatable residue at 988 that facilitated the separation of BRCA1 and CHEK2. It was not restored when cells were transfected with a construct carrying a non-phosphorylatable residue that did not mediate the separation of the two proteins, demonstrating that separation caused by Ser-988 phosphorylation is essential for BRCA1 activation [110]. Heterozygous germline mutations in *CHEK2* have been identified in patients with Li-Fraumeni-like syndrome, characterized by the onset of osteosarcoma, breast cancer, and brain tumors [111].

- Excision Repair Cross-Complementing Rodent Repair Deficiency:

  HGNC Symbol: *ERCC1*

  Chromosome: 19q13.32

ERCC1 was identified in 1983 through genetic transfer experiments in Chinese hamster ovary (CHO) cell lines sensitive to DNA-damaging agents [112]. It encodes a protein involved in nucleotide excision repair (NER) that interacts with ERCC4 (also known as XPF) to form an endonuclease capable of executing the DNA excision necessary for repair. Its primary role is to stabilize and promote the activity of XPF [113,114]. ERCC1, XPF, and XPA assemble into a ternary complex, required for both DNA damage recognition and excision activity [115]. Mutations in *ERCC1* are associated with cerebro-oculo-facio-skeletal syndrome type 4, a prenatal-onset condition characterized by microcephaly, cataracts, facial dysmorphisms, growth delay, and severe psychomotor retardation. This disorder belongs to NER disorders, such as xeroderma pigmentosum, trichothiodystrophy, and Cockayne syndrome.

- mutL homolog 1

  HGNC Symbol: *MLH1*

  Chromosome: 3p22.3

The *MLH1* gene is the human homolog of the MutL gene in E. coli. It was identified in the search for MMR (Mismatch Repair) genes responsible for Hereditary Non-Polyposis Colorectal Cancer (HNPCC), also known as Lynch syndrome. Mutations in *MLH1* are primarily associated with Lynch syndrome type 2 [116]. MLH1 dimerizes with PMS2, forming the MutL-α complex, a key component of MMR [117]. It has been observed to interact with DNA polymerase III, recruiting it to the MMR site. MLH1 also heterodimerizes with MLH3 to form the MutL-γ complex, involved in meiosis[118]. Mutations in *MLH1* are primarily linked to HNPCC. Lynch syndrome type 2 is characterized by an increased risk of tumors in various tissues, including the colon, uterus, ovaries, breast, stomach, intestines, skin, and larynx [119].

- mutS homolog 2 (E. coli)

HGNC Symbol: *MSH2*

Chromosome: 2p21

MSH2 is the human homolog of the MutS gene in E. coli. It was cloned and characterized in 1993 [120]. All MutS proteins share a highly conserved region of about 150 amino acids containing a helix-turn-helix (HTH) structural motif associated with an adenine and Mg2+-binding domain, known as the Walker-A motif, which is essential for MMR [121]. MSH2 is primarily involved in MMR, forming heterodimers with MSH6 and MSH3. Heterozygous mutations in *MSH2* cause Lynch syndrome type 1, particularly associated with microsatellite instability [122]. Lynch syndrome follows an autosomal dominant inheritance pattern.

- mutS homolog 6 (E. coli)

  HGNC Symbol: *MSH6*

  Chromosome: 2p16

MSH6 is a major component of MMR, dimerizing with MSH2 to form the MutS-α complex, which binds DNA mismatches to initiate repair. It contains the Walker-A ATPase motif. Heterozygous mutations in *MSH6* are primarily associated with Lynch syndrome.

- Partner and Localizer of BRCA2

  HGNC Symbol: *PALB2*

  Chromosome: 16p12.1

PALB2 was identified and cloned through mass spectrometry analysis to identify proteins that co-immunoprecipitated with BRCA2. *PALB2* encodes a protein that co-localizes with BRCA2 in nuclear foci, promoting its localization and stability within nuclear structures, thus facilitating its proper function [123]. PALB2 essential role is to act as a scaffold for the formation of the BRCA1-PALB2-BRCA2 complex, promoted by CHK2-mediated phosphorylation of BRCA1 at Ser-988. A coiled-coil domain in the N-terminal domain (NTD) interacts with the coiled-coil domain of BRCA1, while the C-terminal domain (CTD) of PALB2 interacts with the NTD of BRCA2 [124]. In conclusion, *PALB2* mutations impair homologous recombination (HR). Depletion of PALB2 results in a phenotype similar to that caused by *BRCA2* mutations since the absence of PALB2 protein product prevents the localization of BRCA2 but does not impact the activation of the S-

phase checkpoint mediated by BRCA1[125-126]. Heterozygous germline mutations in *PALB2* increase susceptibility to breast/ovarian cancer, while biallelic mutations are responsible for Fanconi anemia of the N complementation group [127].

- Poly (ADP-ribose) Polymerase 1

  HGNC Symbol: *PARP1*

  Chromosome: 1q41-q42

The protein encoded by *PARP1* is primarily involved in base excision repair (BER). It catalyzes the poly(ADP-ribosyl)ation, consuming NAD+, of a limited number of acceptor proteins involved in chromatin architecture, such as core nucleosome histones and histone H1, proteins with high mobility group (HMG) domains, and topoisomerases I and II. This modification appears to be necessary for DNA damage repair [128].

- PMS1 Postmeiotic Segregation Increased 1 (S. cerevisiae)

  HGNC Symbol: *PMS1*

  Chromosome: 2q31-q33

Identified during the search for genes homologous to bacterial and yeast MutL, the involvement of PMS1 in mismatch repair (MMR) is unclear [129]. However, dimer formation with MLH1 has been demonstrated. Mutations in *PMS1* can account for cases of hereditary non-polyposis colorectal cancer (HNPCC) in the absence of mutations in *MSH2* or *MLH1* [130,131].

- PMS2 Postmeiotic Segregation Increased 2 (S. cerevisiae)

  HGNC Symbol: *PMS2*

  Chromosome: 7p22.1

Initially identified as PMS1 during the search for genes homologous to bacterial and yeast MutL, the protein product of *PMS2* associates with MLH1, forming the MutL-α heterodimer required in MMR. Fourteen pseudogenes for *PMS2* have been identified [132]. Mutations in this gene lead to HNPCC phenotypes and a syndrome known as "Mismatch Repair Cancer Syndrome," characterized by the occurrence of a primary brain tumor followed by multiple colorectal adenomas [133,134].

- Phosphatase and tensin homolog (PTEN)

  HGNC Symbol: *PTEN*

  Chromosome: 10q23

Identified through the observation of a frequent loss of heterozygosity associated with the locus 10q23, occurring in 70% of glioblastomas and 60% of advanced prostate cancers [135], *PTEN* encodes a ubiquitously expressed protein. It functions as a tumor suppressor by its ability to antagonize the PI3K pathway through lipid phosphatase activity. Furthermore, it counteracts MAPK signaling through phosphatase activity targeting tyrosine, serine, and threonine-phosphorylated proteins. The loss of *PTEN* expression has been associated with basal-like breast cancer, both in non-hereditary and hereditary cases with *BRCA1* defects. The loss of PTEN in BRCA1-defective basal-like tumors is linked to significant mutations such as intragenic chromosomal breaks, inversions, deletions and copy number alterations, which are consistent with DNA double-strand break repair defects. This indicates a specific and recurrent oncogenic consequence of BRCA1 dysfunctions and implies that the PTEN pathway is directly involved in the transformation of progenitor cells into a basal-like phenotype [136]. Alterations in PTEN function are also implicated in the development of melanomas, cervical, endometrial, and prostate cancers. Although germline *PTEN* mutations do not seem to play a significant role in determining prostate cancer susceptibility. *PTEN* mutations cause Cowden syndrome, or "multiple hamartoma syndrome," which confers a 25-50% lifetime risk of developing breast carcinoma in affected women. *PTEN* mutations are rare in sporadic carcinomas, but loss of heterozygosity is detected in 11-41% of cases.

- RAD50 Homolog (S. cerevisiae)

HGNC Symbol: *RAD50*

Chromosome: 5q23-q31

Identified through the selection of cDNA from the chromosomal interval 5q23-q31, *RAD50* is the human homolog of the Rad50 gene in S. cerevisiae [137]. The RAD50 protein exhibits Mn2+-dependent ssDNA endonuclease activity and 3' > 5' exonuclease activity, which are crucial for recombination, repair, and the maintenance of genomic stability. RAD50 is a part of the MRN complex. Heterozygous mutations in *RAD50* are associated with Nijmegen breakage syndrome (NBS) [138,139].

- RAD51 Paralog C

  HGNC Symbol: *RAD51C*

  Chromosome: 17q25.1

The *RAD51* gene family, identified in both yeast and humans, encodes strand-transfer proteins involved in DNA repair by recombination and in meiosis. In mammals, numerous genes in this family have been identified [140]. Human RAD51 paralogs are involved in the processing of Holliday junctions [141]. RAD51, RAD51C, and XRCC3, proteins of homologous recombination, are also required for maintaining mitochondrial genomic stability [142]. Mutations in *RAD51C* are associated with DNA damage response (DDR) defects and Fanconi anemia in the O complementation group. Recent studies link *RAD51*C mutations to hereditary breast cancer [143-146].

- RAD52 Homolog (S. cerevisiae)

  HGNC Symbol: *RAD52*

Chromosome: 12p13-p12.2

The human RAD52 protein forms heptameric rings that catalyze the pairing of complementary ssDNA strands. RAD52 is primarily involved in the repair of double-strand breaks (DSBs) and plays a central role in recombination [147].

- Serine/Threonine Kinase 11 (STK11)

  HGNC Symbol: *STK11*

  Chromosome: 19p13.3

STK11 was identified and characterized in 1998 as a gene located within a locus associated with Peutz-Jeghers syndrome. This rare autosomal dominant hereditary disease is characterized by pigmentation spots in the oral area, intestinal polyp formation, and an increased risk of cancer [148]. STK11 is a tumor suppressor protein with Ser/Thr kinase activity that regulates AMP-activated protein kinase (AMPK) members' activity. It plays a regulatory role in various processes such as cellular metabolism, cell polarity (cytoskeletal remodeling), apoptosis, and DNA damage response [149]. STK11 physically associates with p53 and regulates its apoptotic pathways. It acts upstream of AMPK by mediating the phosphorylation and activation of the catalytic subunits PRKAA1 and PRKAA2, thereby regulating the inhibition of cell signaling that promotes cell growth and proliferation when cellular energy availability is low. It also regulates glucose homeostasis in the liver and autophagy activation [150]. In addition to its association with Peutz-Jeghers syndrome, *STK11* is linked to testicular cancer and is a gene with lower frequency and penetrance among the ones that increases susceptibility to breast cancer [151].

- Tumor Protein p53 (TP53)

  HGNC Symbol: *TP53*

  Chromosome: 17p13.1

TP53 encodes the p53 protein, one of the most crucial transcription factors with tumor suppressor activity. It responds to various cellular stresses and regulates proteins to induce cell cycle arrest, apoptosis, senescence, DNA repair, and metabolic changes. The p53 is also involved in apoptosis induction through non-transcriptional cytoplasmic mechanisms. The p53 is kept inactive by MDM2-mediated polyubiquitination, leading to proteasome-dependent degradation. The p53 functions as a tetramer; therefore, missense mutations that block its aggregation can result in dominant-negative mutants [152-154]. Mutations in *TP53* primarily cause Li-Fraumeni syndrome, an autosomal dominant syndrome characterized by a high risk of developing various cancers, including sarcomas, breast tumors, brain tumors (astrocytomas), and adrenocortical carcinomas. Along with *BRCA1* and *BRCA2*, it is one of the high-penetrance genes in hereditary breast cancer. Numerous *TP53* mutations have been found in many triple-negative breast cancers (TNBC), making it a potential future therapeutic target for this category of tumors [155].

PROJECT OBJECTIVES

BRCA1 and *BRCA2* are major breast cancer susceptibility genes whose pathogenic variants are associated with a significant increase in the risk of breast and ovarian cancers. Current genetic screening is generally limited to *BRCA1/2* exons and intron/exon boundaries. Some studies have highlighted that, thanks to the wide use of Next-Generation Sequencing (NGS), the number of genes suspected to be involved in cancer predisposition has dramatically increased. Since limited information currently exists about the impact of variants in *BRCA1/2* non-coding regions, the majority of variants that were identified in these regions remain unclassified. Therefore, about 80% of germline *BRCA1/2* tests result to be "negative", while introns and proximal untranslated regions remain relatively unexplored. However, evidence of non-coding variants' impact on cancer risk and response to treatment begins to emerge. We would investigate the prevalence of non-*BRCA* pathogenic germline variants in patients with risk factors: triple-negative breast cancer, early onset (< 35), family history of breast and/or ovarian cancers, bilateral breast cancer and male breast cancer, using an NGS custom panel of promoter regions of 62 genes involved into cancer predisposition. We enrolled 144 consecutive triple-negative breast cancer patients subjected to germline *BRCA1/2* test after selection by the Genetic Counseling Service of IRCCS IRST. Molecular analyses were performed on DNA extracted from peripheral blood samples of the patients enrolled. The genetic analysis was performed by next-generation sequencing (NGS) on the Illumina NextSeq 550 platform, using a custom panel including the promoter regions of 62 genes involved in cancer predisposition. Genetic information obtained from patient samples remained confidential. All patients enrolled in the study were assigned an identification code in order to maintain rigorous confidentiality standards. The biomolecular characterization of biological samples was performed at the Biosciences Laboratory of IRST IRCCS. All clinical-pathological data, together with follow-up and treatment information was collected and analyzed in association with the identified genetic variants.

MATERIALS AND METHODS

Ethics statement

The study was performed in accordance with the Good Clinical Practice and the Declaration of Helsinki and approved by the AVR Ethics Committee (protocol L3P2210, GifT). All the patients enrolled in the study have signed informed consent for the genetic analyses and for the use of the results for research purposes.

Patients and samples

Patients with a diagnosis of triple-negative breast cancer referring to the IRST Genetic Counseling service or to the Oncology units of the Area Vasta Romagna (AVR) catchment area in the years 2019-2021 were included in this study. To be considered eligible for this multicenter, retrospective study, patients must have a histologically confirmed diagnosis of triple-negative breast cancer and patients must have performed a blood sample withdrawal for *BRCA1/2* germline alteration; for those patients with a clinical history available, we collected and merged clinical and genetic data. Patients were excluded if information on *BRCA* status was not available.

All the consecutive triple-negative breast cancer patients referred from 1st January 2019 to 31st December 2021 were considered eligible for this multicenter, retrospective study. The following data were collected from all consenting patients after registration:

- demographic data: birthday, weight and height at the time of treatment initiation, ECOG performance status;
- Tumor information: date of diagnosis, ovarian cancer histology, grade and stage, date of second malignancy onset, type of tumor and its main characteristics;
- Treatment information: use of neoadjuvant treatment, date of start and end of chemotherapy, chemotherapeutic regimen with doses, number of cycles administered, type of surgery, date of surgery, PDL1 status, date of progression/relapse (if any), number and types of further therapeutic regimens;
- Date of death or last follow-up (if still alive).

Next-generation sequencing (NGS) analysis on blood samples

Blood was stored at −80 °C until genomic DNA was extracted. DNA was purified by QIAamp DNA Mini Kit (Qiagen) and quantified using Qubit fluorometer (Thermo Fisher Scientific) with Qubit dsDNA BR Assay Kit.

Figure 5. Illumina NextSeq 550 sequencer.

Sequencing libraries were created starting from 200 ng of genomic DNA, following the protocol Illumina DNA Prep with Enrichment (Illumina) with an NGS custom panel (Integrated DNA Technologies).

ABRAXAS1, APC, ATM, AXIN2, BAP1, BARD1, BLM, BMPR1A, BRCA1, BRCA2, BRIP1, CDH1, CDK4, CDKN2A, CHEK2, CTNNA1, CTNNA2, CTNNA3, CTNNB1, CTNND1, CTNND2, EPCAM, GALNT12, GREM1, HOXB13, JUP, MEN1, MLH1, MLH3, MRE11A, MSH2, MSH3, MSH6, MUTYH, NBN, NF1, NF2, NTHL1, PALB2, PIK3CA, PMS1, PMS2, POLD1, POLE, PTEN, RAD50, RAD51C, RAD51D, RB1, RECQL4, RET, RNF43, RPS20, SMAD4, STK11, TERC, TERT, TP53, TSC2, VCL, WRN, XRCC2, + enhancers CDH1gene.

Figure 6. List of 62 genes involved in cancer predisposition, included in an NGS custom panel of promoter regions.

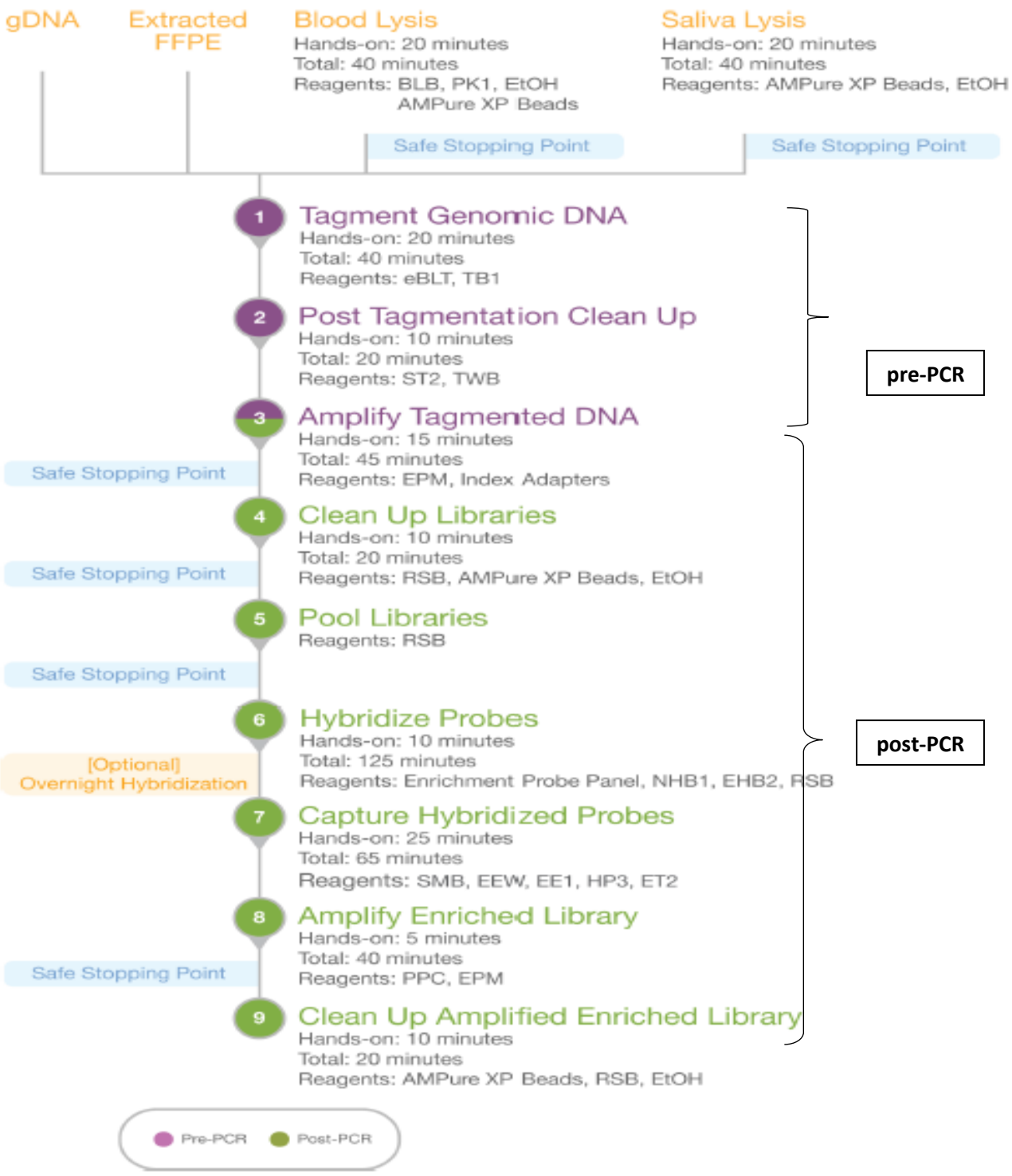

Figure 7. Illumina DNA Prep with Enrichment Workflow.

The custom panel included the promoter regions of 62 genes associated with a predisposition towards common and rare cancers. The genomic coordinates of the promoters were obtained from UCSC and Ensembl genome browsers.

Sequencing was performed by using the NextSeq550 platform (Illumina) with NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles) configured 2 × 151 cycles, according to the manufacturer's instructions.

The bioinformatics analysis of 144 consecutive triple-negative breast cancer patients was performed with a customized pipeline. Raw reads were analyzed with Illumina DRAGEN Bio-IT Platform v4.0 (1), on hg19 reference genome, using the following command line parameters:

--read-trimmers quality,adapter --trim-min-quality 20 --trim-adapter-read1 <adapter_file> --trim-adapter-read2 <adapter_file>

--enable-duplicate-marking true

--enable-variant-caller true

--vc-target-bed <bed_file>

--vc-target-bed-padding 50

Resulting variant calls were annotated with ANNOVAR v2020-06-07 (2) on the databases refGene, avsnp150, gnomad211_exome, clinvar_20221231, intervar_20180118, mcap13, dbnsfp42a.

Then the variants in the promoters of 28 genes *BRCA1*, *BRCA2*, *CDH1*, *PTEN*, *STK11*, *TP53*, *ATM*, *CHEK2*, *BARD1*, *NF1*, *NBN*, *MRE11*, *RAD50*, *PALB2*, *BRIP1*, *RAD51C*, *RAD51D*, *MSH2*, *MLH1*, *MSH6*, *PMS2*, *BLM*, *WRN*, *RECQL4*, *ABRAXAS1* (*FAM175A*), *BAP1*, *NTHL1* and *XRCC2* genes were filtered on the basis of quality (coverage>10X), frequency in the population from gnomAD (AF<0.05 or NA) and frequency in our dataset (variants present in more than 5% of the patients were excluded as common polymorphisms).

Filters applied to identify these variants include:

- Variants present in more than 5 patients.
- Variants with a population frequency exceeding 5% (based on gnomAD database)
- Variants with a Variant Allele Frequency (VAF) of less than 0.3 (considered unreliable calls on germline DNA).
- Variants with a coverage of less than 10 (deemed unreliable calls).

- Variants classified as benign/likely benign on ClinVar.
- Variants located in highly repetitive regions (microsatellites).

Statistical Analysis Description

Appropriate descriptive statistics were employed to characterize the enrolled patients in the study based on demographic attributes and tumor characteristics. Median values (with minimum and maximum values) were reported for continuous variables, while absolute values and percentages were reported for non-continuous variables. Disease-free survival (DFS) was calculated in months as the difference between the date of diagnosis and the date of disease progression for patients experiencing progression. For patients who did not experience progression or had no evidence of disease at the time of death, DFS was calculated as the difference between the date of diagnosis and the date of the last disease re-evaluation or the date of death. Events were defined as instances of disease progression or deaths without evidence of disease. Overall Survival (OS) was calculated in months as the difference between the date of diagnosis and the date of death for deceased patients. For living patients, OS was calculated as the difference between the date of diagnosis and the date of the last follow-up. Events were represented by deaths. The association between clinical characteristics and genetic variants was assessed using the Chi-square test. The probability percentages for Disease-free survival and Overall Survival were calculated using the Kaplan-Meier product limit method (Kaplan EL, Meier P: Nonparametric estimation for incomplete observations. J Am Stat Assoc 1958;53:457-481). The proportional hazards Cox regression model was used to calculate Hazard Ratios (HR) and their corresponding 95% confidence intervals (95% CI) for covariates, both in univariate and multivariate analyses. All *p*-values were determined using two-tailed tests, and the statistical analyses were conducted using the SAS statistical software, version 9.4 (SAS Institute, Cary, NC, USA).

RESULTS

Between January 2019 and December 2021, 144 patients were recruited in this study. Principal clinical characteristics of our study population were available in 75 and are presented in Table 2. We have identified 635 rare variants in the 28 genes among the 144 patients: 54 small deletions, 28 small insertions and 553 nucleotide changes, as shown in table 3. Out of these 635 rare variants, 621 were classified as 'unclassified variants, according to ClinVar. Among the 75 patients, with a mean age of 53 years (range, IQR 34-71, 47-60), 29 (38.7%) had family history, 3 (4%) presented bilateral tumors, 22 (29.3%) had a residual disease after neoadjuvant chemotherapy, only 3 patients (4%) developed a second tumor. Most of the patients received anthracycline therapy in (neo) adjuvant setting (85.3%); relapse occurred in 22 patients (29%).

Table 2. Patient Clinical Characteristics

	N. (%)
Age: median value (range, IQR)	53 (34-71, 47-60)
Family history	
No	46 (61.3)
Yes	29 (38.7)
Bilateral	
No	72 (96.0)
Yes	3 (4.0)
Stage	
I	25 (33.3)
II	31 (41.4)
III	19 (25.3)
Anthracycline:	
No	11 (14.7)
Yes	64 (85.3)
Neoadjuvant chemotherapy	
No	42 (56.0)
Yes	33 (44.0)
Pathological Complete Response (pCR)	

Yes	11 (33.0)
No	22 (67.0)
Adjuvant chemotherapy	
No	23 (30.7)
Yes	52 (69.3)

Table 3. Rare germline variants in 28 genes related to breast cancer predisposition

Gene	n. of rare variants
BRCA1	22
BRCA2	9
CDH1	72
PTEN	47
STK11	71
TP53	15
ATM	68
CHEK2	17
BARD1	28
NF1	20
NBN	6
MRE11	21
RAD50	14
PALB2	11
BRIP1	15
RAD51C	9
RAD51D	6
MSH2	29

MLH1	23
MSH6	13
PMS2	32
BLM	17
WRN	8
RECQL4	18
ABRAXAS1 (FAM175A)	12
BAP1	6
NTHL1	5
XRCC2	21
TOT	**635**

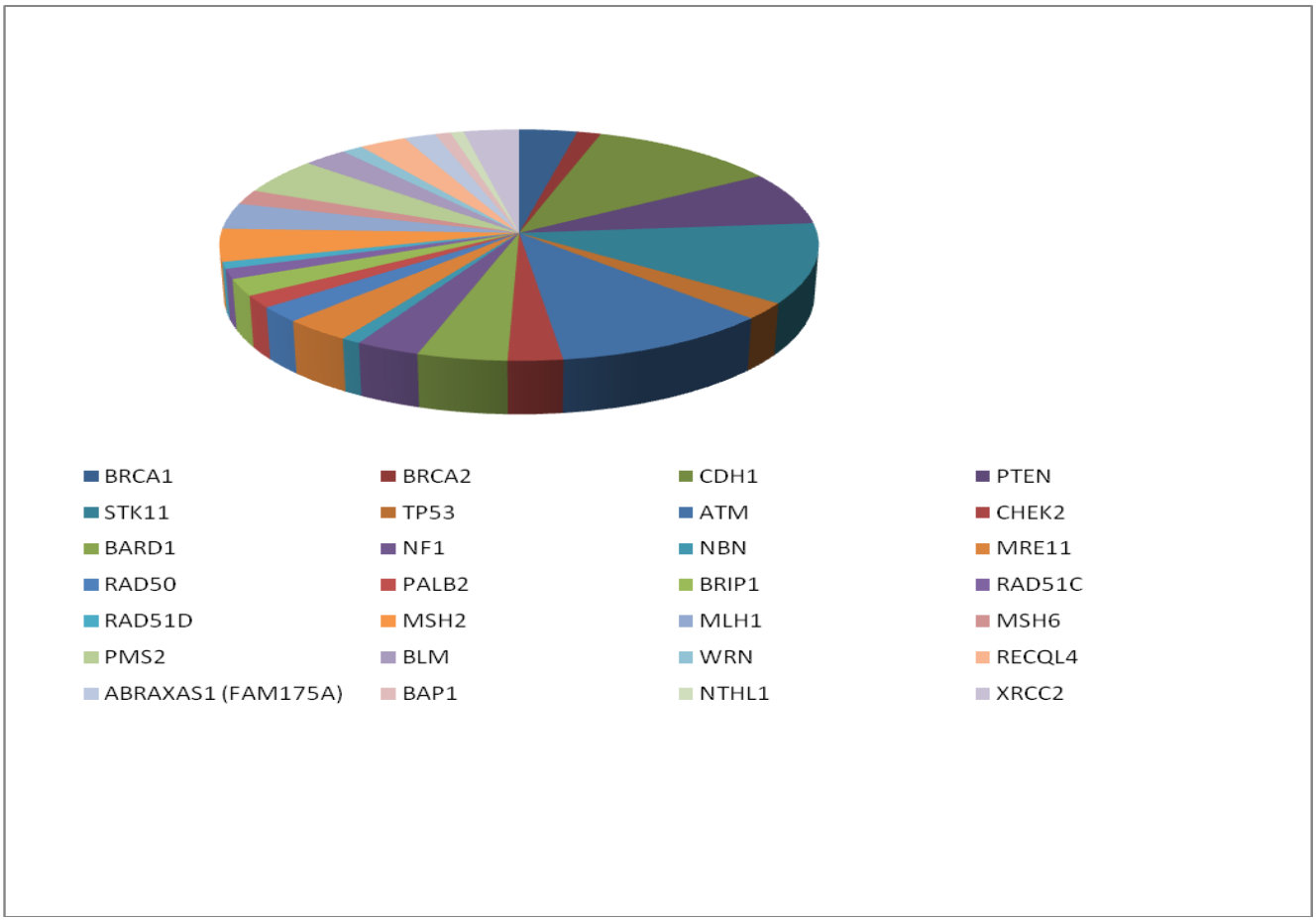

Figure 8. Distribution of 635 rare variants in the non-coding regions of 28 genes predisposing to breast cancer was analyzed.

Table 4. Number of germline rare variants and germline rare variants among 28 genes

Number of Variants: median (range, IQR)	4 (1-19, 3-5)
N. of variants	
1	4 (5.3)
2	12 (16.0)
3	16 (21.3)
4	11 (14.7)
5	14 (18.7)
6	3 (4.0)
7	7 (9.3)
8	4 (5.3)
16	1 (1.3)
19	3 (4.0)
ABRAXAS1	
WT	70 (93.3)
mutated	5 (6.7)
ATM	
WT	47 (62.7)
mutated	28 (37.3)
BAP1	
WT	72 (96.0)
mutated	3 (4.0)
BARD1	
WT	62 (82.7)
mutated	13 (17.3)
BLM	
WT	69 (92.0)
mutated	6 (8.0)
BRCA1	
WT	64 (85.3)
mutated	11 (14.7)
BRCA2	
WT	70 (93.3)
mutated	5 (6.7)
BRIP1	
WT	67 (89.3)
mutated	8 (10.7)
CDH1	
WT	42 (56.0)
mutated	33 (44.0)
CHEK2	
WT	68 (90.7)

mutated	7 (9.3)
MLH1	
WT	63 (84.0)
mutated	12 (16.0)
MRE11A	
WT	66 (88.0)
mutated	9 (12.0)
MSH2	
WT	63 (84.0)
mutated	12 (16.0)
MSH6	
WT	68 (90.7)
mutated	7 (9.3)
NBN	
WT	72 (96.0)
mutated	3 (4.0)
NF1	
WT	65 (86.7)
mutated	10 813.3)
NTHL1	
WT	73 (97.3)
mutated	2 (2.7)
PALB2	
WT	68 (90.7)
mutated	7 (9.3)
PMS2	
WT	57 (76.0)
mutated	18 (24.0)
PTEN	
WT	59 (78.7)
mutated	16 (21.3)
RAD50	
WT	68 (90.7)
mutated	7 (9.3)
RAD51C	
WT	71 (94.7)
mutated	4 (5.3)
RAD51D	
WT	73 (97.3)
mutated	2 (2.7)
RECQL4	
WT	65 (86.7)
mutated	10 (13.3)
STK11	
WT	51 (68.0)
mutated	24 (32.0)
TP53	
WT	68 (90.7)
mutated	7 (9.3)
WRN	
WT	72 (96.0)
mutated	3 (4.0)

XRCC2	
WT	64 (85.3)
mutated	11 (14.7)

Rare germline variants in *BRCA2* were statistically significantly related to worse overall survival (*p-value*=0.017 HR=4.76 (1.32-17.15)). No differences in Disease-free survival and overall survival were found for other genes. *CDH1* rare variants were related to the highest percentage of non-pathological complete response and bilateral tumors (*p-value*=0.0273). *MLH1* and *PALB2* rare variants were found to be related to bilateral breast cancer (*p-value*=0.0146 and p=0.0005, respectively). Rare variants of the *ATM* gene were associated with a positive family history (*p-value* 0.0408).

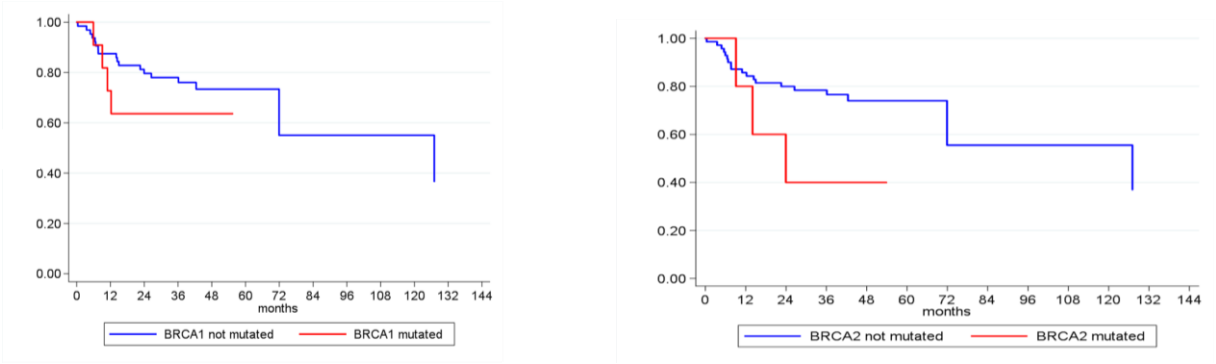

Figure 9. Disease-free survival (DFS) according to *BRCA* status: a) DFS and *BRCA1*; b) DFS and *BRCA2*.

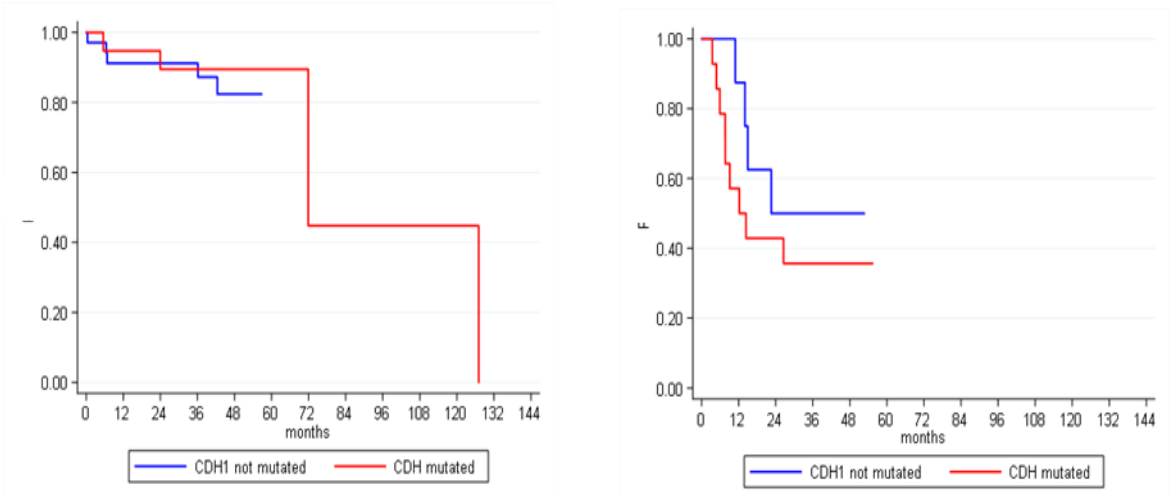

Figure 10. Disease-free survival (DFS) according to *CDH1* status and pathological complete response (pCR): on the left no pCR, on the right pCR.

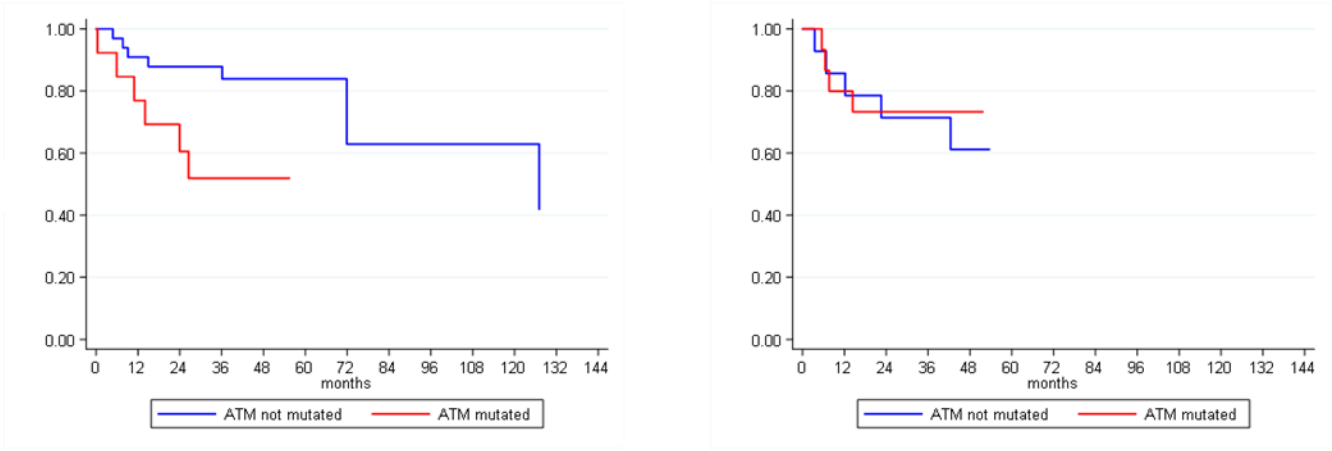

Figure 11. Disease-free survival (DFS) according to *ATM* status and family history: a) No family history; b) Family history.

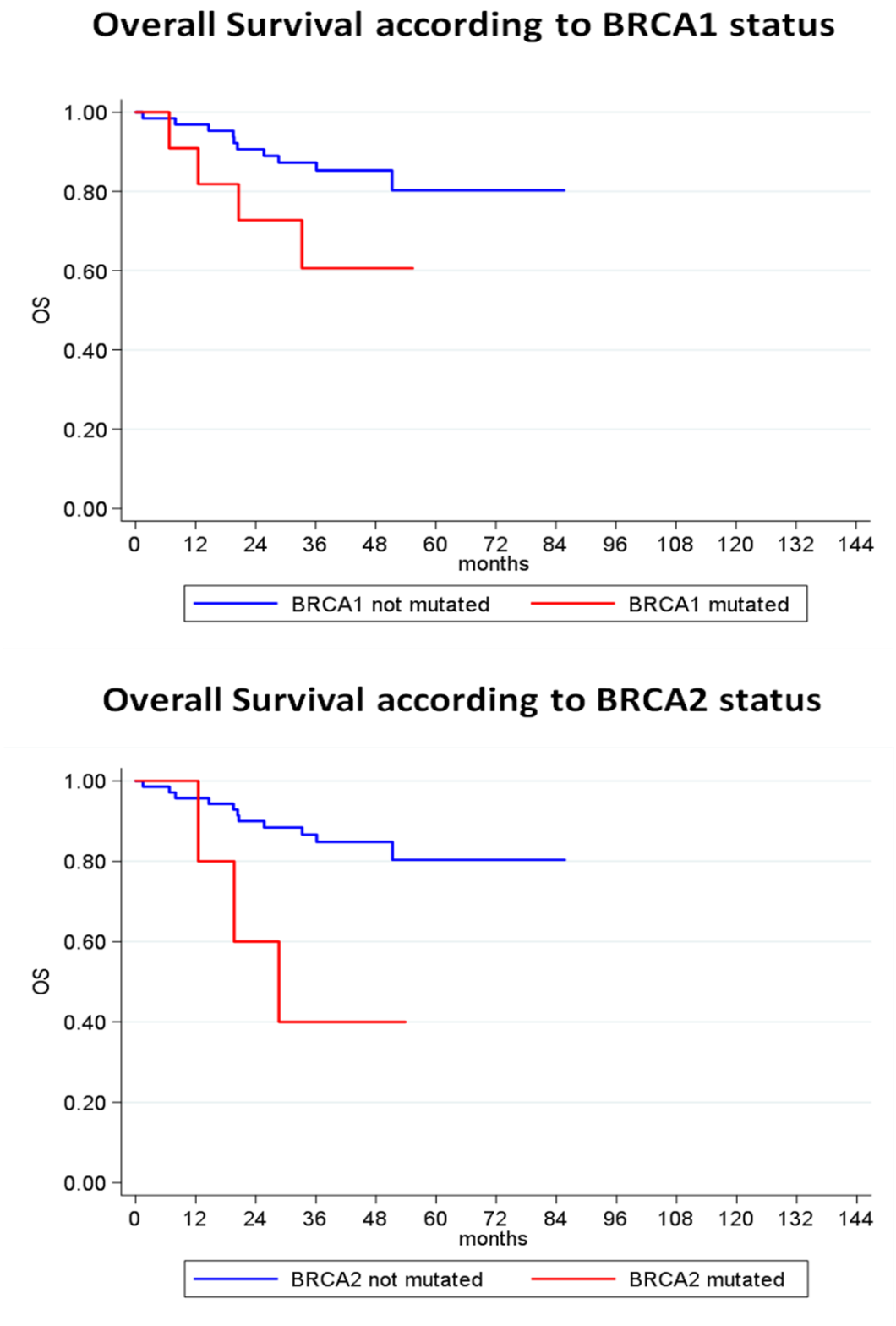

Figure 12. Overall Survival according to BRCA status: a) Relationship between *BRCA1* and overall survival (OS); b) Relationship between *BRCA2* and overall survival (OS).

Table 5. DFS and OS and Clinical Characteristics

	DFS		OS	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
Family history				
No	1.00		1.00	
Yes	1.34 (0.56-3.24)	0.511	0.88 (0.29-2.63)	0.822
Bilateral				
No	1.00		1.00	
Yes	NE	-	NE	-
Residual disease				
No	1.00		1.00	
Yes	6.09 (2.41-15.38)	0.0001	3.80 (1.31-10.98)	0.014
Stage				
I	1.00		1.00	
II	3.57 (0.99-12.90)		NE	
III	7.42 (1.94-28.42)	0.013	NE	-

Abbreviations: NE: not estimable

Table 6. Multivariate Analysis of DFS and OS according to pCR and germline *BRCA2* rare variants.

	DFS		OS	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
Residual disease				
No	1.00		1.00	
Yes	4.43 (1.65-11.89)	0.003	3.22 (1.00-10.38)	0.050
BRCA2				
WT	1.00		1.00	
mutated	4.48 (1.20-16.68)	0.025	7.23 (1.82-28.73)	0.005

Table 7. Multivariate Analysis of DFS and OS according to pCR and germline *MSH6* rare variants.

	DFS		OS	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
Residual Disease				
No	1.00		1.00	
Yes	4.32 (1.61-11.59)	0.004	3.23 (0.98-10.64)	0.054
MSH6				
WT	1.00		1.00	
mutated	1.30 (0.38-4.40)	0.677	2.60 (0.66-10.14)	0.170

Table 8. Multivariate Analysis of DFS and OS according to pCR, germline *BRCA2* rare variants and *MSH6*.

	DFS		OS	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
Residual disease				
No	1.00		1.00	
Yes	4.41 (1.64-11.89)	0.003	3.46 (1.03-11.65)	0.045
BRCA2				
WT	1.00		1.00	
mutated	4.39 (1.17-16.52)	0.028	6.23 (1.49-26.09)	0.012
MSH6				
WT	1.00		1.00	
mutated	1.15 (0.35-3.84)	0.814	1.84 (0.46-7.33)	0.384

Abbreviations: DFS, Disease-free survival; OS, overall survival; WT wild type)

In multivariate analysis, rare germline variants of *BRCA2* are confirmed to be an independent prognostic factor correlated with shorter DFS and OS. Compared to WT patients for *PALB2* mutation, patients with rare variants in the promoter of *PALB2* gene have a greater tendency to develop bilateral breast tumors. The stage at diagnosis is confirmed to be an independent prognostic factor. When grouped by the number of mutations in order to find a cut-off, no differences were observed in terms of DFS and OS.

Table 9. Univariate analysis of DFS and OS and genetic variants

	DFS		OS	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
ABRAXAS1				
WT	1.00		1.00	
mutated	0.69 (0.09-5.16)	0.718	ns	-
ATM				
WT	1.00		1.00	
mutated	1.85 (0.77-4.45)	0.169	1.89 (0.66-5.42)	0.233
BAP1				
WT	1.00		1.00	
mutated	ns	-	ns	-
BARD1				
WT	1.00		1.00	
mutated	0.55 (0.16-1.89)	0.347	0.63 (0.14-2.90)	0.558
BLM				
WT	1.00		1.00	
mutated	ns	-	ns	-
BRCA1				
WT	1.00		1.00	
mutated	1.69 (0.56-5.07)	0.349	2.73 (0.85-8.74)	0.090
BRCA2				
WT	1.00		1.00	
mutated	2.66 (0.78-9.12)	0.118	4.76 (1.32-17.15)	0.017
BRIP1				
WT	1.00		1.00	

mutated	1.01 (0.23-4.35)	0.993	0.69 (0.09-5.32)	0.722
CDH1				
WT	1.00		1.00	
mutated	2.02 (0.86-4.73)	0.105	1.30 (0.46-3.72)	0.619
CHEK2				
WT	1.00		1.00	
mutated	ns	-	ns	-
MLH1				
WT	1.00		1.00	
mutated	1.31 (0.44-3.91)	0.632	1.52 (0.42-5.47)	0.518
MRE11A				
WT	1.00		1.00	
mutated	0.82 (0.19-3.55)	0.795	ns	-
MSH2				
WT	1.00		1.00	
mutated	1.30 (0.43-3.89)	0.639	0.95 (0.21-4.24)	0.944
MSH6				
WT	1.00		1.00	
mutated	2.67 (0.89-8.00)	0.079	2.95 (0.82-10.60)	0.097
	DFS		**OS**	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
NBN				
WT	1.00		1.00	
mutated	ns	-	ns	-
NF1				
WT	1.00		1.00	

mutated	0.64 (0.15-2.78)	0.555	0.49 (0.06-3.74)	0.491
NTHL1				
WT	1.00		1.00	
mutated	1.72 (0.23-12.88)	0.598	2.62 (0.34-20.22)	0.354
PALB2				
WT	1.00		1.00	
mutated	0.56 (0.13-2.50)	0.449	1.66 (0.44-6.24)	0.454
PMS2				
WT	1.00		1.00	
mutated	1.01 (0.36-2.76)	0.998	1.25 (0.39-3.98)	0.707
PTEN				
WT	1.00		1.00	
mutated	0.90 (0.33-2.40)	0.828	0.45 (0.10-2.02)	0.295
RAD50				
WT	1.00		1.00	
mutated	0.94 (0.22-4.05)	0.933	1.31 (0.29-5.90)	0.723
RAD51C				
WT	1.00		1.00	
mutated	1.01 (0.13-7.54)	0.994	1.51 (0.20-11.58)	0.690
RAD51D				
WT	1.00		1.00	
mutated	ns	-	ns	-
RECQL4				
WT	1.00		1.00	
mutated	0.77 (0.22-2.66)	0.676	1.21 (0.33-4.46)	0.776
STK11				

WT	1.00		1.00	
mutated	1.84 (0.78-4.38)	0.166	1.20 (0.40-3.58)	0.748
TP53				
WT	1.00		1.00	
mutated	0.42 (0.06-3.16)	0.401	ns	-
WRN				
WT	1.00		1.00	
mutated	1.09 (0.14-8.12)	0.936	1.58 (0.21-12.09)	0.660
XRCC2				
WT	1.00		1.00	
mutated	2.36 (0.86-6.51)	0.096	0.99 (0.22-4.44)	0.991
N. rare variants/patient				
<4	1.00		1.00	
≥4	1.43 (0.60-3.37)	0.418	2.07 (0.66-6.51)	0.214

DISCUSSION

Breast cancer is a complex disease that can be influenced by both genetic and environmental factors. Inherited pathogenic variants in the *BRCA1* and *BRCA2* genes are well-established risk factors for breast cancer, particularly in women with a family history of the disease. TNBC is a subtype of breast is an aggressive form of breast cancer that is associated with a poorer prognosis compared to other subtypes. Recent studies have shown that rare germline variants in non-coding regions of *BRCA* and other genes can contribute to the development of triple-negative breast cancer. However, recent studies have shown that non-coding variants in these genes and other genes can also contribute to breast cancer predisposition, particularly in triple-negative breast cancer [156]. This study found that non-coding variants in the 5' region of *BRCA1* and *BRCA2* genes can alter promoter activity and protein binding, which can affect gene expression and ultimately impact breast cancer risk. Non-coding variants in *BRCA* and other genes can affect gene expression, splicing, and protein binding, which can ultimately impact breast cancer risk. Another study found that non-coding variants in *BRCA1* and *BRCA2* genes can affect the splicing of RNA, which can lead to the production of abnormal proteins and contribute to cancer development [157]. These variants are spread throughout the non-coding regions of the genes and can be difficult to detect using traditional genetic testing methods. Recent advances in sequencing technology have made it possible to identify these rare germline variants and study their impact on breast cancer predisposition [158]. Based on the search results, there is limited information on ongoing clinical trials investigating the impact of non-coding variants in *BRCA1* and *BRCA2* genes on breast cancer prognosis. While these studies suggest that non-coding variants in *BRCA1* and *BRCA2* genes can impact breast cancer risk, there is limited research on their impact on breast cancer prognosis [159-161]. To date, the risks associated with rare variants in breast cancer predisposition genes have been largely unclear. Our aim was to investigate the impact of rare germline variants in non-coding regions of *BRCA* and other cancer predisposition genes on the TNBC. In our series of 144 patients with early TNBC found to be wild type for germline variants in the coding regions of *BRCA1/2* and other cancer predisposition genes, were found to have at least one variant in the promoter regions of 28 breast cancer predisposition genes, with a median of 4 rare mutations for each patient. For 75 patients, clinical data were available and we correlated the presence of these rare variants in the non-coding regions of 28 genes predisposing to breast cancer with the clinical variables of interest and disease aggressiveness. Rare germline variants in *BRCA2* were found to significantly worsen overall survival (*p-value* = 0.017; HR = 4.76, 95% CI 1.32-17.15). In the POSH study [162], Copson and colleagues reported that patients with a *BRCA1* or *BRCA2* mutation had a similar

prognosis as patients without these mutations, but several studies have indicated that mutations in *BRCA1* and *BRCA2* may impact the effectiveness of chemotherapy. The GeparSixto Study [163], which randomly assigned patients to either standard chemotherapy containing anthracycline and taxane alone or with the addition of carboplatin, demonstrated that in patients with triple-negative breast cancer, the presence of a *BRCA1* or *BRCA2* mutation has been linked to a higher percentage of patients achieving a pathological complete response and improved survival when compared to those without a mutation, irrespective of other factors (such as kind of chemotherapy used). Patients without a mutation who received standard chemotherapy without carboplatin had lower disease-free survival rates than those who received chemotherapy plus carboplatin. Therefore, patients with triple-negative breast cancer and a *BRCA1* or *BRCA2* mutation might have a survival advantage because of the higher efficacy of systemic chemotherapy. None of our patients received platinum salts as chemotherapy. In addition, a Sardinian study observed a lower breast cancer-specific overall survival rate in *BRCA2* mutation carriers after the first two years from diagnosis. Most of the deaths in our case series were observed in the first two years from diagnosis [164]. Rare variants in *PALB2* presented in 10% of all consecutive patients, and 29% had bilateral tumors with a positive statistical association (*p-value*=0.0005). Similarly, *MLH1* was found in 16% of our cases and was related to a higher risk of bilateral tumors (*p-value*=0.0146). Adding these findings, *ATM* variants were strongly associated with positive family history (*p-value*=0.0408) whilst *CDH1* was strongly associated with residual after neoadjuvant chemotherapy (*p-value*=0.0273) being present in 44% of all cases. *BRCA2* was confirmed to be an independent variable, associated with worse outcomes. Our findings could help to underline the importance of extended genetic testing using panels including the promoter regions of genes involved in cancer predisposition, especially in breast cancer patients lacking mutations in the coding regions of *BRCA1/2*. Further research is needed to fully understand the role of non-coding variants in these genes in breast cancer prognosis and could help the choice of the best treatment. The last American College of Medical Genetics guidelines [165] do not provide specific recommendations for the reporting and classification of variants identified in *BRCA1/2* promoters and intronic and untranslated regions. Therefore, carriers should be managed exclusively based on their personal and family history, which allows for the estimation of cancer risk. Software-based models, such as BRCAPRO, are useful for estimating the risk of a woman developing cancer in the course of her life, regardless of *BRCA* status [166]. A lifetime risk of >20% justifies intensive surveillance, including annual MRI and discussion of prophylactic surgery. Nevertheless, the existing data are insufficient to substantiate the use of chemoprophylaxis with tamoxifen and risk-reducing salpingo-oophorectomy in this particular scenario. Variants of

uncertain significance (VUS) constitute a challenge for carriers and their doctors. They occur at a frequency between 5% for Caucasian Americans and up to 20% for Afro-Americans. In Europe, they are present in about 10% of *BRCA* screenings. Since the disease risk associated with VUS is unknown, the risk is not interpretable, but it may be over-interpreted or misinterpreted. As a result, it should not be used for clinical decision-making. To date, there is limited available data concerning sequence alterations in non-coding regions of *BRCA1/2*. Even less information is available about the outcome of carriers who should be managed based on their lifetime cancer risk once their genetic screening remains inconclusive.

CONCLUSION

To summarize, our study underscores the growing significance of rare germline variants in non-coding regions of genes like *BRCA1/2* in contributing to triple-negative breast cancer predisposition. Understanding the impact of these variants is crucial for the development of more effective screening and prevention strategies. Identifying individuals at increased risk due to these variants can guide clinical management, potentially improving patient outcomes. Further research is necessary to fully understand their role in breast cancer risk and to develop enhanced screening and prevention approaches for at-risk individuals. Given the limitations, our analyses should be regarded as preliminary, and larger studies are needed to validate these findings.

REFERENCES

1. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin. 2023 Jan;73(1):17-48. doi: 10.3322/caac.21763. PMID: 36633525.

2. Lopez, M.G., N.; Cascinu, S.; Marchetti, P., Oncologia Medica Pratica. III ed. Vol. Unico. 2010: SEU - Società Editrice Universo.

3. AIOM, Linee guida NEOPLASIE DELLA MAMMELLA. 2021.

4. AIRTUM 08/07/2022]; Available from: http://www.registri-tumori.it.

5. Berrino, F.M., A., Ormoni, fattori costituzionali, dieta e carcinoma mammario. Attualità in Senologia 2005. 44: p. 12-21.

6. Siliprandi, N.T., G., Biochimica medica strutturale, metabolica e funzionale. IV ed. 2013: PICCIN.

7. Karim, S., et al., Low expression of leptin and its association with breast cancer: A transcriptomic study. Oncol Rep, 2016. 36(1): p. 43-8.

8. Merenbakh-Lamin, K., et al., D538G mutation in estrogen receptor-alpha: A novel mechanism for acquired endocrine resistance in breast cancer. Cancer Res, 2013. 73(23): p. 6856-64.

9. Herynk, M.H., et al., Association between the estrogen receptor alpha A908G mutation and outcomes in invasive breast cancer. Clin Cancer Res, 2007. 13(11): p. 3235-43. 10. Lai, F.M., et al., A case-control study of parity, age at first full-term pregnancy, breast feeding and breast cancer in Taiwanese women. Proc Natl Sci Counc Repub China B, 1996. 20(3): p. 71-7.

11. Smith, D.C., et al., Association of exogenous estrogen and endometrial carcinoma. N Engl J Med, 1975. 293(23): p. 1164-7.

12. Ziel, H.K. and W.D. Finkle, Increased risk of endometrial carcinoma among users of conjugated estrogens. N Engl J Med, 1975. 293(23): p. 1167-70.

13. Hemminki, E., et al., Prescribing of noncontraceptive estrogens and progestins in the United States, 1974-86. Am J Public Health, 1988. 78(11): p. 1479-81.

14. Gambrell, R.D., Jr., Clinical use of progestins in the menopausal patient: dosage and duration. J Reprod Med, 1982. 27(8 Suppl): p. 531-8.

15. Ross, R.K., et al., Effect of hormone replacement therapy on breast cancer risk: estrogen versus estrogen plus progestin. J Natl Cancer Inst, 2000. 92(4): p. 328-32.

16. Pike, M.C., et al., Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol Rev, 1993. 15(1): p. 17-35.

17. Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast

cancer. Collaborative Group on Hormonal Factors in Breast Cancer. Lancet, 1997. 350(9084): p. 1047-59.

18. Steinberg, K.K., et al., A meta-analysis of the effect of estrogen replacement therapy on the risk of breast cancer. JAMA, 1991. 265(15): p. 1985-90.

19. Pike, M.C.B., L.; Spicer, D.V., The relationship of exogenous hormones and to breast cancer risk. Current therapy in oncology, ed. N. JE. 1995, Philadelphia (PA): Mosby Year Book.

20. Pharoah, P.D., et al., Family history and the risk of breast cancer: a systematic review and meta-analysis. Int J Cancer, 1997. 71(5): p. 800-9.

21. Perou, C.M., et al., Molecular portraits of human breast tumours. Nature, 2000. 406(6797): p. 747-52.

22. Sorlie, T., et al., Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A, 2001. 98(19): p. 10869-74.

23. van de Vijver, M.J., et al., A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med, 2002. 347(25): p. 1999-2009.

24. Sotiriou, C., et al., Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci U S A, 2003. 100(18): p. 10393-8.

25. Prat, A. and C.M. Perou, Deconstructing the molecular portraits of breast cancer. Mol Oncol, 2011. 5(1): p. 5-23.

26. Carey, L.A., Directed therapy of subtypes of triple-negative breast cancer. Oncologist, 2010. 15 Suppl 5: p. 49-56.

27. Prat, A., et al., Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res, 2010. 12(5): p. R68.

28. Cheang, M.C., et al., Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst, 2009. 101(10): p. 736-50.

29. Lips, E.H., et al., Triple-negative breast cancer: BRCAness and concordance of clinical features with BRCA1- mutation carriers. Br J Cancer, 2013. 108(10): p. 2172-7.

30. Mavaddat N, Rebbeck TR, Lakhani SR, Easton DF, Antoniou AC. Incorporating tumour pathology information into breast cancer risk prediction algorithms. Breast Cancer Res. 2010;12(3):R28. doi: 10.1186/bcr2576. Epub 2010 May 18. PMID: 20482762; PMCID: PMC2917017.

31. Roy, R., J. Chun and S. N. Powell, BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer, 2012. 12(1): 68-78.

32. Weterings, E. and D. J. Chen, The endless tale of non-homologous end-joining. Cell Res, 2008. 18(1): 114-124.

33. Bryant, H. E., E. Petermann, et al., PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. Embo j, 2009 28(17): 2601-2615.

34. Liu, Y. and S. H. Wilson. DNA base excision repair: a mechanism of trinucleotide repeat expansion. Trends Biochem Sci, 2012. 37(4): 162-172.

35. de Laat, W. L., N. G. Jaspers and J. H. Hoeijmakers. Molecular mechanism of nucleotide excision repair. Genes Dev, 1999. 13(7): 768-785.

36. Martin, A. and M. D. Scharff. AID and mismatch repair in antibody diversification. Nat RevImmunol, 2002. 2(8): 605-614.

37. Eisinger, F., et al., Hereditary breast cancer, circa 1750. Lancet, 1998. 351(9112): p. 1366.

38. Holm, N.V., M. Hauge, and B. Harvald, Etiologic factors of breast cancer elucidated by a study of unselected twins. J Natl Cancer Inst, 1980. 65(2): p. 285-98.

39. Williams, W.R. and D.E. Anderson, Genetic epidemiology of breast cancer: segregation analysis of 200 Danish pedigrees. Genet Epidemiol, 1984. 1(1): p. 7-20.

40. Newman, B., et al., Inheritance of human breast cancer: evidence for autosomal dominant transmission in high-risk families. Proc Natl Acad Sci U S A, 1988. 85(9): p. 3044-8.

41. Houlston, R.S., et al., Family history and risk of breast cancer. J Med Genet, 1992. 29(3): p. 154-7.

42. Hall, J.M., et al., Linkage of early-onset familial breast cancer to chromosome 17q21. Science, 1990. 250(4988): p. 1684-9.

43. Wooster, R., et al., Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. Science, 1994. 265(5181): p. 2088-90.

44. Hirotsu, Y., et al., Detection of BRCA1 and BRCA2 germline mutations in Japanese population using next-generation sequencing. Mol Genet Genomic Med, 2015. 3(2): p. 121-9.

45. Roy, R., J. Chun, and S.N. Powell, BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer, 2012. 12(1): p. 68-78.

46. Couch, F.J., K.L. Nathanson, and K. Offit, Two decades after BRCA: setting paradigms inpersonalized cancer care and prevention. Science, 2014. 343(6178): p. 1466-70.

47. Rudolph, A., J. Chang-Claude, and M.K. Schmidt, Gene-environment interaction and risk of breast cancer. Br J Cancer, 2016. 114(2): p. 125-33.

48. Bulletins, A.C.o.P., Hereditary breast and ovarian cancer syndrome. Gynecol Oncol, 2009. 113(1): p. 6-11.

49. Matros, E., et al., BRCA1 promoter methylation in sporadic breast tumors: relationship to gene expression profiles. Breast Cancer Res Treat, 2005. 91(2): p. 179-86.

50. ISS. Che cos'è un test genetico. 08/07/2016]; Available from: http://www.iss.it/tege/index.php?lang=1&id=79&tipo=10.

51. Kriege, M., et al., Efficacy of MRI and mammography for breast-cancer screening in women with a familial or genetic predisposition. N Engl J Med, 2004. 351(5): p. 427-37.

52. Hartmann, L.C., et al., Efficacy of bilateral prophylactic mastectomy in women with a family history of breast cancer. N Engl J Med, 1999. 340(2): p. 77-84.

53. Narod, S.A., et al., Tamoxifen and risk of contralateral breast cancer in BRCA1 and BRCA2 mutation carriers: a case-control study. Hereditary Breast Cancer Clinical Study Group. Lancet, 2000. 356(9245): p. 1876-81.

54. Alsop, K., et al., BRCA mutation frequency and patterns of treatment response in BRCA mutation-*positive* women with ovarian cancer: a report from the Australian Ovarian Cancer Study Group. J Clin Oncol, 2012. 30(21): p. 2654-63.

55. Weil, M.K. and A.P. Chen, PARP inhibitor treatment in ovarian and breast cancer. Curr Probl Cancer, 2011. 35(1): p. 7-50.

56. Calo, V., et al., The Clinical Significance of Unknown Sequence Variants in BRCA Genes. Cancers (Basel), 2010. 2(3): p. 1644-60.

57. Easton, D.F., et al., A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the BRCA1 and BRCA2 breast cancer-predisposition genes. Am J Hum Genet, 2007. 81(5): p. 873-83.

58. Walsh, T., et al., Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. JAMA, 2006. 295(12): p. 1379-88.

59. Moulder, S. and G. N. Hortobagyi. Advances in the treatment of breast cancer. Clin Pharmacol

Ther, 2008. 83(1): 26-36.

60. McHugh, P. J., W. R. Sones and J. A. Hartley (2000). "Repair of intermediate structures produced at DNA interstrand cross-links in Saccharomyces cerevisiae." Mol Cell Biol 20(10): 3425-3433.

61. Tercero, J. A. and J. F. Diffley (2001). "Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint." Nature 412(6846): 553-557.

62. Shao, R. G., C. X. Cao, H. Zhang, K. W. Kohn, M. S. Wold and Y. Pommier (1999).

"Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes." Embo j 18(5): 1397-1406.

63. Minotti, G., P. Menna, E. Salvatorelli, G. Cairo and L. Gianni (2004). "Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity." Pharmacol Rev 56(2): 185-229.

64. Zdraveski, Z. Z., J. A. Mello, M. G. Marinus and J. M. Essigmann (2000). "Multiple pathways

of recombination define cellular responses to cisplatin." Chem Biol 7(1): 39-50.

65. McHugh, P. J., V. J. Spanswick and J. A. Hartley (2001). "Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance." Lancet Oncol 2(8): 483-490.

66. D'Andrea, A. D. and W. A. Haseltine (1978). "Sequence specific cleavage of DNA by the antitumor antibiotics neocarzinostatin and bleomycin." Proc Natl Acad Sci U S A 75(8): 3608-3612.

67. Abal, M., J. M. Andreu and I. Barasoain (2003). "Taxanes: microtubule and centrosome targets, and cell cycle dependent mechanisms of action." Curr Cancer Drug Targets 3(3): 193-203.

68. Hagiwara, H. and Y. Sunada (2004). "Mechanism of taxane neurotoxicity." Breast Cancer 11(1): 82-85.

69. Gasparini, G., O. Caffo, S. Barni, L. Frontini, A. Testolin, R. B. Guglielmi and G.

Ambrosini (1994). "Vinorelbine is an active antiproliferative agent in pretreated advanced breast cancer patients: a phase II study." J Clin Oncol 12(10): 2094-2101.

70. Wang, T. H., H. S. Wang and Y. K. Soong (2000). "Paclitaxel-induced cell death: where the cell cycle and apoptosis come together." Cancer 88(11): 2619-2628.

71. Jordan, V. C. (2003). "Tamoxifen: a most unlikely pioneering medicine." Nat Rev Drug Discov 2(3): 205-213.

72. Swain SM, Baselga J, Kim SB, Ro J, Semiglazov V, Campone M, Ciruelos E, Ferrero JM, Schneeweiss A, Heeson S, Clark E, Ross G, Benyunes MC, Cortés J; CLEOPATRA Study Group. Pertuzumab, trastuzumab, and docetaxel in HER2-*positive* metastatic breast cancer. N Engl J Med. 2015 Feb 19;372(8):724-34. doi: 10.1056/NEJMoa1413513. PMID: 25693012; PMCID: PMC5584549.

73. Palleschi M, Tedaldi G, Sirico M, Virga A, Ulivi P, De Giorgi U. Moving beyond PARP Inhibition: Current State and Future Perspectives in Breast Cancer. Int J Mol Sci. 2021 Jul 23;22(15):7884. doi: 10.3390/ijms22157884. PMID: 34360649; PMCID: PMC8346118.

74. Iglehart, J. D. and D. P. Silver (2009). "Synthetic lethality--a new direction in cancer-drug development." N Engl J Med 361(2): 189-191.

75. Robson M, Im SA, Senkus E, Xu B, Domchek SM, Masuda N, Delaloge S, Li W, Tung N, Armstrong A, Wu W, Goessl C, Runswick S, Conte P. Olaparib for Metastatic Breast Cancer in Patients with a Germline BRCA Mutation. N Engl J Med. 2017 Aug 10;377(6):523-533. doi: 10.1056/NEJMoa1706450. Epub 2017 Jun 4. Erratum in: N Engl J Med. 2017 Oct 26;377(17):1700. PMID: 28578601.

76. Tutt ANJ, Garber JE, Kaufman B, Viale G, Fumagalli D, Rastogi P, Gelber RD, de Azambuja E, Fielding A, Balmaña J, Domchek SM, Gelmon KA, Hollingsworth SJ, Korde LA, Linderholm B, Bandos H, Senkus E, Suga JM, Shao Z, Pippas AW, Nowecki Z, Huzarski T, Ganz PA, Lucas PC,

Baker N, Loibl S, McConnell R, Piccart M, Schmutzler R, Steger GG, Costantino JP, Arahmani A, Wolmark N, McFadden E, Karantza V, Lakhani SR, Yothers G, Campbell C, Geyer CE Jr; OlympiA Clinical Trial Steering Committee and Investigators. Adjuvant Olaparib for Patients with BRCA1- or BRCA2-Mutated Breast Cancer. N Engl J Med. 2021 Jun 24;384(25):2394-2405. doi: 10.1056/NEJMoa2105215. Epub 2021 Jun 3. PMID: 34081848; PMCID: PMC9126186.

77. Litton JK, Rugo HS, Ettl J, Hurvitz SA, Gonçalves A, Lee KH, Fehrenbacher L, Yerushalmi R, Mina LA, Martin M, Roché H, Im YH, Quek RGW, Markova D, Tudor IC, Hannah AL, Eiermann W, Blum JL. Talazoparib in Patients with Advanced Breast Cancer and a Germline BRCA Mutation. N Engl J Med. 2018 Aug 23;379(8):753-763. doi: 10.1056/NEJMoa1802905. Epub 2018 Aug 15. PMID: 30110579.

78. Xu, X., Z. Weaver, S. P. Linke, C. Li, J. Gotay, X. W. Wang, C. C. Harris, T. Ried and C.

X. Deng (1999). "Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells." Mol Cell 3(3): 389-395.

79. Chappuis, P. O., J. Goffin, N. Wong, C. Perret, P. Ghadirian, P. N. Tonin and W. D. Foulkes (2002). "A significant response to neoadjuvant chemotherapy in BRCA1/2 related breast cancer." J Med Genet 39(8): 608-610.

80. Egawa, C., K. Motomura, Y. Miyoshi, Y. Takamura, T. Taguchi, Y. Tamaki, H. Inaji, H.

Koyama and S. Noguchi (2003). "Increased expression of BRCA1 mRNA predicts favorable response to anthracycline-containing chemotherapy in breast cancers." Breast Cancer Res Treat 78(1): 45-50.

81. Kennedy, R. D., J. E. Quinn, P. B. Mullan, P. G. Johnston and D. P. Harkin (2004). "The

role of BRCA1 in the cellular response to chemotherapy." J Natl Cancer Inst 96(22): 1659-1668.

82. Quinn, J. E., C. R. James, G. E. Stewart, J. M. Mulligan, P. White, G. K. Chang, P. B.Mullan, P. G. Johnston, R. H. Wilson and D. P. Harkin (2007). "BRCA1 mRNA expressionlevels predict for overall survival in ovarian cancer after chemotherapy." Clin Cancer Res 13(24): 7413-7420.

83. Eckstein, N. and B. Haas (2012). "[Platinum-based chemotherapy in triple negative breast

cancer]." Dtsch Med Wochenschr 137(7): 333-336.

84. Harkin, D. P., J. M. Bean, D. Miklos, Y. H. Song, V. B. Truong, C. Englert, F. C. Christians, L. W. Ellisen, S. Maheswaran, J. D. Oliner and D. A. Haber (1999). "Induction of GADD45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1." Cell 97(5): 575-586.

85. Mullan, P. B., J. J. Gorski and D. P. Harkin (2006). "BRCA1--a good predictive marker of

drug sensitivity in breast cancer treatment?" Biochim Biophys Acta 1766(2): 205-216.

86. Matros, E., Z. C. Wang, G. Lodeiro, A. Miron, J. D. Iglehart and A. L. Richardson

(2005). "BRCA1 promoter methylation in sporadic breast tumors: relationship to gene expression profiles." Breast Cancer Res Treat 91(2): 179-186.

87. Lips, E. H., L. Mulder, A. Oonk, L. E. van der Kolk, F. B. Hogervorst, A. L.Imholz, J. Wesseling, S. Rodenhuis and P. M. Nederlof (2013). "Triple-negative breast cancer: BRCAness and concordance of clinical features with BRCA1-mutation carriers." Br J Cancer 108(10): 2172- 2177.

88. Hsu, N. C., Y. F. Huang, K. K. Yokoyama, P. Y. Chu, F. M. Chen and M. F. Hou (2013). "Methylation of BRCA1 promoter region is associated with unfavorable prognosis in women with earlystage breast cancer." PLoS One 8(2): e56256.

89. Bal, A., S. Verma, K. Joshi, A. Singla, R. Thakur, S. Arora and G. Singh (2012). "BRCA1-methylated sporadic breast cancers are BRCA-like in showing a basal phenotype and absence of ER expression." Virchows Arch 461(3): 305-312.

90. Vollebergh, M. A., E. H. Lips, et al., (2011). "An aCGH classifier derived from BRCA1-mutated breast cancer and benefit of high-dose platinum-based chemotherapy in HER2-negative breast cancer patients." Ann Oncol 22(7): 1561-1570.

91. Carey, L. A., E. C. Dees, L. Sawyer, L. Gatti, D. T. Moore, F. Collichio, D. W. Ollila, C. I. Sartor, M. L. Graham and C. M. Perou (2007). "The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes." Clin Cancer Res 13(8): 2329-2334.

92. Rodriguez, A. A., A. Makris, et al., (2010). "DNA repair signature is associated with anthracycline response in triple negative breast cancer patients." Breast Cancer Res Treat 123(1): 189-196.

93. Foulkes, W. D. (2008). "Inherited susceptibility to common cancers." N Engl J Med 359(20):2143-2153.

94. Walsh, T., M. K. Lee, S. Casadei, A. M. Thornton, S. M. Stray, C. Pennil, A. S. Nord, J. B. Mandell, E. M. Swisher and M. C. King (2010). "Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing." Proc Natl Acad Sci U S A 107(28): 12629-12633.

95. Fackenthal, J. D. and O. I. Olopade (2007). "Breast cancer risk associated with BRCA1 and BRCA2 in diverse populations." Nat Rev Cancer 7(12): 937-948.

96. Smith, T. M., M. K. Lee, C. I. Szabo, N. Jerome, M. McEuen, M. Taylor, L. Hood and M. C. King (1996). "Complete genomic sequence and analysis of 117 kb of human DNA containing the gene BRCA1." Genome Res 6(11): 1029-1049.

97. Thompson, M. E., R. A. Jensen, P. S. Obermiller, D. L. Page and J. T. Holt (1995). "Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression." Nat Genet 9(4): 444-450.

98. Wang, Y., D. Cortez, P. Yazdi, N. Neff, S. J. Elledge and J. Qin (2000). "BASC, a

super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures." Genes Dev 14(8): 927-939.

99. Esteller, M. (2003). "Cancer epigenetics: DNA methylation and chromatin alterations in human cancer." Adv Exp Med Biol 532: 39-49.

100. Elledge, S. J. and A. Amon (2002). "The BRCA1 suppressor hypothesis: an explanation for the tissue-specific tumor development in BRCA1 patients." Cancer Cell 1(2): 129-132.

101. Konishi, H., et al., (2011). "Mutation of a single allele of the cancer susceptibility gene BRCA1 leads to genomic instability in human breast epithelial cells." Proc Natl Acad Sci U S A 108(43): 17773- 17778.

102. Roy, R., J. Chun and S. N. Powell (2012). "BRCA1 and BRCA2: different roles in a common

pathway of genome protection." Nat Rev Cancer 12(1): 68-78.

103. Antoniou, A., et al., (2003). "Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies." Am J Hum Genet 72(5): 1117-1130.

104. Wooster, R., G. Bignell, J. Lancaster, S. Swift, S. Seal, J. Mangion, N. Collins, S. Gregory, C. Gumbs and G. Micklem (1995). "Identification of the breast cancer susceptibility gene BRCA2." Nature 378(6559): 789-792.

105. Yang, H., P. D. Jeffrey, J. Miller, E. Kinnucan, Y. Sun, N. H. Thoma, N. Zheng, P. L. Chen, W. H. Lee and N. P. Pavletich (2002). "BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure." Science 297(5588): 1837-1848.

106. Cantor, S. B., D. W. Bell, S. Ganesan, E. M. Kass, R. Drapkin, S. Grossman, D. C. Wahrer, D. C. Sgroi, W. S. Lane, D. A. Haber and D. M. Livingston (2001). "BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function." Cell 105(1): 149-160.

107. Mansouri, A., N. Spurr, P. N. Goodfellow and R. Kemler (1988). "Characterization and chromosomal localization of the gene encoding the human cell adhesion molecule uvomorulin." Differentiation 38(1): 67-71.

108. Masciari, S., N. Larsson, et al., (2007). "Germline E-cadherin mutations in familial lobular breast cancer." J Med Genet 44(11): 726-731.

109. Matsuoka, S., M. Huang and S. J. Elledge (1998). "Linkage of ATM to cell cycle regulation

by the Chk2 protein kinase." Science 282(5395): 1893-1897.

110. Lee, J. S., K. M. Collins, A. L. Brown, C. H. Lee and J. H. Chung (2000). "hCds1- mediated phosphorylation of BRCA1 regulates the DNA damage response." Nature 404(6774): 201-204.

111. Bell, D. W., et al., (1999). "Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome." Science 286(5449): 2528-2531.

112. Rubin, J. S., A. L. Joyner, A. Bernstein and G. F. Whitmore (1983). "Molecular identification of a human DNA repair gene following DNA-mediated gene transfer." Nature 306(5939): 206- 208.

113. Gregg, S. Q., A. R. Robinson and L. J. Niedernhofer (2011). "Physiological consequences of defects in ERCC1-XPF DNA repair endonuclease." DNA Repair (Amst) 10(7): 781-791.

114. Kashiyama, K., et al., (2013). "Malfunction of nuclease ERCC1-XPF results in diverse clinical manifestations and causes Cockayne syndrome, xeroderma pigmentosum, and Fanconi anemia." Am J Hum Genet 92(5): 807-819.

115. Park, C. H. and A. Sancar (1994). "Formation of a ternary complex by human XPA, ERCC1, and ERCC4(XPF) excision repair proteins." Proc Natl Acad Sci U S A 91(11): 5017-5021.

116. Papadopoulos, N., N. C. Nicolaides, Y. F. Wei, S. M. Ruben, K. C. Carter, C. A. Rosen, W. A. Haseltine, R. D. Fleischmann, C. M. Fraser, M. D. Adams and et al. (1994). "Mutation of a mutL homolog in hereditary colon cancer." Science 263(5153): 1625-1629.

117. Kadyrov, F. A., L. Dzantiev, N. Constantin and P. Modrich (2006). "Endonucleolytic function of MutLalpha in human mismatch repair." Cell 126(2): 297-308.

118. Holloway, J. K., J. Booth, W. Edelmann, C. H. McGowan and P. E. Cohen (2008). "MUS81 generates a subset of MLH1-MLH3-independent crossovers in mammalian meiosis." PLoS Genet 4(9): e1000186.

119. Clyne, M., J. Offman, S. Shanley, J. D. Virgo, M. Radulovic, Y. Wang, A. Ardern-Jones, R. Eeles, E. Hoffmann and V. P. Yu (2009). "The G67E mutation in hMLH1 is associated with an unusual presentation of Lynch syndrome." Br J Cancer 100(2): 376-380.

120. Fishel, R., M. K. Lescoe, M. R. Rao, N. G. Copeland, N. A. Jenkins, J. Garber, M. Kane and R. Kolodner (1993). "The human mutatedr gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer." Cell 75(5): 1027-1038.

121. Gradia, S., S. Acharya and R. Fishel (1997). "The human mismatch recognition complex hMSH2-hMSH6 functions as a novel molecular switch." Cell 91(7): 995-1005.

122. Fishel, R., A. Ewel, S. Lee, M. K. Lescoe and J. Griffith (1994). "Binding of mismatched

microsatellite DNA sequences by the human MSH2 protein." Science 266(5189): 1403-1405.

123. Xia, B., J. C. Dorsman, N. Ameziane, Y. de Vries, M. A. Rooimans, Q. Sheng, G. Pals, A. Errami, E. Gluckman, J. Llera, W. Wang, D. M. Livingston, H. Joenje and J. P. de Winter (2007). "Fanconi anemia is associated with a defect in the BRCA2 partner PALB2." Nat Genet 39(2): 159-161.

124. Oliver, A. W., S. Swift, C. J. Lord, A. Ashworth and L. H. Pearl (2009). "Structural basis for recruitment of BRCA2 by PALB2." EMBO Rep 10(9): 990-996.

125. Zhang, J., H. Willers, Z. Feng, J. C. Ghosh, S. Kim, D. T. Weaver, J. H. Chung, S. N. Powell and F. Xia (2004). "Chk2 phosphorylation of BRCA1 regulates DNA double-strand break repair." Mol Cell Biol 24(2): 708-718.

126. Zhang, F., J. Ma, J. Wu, L. Ye, H. Cai, B. Xia and X. Yu (2009). "PALB2 links BRCA1

and BRCA2 in the DNA-damage response." Curr Biol 19(6): 524-529.

127. Rahman, N., S. Seal, et al., (2007). "PALB2, which encodes a BRCA2-interacting protein, is a

breast cancer susceptibility gene." Nat Genet 39(2): 165-167.

128. Kim, M. Y., S. Mauro, N. Gevry, J. T. Lis and W. L. Kraus (2004). "NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1." Cell 119(6): 803-814.

129. Cannavo, E., B. Gerrits, G. Marra, R. Schlapbach and J. Jiricny (2007). "Characterization of the interactome of the human MutL homologues MLH1, PMS1, and PMS2." J Biol Chem 282(5): 2976-2986.

130. Nicolaides, N. C., N. Papadopoulos, B. Liu, Y. F. Wei, K. C. Carter, S. M. Ruben,

C. A. Rosen, W. A. Haseltine, R. D. Fleischmann, C. M. Fraser and et al. (1994). "Mutations

of two PMS homologues in hereditary nonpolyposis colon cancer." Nature 371(6492): 75-80.

131. Silva, F. C., M. D. Valentin, O. Ferreira Fde, D. M. Carraro and B. M. Rossi (2009).

"Mismatch repair genes in Lynch syndrome: a review." Sao Paulo Med J 127(1): 46-51.

132. Hamilton, S. R., B. Liu, R. E. Parsons, N. Papadopoulos, J. Jen, S. M. Powell, A.

J. Krush, T. Berk, Z. Cohen, B. Tetu and et al. (1995). "The molecular basis of Turcot's syndrome."

N Engl J Med 332(13): 839-847.

133. Li, J., C. Yen, D. Liaw, et al., (1997). "PTEN, a putative protein tyrosine phosphatase gene

mutated in human brain, breast, and prostate cancer." Science 275(5308): 1943-1947.

134. Pezzolesi, M. G., K. M. Zbuk, K. A. Waite and C. Eng (2007). "Comparative genomic

and functional analyses reveal a novel cis-acting PTEN regulatory element as a highly conserved functional E-box motif deleted in Cowden syndrome." Hum Mol Genet 16(9): 1058-1071.

135. Saal, L. H., S. K. Gruvberger-Saal, et al., (2008). "Recurrent gross mutations of the PTEN

tumor suppressor gene in breast cancers with deficient DSB repair." Nat Genet 40(1): 102-107.

136. Xie, C. C., L. Lu, J. Sun, S. L. Zheng, W. B. Isaacs, H. Gronberg and J. Xu (2011).

"Germ-line sequence variants of PTEN do not have an important role in hereditary and non-hereditary prostate cancer susceptibility." J Hum Genet 56(7): 496-502.

137. Dolganov, G. M., R. S. Maser, A. Novikov, L. Tosto, S. Chong, D. A. Bressan and

J. H. Petrini (1996). "Human Rad50 is physically associated with human Mre11: identification of a conserved

multiprotein complex implicated in recombinational DNA repair." Mol Cell Biol 16(9): 4832-4841.

138. Carney, J. P., R. S. Maser, H. Olivares, E. M. Davis, M. Le Beau, J. R. Yates, 3rd,

L. Hays, W. F. Morgan and J. H. Petrini (1998). "The hMre11/hRad50 protein complex and

Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response." Cell 93(3): 477-486.

139. Waltes, R., R. Kalb, M. Gatei, A. W. Kijas, M. Stumm, A. Sobeck, B. Wieland, R.

Varon, Y. Lerenthal, M. F. Lavin, D. Schindler and T. Dork (2009). "Human RAD50 deficiency

in a Nijmegen breakage syndrome-like disorder." Am J Hum Genet 84(5): 605-616.

140. Dosanjh, M. K., D. W. Collins, W. Fan, G. G. Lennon, J. S. Albala, Z. Shen and D.

Schild (1998). "Isolation and characterization of RAD51C, a new human member of the RAD51 family of related genes." Nucleic Acids Res 26(5): 1179-1184.

141. Liu, Y., J. Y. Masson, R. Shah, P. O'Regan and S. C. West (2004). "RAD51C is required for Holliday junction processing in mammalian cells." Science 303(5655): 243-246.

142. Sage, J. M., O. S. Gildemeister and K. L. Knight (2010). "Discovery of a novel function for

human Rad51: maintenance of the mitochondrial genome." J Biol Chem 285(25): 18984-18990.

143. Vaz, F., H. Hanenberg, et al., (2010). "Mutation of the RAD51C gene in a Fanconi anemialike

disorder." Nat Genet 42(5): 406-409.

144. Pelttari, L. M., T. Heikkinen, D. Thompson, A. Kallioniemi, J. Schleutker, K. Holli, C. Blomqvist, K. Aittomaki, R. Butzow and H. Nevanlinna (2011). "RAD51C is a susceptibility gene for ovarian cancer." Hum Mol Genet 20(16): 3278-3288.

145. Loveday, C., C. Turnbull, E. Ruark, R. M. Xicola, E. Ramsay, D. Hughes, M. Warren-Perry, K. Snape, D. Eccles, D. G. Evans, M. Gore, A. Renwick, S. Seal, A. C. Antoniou and N. Rahman (2012). "Germline RAD51C mutations confer susceptibility to ovarian cancer." Nat Genet 44(5): 475-476; author reply 476.

146. Thompson, M. E., R. A. Jensen, P. S. Obermiller, D. L. Page and J. T. Holt (1995).

"Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression." Nat Genet 9(4): 444-450.

147. Kagawa, W., H. Kurumizaka, R. Ishitani, S. Fukai, O. Nureki, T. Shibata and S. Yokoyama (2002). "Crystal structure of the homologous-pairing domain from the human Rad52 recombinase in the undecameric form." Mol Cell 10(2): 359-371.

148. Jenne, D. E., H. Reimann, J. Nezu, W. Friedel, S. Loff, R. Jeschke, O. Muller, W.

Back and M. Zimmer (1998). "Peutz-Jeghers syndrome is caused by mutations in a novel serine threonine kinase." Nat Genet 18(1): 38-43.

149. Karuman, P., O. Gozani, R. D. Odze, X. C. Zhou, H. Zhu, R. Shaw, T. P. Brien, C. D. Bozzuto, D. Ooi, L. C. Cantley and J. Yuan (2001). "The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death." Mol Cell 7(6): 1307-1319.

150. Ollila, S. and T. P. Makela (2011). "The tumor suppressor kinase LKB1: lessons from mouse models." J Mol Cell Biol 3(6): 330-340.

151. van der Groep, P., E. van der Wall and P. J. van Diest (2011). "Pathology of hereditary breast cancer." Cell Oncol (Dordr) 34(2): 71-88.

152. Toledo, F. and G. M. Wahl (2006). "Regulating the p53 pathway: in vitro hypotheses, in vivo veritas." Nat Rev Cancer 6(12): 909-923.

153. Bourdon, J. C. (2007). "p53 and its isoforms in cancer." Br J Cancer 97(3): 277-282.

154. Vousden, K. H. and D. P. Lane (2007). "p53 in health and disease." Nat Rev Mol Cell Biol 8(4): 275-283.

155. Turner, N., E. Moretti, O. Siclari, I. Migliaccio, L. Santarpia, M. D'Incalci, S. Piccolo, A. Veronesi, A. Zambelli, G. Del Sal and A. Di Leo (2013). "Targeting triple negative breast cancer: is p53 the answer?" Cancer Treat Rev 39(5): 541-550.

156. Burke LJ, Sevcik J, Gambino G, Tudini E, Mucaki EJ, Shirley BC, Whiley P, Parsons MT, De Leeneer K, Gutiérrez-Enríquez S, Santamariña M, Caputo SM, Santana Dos Santos E, Soukupova J, Janatova M, Zemankova P, Lhotova K, Stolarova L, Borecka M, Moles-Fernández A, Manoukian S, Bonanni B; ENIGMA Consortium; Edwards SL, Blok MJ, van Overeem Hansen T, Rossing M, Diez O, Vega A, Claes KBM, Goldgar DE, Rouleau E, Radice P, Peterlongo P, Rogan PK, Caligo M, Spurdle AB, Brown MA. BRCA1 and BRCA2 5' noncoding region variants identified in breast

cancer patients alter promoter activity and protein binding. Hum Mutat. 2018 Dec;39(12):2025-2039.

157. Santana Dos Santos E, Lallemand F, Burke L, Stoppa-Lyonnet D, Brown M, Caputo SM, Rouleau E. Non-Coding Variants in BRCA1 and BRCA2 Genes: Potential Impact on Breast and Ovarian Cancer Predisposition. Cancers (Basel). 2018 Nov 16;10(11):453. doi: 10.3390/cancers10110453. PMID: 30453575; PMCID: PMC6266896.

158. Colas C, Golmard L, de Pauw A, Caputo SM, Stoppa-Lyonnet D. "Decoding hereditary breast cancer" benefits and questions from multigene panel testing. Breast. 2019 Jun;45:29-35. doi: 10.1016/j.breast.2019.01.002. Epub 2019 Jan 8. PMID: 30822622.

159. Lee A, Moon BI, Kim TH. BRCA1/BRCA2 Pathogenic Variant Breast Cancer: Treatment and Prevention Strategies. Ann Lab Med. 2020 Mar;40(2):114-121. doi: 10.3343/alm.2020.40.2.114. PMID: 31650727; PMCID: PMC6822003.

160. Pohl-Rescigno E, Hauke J, Loibl S, et al. Association of Germline Variant Status With Therapy Response in High-risk Early-Stage Breast Cancer: A Secondary Analysis of the GeparOcto Randomized Clinical Trial. *JAMA Oncol.* 2020;6(5):744–748. doi:10.1001/jamaoncol.2020.0007

161. Cragun D, Weidner A, Tezak A, Clouse K, Pal T. Cancer risk management among female BRCA1/2, PALB2, CHEK2, and ATM carriers. Breast Cancer Res Treat. 2020 Jul;182(2):421-428. doi: 10.1007/s10549-020-05699-y. Epub 2020 May 22. PMID: 32445176.

162. Copson ER, Maishman TC, Tapper WJ, Cutress RI, Greville-Heygate S, Altman DG, Eccles B, Gerty S, Durcan LT, Jones L, Evans DG, Thompson AM, Pharoah P, Easton DF, Dunning AM, Hanby A, Lakhani S, Eeles R, Gilbert FJ, Hamed H, Hodgson S, Simmonds P, Stanton L, Eccles DM. Germline BRCA mutation and outcome in young-onset breast cancer (POSH): a prospective cohort study. Lancet Oncol. 2018 Feb;19(2):169-180. doi: 10.1016/S1470-2045(17)30891-4. Epub 2018 Jan 11. PMID: 29337092; PMCID: PMC5805863.

163. Loibl S, Weber KE, Timms KM, Elkin EP, Hahnen E, Fasching PA, Lederer B, Denkert C, Schneeweiss A, Braun S, Salat CT, Rezai M, Blohmer JU, Zahm DM, Jackisch C, Gerber B, Klare P, Kümmel S, Schem C, Paepke S, Schmutzler R, Rhiem K, Penn S, Reid J, Nekljudova V, Hartman AR, von Minckwitz G, Untch M. Survival analysis of carboplatin added to an anthracycline/taxane-based neoadjuvant chemotherapy and HRD score as predictor of response-final results from GeparSixto. Ann Oncol. 2018 Dec 1;29(12):2341-2347. doi: 10.1093/annonc/mdy460. PMID: 30335131.

164. Budroni, M., Cesaraccio, R., Coviello, V. et al. Role of BRCA2 mutation status on overall survival among breast cancer patients from Sardinia. BMC Cancer 9, 62 (2009). https://doi.org/10.1186/1471-2407-9-62

165. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL; ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology.

Genet Med. 2015 May;17(5):405-24. doi: 10.1038/gim.2015.30. Epub 2015 Mar 5. PMID: 25741868; PMCID: PMC4544753.

166. Berry DA, Iversen ES Jr, Gudbjartsson DF, Hiller EH, Garber JE, Peshkin BN, Lerman C, Watson P, Lynch HT, Hilsenbeck SG, Rubinstein WS, Hughes KS, Parmigiani G. BRCAPRO validation, sensitivity of genetic testing of BRCA1/BRCA2, and prevalence of other breast cancer susceptibility genes. J Clin Oncol. 2002 Jun 1;20(11):2701-12. doi: 10.1200/JCO.2002.05.121. PMID: 12039933.