ELIZABETH SANTANA DOS SANTOS

Contribuição das variantes missense e não codificantes dos gener BRCA1/2 para a predisposição hereditária e resposta ao tratamento dos cânceres de mama e ovário

Tese apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Programa de Oncologia

Orientadora: Profa. Dra. Maria Aparecida Azevedo Koike Folgueira

Ecole Doctorale de Cancérologie

Orientador: Dr. Etienne Rouleau

SÃO PAULO

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RESUMO

Santos ES. *Contribuição das variantes missense e não codificantes dos gener BRCA1/2 para a predisposição hereditária e resposta ao tratamento dos cânceres de mama e ovário* [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2020.

O câncer de mama e de ovário são atualmente definidos de acordo com as principais vias envolvidas na tumorigênese. Nos cânceres de mama/ovário hereditários (HBOC), os tumores com variantes patogênicas (PV) BRCA1/2 apresentam comprometimento na via de reparo do DNA por recombinação homóloga (HR). Por muitos anos, as variantes patogênicas dos genes BRCA1/2 foram pesquisadas apenas no DNA germinativo. Atualmente, essas informações são igualmente pesquisadas no tumor com o intuito de personalizar o tratamento. Porém, a razão da inativação desta via permanece incerta na maioria dos casos, mesmo na presença instabilidade genética confirmada por diferentes assinaturas. Oss estudos indicam que variantes patogênicas que inativam aproteína pode não ser o único mecanismo da disfunção da RH. Nesse contexto, o principal objetivo desta tese é identificar mecanismos alternativos de inativação da RH para melhorar: aconselhamento genético e otimizar a resposta terapêutica. Para esse fim, buscamos contribuir para a classificação de variantes BRCA1/2 não codificantes e e variantes missense de significado incertoe pesquisamos novos biomarcadores de resposta terapêutica aos agentes que danificam o DNA em outros genes da RH. Identificamos variantes germinativas nos principais elementos reguladores da transcrição do BRCA1 e BRCA2 e demonstramos que parte delas eram funcionalmente ativas e apresentavam argumentos adicionais sugerindo patogenicidade. Exploramos também as características moleculares de tumores da mama e do ovário de portadores da variantes *BRCA1* e observamos um predomínio da perda do alelo selvagem no grupo de tumores com variantes patogênicas. Frente a este achado, propusemos incorporar as informações da LOH no modelo multifatorial para a classificação das variantes BRCA1. Finalmente, descrevemos mecanismos alternativos de inativação da recombinação homóloga em uma população de pacientes com câncer de ovário que apresentaram ótima resposta à quimioterapia à base de platina, incluindo hipermetilação do promotor *BRCA1* emutações em outros genes da via.

Descritores: Recombinação homóloga; BRCA1; BRCA2; Variantes não codificantes, Variantes BRCA1⁄2 de significado incerto; Câncer de mama; Câncer de ovário.

ABSTRACT

Santos ES. *Contribution of the missense and non-coding BRCA1/2 variants for the hereditary predisposition and response to treatment of breast and ovarian cancers* [thesis]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; 2020.

Ovarian and breast cancers are currently defined by the main pathways involved in the tumorigenesis. In hereditary breast/ovarian cancers (HBOC), tumors with BRCA1/2 pathogenic variants (PV) present an impairment of DNA repair by homologous recombination (HR). For many years, BRCA1/2 PV were only searched on germline DNA. Currently, this information is also searched at tumor level to personalize treatment. Even so, the reason of the inactivation of this pathway remains uncertain for most cases, even in the presence of HR deficient signature. Gathered evidence indicates that protein inactivating PV may not be the only mechanism of HR dysfunction. In this context, the main objective of this thesis is to identify alternative mechanisms of HR inactivation to improve both: genetic counseling and therapeutic response. For this purpose, we have attempted to contribute to non-coding and missense (other than premature stop codon) BRCA1/2 variant classification and searched for new biomarkers of therapeutic response to DNA damage agents in other HR genes. We identified germline variants in key transcriptional regulatory elements of BRCA1 and BRCA2, and demonstrated that part of them were functionally active and had additional arguments suggesting pathogenicity. We also explored molecular features of breast and ovarian tumors from BRCA1 variant carriers and observed a predominance of loss of the wild-type allele. Conforming to this evidence, we propose to incorporate LOH information, into the multifactorial model for BRCA1 variant classification. Finally, besides the enrichment of BRCA1/2 germline and somatic PV, we described alternative mechanisms of HR inactivation in a OC population presenting optimal response to platinum-based chemotherapy, including BRCA1 promoter hypermethylation and also mutations in other genes of HR pathway.

Descriptors: Homologous recombination; Genes, BRCA1; Genes, BRCA2; Non-coding variants; BRCA1⁄2 variant of uncertain significance; Breast câncer; Ovarian neoplasm.

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
BARD1	BRCA1 Associated RING Domain 1
BER	Base excision repair
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
BRCT	BRCA1 C Terminus
BRIP1	BRCA1 interacting protein C-terminal helicase 1
CASP	CAspase
CCNE	Cycline E
CDK	Cyclin dependent kinase
CIMBA	Consortium of Investigators of Modifiers of *BRCA1/2*
CHEK2	Checkpoint kinase 1
CHEK2	Checkpoint kinase 2
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleoside 5'-triphosphate
DSB	Double-strand Breaks
ER	Estrogen receptor
ERCC1	Excision repair cross-complementing protein 1
HBOC	Hereditary breast and ovarian cancer
HER2	Human epidermal growth factor Recept 2
HGSOC	High-grade serous ovarian carcinomas
HR	Homologous recombination
HRD	Homologous recombination deficiency
LOH	Loss of heterozygosity
LR	Likelihood ratio
MMR	Mismatch repair
MRI	Magnetic resonance imaging

MRN complex	**M**RE11-**R**AD50-**N**BS1 complex
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NMD	Nonsense-mediated mRNA decay
ORR	Overal response rate
PALB2	Partner and localizer of BRCA2
PARP	Poly (ADP-ribose) polymerase
PR	Progesteron receptor
PROSE	Prevention and observation of surgical endpoints
PTEN	Phosphatase and tensin homolog
RPA	Replication protein A
RRM	Risk reducing mastectomy
RRSO	Risk-reducing salpingo-oophorectomy
STIC	Serous tubal intraepithelial carcinomas
TNBC	Triple negatif breast cancer
VUS	Variant of unknown significance

SUMMARY

1 INTRODUCTION

This introduction will resume the functional mechanisms and consequences of DNA damage repair failure in the cell, with focus on homologous recombination deficiency. The understanding of the mechanism at the cell level will be faced to the current knowledge from clinical data about HR deficiency in cancer predisposition and treatment. To elucidate the dysfunction mechanism observed at clinical level, it is very important to contemplate the mechanisms involved at the cellular level. In Figure 1, we try to represent the anallytical approach used in this work to better understand the mechanism of inactivation of homologous recombination pathway through the search for new alterations.

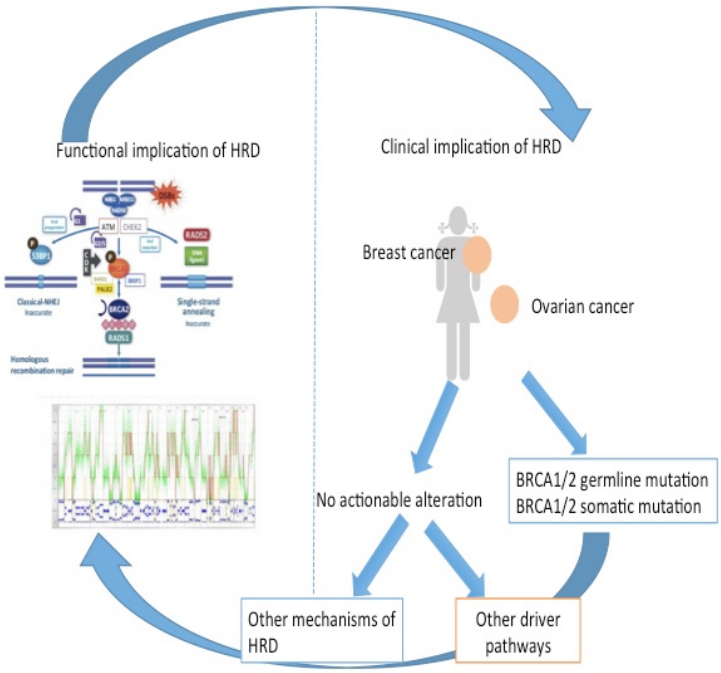

Figure 1 Homologous recombination deficiency: elucidating the HRD pattern in breast and ovarian cancers beyond BRCA1/2 coding mutations requires efforts to correlate the clinical data to functional knowledge.

Fonte:

1.1 HRness in ovarian and breast cancers

1.1.1 Cell cycle, DNA reparation and aptoptosis pathways

In this part, we will explain the different connexions between cell cycle, DNA reparation, and apoptosis. The cell cycle, once initiated, could faceDNA damage. If so, cells have to stop their progression through the cell cycle to allow for DNA repair. After this, the cells can restart their progression through the G2 and M phases of the cell cycle. However, if the DNA damages are too important, the cells undergo apoptosis.

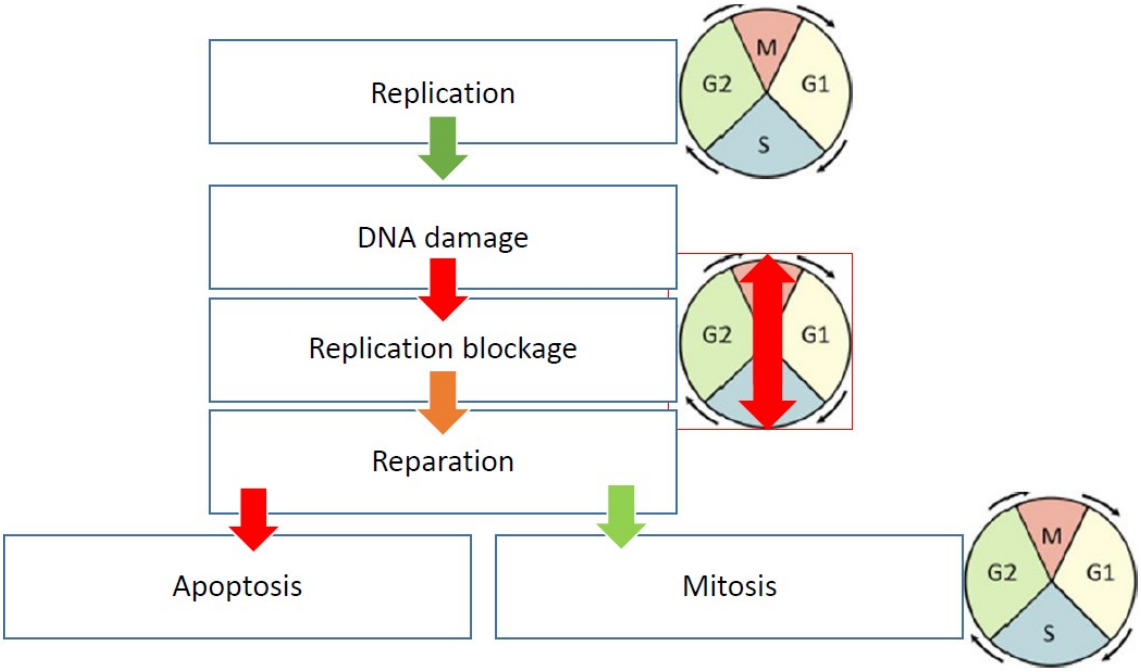

Figura 2 Schematic view of cell replication, DNA repair and apoptosis pathways.
Fonte:

1.1.1.1 Cell cycle

Since cells divide constantly, to maintain genome integrity of cells and tissues in development, DNA must be duplicated precisely before cell division occurs, with correction of any mistakes. There are checkpoints in the cell cycle involved in maintenance of DNA integrity.

The cell cycle is divided into 4 phases: G1 (preparation of the DNA replication), S (DNA replication), G2 (preparation of the mitosis), and M (Mitosis). When a cell is out of the cell cycle, it is in the G0 phase. The cell cycle is controlled by different cyclin-dependent kinases (CDK). Each CDK is specificaly linked to a cyclin which is crucial for its kinase activity. The different dimers CDK-cycline modulate the progression of cells through the cell cycle. Each CDK-cyclin complex is specific of one or several phase(s) of the cell cycle. For instance, the dimer CDK2-cycline E modulates the G1/S transition, the dimer CDK1-cycline A modulates the G2 and S phases, and the dimer CDK1-cycline B modulates the M phase (CHIRACKAL MANAVALAN et al., 2019). During the G1 phase, CDK4-cyclin D and CDK6-cyclin D phosphorylate the protein RB. This phosphorylation inhibits the RB-E2F association. Once liberated from RB, the transcriptional activator E2F activates the transcription of genes indispensable fot the DNA replication or S phase (KENT; LEONE, 2019). The transition to each phase is controlled by proteins such as CDK12 (CHIRACKAL MANAVALAN et al., 2019). The regulation of CDK-cyclin complexes are mainly assured through phosphorylation and dephosphorylation cycles (NISHITANI; LYGEROU, 2002).

During S phase, the quantity of DNA is doubled with the replication forks. The double-stranded DNA is separated into single-stranded DNA, allowing the recruitment of replication protein A (RPA) and then the loading of the replicative DNA polymerases and PCNA sliding clamps (TAKEDA; DUTTA, 2005). Three DNA polymerases - Pol α, Pol δ, and Pol ε - are essential for DNA replication. After Pol α initiatesDNA synthesis by, Pol δ takes over on the lagging and Pol ε takes over on the leading strand, performing the bulk of replication with very high fidelity (HEITZER; TOMLINSON, 2014). The main actors are the polymerases Pol ε (coded by the POLE gene) and Pol δ (codeded by the POLD1 gene). Dysfunction of these proteins generates intrinsic DNA errors (BELLIDO et al., 2016).

Three canonical S-phase "checkpoint pathways" are involved in the maintenance of DNA integrity: replication checkpoint detects stalled replication forks, S-M checkpoint blocks mitosis until the entire genome has been successfully duplicated, and intra-S Phase checkpoint which is sensitive to double-strand-breaks (DSBs) and whose main regulators are ATR and ATM kinases (BARTEK; LUKAS; LUKAS, 2004). This system will detect DNA errors, leading to the stop of the cell cycle and the direct reparation of these errors.

The most sensitive phase is the S phase. The proteins involved in this phase also participate in the replication cycle, such as BRCA1. BRCA1 also participates in the maintenance of centrosome number during late S and G2/M phase (SHAO et al., 1996).

Contrary to BRCA1, the role of BRCA2 in transcriptional and cell cycle regulation is less certain, but some studies support such roles (MARMORSTEIN; OUCHI; AARONSON, 1998).

1.1.1.2 Reparation mecanisms

Each replication faces to 10,000 to 100,000 DNA damage lesion events per day (DEXHEIMER, 2013). Those DNA damages can be caused by environmental agents such as environmental chemicals, cigarette smoke, and ionizing radiation from sunlight, or also from endogenous processes such as normal cell metabolism which generates reactive oxygen species that can oxidize DNA bases and cause single strand bases damage. Some enodogenous DNA damages can also be caused by defaults in regulation proteins or repair proteins.

However, genomic integrity is maintained by the complex network of DNA repair response which includes cell-cycle checkpoints and DNA repair pathways activated by endogenous and exogenous cell stressors. The type of repair mechanism is determined by the type of lesions and position in the cell cycle. It is also important to understand that the reparation system needs to stop the cell cycle to correctly change the error in the DNA. That is why there is direct interaction between the CDKs and the proteins sensitive to DNA alterations.

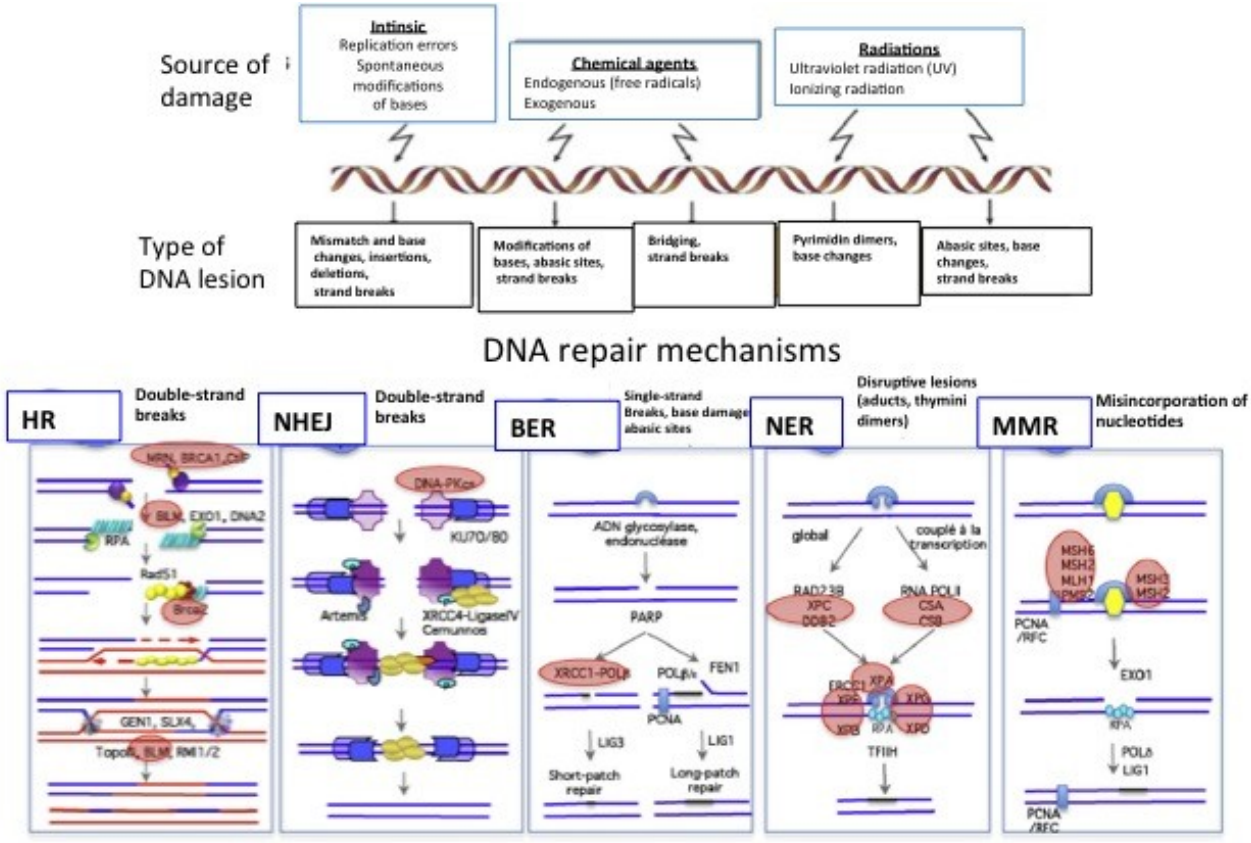

Figure 2 Mechanisms of DNA repair according to the type of lesion.
Adapted from (RASS et al., 2012).

The reparation systems depend on the nature of the break. The single-strand breaks, are repaired by base excision repair (BER) (David et al., 2007). This mechanism is based on removal of damaged base from the double helix. The injured section of DNA is then excised and replaced with newly synthesized DNA. Poly (ADP-ribose) polymerase 1 and 2 (PARP 1 and 2) are key enzymes in this process, acting as sensors and signal transducers for lesions.

Nucleotide Excision Repair (NER) is responsible for repairing bulkier single-strand lesions that distorts the DNA helical structure and disrupts base pairing (SPIVAK, 2015). NER involves recognition of the lesions, adducts, or structures that disrupt DNA, removal of short oligonucleotides containing the lesion, synthesis of a repair patch copying the opposite undamaged strand, and ligation to restore the DNA to its original form. Excision repair cross-complementing protein 1 (ERCC1) is a key protein in this DNA repair pathway.

DNA damages can also occur during DNA replication by misincorporation of nucleotides (deoxyribonucleoside 5'-triphosphate or dNTP) forming insertion and deletion loops. Mismatch Repair (MMR) system detects the resulting mismatches in the DNA sequence, excises the newly synthesized DNA encompassing the mismatch site, and replaces

it with newly synthesized DNA. MMR is one of the most important guardians of genomic stability. It maintains DNA integrity during DNA replication, aborts illegitimate recombination, and affects the outcome of several other processes of DNA metabolism. The deficiency of MMR gives rise to a mutator phenotype and microsatellite instability that leads to cancer. Proteins encoded by the MutS and MutL homologous genes, such as MSH2 and MLH1, are central in the process of mismatch repair (LI; PEARLMAN; HSIEH, 2016).

DNA double-strand breaks (DSBs) are carried out by two major mechanisms that differ in their fidelity and template requirement: Non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ is an error-prone pathway that does not use a template for DNA repair but simply ligates the broken DNA ends together, which leads to an accumulation of errors such as little insertions or deletions (CHANG et al., 2017). On the other hand, HR repair is a highly conserved mechanism that enables the accurate repair of DNA double-strand breaks by using the intact sister chromatid as a template for repair, thereby maintaining the sequence integrity. As it requires a template for repair, it occurs mainly during the late S and G2 phases of cell cycle. BRCA1 and BRCA2 are key components of this pathway that involves the coordinated interaction of BRCA1 and BRCA2 with other DNA repair proteins such as ATM, CHEK2, BARD1, BRIP1, MRE11, RAD50, NBN/NBS1, RAD51C, RAD51D, and PALB2. In cells that are deficient of BRCA1⁄2, the repair of DNA double-strand breaks relies on the error-prone NHEJ pathway (O'KANE; CONNOR; GALLINGER, 2017).

The reparation mechanisms of NHEJ and HR are not involved equally during the cell cycle (Figure 3). In G0 and M phases, CDK activity is low and no sister chromatide is available, favoring NHEJ pathways. NHEJ is mainly present during G1 phase, whereas the HR is mainly present during the S phase. In the M phase, both HR and NHEJ repair are blocked, and DSB that arise during mitosis are repaired by single-strand annealing (a DNA damage repair mechanism which we do not describe in this manuscript), resulting in large-scale chromosomal rearrangements. CDK activity, which increases in the S and G2 phases of the cell cycle, favors also BRCA1 activation and DNA repair by HR.

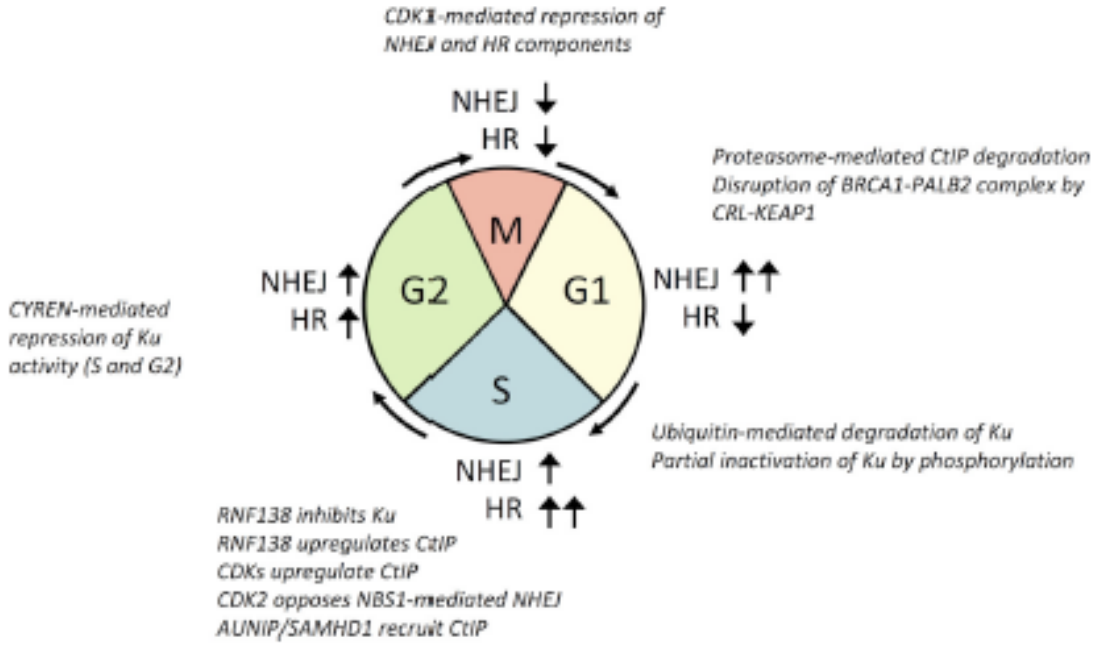

Figure 3 Pathways for DNA repair are active at different rates at different phases of the cell cycle. Adapted from (HER; BUNTING, 2018).

Briefly, HR DNA repair begins after recognition of the 5'ends of the double-strand DNA break by the MRN complex (MRE11-RAD50-NBS1). This complex recruits Ataxia telegiectasia mutated (ATM), a protein kinase. ATM subsequently phosphorylates downstream proteins, particularly BRCA1, and CHEK1 and CHEK2, which are two serine/threonine-protein kinases inducing cell cycle arrest at the G1/S and G2/M cell cycle checkpoint allowing DNA damages repair (STELZER et al., 2016). The phosphorylation of BRCA1 by ATM induces its recruitment to DNA damage sites and its binding to BARD1, a E3 ubiquitin-protein ligase essential for BRCA1 stability (STELZER et al., 2016). CDK activity, which increases in the S and G2 phases of the cell cycle, also favors BRCA1 activation and DNA repair by HR. BRCA1 activation then allows extentive 5' end ressection to produce 3' single-stranded DNA and the induction of the RAD51 loading to the single-stranded DNA by the BRCA1/BRCA2/PALB2 complex. PALB2 recruits BRCA2 and RAD51 to DNA break sites by enhancing BRCA1-BRCA2 interaction and binding DNA with high affinity for D loop (STELZER et al., 2016). DNA is then repaired using the homologous region of the chromatid as a replicative template.

Besides the maintenance of genomic integrity, BRCA1 and BRCA2 proteins have multiples functions in the cell, whose failure might also be related to carcinogenesis. BRCA1

is involved in checkpoint regulation during cell cycle; a strategy that transiently inhibits DNA synthesis allowing for the repair of DNA lesions (YU; CHEN, 2004). Moreover, several lines of evidence suggest that BRCA1 regulates its expression at RNA transcription level and through participation on chromatin remodeling (BOCHAR et al., 2000; SCULLY et al., 1997). BRCA1 also participates in the maintenance of centrosome number during late S and G2/M phase and also in the regulation of apoptosis (SHAO et al., 1996). Contrary to BRCA1, the role of BRCA2 in transcriptional and cell cycle regulation is less certain but some studies support such roles (MARMORSTEIN; OUCHI; AARONSON, 1998).

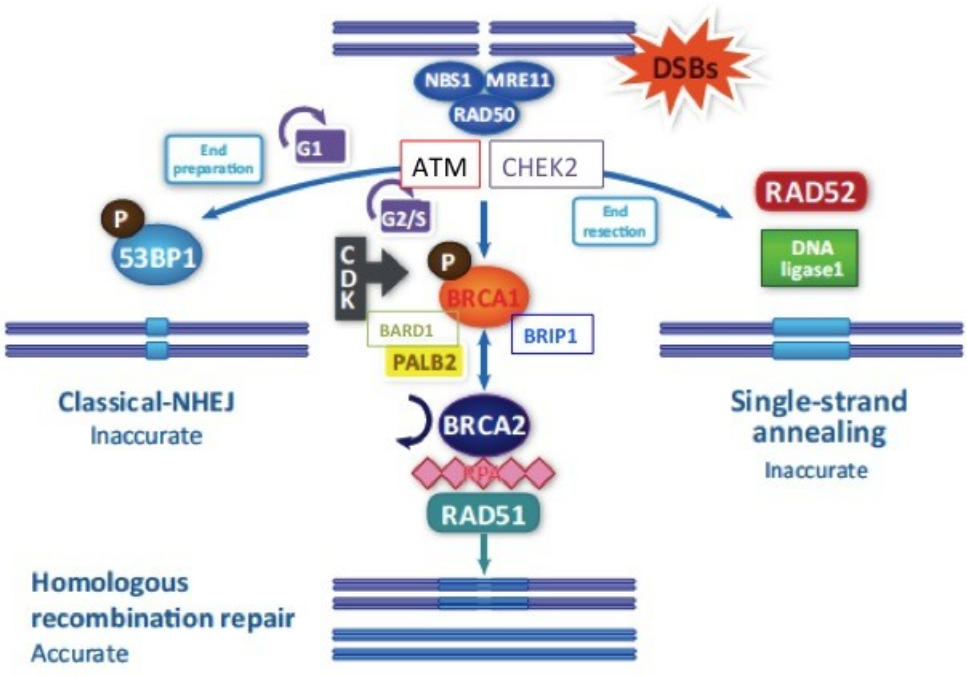

Figure 4 DNA double strand break repair by homologous recombination. Coordinated interaction of BRCA1 and BRCA2 with other HR proteins to repair double strand breaks.
Adapted from (O'KANE; CONNOR; GALLINGER, 2017)

1.1.1.3 Failure of DNA repair and cell death

Once submitted to high level of stress, the cell risks death. It may happen either through programmed cell death or through accidental cell death (TANG et al., 2019).

Apoptosis is the programmed cell death type 1 (GREEN; LLAMBI, 2015). In autophagy (cell-death type 2), the cell consumes itself. If the mechanism of apoptosis is not in place, the destruction will lead to necrosis (cell-death type 3).

Apoptosis can involve an intrinsic pathway with mitochondria or extrinsic pathway with death receptors. In the intrinsic pathway, the DNA damage can be a cause of the stress. Apoptosis is also dependent of proteins called caspases. Pro-apoptotic (BAX, BAK1) and antiapoptotic (BCL2, BCL2L1) are involved in the regulation of the caspases (CASP9, 3, 6, 7). Proteins implicated in DNA repair can also be part of the apoptosis process. BRCA1 also participates in the regulation of apoptosis (SHAO et al., 1996). PARP1 is related to oxidative DNA damage which can lead to parthanatos (a PARP1-dependent form of cell death that relies on the AIFM1-MIF pathway) with chromatinolysis. Here, the oxidative stress-induced DNA damage leads to chromatinolysis (TANG et al., 2019). The protein p53 is a multifunctional partner which can also promote and activate DNA repair proteins, arrest growth in the replication cycle, and initiate apoptosis.

1.1.1.4 BRCA1 and BRCA2 proteins

Of the main five overlapping pathways of DNA damage response described above, Homologous Recombination (HR) is the one for error-free repair of double-strand breaks. BRCA1 and BRCA2 are essential proteins involved in HR. Their dysfunction leads to genomic instability, which is a hallmark of cancer (HANAHAN; WEINBERG, 2011).

BRCA1 gene is located on chromosome 17 (17q21) and encompasses 24 exons. It was originally mapped in 1990 and subsequently cloned in 1994 (MIKI et al., 1994). This gene encodes a 1,863 amino acid long protein that contains at the N-terminus, a nuclear export signal (NES), and a RING domain. The RING domain heterodimerizes with the partner protein BRCA1-associated RING domain (BARD1) to form an E3 ubiquitin ligase. The main function of BRCA1⁄BARD1 complex is its E3 ubiquitin ligase activity (post damage) at double-strand break sites, which results in the ubiquitination of other proteins involved in DNA damage repair, such as CtIP and H2AX (JOUKOV et al., 2001; YU et al., 2003a, 2006). Ubiquitinated CtIP binds to chromatin to manage G2⁄M checkpoint control. Ubiquitinated H2AX remodels chromatin so that it becomes accessible for DNA repair machinery. In its carboxyl (C)- terminus, there are tandem repeats of two BRCA1 carboxyl-terminal (BRCT) domains. Each comprises of about 100 amino acids, and engages in forming functional

macromolecules complexes with partner proteins. More central, BRCA1 has two nuclear localization signals (NLS): one DNA binding domain and one SQ cluster domain containing several threonine and serine residues which can become phosphorylated (Figure 4) (TAKAOKA; MIKI, 2018). BRCA1 also interacts with BRCA2 via the bridging protein PALB2 (partner and localizer of BRCA2) through BRCA1 coiled-coil domain during RAD51 recruitment to double-strand breaks (ZHANG et al., 2009).

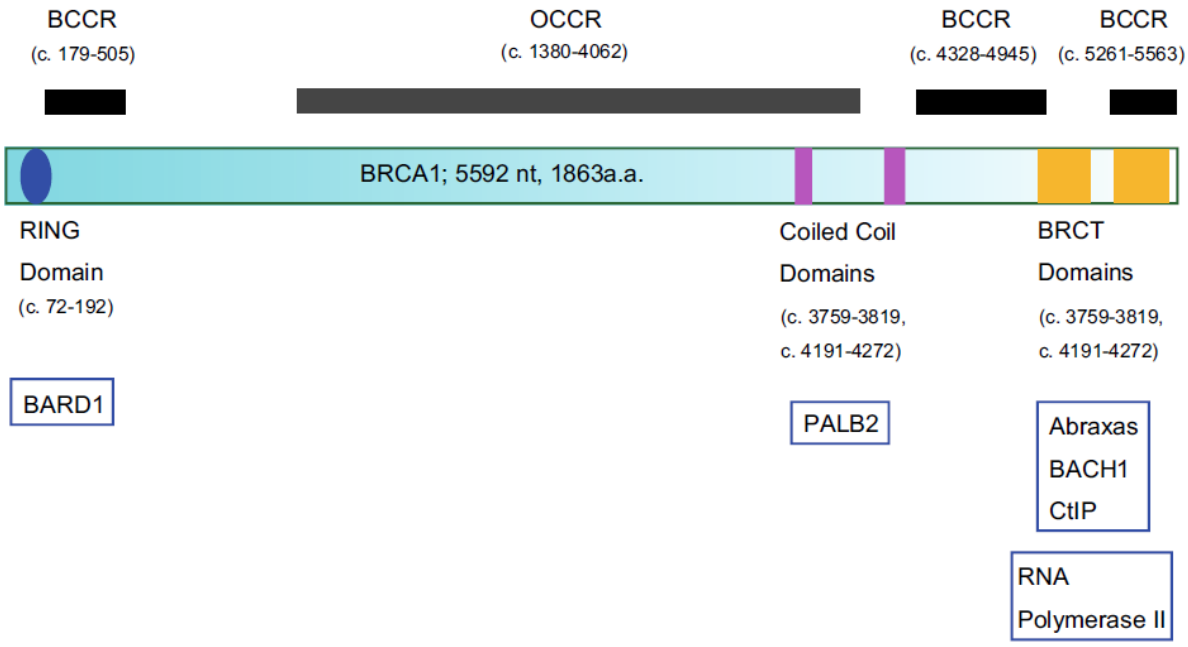

Figure 5 BRCA1 structure and its domains of interaction with other proteins. Breast (BCCRs) and ovarian (OCCR) inferred cancer cluster regions are highlighted.
Adapted from (TAKAOKA; MIKI, 2018)

BRCA2 gene is located on chromosome 13 (13q12.3) and encompasses 27 exons. BRCA2 encodes a 3,418 amino acid protein that also contains motifs that mediate its interaction with partner proteins. Currently, three BRCA2 regions have been described as particularly important for HR function: (1) an N-terminal PALB2-binding site; (2) the BRC repeat which corresponds to eight consecutive motifs located in the central region of the protein (in the exon 11), with a well described function of interaction with RAD51 and other partners; and (3) the C-terminal region, composed of three oligosaccharide binding folds (or OB folds), a helical domain and a tower domain that together constitute the DNA binding region and a RAD51 binding domain (MARTINEZ; BALDEYRON; CARREIRA, 2015; VON NICOLAI et al., 2016a). Recently, the team of A. Carreira showed a new DNA

interaction with the N-teminal (VON NICOLAI et al., 2016b). BRCA2 has been shown to play a role in a number of mitotic processes including the spindle assembly checkpoint, cytokinesis, and daughter cell abscission (VENKITARAMAN, 2014, p. 201).

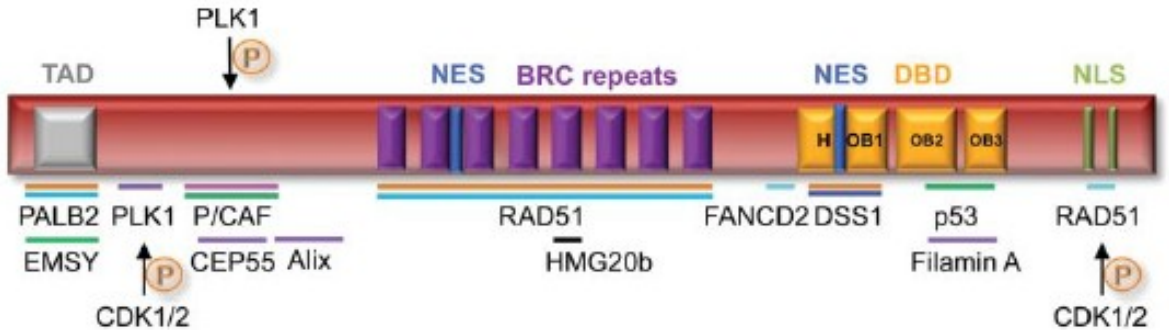

Figure 6 Structure of BRCA2 protein and its domains of interaction with protein partners.
Adapted from (MARTINEZ; BALDEYRON; CARREIRA, 2015)

1.1.1.5 Dysfunction of the reparation pathways

1.1.1.5.1 Absence of protein due to mutations

Most of the genes implied in the reparation pathways have been classified as tumor suppressor genes. Their dysfunction can be related to the absence of the protein or to the inactivation of functional domains. The main cause of dysfunction is the absence of protein due to mutations. Mutations in HR genes have been reported in breast and ovarian cancers (ANTONIOU et al., 2014a, p. 2; PELTTARI et al., 2011, 2012) and in recent years several studies have evaluated the consequences of the absence of BRCA1/2 expression.

The immunochemistry should confirm the absence of the protein in the tumor with *BRCA1/2* mutations. However, the performance of the antibodies is not good enough to propose the testing in a routine basis. Some studies showed no correlation between *BRCA1* mutation status and protein expression (clone MS110, Millipore). The same was found for BRCA2 (Sigma) expression in prostate cancer (NIENTIEDT et al., 2017). Nevertheless there are a few examples of interest for performing immunohistochemistry to evaluate BRCA1 protein (GARG et al., 2013). The impact of the alteration has also been shown on the RAD51 foci and nuclear expression for BRCA2-associated tumor (HONRADO et al., 2005). Other

indirect impacts have been estimated on the CDK expression. The expression of the cell-cycle proteins cyclins A, B1, and E is associated with a BRCA1 tumor, whereas cyclin D1 expression is associated with BRCA2 tumors.

RAD51 foci have been used to identify the reactivation of the HR pathway in resistance mechanism (CRUZ et al., 2018). Furthermore, some HR expression profile have related to clinical prognosis impact as loss of NBS1, BRCA1, ATM, and abnormal p53 expression in breast cancer.

1.1.1.5.2 Loss of function

The presence of missense variant in functional domains can hurdle the pathway of reparation. Pathogenic missense variants generally have impact on domains directly implied in the DNA reparation activity, such as the BRCT and RING domains of BRCA1 gene.

A genetic variant can be defined as an alteration in the most common DNA nucleotide sequence (of reference sequence). They can be inferred as pathogenic since they result in predicted truncating or null proteins, and/or are frequent enough in breast–ovarian cancer families that their risk of disease can be estimated directly (SPURDLE et al., 2012a). In addition to pathogenic variants in *BRCA1/2* genes, the presence of genetic instability may be a consequence of mutations or epigenetic silencing of *BRCA1/2* or other HR genes. This genomic instability leads to the accumulation of genetic alterations, which are essential for cancer development.

1.1.1.5.3 Expression regulation

The regulation of the protein expression can be related to level of expression without any mutation in the coding sequence. The main possibility is related to promoter methylation.

Along with epigenetic silencing, they can participate in the carcinogenesis of a significant number of sporadic and hereditary cancers. Aberrant *BRCA1* promoter methylation is seen in 5-30% of ovarian cancers (ESTELLER et al., 2000; GEISLER et al., 2002) and in 11-14% of sporadic breast cancer. It is more frequent (approximately 30%) among triple-negative breast cancer (TNBC), a breast cancer which does not express human growth factor receptor 2 (HER2), estrogen receptor, or progesterone receptor (ESTELLER et al., 2000; LIPS et al., 2013). Promoter hypermethylation in ovarian and TNBC samples have also been

described in other HR genes, such as PALB2, ATM, RAD50, RAD51C, and FANCF, (BERNARDS et al., 2018, 2018; DITE et al., 2012). In contrast, *BRCA2* promoter hypermethylation is a very rare event in breast and ovarian tumorigenesis. Additionally, BRCA post-transcriptional downregulation through miRNA has been described in breast and ovarian cancinogenesis, which could also explain cases sharing BRCA histopathological features yet with no mutation identified (MOSKWA et al., 2011).

1.1.1.5.4 Transcription factor deregulation

Those genes are also regulated by transcription factor and some of them have been well described. The role of CDK12 has been described as an activator of the HR genes promoters (PACULOVÁ; KOHOUTEK, 2017). The protein EMSY is more inclined to have a negative impact on the expression, which explains the role in the amplification of this protein (HOU et al., 2014). Contrary to many studies, the absence of ER site on the regulation site of BRCA1 has been confirmed.

Another level of alteration could happen in the post-translational regulation. Some post-translational alterations, such as phosphorylation can be clearly responsible for activation or inactivation of a pathway (DERIBE; PAWSON; DIKIC, 2010). The mechanism implying ubiquitin modification is also known to limit the activity of some proteins by accelerating the intracytoplasmic destruction of them. This mechanism has been described with HR proteins (LEE et al., 2018).

1.1.1.5.5 Compensatory mechanisms to other reparation pathways

Since there are several DNA repair pathways, some compensatory mechanisms have been observed (VANDERSTICHELE et al., 2017). For example, if the HR pathway is inactivated, the BER and the alternative non-homologous end joining DNA repair (NHEJ) will try to compensate for it imperfectly. Moreover, since HR requires a full coordination of different proteins, the impact on the pathway will probably differ according to the protein affected.

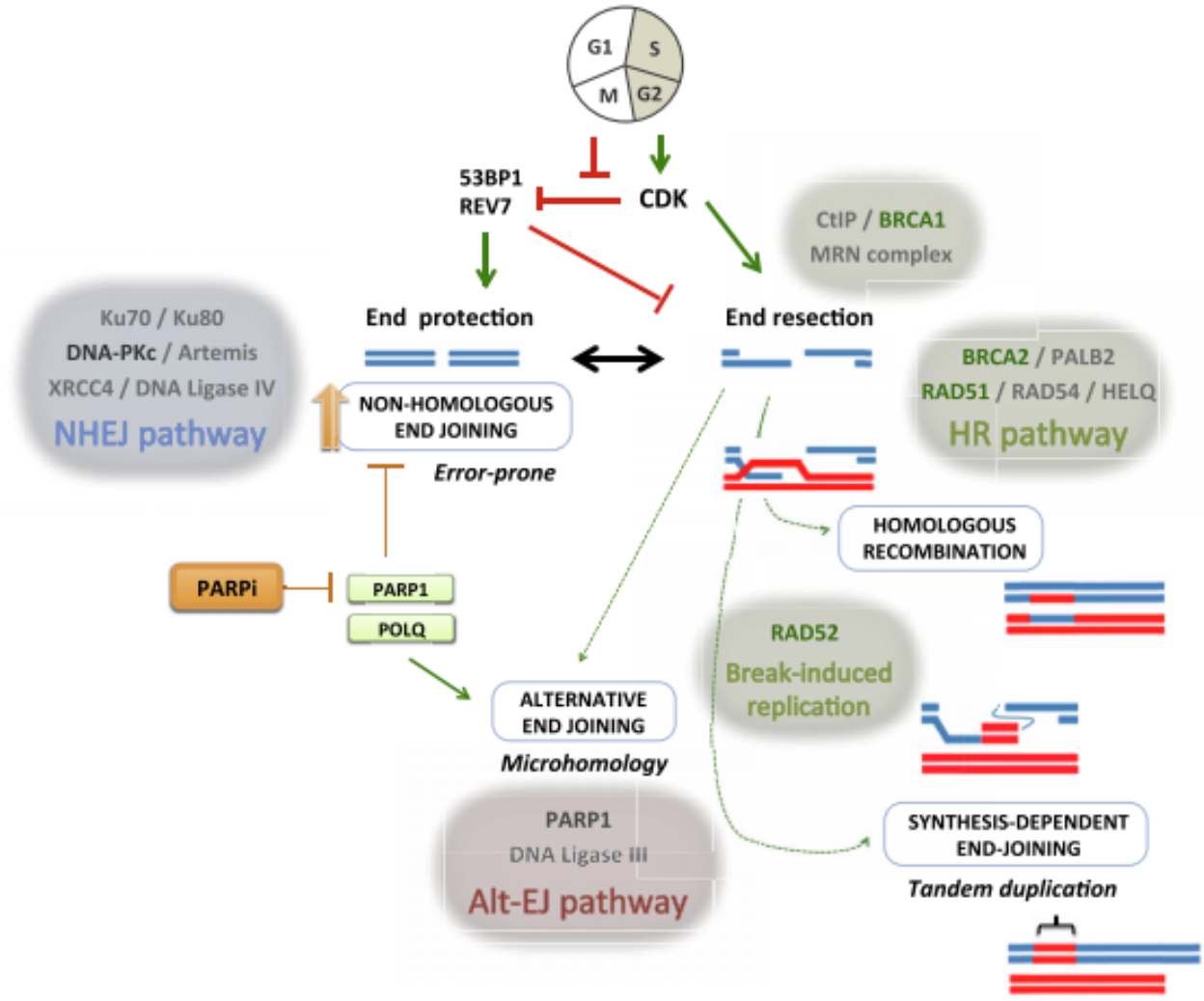

Figure 7 Compensating mechanisms of DNA repair.
Addapted from (VANDERSTICHELE et al., 2017).

1.1.1.6 Signature

The inefficiency of DNA repair mechanism generates a scenario of genetic instability. Mutational signatures are then designed to identify homologous recombination deficient (HRD) phenotype and characterize a larger population which can benefit from DNA damaging agents, extending beyond BRCA mutant tumors.

This includes signatures based on the evaluation of the following: (1) Copy number alteration (CNA) profiles, which are determined by the identification of DNA gains or losses in the tumor. CNA can be evaluated by comparative genomic hybridization array (aCGH), multiplex ligation-dependent probe amplification (MLPA), or single-nucleotide

polymorphism (SNP) arrays (GROSS et al., 2016); (2) Loss of heterozygosity (LOH) score, evaluated by the imbalance in the ratio of parental alleles from the normal 1:1 (WANG et al., 2012); (3) Telomeric allelic imbalance, which calculates the allelic imbalance extending from the site of DNA damage to the telomere (BIRKBAK et al., 2012a; ROTTENBERG et al., 2008); (4) Large-scale transitions, that consist of chromossome breaks (translocations, inversions, or deletions) of at least 10Mb between adjacent regions (POPOVA et al., 2012a); (5) mRNA and miRNA expression (KONSTANTINOPOULOS et al., 2010). Finally, two scores were developed combining different methods to improve the sensitivity to identify the BRCAness phenotype: myChoice HRD test (Myriad Genetics) combines measures of LOH, TAI, and LST (TIMMS et al., 2014), and Foundation Medecine HR score that combines measures of *BRCA1/2* mutation status and percentage of LOH (SWISHER et al., 2017a). The threshold of composite scores have been described and validated in prospective clinical trials (TELLI et al., 2016).

HRD tumors represent up to 50% of HGSOC and more than 20% of basal breast cancers, but a BRCA mutation is identified in only about 20% of them (CANCER GENOME ATLAS RESEARCH NETWORK, 2011; KOBOLDT et al., 2012b). According to the results of recent trials, even if the tumor is sporadic, the identification of an HRD phenotype helps in personalizing therapy. The comprehension of breast and ovarian related carcinogenesis has evolved from solely mutation identification in candidate genes onwards to the integration of large volume of genomics and transcriptome data, revealing recurrently altered pathways and signatures of mutational processes. All of these methods described above (individually or in combination) were able to discriminate HR deficient tumors which were correlated with responsiveness to platinum and PARPi, and resulted in improved outcome.

HRD signatures also predict sensitivity to immunotherapy. Indeed, *BRCA1/2*-mutated high-grade serous ovarian cancers exhibit a higher mutational load and a specific mutational signature with an elevated number of larger indels up to 50. This is a group of *BRCA1/2*-mutated tumors with high number of TILs (tumor-infiltrating lymphocytes) and with good prognosis (STRICKLAND et al., 2016).

1.1.2 Tumorigenesis in ovarian and breast cancer

1.1.2.1 Early molecular stage of development in ovarian and breast cancer

1.1.2.1.1 Breast cancers

Breast cancers arise in the terminal duct lobular units of the collecting ducts (the functional unit of the breast), as a consequence of a continuum of lesions and molecular alterations from normal glands to malignant tumors. The two main localizations of invasive cancers are inside the milk duct for ductal carcinomas and inside the milk glands (lobules) for lobular carcinomas.

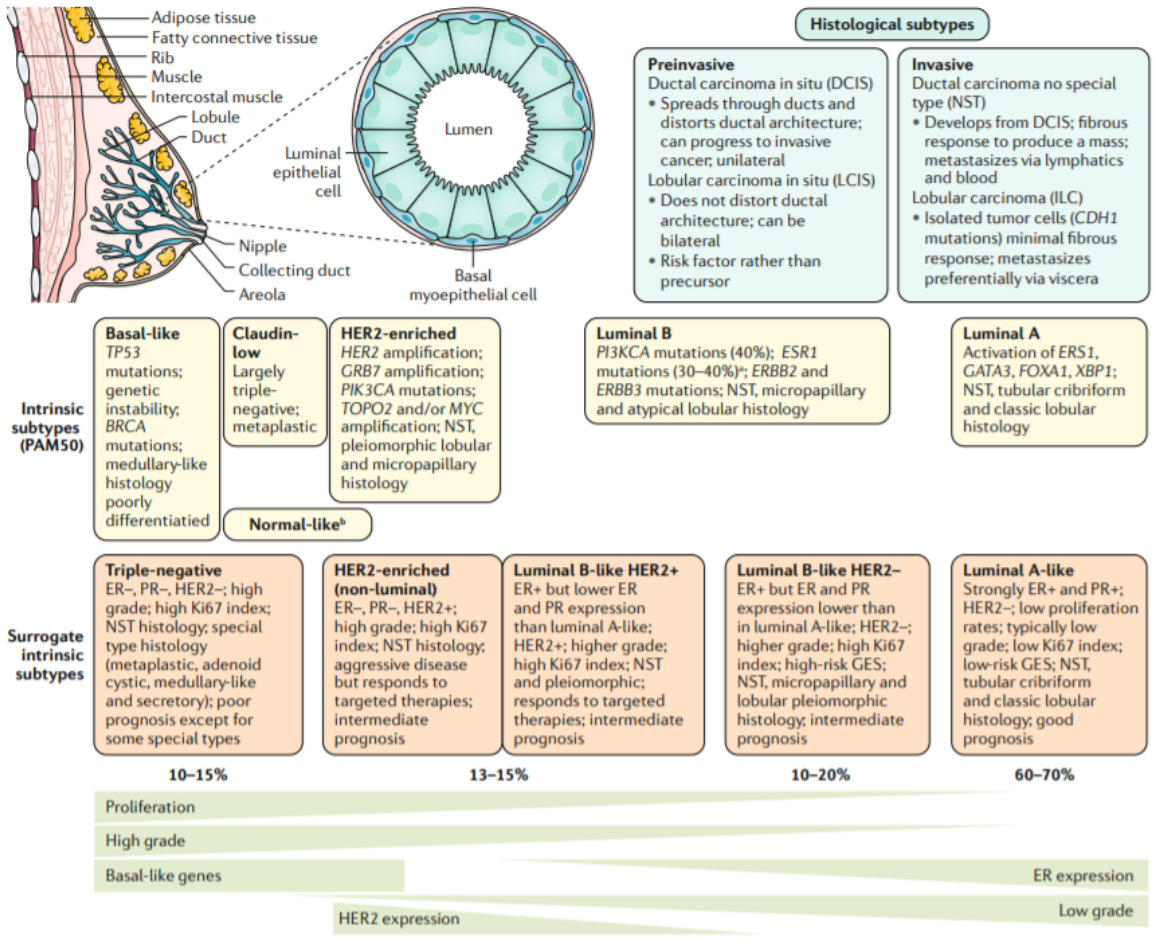

Figure 8: Histological and molecular subtypes of breast carcinomas and their pre-invasive counterparts. Adapted from (HARBECK et al., 2019)

The cell origin and the molecular alterations that drive breast carcinogenesis differ among the different subtypes. In 2000, the work of Perou and Sorlie allowed the subdivision of breast cancers in four subtypes, distinguished by differences in their gene expression patterns with distinct clinical behaviours (PEROU et al., 2000).

<table>
<tr><td>Luminal A</td><td>Luminal B</td><td>Basal-like</td><td>HER2 amplified</td></tr>
<tr><td colspan="2">Expression of luminal cell markers</td><td>High expression of genes typical of the epithelial basal cell layer and absence of the expression of hormonal receptors and ERBB2</td><td>Over-expression of ERBB2 and multiple genes of 17q11 amplicon, and a negativity for hormonal receptors and basal cell markers</td></tr>
<tr><td colspan="2">Low-grade like pathway</td><td colspan="2">High-grade like pathway</td></tr>
</table>

Table 1: Molecular subtypes of breast cancers and their different pathways of cancer development (Perou et al, 2000)

Evidence suggests that at the molecular level, these different molecular subtypes evolve along two different pathways of progression: (1) low-grade-like pathway, characterized by gain of 1q; loss 16q; infrequent amplification of 17q12; gene expression signature associated with ER phenotype, diploid or near-diploid karyotypes; and low tumor grade, including luminal A and part of luminal B tumors, and (2) the high-grade-like pathway, characterized by loss of 13q; gain of chromossomal region 11q13; amplification of 17q12 (region of ERBB2 gene that encodes HER2 protein); and expression of signature genes involved in the cell cycle and cellular proliferation, including intermediate-high grade tumors such as HER2 positive and TNBC (LOPEZ-GARCIA et al., 2010).

A large-scale genome sequencing study including all subtypes at the initial diagnosis revealed that the most frequently mutated and⁄or amplified genes in breast tumor cells are TP53 (41%), PIK3CA (30%), MYC (20%), PTEN (16%), CCND1 (16%), ERBB2 (13%) FGFR1 (11%) and GATA2 (10%). Luminal A tumors have a high prevalence of *P1K3CA* mutations (49%), whereas basal-like tumors present a high prevalence of *TP53* mutation (84%) (NIK-ZAINAL et al., 2016). This work also detected some recurrent mutations in the promoter of a few genes (WDR74, TBC1D12, PLEKHS1), and in two long non-coding RNAs (MALAT1 and NEAT1). The impact of these mutations is still unclear. This study had no information on methylation. In addition, during breast cancer development genes can either be

globally hypomethylted (leading to upregulation of oncogenes and genetic instability) or less frequently focally hypermethylated (leading to silencing of DNA repair genes and genetic instability).

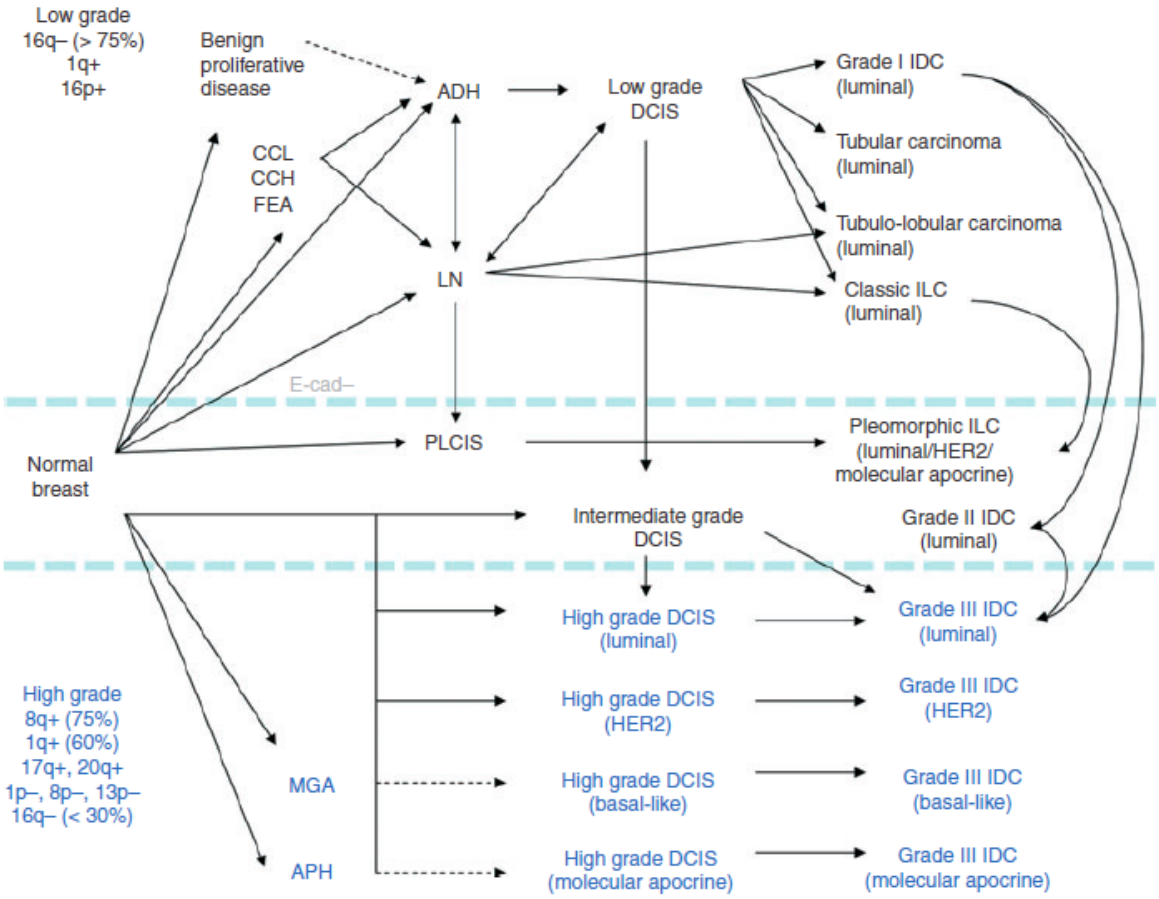

Figure 9: The high-grade and low-grade multistep model of breast cancer progression based on morphological, immunophenotypical and molecular features.
Adapted from (LOPEZ-GARCIA et al., 2010).[1]

1.1.2.1.2 Ovarian cancers

Ovarian carcinomas correspond to 90% of ovarian cancers and comprise different subtypes of disease with specific morphologies and molecular patterns.

Histological subtype	Clinical findings	Repair pathway	Mutations

[1] ADH: atypical ductal hyperplasia; APH: atypical apocrine hyperplasia; CCH: columnar cell hyperplasia; CCL: columnar cell lesion; DCIS: ductal carcinoma in situ; E-cad: E-cadherin; FEA: flat epithelial atypia; IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma; LN: lobular neoplasia; MGA: microglandular adenosis; PLCIS: pleomorphic lobular carcinoma in situ

High-grade serous carcinoma	Highly aggressive tumours • Papillary or solid growth pattern • High proliferative rate • Initial chemosensitivity with subsequent acquisition of increasing resistance	HR pathway	BRCA1/2, HR genes, TP53
Low-grade serous carcinoma	Indolent behaviour • Micro-papillary pattern • Low proliferative rate • Relative chemoresistance	Stability	BRAF, KRAS, NRAS, PIK3CA
Endometrioid	Solid and cystic patterns • Frequently associated with endometriosis • Similar profile to serous carcinoma	MMR pathway POLE pathway	PIK3CA, PTEN, ARID1A, POLE
Clear-cell carcinoma	Glycogen-containing cells with clear cytoplasm • Tubulo-cystic, papillary, solid, or mixed patterns • Frequently associated with endometriosis • Early-stage diagnosis • Poor prognosis and resistance to chemotherapy	-	ARID1A, PIK3CA, PTEN
Mucinous carcinoma	Large size tumours filled with mucus-like material • Early-stage diagnosis • Chemoresistant	-	KRAS, PIK3CA – HER2 amplification

Table 2: Different histological subtypes of epithelial ovarian cancers and their molecular feature. Adapted from (Lheureux et al., 2019).

In the perspective of HR alterations, high-grade serous carcinomas are the most common subtype, in which HRD is most commonly related to *BRCA1/2* mutations. It has been hypothesized that high-grade serous ovarian carcinomas (HGSOC) originates from pre-malignant lesions in the tubas (serous tubal intraepithelial carcinoma) instead of the ovary itself, since both share the same morphological and molecular features which involves mutations in *TP53* gene as an early event (DUCIE et al., 2017; KINDELBERGER et al., 2007). Atypical lesions within the fimbriated end of the fallopian tube (serous tubal intraepithelial carcinomas [STIC]) display similar morphology and *TP53* signatures as HGSOC, suggesting the neoplastic process may originate at these tubal lesions and shed into the ovary, where they aggressively progress (KUHN et al., 2012). Compelling data suggests the same origin for low-grade serous carcinomas, but that they progress from benign serous cystadenoma to borderline serous tumors and then on to low-grade carcinomas.

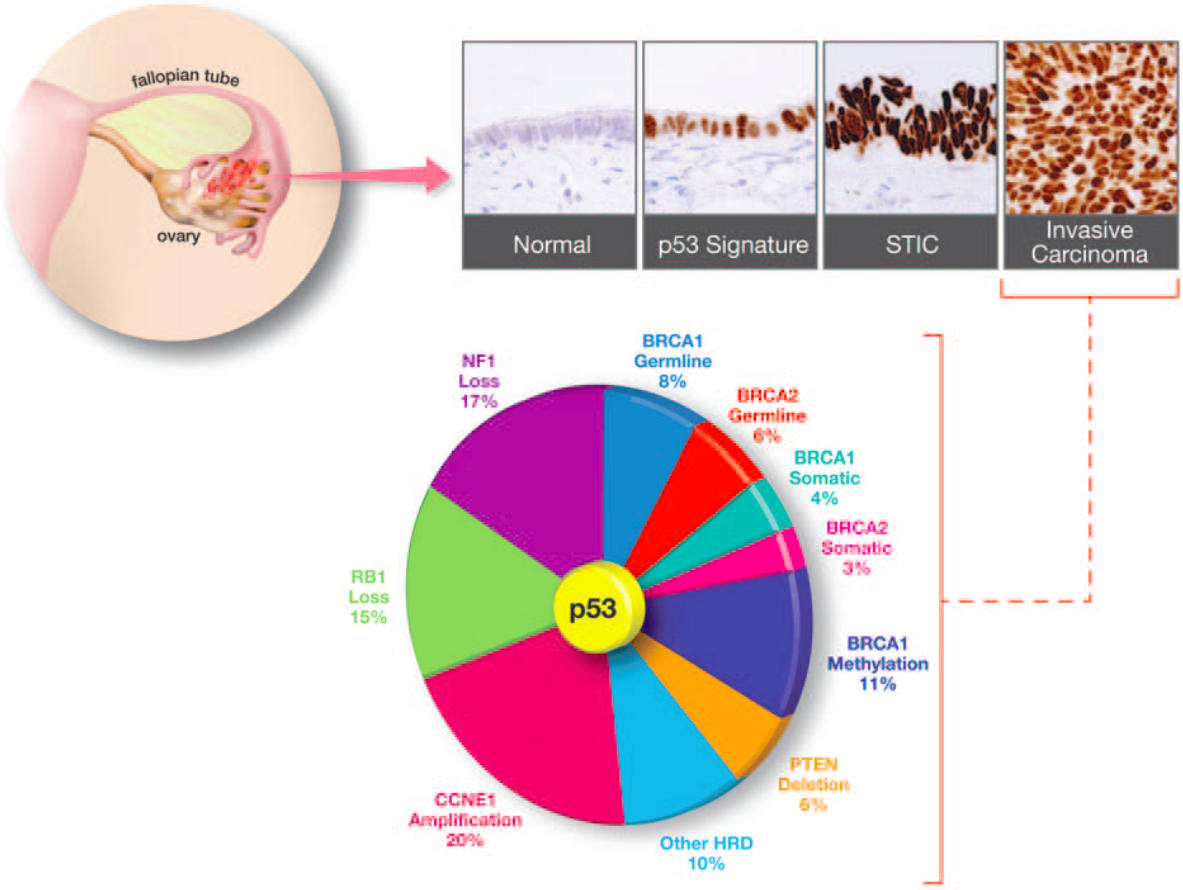

Figure 10 Pathogenesis of HGSOC. Pathological and molecular alterations and the evolution from precursor lesions in fallopian tube ephitelium.
Adapted from serous tubal intraephitelial carcinoma-STIC.

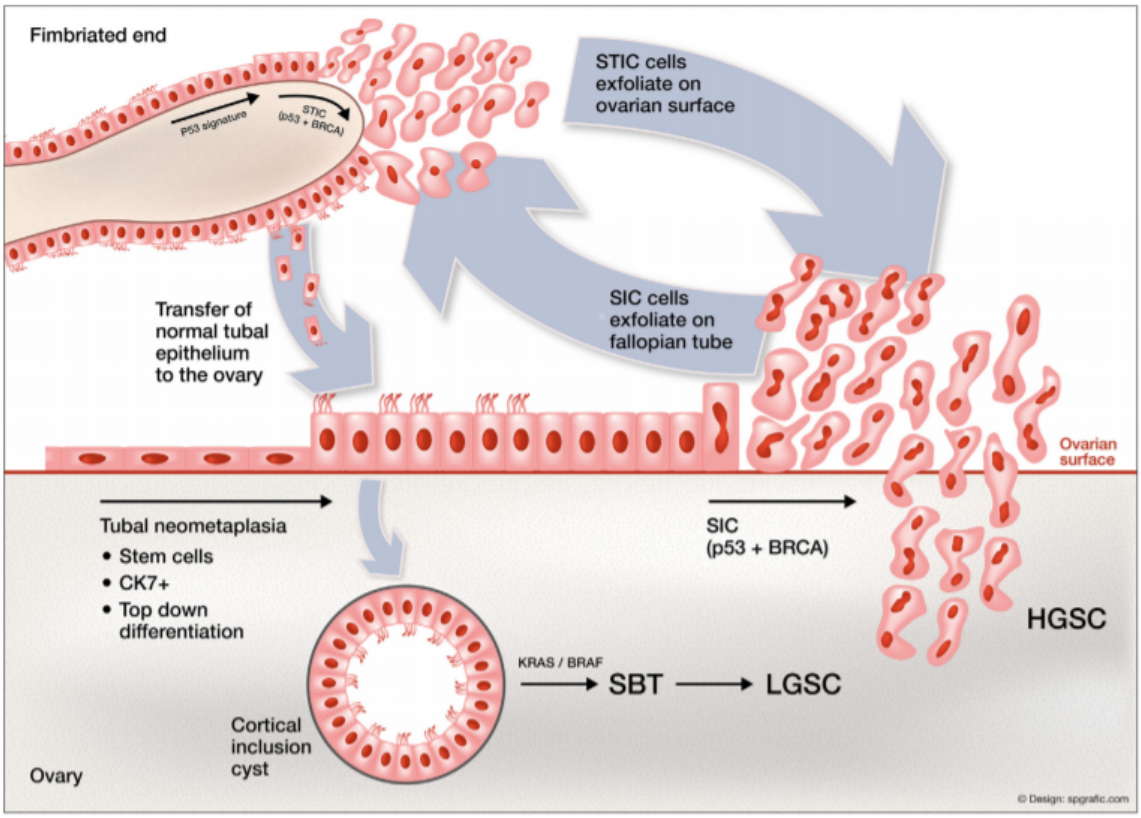

Figure 11 Different histological and molecular features of ovarian carcinomas.
Adapted from (PRAT; D'ANGELO; ESPINOSA, 2018).

Integrated genomic analysis led to the shift that ovarian cancer was not just one disease, but rather several distinct diseases presenting different histological and molecular features. HGSOC are characterized by nearly universal *TP53* abnormalities, also detected in endometrioid and other high-grade diseases. This subtype also presents high genomic instability, somatic DNA copy-number changes, and whole genome duplications. As stated before, HRD is present in about 50% of HGSOC. Overall, *TP53* mutations occur in 96% of the cases; *BRCA1* and *BRCA2* mutations in 22% of the cases (15-20% of these are germline); and additional somatic mutations in six other genes are identified in 2-6% of the cases (*NF1, RB1, CDK12, FAT3, CSMD3 and GABRA6).* Recent molecular analysis, which was based on the profile of RNA and microRNA expression, stratified HGSOC into four different prognostic subtypes (C1-mesenchymal, C1-immune, C4-differentiated, C5-proliferative) and seven copy-number signatures. However, different from breast cancer, the molecular stratification is not yet validated to be used for accurate prediction of drug sensitivity and/or resistance to treatment (ANTONIOU et al., 2014b; CANCER GENOME ATLAS

RESEARCH NETWORK, 2011; KONECNY et al., 2014; MACINTYRE et al., 2018; TOTHILL et al., 2008; WANG et al., 2006).

1.1.2.1.3 Hereditary breast and ovarian cancers

Evidence suggests that genomic instability is present in both hereditary and sporadic cancers, but occuring in different stages of cancer development and with different molecular basis. While in hereditary cancers genetic instability probably precedes the acquisition of mutations in oncogenes and tumor supressor genes, and therefore precedes the acquisition of other hallmarks of cancer (HANAHAN; WEINBERG, 2000), in sporadic cancers, studies suggest that the first hallmark acquired may be activation of growth signalling secondary to mutations in oncogenes or anti-oncogenes. In hereditary cancers, genomic instability is related to mutations in DNA repair genes, such as mutations of mismatch repair genes in hereditary non-polyposis colon cancer; biallelic germline mutations in *MUTYH* (a DNA base excision repair gene) resulting in hereditary polyposis and increased risk of colon cancer; and mutations in HR genes (including *BRCA1* and *BRCA1*) that predisposes to various cancers, including breast and ovarian cancer. In sporadic cancer, genetic instability is probably related to an oncogenic-induced collapse of DNA replication forks.

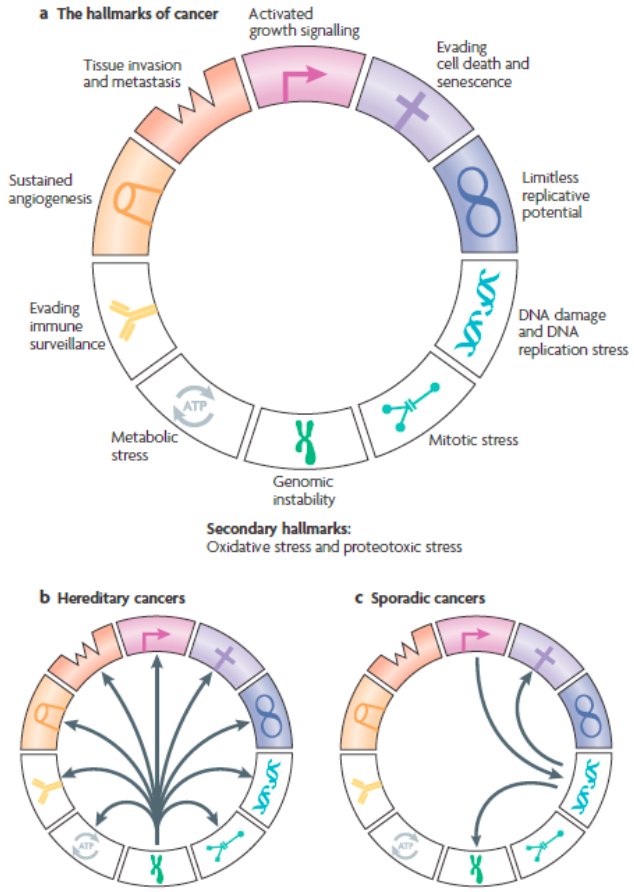

Figure 12 A proposed revision of the hallmarks of cancer and the diferent temporal orders of events in hereditary vs sporadic cancers.
Adapted from (NEGRINI; GORGOULIS; HALAZONETIS, 2010)

According to the mutator hypothesis, genomic instability in hereditary cancers is related to mutations in caretaker genes (genes involved in maintaining genomic stability) that happens during early carcinogenesis. Classical caretaker genes are DNA repair genes, including *BRCA1/2*, and mitosis checkpoint genes. Chromossomal abnormalities are present from the stage of precancerous lesions and participate in cancer development by increasing of the spontaneous mutation rate (LOEB, 1991). The observation that only a part of chromossomal abnormalities are seen in all tumor cells, is in line with the hypothesis that tumor cells originate from a single genetic unstable cell which continues to accumulate mutations during cancer development. The results of high-through put sequencing studies showed that mutations in caretakers genes were infrequent in sporadic cancers (JONES et al., 2008; PARSONS et al., 2008; SJÖBLOM et al., 2006; WOOD et al., 2007). However, those inactivation of caretaker genes can also be purely sporadic and define a specific subtype with comparable features of tumors carrying germline mutations.

1.1.2.2 Temporal order and somatic tumor-driving events of BRCA-associated tumorigenesis

In relation to tumorigenesis specifically related to *BRCA1* and *BRCA2,* data suggest that *BRCA* biallelic inactivation renders the cell vulnerable to genomic instabilIty, being the background for sucessive mutations that culminate in cancer development. Conforming to this theory, Von Waldhe et al recently demonstrated concordance between HRD scores across different regions of the same BRCA related breast cancer, indicating that HRD affects the entire primary tumor and corresponds to a founding event (WAHLDE et al., 2016).

As typical tumor supressor genes, the inactivation of the second allele of *BRCA1/2* is presumed to be a rate-limiting step (KNUDSON, 1971). LOH is the most common second hit event of breast and ovarian *BRCA1/2*-associated carcinogenesis. It is either as a consequence of large deletions, genomic rearrangements, incorrect mitosis or deficient DNA repair. It has been reported in 90% (breast) and 91% (ovarian) of *BRCA1*-associated cancers and in 54 % (breast) and 84%(ovarian) *BRCA2*-associated cancers (MAXWELL et al., 2017a). Alternative second-hit mechanisms, such as somatic inactivating point mutations, have been described in a small minority of BRCA1-associated breast and ovarian cancers (PENNINGTON et al., 2014a; WINTER et al., 2016). Furthermore, hypermethylation of *BRCA1* promoter has also been responsible for the silencing of the wild-type allele, but also in only a minority of the cases (DWORKIN et al., 2009; ESTELLER et al., 2001).

Genetic inactivation of the wild-type allele is expected to happen early in carcinogenesis. Unexpectedly, however, a study performed in a single-cell level in *BRCA1* breast cancer model demonstrated that loss of wild-type allele may not be the first event in the majority of associated breast tumors and may not be present in all cancer cells in a given tumor. Two main evolutionary trajectories were found in *BRCA1* tumors defined by the presence or absence of PTEN. In the majority of tumors (51%), loss of *PTEN* was probably the first event, followed by mutation in *TP53* or *BRCA1* LOH with similar probability. *TP53* mutation was the second most common first event (31%), and it was almost always followed by *BRCA1* LOH. *BRCA1* LOH was the least common first event (18%), and the majority of the cases presented only *TP53* mutation only as an additional alteration. Further, it was proved that the relative order of events during tumorigenesis were associated with tumor subtype: TNBC almost always had *PTEN* loss as the first event, while luminal tumors showed *TP53* or *BRCA1* LOH as the first event (Martins et al., 2012). Therefore, it appears that *PTEN* and

TP53 mutations happen early during tumor development and are followed by the loss of BRCA function, which plays a relevant role in increasing genetic instability.

Van Heetvelde and colleagues described the panorama of second-hit events in breast and ovarian cancers from patients harboring germline *BRCA1/2* mutations. Indeed, copy neutral LOH was the most prevalent mechanism of wild-type (wt) allele inactivation (detected in 69% of breast cancers and 67% in ovarian cancers). Mots intriguingly however, only a minority of tumors (35% breast and 47% ovarian cancers) presented loss of the wild-type allele in all cancerous cells but in the majority of the cases different mechanisms of wt allele inactivation were present in the same tumor (VAN HEETVELDE et al., 2018). Moreover, somatic intragenic deletions and methylated subclones were found in combination with partial LOH.

It has been suggested that heterozygous mutations affecting *BRCA1* and *BRCA2* might be enough for carcinogenesis, even when the remainig wild-type allele remains expressed. Indeed, recent genomic studies have showed that a significant fraction of cancers arising in BRCA mutation carriers retain a functional wild-type allele. Plon et al observed this in 24% of breast and ovarian cancers with *BRCA1/2* pathogenic variants. In line with this, Maxwell et al. showed that retention of the wild-type allele was observed in almost half (46%) and in 16% of breast and ovarian *BRCA2*-associated cancers, respectively. However, it was less frequent for *BRCA1* breast (7%) and ovarian (10%) cancers. (MAXWELL et al., 2017a). The prevalence of 8% was globally estimated in a large panorama of tumors and was clearly more frequent in lung cancer, which was up to 20% (Jonsson et al., 2019). Furthermore, some lines of evidence suggest that heterozygous truncating *BRCA1* and *BRCA2* mutations may render cells vulnerable to happloinsufuciency, when exposed to replication stress (TAN et al., 2017).

Noteworthy is that both the loss of the wild-type allele and the phenotypic evidence of *BRCA* dependence are significantly higher in breast and ovarian cancers with *BRCA* mutations compared to cancers not associated with *BRCA1/2* germline carrier status (YOST et al., 2019). This data suggests that *BRCA* pathogenic mutations in patients with non-BRCA-associated cancers are often incidental findings, as well as that these tumors are often not BRCA-driven cancers and that *BRCA* mutations should be secondary in the tumorigenesis in these cases. In line with this, Jonnson and colleagues demonstrated that the impact of *BRCA1/2* mutations in cancer development is lineage dependent. They assessed the dependance of BRCA dysfunction through determination of selective pressure for wild-type BRCA allele loss in tumors with germline or somatic *BRCA* mutations and found that the

prevalence of loss of the wt allele was significantly higher in BRCA-associated cancer types for both germline and somatic mutation (JONSSON et al., 2019). The reason why *BRCA1/2* mutations predispose mainly to breast and ovarian cancers, even if their physiological functions are relevant in all tissues, is probably related to the genotoxic effects of tissue-specific hormones such as estrogen especially in the breast but also in the ovaries.

1.1.2.3 Description of mutations in HR pathways in breast and ovarian cancers

In 2018, the CIMBA consortium presented an inventory of the current state of *BRCA1* and *BRCA2* mutations. There are 1,650 unique *BRCA1* and 1,731 unique *BRCA2* mutations distributed whithin these genes (REBBECK et al., 2018a). Different types of mutations are repertoried: frameshift, nonsense, missense, and splice. Frameshift are the most common type, followed by nonsense mutations. The most common effect of the mutations was premature translation termination and the majority of mutant mRNAs were predicted to undergo nonsense-mediated mRNA decay (NMD) (ANCZUKÓW et al., 2008). Despite having the same spectrum of mutations, the frequency distribution by mutation type, effect, or function differed significantly ($p<0.05$) between *BRCA1* and *BRCA2* mutation carriers in the CIMBA cohort (REBBECK et al., 2018a). These differences are largely because genomic rearrangements and missense mutations account for a much higher proportion of alterations in *BRCA1* when compared to BRCA2, as previously described (WELCSH; KING, 2001). In the 2000s, large rearrangement (deletion or duplication of one or more exons) were also highlighted (ROULEAU et al., 2012).

Mutations in HR genes beyond BRCA have been reported in breast and ovarian cancers (ANTONIOU et al., 2014a, p. 2; PELTTARI et al., 2011, 2012). As expected, *BRCA1/BRCA2* were the most commonly altered genes, followed by several genes including *CHEK2, PALB2, RAD51C*, and *RAD51D*. Some of them were preferentially affected by germline alterations (e.g., *BRCA1/2, CHEK2, FANCM, PALB2*), whereas others (e.g., *ATM, BAP1, CDK12*) were preferentially affected by somatic events.

The prevalence of germline HR genes alterations in patients with breast cancer is about 10%. After *BRCA1/2*, the main HR genes affected are: *CHEK2*, *ATM*,*BRIP1*, *PALB2*, *PTEN*, *NBN*, *RAD51C*, *RAD51D*, *MSH6*, and *PMS2* (TUNG et al., 2016). For ovarian cancer, HR mutations are identified in more than 25% of the

cases(PENNINGTON et al., 2014a; RIAZ et al., 2017). Beyond *BRCA1/2*, the main HR genes affected are: *RAD51D, BRIP1, RAD51C, CHEK2, PALB2* and *BARD1*.

Analysis of TCGA data confirmed the prevalence of HR pathway alterations in 10% and 25% of breast and ovarian cancers, respectively. It was demonstrated that bi-allelic alterations in HR genes are mutually exclusive of each other (RIAZ et al., 2017). Moreover, biallelic inactivation of BRCA1 or BRCA2 is associated with a pattern of genome-wide mutations known as signature 3 that reflects the lack of competence to repair DNA double-strand breaks. Analysis of ~1,000 samples confirmed the same pattern of HRD in breast cancer samples of germline (nonsense and frameshift) PALB2 variants carriers, while for ATM or CHEK2 it was not observed.

1.2 Usual suspect for breast and ovarian cancer - *BRCA1* and *BRCA2* variants

1.1.2 Hereditary breast and ovarian cancer (HBOC) syndrome

1.2.1.1 Pathogenic variants in *BRCA1/BRCA2* genes

In 1866, the French physician Paul Broca introduced the concept of familial risk for breast cancer by describing an aggregate of cases in his wife family (BROCA, 1866). Since then, successive cases of families with multiple cases of breast cancers have been documented in literature. Decades after the clinical description of the syndrome, the region of chromosome 17 (17q21) implicated with the hereditary nature of 146 early-onset breast cancer cases in 23 families was first identified by King and colleagues in 1990 (HALL et al., 1990). *BRCA1* gene was finally cloned in 1994. One year later, *BRCA2* was identified on chromosome 13 and cloned. Hereditary breast and ovarian cancer (HBOC) syndrome is an inherited cancer-predisposing syndrome, mainly related to mutations or pathogenic variants in *BRCA1* and *BRCA2* genes. This syndrome is originally characterized by multiple females affected by breast and ovarian cancers at an early age. There is also an increased risk of other cancers such as male breast, prostate, pancreatic and melanoma (MERSCH et al., 2015).

For a patient suspected of having HBOC, it is an important first step to determine her chances of carrying a mutation in a high penetrance gene, such as *BRCA1* and *BRCA2*, as well as the impact of the pathogenic variant on her risk of developing breast cancer. Multiple score systems exist to estimate the likelihood that an individual or family has a germline pathogenic

variant in *BRCA1* and *BRCA2* and help further discriminate the eligible patients for *BRCA1/2* screening, such as BRCAPRO, BOADICEA, Tyrer-Cuzick (ANTONIOU et al., 2004; EVANS et al., 2004; TYRER; DUFFY; CUZICK, 2004), with different degrees of validation (NELSON et al., 2019). Once the risk is estimated, it is appropriate to initiate genetic testing in a family member who is most likely to test positive for a pathogenic variant. The likelihood of detecting an underlying disease-causing pathogenic variant is highest in the most severely affected families, especially in those with ovarian cancer and with cancer detected at a young age. Guidelines providing criteria for *BRCA1* and *BRCA2* screening varies between countries (DALY et al., 2017). Current recommendations for the screening continue to expand and include, but are not limited to the following; all patients with epithelial ovarian cancer; breast cancer diagnosed younger than 45 years, TNBC younger than 60 years, breast cancer with Ashkenazi Jewish ancestry, all individuals with pancreatic cancer, and all men with breast cancer or metastatic prostate cancer. In addition to the predictive models mentioned above, other clues to the presence of a pathogenic variant include early disease onset, bilateral tumors development, synchronous or metachronous lesions, clustering of multiple breast cancers in 2-3 successive generations, male breast cancer, presence of rare histopathological diagnoses (TNBC, medullary breast cancer), cluster of breast cancer in families, and cancer multiplicity in the same individual. In some countries, a probability > 10-20% is necessary in order to obtain a prescription of *BRCA1/2* molecular screening. Therefore, the prevalence of *BRCA1* and *BRCA2* germline pathogenic variants vary considerably among different ethnic groups, geographic areas, and according to different testing criteria.

While hereditary breast cancers constitute about 5-10% of all breast cancer cases and *BRCA1/2* mutations account for half of these cases (FACKENTHAL; OLOPADE, 2007), 14% of all ovarian cancer patients harbor a germline pathogenic *BRCA1/2* variant, which is inherited in an autosomal dominant fashion (Alsop et al., 2012). In general, pathogenic variants in *BRCA1* and *BRCA2* are identified in a minority of index cases tested, with 5-10% of breast and 15% of ovarian cancer patients (ANGLIAN BREAST CANCER STUDY GROUP, 2000; WHITTEMORE et al., 2004). Because of different methodologies and populations, there is a high variability in the estimate prevalence of *BRCA1/2* pathogenic mutation. The prevalence is 10 times higher in the Ashkenazi Jewish population (ROA et al., 1996).

Despite recent advances concerning the molecular mechanism of HBOC, the two major predisposing genes remain *BRCA1* and *BRCA2*, inherited in an autosomal dominant

fashion. Germline monoallelic pathogenic variants significantly elevate the risk of mainly breast and ovarian but also of pancreatic, prostatic, and other cancers. Bi-allelic mutations of these genes cause congenital syndromes associated with developmental abnormalities, chromosome fragility, and cancers at various sites. Since their cloning in the early 1990s, multiple different variants have been detected in *BRCA1* (more than 1,800) and *BRCA2* (more than 2,000), but only a fraction of them are known to cause cancer susceptibility (CAPUTO et al., 2012; CLINE et al., 2018; COUCH; NATHANSON; OFFIT, 2014). Some founder mutations are relatively frequent in particular ethnic groups, such as *BRCA1* 185delAG (c.68_69del), *BRCA1* 5382insC (c.5266dup), and *BRCA2* 6174delT (c.5946del) in Ashkenazi, and *BRCA2* 999del (c.771_775del) in Icelanders. Multiple missense variants have been reported in both genes, but most recognized disease-associated pathogenic variants are those that result in premature protein truncation and include nonsense mutations, insertion/deletion resulting in translational frameshifts, and mutations that affect splice sites. A few missense mutations located in domains of interaction with other proteins abrogates function and confers risks comparable to truncating mutations. In addition, it has recently been acknowledged that the magnitude of risk (penetrance) of developing breast or ovarian cancer depends on the location and the type of a particular pathogenic variant (REBBECK et al., 2015a), co-occurence with single-nucleotide polymorphisms, ethnicity, and environmental factors.

Gene	Frequency in Breast Cancer (%)	Frequency in Ovarian Cancer (%)
BRCA1	2-3.7	3.6-24
BRCA2	1-2.8	3.1-6.5
ATM	0.3-1.1	0.57
ATR	0.15	—
BARD1	0.14-0.15	0.12-0.48
BLM	0.25	—
BRIP1	0.1-0.8	0.9-1.4
CDH1	0.05-0.38	—
CHEK2	0.4-2.1	0.57-1.4
FAM175A	—	0.16-0.48
Mismatch repair genes	0.4-0.91	0.4-0.66
MRE11A	0.2-0.25	0.10-0.28
NBN	0.1-0.25	0.24-0.47
PALB2	0.2-1.3	0.28-0.62
PTEN	0.05-0.2	—
RAD50	0.1	0.16-0.29
RAD51B	—	0.06
RAD51C	0.2-0.29	0.41-2.9
RAD51D	0.2	0.35-1.2
STK11	0	—
TP53	0.25-0.53	0.31-0.84

Table 3 Frequency of Germline mutations in patients screened for HBOC syndrome.

Adapted from (HOANG; GILKS, 2018).

1.2.1.2 Other genes

Since the discovery of *BRCA1* and *BRCA2* genes 25 years ago, several other breast cancer susceptibility genes have been identified. With the current popularization of next generation sequencing, the single-gene strategy is used in selected circumstances. Most services are now sequencing *BRCA1/2*, along with other genes related to breast and ovarian cancer hereditary predisposition in the context of a HBOC gene panel (Table 1). Pathogenic variants in other high-risk genes, such as *TP53*, *STK11*, *PTEN* and *CDH1* explain approximately additional 5% of HBOC cases. Some of them have found to increase the risk of cancer similar to *BRCA1/2*, sharing the same care guidelines for cases where a *BRCA1/2* pathogenic variant has been identified. *BRCA1/2* HBOC can be distinguished from these other disorders based on the spectrum of tumors present in the family and with the aid of germline genetic screening. *PALB2* variants have now been determined to be of high penetrance (ANTONIOU et al., 2014a). *ATM, CHEK2*, and *BARD1* genes are considered to have a moderate increase in risk (COUCH et al., 2017). Numerous other genes, such as *NF1, RAD51C, RAD51D, BRIP1, NBN, MRE11A, FANCM, RECQL, MLH1, MSH2*, *MSH6,* and *PMS2,* are suspected to be related to the risk of breast cancer, but they still need confirmation given their low penetrance and divergent results between studies. These previously cited genes are usually included in HBOC panels (COUCH et al., 2017). Still, some of the commercial panels mix genes related to different hereditary cancer syndromes, adding the challenge of interpreting the clinical risk of mutations related to other syndromes when they are identified in HBOC patients. This leads to the increase of uninterpretable results, since the number of variants of uncertain significance increases when multiple genes are tested. For multi-gene panel testing, a pathogenic mutation is identified in approximately 30% of HBOC patients, most commonly in *BRCA1* and *BRCA2* (COUCH; NATHANSON; OFFIT, 2014). It is thus worth mentioning that despite technology advances and recent democratization of access to genetic screening, the predisposition mechanism remains undefined for about two thirds of families meeting the clinical criteria for HBOC.

1.2.1.3 Cancer risk estimation

The optimal management of individuals with HBOC depends on accurate age-specific cancer risk estimates. Women carrying a germline pathogenic variant in the *BRCA1* gene have a cumulative lifetime risk of developing breast and ovarian cancers of 72% and 44%, respectively. For *BRCA2,* the lifetime risk for breast and ovarian cancer is 69% and 17%, respectively (KUCHENBAECKER et al., 2017). Initially, retrospective studies estimated the cumulative breast cancer risk at 70 years from 40-87% and 27-84% for *BRCA1* and *BRCA2* carriers, respectively. These studies pointed out that the peak of incidence occurs slightly earlier for *BRCA1* mutations, when compared to *BRCA2* carriers (41-50 years versus 51-60 years). Broadly, *BRCA2* carriers have a lower penetrance for ovarian cancer than do *BRCA1* carriers. Ovarian cancer risk ranged from 16-68% for *BRCA1* and from 11-30% for *BRCA2* carriers. More recently, prospective epidemiological studies have provided a more accurate estimation of the risk. EMBRACE study found that carriers of *BRCA1* and *BRCA2* pathogenic mutations have a mean cumulative risk of breast cancer at age 70 years of 60% and 55%, respectively. The equivalent mean cumulative ovarian cancer risk was 59% and 16.5% in the presence of a BRCA1 and *BRCA2* mutation, respectively (MAVADDAT et al., 2013). These findings were confirmed by a larger cohort that included 6,036 *BRCA1* and 3,820 *BRCA2* female mutation carriers and estimated the cumulative breast cancer risk at age 80 years of 72% for *BRCA1* and 69% for *BRCA2* carriers. The cumulative ovarian cancer risk at age 80 years was 44% for *BRCA1* and 17% for *BRCA2* carriers (KUCHENBAECKER et al., 2017). The later study also reported that breast cancer incidences increased rapidly until ages 30-40 years for *BRCA1* and 40-50 years for *BRCA2*, then remained a similar constant incidence remained until age 80 years. Also, the cumulative 20-year risk of contralateral breast cancer after breast cancer diagnosis was 40% for *BRCA1* and 26% for *BRCA2*. Concerning male breast cancer, the relative risk of male breast cancer is elevated for both genes, particularly *BRCA2*. While in general population the estimated risk is 0.1%, the risks for men varies between 7-14% for *BRCA2* mutations and corresponds to 1% for *BRCA1* carriers (EVANS et al., 2010). Aditionally, the lifetime risk for prostate cancer is about 15-25%, which is much higher than the average risk in men and tends to occur at younger age, when it is more aggressive (PRITCHARD et al., 2016).

Recent evidence helped refine carrier's risk of developing cancer, based on the location of the mutation in the gene. These trials identified clusters of breast and ovarian

susceptibility, suggesting that individualized counseling should incorporate mutation location for assessment of cancer risk since variants in different regions of *BRCA1/2* genes generate different cancer risk. For example, *BRCA2* variants located in 5' to c.2830 (including exon3 and the domain of interaction with PALB2) and c.6402 to 3'(including BRCA2 C-terminus and the domain of interaction with DNA) were associated with a significant higher risk of breast cancer when compared with central variants (KUCHENBAECKER et al., 2017; REBBECK et al., 2015b). In addition, evidence suggests that different types of variants (e.g. missense x loss of function) within the same gene can lead to different risk estimates.

1.2.1.4 Management

The importance of identifying at-risk individuals lies in providing appropriate screening, surveillance, and risk reduction interventions. The individualized approach should include discussion about the risks and benefits of risk-reduction surgeries, taking into account patient's age, priorities, previous cancer history, comorbidities, and cancer-related anxiety. Prospective studies demonstrated that for *BRCA* carriers without a personal history of cancer, bilateral risk-reducing mastectomy (RRM) is associated with 90% or more decreased risk of breast cancer with a residual risk of 1-2% (CARBINE et al., 2018; DOMCHEK et al., 2010). But the decision to undergo RRM and the ideal time can be influenced by life events, beeing uncertain for some women. For individuals with HBOC who choose not to undergo risk-reducing surgery, proper follow up with intensive cancer screening has an impact on early detection of cancer with increased cure rate. It is important to mention that for patients with a strong familiar breast cancer risk, even if a pathogenic mutation is not identified, appropriate follow-up and awareness training with monthly self-breast examination should begin at 18 years with clinical breast examination recommended at 25 years, and from then on every 6 months. Between 25-29 years, radiographic screening is suggested. From 30 to 75 years, annual MRI and mammography are recommended. A recent prospective randomized study that performed paired MRI and mammography in women with high risk for breast cancer confirmed the benefit of adding MRI to the screening of this population. This study showed that 61% of the tumors would not have been diagnosed by only a mammography, and it also demonstrated that MRI allowed the diagnosis of cancer at an earlier stage. In the group that underwent MRI, the identified tumors presented a smaller size (9x17mm, p=0.014) and were mostly under 1 cm (58%) with less involvement of the regional lymph node (11% x 63%,

p=0.014) (SAADATMAND et al., 2019). Also for *BRCA1/2* mutation carriers older than 50 years, the addition of MRI to mammography improves screening sensitivity by a magnitude similar to that observed in younger women (PHI et al., 2015).

Awareness of ovarian cancer risk should also exist, but screening for ovarian cancer is more challenging due to low sensitivity of the exams. It is advised that risk-reducing salpingo-oophorectomy (RRSO) be offered between age 35 and 40 years for women with *BRCA1* mutations who have completed childbearing. For *BRCA2* carriers, it can be delayed until age of 45 years, since only 1% of this population presents ovarian cancer by age 50. Nevertheless, health considerations related to premature surgical menopause, including an increased risk of osteoporosis and cardiovascular disease, should be discussed with women considering surgery. RRSO is the only evidenced-based strategy to prevent ovarian and fallopian tubes cancer. It is associated with an 80% of reduction of ovarian cancer risk, a 50% reduction of breast cancer risk in premenopausal women and of breast and ovarian-cancer specific mortality (HARTMANN; LINDOR, 2016). Annual screening with CA125 and transvaginal ultrasound may be considered for women who refuse prophylactic surgery, with limited sensitivity (less than 50%) and positive predictive value (less than 17%) (STIRLING et al., 2005). Studies show that both are ineffective in detecting tumors during the very early stage to influence prognosis. The PROSE (Prevention and Observation of Surgical Endpoints) study evaluated the effect of risk-reducing salpingo-oophorectomy on mortality and confirmed that the surgical group had lower all-cause mortality (HR 0.40; 95% CI 0.26-0.61), breast-cancer specific mortality (HR 0.44; 95% CI 0.26-0.76), and ovarian cancer-specific mortality (HR 0.21; 95% CI 0.06-0.80) (DOMCHEK et al., 2010) . Some questions about the extent of surgery remain unanswered, such as whether adding hysterectomy to the procedure has survival benefits, and even if just performing salpingectomy alone would be sufficient for risk reduction. The latter is based on the pathophysiology of ovarian cancer and its likely origin is *in situ* lesions located in fallopian tubes. Recent data has suggested that women with *BRCA1/2* mutations present an increased risk for uterine serous carcinoma, which generated an extensive discussion whether hysterectomy should be performed at the time of prophylactic surgery (HAVRILESKY et al., 2017; SHU et al., 2016). However, current standard guidelines do not include hysterectomy as part of risk reducing surgery.

Since germline *BRCA2* pathogenic variants are associated with a five-to-eight increase in the risk of developing prostate cancers, which are more aggressive and with a shorter survival rate; male carriers should begin prostate screening at age 45. Additionally, *BRCA2*

mutations are present in 7% of pancreatic cancers irrespective of familial history, and account for approximately 10% of hereditary pancreatic cancers. Therefore, an individualized screening should be advised, preferably in the context of a clinical screening protocol because there is no consensus for pancreatic cancer screening in most institutions so far. A full body and eye examination for melanoma is also recommended because of increased risks of uveal melanoma (DALY et al., 2017).

In addition to intensified screening and risk reduction surgeries, some pharmacological measures have proven to positively impact the management of *BRCA* carriers. Chemoprevention with Tamoxifen may be offered for breast cancer primary prevention of *BRCA2* carriers, since 75% of *BRCA2*-associated breast cancer are ER positive (KING et al., 2001). However, for *BRCA1* carriers, the current use of tamoxifen is less studied and data is inadequate to support the use of tamoxifen, since they present mainly TNBC. Beyond Tamoxifen, observational studies have shown that oral contraceptives reduce the risk of ovarian cancer by 30% and 40% in the general and BRCA population, respectively. The concern about theoretical increased risk of breast cancer was not confirmed in studies of women with HBOC syndrome. However, data from randomized controlled trials is lacking and therefore the use of oral contraceptives for prevention of ovarian cancer in women who have not undergone risk-reducing salpingo-oophorectomy is controversial (HAVRILESKY et al., 2013; MOORMAN et al., 2013).

1.2.2 Breast and ovarian cancer related to *BRCA1/2* genes

1.2.2.1 Breast cancer related to *BRCA1/2* variants

About 70% of breast tumors arising in *BRCA1* mutation carriers are “triple negative” (MAVADDAT et al., 2012). On the other hand, only 10% to 20% of TNBCs carry a *BRCA1* mutation (COUCH et al., 2015; GONZALEZ-ANGULO et al., 2011). *BRCA1*-associated tumors generally present a higher mitotic rate and are peculiarly higher-grade tumors, presenting greatly increased mitotic count, pushing margins, lymphocytic infiltrate, trabecular growth pattern, and necrosis (FOULKES et al., 2003; LAKHANI et al., 2002; SOUTHEY et al., 2011). These tumors generally express mioepithelial cell-type cytokeratins (CK5⁄6, CK14 and CK17) and present a basal-like gene expression profile (FOULKES et al., 2003). A previous study showed that reduced expression of CK8⁄18 could help discriminate the basal

tumors from *BRCA1* carriers from those sporadic tumors (MULLIGAN et al., 2011). Moreover, loss of PTEN is generally found and seems to be an early event in *BRCA1*-related TNBC tumorigenesis, while *TP53* mutations occurs first in most luminal BRCA1 tumors (MARTINS et al., 2012a).

BRCA2 breast carcinomas are most closely like sporadic tumors, generally expressing the estrogen receptor (77%) and are in the minority triple negative (MAVADDAT et al., 2012; SPURDLE et al., 2014a). RNA tumor profiling demonstrated that *BRCA2* tumors are mainly of the luminal B subtype and are more likely than non-BRCA2 tumors to be ER positive and of high grade , with pushing margins (BANE et al., 2007; LARSEN et al., 2013).

Mavaddat and colleagues evaluated the histopathological characteristics of the largest cohort of breast cancer patients harboring *BRCA1/2* germline mutations. This included 4,325 patients with *BRCA1* mutations and 2,568 patients with *BRCA2* mutations (MAVADDAT et al., 2012). Breast tumors were mostly invasive ductal carcinomas (occuring in the milk ducts) for both *BRCA1* (80%) and *BRCA2* (83%) carriers. Lobular carcinoma (occuring in the breast lobules) was the second most common subtype for *BRCA2* carriers (8.4%), and medullary carcinoma (a subtype of invasive ductal carcinoma) for *BRCA1* carriers (9.4%). The frequency of TNBC was 69% for *BRCA1* and 16% for *BRCA2*. Thirteen percent of *BRCA1* tumors were HER2 positive, while 10% for *BRCA2*. *BRCA1* tumors were a majority grade 3 (77%), while for *BRCA2* half of the tumors were grade 3. For *BRCA1* carriers, the grade of the tumor decreased with increasing age, as well as the proportion of estrogen receptor-negative tumors. In contrast, the grade and the proportion of ER negative tumors increased with age for *BRCA2*. Such findings are in agreement with previous studies with a smaller number of participants (FOULKES et al., 2004; TUNG et al., 2010). Pathology data was available for 702 *BRCA1* and 302 *BRCA2* mutation carriers in the same cohort who developed a contralateral breast cancer (MAVADDAT et al., 2012). The median interval for a second breast cancer was 5.2 years. Interestingly, the ER/PR status of the first breast tumor was predictive of the ER/PR of the second cancer for both *BRCA1* and *BRCA2* carriers, suggesting that the second tumor arises in the same genetic and environmental background has the same pathology.

Concerning the prognosis of *BRCA*-associated breast cancers, recently the POSH study showed no difference in survival for patients carrying a *BRCA* mutation when compared to those with sporadic breast cancer (COPSON et al., 2018). However, in TNBC subgroup, *BRCA* carriers had a better survival than non-carriers, which may be related to better

sensitivity to chemotherapy. This survival advantage of TN *BRCA* mutant subgroup was also confirmed in a recent meta-analysis (BARETTA et al., 2016).

1.2.2.2 Ovarian cancer related to *BRCA1/2* mutations

The majority of ovarian tumors related to *BRCA1* and *BRCA2* constitutional pathogenic variants are serous carcinomas (67%), followed by endometrioid (12%), clear-cell (2%), and mucinous carcinomas (1%) (MAVADDAT et al., 2012). Tumors in *BRCA1/2* carriers are more likely than tumors in age-matched controls to be invasive serous adenocarcinomas and unlikely to be borderline or mucinous tumors. They are of higher grade, with a higher percentage of solid components and are more likely to stain strongly to TP53 (LAKHANI et al., 2004). There are no significant differences in ovarian cancer morphology or grade between *BRCA1* and *BRCA2* tumors (MAVADDAT et al., 2012). However, *BRCA1* carriers present a higher ovarian cancer lifetime risk than *BRCA2*. The cumulative ovarian cancer risk to age 80 years is around 44% and 17% for *BRCA1* and *BRCA2* carriers, respectivelly.

1.2.3 Implications for treatment response *BRCA1/2* – predictive biomarker

1.2.3.1 Sensitivity to platinum salts

Platinum salts, such as cisplatin and carboplatin, are effective breast and ovarian cancer treatments. They act as DNA cross-linking agents forming intra-strand crosslinks, and are especially active in cells lacking HR function. Although their clinical effectiveness as first-line chemotherapy for breast cancers has been confirmed (ORR of 50% for cisplatin and 30% for Carboplatin), studies have shown that they have only modest activity in previously treated metastatic breast cancers (MARTÍN, 2001; SLEDGE et al., 1988). Tutt and colleagues were able to demonstrate that the presence of a germline *BRCA* mutation was predictive of a greater benefit in the metastatic scenario. The trial included 376 unselected TNBC patients after first-line treatment failure who were randomized to receive either Carboplatin or Docetaxel. While there was no difference between ORR to carboplatin and ORR to Docetaxel in the overall population (ORR 31.4% x 34%), subjects with a deleterious *BRCA1/2* germline mutation had a significantly better response to carboplatin than to docetaxel, doubling the

overal response rate (ORR 68% x 33.3%, p=0.03). However, the highest platinum sensitivity was limited to BRCA mutation carriers. Such benefit was neither observed for subjects with a high HRD score, nor for tumors presenting *BRCA1* promoter (TUTT et al., 2018a). These results were consistent with previous results from a smaller phase 2 trial in methastatic TNBC in which platin agents were active specially in the presence of *BRCA1/2* mutations but not in the presence of *BRCA1* promoter methylation (ISAKOFF et al., 2015).

The activity of platinum salts was also evaluated in early breast cancer, with proven benefits in the neoadjuvant scenarium for TNBC subtype regardless of *BRCA1* status (VON MINCKWITZ et al., 2014). Data from a retrospective study support the use of platinum salts in the neoadjuvant treatment of women with a *BRCA* mutation. Expressive response rates have been observed for *BRCA1* mutation carriers treated with cisplatin monotherapy compared with standard regimens based on antracycline and taxanes (pCR 83% x 8-22%) (BYRSKI et al., 2010). However, the usefulness of *BRCA1/2* mutations as predictive biomarkers of platinum response in the neoadjuvant scenario is still questioned. Some authors advocate that gBRCA1⁄2 mutation carriers have a higher likelihood of achieving pCR thanks to a higher sensitivity to cytotoxic agents in general, regardless of the addition of platinum salts (WANG et al., 2015). GeparSixto was a phase II study which confirmed the benefit of adding carboplatin to neoadjuvant chemotherapy with increase of pathological complete response rate (53.2%x 36.9%, p=0.005), an advantage translated in a superior disease-free survival rate at 3 years. The secondary analysis of GeparSixto trial, performed to evaluate if *BRCA1/2* status were predictive of response to chemotherapy, could not confirm this hypothesis. It found that the addition of carboplatin did not increase pCR rate in mutation carriers (65.4% x 66.7% in treated vs untreated, respectively). Surprisingly, in the wild-type population, neoadjuvant carboplatin significantly increased it (55% x 36.4% OR 2.14, 95% CI 1.28-3.58, p=0.004). Additionally, g*BRCA1/2* mutation carriers experienced a better DFS, which was not significantly improved by the addition of carboplatin (82.5% in carboplatin treated x 86.3% untreated patients).

Little data is available in the adjuvant setting for platinum salts in *gBRCA1/2* associated breast cancers. In 2014, Dwadasi and colleagues randomized TNBC patients who had residual disease after neoadjuvant chemotherapy based on antracicline and taxanes, receiving four additional cycles of adjuvant cisplatin (75 mg⁄m2), with and without the PARPi rucaparib. The primary end point (DFS in 1 year) was similar in both arms and was not different between patients with BRCA-associated and sporadic tumors (85% x 79%,

respectively). Yet it is noteworthy that there was no relapse in any of the eight patients with g*BRCA* mutation.

1.2.3.2 HR deficiency and development of a targeted therapy: PARP inhibitor treatments

Breast cancers associated with *gBRCA1/2* mutations represent 3-5% of cases. The percentage of somatic *BRCA1/2 (sBRCA1/2)* mutations in breast cancer is not well established. However, two studies found that approximatelly 3% of unselected cases present *sBRCA1/2* mutations (NIK-ZAINAL et al., 2016; WINTER et al., 2016). Ovarian epithelial cancers associated with *gBRCA1/2* mutations represent ~22% of the cases, with 15% germline and ~7% somatic mutations (CANCER GENOME ATLAS RESEARCH NETWORK, 2011). In the absence of BRCA1 or BRCA2 protein function, the preferential use of error-prone DNA repair mechanisms leads to genomic instability, a peculiar feature of breast and ovarian cancers arising from *BRCA* mutations that may favor carcinogenesis. As stated before, the rational to use Poly (ADP-ribose) polymerase (PARP) inhibitors to treat tumors arising in *gBRCA1/2* mutation carriers is based on the principle of synthetic lethality, a concept in which if only one of the two genes is mutated, then it is compatible with viability, while a mutation in both leads to cellular death (KAELIN, 2005; LORD; ASHWORTH, 2017).

PARPs are a large family of multifunctional enzymes that play a key role in the repair of single-strand breaks (SSB) through base excision repair. Of the 17 members of the PARP protein family, PARP1 is best characterized (VYAS et al., 2013). PARP1 is the major target of PARPi. The inhibition of PARP impairs the repair of SSBs through disruption of the base excision repair pathway and PARP1 trapping that happens through inhibition of auto-PARylation and/or PARP release from DNA. These events lead to accumulation of SSB, which lead to DSBs at the replication fork and thus to the death of homologous recombination deficient cells such as *BRCA1/2* mutants in a process named “synthetic letality”. This concept has moved from the field of genetics to medical oncology, opening new perspectives for treating tumors containing the BRCAness HR deficient phenotype.

The first trial evaluating the efficacy of PARPi (olaparib) in breast cancer was published in 2009 (FONG et al., 2009). This phase I trial included 60 heavily pretreated women, 3 of them carrying a *BRCA* pathogenic variant. One out of these 3 patients presented a complete response for 60 months. The second one had stable disease for 7 months (FONG et al., 2009). These results led to the approval of 2 phase II trials including women with

gBRCA1/2 pathogenic variants with advanced breast cancer, who presented response rates ranging from 12-41% (KAUFMAN et al., 2015; TUTT et al., 2010). Recently, a prospective phase III trial compared olaparib versus standard of care chemotherapy in patients with metastatic breast cancer harboring a *gBRCA1/2* pathogenic variant. The PFS was significantly longer in the olaparib group (7 x 4.2 months HR 0.58, 95%CI 0.43-0.80, p<0.001), as well as improvement of quality of life. No significant benefit in overall survival has been proved yet (ROBSON et al., 2017).

More recent studies have investigated the benefit of adding platinum salts in comparison and in combination with PARPi for the treatment of BRCA related early breast cancer. Telli and colleagues reported a pCR of 36% in a single-arm phase II study that evaluated the combination of iniparib, gemcitabine and carboplatin for the neoadjuvant treatment of *BRCA* pathogenic variant. The study confirmed that a high loss of heterozygosity score was a predictor of better response (TELLI et al., 2015). Next, the combination of a PARPi (Veliparib) with carboplatin in addition to standard neoadjuvant chemotherapy with Docetaxel was evaluated in the BrighTNess trial, a phase III randomized study that included stage II-III TNBC. In this trial, the addition of carboplatin and veliparib increased pCR rate in both *gBRCA1/2* pathogenic variant carriers (57%) and wild-type patients (53%), but no significant differences in patients who received only carboplatin (LOIBL et al., 2018).

Just like for early breast cancer, several studies are now comparing platinum salts to PARPi and evaluating them in combination for advanced breast cancer. A recently published phase II trial evaluated the efficacy of adding the PARPi veliparib to chemotherapy regimens (carboplatin and paclitaxel or temolzolamide) in patients with *gBRCA1/2* mutated metastatic breast cancer. A numerical but not statistically significant increase in PFS and OS was observed with the addition of veliparib to the platinum-based regimen carboplatin and paclitaxel (HAN et al., 2018).

Beyond breast cancer, PARPis have been widely tested for ovarian cancer treatment in different settings. High-grade serous carcinoma (HGSOC), the most common subtype of ovarian cancer, is characterized by nearly universal TP53 mutations (96%) and high genomic instability. As stated before, one half of HGSOC displays defects in HR DNA repair pathway, with mutations identified in *BRCA1/2* in ~22% of the cases with ~15% germline and ~7% of tumoral mutations) (CANCER GENOME ATLAS RESEARCH NETWORK, 2011). Pathogenic variants in other HR genes are less common and are present in about 3% of the cases. Sporadic tumors also display HR defects as *BRCA* mutants (the BRCAness phenotype),

and consequently higher response rate to platinum-based chemotherapy and PARPi. Most patients with advanced-stage ovarian carcinoma are initially treated with platinum-based chemotherapy, but the majority of them will ultimately relapse. Longer treatment-free intervals and improved overall survival rates observed in this group are related to their inability to repair DNA damage. Based on this rational, two phase I studies tested the safety and benefit of olaparib for treatment of ovarian cancer harboring *gBRCA1/2* pathogenic variants (FONG et al., 2009, 2010). In the first study, Fong et. al enroled 60 solid tumor patients, in which ovarian tumors led with 21 cases. Of the 21 ovarian tumors, 16 had *gBRCA1/2* pathogenic variants, who had received at least one line of chemotherapy. Response was documented only in patients harboring g*BRCA1/2* pathogenic variants (16 of 21) in both platinum-sensitive (61.5%) and platinum-resistant (41.7%) cohorts (FONG et al., 2010). This study supported the anti-tumor activity of PARP inhibition for the treatment of ovarian cancer. Subsequently, in the expansion phase, only ovarian cancer carriers of *BRCA1* or *BRCA2* mutations were enrolled. Of the 50 patients, 20 (40%) presented partial or complete response and 3 (6%) presented disease stabilization. The authors again confirmed a significant association between the clinical benefit rate and platinum-free interval.

Subsequent phase II studies confirmed the efficacy of olaparib as monotherapy for the treatment of metastatic HGSOC patients harboring *gBRCA1/2* pathogenic variants, with ORR ranging from 33-41% and a median response duration of 8.8 months (KAYE et al., 2012).

Next, the trials focused on the use of olaparib in the maintenance scenario for platinum-sensitive relapsed ovarian cancer. Ledermann et al. confirmed the improvement of PFS by olaparib initially in a retrospective pre-planned analysis of a phase II trial, and subsequently in a prospective trial (8.4 months vs. 4.8 months; HR 0.35; 95% CI 0.25-0.49; P<0.001). The benefit was even greater in the presence of *BRCA1/2* germline or somatic mutations (LEDERMANN et al., 2014). Also, SOLO 2 phase III trial met its primary end point, with improved PFS with olaparib than with placebo (19.1 months vs. 5.5 months, HR0.30; 95% CI 0.22-0.41) (PUJADE-LAURAINE et al., 2017a). Following these results, olaparib was also tested in newly diagnosed ovarian cancer patients, after administration of platinum-based adjuvant chemotherapy. Also, in the adjuvant scenario, olaparib significantly reduced the risk of disease progression or death by 70% (MOORE et al., 2018a).

Therefore, olaparib was initially approved by the Food and Drug Administration (FDA) in 2014 for the maintenance treatment of *BRCA1/2*-mutated ovarian cancer. Subsequently, in 2018 the approval was extended to all platinum-sensitive patients regardless

of *BRCA1/2* status, because it was realized that the benefit extended to all HRD tumors. Following SOLO1 trial, olaparib was also approved in first-line maintenance for *BRCA*-mutated (BRCAm) advanced ovarian cancer. Currently, two other PARPi have been approved by the FDA for the treatment of ovarian cancer: niraparib and rucaparib. Other PARPi are under development and test, such as veliparib and talozaparib, based on the rational described above.

The phase III NOVA study confirmed the benefit of niraparib in the maintenance setting of platinum sensitive HGSOC. In this study, the authors stratified the analysis by the presence of *BRCA1/2* pathogenic variant and in the wild type group, by the presence of HR deficiency. The benefit of niraparib was more pronounced among patients with *gBRCA1/2* pathogenic variant (PFS 21 vs. 5.5 months, HR 0.27; 95% CI -.0.17-0.41). However, it was not negligible among *gBRCA1/2* wild-type patients with HR deficient tumors (12.9 vs. 3.8 months, HR 0.38; 95% CI 0.24-0.59). These results led to FDA approval of niraparib in the maintenance setting, regardless of *BRCA1/2* status. Additionally, niraparib antitumor activity was also documented for late-line treatment of ovarian cancer patients, with greater benefit among HRD-positive tumors, regardless of relation to a *BRCA1/2* pathogenic variant (MOORE et al., 2019).

Ultimately, rucaparib was also approved by FDA for maintenance treatment of ovarian cancer, based on the results of ARIEL2 and ARIEL3 trias (COLEMAN et al., 2017b; SWISHER et al., 2017a). As for niraparib, a preplanned analysis of PFS according to a tumor genomic profiling test for homologous recombination and loss of heterozygosis analysis confirmed that the benefit of the PARPi was bigger but not restricted to *BRCA* mutant tumors. The PFS was 16.6 months and 13.4 months in patients with BRCAm and homologous recombination deficient ovarian carcinomas, respectively (vs. 5.4 months for patients who received placebo; $p<0.0001$).

In line with these findings, it is clear that the population with potential benefit from PARPi is likely wider than germlin *BRCA* mutation-associated disease. However, it is known that part of the patients even carrying the mutation will present primary or secondary resistance to the treatment. For this reason, biomarkers to broaden the selection of patients, with the potential clinical benefit from these agents, are in development.

1.3 Unidentified *BRCA1/2* variants in ovarian and breast cancer

As stated before, the identification of a *BRCA1/2* variant and determining its clinical significance now has an impact on genetic counseling , in addition to the therapeutic decision. However, although sequencing of *BRCA1/2* has been available for over 25 years, after a period of intense research, a pathogenic variant is identified in approximately 10% of tested families (CAPUTO et al., 2012). Thousands of *BRCA1/2* variants are identified in HBOC patients, but only some are actually related to cancer susceptibility (Caputo et al., 2012). In a majority, they are (likely) pathogenic truncating variants that generate a premature stop codon, which truncates the encoded protein and decreases protein expression through nonsense-mediated mRNA decay (ANCZUKÓW et al., 2008). Ten percent of individuals undergoing genetic *BRCA1/2* screening receive test results reporting variants of uncertain clinical significance (VUS). A much higher proportion is seen in non-Caucasian populations (FRANK et al., 2002; HAFFTY et al., 2006; KURIAN et al., 2019; NANDA et al., 2005; WEITZEL et al., 2005). About 10% of identified variants in *BRCA1/2* genes are either in-frame deletions/insertions, missense, silent variants, or variants in intronic and regulatory regions that may influence splicing or translation. These sequence variations present unknown functional effect on *BRCA1* and *BRCA2* and cannot currently be classified as either pathogenic or of low clinical significance. A large number of missense variants and virtually all non-coding deep intronic or promoter variants remain of unknown significance (VUS) since they cause subtle changes in protein structure (for missense variants) or in the amount of produced protein (for non-coding variants), being generally difficult to reliably determine their pathogenicity merely from clinical genetic information. A VUS finding should be considered clinically as not useful, and should not be taken into account into clinical decision until further evidence emerges to shift interpretation. Medical advice should be solely based on family and personal medical presentation. But in some cases, they are managed inappropriately as pathogenic mutation leading to psycological distress and inappropriate interventions in patients (REBBECK et al., 2018b). Even though individual VUS are rare, the identification of a VUS is not a rare event and has a tendency to increase with concomitant sequencing of several genes in NGS panels. Information about VUS is collected in different public databases. Attempts to evaluate the clinical significance of these variants include frequency analysis in case-control studies, personal and familial history, co-segregation of the

variant with disease in affected families, co-occurrence *in trans* with deleterious variants, *in silico* prediction models, and functional and tumoral data.

Thus, despite the remarkable advances seen in the past years, for the majority of HBOC families, little is understood about the underlying molecular mechanisms of cancer susceptibility. New technologies are being developed to extensively search in parallel for a pathogenic variant in a panel of other genes related to the syndrome, some of them also related to DNA repair. These high to moderate penetrance variants in suspected or known breast cancer related genes, such as *TP53, PTEN, STK11, CDH1, ATM, BRIP1, PALB2, RAD51* isoforms (*RAD51C, D, B*) may also contribute to hereditary predisposition, but altogether these variants only explain about 5% of the unsolved cases (CASTÉRA et al., 2018) and VUS are also identified in these new genes.

BRCA1/2 VUS classification is particularly challenging. This is why, in 2009 an international consortium was created for this purpose, which allowed the classification of a certain number of variants (DE LA HOYA et al., 2016; MOGHADASI et al., 2018; SPURDLE et al., 2012a, 2012b). This consortium has recently extended the scope of this study for other HBOC genes.

1.3.1 Multifactorial model for variant classification

Currently, *BRCA1/2* VUS classification is based on a posterior probability score calculated from a multifactorial likelihood model that combines multiples lines of data considering that each feature is an independent predictor of variant pathogenicity (GOLDGAR et al., 2008). This model combines the prior probability of pathogenicity derived from an evolutionary sequence conservation model (Align-GVGD) and from bioinformatic prediction of the variant effect on protein sequence or RNAm splicing, with likelihood ratios for pathogenicity estimated from (1) how variant co-segregates with cancer in families, (2) whether the variant is seen in co-occurrence *in trans* with a pathogenic variant in the same gene (which should be lethal or cause Fanconi Anemia if the VUS is pathogenic), (3) personal and family history of cancer associated with the VUS, and (4) tumor pathology of the associated breast tumor.

1.3.2 Co-segregation studies and personal/family history

Co-segregation studies are based on the odds that a VUS is linked to breast or ovarian cancer in families more than expected by chance. In general, it requires complex statistical analysis to combine segregation analysis from several families (THOMPSON; EASTON; GOLDGAR, 2003a).

Also, the observation from phenotype aspects of *BRCA1/2* families (in comparison to families without such mutation), such as age at onset and the number of malignant tumors of specific subtypes, allowed the calculation of likelihood that a pathogenic mutation is present and should be applied to the VUS. The analysis of personal history of individuals who carry a specific VUS (e.g, age at diagnosis, tumor type, number of affected first and second-degrees relatives, age at diagnosis and tumor types) should allow for calculation of the probability that the VUS is pathogenic (Goldgar et al., 2004).

Co-segregation and the summary of personal history and family history analysis provide direct mesure of disease susceptibility. However, they require analysis of genomic data from many individuals in a family, which is rarely available, or analysis of a very large dataset. Thus the information derived from both methods is rarely conclusive.

1.3.3 Contribution of tumoral analysis for variant classification

It is known that breast tumors from *BRCA2* carriers resemble sporadic tumors and are less distinctive than that of *BRCA1* carriers (LAKHANI et al., 2002). *BRCA2* tumors are predominantly luminal whereas *BRCA1* breast cancers are more likely to be triple-negative. Based on histopathological characteristics of tumors containing known pathogenic variants, statistical weighting has been applied to tumors of VUS carriers (BANE et al., 2009; LAKHANI et al., 2002; MAVADDAT et al., 2010; PHUAH et al., 2012). The fact that there is not a breast cancer phenotype restricted to *BRCA1/2* carriers and that these tumors do not present uniform characteristics have hindered this approach. Recently, however, LR pathology for breast cancer has been refined with the analysis of a large pathology dataset of *BRCA1/2* variant carriers (4,477 *BRCA1* and 2,565 *BRCA2*) in comparison with that of the 47,000 sporadic breast cancers (Spurdle et al., 2014). The authors concluded that triple-negative phenotype was highly predictive of *BRCA1* mutation status, regardless of age (LR for women under 50 years was 3.73; LR for women 50 years or older was 4.41). In contrast, triple-

negative status modestly predicted *BRCA2* mutation, and only for women of 50 years and older (LR 1.79). ER-positive grade 3 tumors modestly predicted *BRCA2* mutation status irrespective of age (LR 1.7), while for *BRCA1*, ER-positive phenotype negatively predicted *BRCA1* mutation status, irrespective of grade (LR 0.08-0.9).

Other attempts for breast cancer have been made to estimate the LR of *BRCA1* mutation based on: cytokeratin staining in combination with ER status and morphology (LAKHANI et al., 2005), CGH array to identify a BRCA1 or BRCA2-like profiles, and *BRCA1* promoter methylation tests (based on the mutual exclusivity of *BRCA1* germline mutations and *BRCA1*-Promoter methylation). Additionaly, recent research has also shown the utility of extending the analysis of histopathological features of ovarian cancer (such as modified Nottingham grade 3, serous⁄undifferentiated histology, prominent intraepithelial lymphocytes, marked nuclear atypia with giant forms, and abundant mitotic figures) to predict *BRCA* mutation status. However, the number of analyzed samples in the respective studies was limited, so further analysis in a larger cohort is required. Currently, only breast pathology data such as grade and ER⁄PR⁄HER2 status are included in likelihood and posterior probability model score (PARSONS et al., 2019).

Moreover, incorporation of LOH analysis in the posterior probability model has been proposed. Since LOH of the wild-type allele is the most frequent second-hit event in *BRCA*-related carcinogenesis, it has been proposed that the observation of loss wild-type allele in tumors of *BRCA1/2* carriers would argue in favor of the VUS pathogenicity. The frequency of loss of the wild-type allele among pathogenic *BRCA1/2* variants varies for breast and ovarian cancers: a proportion of loss of wild-type allele in ovarian tumors as high as 93% for *BRCA1* and 90% for *BRCA2* carriers (MAXWELL et al., 2017a). A similar percentage of 90% occurred for *BRCA1* breast cancers, but was less evident (54%) for *BRCA2* breast cancers (MAXWELL et al., 2017a; NONES et al., 2019). Nevertheless, some studies have argued in favor of LOH as a useful tool to predict variant pathogenicity (Chenevix-Trench et al., 2006; Spearman et al., 2008; Yang et al., 2018), while others warned that it should be applied with caution (BERISTAIN et al., 2010; SPURDLE et al., 2008a; VAN HEETVELDE et al., 2018). To confirm this hypothesis, analysis of a larger number of samples may be required.

1.3.4 Multifactorial model and VUS classification

The combination of both, the prior probability and the likelihood component allows the calculation of a posterior probability of causality, which enables the classification of the individual VUS as pathogenic or likely pathogenic if its probability of being pathogenic is greater than 0.95 and 0.99 respectively. The variant is classified as neutral or likely neutral if this probability is less than 0,001 or between 0.001 and 0.049, respectively. All variants whose probability of being deleterious is between 0.05 and 0.949 remain of uncertain significance (PLON et al., 2008a) For now, results from functional studies are not integrated into the algorithm and the use of tumoral data is limited to morphological and immunohistochemical breast cancer data. Moreover, the low frequency of these variants and the limited access to family history, genetic, and tumoral information are limitations of the method.

Recently, a new classification has been created by the American College of Medical Genetics (ACMG) (RICHARDS et al., 2015) to apply to all genes in order to standardize the classification criteria. These criteria do not apply for *BRCA1/2* genes because the multifactorial model, mentioned above, remains more accurate when co-segregation data is included.

In this thesis, we are particularly interested in *BRCA1/2* missense and non-coding variants, which will be described in the following paragraphs. We were also interested in understanding the potential impact of the tumoral and therapeutic information for the VUS classification.

1.3.5 *BRCA1/2* splicing variants

1.3.5.1 *In silico* tool predictions

A number of *in silico* tools are available to help understand if a given intronic or exonic variant leads to an improper exon and intron recognition on messenger RNA and results in the generation of an aberrant transcript of the mutated gene. Four examples of these tools are: Neural network splice (NNSplice) is based on machine learning technique, i.e. artificial neural network (REESE et al., 1997, p. 199); Splice site finder (SSF) and human splicing finder (HSF) score calculation is based in the matrix and its homologous percentage with the tested sequence (DESMET et al., 2009; SHAPIRO; SENAPATHY, 1987, p. 198);

Max-EntScan (MES) is based on maximum entropy of a nucleotide sequence with a set of constraints fixed by the MES model (YEO; BURGE, 2004); and GeneSplicer (GS) (PERTEA; LIN; SALZBERG, 2001) is based on a decision tree method and captures potential strong dependencies between signal positions by dividing the dataset into subsets based on pairwise dependency between positions and modeling each subset separately. These tools are generally freely available, allowing high-throughput submission. They can be used either as stand-alone programsor as part of commercial deal (Alamut, Interactive Biosoftware, Rouen, France) or free Web-based applications (HSF). A major advantage of MES running under Alamut (hereinafter referred to as MES-A) is that the user no longer needs to indicate a dedicated analysis window with intron/exon junctions. MES-A scores the entire sequence, automatically moving the window with a 1 bp shift. As a result, all positions can be analyzed with the MES-A implementation, as opposed to the stand-alone program. This point must be stressed, as it circumvents the limitation of the stand-alone program, which cannot always be used as a first-line tool (HOUDAYER et al., 2008). Recently, a new tool emerged from a international collaboration for predicting variant spliceogenicity: SPiCE (Splicing Prediction in Consensus Elements) (LEMAN et al., 2018). SPiCE combines *in silico* predictions from SpliceSiteFinder-like and MaxEntScan and uses logistic regression to define optimal decision thresholds for RNA experiments.

1.3.5.2 Functional assay: Assays to measure splicing

These assays evaluate the impact of VUS on RNA splicing focus on the gene region carrying the variant, and compare the wild type with the variant sequencing providing proofs of the involvement of the variant in the splicing alteration. These assays complement the use of *in silico* prediction tools and can be based either on a minigene construction or by an investigation of DNA transcripts derived from blood or tissue samples from patients performed by RT-PCR, qPCR and droplet digital PCR (VAN HEETVELDE et al., 2017). During these experiments, the presence of both alleles can be considered an indication of no effect of the VUS on splicing, whereas absence of the mutant allele in the full-length product can be an evidence of a complete effect. But for RNA assays, quality control is an issue, as loss of splicing fidelity has been reported in cells analyzed under non-physiological conditions (WIMMER et al., 2000).

1.3.6 *BRCA1/2* missense variants classification

Missense variants alter DNA sequencing, making a different codon of 3 nucleotides that leads to a single amino acid residue change in the final protein, encoding a stable yet mutant protein. Their classification is particularly challenging since it is difficult to estimate the impact of subtle changes in protein structure and in its function.Whether it is clinicaly useful to identify a missense variant in *BRCA1* or *BRCA2* genes is clinically useful remains questionable for a great number of cases. Only a small proportion are pathogenic, which are generally restricted to BRCA1/2 functional domains such as the Ring finger and BRCT domains of BRCA1 as well as the carboxy terminal domain of BRCA2 containing the DNA binding domain.

1.3.6.1 *In silico* tool predictions

Based on the assumption that a high level of conservation of gene sequence through evolution indicates that the DNA sequence of a specific functional domain must be maintained for it to work properly, a number of *in silico* tools are available to allow phylogenetic analysis (i.e to analyze if there is evolutionary conservation of nucleotide base pairs or individual amino acids across species). If the VUS is located in a highly conserved area of the gene, it is inferred that deviation of almost any type would be harmful.

Sorting Intolerant from Tolerant (SIFT) (NG; HENIKOFF, 2001) is a sequence homology-based tool that predicts variant pathogenicity using normalized probabilities calculated from the input multiple sequence alignment. These multiple sequence alignments are obtained by internally generating it or by allowing the user to submit their own FASTA-formatted alignment.

Polymorphism Phenotyping v2 (Poly Phen2.1) (RAMENSKY; BORK; SUNYAEV, 2002) predicts variant as “benign”, “possibly damaging” or “probably damaging” based on eight sequence-based and three structure-based predictive features used by the probabilistic classifier based on machine learning methods.

Align-Grantham Variation Grantham Deviation (Align-GVGD) (TAVTIGIAN et al., 2006, 2008) is a method that predicts variant pathogenicity based on a combination of Grantham Variation (measures the amount of observed biochemical evolutionary variation at a particular position in the alignment) and Grantham Deviation (measures biochemical

difference between the reference and the amino acid encoded by the variant). Recently, Align-GVGD has been modified to take into account the impact of missense variants on splicing (VALLÉE et al., 2016).

Aditionally, protein conformational modeling provides another *in silico* tool to evaluate whether a specific amino acid change may impact protein function. It is important to emphasize that comparisons of these different computer programs show that they can result in divergent conclusion (HICKS et al., 2011). Thus, none of them is sufficientlly robust for reinterpreting a VUS.

1.3.6.2 Functional tests for assessing missense variant

Several functional assays have been proposed to evaluate the impact of a single amino acid substitution on BRCA1/2 biological roles and biochemical properties. The purpose of functional assays is to serve as independent classifiers of VUS by assessing, directly or indirectly, their influence on protein conformation or function and generating additional information that could be integrated with available genetic and epidemiological data into multifactorial likelihood models in the future. According to ACMG, they are considered as strong evidence in determining pathogenicity of a given VUS. Although their results are not integrated into the multifactorial model for variant classification, they are useful for screening and stratification of variants for which additional analysis is cost-effective.

Different functional tests have been developed for years in human cells, yeasts or bacteria, and on whole proteins or specific domains by different strategies: cellular, biochemical, biophysical, etc... (GUIDUGLI et al., 2014; MILLOT et al., 2012).

1.3.6.2.1 Functional assays for BRCA1 missense variants

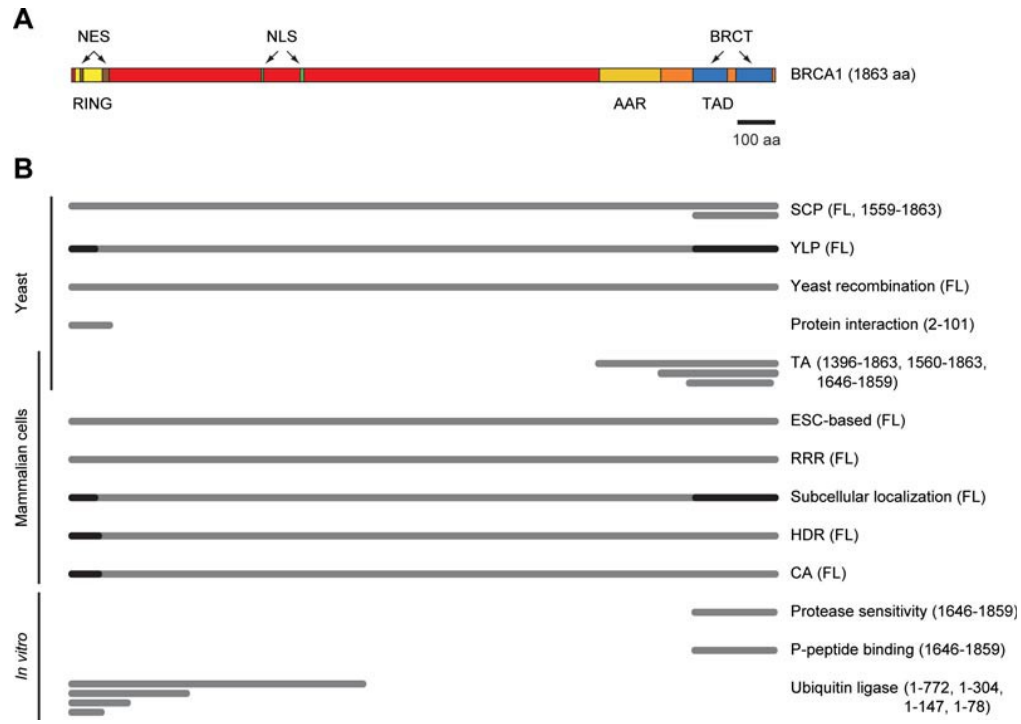

Figure 13: Examples of functional assays for BRCA1
(MILLOT et al., 2012)

Many functional assays are currently available for analysis of BRCA1 function. The main assay is the Homology-Directed Recombination (HDR) Assay. It has been described and performed by several teams in different cell lines in recent years. This assay assesses the ability of BRCA1 to perform HDR in the presence of a given VUS (PETITALOT et al., 2019; RANSBURGH et al., 2010; TOWLER et al., 2013). Many variants have been evaluated through this assay by Monteiro et al (CARVALHO et al., 2007; FERNANDES et al., 2019). Other functional tests are available, such as Ubiquitin Ligase Activity and Protein Interaction assay (UBcH5a/c, BARD1, BACH1, CtIP, Abraxas) (BRZOVIC et al., 2001; CLAPPERTON et al., 2004; MORRIS et al., 2006; NIKOLOPOULOS et al., 2007; PETITALOT et al., 2019; ROWLING; COOK; ITZHAKI, 2010), Protease Sensitivity Assay (WILLIAMS et al., 2003, 2004; WILLIAMS; GREEN; GLOVER, 2001), Phosphopeptide Binding Assays (BOTUYAN et al., 2004; CLAPPERTON et al., 2004; LEE et al., 2010; PETITALOT et al., 2019; SHIOZAKI et al., 2004; WILLIAMS et al., 2004; YU et al., 2003b), Small Colony Phenotype Assay (COYNE et al., 2004; MILLOT et al., 2011; MONTEIRO; HUMPHREY, 1998), Yeast Localization Phenotype Assay (MILLOT et al., 2011), Embryonic Stem Cell-Based Functional Assay (CHANG et al., 2009), Restoration of Radiation Resistance (SCULLY et al., 1999), Centrosome Amplification (KAIS et al., 2012, p. 20; STARITA et al., 2004), Yeast

Recombination Assay (CALIGO et al., 2009, p. 20019), and Subcellular Localization Assay (AU; HENDERSON, 2005; FENG et al., 2004; PETITALOT et al., 2019; RODRIGUEZ; AU; HENDERSON, 2004; WANG et al., 2010). Recently, the CRISPR/Cas9 technology has been used to systematically assess the functionality of BRCA1 VUS. However the authors focused on the RING and BRCT domains only (FINDLAY et al., 2018; STARITA et al., 2018).

1.3.6.2.2 Functional assays for BRCA2 missense variants

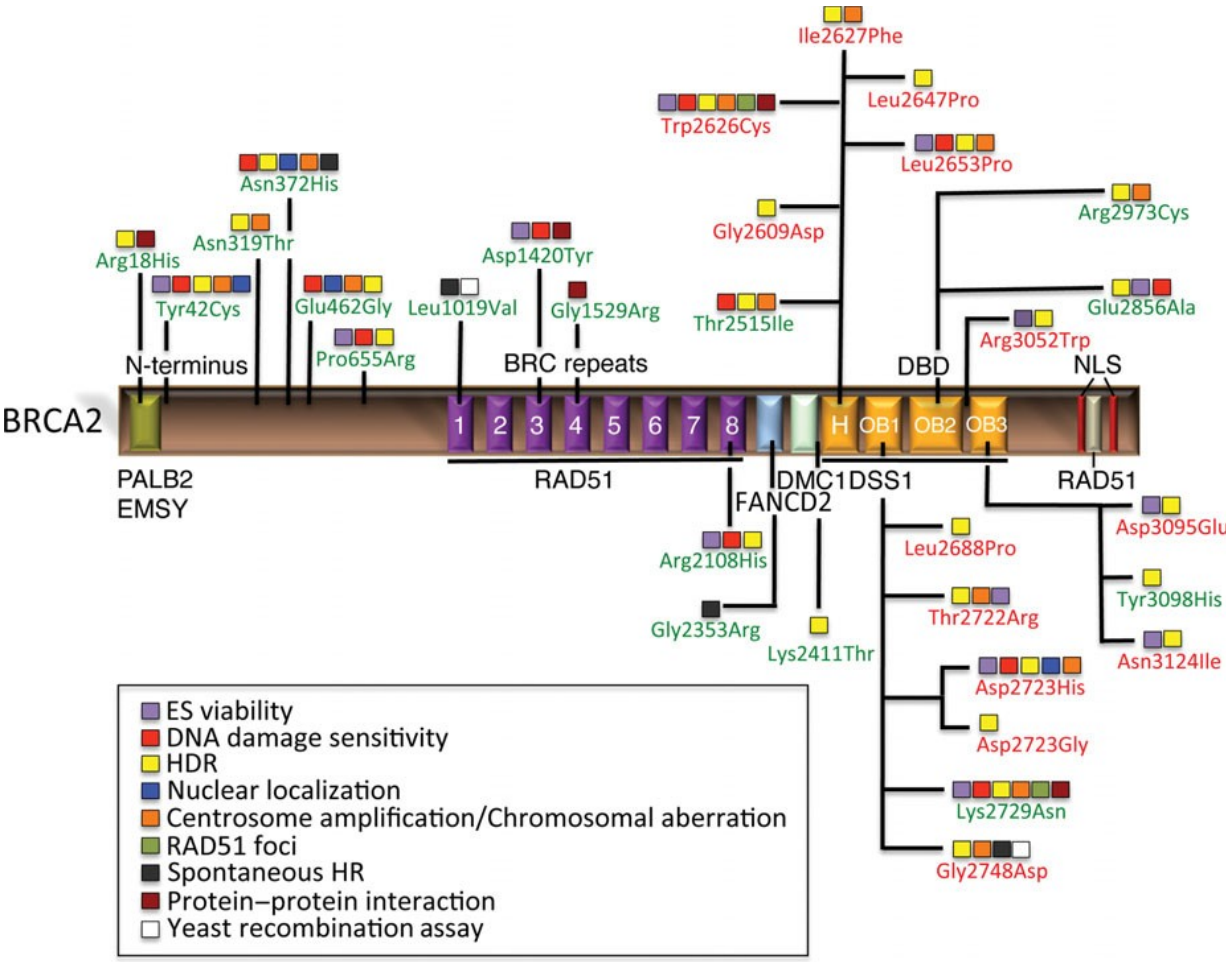

Figure 14: Examples of functional assays for BRCA2
(GUIDUGLI et al., 2014)

As for BRCA1, several functional assays are available for BRCA2. The most commonly used is also the HDR Assay. This test has been performed in different cell line: hamster (VC8, BRCA2-/-), yeast, and human cells (FARRUGIA et al., 2008; GUIDUGLI et al., 2013; MOYNAHAN; PIERCE; JASIN, 2001; SHIMELIS et al., 2017; WU et al., 2005). Another example is the Embryonic Stem Cell-Based Functional Assay which provides

information about the impact of the variant at the splicing and protein level (BISWAS et al., 2011, 2012; KUZNETSOV; LIU; SHARAN, 2008; MESMAN et al., 2019). Lastly, the Centrosome Amplification Assay (FARRUGIA et al., 2008; WU et al., 2005), mytomicin or anti-PARP Survival Assay (CAPUTO et al., 2018; WU et al., 2005), Syngeneic Human Cancer BRCA2 Knockout Cell LineModel (SyVal Model) (HUCL et al., 2008, p. 200), Nuclear Localization Assay (BISWAS et al., 2012; WU et al., 2005), BRCA2 Protein–Protein Interaction-Based Assays (BISWAS et al., 2012; SHIMELIS et al., 2017; VON NICOLAI et al., 2016b; XIA et al., 2006), and Phenotype in Heterozygous Carriers allows the evaluation of different functions of BRCA2.

To date, the results of a functional test are not sufficient to classify a VUS because the result obtained reflects only one of the functions of BRCA1 and BRCA2 proteins. However, they have multiple functions. New functional assays are still emerging. The most promising seems to be the essays using CRISPR/Cas9 technology (FINDLAY et al., 2018). This test includes BRCA1/2 regions to evaluate the impact of the variant on splicing and at the protein level. The results of functional tests may be integrated in the multifactorial model soon, but this has not yet been completed (IVERSEN et al., 2011; WOODS et al., 2016).

1.3.7 BRCA1/2 expression regulation and non-coding variants

1.3.7.1 Regulatory regions of *BRCA1* and *BRCA2* genes

BRCA1 and *BRCA2* expression are controlled at the transcriptional and post-transcriptional levels. The key transcriptional regulatory elements are housed in gene promoters, introns and long-range elements, while the key post-transcriptional control elements are predominantly located in 5’ and 3’ untranslated regions (UTRs). Both genes are expressed in a cell cycle regulated manner, with low levels of proteins being observed in G0 and early G1 phases before entry into S phase, and high levels are maintained through S and G2 phases of the cell cycle (MISRA et al., 2010; VAUGHN et al., 1996).

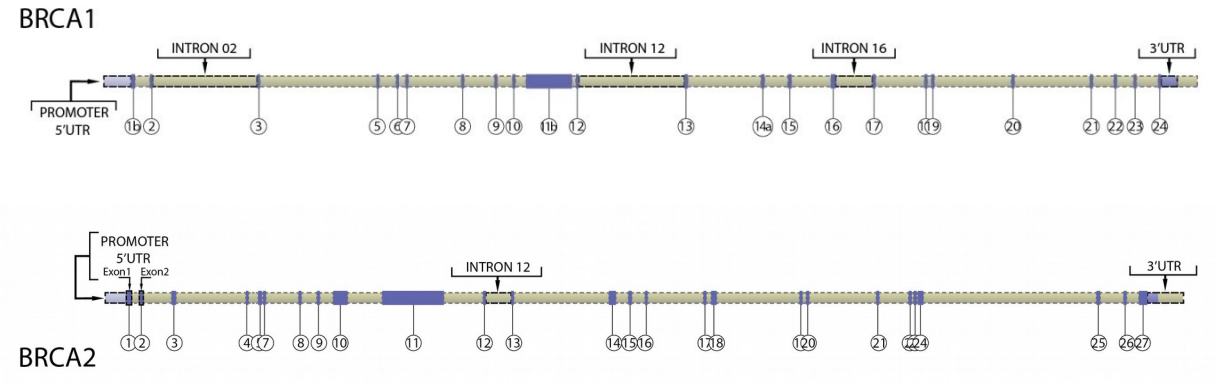

Figure 15 Non-coding regions of BRCA1⁄2 genes studied to date.

The core promoter of *BRCA1* includes the non-coding exon 1 and part of intron 1 of *BRCA1,* as well as the exon 1 and part of intron 1 of the neighboring gene *NBR2* (chr17:43,168,800-43,172,601). BRCA1 expression is complex with its transcription controlled by two different promoters, α and β, respectively located upstream from the alternative first exon 1A (121bp) and 1B (378bp). These two promoters encode 5'UTR-a and 5'UTR-b (XU et al., 1995; XU; CHAMBERS; SOLOMON, 1997), which share the same translation start codon (located in exon 2). These transcripts differ by the 5'UTR (exon 1) and are expressed in a tissue specific fashion: exon 1B is only expressed in breast cancer while exon 1A transcripts are present in both normal and tumor tissue. The maintenance of the correct ratio between the two transcripts has the potential to be important for normal regulation and function. *In vitro* studies show that this structural difference is related to a lower translation efficiency of 5'UTR-a in comparison with 5'UTR-b (SOBCZAK; KRZYZOSIAK, 2002).

The more efficient *BRCA1* promoter (α) consists of a region of 200 base pairs, upstream of the start site, which functions as a bidirectional transcriptional element able to direct expression in either the *BRCA1* or *NBR2* direction. There is some evidence to suggest that these two genes, separated by little more than 200bp, are reciprocally regulated and present divergent transcription (SUEN; TANG; GOSS, 2005). However, gene expression data from TCGA confirm the co-expression regulation for ovarian serous carcinomas but not in the breast cancer data set (CURTIS et al., 2012; NETWORK, 2011). BRCA1 promoter contains: RIBS element that acts as an activator and possesses multi subunit EtsGA-binding protein binding sites (ATLAS et al., 2000), CREB binding site that is a strong positive transcriptional

element (ATLAS; STRAMWASSER; MUELLER, 2001), CAAT box (XU; CHAMBERS; SOLOMON, 1997); and an E2F binding site (Wang et al.,2000). No estrogen responsive element (ERE) was identified in *BRCA1* promoter α, therefore the stimulation of BRCA1 expression by estrogen seems to result from an indirect effect of estrogen. In contrast, an ERE was described in *BRCA1* promoter β, so in this case, estrogen stimulation effect is due to estrogen bound to the DNA and subsequent interaction with the transcription machinery to stimulate transcription (NORRIS et al., 1995; XU; CHAMBERS; SOLOMON, 1997). In addition to promoter elements, upstream repressor elements were also described in regions upstream of the start of transcription and translation (SUEN; GOSS, 2001a).

There is limited information about regulatory elements outside of the *BRCA1* promoter. Suen and Goss localized a 36-bp repressor element in the first intron of *BRCA1* (SUEN; GOSS, 2001b). Wardrop and Brown subsequently described two evolutionarily conserved regions rich of TF binding sites in the second *BRCA1* intron that mediate both activation or repression of the *BRCA1* gene (WARDROP; BROWN; KCONFAB INVESTIGATORS, 2005a). The *BRCA1* 3' untranslated region (3'UTR) has been shown to be important for post-transcriptional regulation and exemplified by a variety of variants located there that negatively regulate mRNA translation, probably by disruption or creation of complementary MicroRNAs binding sites (BREWSTER et al., 2012; GARCIA et al., 2016; LHEUREUX et al., 2011; PONGSAVEE et al., 2009).

BRCA2 core promoter was first described four years after *BRCA2* gene cloning (DAVIS et al., 1999). It is located -66 to +129 from the transcriptional start site, and corresponds to a region rich in CG nucleotides and with several TF binding sites including E-box, Ets/E2F and SP1. *BRCA2* promoter is induced by NFkB and Elf1 (DAVIS et al., 1999; WU et al., 2000), and repressed byp53, PARP1, and SLUG (SHARAN et al., 1999; WANG et al., 2008; WU; JIANG; COUCH, 2003). Recently, functional studies based on micro deletions mapped other regulatory promoter regions with up and down-regulating elements (FRAILE-BETHENCOURT et al., 2018). As for *BRCA1*, it is expressed in a cell cycle regulated manner and the estrogen induction is also an indirect effect of mitogenic activity. Low protein levels are observed in G0 and early G1 phases while peak levels are reached in late G1, S and G2 phases of the cell cycle. Misra et al described the bi-directional activity of *BRCA2* promoter, similar to that of *BRCA1*. It was shown that the forward and reverse promoter activity regulates both *BRCA2* and *ZAR2* transcription, respectively. Interestingly, during G0 and G1 phase of the cell cycle, this promoter is 8-20 times more active in the reverse orientation

increasing the production of ZAR2 protein that binds to the promoter and silences BRCA2 expression. Whereas during the pre-division phases (S/G2), the forward activity is 5-8 times higher and the ZAR2 is trapped in the cytoplasm (MISRA et al., 2010). Nevertheless, TCGA gene expression data do not confirm this co-expression regulation in the breast cancer data set and no data is available for ovarian serous carcinomas (CURTIS et al., 2012; NETWORK, 2011).

Currently, little information is available on *BRCA2* non-coding regions is available. A few cis-acting intronic polymorphisms that alter the binding of transcription factors at regulatory sites have been described (MAIA et al., 2012) as well as one 3'UTR variant (*BRCA2* c.*172G4A) but with no clear evidence of pathogenicity (GARCIA et al., 2016).

1.3.7.2 Assay for assessing *BRCA1/2* variants in non-coding regions

1.3.7.2.1 Assays to measure gene expression and protein function (Functional Assays)

Variants can potentially affect normal pre-mRNA splicing and be deleterious either via disruption of consensus sequences, creation of de novo sequences, or alteration of splicing regulatory elements (SPURDLE et al., 2008b). Deep intronic variants can also impact splicing, such as altering the function of branch sites, although the significance and mechanisms of such events remain unclear (ANCZUKÓW et al., 2012; DUTIL et al., 2018).

Functional assays can evaluate the variant's impact on the ability of the protein to perform some key cellular functions, which in the case of non-coding variants may be related to deficient gene expression.

Luciferase reporter assay is a standard method to evaluate the impact of non-coding variants on gene expression. This assay consists of transfecting cells with a plasmid containing the luciferase gene under the control of DNA regulatory regions (promoter, enhancer and repressor) with and without the variant of interest. The comparison between luciferase activities of cells transfected with the variant-containing plasmid and cells transfected with the plasmid containing the wild-type sequence, allow for the determination of the variant impact on the biological function of regulatory regions. This assay is also used to evaluate 3'UTR functional regions on gene expression.

It is challenging to integrate calibrated functional assay data into multifactorial models since pathogenic mutations do not affect the functional endpoints in the same way. Another

issue is the low reproducibility between experiments, less prominent for variants with a greater effect. Plasmid DNA is placed in an artificial environment which may fail to reproduce the expression pattern of its endogenous equivalent due to differences on chromatin context. Regarding *BRCA1/2* non-coding variants, although Luciferase assay is the current standard, the ideal cutoff that abrogates the allele expression has yet to be determined. For Lynch syndrome it was suggested that 50% reduction of gene expression makes MMR function insufficient (HINRICHSEN et al., 2013).

1.3.7.2.2 Assays to investigate the underlying mechanism of variant impact

Transcription factors (TF) and microRNAs operate via base-paring interactions with DNA and mRNA, respectively. The majority of TF binding sites are located in promoter, enhancer and repressor elements (some of which overlap with the 5'UTR), while the majority of microRNAs binding sites are placed in 3'UTR. Some *in silico* tools are available to investigate if the variant can create or disrupt one of these. For this purpose, microRNA and TF binding site prediction software, ENCODE ChIP-sep data and information theory analysis can all provide clues that may be confirmed with *in vitro* experiments.

In vitro experiments are generally the next step to elucidate the underlying mechanism through which the variant can interfere. For 3'UTR variants, the correspondent miRNA vector (synthetic or plasmid) is co-transfected with the Luciferase *BRCA1/2* 3'UTR reporter, containing the variant or not. The results are then compared to determine if the variant has an impact. For promoter variants, several methods have been used for the characterization of protein-DNA interaction, including electrophoretic mobility shift assay (EMSA) (GARNER; REVZIN, 1981) and Chromatin immunoprecipitation assays (ChIP)(ORLANDO; STRUTT; PARO, 1997). EMSA is based on the principle that a protein-DNA complex migrates more slowly through an electrophoresis gel than the corresponding free DNA. Differences in binding patterns between the wild-type and mutant DNA sequences that labeled with a radioactive or luminescent tag, are indicative of TFs interacting with the DNA sequence in question. The candidate TF can then be identified by the use of an antibody against itself, using a 'supershift' assay. ChIP assays are an alternative method for directly visualizing an *in vivo* interaction between a specific protein and a regulatory element. After DNA cleavage by restriction enzymes, protein-DNA complexes are purified by immuno-precipitation with antibodies directed against the protein of interest. Then, to confirm that the protein was linked

to the TF binding site, the bound antibody is neutralized, proteins are digested and DNA is analyzed for the presence of the regulatory element by PCR. Interacting proteins can also be identified using mass spectrometry.

Finally, promoter methylation has been described as alternative mechanism of BRCA1 and BRCA2 silencing (VOS; VAN DIEST; MOELANS, 2018). This is another mechanism of disrupting transcriptional regulation, which can be evaluated through pyrosequencing or Next Generation Sequencing.

1.3.7.3 Impact of *BRCA1/2* non-coding alterations on breast and ovarian cancer predisposition

The incorporation of next generation sequencing analysis for germline tests has expanded the availability of information, including a greater number of sequence variants whose biologic impact remains unknown Bioinformatic analysis of the entire normal *BRCA1* and *BRCA2* genes have been performed to identify those non-coding regions most likely to be functional. This analysis has incorporated publically available data including population frequency (from dbSNP, 1000 genome, EVS, and case-control studies performed by ENIGMA groups), evolutionary conservation, and where relevant, transcription factor binding sites (predicted and actual [from ChIP-seq] from ENCODE) (Table 1). Recent data originating from HBOC population screening confirm the presence of variants in these regions. Some of these variants are functionally active, which reinforces their possible link with hereditary predisposition. But currently, except for some non-coding variants identified in intron and exon boundaries with impact on splicing, all the sequence alterations identified in *BRCA1/2* non-coding regions remain unclassified.

BRCA1 and *BRCA2* promoters of predisposed patients with no pathogenic variant identified have been screened in search for potential 5'UTR mechanisms of gene deregulation (BURKE et al., 2018; DOS SANTOS et al., 2017; FRAILE-BETHENCOURT et al., 2018). These studies led to the identification of some variants with an impact on transcriptional regulation. For some of these, the underlying mechanism of down regulation is related to disruption of interactions between transcription factors and their binding sites.

Promoter variants can also reduce gene expression through interference of CpG islands and consequent methylation-associated epigenetic silencing of the correspondent allele. Recently, this mechanism was described in two families carrying a *BRCA1* promoter variant (c.-107A>T). RNA sequencing revealed that the heterozygous variant segregated with the

hypermethylated *BRCA1* allele, resulting in the allelic loss of *BRCA1* expression (EVANS et al., 2018). Similar to Lynch syndrome (HITCHINS et al., 2007; WARD et al., 2013), this example raises the question of whether constitutional *BRCA1/2* epimutations can represent an alternative mechanism for cancer predisposition.

Region of Interest	Hg19 Coordinates	Length	Comments
BRCA1 promoter	chr17: 41,277,500–41,278,500	1000 bases	Comprises 1 kb upstream on transcription start site
BRCA1 5′UTR (exon 1A)	chr17: 41,277,287–41,277,500	223 bases	Exon 1A
BRCA1 5′UTR (exon 1B)	chr17: 41,277,340–41,277,197	145 bases	Exon 1B
BRCA1 5′UTR + ATG (exon 2 to ATG)	chr17: 41,276,110–41,276,133	22 bases	5′ end of Exon 2
BRCA1 intron 2	chr17: 41,271,250–41,272,100	850 bases	Includes validated enhancer and repressor elements that participate in gene looping and are conserved. Also contains sequences that UCSC/ENCODE indicates this region contains transcription factor binding sites, DnaseHS sites
BRCA1 intron 12 (region 1)	chr17: 41,237,500–41,237,850	350 bases	UCSC/ENCODE indicates this region contains transcription factor binding sites, DnaseHS sites and is conserved.
BRCA1 intron 12 (region 2)	chr17: 41,236,600–41,236,960	360 bases	UCSC/ENCODE indicates this region contains transcription factor binding sites, DnaseHS sites and is conserved.
BRCA1 intron 16	Chr17: 41,220,900–41,221,250	350 bases	UCSC/ENCODE indicates this region contains transcription factor binding sites, DnaseHS sites
BRCA1 3′UTR (exon 24)	chr17: 41,196,311–41,197,698	1387 bases	From and including stop codon
BRCA2 promoter	chr13: 32,888,616–32,889,616	1000 bases	Comprises 1 kb upstream on transcription start site
BRCA2 5′UTR (exon 1)	chr13: 32,889,616–32,889,805	189 bases	Exon 1 (Refseq)
BRCA2 5′UTR (exon 2 to ATG)	chr13: 32,890,558–32,890,600	42 bases	Includes translation start codon
BRCA2 3′UTR	chr13: 32,972,904–32,973,809	905 bases	From and including stop codon

Table 4: Priority regions of *BRCA1/2* genes for screening

A 5'UTR variant may also impact translation efficiency by interfering in the consensus motif for the start of protein translation. Wang et al. described a variant located 2 bases downstream *BRCA1* start codon that reduced the protein expression in this way. In the presence of the 5'UTR variant (+118A>T, c.-2A>T), luciferase activity was significantly reduced compared to the wild type, while transcription efficiency and mRNA stability were assured by equal mRNA levels. Immuno-histochemical staining of the tumor could confirm the reduced expression of *BRCA1* protein for the variant carriers. Signori et al also described a variant at position -3 from the *BRCA1*start codon associated with a significant decrease in mRNA translation through the same mechanism (SIGNORI et al., 2001).

Germline variants have been described in the 3'UTR region of the *BRCA1/2* genes, some of them with a proven impact on gene expression (MOGILYANSKY et al., 2016a; PONGSAVEE et al., 2009; SAUNUS et al., 2008a). MicroRNA is small non-coding RNA which negatively regulates mRNA translation by recognizing complementary sites, most located in this region. They can induce mRNA degradation or inhibit their translation resulting in gene down regulation. 3'UTR variants can disrupt pre-existing or create new cis-regulatory elements or binding sites for trans-acting RNA binding proteins or micro-RNAs.

However, there still exists a paucity of data on *BRCA1/2* 3'UTR regions. Brewster et al carried-out a screening of *BRCA1* 3'UTR in a large population of breast cancer cases *BRCA*-mutation negative. This study put in evidence 15 novel *BRCA1* 3'UTR variants, one of them (c.*1340_1342delTGT) related to the creation of a new microRNA binding site: miR-103. Another 3'UTR screening of 716 index cases negative for *BRCA1/2* pathogenic mutations also detected SNPs and 6 rare variants in these region, 3 of which are novel (GARCIA et al., 2016).

Although intronic data is even scarcer, a few intronic variants have been described in the intron 2 of *BRCA1* (c.81-3980A>G), which were able to revert the enhancing impact of these regions over *BRCA1* promoter activity. Although these regions are situated several kilobases downstream of the promoter region, it is hypothesized that they regulate *BRCA1* expression at the transcriptional level, most likely via gene looping (DOS SANTOS et al., 2017; WARDROP; BROWN; KCONFAB INVESTIGATORS, 2005b).

Currently, it is difficult to predict the risk attributed to the presence of these variants, given the scarcity of data and the fact that they could have impact in different steps of gene expression. However, contrary to coding mutation, they may not impact protein function. Non-coding variants are expected to have more subtle quantitative effects and may probably be associated with a lower but still important impact on cancer risk. This would impacts on the relative risk.

However, there is currently no formal recommendation for classifying *BRCA1/2* non-coding variant carriers, nor guidelines for managing patients carrying these variants. As stated previously, except for some variants located in the intron/exon transition that impact on splicing, the significance of nearly all variants identified in *BRCA1/2* non coding regions remains uncertain. These sequence changes do not clearly affect the protein, but they do cause subtle changes which are difficult to interpret. As a quantitative effect is expected, it is a great challenge to define a threshold that classifies the variant as causal or to determine their significance and contribution in breast/ovarian cancer susceptibility. Thus, it is still difficult to reach accurate conclusions useful for genetic counseling.

The last American College of Medical Genetics guideline provides no specific recommendation for reporting and classification of variants identified in *BRCA1/2* promoters, intronic and untranslated regions (RICHARDS et al., 2015).To date, as well as for missense unclassified variants, carriers should be managed exclusively based on their personal and family history which allows for the estimation of cancer risk.

1.3.8 HR deficiency beyond *BRCA1/2* pathogenic variants

1.3.8.1 HR genes and cancer predisposition

HBOC families with unsolved molecular mechanism of predisposition remain some of the most challenging in oncogenetic clinics. In the last years, the introduction of multigene panel sequencing generated an accumulation of data about germline and somatic pathogenic variants (PV) in HR genes beyond *BRCA1* and *BRCA2*. However, precise risk estimate are underway for most genes. Some of them have have proven association with breast and ovarian cancer predisposition as moderate (with a relative risk of two-to-five fold) or high penetrance gene (with a relative risk of eight-fold). However, the conclusion from different studies regarding the magnitude of the risk is often contradictory.

Couch and colleagues performed a case-control study that included 65.000 breast cancer patients to estimate the risk of pathogenic variants in non-*BRCA1/2* predisposition genes (after exclusion of syndromic breast cancer genes such as *CDH1*, *PTEN* and *TP53*). This study confirmed that 5 out of 16 HR genes were associated with moderate-high increased risk of breast cancer: *ATM* (OR 2.78; 95% IC 2.22-3.62), *BARD1* (OR 2.16; 95% IC 1.31-3.63), *CHEK2* (OR 1.48; 95% IC 1.31-1.67), *PALB2* (OR 7.46; 95% IC 5.12-11.19), and *RAD51D* (OR 3.07; 95%IC 1.21-7.88). In contrast, mutations in *BRIP1*, *RAD51C*, *MRE11A*, *RAD50*, *NBN-MRN* complex, *MLH1* and *PMS2* mismatch repair genes, and *NF1* were not associated with breast cancer risk (COUCH et al., 2017).

Gene	Ambry Genetics Inc Cases			ExAC Controls			Cancer Risk	
	Mutated Alleles, No.	Cases, No.	Mutation Frequency, %	Mutated Alleles, No.	Individuals, No.	Mutation Frequency, %	OR (95% CI)	*P* Value
ATM	274	29 229	0.94	90	26 644	0.34	2.78 (2.22-3.62)	2.42×10^{-19}
BARD1	52	28 536	0.18	22	26 078	0.08	2.16 (1.31-3.63)	2.26×10^{-3}
BRIP1	71	28 536	0.25	41	26 840	0.15	1.63 (1.11-2.41)	.01
CDKN2A	6	8457	0.07	7	24 312	0.03	2.47 (0.83-8.16)	.11
CHEK2	424	29 090	1.46	163	25 215	0.65	2.26 (1.89-2.72)	1.75×10^{-20}
CHEK2 1100delC	338	29 090	1.16	127	25 215	0.50	2.31 (1.88-2.85)	3.04×10^{-17}
*CHEK2**	721	29 090	2.48	424	25 215	1.68	1.48 (1.31-1.67)	1.11×10^{-10}
MLH1	4	15 475	0.03	6	26 639	0.02	1.15 (0.30-4.19)	>.99
MRE11A	21	28 536	0.07	23	26 767	0.09	0.86 (0.46-1.57)	.65
MSH2	9	15 475	0.06	6	25 329	0.02	2.46 (0.81-6.93)	.11
MSH6	32	15 475	0.21	28	26 151	0.11	1.93 (1.16-3.27)	.01
NBN	48	28 536	0.17	39	26 264	0.15	1.13 (0.73-1.75)	.59
NF1	27	25 950	0.10	29	26 130	0.11	0.94 (0.55-1.62)	.89
PALB2	241	30 025	0.80	29	26 869	0.11	7.46 (5.12-11.19)	4.31×10^{-38}
PMS2	17	15 475	0.11	33	24 674	0.13	0.82 (0.44-1.47)	.56
RAD50	45	28 536	0.16	54	26 474	0.20	0.77 (0.52-1.61)	.23
RAD51C	26	28 536	0.09	31	26 647	0.12	0.78 (0.47-1.37)	.43
RAD51D	18	25 950	0.07	6	26 555	0.02	3.07 (1.21-7.88)	.01

Abbreviations: ExAC, Exome Aggregation Consortium; OR, odds ratio.

CHEK2*: Inclusion of common missense variants p.Ile157Thr and p.Ser428Phe.

Figure 16 Breast cancer mutation frequency and relative estimation for HR gene mutations beyond *BRCA1/2* Adapted from Couch et al 2017 (COUCH et al., 2017)

More recently, LaDuca and colleagues performed an even larger case-control study to estimate frequency and cancer risk association of 32 cancer predisposition genes in 165,000 individuals referred for multigene panel genetic testing in the United States. In partial agreement with Couch's work, *ATM*, *BARD1*, *CHEK2*, *PALB2*, and *RAD51D* demonstrated statistically significant association with breast cancer, with similar hazard ratios. However, in this work, other genes were associated with increased breast cancer risk less than twofold (*BRIP1, MSH6, NBN*, and *RAD51C*). In addition, authors could demonstrate that pathogenic variants in nine of these genes with elevated breast cancer risk were also associated with increased risk for ovarian cancer (*BRCA1/2, ATM, BRIP1, RAD51C/D, NBN, TP53*, and *MSH6*), along with *MSH2* and *PMS2*. Odds ratios for ovarian cancer across these 11 genes ranged from 1.91 for *ATM* to 13.8 for *BRCA1*. Pathogenic variants in *BRCA2*, *PALB2*, and *ATM* were significantly associated with increased risk for pancreatic cancer (LADUCA et al., 2019). It is noteworthy that this study was performed on patients refered for genetic testing but 5,5% of patients with pathogenic variants identified in BRCA1⁄2 genes did not meet criterias for testing.

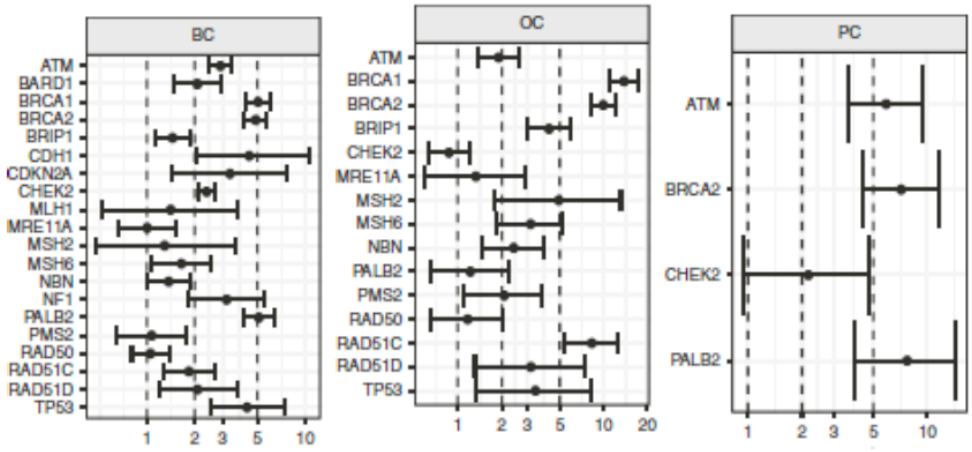

Figure 17 Gene estimation cancer risk derived from a case-control study in 165,000 individuals refered for multigene panel genetic testing.
Adapted from (LADUCA et al., 2019)).

1.3.8.2 HR genes and response to treatment: Targetable HR genes

Considering that HR is a multigene pathway of DNA repair, mutations in HR genes beyond *BRCA1/2* should explain the HRD phenotype of some tumor cells despite being *BRCA1/2* wild-type. Evidence of genomic scar of HRD can be searched through different genomic scores and functional assays. In general, different trials show that a high HRD scores is predictive of a better response to PARPi, showing that the benefit can be extended to HRD carcinomas BRCA wild-type. However, the value of identifying an HR gene mutation itself is not currently clear. A recent study performed on 17,566 sporadic tumors showed an overall frequency of somatic HR gene mutations of 17.4% across all types of cancers. Endometrial (34%), biliary tract (29%), and bladder cancers (24%) harbored the most elevated rates and *ARID1A (7.2%*) followed by *BRCA2* (3%), *BRCA2* (2.8%) and *ATM* (1.3%) were the most commonly mutated genes. Specifically for breast and ovarian cancer, the frequency of HR gene mutations was 17% and 20% respectively (Heek et al, 2018). In the TCGA of HGSOC 26% of tumors presented HR genetic or epigenetic alterations in genes other than *BRCA1/2*, including *RAD51C* promoter methylation (3%), pathogenic variants of ATM/ATR (2%), EMSY (8%) and other genes (5%).

Clinical trials	Phase	Type of cancer	Type of drugs	N of BRCA wild patients/HR deficient pathway
NCT01891344 (ARIEL2)	II	relapsed platinum sensitive HGOC	Rucaparib	154/20
NCT01682772 (TOPARP A)	II	mCRPC	Olaparib	39/9
NCT01682772 (TOPARP B)	II	mCRPC	Olaparib	66/66
NCT02952534 (TRITON2)	II	mCRPC	Rucaparib	21/21*
NCT 03140670	II	platinum-sensitive advanced PC	Rucaparib	2/2
NCT02401347	II	HER2-negative breast cancer or other solid tumors	Talazoparib	19/19
NCT01968213 (ARIEL3)	III	recurrent platinum sensitive OC	Rucaparib	368/43
NCT00753545 (Study 19)	II	recurrent platinum sensitive OC	Olaparib	118/21
NCT01847274 (ENGOT-OV16/NOVA)	III	recurrent platinum sensitive OC	Niraparib	249/115†
PROfound	III	mCRPC	Olaparib	228/228

Table 5: Clinical trials that evaluated PARPi efficacy in tumors with HR mutations beyond *BRCA1/2*[2]

Tumoral sequencing has revealed that ATM is among the most mutated genes in sporadic cancers. They occur in many tumor types, but are more often found in hematologic malignancies, prostate (8%), pancreatic (8%) and lung adenocarcinomas. (9%) (BIANKIN et al., 2012; FORBES et al., 2017; THE CANCER GENOME ATLAS RESEARCH NETWORK, 2014). In breast cancers, point somatic mutation is identified in about 5% of incidence, but copy number variation is more commonly found (46%) (FORBES et al., 2017). Ovarian cancers present ATM mutations in 1-5% of the cases. For PALB2, the prevalence of PV is 0.1 %, 0.6%, 0.2%, and 0.3% in breast, ovarian, prostate, and pancreatic cancer, respectively (CERAMI et al., 2012; GAO et al., 2013). Somatic PV have been reported with a frequency of: 0.1% and 0.9% in breast and ovarian cancers, respectively, for RAD51B; of 0.2 %, 1.3%, 1.3% in breast, prostate, and pancreatic cancers respectively for RAD51C; and of

[2] DSB: double stranded breaks; HRR: homologous recombination repair; RPA: replication protein A; SSB: single stranded break; HGOC: high grade ovarian cancer; PC: pancreatic cancer. *forty patients were included but 21 were evaluable for response †the study defined the 115 patients based on the HRD score

0.9 % and 0.6% in ovarian and prostate cancers respectively for RAD51D (CERAMI et al., 2012; GAO et al., 2013). *BRIP1* Somatic PV have been reported in 0.3 % of breast, ovarian and prostate cancer (CANTOR; XIE, 2010, p. 201; CERAMI et al., 2012; GAO et al., 2013). BARD1Somatic alterations have been reported in 0.2 % and 0.6% in breast and prostate cancers respectively (CERAMI et al., 2012; GAO et al., 2013). Somatic pathogenic variants in CHEK1 have been reported in 0.1 %, 1.3% and 0.9% of breast, ovarian, and prostate cancers respectively, while pathogenic variants affecting CHEK2 have been described in 0.3 %, 0.6%, and 1.3% in breast, ovarian, and prostate cancers respectively (CERAMI et al., 2012; GAO et al., 2013)

Most likely, mutations in genes encoding proteins with a more proeminent role in HR pathway will have greater impact. Currently, multiple studies are under way in addressing this question. There is still little data available and the results from individual studies in terms off long-term benefit are inconsistent. Currently, HR genes mutations beyond *BRCA1/2* are not taken into consideration in clinical practice. The rationale was reinforced in preclinical studies, but although some results from clinical trial are already available, it is still too early to draw a conclusion (Table 2).

ARIEL2 (NCT01891344 phase II trial) (SWISHER et al., 2017b) evaluated the efficacy of rucaparib in relapsed platinum sensitive high-grade ovarian cancer. Among the 154 included patients with somatic wtBRCA 20 harbored pathogenic variants in HR genes (2 *ATM*, 2 *BRIP1*, *2 CHEK2, 1 FANCA, 1 FANCI, 2 FANCM, 2 NBN, 1 RAD51B, 4 RAD51C, 2 RAD51D and 1 RAD54L*) and were evaluable for response. Overall response rate (ORR) and disease control rate (DCR) in this subgroup were equal to 21% and 89.5 % respectively. Survival data (overall survival-OS and progression free survival-PFS) were not available in the published report. It should be noted that the ORR in the subgroup of patients with mutated BRCA was equal to 80% (SWISHER et al., 2017b).

TOPARP (NCT01682772 phase II trial) (MATEO et al., 2019) evaluated the efficacy of olaparib in patients with metastatic castration-resistant prostate cancer (mCRPC). Phase A and B included unselected and selected patients, respectively, for likely pathogenic variants in HR genes. Among 39 wtBRCA patients included in phase A, 9 had HR-genes pathogenic variants (*6 ATM, 1 FANCA/CHEK2, 1 PALB2,* and *1 RAD51)* among whom 5 (55%) responded to olaparib. Survival data was not reported (MATEO et al., 2015b). The phase B included 66 wtBRCA patients harboring the following HR-gene pathogenic variants: *7 PALB2, 19 ATM, 20 CDK1*2, and 20 patients with variants in other HR genes. ORR was 57%

for *PALB2* subgroup (median PFS 5.3 months), 37% for *ATM* (mPFS 6.1 months), 25% for *CDK12* (mPFS 2.9 months), and 20% among the last subgroup (mPFS 2.8 months**).** In comparison, the ORR in *BRCA*-mutated patients was equal to 80% with a median PFS of 8.1 months.

TRITON2 (NCT02952534 phase II trial) (ABIDA et al., [s.d.]) evaluated the efficacy of rucaparib in mCRPC. Of the 40 wtBRCA patients harboring HR genes pathogenic variants, 21 were evaluable for response (5 *ATM*, 8 *CDK12*, and 8 in other genes). Two patients (1 with *BRIP* and 1 with *FANCA*) presented partial response and 5 patients presented stable disease as their best response (DCR 87.5%). Although no patient in both *ATM* and *CDK12* subgroups had partial or complete response to rucaparib, DCR was equal to 80% and 62.5% respectively. Survival data were not reported. It should be noted that ORR and DCR in patients having *BRCA* pathogenic variants were equal to 44% and 80% respectively.

NCT 03140670 (phase II trial) (BINDER et al., 2019) evaluated the efficacy of rucaparib in patients with platinum-sensitive advanced pancreatic cancer with a pathogenic germline or somatic variant in *BRCA1, BRCA2* or *PALB2*. Among 19 patients evaluable for PFS at the time of interim analysis, both patients harboring only *PALB2* germline pathogenic variants responded to treatment. No further information was available.

NCT02401347 (phase II trial) (GRUBER et al., 2019) evaluated the efficacy of talazoparib in wtBRCA with advanced HER2-negative breast cancer or other solid tumors harboring pathogenic variants in HR genes. The study enrolled 12 breast cancer patients and 7 patients with other solid tumors (including pancreatic cancer) evaluable for response. In the former group, 3 had a response (2 *PALB2*, 1 *CHEK2*/*FANCA/PTEN*) and 3 had SD ≥ 6 months (1 *PALB2*, 1 *ATR*, 1 *PTEN*). Thus, the ORR and CBR were equal to 25% and 50% respectively. Survival data were not reported.

ARIEL3 (NCT01968213 - randomized controlled double-blind phase III trial) (COLEMAN et al., 2017c; O'MALLEY et al., 2018) evaluated the response to rucaparib in patients with recurrent platinum sensitive ovarian cancer. Forty-three wtBRCA patients harboring pathogenic variants in HR genes were randomized 2:1 to receive rucaparib (n=28) or placebo (n=15). Among the patients in the former group, *RAD51C* (n=6), *RAD51D* (n=4), and *RAD54L* (n=3) were the communly altered genes, followed by ATM (n=2), *ATR* (n=2), *CHECK2* (n=2), *FANCD2* (n=2), *RAD50* (n=2), *FANCL* (n=2), *BARD1* (n=1), *FANCI* (n=1), and *FANCM* (n=1). Hazard ratio favored rucaparib with nearly 80% reduction of progression risk compared to placebo (HR 0.21 p=0.005) and the median PFS doubled in the group

receiving PARPi (11.1 months versus placebo 5.5 months). It is noteworthy that the risk reduction was similar to that found in the subgroup of patients with BRCA pathogenic variants (HR: 0.20 p <0.001).

Study 19 (NCT00753545 - phase II trial) (HODGSON et al., 2018; LEDERMANN et al., 2016) evaluated the efficacy of olaparib as maintenance treatment for platinum sensitive recurrent ovarian cancer. Twenty-one out of 118 wtBRCA patients had pathogenic variants in HR genes (*5 BRIP1, 3 CDK12, 3 RAD54L, 2 RAD51B, 1 ATM, 1 FANCA, 1 FANCD2, 1 FANCI, 1FANCL, 1 RAD51C, 1 RAD52,* and *1 XRCC3*). In this subgroup, the magnitude of risk reduction of progression with olaparib corresponded to approximately 80% (HR: 0.21, p value= 0.03) and was similar to that seen in patients with BRCA pathogenic variants (HR: 0.18 p < 0.001). On the other hand, olaparib did not add any statistically significant benefit in patients with wtBRCA/wtHR-genes. Individual data was not reported.

ENGOT-OV16/NOVA (NCT01847274 - randomized controlled double-blind phase III trial) (MIRZA et al., 2016a) evaluated the efficacy of niraparib versus placebo as maintenance for patients with platinum-sensitive, recurrent ovarian cancer. The authors performed a retrospective exploratory analysis of 331 patients enrolled in the NOVA cohort germline wtBRCA. The results showed an added benefit of niraparib over placebo in all subgroups with an HR of 0.27, 0.31 and 0.49 in patients with somatic BRCA pathogenic variants, somatic wild-type BRCA/HR-genes pathogenic variants, and wtBRCA/wtHR-genes respectively. Neither individual data nor gene level analysis were reported.

PROfound (NCT02987543 -open label randomized controlled phase III trial) (FIZAZI et al., 2019) evaluated the efficacy of olaparib versus physician's choice (enzalutamide or abiraterone) in castration-resistant metastatic prostate cancer harboring HR gene alterations. The study a cohort A with *ATM, BRCA1*, or *BRCA2* mutation and a cohort B with mutations in other 12 HR genes (*CDK12, CHEK2, PPP2R2A, PALB2, BRIP1, RAD54L, BARD1, RAD51B, RAD51D, CHEK1, FANCL,* and *RAD51C*). The PFS benefit of olaparib was confirmed in both cohorts with an HR of 0.34 (p<0.0001) and 0.44 (p=0.0192) in cohort A and B respectively. Despite the high rate of cross-over (80% of patients in the antiandrogen arm finally received olaparib), the interim analysis had a favorable trend in OS for olaparib arm considering the entire population. As for gene-level exploratory analysis, 89 patients harbored *CDK12* pathogenic variants (61 olaparib versus 28 control), 86 *ATM* (62 versus 24), 12 *CHEK2* (7 versus 5), 5 *RAD51B* (4 versus 1) and 5 *RAD54L* (3 versus 2). The median

radiologic PFS was equal to 5 for olaparib versus 2.2 months for the control in *CDK12* subgroup, 5.4 versus 4.7 for *ATM*, 5.5 versus 3.4 in *CHEK2*, 10.7 vs 1.8 for *RAD51B*, and 7.2 vs 2.4 for *RAD54L*.

2 THESIS OBJECTIVES

Ovarian and breast cancers are now defined by the main pathways involved in the tumorigenesis. Dysfunction in DNA repair by homologous recombination plays a major role in some subtypes of these cancers. In hereditary breast and ovarian cancer (HBOC) predisposition, tumors with *BRCA1/2* pathogenic variants present an impairment of this reparation pathway which is facilitated by the pre-existing germline mutation. For many years after the discovery of the *BRCA1* and *BRCA2* genes, variants were only searched on germline DNA. With the technical improvement (e.g. arrival of the NGS, as mentioned before) and with the establishment of *BRCA1/2* pathogenic variants as a target for treatment, laboratories have developed screening for *BRCA1/2* genes on tumors, increasing also the number of tumoral data. Even so, the reason of the inactivation of this pathway remains uncertain sometimes.

In the context of HBOC syndrome, most medical genetics laboratories currently perform germline sequencing through gene panels with a restricted number of genes, and generally limited to defined coding regions within these genes with regards to the medical management of these results. Although the number of screened genes has increased from 2 to nearly 100 genes in some panels, there are still many families whose HBOC predisposition mechanism remains unexplained and with a missing heritability. Currently, in screened HBOC cases, ~10% have pathogenic variants (~15% if the other genes are also studied) and ~10% of patients present VUS. One major limit in this diagnosis, and consequently in the management of patients, is the detection of an increasing number of nucleotide variants of unknown biological/clinical significance (VUS). VUS remains unusable in patient and family management care. Tools for classification are more and more important in a context of a production of massive genomic information. But what about the remaining 75-80% of families with a diagnosis of HBOC?

Concerning response to PARP inhitors, a group of patients harboring *BRCA1/2* pathogenic variants are particularly sensitive. However, many patients seem to be good responders even without any *BRCA1/2* pathogenic variants. They constitute a missing predictive biomarker group. Genetics labortories are also involved in the extension of the panel and signature analysis to understand this sensitivity background.

In order to try to make progress on this point, the main objective of this thesis is to identify alternative mechanisms of homologous recombination (HR) pathway inactivation beyond BRCA1/2 coding and premature stop pathogenic variants, to optimize both cancer predisposition management and therapeutic response. The purpose of identifying such mechanisms is to improve genetic counseling and to broaden the population that benefits from target therapies, known to be more effective in HRD tumors.

Regardless of the different types of genetic panels available, there has been minimal exploration of non-coding regions. These regions represent 98% of the human genome and exploring them is a project in itself, with limitations when compared to the study of the coding regions because all the uncontrollable gene regulation mechanisms that come into play. This study began with the screening of non-coding regions in non-*BRCA1/2* patients to demonstrate that variants in these regions really existed. After confirming their presence, the next step aimed to understand which variants had a potential impact, and there again a new problem arose. The frequency databases in the control populations either poorly referenced or did not reference at all the non-coding variants. As a result, we could not easily discriminate between a polymorphism and VUS. A second problem was with *in silico* predictive software, since it is largely developed to evaluate variants only located in the coding regions or intron/exon junctions. The support of the international consortium ENIGMA allowed us to meet people who could help us on this point. Once the variants were obtained and selected, new functional tests were investigated because the majority of the functional tests were developed for missense variants. Once the various problems were solved, 20 non-coding *BRCA1* and *BRCA2* variants were evaluated. This part is discussed in an article published by Santana E et al ***"Assessment of the functional impact of germline BRCA1/2 variants located in non-coding regions in families with breast and/or ovarian cancer predisposition."***in Breast Cancer and research treatment.

During *BRCA1/2* genes screening, beyond the thousands of variants that are already identified, many new variants are continually being identified. The following classification strategies were implemented: screening of the control population, development of *in silico* prediction and functional tests, and co-segregation in families (in particular by national and international consortia). Since more and more information is being accumulated with tumor sequencing, it will be important to consider tumor information. Currently, this information is underutilized. In 2014, a multi-institutional study established the likelihood-ratio (LR) pathology, which has been integrated in the multifactorial model. However, LOH information

is not used for variant interpretation. This could help to classify missense variants since they are rare, and therefore with limited information to interpret them. This part is discussed in the second article ***"Contribution of the loss of heterozygosity to BRCA1 variant classification"*** that will soon be submitted.

Finally, the main question of this thesis was adressed by analyzing the therapeutic response. Neoadjuvant chemotherapy (NAC) followed by interval debulking does not present inferior results to those of primary cytoreduction and offers the opportunity to evaluate chemo-sensitivity in vivo. Chemotherapy response scores (CRS) have been shown to correlate with outcome with a complete (or near complete) (CRS3) response predicting improved progression-free survival. The recruitment specifically in these responders could bring us an increase in the yield of identification of new mechanisms of inactivation. As before with large familial cosegregation, the therapeutic strategy developed on tumor material will be applied to the screeeing on the HR pathway in non-coding regions *BRCA1/2* genes and other coding HR genes. This part is discussed in the last article ***"Mutation analysis of ovarian carcinoma patients presenting optimal response to neoadjuvant chemotherapy"*** in preparation.

This thesis is presented in three main parts: parts one and two contribute to the advance of non-coding and missense *BRCA1/2* variant classification in cancer predisposition and the last part focuses on the exploration of new biomarkers of therapeutic response to DNA damage agents beyond *BRCA1/2* coding mutations.

3 RESULTS

3.1 Non-coding regions

3.1.1 Background

3.1.1.1 Non coding regions and regulatory impact

Current technological sequencing advancements and development of bioinformatics tools have enabled the exploration and elucidation of the genome structure and non-coding DNA regions. The description of the functional elements of human genome by the encyclopedia of DNA elements provided a better understanding of the human genome expression regulation and how regulatory data is encoded. This effort demonstrated that most of the human genome is involved in gene expression regulation, while the small minority of the nucleotides (1.2%) encodes proteins within humans. The ENCODE project has also described thousands of regulatory active regions and showed that 90% of common variants fall outside the coding regions of the genes (ENCODE PROJECT CONSORTIUM, 2012). Nevertheless, the majority of the studies to date have focused on the coding regions of the cancer associated genes.

3.1.1.2 Germline cancer-associated variants in the regulatory regions

Until recently, attention had been focused on coding regions of the genes associated with cancer risk. Exome sequencing of human genome and co-segregation studies have made evident that lots of disease-associated variants play a role in hereditary susceptibility. Since coding changes do not explain all of the predisposition cases, the importance of the non-coding regions (including promoters, introns, intergenic sequences and non-coding RNAs) in biological functions and hereditary predisposition must be considered.

Gathered evidence indicates that genetic variants in the non-coding but functional elements can contribute to the development of hereditary cancers. The presence of variants in these regions can impact gene transcription by the creation or disruption of transcription

factors binding sites, or by interfering with CpG island methylation which leads to an aberrant methylation pattern. In addition, variants may have an impact at the post-transcriptional level, creating or disrupting microRNA 3' complementary binding sites in 3'UTRs and interfering with the stability of RNAs and microRNAs. Moreover, the elucidation of 3D chromatin structure reveals a complex network of interaction between the regulatory regions of the genome which includes long-range interactions between functionally coordinated domains lying hundreds of kilobases upstream or downstream of their target (HEIDARI et al., 2014; HUGHES et al., 2014). Therefore, non-coding sequence alterations may also influence this model of regulation.

There is increasing data associating germline non-coding variants with cancer risk. Additionally, most cancer-associated single nucleotide variants (SNVs) identified through genome-wide association studies are located in non-coding regions, with some of them having a proven role in gene expression regulation (STACEY et al., 2007; ZHANG; LUPSKI, 2015). Two examples: (i) a germline variant in the promoter of *TERT* (telomerase reverse transcriptase) gene (c.-57T>G) significantly increased promoter activity. This variant co-segregated with cancer in a family with 14 melanoma cases who were not carriers of germline mutations in the two known melanoma genes, *CDKN2A* and *CDK4* (HORN et al., 2013). The variant increases *TERT* expression probably by the creation of a new binding site for Ets, Elk1, and Elk4 transcription factors. The increase of *TERT* expression is a fundamental requirement for cell transformation and immortality (CONG; WRIGHT; SHAY, 2002; XU; LI; STOHR, 2013).; and (ii) constitutional germline mutations have also been described in *MLH1* and *PTEN* promoters and correlated with the risk of cancer (HITCHINS et al., 2007, 2011; WARD et al., 2013; ZHOU et al., 2003). Interestingly, the 5'UTR *MLH1* variant c.-27C>A is an example of a non-coding sequence change associated with an epigenetic modification. The presence of the variant generates aberrant methylation of the promoter and silences of the affected allele (HITCHINS et al., 2007, 2011; WARD et al., 2013; ZHOU et al., 2003)

Since *BRCA1/2* coding or intron/exon junctions pathogenic variants only explain 10% of the predisposed families, exhaustive efforts have been undertaken for more than 20 years to identify other loci contributing to breast cancer susceptibility. It remains possible that some of the remaining risk maybe related to the main HBOC genes *BRCA1/2,* potentially by variants causing deregulation of expression. Until now, few studies have analyzed *BRCA1/2* non-coding regions.

3.1.2 Hypothesis

Our hypothesis was that unclassified variants located in regulatory regions of *BRCA1* and *BRCA2* should have an impact in breast and ovarian cancer predisposition.

3.1.3 Summary results and concluding remarks

Massively parallel sequencing and the use of whole-genome sequencing for screening have led to a substantial increase of variants identified in the *BRCA1/2* non-coding regions. To date, Genome aggregation database (gnomAD) has collected more than 1,000 *BRCA1/2* non-coding variants, for which functional impact is presently unknown. It is hard to measure since they impact both transcription regulation and reduction of expression of functionally active protein, and not in its extinction. A priority now is to weight the contribution of these variants in cancer risk. Indeed, as subtle quantitative effects are expected, it is challenging although important to define a threshold of effect that classifies these non-coding variants as "pathogenic variants" to allow accurate genetic counseling. Their classifcation is challenging since they reduce gene expression by changes *in trans* acting factors or cis-regulatory region and result in subtle change in the final protein. They may explain the remainder of the risk by themselves or in combination of not yet identified high, moderate or low risk variants located in *BRCA1/2* and/or in other cancer risk loci, with the potential to achieve the same end as truncating mutations in the gene itself.

This article brings insights to the increasing need of the medical community to explain the hereditary predisposition to breast and or ovarian cancers. This approach is innovative as it explores non-coding regulatory *BRCA1/2* elements and functional impact of variants there located in these regions which may represent an important but unexplored tumorigenic mechanism.

We were able to screen regulatory regions with the greatest potential for regulating BRCA1/2 expression of approximatelly 4,500 women who met the clinical criteria of HBOC syndrome and negative for pathogenic variant of *BRCA1/2*. This screening allowed the identification of 117 variants, some of them with proven impact on promoter activity and supposed impact on gene expression. For a portion of them, clinical arguments were available

to reinforce the hypothesis of their relationship with cancer predisposition. We analysed 20 of them by functional assays to stratify these variants. In addition, we reported the enhancer property of an intronic sequence located in the intron 12 of *BRCA1* and confirmed the enhancer property of a previously described region in the intron 2 of the same gene.

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PRECLINICAL STUDY

Assessment of the functional impact of germline *BRCA1/2* variants located in non-coding regions in families with breast and/or ovarian cancer predisposition

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Abstract

Purpose The molecular mechanism of breast and/or ovarian cancer susceptibility remains unclear in the majority of patients. While germline mutations in the regulatory non-coding regions of *BRCA1* and *BRCA2* genes have been described, screening has generally been limited to coding regions. The aim of this study was to evaluate the contribution of *BRCA1/2* non-coding variants.

Methods Four *BRCA1/2* non-coding regions were screened using high-resolution melting analysis/Sanger sequencing or next-generation sequencing on DNA extracted from index cases with breast and ovarian cancer predisposition (3926 for *BRCA1* and 3910 for *BRCA2*). The impact of a set of variants on *BRCA1/2* gene regulation was evaluated by site-directed mutagenesis, transfection, followed by Luciferase gene reporter assay.

Results We identified a total of 117 variants and tested twelve *BRCA1* and 8 *BRCA2* variants mapping to promoter and intronic regions. We highlighted two neighboring *BRCA1* promoter variants (c.-130del; c.-125C > T) and one *BRCA2* promoter variants (c.-296C > T) inhibiting significantly the promoter activity. In the functional assays, a regulating region within the intron 12 was found with the same enhancing impact as within the intron 2. Furthermore, the variants c.81-3980A > G and c.4186-2022C > T suppress the positive effect of the introns 2 and 12, respectively, on the *BRCA1* promoter activity. We also found some variants inducing the promoter activities.

Conclusion In this study, we highlighted some variants among many, modulating negatively the promoter activity of *BRCA1* or *2* and thus having a potential impact on the risk of developing cancer. This selection makes it possible to conduct future validation studies on a limited number of variants.

Keywords BRCA1/2 non-coding variants · Hereditary breast and/or ovarian cancer (HBOC) · BRCA1/2 transcription regulation · Breast and/or ovarian cancer risk

Introduction

At least 10% of the 14 million breast cancer diagnoses made worldwide each year are associated with hereditary predisposition. Breast cancer susceptibility gene 1 (*BRCA1*) and breast cancer susceptibility gene 2 (*BRCA2*) are the two most penetrant genes implicated in hereditary breast and/or ovarian cancer (HBOC) [1, 2]. However, a causal mutation useful for genetic counseling is identified in less than 15% of tested families and, in most cases, little is known about the underlying molecular mechanisms of cancer susceptibility. It would be particularly useful to identify inherited mutations in patients with a family history of cancers to allow

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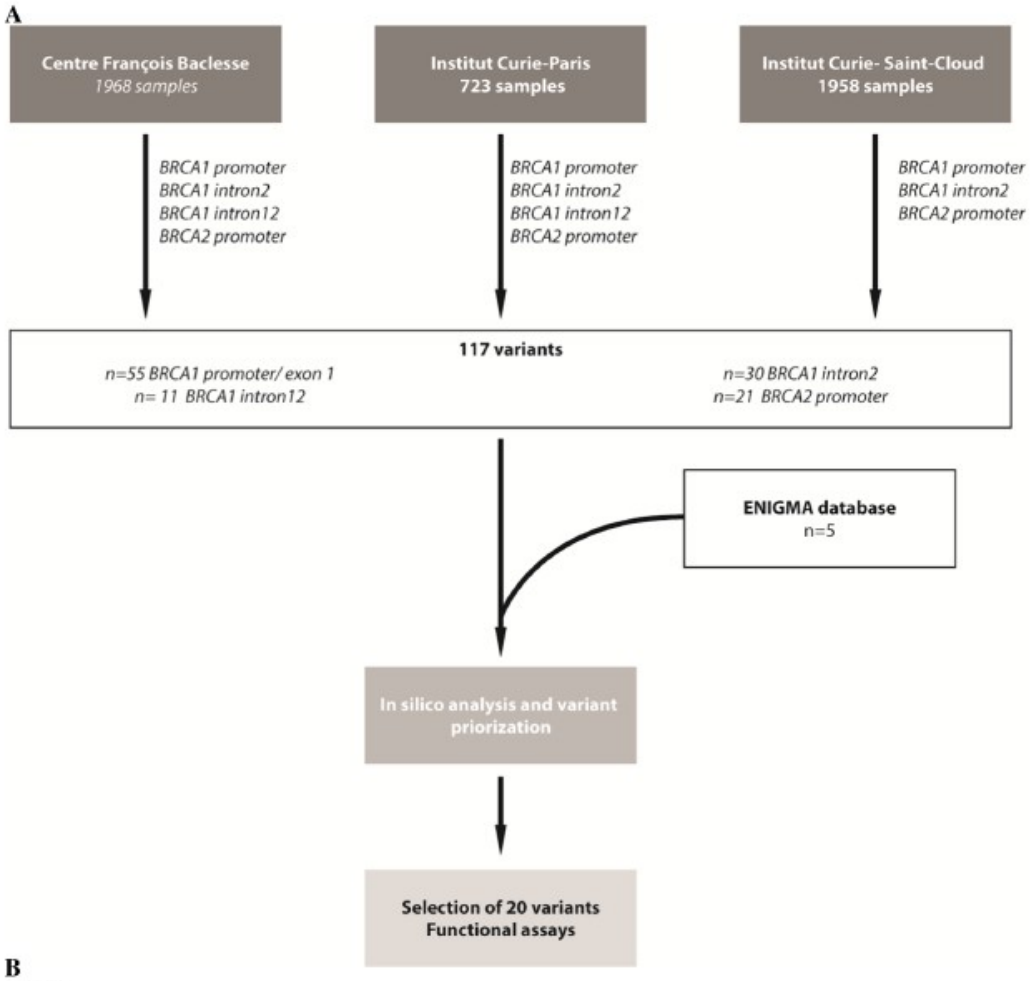

A
Centre François Baclesse
1968 samples
Institut Curie-Paris
723 samples
Institut Curie- Saint-Cloud
1958 samples
BRCA1 promoter
BRCA1 intron2
BRCA1 intron12
BRCA2 promoter
BRCA1 promoter
BRCA1 intron2
BRCA1 intron12
BRCA2 promoter
BRCA1 promoter
BRCA1 intron2
BRCA2 promoter
117 variants
n=55 BRCA1 promoter/ exon 1
n= 11 BRCA1 intron12
n=30 BRCA1 intron2
n=21 BRCA2 promoter
ENIGMA database
n=5
In silico analysis and variant priorization
Selection of 20 variants
Functional assays

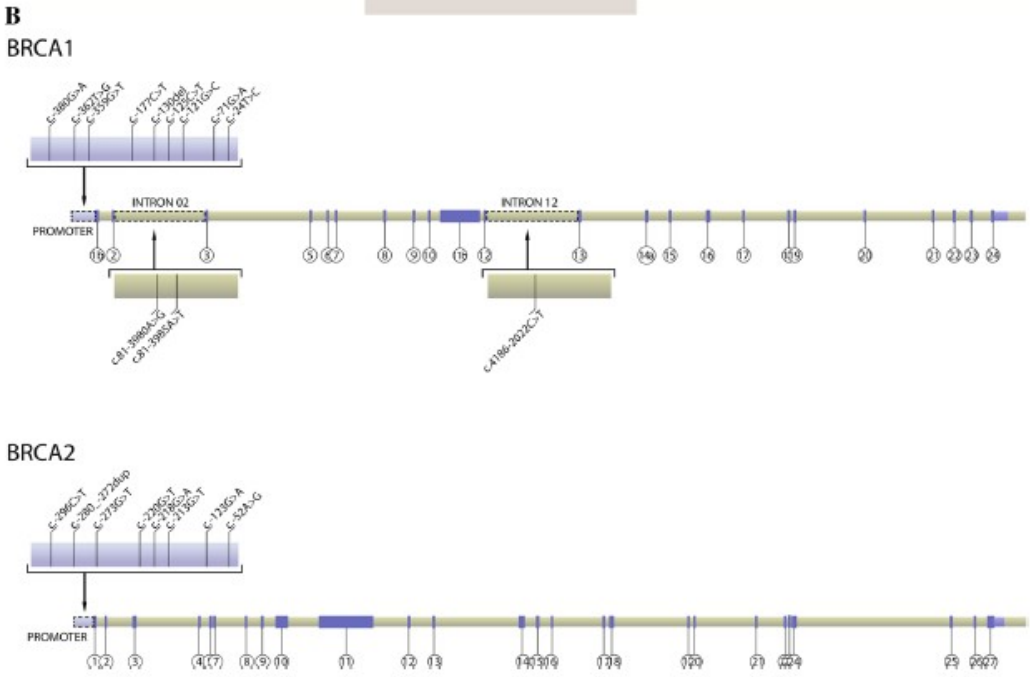

B
BRCA1
INTRON 02
INTRON 12
PROMOTER
BRCA2
PROMOTER

Fig. 1 **a** Work flow diagram describing the screening strategy and variant prioritization. **b** Location of the non-coding regions studied and the respective variants of each region selected for functional analysis

implementation of risk reduction strategies for these patients and their families. New technologies have been proposed to study a panel of genes known or suspected to be involved in breast and/or ovarian cancer predisposition. Other HBOC predisposition genes have also been explored but could represent less than 5% of all causative mutations [3]. *BRCA1/2* coding variants remain the major contributors to HBOC risk and the hypothesis that the remaining predisposition is also related to these genes remains plausible and could be explained by the presence of variants in non-coding regions for which the functional impact is currently unknown.

Progressing sequencing technologies and the development of bioinformatics tools now allow more informed exploration of transcriptional regulation [4, 5]. Germline mutations in the regulatory regions of the genome may represent an important tumorigenic mechanism and the impact of some non-coding regions on transcription regulation of the *BRCA1/2* genes has already been reported. Large genomic deletions involving the *BRCA1* and *BRCA2* promoters increase the risk of cancer [6–8]. Wardrop et al. described two non-coding sequences in intron 2 located 2.5 kb downstream to the *BRCA1* promoter with differential transcriptional regulatory activity [9]. Germline variants in the *BRCA1* and *BRCA2* 5′ and 3′UTRs, resulting in reduced translation efficiency, have also been described [10–15]. Moreover, several examples of variations in the non-coding sequences of other genes have also been correlated with cancer risk. Recently, two different recurrent mutations in the promoter of the telomerase reverse transcriptase (TERT) gene generating telomerase overexpression have been demonstrated to be associated with an increased risk of melanoma [16].

A reasonable mechanism to explain the impact of alterations of non-coding sequences on cancer risk is that the nucleotide change can create or disrupt a binding motif for a given transcription factor, and consequently alter the protein expression in all tissues expressing this factor. However, there is currently a lack of information about the function and polymorphisms of non-coding sequences and genetic screening of *BRCA1/2* genes is generally limited to coding regions and intron–exon junctions. The role of variants in non-coding regions with no splicing effect has not been thoroughly investigated and even less is known about their contribution to transcriptional regulation. Assessment of their impact on cancer predisposition is often more complex. The present study is a first approach to provide data to allow estimations of the impact of these variants on breast and/or ovarian cancer risk.

The primary objective of this study was to assess the significance and contribution of non-coding variants on *BRCA1/2* promoter activity and on breast and/or ovarian cancer risk.

Materials and methods

DNA samples, probands, and cohorts

In order to identify novel germline mutations that could explain hereditary predisposition, patients from three different HBOC cohorts, with eligibility criteria for familial genetic testing according to the French consensus statement and negative for *BRCA1/2* causal mutation, were enrolled [17–19]. A total of 1968 patients were tested at Centre François Baclesse, Caen, 1958 patients were tested at Institut Curie, Saint-Cloud, and 723 patients were tested at Institut Curie, Paris (Fig. 1, Table 1A). The characteristics of each cohort have been previously described [3, 20–22]. The frequency of the variants identified was also evaluated in a control cohort composed of Institut Curie patients with a cancer predisposition other than breast or ovarian cancer. The analysis was done anonymously and the frequency of the variant was only reported to compare with the cases.

DNA was extracted from lymphoblastoid cell lines and 4 *BRCA1/2* non-coding regions were screened by HRM or NGS: *BRCA1* promoter, *BRCA1* intron 2, *BRCA1* intron 12, and *BRCA2* promoter (Fig. 1, Table 1B).

In addition to the variants identified by this screening, we also selected new variants from the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) database [23], in the context of a collaborative study.

Screening of *BRCA1/2* non-coding regions: high-resolution melting analysis and next-generation sequencing

The four regions explored had been previously defined as being regions most likely to be functional and presenting a higher probability of containing disease-associated variants. This analysis comprised bioinformatics, experimental and population-based approaches to identify and validate key non-coding regions in *BRCA1* and *BRCA2* [9, 24, 25]. For example, the regions explored in introns 2 and 12 are highly conserved among mammalian species and contain many potential binding sites for known transcription factors [9].

For HRM screening, PCR reactions were performed in duplicate in a final volume of 15 µl containing 2 ng of DNA, 0.6 µM of each primer (forward or reverse), 1 × LightCycler 480 HRM Master mix (Roche), and LightCycler® 480 Resolight Dye or LCGeen® Plus melting dye for *BRCA1* and

Table 1 Determination of variants in *BRCA1/2* promoters and *BRCA1* introns 2 and 12

A: Cohorts of this study			
Cohorts	Status of BRCA1/2	Technique	Samples or patients
Centre François Baclesse	Negative for causal mutation	NGS	*1968*
Institut Curie—Paris	Negative for causal mutation	HRM or NGS	723
Institut Curie—Saint-Cloud	Negative for causal mutation	HRM	*1958*
B: Number of variants found in each cohort for the target areas of *BRCA 1/2* genes			
Gene	Region	Cohorts (n)	Variants
BRCA1	Promoter/exon 1	3926	55
BRCA1	Intron 2	3624	30
BRCA1	Intron 12	2973	11
BRCA2	Promoter/exon 1	3910	21
Total			117

BRCA2 screening, respectively [26]. Each assay included DNA with known *BRCA1/2* mutation corresponding to the primer set as positive control. The PCR program is available on demand. The non-coding *BRCA1/2* DNA sequences evaluated and the primers selected for this purpose are specified in Supplementary Table 1.

NGS screening was performed with a dedicated panel for cancer predisposition with Illumina sequencers [3, 21, 22]. All known genetic variants detected were confirmed by sequencing PCR products (Sanger sequencing method).

In silico analysis and variant prioritization

For variant prioritization, we first applied a population frequency filter to exclude variants with an allele frequency > 1%. The minor allelic frequency (MAF) was estimated from the Ensembl project or Exome Aggregation Consortium [27, 28]. Information analysis was then performed to identify potentially pathogenic variants. This approach evaluates the effects of the variant on binding sites and whether the variant involves the creation, strengthening, weakening, or abolition of a binding site [5].

All variants were scanned with Shannon Human Splicing Mutation Pipeline, a genome-scale analysis program that predicts the effects of variants on mRNA splicing [29]. Variants were selected according to the following criteria: weakened natural site ≥ 1.0 bits or strengthened cryptic site equal to or greater than the nearest natural site of the same phase. We also analyzed the effects of variants in the 5′UTR region on TF binding using the models previously described by Mucaki et al. [5].

Finally, for functional assays, we prioritized variants located in domains most likely to be functional based on bioinformatics analysis, and for which testing tools were available.

Luciferase reporter gene constructions

Luciferase reporter plasmids containing sequences from the *BRCA1* promoter and *BRCA1* intron 2 have been described previously [9, 25]. For the *BRCA2* luciferase reporter plasmid, a 750 bp region containing the BRCA2 promoter was cloned into the pGL3-Basic vector [9, 25]. In these plasmids, promoter sequences were inserted upstream to the coding sequence of firefly luciferase in the *Xho*I site. The intronic sequences were inserted immediately downstream to the luciferase gene in the *Bam*H1 site (Fig. 2). A new construct was made in order to clone a region of *BRCA1* intron 12 downstream to the luciferase gene, using the Gibson Assembly Method [30]. Variants were introduced into the plasmids by directed mutagenesis. *BRCA1*: c.-287C > T and c.-326_324del variants were used as positive controls. As the *BRCA2* promoter has been less studied, it was not possible to model a positive control for it, and thus the wild-type promoter was used as a reference. The *BRCA2*: c.-52A > G polymorphism was used as negative control. All constructs were verified by DNA sequencing.

Cell culture, transfection, and dual-luciferase reporter assay

The triple-negative breast cancer MDA-MB-231 cell line and the estrogen receptor-positive MCF-7 breast cancer cell line were obtained from American type culture collection (ATCC). MDA-MB-231 was used in every experiment. We confirmed some of the significant results in the MCF-7 breast cancer cell line. All cells were tested regularly for

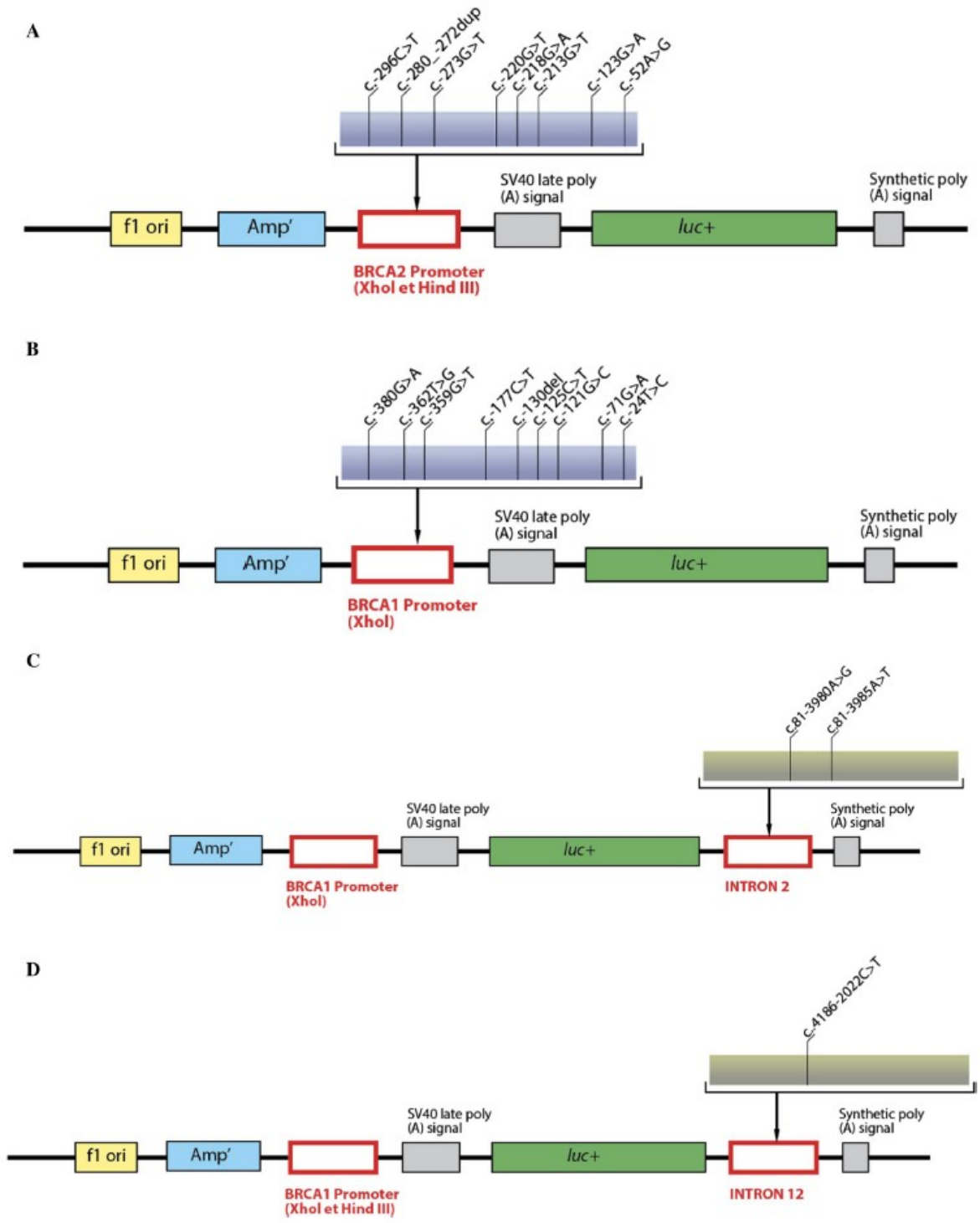


Fig. 2 Representation of the plasmids used in this study. **a** *BRCA2* promoter. **b** *BRCA1* promoter. **c** *BRCA1* promoter and BRCA1 intron 2. **d** *BRCA1* promoter and *BRCA1* intron 12

mycoplasma contamination using plasma Test (invivoGen) and authenticated using the GenePrint 10 system Kit (Promega). MCF-7 and MDA-MB-231 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and antibiotics (37 °C, 5% CO_2). To perform transient transfection, cells were seeded in 24-well plates and were subsequently transfected at 80% confluence using X-treme (QIAGEN) reagent according to the manufacturer's instructions. After 36 h, Firefly and Renilla activities were measured using the dual-luciferase kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity and expressed as mean ± S.D. of triplicates from a representative experiment.

All statistical calculations were performed using PASW Statistics (version 18.0; SPSS Inc., Chicago, IL). Comparisons were performed using a two-sided unpaired Student t test. p values less than 0.05 were considered to be statistically significant.

Clinico-pathological features of variant carriers

When a significant reduction of promoter activity was observed, more evidence for variant classification was sought. Further analysis of the patient's pedigree, allelic imbalance in RNA transcription, and tumor sample features, including Loss of Heterozygosity (LOH) and methylation, were determined, when material was available. LOH analysis was performed by Sanger sequencing or pyrosequencing. The *BRCA1* promoter methylation status was also assessed for variants with functional impact and when the material was available by pyrosequencing assay [31].

Results

Identification of new variants in *BRCA1/2* non-coding regions

The aim of this study was to identify novel germline mutations located in the non-coding regions of *BRCA1* and *BRCA2* genes that could explain hereditary predisposition for breast cancer. To do this, 4 *BRCA1/2* regions of the DNA of patients from 3 different HBOC cohorts were screened: *BRCA1* promoter, *BRCA1* intron 2, *BRCA1* intron 12, and *BRCA2* promoter (Table 1B). This approach allowed the identification of 117 variants in *BRCA1/2* non-coding regions (Fig. 1, Tables 1A, Supplementary Tables 2 and 3).

Five of these 117 variants were identified in more than 4 families: c.81-3625del, c.-20 + 11C > T, c.4186-2050A > G and c.-86C > T in *BRCA1* gene, and c.-175C > T in *BRCA2* gene. Two of them were found exclusively in our cohorts with HBOC predisposition: c.81-3625del and c.-20 + 11C > T in *BRCA1* gene. The remaining three variants were also identified in the control population.

In silico analyses

In silico analysis of these 117 variants identified 3 *BRCA1* variants with a potential impact on splicing: c.-73C > G, c.-86C > T, and c.-19-130insA; 3 *BRCA1* variants with a potential impact on UTR binding site alteration: c.-73C > G, c.-79G > T, and c.-121G > C; and twelve *BRCA1* variants with a potential impact on the TFB site: c.81-3459C > T, c.81-3510C > T, c.-19-479G > T, c.-20 + 131delGGCGTA, c.-20 + 131A > T, c.-20 + 125A > C, c.-177C > T, c.-130del, c.-125C > T, c.-20 + 486insG, c.-19-123insAT, and c.-20 + 11C > T. The impact of the variants on RNA secondary structure was also analyzed and one *BRCA1* variant, c.-130del, displayed a predicted impact on mRNA conformation (Fig. 3).

Moreover, two variants in intron 2 of *BRCA1* could have an impact on the creation of cryptic exons: c.81-4118G > A and c.81-3519G > T. Validation of these cryptic exons would require the development of a dedicated RT-PCR on mRNA. No suspected mRNA splicing effect was detected in silico for these variants.

Six *BRCA2* variants were identified with different potential impacts: c.-112G > A (UTR binding site and splicing factor binding site), c.-123G > A (splicing factor binding site), c.-171G > C (mRNA structure), c.-178insCTGCTG CGCCT (TFB site), c.-213G > T (UTR binding site), c.-296C > T (TFB site). The c.-171G > C variant also displayed a predicted impact on mRNA structure.

Based on these analyses and taking into account the available tools, twenty variants were selected for functional assays [32]. Nine of these 20 variants were located in the *BRCA1* promoter region, two variants were located in *BRCA1* intron 2, one variant was located in *BRCA1* intron 12, and eight variants were located in the *BRCA2* promoter region (Table 2).

Impact of variants on *BRCA2* promoter activity

Among the 8 *BRCA2* variants tested, only c.-296C > T induced a significant reduction (28%) of reporter gene expression, indicating that this variant inhibits the *BRCA2* promoter activity (Fig. 4). Moreover, analysis of the tumor sample harboring this variant identified LOH of the wild-type allele, and the patient's pedigree revealed that one of her 2 sisters had also a diagnosis of breast cancer at the age of 44 years (Table 3, F1), further supporting the potential pathogenic impact of this variant (Fig. 1 supplementary data).

Two variants showed an increase of promoter activity: the eventual role of this positive effect on cancer remains to be

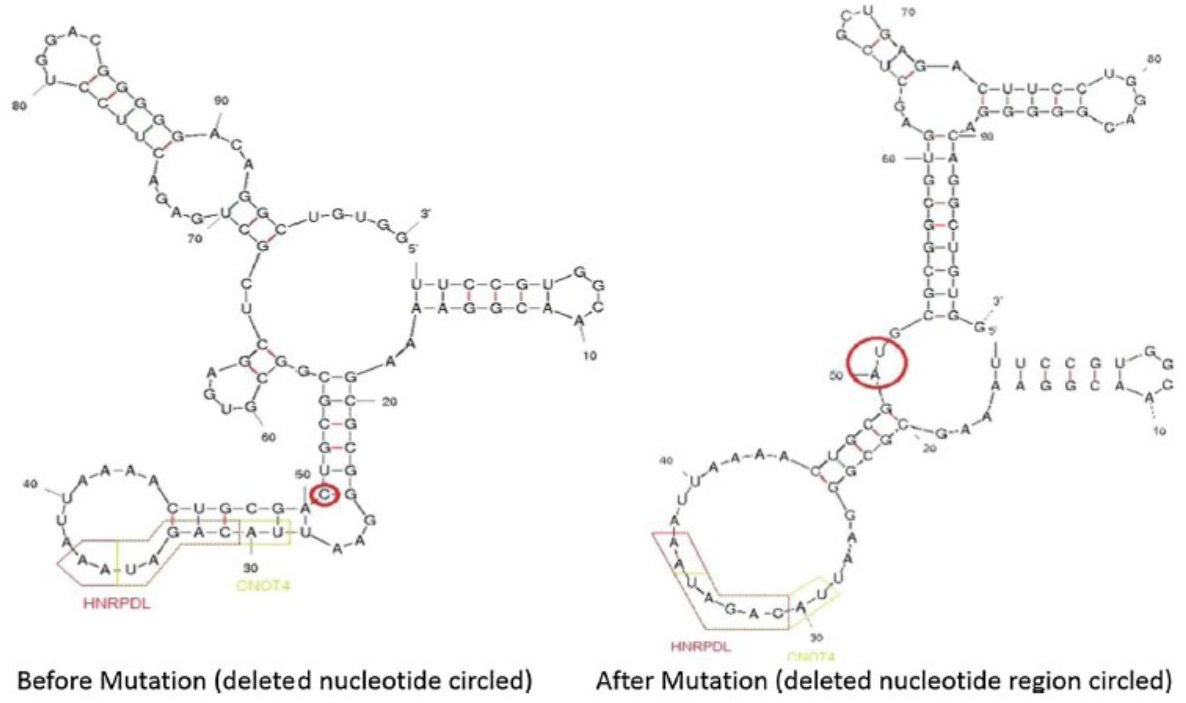


Fig. 3 *BRCA1* variant: c.-130del—structure with mFOLD is significantly changed due to loss of C-G bond

defined. The other variants demonstrated similar levels of activity to that of the wild-type sequence strongly suggesting that these variants are neutral (Fig. 4).

Impact of variants on *BRCA1* promoter activity

The *BRCA1* variants analysis revealed two neighboring variants: c.-125C > T and c.-130del, inducing a strong reduction of promoter activity (60% reduction for c.-130del $p = 0.0002$, and 56% reduction for c.-125C > T $p = 0.0025$) (Fig. 5 and Table 2B). To confirm these results, we repeated the experiment in another breast cancer cell line, MCF-7. We validated our first results (70% reduction for c.-130del, $p = 0.003$, and 30% reduction for c.-125C > T, $p = 0.003$) (Table 2B). One family was available for the *BRCA1* c.-130del with many prostate cancers (Table 3, F2). As for the *BRCA2* promoter, we also found 2 variants increasing weakly the *BRCA1* promoter activity: c.-362T > G; c.-121 G > C (Fig. 5 and Table 2A). The remaining variants were associated with similar reporter gene activity to that of the wild-type sequence (Fig. 5).

We also studied the impact of *BRCA1* intronic variants on *BRCA1* promoter activity: two detected in intron 2 (c.81-3985A > T and c.81-3980A > G) and one detected in intron 12(c.4186-2022C > T). First of all, we confirmed that the presence of a part of intron 2 and also a part of intron 12 increased the activity of the *BRCA1* promoter, 1.48- and 1.72-fold, respectively, confirming that these two introns possess important regulatory sequences (Fig. 6a). The intron 2 effect was already described contrary to the intron 12 [9]. The intronic variant c.81-3985A > T is located in a repressor region previously described in intron 2 [9]. However, we did not detect any influence of this variant on the positive effect of the intron 2 on the *BRCA1* promoter activity. Most importantly, we found that in the presence of the two intronic variants (c.81-3980A > G and c.4186-2022C > T), the introns 2 and 12 had no longer an impact over *BRCA1* promoter activity (Fig. 6b and Table 2B).

We did not detect any *BRCA1* promoter methylation for any functionally active variants.

Discussion

Results statement

Optimal management of hereditary breast and/or ovarian cancer families requires accurate identification of individuals at genuinely high risk. Although it is important to identify new breast and ovarian cancer susceptibility genes, non-coding regions are currently not investigated, with the exception of those intronic variants with an

Table 2 (A) Summary of the 20 variants tested. (B) The effect of the variants tested on luciferase activity

Gene	Variant	Localization	Record	Databases	dbSNP	1000Genomes MAF	ExAC	Conservation*	Putative TF binding site
BRCA1	c.-24T > C	Promoter	1	BIC/ClinVar	–	–	–	− 0,52	–
BRCA1	c.-71G > A	Promoter	1	No	–	–	–	0,93	–
BRCA1	c.-121G > C	Promoter	1	No		–	ALL:C = 0.0019%-	− 1,01	–
BRCA1	c.-125C > T	Promoter	1	No	rs148196794	< 0.01/4ou ALL: T = 0,1%	–	2,14	E2F1
BRCA1	c.-130del	Promoter	1	No	–	–	–	0,37	E2F1, HSF1, TEAD4
BRCA1	c.-177C > T	Promoter	1	No	–	–	–	0,85	CEBPB
BRCA1	c.-359G > T	Promoter	1	No	–	–	–	− 1,17	–
BRCA1	c.-362T > G	Promoter	1	No	–	–	–	1,25	–
BRCA1	c.-380G > A	Promoter	1	No	–	–	–	− 0,28	–
BRCA1	c.81-3985A > T	Intron 2	2	ClinVar	rs543267121	–	–	1,25	–
BRCA1	c.81-3980A > G	Intron 2	1	No	–	–	–	0,21	–
BRCA1	c.4186-2022C > T	Intron 12	1	No	–	–	–	0,85	–
BRCA2	c.-52A > G**	Promoter	1	UMD/LOVD	rs206118	ALL :G = 15%	–	− 0,12	–
BRCA2	c.-123G > A	Promoter	1	No	–	–	–	− 2,14	–
BRCA2	c.-213G > T	Promoter	1	No	rs546292946	–	–	− 0,04	–
BRCA2	c.-218G > A	Promoter	1	No	–	–	–	0,12	–
BRCA2	c.-220G > T	Promoter	1	No	–	–	–	2,38	–
BRCA2	c.-273G > T	Promoter	1	No	–	–	–	0,21	–
BRCA2	c.-280_272dup	Promoter	1	No	–	–	–	2,47	PAX5
BRCA2	c.-296C > T	Promoter	1	No	rs563971900	ALL :T = 0.04%	–	− 0,28	PAX5

Gene	Variant	Localization	Effect on promoter activity MCF-7	Effect on promoter activity MDA-MB231	BRCA1 promoter methylation
BRCA1	c.-24T > C	Promoter	Not tested	NS	–
BRCA1	c.-71G > A	Promoter	Not tested	NS	–
BRCA1	c.-121G > C	Promoter	NS	↗ 1.25x ($p = 0.009$)	No
BRCA1	c.-125C > T	Promoter	↘ 0.7x ($p = 0.003$)	↘ 0.44x ($p < 0.0025$)	NA
BRCA1	c.-130del	Promoter	↘ 0.27x($p = 0.003$)	↘ 0.4x ($p = 0.0002$)↘	No
BRCA1	c.-177C > T	Promoter	Not tested	NS	–
BRCA1	c.-359G > T	Promoter	Not tested	NS	–
BRCA1	c.-362T > G	Promoter	Not tested	↗ 1.74x ($p = 0.0037$)	NA
BRCA1	c.-380G > A	Promoter	Not tested	NS	–
BRCA1	c.81-3985A > T	Intron 2	↗ 1.93x($p < 0.05$)	Not tested	No
BRCA1	c.81-3980A > G	Intron 2	Not tested	NS	–
BRCA1	c.4186-2022C > T	Intron 12	Not tested	NS	–
BRCA2	c.-52A > G	Promoter	Not tested	NS	–
BRCA2	c.-123G > A	Promoter	Not tested	↗ 1.83x ($p < 0.05$)	–

Table 2 (continued)

Gene	Variant	Localization	Effect on promoter activity MCF-7	Effect on promoter activity MDA-MB231	BRCA1 promoter methylation
BRCA2	c.-213G > T	Promoter	Not tested	NS	–
BRCA2	c.-218G > A	Promoter	Not tested	NS	–
BRCA2	c.-220G > T	Promoter	Not tested	NS	–
BRCA2	c.-273G > T	Promoter	Not tested	NS	–
BRCA2	c.-280_272dup	Promoter	Not tested	↗ 1.76x ($p = 0.00084$)	–
BRCA2	c.-296C > T	Promoter	Not tested	↘ 0.72x ($p = 0.0035$)	

NA material not available; *NS* not significant; *p* value was calculated using a two-sided unpaired Student *t* test. *p* values less than 0.05 were considered to be statistically significant

*The phyloP program was used to determinate the conservation score of the variants (http://compgen.cshl.edu/phast/)

**c.-52A > G is a polymorphism used as negative control in all *BRCA2* runs

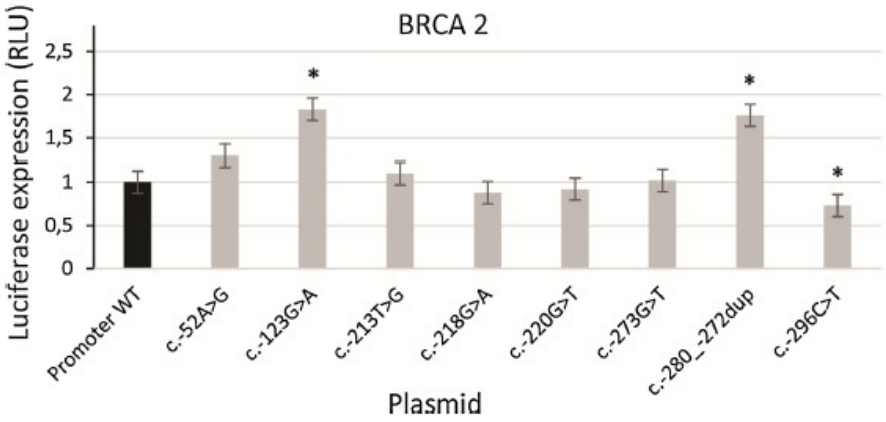


Fig. 4 Impact of different variants on *BRCA2* promoter activity. MDA-MB-231 breast cell line was transfected with the expression vector pRL-TK Renilla in combination with the luciferase reporter plasmids containing the *BRCA2* promoter wild type (Promoter WT) or possessing a variant as indicated. Twenty-four hours later, cell extracts were prepared and luciferase activities quantified

impact on RNA splicing [33, 34]. In the present study, we chose to explore these non-coding regions and carry out functional assays for these variants. Screening of the HBOC population comprising 3926 patients screened for *BRCA1* and 3010 patients screened for *BRCA2* non-coding regions revealed 117 variants (0.5 to 1.4% of the screened population).

We have validated an experimental protocol for the initial functional classification of 20 of these variants that demonstrated 10 non-coding variants with a functional impact on *BRCA1/2* promoter activity. Among these 10 variants, two decreased *BRCA1* promoter activity: c.-130del and c.-125C > T; one decreased *BRCA2* promoter activity: c.-296C > T; and two (c.81-3980A > G and c.4186-2022C > T) suppressed the positive effect of the introns 2 and 12 over the *BRCA1* promoter activity.

Limitations of functional assays for non-coding variants

Fluctuations of the basal reporter activity were observed for both the *BRCA1* and *BRCA2* promoters, which could be explained by poorly controlled parameters of the biological system as well as technical limitations, for example, the quality and conformation of transfected DNA. An internal positive control was always used to ensure correct interpretation of functional results. It is noteworthy that only minor differences were observed for PGL3 basic or Renilla luciferase activity, which confirm transfection efficiency, and that the wild-type promoter was always present to ensure correct interpretation of functional results. Moreover, the results for the potential suppressor variants, *BRCA1* c.-125C > T; *BRCA1* c.-130del; *BRCA2*

Table 3 Summary of clinical and pathological data for the non-coding variants

Gene	Variation (c.)	Localization	Index case data	Co-occurrence of variants in BRCA2 or BRCA1 or in other genes + large rearrangements	Family data	Co-segregation data
BRCA2	c.-296C > T	Promoter	C1: woman with breast cancer (diagnosed at 60)	No	F1: sister with breast cancer (diagnosed at 44)	No information
BRCA1	c.-130del	Promoter	C2: man with prostate cancer (diagnosed at 60)	No	F2: 5 Brothers with prostate cancer (diagnosed at 72, 70, 60, 65, and 64). Maternal grand-mother with breast cancer (diagnosed at 45)	No information
BRCA1	c.81-3985A > T	Intron 2	C3: woman with breast cancer (diagnosed at 48)	No	F3: mother with breast cancer (diagnosed at 60). Brother with prostate cancer (diagnosed at 50)	No information
BRCA1	c.81-3985A > T	Intron 2	C4: woman with breast cancer (diagnosed at 48)	No	F4: mother with breast cancer (diagnosed at 54). Maternal aunt with breast cancer (diagnosed at 45). Maternal cousin with breast cancer (diagnosed at 45). Paternal aunt with ovary cancer (diagnosed at 69). Paternal cousin with breast cancer (diagnosed at 51)	No information
BRCA1	c.81-3985A > T	Intron 2	C5: woman with breast cancer (diagnosed at 40)	No	F5: mother with breast cancer (diagnosed at41). Maternal cousin with breast cancer (diagnosed at 70). Maternal grand-father with pancreatic cancer (diagnosed at 70)	No information

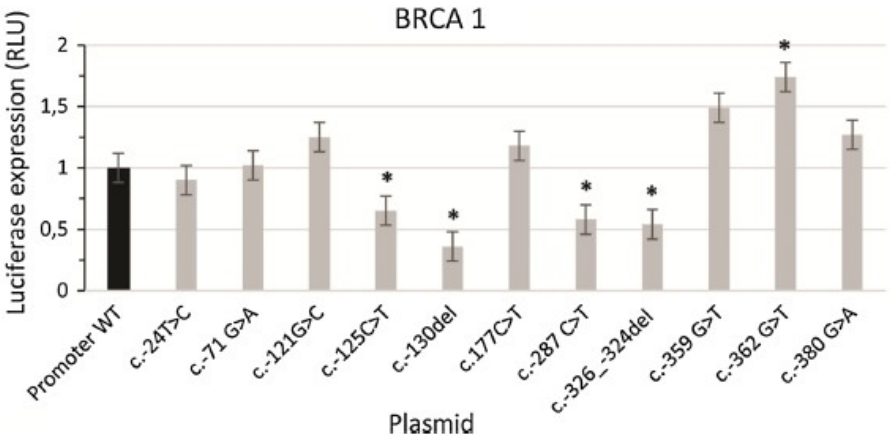
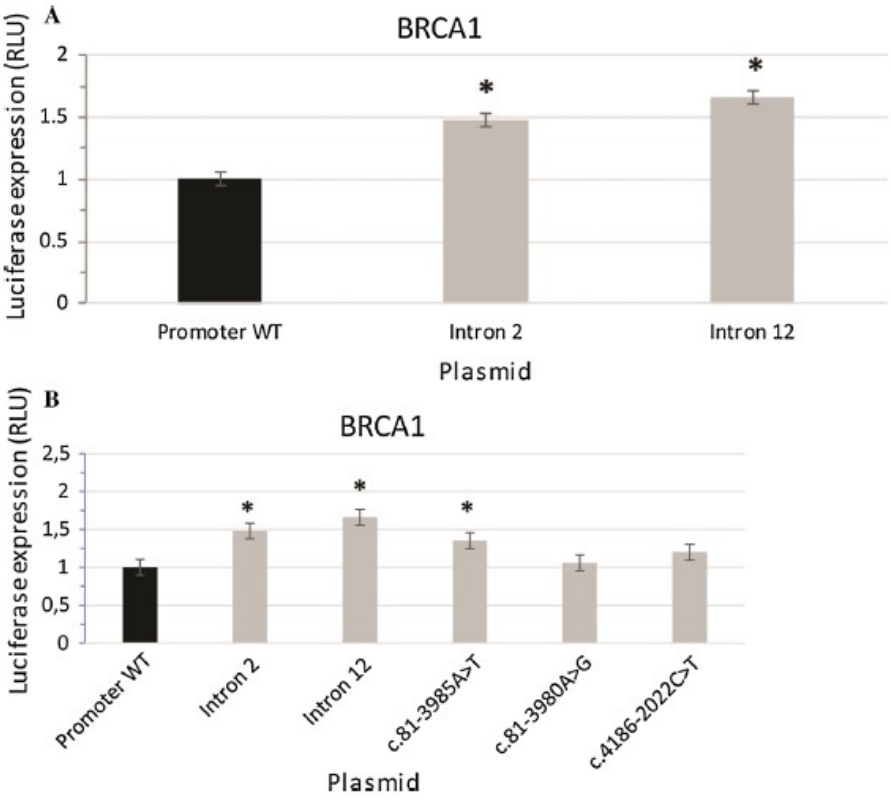


Fig. 5 Impact of different variants on *BRCA1* promoter activity. MDA-MB-231 breast cell line was transfected with the expression vector pRL-TK Renilla in combination with the luciferase reporter plasmids containing the *BRCA1* promoter wild type (Promoter WT) or possessing a variant as indicated. Twenty-four hours later, cell extracts were prepared and luciferase activities quantified. The c.-287C > T and c.-326_324del variants are artificial constructions on CAAT box and on the RIBS element, respectively, used as positive controls

Fig. 6 **a** Impact of different intronic variants on *BRCA1* promoter activity. MDA-MB-231 breast cell line was transfected with the expression vector pRL-TK Renilla in combination with the luciferase reporter plasmids containing the *BRCA1* promoter wild type without (Promoter WT) or with the intron 2 or 12 wild type (**a**) or possessing a variant (**b**) as indicated. Twenty-four hours later, cell extracts were prepared and luciferase activities quantified



c.-296C > T, were always consistent under the various experimental conditions.

Sensitive region in promoter of *BRCA1*

We identified a sensitive region in the *BRCA1* promoter with 3 functionally active variants: c.-125C > T; c.-130del;

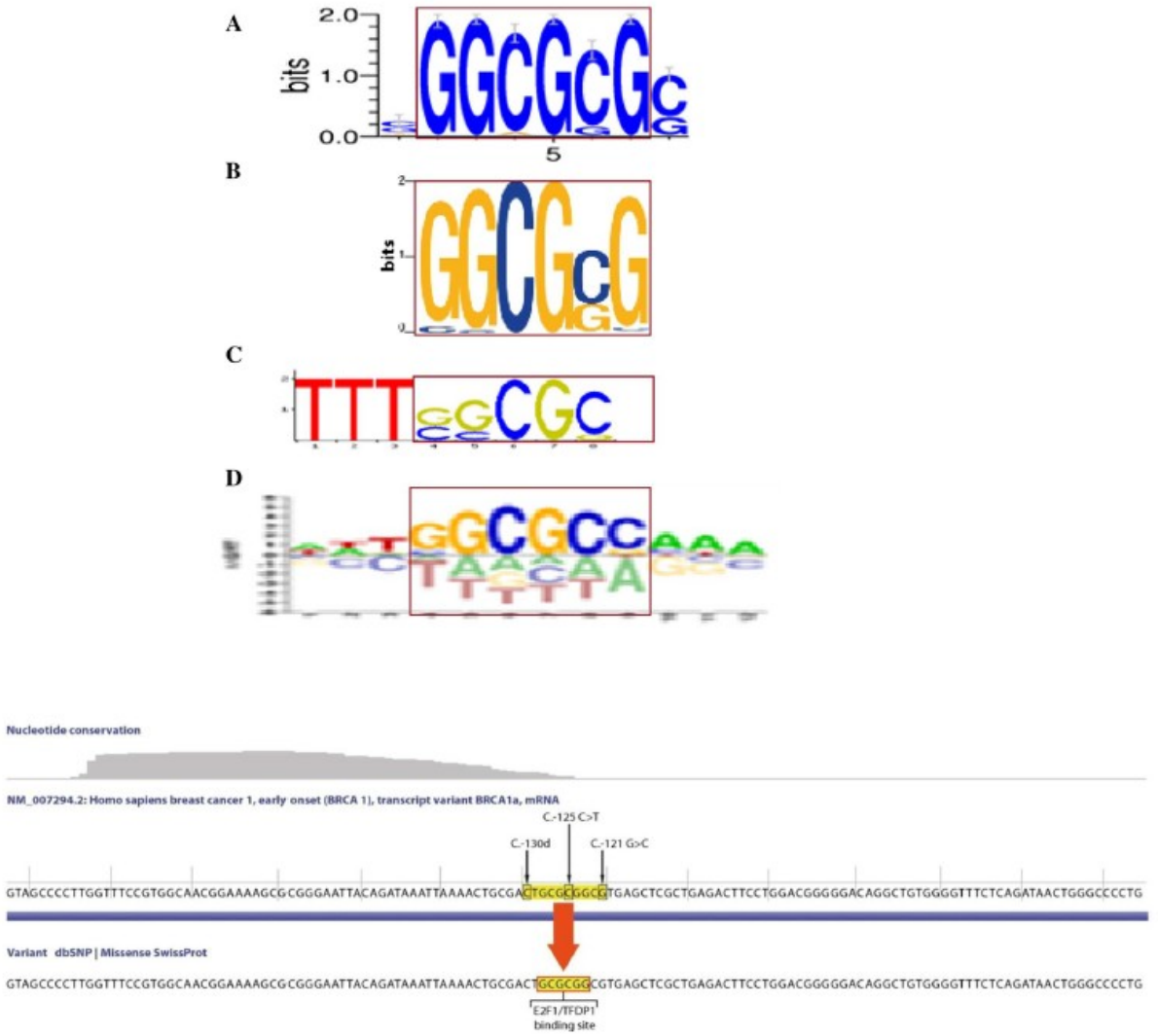


Fig. 7 Identification of a new potential E2F1 binding site in *BRCA1* promoter. **a** Information Models built from publically available ChIP-Seq data (HeLa-S3). **b** Models from SwissRegulon (Fig. 5a)

c.-121G > C, including 2 with a marked repressor impact on promoter activity (Fig. 7). Analysis of the DNA sequence region containing the neighboring *BRCA1* c.-125C > T and *BRCA1* c.-130del promoter variants, using the Swiss Regulon TF database (http://swissregulon.unibas.ch/), revealed that both variants are located in a putative E2F1 transcription factor binding site (TFBS)(Fig. 7). These two variants may thus impact the ability of E2F1 to induce BRCA1 transcription. An E2F1 information model generated using ChIP-Seq data from HeLa-S3 lysates revealed a fairly weak 3.6 bit E2F1 site on the negative strand (Fig. 7a) [35]. When the binding site was analyzed from the negative strand (the orientation of BRCA1 transcription), both mutations were predicted to decrease the strength of the predicted E2F1 site. Variant c.-125C > T was predicted to be a weak variant mainly due to the presence of a 'T' in its sequence when a C or G was expected (TGCGCG; arrow indicates the position of T relative to our model; Fig. 7a). Our analysis also revealed that the c.-130del variant is located in a putative HSF1 and TEAD4 TFBSs. Other transcription factors identified in future studies could therefore increase our understanding of the biological implications of these variants in TFBSs.

Our in silico analysis revealed that the *BRCA1*: c.-130del variant also has a potential impact on the RNA 2D structure. The RNA conformation of the first exon of the *BRCA1* gene

has been described and could have an impact on transcription, as the alternative exon 1b transcript of the *BRCA1* gene has a conformation that could reduce translation of mRNA [36]. This impact cannot be detected with the luciferase assay.

Analysis of the pedigree of the c.-130del index case, looking for more evidence for classification of variants, revealed numerous cases of prostate cancer, usually associated with alterations of the *BRCA2* gene. Patients carrying a *BRCA1* mutation usually present little or no increased cancer risk, but a more aggressive form of prostate cancer [37]. Unfortunately, sequencing of this patient's tumor sample did not reveal any additional useful for classification: neither LOH of the wild-type allele nor promoter methylation was detected. However, recent studies have demonstrated the effect of BRCA1-haploinsufficiency in various cells and tissues, which may explain how mutation in a single *BRCA1* allele conferred increased cancer risk in this patient [38].

BRCA2 promoter

For the first time, a variant of the *BRCA2* promoter has been shown to have a functional impact on transcription (c.-296C > T). This variant is also located close to a region rich in transcription factor binding sites. Analysis of the tumor sample from a carrier of this variant revealed somatic loss of the wild-type *BRCA2* allele, suggesting that loss of heterozygosity may play a role in the tumorigenesis. The other two *BRCA2* variants (c.-280_-272dup and c.-123G > A) showed an enhancer activity, the consequence of which is unknown.

Putative changes in TFBS related to the presence of the variants

The two *BRCA2* variants with a significant impact on transcription (c.-296C > T and *BRCA2*: c.-280_-272dup) were correlated with the TFBS predictions based on the variant prioritization method (Table 2A). These variants alter the binding strength of two PAX5 binding sites. ChIP-Seq experiments have shown that PAX5 binds to the *BRCA2* promoter region. Furthermore, although the *PAX5* gene has not been shown in the literature to have a direct effect on BRCA expression, it has been shown to be hypermethylated in triple-negative breast cancer [39]. Loss of a PAX5 binding site may therefore induce a similar effect to that of an overall reduction of PAX5 gene expression.

TFBS analysis showed weakening of PAX5 binding site from 12.7 to 8.5 bits in the presence of the c.-296C > T variant. Similarly, the promoter activity assay showed an increase in *BRCA2* promoter activity in the presence of the *BRCA2*: c.-280_-272dup event. TFBS analysis predicted that this duplication would create a 5.6 bit PAX5 binding site, which correlates with the reported increase in promoter activity.

Introns 2 and 12 *BRCA1*

Wardrop et al. have described the presence of regulatory regions in the intron 2 sequence of *BRCA1* gene [9]. Although these regions are situated several kb downstream to the promoter region, they regulate *BRCA1* expression at the transcriptional level, most likely via gene looping [25]. We investigated introns 2 and intron 12. Intron 12 locus has been selected for being rich on the transcription factor binding sites and interspecies conservation.

Even if the variant c.81-3985A > T was found in three families (Table 3) suspected for cancer predisposition, we did not detect any influence of this variant on the positive effect of the intron 2 over the *BRCA1* promoter activity. This result strongly suggest that the c.81-3985A > T variant do not inhibit the activity of the *BRCA1* promoter and therefore would have no effect on the breast cancer development. Furthermore, analysis of RNA from the patient's lymphoblastoid cell line showed no allelic imbalance, which support our conclusion that the c.81-3985A > T variant may have no causal impact on cancer (data not shown).

In the other hand, we found that the two intronic variants c.81-3980A > G and c.4186-2022C > T displayed wild-type devoid of intron 2 or 12, respectively. These two variants may inhibit *BRCA1* promoter activity by suppressing the positive effect of the intron 2/12 on the *BRCA1* promoter activity thereby stimulating cancer development. In this study, the regulating impact of intron 12 has been confirmed in vitro and this work highlights the importance of screening this region. Some variants were identified and a variant c.4186-2022C > T has been able to revert the enhancing impact of the intron 12 locus. Unfortunately, there was no material available to work on these variants.

Epigenetics

It is difficult to draw any solid conclusions from these results that could be used for genetic counseling of carriers of variants in *BRCA1/2* non-coding regions. Constitutional epimutation of the promoter has been described for the *MLH1* gene with a cis-acting variant, and a relationship between promoter activity and level of methylation has been established [40–42]. All of these cases presented somatic mosaicism between tissues and family members. No epimutations have been reported in the *BRCA2* gene. However, the promoter of *BRCA1* gene can also be methylated and constitutional epimutations have been reported [43]. No methylation of the promoter was identified on the c.-130del variant.

Conclusion

This study put in evidence the presence of rare variants in the non-coding regions of the *BRCA1* and *BRCA2* genes, and 5 of them induced a significant reduction of transcriptional levels. Our data raise the question whether the presence of these variants in regulatory regions may have an impact on the risk of developing cancer. To be more conclusive, it would be helpful to obtain more information about the frequency of these alterations. The model including the functional assay here described can be a useful tool to highlight the variants requiring further investigation including epimutation or co-segregation analysis, in order to ultimately establish a potential association with cancer risk.

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3.2 Loss of heterozygosity in *BRCA1* variants tumors

3.2.1 Background – Knudson hypothesis

In contrast to rearrangements and nonsense mutations resulting in a premature stop codon, the impact of *BRCA1/2* missense variants is not easily predictable as they result in a single amino-acid change. As a result, the majority of them remain unclassified. Currently, their classification requires a combination of different approaches in a multifactorial model, and tumoral data included in this model is limited to morphological and immunohistochemical features of breast cancers. This model requires access to several families and several carrier cases and it is limited, given the rarity of certain variants.

Unlike other diseases, such as colorectal cancer related to Lynch syndrome, in the case of *BRCA1/2* related-breast and ovarian cancers there are few somatic arguments to confirm the pathogenicity of the variant. The arrival of PARP inhibitors and their promising results in patients with ovarian and breast cancer carrying a *BRCA1/2* somatic or germinal pathogenic variant, has rendered tumoral sequencing data and LOH information readly available. Beyond information of mutational status, estimation of allelic frequency allows inferring the LOH status and whether the wild-type was retained in the course of cancer development.

As a typical tumor suppressor gene, the second allele inactivation of *BRCA1/2* is expected to explain the cancer initiation (KNUDSON, 1971). Indeed, copy neutral LOH is the most prevalent mechanism of second allele inactivation (detected in the majority of breast and ovarian *BRCA* related cancers, reaching 93% of ovarian cancers with *BRCA1* mutations) (Maxwell et al., 2017).

3.2.2 Hypothesis

Tumoral loss of heterozygosity (LOH) analysis can help *BRCA1* missense variants classification

3.2.3 Summary results and concluding remarks

In this article, we analyzed LOH status of 97 malignant tumors (90 breast and 7 ovarian). We observed a relatively stable pattern of LOH (67% of the wild-type allele) for tumors of the pathogenic variant carriers, while allelic balance or loss of variant allele was generally seen for carriers of benign variants. Additionally, we were able to classify 2 VUS (c.4963T>C and c.5497G>A) as pathogenic with tumor allele frequency, histopathologic, and co-segregation data and the samples' LOH analysis was concordant with our hypothesis: Loss of wild-type allele was observed in 4 of 5 samples with c.4963T>C, and all 3 samples with c.5497G>A. It may be noted that 15 cases of pathogenic variant tumors (8 missenses, 7 nonsense/frameshift) presented no allelic imbalance.

We concluded that LOH status cannot be used in isolation to infer variant pathogenicity. However, this information should be useful when being integrated into the multifactorial model for *BRCA1* VUS classification, being complementary to likelihood ratio (LR) pathology. A limitation of this approach is the number of samples containing the same variant required for classification (from 3 to 10 in our estimation), given the rarity of the individual variants. It is also important to understand whether information on wild allele loss is related to response to treatment with PARPi since it is an indirect sign of the implication of *BRCA* mutation in carcinogenesis.

Contribution of the loss of heterozygosity to *BRCA1* variant classification

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Background: At least 10% of the *BRCA1/2* tests identify variants of uncertain significance (VUS) and the distinction between pathogenic and benign variants remains particularly challenging. As a typical tumor suppressor gene, the inactivation of the second wild-type *BRCA1* allele is expected to trigger cancer initiation. Loss of heterozygosity (LOH) of wild-type allele is the most frequent mechanism for the *BRCA1* gene bi-allelic inactivation.

Material and methods: To evaluate if observation of tumor LOH can be an effective tool in predicting the pathogenicity of *BRCA1* missense variants, we carried out a systematic LOH analysis on DNA extracted from 90 breast and 7 ovary tumors diagnosed in 27 benign and 56 pathogenic (n=56) variant carriers samples were analyzed to validate the approach. When an allelic balance was found for a pathogenic variant, the following studies additional analyses were conducted in tumor DNA to evaluate the mechanism of the wild-type allele silencing: *BRCA1* promoter hypermethylation analysis, next-generation sequencing (NGS) of *BRCA1* searching for a another somatic inactivating variant, and BRCAness signature score. Additionnaly, we performed LOH analysis of tumor samples from missense VUS carriers (n=15).

Results: Ninety-seven tumor samples (90 breast and 7 ovarian) were analyzed for 26 different

missense *BRCA1* variant carriers (10 pathogenic, 8 benign and 8 VUS). We observed a relatively stable pattern of LOH (67% of the wild-type allele) for tumors of the pathogenic variant carriers, while allelic balance or loss of variant allele was generally seen for carriers of benign variants. We were able to classify 2 VUS (c.4963T>C and c.5497G>A) as pathogenic with tumor allele frequency, histopathologic, and co-segregation data. Loss of wild-type allele was observed for 4 of 5 samples with c.4963T>C, and 3 of 3 samples with c.5497G>A. It is noteworthy that 15 cases of pathogenic variant tumors (8 missense, 7 nonsense/frameshift) presented an allelic balance, which suggests that genetic instability may be absent despite the presence of a germline pathogenic variant.

Conclusions: LOH data can help clarify the pathogenicity of *BRCA1* VUS. The absence of genetic instability in tumors of pathogenic germline variant carriers further raises the questions whether the presence of an heterozygous germline pathogenic variant is enough to guide the treatment choice and whether the tumor screening is necessary to determine the second-hit event for predicting the efficacy of PARP inhibitors (PARPi).

Introduction

Monoallelic germline *BRCA1/2* pathogenic variants substantially increase the risk of developing breast and/or ovarian cancer, but at least 10% of *BRCA1/2* tests result in VUS. The distinction between germline pathogenic and benign nature of a missense variant remains particularly problematic while the classification of a rare germline missense variant remains challenging (AMENDOLA et al., 2016). In daily practice, in addition to genetic counseling implications, *BRCA1/2* variant classification now has an important impact on therapeutic decisions and in predicting the benefit from PARPi (MIRZA et al., 2016c; MOORE et al., 2018b; ROBSON et al., 2017) and DNA damaging agents (ALSOP et al., 2012b; TUTT et al., 2018b). Recent data suggests that in addition to the germline pathogenic variant, locus-specific LOH may also be necessary to predict sensitivity to DNA damaging agents and better outcomes (MAXWELL et al., 2017b). Several tests assessing different patterns of LOH have also been prospectively evaluated in clinical trials to infer the response to PARPi (BIRKBAK et al., 2012b; COLEMAN et al., 2017a; DONG et al., 2016; POLAK et al., 2017; POPOVA et al., 2012b).

A recent report of LOH analysis in *BRCA1/2* locus of 160 tumors with germline pathogenic variants (94% from patients with truncating variants) confirmed a proportion of

loss of wild-type allele in ovarian tumors as high as 93% for *BRCA1* and 90% for *BRCA2* carriers (MAXWELL et al., 2017b). A similar percentage of 90% occurred for *BRCA1* breast cancers, but was less evident (54%) for *BRCA2* breast cancers. (MAXWELL et al., 2017b; NONES et al., 2019). In contrast, for sporadic tumors, the LOH of the 17p is a more common event than a focal deletion around *BRCA1*. This is found in 20-50% of sporadic breast cancers (KOBOLDT et al., 2012a) and up to 87% of ovarian cancers (BELL et al., 2011).

Many approaches have been proposed to assist the classification of *BRCA1/2* germline missense variants of uncertain significance (VUS), including analysis of splicing effects (GAILDRAT et al., 2012; HOUDAYER et al., 2012; ROULEAU et al., 2010), co-segregation studies within the families (CAPUTO et al., 2018; LINDOR et al., 2012; MOGHADASI et al., 2018; PARSONS et al., 2019; SPURDLE et al., 2012b), co-occurrence in *trans* with a pathogenic mutation, personal/family history, and histopathologic profile (EASTON et al., 2007; GOLDGAR et al., 2004b; PARSONS et al., 2019; SPURDLE et al., 2014b). Currently, LOH data is not included in likelihood and posterior probability model calculations. Although some studies have argued in favor of LOH as a useful tool to predict variant pathogenicity (CHENEVIX-TRENCH et al., 2006, p.; SPEARMAN et al., 2008; YANG et al., 2018a), others warned that it should be applied with caution (BERISTAIN et al., 2010; SPURDLE et al., 2008a; VAN HEETVELDE et al., 2018). Part of the disagreement may be explained by the difference in the methodology used for the analysis. Initially, the presence of LOH was performed using fragment analysis of microsatellite repetition to evaluate if both alleles were present. The distance of the *BRCA1* locus could be a hurdle for this evaluation. Nowadays LOH analysis is performed with more sensitive and precise methods, such as next-generation sequencing or pyrosequencing, which are also able to take into account intratumoral heterogeneity. Furthermore, since the probability of the presence of LOH of the wild-type allele by chance is not null, it seems to be important to explore the LOH status on several tumors with the same germline variant.

We tested the hypothesis that the inactivation of the wild-type allele at the tumor level could argue in favor of *BRCA1* variant pathogenicity. For this purpose, we evaluated 97 tumor samples (90 breast and 7 ovarian) from carriers of 26 distinct *BRCA1* germline variants (10 pathogenic, 8 VUS, and 8 benign/likely benign variants) using a pipeline (pyrosequencing, NGS, methylation, and BRCAness analysis) to identify genomic markers of *BRCA1* locus-specific LOH and other possible mechanisms of gene inactivation.

Material and methods

Patients and Tumor/DNA Samples

The patients were index cases from high-risk breast and/or ovarian cancer from French, Australian, and Brazilian families with eligibility criteria for screening of *BRCA1/2* mutations according to local consensus statement (DALY; KLEIN; REISER, 2017; EISINGER et al., 2006; "kConFab - Eligibility Criteria", [s.d.]), who had consented for genetic testing and use of their samples for research studies.

Paraffin-embedded tumor pretreatment biopsies from 90 breast cancer patients and 7 ovarian cancer patients carrying 26 distinct *BRCA1* variants were obtained from: the kConFab consortium (n=29), French biological resource centers of Institut Curie (n= 67), Centre Oscar Lambret (n= 1), and A.C. Camargo Cancer Center (n=5). Slides of each tumor specimen, stained with hematoxylin and eosin, were reviewed by a local pathologist, who then performed macrodissection to separate tumor epithelium from the surrounding stroma and healthy tissue and estimated the percentage of tumor cellularity. Tumor DNA extraction from 6-10µm-sections of formalin-fixed paraffin-embedded (FFPE) tissues was performed using a NucleoSpin 8⁄96 Tissue Core Kit (Macherey-Nagel) following the manufacturer's protocol. When available, the correspondent constitutive DNA extracted from patient lymphocytes was used as a reference.

Patient medical records were reviewed in order to access clinical and pathological variables, such as age at onset of the cancer, tumor (SBR) grade (ELSTON; ELLIS, 1991), histologic subtype, staging, ER, PR, and Her2 status of the tumors.

Variant Selection

26 distinct *BRCA1* germline variants were included: 10 pathogenic, 8 VUS and 8 likely benign/benign variants (Table 1 of supplementary data). The criteria for classification were based on the French variant database from the Group "Genetic and Cancer" (GGC, Unicancer) (CAPUTO et al., 2012, 2018). The 8 VUS have been reported in ClinVar, but with low or medium review status. To date, they have a discordant ClinVar clinical significance

(VUS, likely pathogenic, and pathogenic) and remain unclassified based on multifactorial analysis (GOLDGAR et al., 2004b; THOMPSON; EASTON; GOLDGAR, 2003b).

Pyrosequencing

Pyrosequencing was the method applied to detect any allelic imbalance of the variant for the majority (n=76) of tumor samples, as described in supplementary data. This method quantifies the level of the nucleotide at a designated variant locus. The DNA of a patient not carrying the variant in question was used as an internal control. The analysis was performed in triplicate. The patient's tumor result was compared with the correspondent germline result when the latter was available. The allelic imbalance was considered once the variant/wild-type imbalance was above 10%.

Assuming that gene inactivation of both alleles is expected for a pathogenic variant in a tumor suppressor gene, observation of an allelic balance identified for a pathogenic variant was subject to three additional assays in order to further explore the mechanism of tumorigenesis and second allele inactivation: 1) *BRCA1* promoter hypermethylation analysis 2) Next-generation sequencing (NGS) of *BRCA1* for an inactivating variant at the somatic level and 3) BRCAness signature analysis**.**

Next-Generation Sequencing (NGS)

For 6 tumors, LOH analysis was performed only by amplicon-based NGS in Ion Proton platform. LOH was considered when the variant/wild-type allele imbalance was above 10%. Furthermore, 15 additional samples underwent full *BRCA1* screening by NGS in search for any further somatic variant that could represent the "second hit" inactivating the *BRCA1* wild-type allele and also validate the level of the nucleotide quantified by pyrosequencing (MiSeq GeneRead / Qiagen *BRCA1/2*).

***BRCA1* Promoter Hypermethylation Analysis**

First, we used the EpiTectBissulfite Kit (Qiagen) for bisulfite conversion of the tumor DNA. Next, pyrosequencing using PyroMark Q96 evaluated the methylation status of four *BRCA1* promoter CpG sites, according to the manufacturer's protocol (TOST; GUT, 2007).

BRCAness Signature

Lastly, when frozen samples were available (n=12), the presence of the homologous recombination deficiency was assessed. The BRCAness signature was developed on the large state transition (HRD-LST) scores from CytoScan data – signature LST – by Popova et al (POPOVA et al., 2012b).

Statistical analyses

We used the chi-squared test to calculate the probability of samples with pathogenic variants being statistically significantly enriched for loss of wild-type allele when compared to samples of benign variants and to calculate the probability of observing more loss of wild-type according to the effect of the variant at the protein level.

Then, we designed a simulation study to estimate the minimum number of cases and the number of LOH cases which would allow the classification of the variant (Figure 1 Supplementary Data). The first scenario was based on the probability for a pathogenic variant to present an LOH if a number of cases were assessed. The second scenario was based on the probability for a benign variant to present an LOH. We mimicked the number of cases and the number of minimum LOH cases to classify the variant. The threshold was determined to have at least 90% probability to reach the number of LOH with a pathogenic variant and less than 10% probability to reach the number of LOH with a benign variant. Those results were modelled by a binomial distribution,

Results

Clinical, Pathological and Genetic Data

We examined 97 breast/ovarian tumor samples from a total of 93 patients. All 7 ovarian tumors were high-grade serous ovarian carcinomas. The majority of samples corresponded to breast carcinomas (n=90; 93%), but with one ductal carcinoma *in situ*. The

90 breast tumors were mostly ductal (90%), high grade (72%), and triple negative breast cancers (60%) (Tables 1A/B).

These 93 patients carried 26 different *BRCA1* variants (Table 1 Supplementary data, Figure 1): 10 pathogenic, 8 (likely) benign and 8 VUS. Among the pathogenic variants, there were 4 missenses, and 6 nonsense/frameshift (with 1 skipping of exon 23 and 1 large duplication). As shown in Table 1B, 76% of the breast tumors from pathogenic variant carriers were estrogen and progesterone receptor negative. The tumors were mainly grade 3 (45/51, 88%) and diagnosed before age 50 years. In the 65 cases whose Her2 status was assessed, 6 (9%) were Her2 positive breast carcinomas, of which 4 were from pathogenic variant carriers (two over 50 years, 2 with an onset at unknown age) (CURTIT et al., 2015).

Somatic Loss of Wild-Type Allele Correlates with Pathogenic Classification of *BRCA1* Germline Variants

An analysis pipeline was established for identification of genomic markers for *BRCA1* locus-specific LOH by pyrosequencing, NGS, and *BRCA1* functional deficiency (termed BRCAness). Considering the entire cohort (Table 2), pathogenic germline variants presented LOH in 72% of tumors, of which 67% presented with a LOH of wild-type allele. The percentage of samples associated with LOH of the wild-type allele was different according to the nature of the variant. Frameshift variants were more likely to present loss of wild-type allele than missense variants (74% vs 57%, not statistically significant) (Table 2).

Benign germline variants presented with LOH in 37% of the breast and ovarian tumors combined, and only 22% of these were due to loss of the wild-type allele (Table 2).

Considering breast cancer samples (Table 3, Figure 1), LOH was observed in 71% of tumors with a germline pathogenic variant, and loss of the wild-type allele was present in 92% of them. Allelic balance was observed in 28% carrying pathogenic variants. In contrast, LOH was observed in 35% of tumors with germline benign variants, and among the 9 samples that presented LOH, 5 (56%) were due to the loss of the wild-type allele (Figure 1, Table 3).

Of note, loss of wild-type allele was present in all 7 ovarian cancer samples (6 pathogenic and 1 benign variant carrier) (Table 4).

Alternative Second Hit Event for Pathogenic Variants without loss of the wt allele

Allelic balance was observed for tumors from 15 pathogenic variant carriers (Table 2). Complete coding sequencing of *BRCA1/2* in tumor DNA by NGS did not reveal any further *BRCA1* somatic pathogenic variant. One single sample with loss of the variant allele was observed with *BRCA1* promoter methylation (Table 5).

Correlation between LOH Presence and High Genomic Instability Score

The analysis of genomic instability was performed for the establishment of BRCAness score in a set of 12 available samples: 6 pathogenic, 3 benign, and 3 VUS (Table 6). BRCAness score showed a strong correlation with LOH analysis.

Of the 6 pathogenic variants, 4 with loss of wild-type allele had a high BRCAness score and 2 with allelic balance had a low BRCAness score. Of the 3 benign variants, all showed allelic balance and low BRCAness score (all luminal breast cancers).

LOH Analysis of Tumors from VUS Carriers

Fifteen tumor samples from germline missense VUS (8 unique variants) were available for analysis (Table 7A/B). Several tumors with the same variant were available for only 2 variants, both located in the BRCT domain: c.5497G>A (3 samples) and c.4963T>C (5 samples). All 3 samples carrying the variant c.5497G>A presented with loss of the wild-type allele. Four out of 5 samples with c.4963T>C variant presented loss of the wild-type allele. These results were consistent with pathology and co-segregation data which allowed us to lastly classify both c.4963T>C and c.5497G>A variants as pathogenic variants (Table 8) (KOTOULA et al., 2017; PAPAMENTZELOPOULOU et al., 2019).

For the BRCT region VUS c.4841C>T, 2 samples were available but with discordant results: one showed loss of variant and the other loss of wild-type allele.

For 5 VUS, only 1 tumor was available. Three VUS presented allelic balance: c.3074C>T, c.5072C>A and c.5177G>T. The 2 remaining BRCT VUS showed loss of the wild-type allele (c.5057A>G and c.5203G>A.).

For VUS carriers with frozen breast tissue samples available (c.4841C>T and c.3074C>T), we also performed analysis of genomic instability. All three tumors (1 luminal and 2 triple negative breast carcinomas) showed low BRCAness score (Table 5).

Simulation of the minimum number of LOH status for classification

A simulation study was used to estimate the number of cases to predict the classification of the variant based by a binomial distribution (Supplementary table A and B). The pathogenicity is considered once the majority of cases have an LOH.

The first scenario was based on the probability for a neutral variant to present an LOH with less than 5% probability to reach the number of LOH. The first situation to exclude the neutrality is 3 samples with 3 LOH. The second scenario was based on the probability for a pathogenic variant to present an LOH. The threshold was determined to have at least 90% probability to reach the number of LOH with a pathogenic variant and less than 5% probability to reach the number of LOH with a benign variant. The optimal number will be then at least 10 samples. Finally, the optimal number of samples should be between 3 to 10 samples. The majority of LOH can be obtained as soon as 3 samples. If it is not obtained, the pathogenicity cannot be excluded. In the data obtained for the VUS in this article, at 5 cases, the probability to reach 4 LOH is under 1% for a neutral variant which help to exclude a neutral variant (c.4963T>C). The probability is exactly the same for 3 variants with 3 LOH (c.5497G>A). Statistically for those two variants, neutrality can be excluded and pathogenicity is confirmed with the majority of LOH.

Discussion

The loss of the remaining wild-type allele is the last event during tumor progression associated with germline pathogenic *BRCA1* variants and this mainly happens through locus-specific LOH (MARTINS et al., 2012b). LOH of the wild-type allele is the most frequent second-hit event in *BRCA1*-related breast carcinogenesis while LOH in sporadic breast and ovarian cancer is not rare, but the lost allele is random. We hypothesized that the repetitive observation of the loss of the wild-type allele for the same variant should argue in favor of the variant pathogenicity. We analyzed pretreatment tumor biopsies of pathogenic (55 samples) and (likely) benign variant carriers (27 samples). Combining NGS and pyrosequencing, a consistent pattern of predominance in pathogenic variants showed 71% of allelic imbalance with 67% loss of wild-type for pathogenic variants, while (likely) benign variants showed 37% allelic imbalance with 22% loss of wild-type allele. Our results were consistent with a

previous report by Maxwell et al. (MAXWELL et al., 2017b) and the recent report of Yost et al (YOST et al., 2019).

In our cohort, we were able to classify 2 VUS (c.5497G>A and c.4963T>C). While both variants remained unclassified based on multifactorial analysis up until then because we did not have enough data to establish a causality score, most data favored causality (FINDLAY et al., 2018; PAPAMENTZELOPOULOU et al., 2019; PETITALOT et al., 2019; TORREZAN et al., 2018). For the variant c.5497G>A, all 3 samples presented loss of the wild-type allele. For the variant c.4963T>C (also in BRCT domain), 4 out of 5 samples (4 breast cancers and 1 ovarian cancer) presented loss of the wild-type allele and the only sample presenting allelic balance had a low tumor cellularity (30%). The binomial distribution of the probability of LOH in neutral variant helped to exclude the neutrality with a probability of error under 1%. Functional assays argued in favor of pathogenicity for both variants (FINDLAY et al., 2018; WOODS et al., 2016). Here, we were able to gather additional data, which allowed us to establish a causality score sufficient to classify these variants pathogenic (Table 8C). We could further demonstrate a functional impact of the variant c.4963T>C through the destabilization of BRCT domain, which would be another argument in favor of its pathogenicity (data not shown) (PETITALOT et al., 2019).

Regarding the evaluation of the loss of heterozygosity for *BRCA1* variant classification, some

LOH analyses have been reported in isolation, with some authors agreeing on the use of LOH data for such while others do not (BERISTAIN et al., 2010; VAN HEETVELDE et al., 2018). We hypothesized that the repetition of LOH of the wild-type for tumors from carriers of the same variant could help the classification of the VUS. Using a more sensitive approach based on NGS and pyrosequencing, we observed a difference in LOH patterns for known pathogenic and (likely) benign variants. This result, if validated with a much larger sample set, would indicate that LOH pattern seen by NGS may provide additional information for classification of VUS in *BRCA1* if the LOH is observed on several cases with the same unclassified variant. LOH information may be complementary to histopathological features, helping to refine the cases with a low pathology likelihood-ratio. However, we were not able to confirm this hypothesis in our cohort since complete immunohistochemical data was missing in one third of the samples, which is indeed one of the limitations of this study. To confirm this hypothesis, multivariate analysis of LOH and pathology data in a larger number of samples for is required. Our results also confirms that the information on a unique case should not be used alone as an argument for VUS reclassification even to give any orientation.

To reduce the risk of a misleading conclusion, the number of tumor samples should be between 3 to 10 cases.

Nevertheless, the application of this methodology could raise some limitations. Some issues such as non-tumor tissue contamination, low tumor cellularity, low quality of tumor DNA, and tumor heterogeneity, could mask the results. So performing macrodissection to separate tumor tissue from healthy breast tissue was an important step for the identification of LOH when present. Tumor cell heterogeneity may also exist. For one sample carrying the variant c.4535G>T, in accordance with tumor morphological heterogeneity, different patterns of LOH were observed when these different regions were analyzed separately (CURTIT et al., 2015). This observation have been already discussed in the litterature [Salomon et al] with sporadic breast cancer in a germline mutant carrier. This observation is also consistent with previous data of Klaes C. et al concluding that different mechanisms inactivating the wild-type allele may be present within the same tumor at various extents (VAN HEETVELDE et al., 2018). This heterogeneity and technical limitations could also be a challenge to assess the correct status for LOH and explain the need to analyze multiple cases with the same variant. For repeated analysis, the rarity of the variants and the difficulty in grouping families with several tumors carrying the same sequence variation can also be a limitation for this analysis where a minimum number of samples were necessary to reach a conclusion. However, this approach can be performed using stored samples from individuals with multiple primary tumors and from families with many affected individuals, which is generally more feasible than co-segregation studies.

Large scale studies confirmed that most but not all tumors with germline *BRCA1* pathogenic variants have locus-specific LOH (CHENEVIX-TRENCH et al., 2006; SPURDLE et al., 2008a; VAN HEETVELDE et al., 2018). It was noteworthy that some tumor samples from pathogenic variant carriers did not present loss of the wild-type allele. We searched for other inactivation mechanisms in tumors with pathogenic variants but without loss of the wild-type allele (Table 6). The LOH status was reclassified in 5 samples by NGS. No further *BRCA1/2* somatic inactivating variant was identified in tumor samples by NGS. Promoter hypermethylation was identified in 1 sample and a PIK3CA mutation in 5 breast cancer samples (2 of them presented luminal phenotype, not typically related to *BRCA1* carcinogenesis, while histopathology data was not available for the remaining 3). We searched for PIK3CA hot spot mutations in parallel to confirm tumoral cellularity and because it is rarely detected in TNBC BRCA1 breast tumors, then would argue against BRCA1-related tumorigenesis (KOTOULA et al., 2017; SEVERSON et al., 2015). The remaining cases may

be explained by stromal contamination related to a low tumor cellularity (that was less than 30% in 2 samples), DNA sample quality, and limitations of pyrosequencing to identify the allelic imbalance.

We went further, and also evaluated the occurrence of LOH according to the effect of the variant on protein level. LOH was reported for 59% and 74% of pathogenic missense and frameshift/nonsense variants, respectively. Although this difference was not statistically significant, this observation could be in favor of some dominant negative effect of *BRCA1* missense pathogenic variants. Vaclova and colleagues showed that lymphoblastoid cell line of heterozygous BRCT missense variants carriers present a lower level of BRCA1 recruitment into DNA-damaged foci and a higher sensitivity to PARPi than cells with truncating variants or normal cells, suggesting that the intact protein is unable to function normally in the presence of mutant BRCA1 (VACLOVÁ et al., 2016). This trend has also been shown for other DNA repair protein such as ATM, and POLE1 as well as for TP53 (CHENEVIX-TRENCH et al., 2002; MULLER; VOUSDEN, 2014; SCOTT et al., 2002). In fact, there is increasing evidence that a heterozygous BRCA1 pathogenic variants lead to haploinsufficiency of some BRCA1 functions even for the homologous recombinaison activity that happens before the loss of heterozygosis (PATHANIA et al., 2014).

Finally, the absence of LOH could be an argument not in favor of platinum salts or PARPi sensitivity. Currently, germline and somatic *BRCA1/2* pathogenic variants are considered biomarkers of response to platinum salts and PARPi without considering the LOH status (COLEMAN et al., 2017a; KAUFMAN et al., 2014; LEDERMANN et al., 2012; MIRZA et al., 2016b; TUTT et al., 2018b). Although clinical trials report better results in patients carrying germline *BRCA1/2* pathogenic variants, the therapeutic benefit of PARPi differs between BRCA-associated cancers, with the best eficacy seen in patients with ovarian cancer which is probably related to their higher HRD scores. Recent data on ovarian cancer suggest that the finding of a pathogenic variant is not enough to predict primary resistance to these agents and confirms that LOH analysis at the tumor level and the presence of BRCAness phenotype may refine this prescription by identifying those patients who will respond positively (MAXWELL et al., 2017b; TUTT et al., 2018b). As we identified loss of the variant allele in 3 (5%) of pathogenic variant carriers and allelic balance in 15 (26%) – from analysis of breast tumor tissue - we believe that LOH analysis of wild-type allele is an important pre-treatment screening method and the absence of inactivation of the wild-type allele is a potential risk for primary resistance.

The data of our cohort confirms that LOH could also be a biomarker for high homologous recombination (HR) score (ABKEVICH et al., 2012). There is a perfect correlation between the presence of loss of the wild-type allele and high BRCAness phenotype. Those samples presenting allelic balance showed a low BRCAness score. It is noteworthy that in the set of pathogenic variants, 19 of 57 tumors analyzed lacked locus-specific LOH and showed low genomic measures of BRCAness. This raised the question if the identification of a *BRCA1/2* pathogenic variant is indeed enough for treatment decisions since a low HR score may exist even in the presence of a pathogenic variant. Also questioned is the benefit of treating these patients with PARPi. The identification of breast cancer clones LOH free after neoadjuvant chemotherapy by Heetvelde et al suggests a primary resistance of subclones with heterozygous state (VAN HEETVELDE et al., 2018). Since LOH is not as common for breast cancer as for ovarian cancer, and since the benefit of PARPi is not as well established for breast tumors as it is for ovarian cancer, the analysis of LOH status should help to identify the subset of breast cancer patients who derive greatest benefit from PARPi (JONSSON et al., 2019). Currently, the official indication of PARPi is based on the identification of germline pathogenic variants in breast cancer and germline or somatic pathogenic variants in ovarian cancer. Even if new clinical trials have now introduced the notion of HR score to extend the indication to non BRCA mutated (GONZÁLEZ-MARTÍN et al., 2019; RAY-COQUARD et al., 2019), there is no mention of the inactivation of the second allele as the proof of the total inactivation of the gene.

In conclusion, these results emphasize that tumors associated with *BRCA1* germline variants should not be considered uniformly from a tissue, pathologic, morphological and genetic point of view. We propose to incorporate LOH data for variant pathogenicity prediction, since tumoral sequencing, LOH information and HRD score is increasingly available with the PARPi indications. Besides being a complementary argument to help in the classification of *BRCA1* variants, LOH could be used as additional biomarkers of response to PARPi even with *BRCA1* pathogenic variants.

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Legends

Figure 1 - Summary of BRCA locus-specific LOH status of breast and ovarian tumors from individuals with germline *BRCA1* variants.

Figure 2 - A/ LOH analysis of benign and pathogenic variant samples. Samples presenting loss of wild-type allele are in green. Samples with allelic balance are in blue. Samples with loss of the variant allele are in red. B/Comparison of wild-type allele loss in samples of pathogenic variants vs samples of benign variants.

Figure 3 - Pedigree of the family carrying the *BRCA1* c.4963T>C variant, showing co-segregation of the variant with breast and ovarian cancers.

Table 1:A/Number of breast and ovarian samples analyzed for each variant category. B/Clinical and pathological data of the breast carcinoma cohort.

A/

	Pathogenic	(likely) Benign	VUS*	Total
Breast	51	26	13	90
Ovary	4	1	2	7

B/

	Pathogenic	(likely) Benign	VUS*	Total
Invasive	51	25	13	89(99%)
In situ	0	1	0	1 (1%)
		TYPE		
Ductal carcinoma	48	22	11	81 (90%)
Other types	3	4	2	9 (10%)
		GRADE		
Grade 1	1	5	0	6 (7%)
Grade 2	4	10	2	16 (17%)
Grade 3	45	10	9	64 (70%)
Unknown	1	1	2	4 (4%)
		ESTROGEN RECEPTOR		
Positive	7	21	5	33 (36%)
Negative	39	4	8	51 (57%)
Unknown	5	1	0	6 (6%)
		PROGESTERONE RECEPTOR		
Positive	5	16	3	24 (26%)
Negative	39	6	9	54 (61%)
Unknown	7	4	1	12 (13%)
		HER2 STATUS		
Positive	4	1	1	6 (7%)
Negative	26	21	10	57 (64%)
Unknown	21	4	2	27 (29%)
		AGE		
<50 years	20	16	6	42 (47%)
>=50 years	4	9	5	18 (20%)
Unknown	27	1	2	30 (33%)

*Variant of uncertain clinical significance

Variant Classification	Total tumors analyzed	Allelic balance	Loss of variant allele	Loss of wt allele	P value
Likely (Benign)	27	17 (63%)	4 (15%)	6 (22%)	0.0001236
Pathogenic	55	15 (26%)	3 (5%)	37 (67%)	- *0.2083*
Nonsense/Frameshift	*34*	*7 (20%)*	*2 (6%)*	*25(74%)*	
Missense	*21*	*8 (36%)*	*1(5%)*	*12 (57%)*	-

Table 2: Proportion of breast/ovarian samples presenting loss of wt allele among pathogenic (considering variant effect at the protein level) and (likely) benign variants

Table 3: LOH **Breast cancer** results from pyrosequencing or NGS experiments for pathogenic (P), benign (B) and likely benign (LB) variants. MS=missense; NS=nonsense; SPL= splicing; SYN=synonymous

Variant nomenclature	Protein nomenclature	Impact	Variant class	Allelic balance	Loss of variant allele	Loss of wt allele	Total	% of allelic imbalance/LOH	% LOH wt
c.68_69del	p.Glu23Valfs*17	FS	P	2	0	7	9	78%	100%
c.131G>T	p.Cys44Phe	MS	P	2	0	1	3	33%	100%
c.181T>G	p.Cys61Gly	MS	P	5	0	6	11	55%	100%
c.962G>A	p.Trp321Ter	NS	P	0	1	1	2	100%	50%
dupEx3-8 (c.81_547dup)	p.Gly183Valfs*4	FS	P	1	0	1	2	50%	100%
c.5095C>T	p.Arg1699Trp	MS	P	1	0	2	3	67%	100%
c.5123C>A	p.Ala1708Glu	MS	P	0	1	0	1	100%	-
c.5266dupC	p.Gln1756Profs*74	NS	P	3	1	13	17	84%	87%
c.5324T>G	p.Met1775Arg	MS	P	0	0	2	2	100%	100%
c.5453A>G	splicing exon 23 (p.(Gly1803Glnfs*11))	FS	P	1	0	0	1	-	-
Total pathogenic	-	-	P	15	3	33	51	71%	92%
c.1067A>G	p.Gln356Arg	MS	B	4	2	1	7	43%	33%
c.2477C>A	p.Thr826Lys	MS	B	1	1	0	2	50%	-
c.4535G>T	p.Ser1512Ile	MS	B	2	1	2	5	60%	66%
c.4812A>G	p.Gln1604Gln	SYN	LB	2	0	1	3	33%	100%
c.4955T>C	p.Met1652Thr	MS	LB	1	0	0	1	-	-
c.4956G>A	p.Met1652Thr	MS	B	5	0	0	5	-	-
c.5117G>C	p.Gly1706Ala	MS	B	1	0	1	2	50%	100%
c.5531T>C	p.Leu1844Pro	MS	B	1	0	0	1	-	-
Total (likely) benign	-	-	B/LB	17	4	5	26	35%	56%

Table 4: LOH **ovarian cancer** results from pyrosequencing or NGS experiments for pathogenic (P), benign (B) and likely benign (LB) variants. MS=missense; FS=Frameshift

Mutation	Protein	Variant class	Impact	Allelic balance	Loss of variant allele	Loss of wt allele	Total	% of allelic imbalance	% LOH wt
c.181T>G	p.Cys61Gly	P	MS	0	0	1	1	100%	100%
c.2477C>A	p.Thr826Lys	B	MS	0	0	1	1	100%	100%
dupEx3-8 (c.81_547dup)	p.Gly183Valfs*4	P	FS	0	0	1	1	100%	100%
c.5266dupC	p.Gln1756Profs*74	P	FS	0	0	1	1	100%	100%
c.5324T>G	p.Met1775Arg	P	MS	0	0	1	1	100%	100%

Table 5: Alternative second allele inactivation mechanism for pathogenic variants of breast cancer samples without loss of the wild-type allele by pyrosequencing. NGS was able to identify 5 additional cases with allelic imbalance.

Variant	Co-occurence BRCA1/2 germline pathogenic variant	Promoter methylation	LOH NGS	Additional variant with tumoral BRCA1/2 sequencing	BRCAness analysis	PIK3CA mutation	TNBC	Conclusion
c.68_69del	No	No	No	No	Low	No	Unknown	Allelic balance
c.68_69del	Unknown	No	-	-	-	No	Yes	Allelic balance
dupEx3-8	No	No	-	-	-	-	Yes	Allelic balance
c.131G>T	Unknown	No	No	No	-	No	Yes	Allelic balance
c.131G>T	Unknown	No	-	-	-	No	Yes	Allelic balance
c.181T>G	No	No	No	-	No	**Yes**	No	Allelic balance
c.181T>G	Unknown	No	No	-	-	**Yes**	Unknown	Allelic balance
c.181T>G	Unknown	No	No	-	-	**Yes**	Unknown	Allelic balance／
c.181T>G	Unknown	No	-	-	-	**Yes**	Unknown	Allelic balance
c.181T>G	Unknown	No	-	-	-	**No**	Unknown	Allelic balance
c.5123C>A	Unknown	**Yes**	-	-	-	No	Yes	Loss of variant allele／Pr methylation
c.5266dup	Unknown	No	No	No	-	No	Yes	Unknown
c.5095C>T	No	No	-	-	-	NE	Unknown	Unknown
c.5266dup	Unknown	NE	NE	NE	NE	No	No	Allelic balance
c.5266dup	No	No	-	No	-	No	Yes	Unknown
c.5266dup	Unknown	No	NE	NE	-	NE	Unknown	Unknown
c.5453A>G	No	No	No	No	Low	**Yes**	No	Allelic balance／

NE= Not exploitable

Table 6: Correlation between the presence of locus-specific LOH and the genomic instability *BRCAness* score

Variant	Variant Classification	Type of tumor	Conclusion LOH (Pyrosequencing /NGS)	BRCAness	LOH BRCA1 (SNP array)	LOH BRCA2 (SNP array)
c.68_69delAG	P	Breast	Allelic balance	Low	No	No
c.5324T>G	P	Breast	Loss of wt	High	Yes	Yes
c.5324T>G	P	Ovary	Loss of wt	High	Yes	Yes
c.5453A>G	P	Breast	Allelic balance	Low	No	No
c.962G>A	P	Breast	Loss of wt	High	Yes	Yes
c.962G>A	P	Breast	Loss of wt	High	Yes	Yes
c.4956G>A (p.Met1652Thr)	B	Breast	Allelic balance	Low	No	No
c.4956G>A (p.Met1652Thr)	B	Breast	Allelic balance	Low	No	No
c.4956G>A (p.Met1652Thr)	B	Breast	Allelic balance	Low	No	No
c.3074C>T (**p.**Thr3025Ile)	VUS	Breast	Allelic balance	Low	Yes	**No**
c.4841C>T (p.Pro1614Leu)	VUS	Breast	Loss of variant allele	Low	Yes	No
c.4841C>T (p.Pro1614Leu)	VUS	Breast	Loss of wt	Low	No	No

P= Pathogenic, B= Benign, VUS=Variant of Uncertain Significance

Table 7: A/ LOH results for tumour samples from variant of uncertain significance (VUS) carriers. B/Available evidence about the VUS analyzed in this study MS=missense

A/

Variant	Protein	Variant class	Impact	Type of tumor	LR pathology	Allelic balance	Loss of wt allele	Loss of variant allele	Total	% of allelic Imbalance/LOH	% LOH wt
c.3074 C>T	p.Thr1025Ile	VUS	MS	Breast	4,41	1	0	0	1	-	-
c.4841C>T	p.Pro1614Leu	VUS	MS	2 Breasts		0	1	1	2	100%	50%
c.4963T>C	p.Ser1655Pro	VUS	MS	4 Breasts, 1 ovary	152.88	1	4	0	5	80%	100%
c.5057A>G	p.His1686Arg	VUS	MS	Breast	3,73	0	1	0	1	100%	100%
c.5072C>A	p.Thr1691Lys	VUS	MS	Breast	4.41	1	0	0	1	-	-
c.5177G>T	p.Arg1726Ile	VUS	MS	Breast	0.21	1	0	0	1	-	-
c.5203G>A	p.Glu1735Lys	VUS	MS	Breast	0.64	0	1	0	1	100%	100%
c.5497G>A	p.Val1833Met	VUS	MS	2 breasts, 1 ovary		0	3	0	3	100%	100%

B/

Variant	Protein nomenclature	Functional domain	dbSNP	Frequency gnomAD (V2.1.1)	SIFT	Prior probability*	References
c.3074C>T	**p.Thr3025Ile**	-	rs397509034	-	0.26	0,02	-
c.4841C>T	**p.Pro1614Leu**	-	rs766305255	ALL:0.0012% - NFE:0.0027%	0.03	0,02	
c.4963T>C	**p.Ser1655Pro**	BRCT1	rs1057518639	-	0.01	0,03	(CARRARO et al., 2013; TORREZAN et al., 2018)
c.5057A>G	**p.His1686Arg**	BRCT1	rs730882166	-	0	0,29	(BOUWMAN et al., 2013; PETITALOT et al., 2019)
c.5072C>A	**p.Thr1691Lys**	BRCT1	rs80357034	-	0	0,81	(BOUWMAN et al., 2013; LEE et al., 2010; PETITALOT et al., 2019; THOMPSON et al., 2016; WOODS et al., 2016)
c.5177G>T	**p.Arg1726Ile**	BRCT1	rs786203547	-	0.07	0,03	(PETITALOT et al., 2019)
c.5203G>A	**p.Glu1735Lys**	BRCT1	rs397509238		0	0,66	(PETITALOT et al., 2019)
c.5497G>A	**p.Val1833Met**	BRCT1	rs80357268	ALL:0.00041% - NFE:0.00090%	0.01	0,03	(CARVALHO et al., 2009; FINDLAY et al., 2018; KARCHIN et al., 2007; NIKOLOPOULOS et al., 2007; PETITALOT et al., 2019; WOODS et al., 2016)

*Vallée 2016

Table 8: Evidence of pathogenicity of the unclassified variant *BRCA1* c.4963T>C. A/ Loss of heterozygosity analysis of breast and ovarian carrier's tumors of family 1; B/ Clinical, pathological and co-segregation data available for the variant *BRCA1* c.4963T>C; C/ Classification of the *BRCA1* VUS c.4963T>C and c.5497G>A on the basis of multifactorial score

A/

Patient	Tumor	% of viable tumor cells	Tumor Histology	VAF Tumor	LOH
1	1	60%	Triple negative breast invasive carcinoma of no special type	65%	Yes
	2	30%	Triple negative breast invasive carcinoma of no special type	49%	No
2	1	70%	Triple negative breast invasive carcinoma of no special type	88%	Yes
	2	90%	Ovarian high grade serous carcinoma	67%	Yes
3	1	90%	Positive Lymph Node from Luminal breast cancer (ER/PR positive, HER 2 negative)	70%	Yes

VAF – Variant allele frequency

B/

Family	Origin	Index case history of cancer	Family history of cancer	Co-segregation data
F1	Brazil	TNBC (29y and 45)	Sister (Luminal BC 29y); 1 paternal aunt (HGSOC 45y and TNBC 60y); 2 paternal aunts with breast cancer (59y and 80y) ; 1 paternal uncle with prostate cancer	The variant was identified in 5 affected individuals and in 4 unaffected individuals
F2	United Kingdom	TNBC (47y)	Paternal aunt (ovarian ca 49y), 2 paternal aunt (Breast ca 50y), paternal cousin (Bilateral breast ca 29y and 37y)	The variant was identified in 2 affected individual
F3	United Kingdom	Breast ca (37y)	Maternal and paternal history of breast cancer	Not available
F4	United Kingdom	Breast ca (31y)	Not available	Not available
F5	United Kingdom	HGSOC	Not available	Not available
F6	United Kingdom	Ovarian ca (48y)	Mother (ovarian ca at 45y); maternal grandmother (ovarian ca 71y); maternal uncle (prostate ca 55y)	The variant was identified in 2 affected individuals

TNBC=triple-negative breast cancers ; HGSOC= high grade serous ovarian carcinoma ; BC=breast cancer ; ca= cancer

C/

Variant	Prior probability	Segregation	Tumor pathology	Family History	Odds for Causality	Posterior Probability of Pathogenicity	Class
c.4963T>C p.Ser1655Pro	0.03	68.44	152.88	8.71	91176,32	0.9996	5
c.5497G>A p.Val1833Met	0.03	In progress					

4

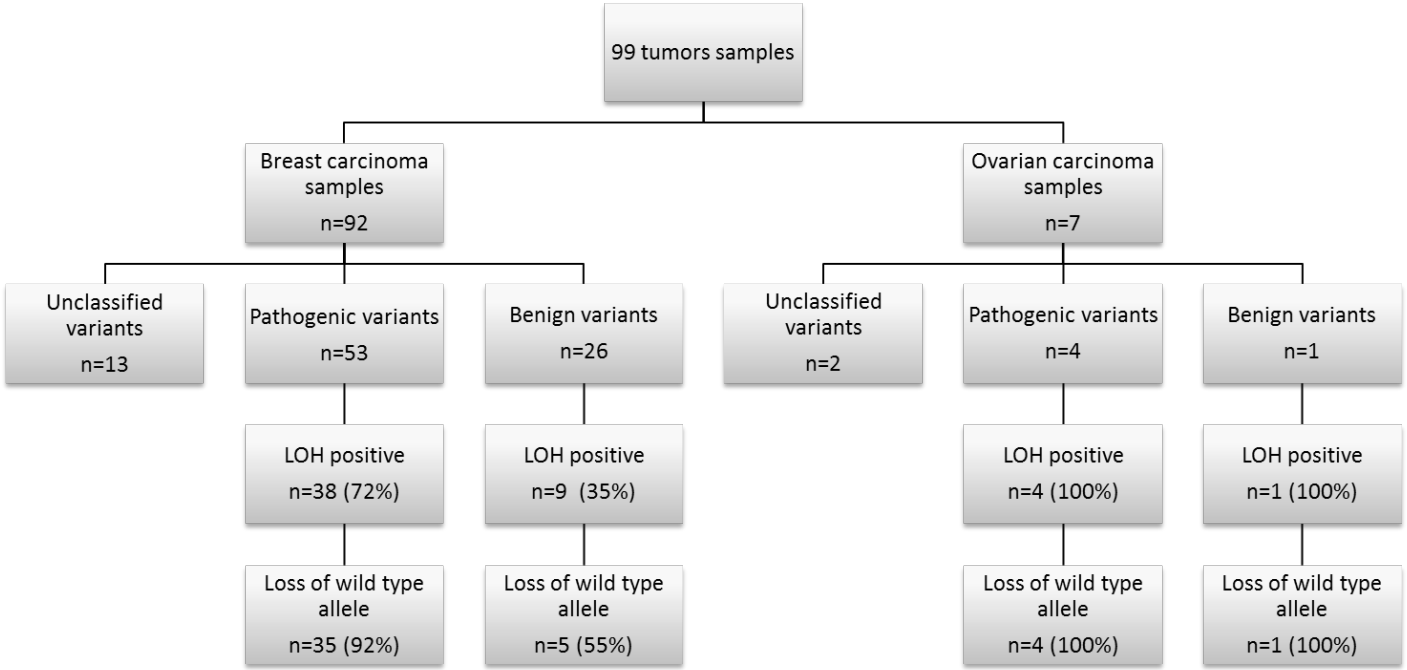


Figure 1. Summary of BRCA locus-specific LOH status of breast and ovarian tumors from individuals with germline BRCA1 variants

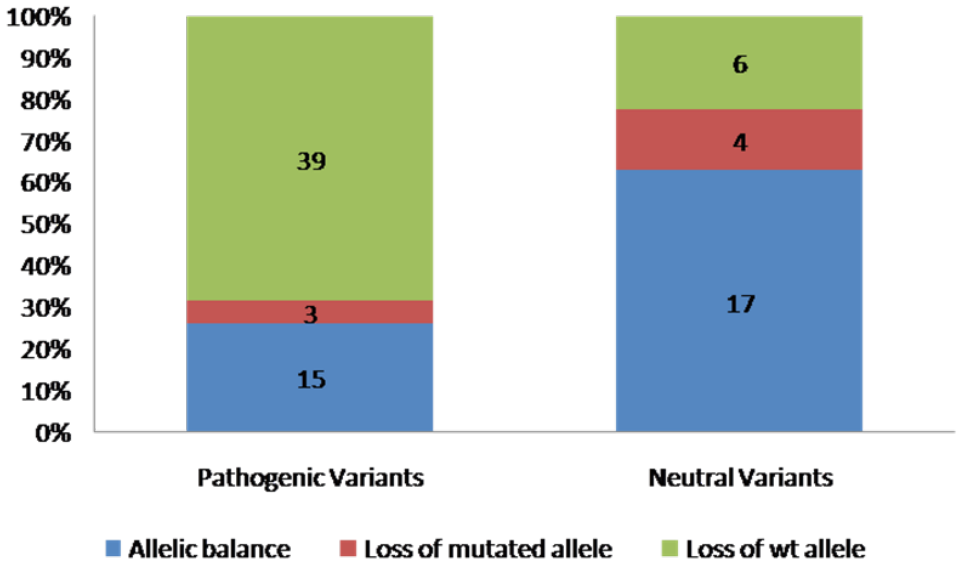


Figure 2A: LOH analysis of neutral and pathogenic variant samples. Samples presenting loss of wild-type allele are in green. Samples with allelic balance are in blue. Samples with loss of mutated allele are in red.

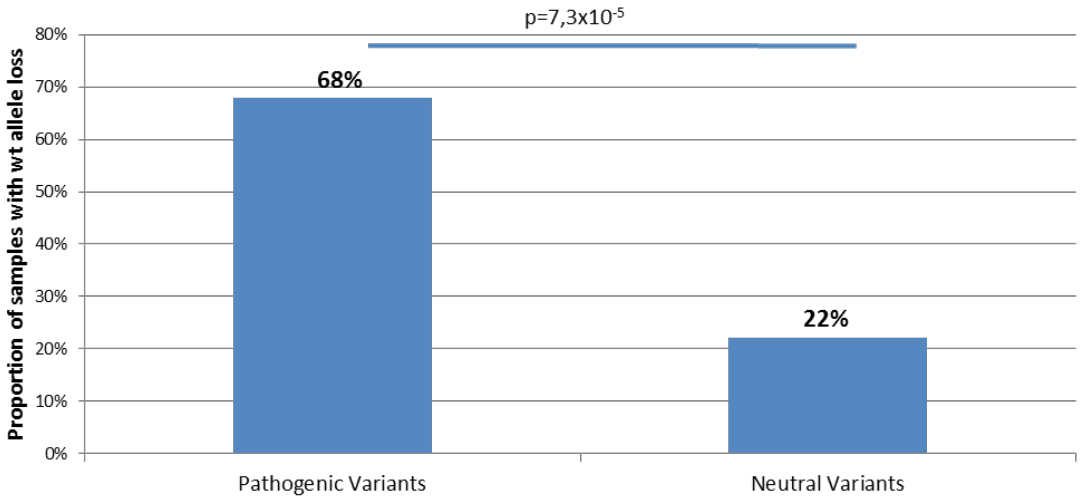


Figure 2B: Comparison of wild-type allele loss in samples of pathogenic variants vs samples of neutral variants. P value of Chi square test is shown.

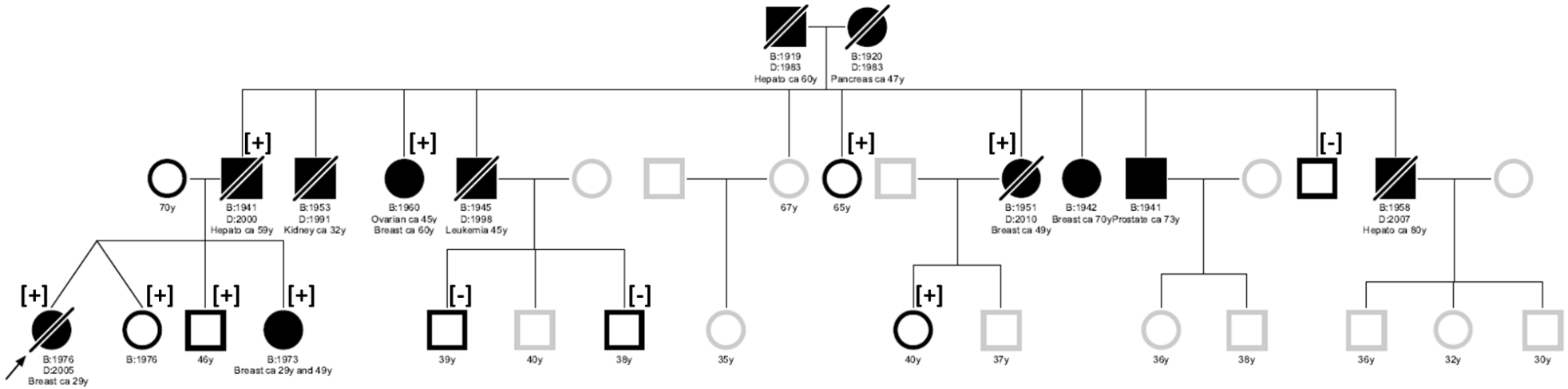


Figure 3: Pedigree of the family carrying the c.4963T>C variant, showing co-segregation of the variant with breast and ovarian cancers.

Supplementay data

Material and methods

Pyrosequencing

The method applied to detect any allelic imbalance of the variant was pyrosequencing for the majority of tumor samples. This method quantifies the level of the nucleotide at a designated variant locus. A mixture (10µl of PCR product, 7µl of Streptavidin Sepharose beads, 25µl of nuclease free water,40 µl of PyroMark binding buffer) was agitated during 10 minutes at 1650rpm to bind PCR products to the beads. The beads were then captured using the vacuum workstation, washed in 40ml of 70% ethanol for 3 s, denatured by denaturation buffer for 3s, and then washed in wash buffer for 5s. The beads were then released and the purified DNA samples were annealed to the sequencing primer in 25µl of annealing buffer for 2 min at 85°C and cooled at room temperature for 10 min. Pyrosequencing was then performed according to manufacturer protocol on a QiagenPyromark Q24 system. Pyrograms were manually interpreted using the Pyromark Q24 software. DNA of a patient known not carrying the variant in question was used as an internal control. The analysis was performed in triplicate. The patient's tumor result was compared with her correspondent germline result when the later was available. The allelic imbalance was considered once the mutant/wild type imbalance was superior to 10%.

PI3K Mutation analysis

The PI3K-AKT-mTOR pathway plays a crucial role in breast tumorigenesis. The presence of a mutation in this pathway could indirect suggest that cancer development was not directly related to BRCA1 mutation. Aiming to put in evidence an alternative mechanism of carcinogenesis for tumors of pathogenic mutation presenting allelic balance, we performed a mutation screening of PIK3CA exons 9 and 20 by high resolution melting (HRM) followed by Sanger sequencing for confirmation if a mutation was found. For HRM analysis 10ng of DNA was amplified in a final volume of 10 µl. PCR reactions were performed using LightCycler 480 High Resolution Melting Master.

Next generation sequencing

For 6 tumors, LOH analysis was performed only by amplicon-based NGS in Ion Proton platform. LOH was considered when the variant/wild-type allele imbalance was above 10%. Primers for the c.4963T>C; p.(Ser1655Pro) *BRCA1* variant were design using Primer3 software (Untergasser et al, 2012). Libraries were prepared using Ion Plus Fragment Library Kit (Thermo Fisher Scientific) after PCR amplification. Sequencing was performed in the Ion Proton platform (Thermo Fisher Scientific), according to the manufacturer's instructions. Mapping of sequencing reads and variant calling were performed using the Torrent Suite Browser and TVC (Thermo Fisher Scientific). Reference/variant bases coverage and frequency were inspected and annotated manually, using the Integrative Genomics Viewer (IGV) software (Robinson et al, 2017).

Simulation study to determine the minimum number of samples that should be analyzed for variant classification

Simulation studies to assess the probability to have a number of LOH depending on number of samples analyzed. The main hypothesis is that the binomial law is followed by the variable.

In our model, the variable "WT LOH" follows the binomial distribution with parameters $n \in \mathbb{N}$ and $p \in [0,1]$ $X \sim B(n, p)$. The probability of getting exactly k WT LOH in n independent samples is given by the formula :

$$f(k,n,p) = \Pr(k;n,p) = \Pr(X = k) = \binom{n}{k} p^k (1-p)^{n-k}$$ with

$$\binom{n}{k} = \frac{n!}{k!(n-k)!}$$

The classification of pathogenicity of the BRCA1 variant in breast and ovarian cancer should be a probability at more than 99% for conclude with a pathogenic variant and less than 1% for neutral variant – cases in grey. For pathogenic variant, the risk is to exclude the

pathogenicity because the majority of LOH is not obtained by change. For a neutral variant, the risk is not to exclude the neutrality due to a majority of LOH obtained by chance.

Table A) binomial distribution of the probability of the Wild-type Loss of heteregozity (WT-LOH) for a neutral variant To exclude a neutral variant in more than 5% (grey cells), there must be at least 3 cases with an LOH – in this case, the probability in a neutral variant is close to 1%.

Table B) binomial distribution of the probability of WT-LOH for a pathogenic variant. The table show that even with a pathogenic variant, there is still a probability to wrongly exclude the pathogenicity. In fact, the goal is to get a majority of samples with LOH (more than 50%). The table conclude that is not possible to achieve the majority with 99% of cases. To conclude to pathogenicity with a majority of LOH in 90% (grey cells), 10 samples are needed. In bold, the cases were both conditions of neutrality (less 5%) et pathogenicity (more than 90%) are achieved.

Finally, the optimal number of samples should be between 3 to 10 samples. The majority of LOH can be obtained as soon as 3 samples.

A)

Number of WT LOH with a neutral variant

Number of analyzed samples

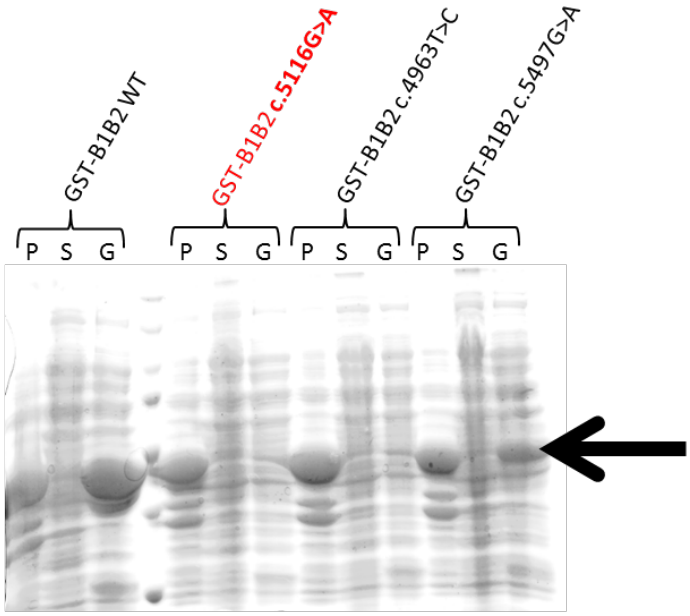
	1	2	3	4	5	6	7	8	9	10
1	0,22	-	-	-	-	-	-	-	-	-
2	0,40	0,05	-	-	-	-	-	-	-	-
3	0,53	0,13	**0,01**	-	-	-	-	-	-	-
4	0,63	0,22	0,04	-	-	-	-	-	-	-
5	0,72	0,31	0,08	0,01	-	-	-	-	-	-
6	0,78	0,40	0,13	0,02	-	-	-	-	-	-
7	0,83	0,48	0,19	0,05	0,01	-	-	-	-	-
8	0,87	0,56	0,25	0,08	0,02	-	-	-	-	-
9	0,90	0,63	0,32	0,12	0,03	0,01	-	-	-	-
10	0,92	0,69	0,39	0,16	**0,05**	0,01	-	-	-	-
11	0,94	0,74	0,46	0,21	0,08	**0,02**	-	-	-	-
12	0,95	0,78	0,52	0,27	0,11	**0,03**	0,01	-	-	-
13	0,96	0,82	0,58	0,32	0,14	**0,05**	**0,01**	-	-	-
14	0,97	0,85	0,63	0,38	0,18	0,07	**0,02**	-	-	-
15	0,98	0,88	0,68	0,44	0,23	0,09	**0,03**	**0,01**	-	-
16	0,98	0,90	0,72	0,49	0,27	0,12	**0,05**	**0,01**	-	-
17	0,99	0,92	0,76	0,54	0,32	0,16	0,06	**0,02**	**0,01**	-
18	0,99	0,93	0,80	0,59	0,37	0,19	0,08	**0,03**	**0,01**	-
19	0,99	0,95	0,83	0,64	0,42	0,23	0,11	**0,04**	**0,01**	-
20	0,99	0,96	0,85	0,68	0,47	0,27	0,14	0,06	**0,02**	**0,01**

B)

Number of WT LOH with a pathogenic variant

Number of analyzed samples

	1	2	3	4	5	6	7	8	9	10
1	0,68	-	-	-	-	-	-	-	-	-
2	0,90	0,47	-	-	-	-	-	-	-	-
3	0,97	0,76	0,32	-	-	-	-	-	-	-
4	0,99	0,90	0,62	0,22	-	-	-	-	-	-
5	1,00	0,96	0,82	0,50	0,15	-	-	-	-	-
6	1,00	0,99	0,92	0,71	0,39	0,10	-	-	-	-
7	1,00	0,99	0,96	0,85	0,61	0,30	0,07	-	-	-
8	1,00	1,00	0,99	0,93	0,78	0,51	0,23	0,05	-	-
9	1,00	1,00	0,99	0,97	0,88	0,69	0,42	0,17	0,03	-
10	1,00	1,00	1,00	0,99	**0,94**	0,82	0,61	0,34	0,13	0,02
11	1,00	1,00	1,00	0,99	0,97	**0,90**	0,75	0,52	0,27	0,09
12	1,00	1,00	1,00	1,00	0,99	**0,95**	0,86	0,68	0,44	0,22
13	1,00	1,00	1,00	1,00	0,99	**0,97**	**0,92**	0,80	0,61	0,37
14	1,00	1,00	1,00	1,00	1,00	0,99	**0,96**	0,88	0,74	0,53
15	1,00	1,00	1,00	1,00	1,00	0,99	**0,98**	**0,93**	0,84	0,67
16	1,00	1,00	1,00	1,00	1,00	1,00	**0,99**	**0,96**	**0,90**	0,79
17	1,00	1,00	1,00	1,00	1,00	1,00	0,99	**0,98**	**0,95**	0,87
18	1,00	1,00	1,00	1,00	1,00	1,00	1,00	**0,99**	**0,97**	**0,92**
19	1,00	1,00	1,00	1,00	1,00	1,00	1,00	**1,00**	**0,98**	**0,95**
20	1,00	1,00	1,00	1,00	1,00	1,00	1,00	1,00	**0,99**	**0,97**



Supp Figure 1: **Classification of the VUS as a function of the impact of the corresponding missense variations on BRCT domain expression in *E. coli*.** Mutated BRCT domains fused to GST were expressed in *E. coli* and purified by affinity chromatography using glutathione beads. This figure shows a SDS-PAGE gel with samples from the bacterial pellet (P), supernatant (S) and the glutathione beads (G) after incubation with the supernatant and washing. The arrow indicates the position of the protein GST-B1B2.

4.1 3.3 Mutation analysis in optimal responders to chemotherapy

3.3.1 Background – Exceptional responders and biomarkers

Dysregulation of DNA damage repair (DDR) processes is a common phenomenon in cancers, known to be associated with breast and ovarian hereditary cancers. Interestingly, DDR defects are not only important to understand the carcinogenic process, but may also be used to optimize therapy response, providing options for therapeutic intervention. HR defects have been frequently described in hereditary breast and ovarian cancers, and have been associated with optimal response to DNA damaging targeting agents, such as platinum salts and PARPi. But to date, only *BRCA1/2* coding pathogenic variants have been validated as biomarkers for treatment choice for ovarian, breast, pancreatic and prostate cancers.

However, it has been demonstrated that a wider population, currently identified by high genomic instability scores, present optimal response to these agents. Signatures of HR deficiency including the analysis of genomic rearrangements as well as RNA expression have been proposed for this purpose (Popova et al., 2012). Clinical trial results, although incipient, suggest that other HR genes alterations are also predictors of optimal treatment response. We were therefore interested in exploring alternative mechanisms to *BRCA1/2* coding pathogenic variants for inactivating DNA repair, to better understand the hereditary predisposition mechanism (when it exists) and to expand the population of cancer patients who benefit from targeting DNA damage response in cancer therapy.

3.3.2 Hypothesis

The choice of exceptional responders should enrich in causal alterations. In view of the optimal response to platinum-based chemotherapy, the molecular alterations identified in this population have high chances of being responsible for homologous recombination pathway inactivation.

3.3.3 Summary results and concluding remarks

To validate our hypothesis, we worked on a cohort of 43 ovarian tumors of patients who experienced a complete or near complete response to platinum based neoadjuvant

chemotherapy and without *BRCA1/2* germline pathogenic variants. We were able to confirm the hypothesis that this is indeed a population enriched of DDR related genes alterations, mainly in HR genes. Not only a higher rate of germline and somatic *BRCA1* and *BRCA2* pathogenic variants were identified, but also tumors with defects in other genes of the same genetic pathway and epigenetic forms of gene silencing. We mainly identified point mutations even if the technique was able to detect large rearrangements. Moreover, it is a population with a high prevalence of HR variants of uncertain significance that, once classified, may further increase the rate of inactivating DNA damage repair mechanism elucidation. These results are useful for selecting additional patients for ongoing and future clinical trials with PARP inhibitors. In addition to this relatively short-term translational achievement, HR alterations beyond *BRCA1/2* may be be useful to select patients for PARP inhibitor treatment in general oncology practice for breast and ovarian cancers, as well as other tumor types. Furthermore, the molecular alterations here identified may help to distinguish patients with greater benefit from receiving chemotherapy as their first treatment (neoadjuvant) even if they have potentially ressectable disease.

This study explored tumor molecular alterations of patients presenting optimal response to platinum- based treatment and reinforced the relevance of exploring new biomarkers of response to improve the selection of patients with therapeutic benefit. In view of our results, it seems relevant to check also epigenetic HR alterations, such as *BRCA1* promoter hypermethylation status and probably hypermethylation of other promoter genes, such as RAD51C. It would be also interesting to confirm the inactivation of HR pathway through analysis of HRD scores and to check if microssatelite instability is present in correlation with pathogenic variants of MMR genes. All these raised work points are in progress.

Mutation analysis of ovarian carcinoma patients presenting optimal response to neoadjuvant chemotherapy

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Background: Neoadjuvant chemotherapy (NAC) followed by interval debulking does not present inferior results to those of primary cytoreduction and offers the opportunity to evaluate chemo-sensitivity *in vivo*. Chemotherapy response score (CRS) have been shown to correlate with outcome with a complete or near-complete (CRS3) response predicting improved progression-free survival. Approximately 20% of ovarian cancers present *BRCA1/2* mutations, which predict a better response to platinum salts. Our proposal is to determine the prevalence of *BRCA1/2* or other homologous recombination (HR) gene mutations, and search for other molecular mechanisms of HR inactivation which could explain the great sensitivity to platinum salts.

Methods: Retrospective analysis of clinical, pathological and sequencing data of patients who experienced a complete or near-complete response to platinum-based NAC was performed., When tumor samples were available for patients with no *BRCA1/2* pathogenic variant identified, tumoral analysis was performed based on Next Generation Sequencing (NGS) comprising a DNA damage repair (DDR) related panel, mainly associated with homologous recombination DNA repair pathway (*BRCA1, BRCA2, ATM, BARD1, BRIP1, CCNE1, CDK12, CHEK2, PALB2, RAD51C, RAD51D, MLH1, MSH2, PMS2, TP53,* including non-coding regulatory regions of *BRCA1*, *BRCA2* and *RAD51C*). In parallel, we performed *BRCA1* promoter methylation analysis by ddPCR, immunohistochemistry on tumoral samples to determine immune co-regulators expression, and a functional RAD51 assay able to discriminate homologous recombination deficient (HRD) tumors.

Results: A total of 43 patients were identified who demonstrated CRS3 post-NAC. A majority of patients had stage III disease, (67%), of either serous histology or poorly differentiated

adenocarcinoma (70%). The median progression free survival (PFS) of the entire cohort was 48 months and the PFS of patients presenting complete response was significantly higher when compared to those presenting near-complete pathological response (24 months x not reached; p=0.0076). No difference in overall survival (OS) was observed. To date, germline and/or somatic analyses were available for 30 patients. The prevalence of pathogenic *BRCA1/2* variants is higher than expected in this cohort of patients presenting a CRS3. In total, 11 of 30 patients (36%) had a germline or somatic pathogenic *BRCA1/2* variant (4 germline, 4 surely somatic, and 3 variants for which origin could not be specified). In addition, among the 17 *BRCA1/2* wild-type WT patients subjected to further NGS analysis, the following alterations were identified in 5 samples (29%): 1 case presenting an ATM nonsense mutation (c.2465T>G; p.Leu822*), 1 pathogenic mutation of CDK12 gene (c.3G>A;p.Met1?), 2 cyclin amplifications, and 1 CHEK2 mutation (c.1671+1_1671del) co-occurent with a MSH2 mutation (c.2021G>A,p.Gly674Asp). Moreover, this cohort is enriched of variants of uncertain significance (VUS), part of them located in *BRCA2* 3'UTR. One *BRCA1* VUS is located on BRCT domain (c.5165C>T p.Ser1722Phe) and could have a deleterious impact. Analysis of *BRCA1* promoter hypermethylation revealed also a higher rate (3/9; [33%]) than the expected.

Conclusion: HGOC patients presenting CRS3 response to neoadjuvant chemotherapy are enriched of BRCA germline/somatic *BRCA* mutations and BRCA1 promoter hypermethylation. In addition, among the subset of BRCA WT CRS3 tumors, additional DDR-related alterations were identified. The prevalence of HR gene mutations can be underestimated in a context of pCR since the somatic screening is then impossible on the debulking material. Somatic *BRCA1/2* and complete DDR related genes sequencing on the initial biopsy may be useful to select EOC patients for PARPi treatment in general oncology practice.

Introduction

Ovarian cancer (OC) is the 7th cause of cancer among women in the world (BRAY et al., 2018) and remains the most lethal gynecological malignancy. Because of its asymptomatic nature, the diagnosis typically takes place in advanced stages, which is reflected in the evolution and lifespan of patients. The overall 5-year survival for stage IIIC patients is only 25-30%, whilst the rate for stage IV stands at 10-15%. Debulking surgery preceded or followed by chemotherapy remains the cornerstone of treatment. Complete cytoreduction, defined by the absence of residual tumor after surgery is the most important prognostic factor for survival (DU BOIS et al., 2009). Two randomized trials have shown that neoadjuvant chemotherapy does not relate to inferior results if compared to those of primary cytoreduction, with the advantage of reduced postoperative morbidity and the opportunity to evaluate *in vivo* chemo-sensitivity (KEHOE et al., 2015; VERGOTE et al., 2010). However, criteria to select patients more likely to present an optimal response, and with greater benefit from neoadjuvant chemotherapy is not well established. Therefore, we sought for biomarkers for treatment response in this context.

The Cancer Genomics Atlas (TCGA) of Ovarian Cancer characterized the main molecular alterations of high-grade serous ovarian carcinomas (HGSOC), which accounts for 90% of epithelial ovarian cancers. These tumors are characterized by high genetic instability, high incidence of DNA copy number variations, and point mutations. Ninety-six percent of tumors have mutations in *TP53* (whose loss of function favors genetic instability) and approximately half of tumors present homologous recombination (HR) pathway dysfunction (CANCER GENOME ATLAS RESEARCH NETWORK, 2011; GURUNG et al., 2013; MITTEMPERGHER, 2016). Clinical data has highlighted an increased sensitivity of *BRCA1/2* deficient tumors to PARP inhibitors and platinum salts, as well as the utility of BRCA mutation in selecting ovarian cancer patients who will better benefit from these treatments. Beyond *BRCA*-mutated group, a significant proportion of ovarian cancers with BRCA-like functional abnormalities (*BRCAness* tumors) also present similar benefit. Approximately a quarter of ovarian-tumors HR deficiency is related to mutations in non-BRCA HR genes, such as *PALB2, BARD1, BRIP1, RAD51C, RAD51D, PTEN, CHEK2* and *CDK12* (PENNINGTON et al., 2014b). Dysfunction in HR pathway also includes aberrant methylation of CpG islands of HR genes. *BRCA1* promoter hypermethylation, and the ulterior reduction or loss of protein expression, are found in 5-31% of ovarian cancers (CATTEAU et al., 1999; ESTELLER et al., 2000; GEISLER et al., 2002). For *BRCA2*, this event is very rare

(HILTON et al., 2002). Other epigenetic defects in ovarian cancer samples, such as promoter hypermethylation of *RAD51C* and *FANCF* have been described (CANCER GENOME ATLAS RESEARCH NETWORK, 2011; CUNNINGHAM et al., 2014; WANG et al., 2006).

Besides DNA repair alterations, a tumor immune profile has emerged as a prognostic marker in ovarian carcinoma. Studies of long-term survivors patients have shown the association of long-term survival with CD8+ and CD3+ tumor infiltrating lymphocytes (TILs) and high expression of MHC2 (DARB-ESFAHANI et al., 2018; GARSED et al., 2018). At least one study showed that high CD3+ TILs and high CD68+ tumor associated macrophages (TAMs) were related to longer overall survival (MORSE et al., 2019). Understanding the association of different immune profiles with neoadjuvant chemotherapy response could help not only to better select patients for neoadjuvant chemotherapy, but could also identify subgroups of patients suitable for immunotherapy combinations.

Given this context, our proposal is to explore the molecular alterations found in tumors of patients who had optimal response to neoadjuvant chemotherapy in the search of biomarkers of response and to select the patients who will better benefit from the administration of platinum salt-based neoadjuvant chemotherapy.

MATERIAL AND METHODS

Patients and Tumor/DNA Samples

Patients were eligible if they had been diagnosed with stage III-IV epithelial ovarian cancer (EOC) and presented CRS3 response after neoadjuvant chemotherapy. The three-tier chemotherapy response score (CRS) has been proposed to stratify EOC patients into complete/near-complete (CRS3), partial (CR2) and no/minimal (CRS1) response after neoadjuvant chemotherapy followed by debulking surgery (BÖHM et al., 2015). CRS3 is a marker of better prognosis and has been defined as complete or near-complete response with no residual tumor in the peritoneum or minimal irregularly scattered tumor foci seen as individual cells, cell groups, or nodules up to 2 mm maximum size (BÖHM et al., 2015; COHEN et al., 2019). Patients included were treated in the department of Gynecologic oncology of Institut Gustave Roussy, Villejuif, France (n=33) and A.C. Camargo Cancer Center, Sao Paolo, Brazil (n=10), between January 1999 and July 2019. Clinical, pathological, and germline sequencing data were retrospectively reviewed.

Paraffin-embedded tumor pretreatment biopsies were used for analysis. Slides of each tumor specimen, stained with hematoxylin and eosin, were reviewed by a local pathologist, who then performed microdissection to separate the tumor epithelium from the surrounding

stroma and healthy tissue in order to estimate the percentage of tumor cellularity. Tumor DNA extraction from 6-10µm-sections of formalin-fixed paraffin-embedded (FFPE) tissues was performed using the tissue preparation system (Siemens Healthcare Diagnostics), as described previously (VAN EIJK et al., 2013). The Qubit dsDNA HS Assay kit (Qubit 2.0 Fluorometer, Life Technologies) and Genomic DNA ScreenTape Analysis (Agilent, Santa Clara, CA, USA) were used for DNA quantification, according to manufacturer's instruction.

Development and validation of the DNA damage response (DDR) related panel

We developed a DDR related panel comprising *BRCA1, BRCA2* and 13 genes frequently mutated in epithelial ovarian cancer (EOC), mainly related to homologous recombination DNA repair pathway (*BRCA1, BRCA2, ATM, BARD1, BRIP1, CCNE1, CDK12, CHEK2, PALB2, RAD51C, RAD51D, MLH1, MSH2, PMS2, TP53*). All genes had been previously identified as germline or somatic mutated in relation to EOC. Regions of interest spanned all protein coding regions and intron-exon boundaries, as well as targeted non-coding regions of *BRCA1, BRCA2* and *RAD51C* promoters and intronic regions of BRCA1 (intron 2 and intron 12). These non-coding regions had previously been defined as most likely functional and then presented a higher probability of containing disease-associated variants (DOS SANTOS et al., 2018; SAUNUS et al., 2008b; TAN-WONG et al., 2008; WARDROP; BROWN; KCONFAB INVESTIGATORS, 2005a). Additionally, to delimit the regions of RAD51C promoter that should be screened, we performed an *in silico* analysis of *RAD51C* promoter in search of conserved regions and of regions with many potential transcription factor binding sites. We also considered experimental data from functional studies available (HINE et al., 2014). The non-coding regions of *BRCA1, BRCA2* and *RAD51C* genes screened in tumoral samples are described in the Table 1.

Tumoral sequencing

For patients with germline *BRCA1/2* wild-type (WT) or unknown, Next-generation sequencing (NGS) was performed using FFPE-isolated tumor DNA with a total input of 200 ng per sample. The fragmentation was mechanic with a Covaris E220 and 240 seconds per sample (Covaris Massachusetts, USA). The mean tumor cell percentage of the samples was higher than 30%. An Agilent Sureselect Custom panel made in SureDesign (Agilent technologies) was used for variant detection with the following gene design : *BRCA1,* exons

1-24, *BRCA2* exons 1-27, *ATM* exons 2-63, *BARD1* exons 1-10, *BRIP1* exons 2-20, *CCNE1 (*only for amplification detection*)* , *CDK12* exons 1-14, *CHEK2* exons 2-15, *PALB2* exons 1-3, *RAD51C* exons 1-9, *RAD51D* exons 1-14, *TP53* exons 1-12, and targeted non-coding regions of *BRCA1, BRCA2* and *RAD51C* genes, which are specified in Table 2.

Library preparation and target enrichment was performed using the SureSelect XT HS Target Enrichment System for Illumina Paired-End Multiplexed Sequencing library reagent kit (Agilent technologies, Santa Clara, California, United States) according to manufacturer's instructions. The captured DNA libraries were sequenced with 16 samples per run using the Illumina MiSeq (Illumina, San Diego, California, United States). The average depth of sequencing was 300X to assure a limit of detection of at least around 5% and coverage of at least 90% at 200X and 100% at 100X. The data analysis pipeline included the following algorithms: BWA-MEM v-0.7.12 for read alignment to the hg19 human reference genome and Samtools v-1.2 and Picard-tools v-1.139 for PCR duplicate quantification and removal. GATK Haplotype v-3.4-46, snpEff v-4.0 and MutaCaller-1.7 were used for variant calling and classification. The pipeline was mainly developed in-house and validated with internal quality compliant to the ISO15189 requirements. Variants were called with a minimum allelic frequency threshold of 1% for already classified variants (those known in the internal database) and 5% for non-classified variants, with a read depth threshold of 30X for the total reads at the variant location and at least 10X for the variant. Several filters were applied to further select for potentially relevant variants among the called variants. The population databases Exac and gnomAd were used to automatically filter out polymorphism as soon as the population frequency was higher than 0.5%. Non-classified variants (not known in the internal database) were excluded if the intra-run recurrence within the 16 analyzed samples per illumina run was superior to 4/16 (25%), as this may be an indicator for an artefact or polymorphism.

Variants were categorized using 5-tier pathogenicity classification : class 1=benign, class 2= likely benign, class 3= variant of uncertain significance (VUS), class 4= likely pathogenic, class 5= pathogenic(PLON et al., 2008b). Variants were annotated on the basis of build GRCH (hg19) using the following transcript numbers : BRCA1, NM_007294.3; BRCA2, NM_000059.3 ; ATM, NM_000051.3; BARD1: NM_000465.4 BRIP1, NM_032043.2; CDK12, NM_016507.3; CHEK2, NM_007194.3; *PALB2: NM_024675.3 ; RAD51C; NM_058216.3* RAD51D, NM_002878.3 and *TP53 : NM_000546.5*.

BRCA1 hypermethylation analysis

DNA was converted with a bisulfite approach using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Detection of BRCA1 promoter methylation was performed on the Naica digital PCR system (Stilla Technologies, France). The principle of the development of the duoplex dPCR assay for the detection of BRCA1 promoter hypermethylation was previously described (JOVELET et al., 2017). Digital PCR reactions were assembled using PerFecTa Multiplex qPCR ToughMix (Quanta Biosciences, Gaithersburg, MD, USA), 40nM FITC (Saint Louis, MO, USA), 1µl of primer and probes multiplex mix, and 3 µl of DNA template. Sapphire prototype (v.1) chips (Stilla Technologies, Villejuif, France) were first primed with PCR oil using the Stilla-loading device. A total of 4 PCR reactions of 20µl each were then loaded per Sapphire chip before being compartmentalized into 15,000 to 20,000 droplets using the Stilla loading device. Finally, the inlet and outlet ports of the Stilla chips were overlaied with Capping oil (Stilla Technologies), prior to thermocycling using the Naica Geode prototype thermocycler. Cycling conditions were 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds and 62°C for 15 seconds. The duoplex was composed with primers: a set was orientated to methylated CpG in BRCA1 promoters and another set was oriented to the same region but not methylated (Figure 1, supplementary data). Sapphire chips containing the 2D crystals of droplets generated were imaged using the Naica Prism3 reader and fluorescent data were analyzed using Crystal Miner software (Stilla Technologies). Each sample was analyzed in duplicate. Standard non-methylated DNA and methylated DNA (EpiTect Control DNA kit, Qiagen, Hilden, Germany) were used as negative and positive controls, respectively. Negatives and positives droplets were discriminated using manual thresholding according to the signal given bythe negative and positive controls included in each individual experiment.

Functional RAD51 assay to determine HR capacity

We obtained formalin-fixed, paraffin-embedded biopsy tissue blocks from the Department of Pathology of both institutions. After pathology review to choose the most representative region of each tumor, we constructed a tissue microarray (TMA) using a 2-mm tissue sections (in triplicate) of each block from pre-treatment biopsy tissue specimens.

Then, we performed immunofluorescence (IF) analysis on 2µm-thick TMA sections following the IF protocol described by Serra et al. Briefly, all sections were heated at 60° C for 1 hour, deparaffinized with xylene and hydrate with decreasing concentrations of ethanol. For antigen retrieval, we used antigen retrieval buffer ph9 (DAKO) heating in a convectional microwave for 20 min, leaving it to cool down at RT for 30 min. Slides were incubated at RT for 60 min with the primary antibody and for 30 min at RT with the secondary fluorescent antibody. DAPI was added before mounting in Dako fluorescence media.

For the moment, RAD51 foci were quantified on 6 TMA best responders tumor samples, by scoring the percentage of geminin-positive cells with ≥5 RAD51 foci par nucleus. Geminin is a cell-cycle regulator that prevents DNA replications, and it is used as counterstaining to mark S/G2- cell cycle phase. Scoring was blindly performed using a 60X/1.3 immersion oil lens (Olympus DP72 microscope). We counted 100 geminin-positive cells, when it was possible, from at least 4-5 representative areas of each sample. The amount of DNA damage was also quantified in all EOC tumor samples by scoring the percentage of geminin-positive cells with γH2AX as described for RAD51 scoring but counting only 50 geminin-positive cells. Similarly, BRCA1 scoring was also done on 50 geminin-positive cells. High RAD51 tumors were considered when the tumor presented ≥ 10% $Geminin^+$ $RAD51^+$ cells. Primary and secondary antibodies used for the detection of the different HR markers are described in Table 2.

Immune co-regulators expression

We performed chromogen-based IHC analysis for the detection of PD-L1, IDO-1, TIM-3 and LAG-3 coregulator immune markers on 2µm-thick TMA sections by using the ultra-automated Discovery Ultra staining system (Ventana Medical Systems, Roche). Briefly, all TMA sections were deparaffinized at 69° C using the EZ Prep, heat pre-treated at 98°C for 4 min in cell conditioning media I (CCI) for antigen retrieval, incubated at RT for 8 min with DISC inhibitor media for endogenous peroxidase inactivation and incubated at RT for 60 min with the primary antibody. Primary antibodies used for the detection of the different immune coregulators are specified in the Table 3. Later, the slides were incubated for 16 min at RT with the secondary antibody followed by the application of HRP multimer for 8 min. The antigen-antibody complexes were detected using a chromogen Ventana detection kit. For each staining run, tonsil was used as positive control and primary omission antibody as negative

control.

Statistical analysis

Descriptive statistics was used to characterize the samples with determination of frequencies, means, medians and measures of central dispersion. Chi-square or Fisher's exact test will be used for the analysis of interaction between categorical variables. Student's t-test or Mann-Whitney test will be used to compare the medians of continuous variables according to the categories of categorical variables. Kaplan-Meier curves will be generated for survival analysis. The impact of different variables, including the presence of homologous recombination gene mutations, will be assessed by the log-rank test. Progression-free survival will be defined as the time from diagnosis to recurrence or death. Overall survival will be defined as the time from diagnosis to death for any cause. Multivariate analysis will be employed to predict the combined impact of independent variables on survival using the proportional hazards Cox regression model. We considered statistically significant the analyzes whose p values were less than 0.05.

Results

Clinicopathological characteristics of the cohort

A total of 43 patients with stage III-IV epithelial ovarian cancer (EOC) presenting CRS3 response after neoadjuvant chemotherapy were retrospectively identified and included in the study: 29 individuals with high-grade serous carcinoma, 7 with low-grade serous carcinoma, 5 with endometrioid carcinoma, 1 with poorly differentiated carcinoma and 1 with clear-cell carcinoma. Table 4 provides clinico and pathological characteristics of the entire cohort, as well as the treatment details.

Most cases were stage III (n=26, 67%), of either serous histology or poorly differentiated adenocarcinoma (70%), and all patients were optimally cytoreduced (to <1 cm maximal residual tumor diameter) at the time of primary surgery. All primary carcinomas received platinum-based chemotherapy, with similar proportions receiving 3 or 6 cycles before surgery. The total number of cycles of NAC depended upon the extent of disease and patient’s tolerance. Only 5 patients (12%) received a NAC regimen including bevacizumab.

Overall homologous-recombination mutation rate

A summary of molecular analyzes performed in the cohort so far, is presented in Figure 1. Results are summarized in Figure 2 and 3. Thirty out of 43 patients had pre-treatment biopsy available for analysis. The prevalence of pathogenic *BRCA1/2* variants in this cohort was higher than expected. In total, 11 out of 30 (36%) patients had a *BRCA1* (n=5) or a *BRCA2* (n=6) pathogenic variants (Figure 2 and table 6). Four out of 11 (13%) were known germline pathogenic variants, while 7 were identified during tumoral sequencing. Four out of 7 were certainly somatic (for the remaining 3 neither germline sequencing nor allelic frequency allowed to conclude whether the variant was surely somatic). For the remaining 19 patients with wild-type *BRCA1/2* sequencing, 2 samples were excluded from the analysis because they did not pass the quality control. Five out of 17 samples (29%), had other DDR related alterations which were mostly HR pathogenic variants: 1 case presenting an ATM nonsense mutation (c.2465T>G; p.Leu822*), 1 pathogenic mutation of CDK12 gene (c.3G>A;p.Met1?), 1 CHEK2 mutation (c.1671+1_1671del) concomitant with a MSH2 mutation (c.2021G>A, p.Gly674Asp), and 2 cyclin amplification (Figure 2 and Table 7). Next, we investigated the presence of variants in non-coding regions of *BRCA1*, *BRCA2* and *RAD51C* genes as as well as coding VUS in *BRCA1/2* and other HR genes that could possibly explain the optimal response to treatment. Finally, 7 out of 13 (54%) wild-type samples presented 9 different variants of uncertain significance (VUS) in the screened genes (Table 4): 2 *BRCA1* (one of them located on BRCT domain (c.5165C>T p.Ser1722Phe) that should be highlighted, and the other in the untranslated transcribed region or 3'UTR), 5 *BRCA2* (2 of them in 3'UTR), 1 *BRIP1* and 1 *CHEK2*. In addition, other non-coding variants were also identified, but this time, in association with pathogenic variants in the coding region of HR genes (Table 5).

Survival data

The median progression-free survival (PFS) of the entire cohort was 48 months (Figure 6). The PFS of patients presenting complete response was significantly higher when compared to those presenting near-complete pathological response (24 months x not reached; p=0.0076) (Figure 7A). Nevertheless, no difference in PFS (p=0.92) or OS (0.78) was observed when we compared patients presenting a *BRCA1/2* mutation, other DDR related or no molecular alteration.

The OS of the entire cohort was not reached. No difference in OS was observed when we compared patients presenting complete or near-complete response to neoadjuvant treatment (p=0.1) (Figure 7B).

Immunoprofiling and co-regulators expression

To date, results concerning the characteristics of lymphocyte infiltrate are pending. Concerning co-regulators receptor expression, data is available for 8 samples. Expression of PD-L1, IDO-1 and LAG-3 is low in this cohort. TIM3 is the more abundant co-inhibitory receptor expressed, present in 6 out of 8 (75%) samples.

BRCA1 promoter hypermethylation

Analysis of BRCA1 promoter hypermethylation also revealed a higher than expected rate (3 out of 9 samples or 33%) (Table 10), when compared to TCGA data (that identified hypermethylation in 15% of the cases). Both hypermethylated samples presented an upper methylation rate higher to 80% which could be considered as biallelic alteration. Both samples were from high grade serous carcinoma with no other deleterious mutation in the HR pathway.

RAD51C functional assay

The RAD51C assay has proven to be highly discriminative of HRD (CASTROVIEJO-BERMEJO et al., 2018). Impaired RAD51 foci formation is predictive of HRD. Thus, we were interested in scoring the percentage of RAD51-positive cells in S/G2-phase of the cell cycle (geminin-positive). Based on previous studies, a 10% RAD51 and BRCA1 score cutoff was used to consider a sample as positive. For the moment we were able to score 6 FFPE samples of the cohort. Three out of 6 samples (50%) scored negative for RAD51 foci: one sample with a *BRCA1* mutation, one with a *BRCA2* mutation and one with no mutation identified. Three out of 6 samples (50%) scored positive for RAD51 foci: one harbored a BRCA2 mutation, another one two concomitant mutations in CHEK2 and MSH2, while in the third one no mutation was identified.

DISCUSSION

Understanding the inactivation mechanism of HR pathway of patients presenting optimal response to platinum-based chemotherapy has the potential to broaden the population who will benefit from therapies targeting DNA damage response, such as platinum salts and PARPi. With this in mind, we performed molecular analysis of pre-treatment biopsy of EOC patients with optimal response to treatment, searching for alternative predictive biomarkers of response beyond *BRCA1/2* mutations. We present here the preliminary data from experiments performed so far. Our results confirmed that this population is indeed enriched of DDR related alterations, with higher rates of *BRCA1/2* mutations than expected in general EOC population, as well as in other HR genes. Seventeen out of 30 (57%) patients presented an alternation (11 in *BRCA1/2*, 2 promoter methylation of *BRCA1*, and 3 other HR genes). This confirms the need to perform an extensive molecular analysis on the initial biopsis of those patients.

The population under study was also enriched of VUS (12 of 30 samples, either in wild-type samples or in association with pathogenic HR variants), notably in 3'UTR of *BRCA1* and *BRCA2*. This finding reinforces the interest to explore non coding variants with potential impact on transcription (being either in pre or post-transcriptional level) related to HR pathway dysfunction. Given the context in which these VUS were identified, inferred deficiency, additional studies should be performed to conclude on their biological significance.

The mutation distribution sounds specific in this cohort of EOC patients with optimal response. In our cohort, we were not able to confirm the somatic origin of *BRCA1/2* mutations in 3 samples. Regardless, the rate of somatic mutations was higher (4 of 30 samples, 12%) than previously described in EOC population. Results from previous studies demonstrate that apparent somatic or germline *BRCA1/2* mutations have the same predictive value of sensitivity to olaparib and platin-based chemotherapy (LEDERMANN et al., 2012). Also noteworthy, is the inversion in the relationship of prevalence of *BRCA1/2* mutations found in this cohort, with a higher prevalence of *BRCA2* than *BRCA1* mutations, for both pathogenic and unclassified variants. If the somatic mutations prevalence is confirmed, this result stresses the importance to analyze samples before any chemotherapy to limit false negative.

Concerning other HR mutations identified, CDK12 is a multipurpose cyclin whose relationship with carcinogenesis is not yet fully established. However, it functions mainly as a transcriptional regulatory factor of several genes through interaction with RNA polymerase II. Studies have concluded association of *CDK12* mutations with homologous recombination deficiency and genomic instability (LUI; GRANDORI; KEMP, 2018). Concerning *ATM* and

CHEK2, they are more "damage sensor" than effector genes. It is intuitive to think that depending on the level of HR pathway inactivation, mutations in different proteins would present higher or lower impact in DNA damage response. Furthermore, PARPi have also shown preclinical and clinical activity for a wider group cancers harboring dysfunctional HRR (KAUFMAN et al., 2014; MATEO et al., 2015a; MCCABE et al., 2006; MIRZA et al., 2016b; PUJADE-LAURAINE et al., 2017b). These mutations may explain better results of DNA damaging agents in BRCAwt population presenting high BRCAness scores. Prospective studies and accumulation of data should help clarify this issue.

Regarding the identification of a MSH2 mutation, although it is a known prognostic biomarker for endometrium cancer, as far as we know it has never been correlated to better response in EOC. In our study, one MSH2 germline mutation (c.2021G>A) was identified, in association with a somatic CHEK2 mutation (c.1671+1_1671del) and 3 VUS (BRCA1:c.5074+7C>T, BRCA1:c.5432A>C, p.Gln1811Pro, and MLH1:c.424T>C, p.Cys142Arg in a sample of HGSOC with no TP53 mutation. To date, we assume that HGSC MMR deficient have the same prognosis of MMR proficient tumors. Despite the morphology of HGSC, and considering the absence of TP53 mutation, this tumor may have molecular similarities with endometrioid carcinomas. For this tumor, a pathology review is ongoing including the evaluation of the microsatellite status.

Lastly, the 2 samples showing cyclin amplification go against what has been described in most studies so far (around 7% in this cohort). Approximately 30% of HGSC tumors have alterations in the Rb pathway and cell cycle control, including amplification of CCNE1 (∼20%), loss of RB1 (∼10%), or gain of RBBP8 (∼4%). Strikingly, activation of the RB1/CCNE1 pathway is largely exclusive of BRCA1/2 mutation. Both BRCA1/2 dysfunction and CCNE1 amplification are known to promote genomic instability and tumor progression (ETEMADMOGHADAM et al., 2013). The presence of this CCNE1 amplication remains controversal in terms of actionability or prognosis significance. In fact, CCNE1 amplification has also been observed in long-term survivors within the TCGA cohort at a 10% (1⁄10) frequency and at the same frequency (2⁄20) in the work of Yang and colleagues (YANG et al., 2018b). There should be other cofactors which could explain the sensitivity of those tumors and which were not identified in the HR screened here.

In addition, we performed RAD51 functional assay in parallel 6 samples for the moment. This assay can provide a more comprehensive and dynamic readout of tumor HR capacity throughout disease evolution and at a specific moment of treatment. Three out of 6 (50%) samples scored negative for RAD51 foci and then HR deficient: 1 sample with a

BRCA1 mutation, 1 with a *BRCA2* mutation, and 1 with no mutation or VUS identified. Further analysis need to be performed on this sample as epigenetic analysis. For the positive RAD51 foci, the result on the CHEK2/MSH2 is certainly coherent regarding the tumorigenesis. However, the case with the BRCA2 mutation should also need more analysis as HRD score to confirm the proficiency of the HR pathway. For the moment, results are preliminary so it is difficult to reach a conclusion.

Finally, our study showed a better median PFS of patients presenting complete when compared to those presenting near-complete pathological response (24 months x not reached; p=0,0076), questioning the value 3 tier chemotherapy response score in stratifying patients. This contradicts previous studies. We also demonstrated that patients presenting optimal response to platinum-based chemotherapy present a higher PFS (48 months), irrespective of having a *BRCA1/2* mutations. This questions the prognostic value of these mutations in the context of pCR. Previous studies evaluating the prognostic role of BRCA mutated tumors showed that it does not increase the chance of cure. Put differently, the prognosis of patients in 5 years is better but in 10 years it becomes similar to that of BRCA non-mutated tumors. For example, initially the disease is indeed more sensitive to chemotherapy, but one relapsed the prognostic value of BRCA mutations remains questionable. Though it is intuitive to think that the longer initial PFS seen in *BRCA1/2*-related cancers may reflect in a better OS, at this point, this study does not allow us to conclude this. Analysis in a larger sample is necessary to drawn any conclusion.

To conclude, this study helped to understand the inactivation mechanism of HR pathway of patients presenting optimal response to platinum-based chemotherapy. There were a enrichment in the alteration identified. The clinical impact still need to be assessed for the management of those patients.

Acknowledgments

We thank the staff of Department of Medical Biology for technical support on ddPCR methylation design and NGS analysis. Special thanks to Roseline, Catherine Richon,Voreak Suybeng and Odile Leopold .

Tables

Table 1 Non-coding regions of BRCA1, BRCA2 Aand RAD51C genes screened in tumoral samples

Region screened	Hg19* coordinates	Length
BRCA1 promoter	chr17: 41.277.273 - 41.277.527	255bp
BRCA1 intron 2	chr 17: 41 271 752-41 272 078	326bp
BRCA1 intron12	chr17:41,236,600 –41,236,960	360bp
BRCA2 promoter	chr13:32.889.482-32.889.861	380bp
RAD51C promoter	chr 17: 56.764.394-56.770.005	5.611bp

Table 2: Primaryantibodies used for the detection of the different HR markers

Primary antibodies	Species	Dilution	Company
Rad51	rabbit	1/1000	Abcam ab133534
Geminin	mouse	jan/60	DAKO 2022-05-31
Geminin	rabbit	1/400	Proteintech 10802-I-AP
gH2AX	mouse	1/200	Millipore JBW 301
BRCA1	mouse	jan/50	Santacruz 1/50

Table 3: Secondary antibodies used for the detection of the different HR markers

Secondary antibodies	Species	Dilution	Company
AlexaFluor 568	mouse	1/500	Invitrogen A10037
AlexaFluor 568	rabbit	1/500	Invitrogen A11011
AlexaFluor 568	mouse	1/500	Invitrogen A11001
AlexaFluor 568	rabbit	1/500	Invitrogen A11008

Table 4 Primary antibodies used for the detection of the different immune coregulators. WB: **water bath at 98° C for 30 min.** TE: **Tris-EDTA pH9.** CIT: **sodium citrate pH6.**

Antibody	Clone	Dilution	Pretreatment	Company
PD-L1	E1L3N	1/200	WB+TE	Cell signaling
TIM-3	D5D5R	1/200	CC1	Cell signaling
LAG-3	D2G40	1/300	CC1	Cell signaling
IDO-1	SP260	1/20 000	WB+CIT	Spring Bioscience

Table 5 Clinical and pathological characteristics of the cohort of patients presenting optimal response to neoadjuvant chemotherapy. Bv=bevacizumab ; mut=mutated ; CRS3=chemotherapy response 3 ; pCR=pathological complete response

	All subjects	BRCA mut	HR mut	Wild-type	Unknown
Total	43	11	4	13	15
Median age	70	68	68	67	67
Range (years)	47-88	47-73	48-83	53-82	51-88
Site					
Ovary	42	11	4	12	15
Other	1	0	0	1	0
Histology					
High-grade serous carcinoma	29	8	3	10	8
Low-grade serous carcinoma	7	1	0	4	2
Poorly diferentiated	1	0	1	0	0
Clear cell	1	0	0	1	0
Endometrioid	5	2	1	0	2
Stage					
III	26	7	3	11	5
IV	17	4	2	5	6
Complete cytoreduction					
Yes	43	11	4	13	15
No	0	0	0	0	0
Chemotherapy regimen					
carboplatin+paclitaxel 3-4x	19	5	2	5	6
carboplatin+paclitaxel 6x	18	3	1	10	4
carboplatin+paclitaxel + Bv 4x-->Bv	2	1	0	1	0
carboplatin+paclitaxel+Bv 6x-->Bv	3	0	0	2	1
missing	1	0	0	1	0
Chemotherapy response					
CRS3	21	5	1	7	8
pCR	22	6	4	9	3

Table 6*: BRCA1* and *BRCA2* pathogenic variants identified in the cohort.

Patient	Pathology	Gene	Variant	Type of variant	Domain	Class
P2	HGSOC	BRCA1	c.3477_3480del;p.Ile1159fs	G	No (exon 11)	5-deleterious
P5	HGSOC	BRCA1	c.427G>T;p.Glu143*	G	No (exon 7)	5-deleterious
P12	Endometrioid carcinoma	BRCA2	c.3931G>T;p.Glu1311*	G	No (exon 11)	5-deleterious
P13	HGSOC	BRCA1	c.1789G>T;p.Glu597*	S	No (exon 11)	5-deleterious
P16	HGSOC	BRCA1	c.5266dup;p.Gln1756fs	S	BRCT (exon 20)	5-deleterious
P17	HGSOC	BRCA2	c.358del; p,Val120fs	S	No (exon 4)	5-deleterious
P31	HGSOC	BRCA2	c.409_413del;p.Ser137FS	S	No (exon 4)	5-deleterious
P34	Endometrioid carcinoma	BRCA2	c.7060C>T; p.Gln2354*	S	No (exon 14)	5-deleterious
P35	HGSOC	BRCA2	c.1323delT,p.Thr441fs-47	S	No (exon 10)	5-deleterious
P37	HGSOC	BRCA1	c.2662_2670delinsAC; p.(His888Thrfs*3)-23	S	No (exon 11)	5-deleterious
P40	HGSOC	BRCA2	c.8488-1G>A	S	No (exon 19)	5-deleterious

Table 7 Non-BRCA pathogenic variants identified in the cohort. Figure 1 HGSOC= high grade serous ovarian carcinoma; G= germline; S= somatic

Patient	Pathology	Gene	Alteration	Allelic frequency	Type of variant	Class
P8	HGSOC	CDK12	c.3G>A;p.Met1?	0,28	T	4-probably deleterious
P10	Endometrioid carcinoma	ATM	c.2465T>G; p.Leu822*	0,34	T	5-deleterious
P11	HGSOC	CCNE1	amplification		T	5-deleterious
P22	HGSOC	CCNE1	amplification		T	5-deleterious
P38	HGSOC	CHEK2	CHEK2:c.1671+1_1671del,-12	0,12	T	5-deleterious
P38	HGSOC	MSH2	MSH2:c.2021G>A,p.Gly674Asp	0,43		5-deleterious

Table 8 Variants of uncertain significance (VUS) identified in wild-type samples of the cohort

Patient	Mutation status	Pathology	Gene	Variant	Type of variant	Domain	Class
P6	WT	HGSOC	BRCA1	c.5165C>T p.Ser1722Phe	T	BRCT (exon19)	3 - unclassified
P42	WT	HGSOC	BRCA1	c.*838C>A	T	3'UTR	3- unclassified
P1	WT	Clear cell	BRCA2	c.9652G>A	T	No (exon 27)	3- unclassified
P7	WT	HGSOC	BRCA2	c.*14C>T	G	3'UTR	3- unclassified
P11	WT	HGSOC	BRCA2	c.*72A>G	G	3'UTR	3- unclassified
P43	WT	HGSOC	BRCA2	c.1343G>A,p.Arg448His	T		3- unclassified
P43	WT	HGSOC	BRCA2	c.*839T>C,-67	T		3- unclassified
P9	WT	HGSOC	BRIP1	c.2932G>C, p.Gly978Arg	T	-	3- unclassified
P43	WT	HGSOC	CHEK2	c.1590+62A>G	T	-	3- unclassified

Table 9 Variants of uncertain significance (VUS) identified in association with pathogenic variants of HR gene

Patient	Mutation status	Pathology	Gene	Variant	Origine of variant	Domain	Class
P12	BRCA2 mut	Endometrioid	BRCA1	c.4186-2152C>G	T	Intron 12	3- unclassified
P12	BRCA2 mut	Endometrioid	BRCA2	c.*623C>T	T	3'UTR	3- unclassified
P12	BRCA2 mut	Endometrioid	RAD51C	c.*283A>G	T	-	3- unclassified
P38	CHEK2mut and MSH2 mut	HGSOC	BRCA1	c.5432A>C,p.Gln1811Pro-67	T	BRCT(exon 23)	3- unclassified
P38	CHEK2mut and MSH2 mut	HGSOC	BRCA1	c.5074+7C>T,-34	T	-	3- unclassified
P38	CHEK2mut and MSH2 mut	HGSOC	MLH1	c.424T>C,p.Cys142Arg	T	-	3- unclassified
P10	ATM mut	Endometrioid	BRIP1	c.2220G>T; p.Gln740His	T	-	3- unclassified
P35	BRCA2 mut	HGSOC	RAD51D	c.*366C>T,-67	T	-	3- unclassified
P37	BRCA1 mut	HGSOC	CHEK2	c.649C>T,p.Leu217Phe-28	T	-	3- unclassified

Table 10: Results of BRCA1 hypermethylation analysis

Patient	Mutation status	Pathology	Promoter hypermethylation
P1	WT	Clear cell carcinoma	Negative
P9	WT	HGSOC	Positive
P10	WT	Endometrioid	Negative
P12	BRCA2 mut	Endometrioid	Negative
P19	WT	HGSOC	Negative
P37	BRCA1 mut	HGSOC	Negative
P41	WT	HGSOC	Positive
P42	WT	HGSOC	Negative
P43	WT	HGSOC	Positive

Figures

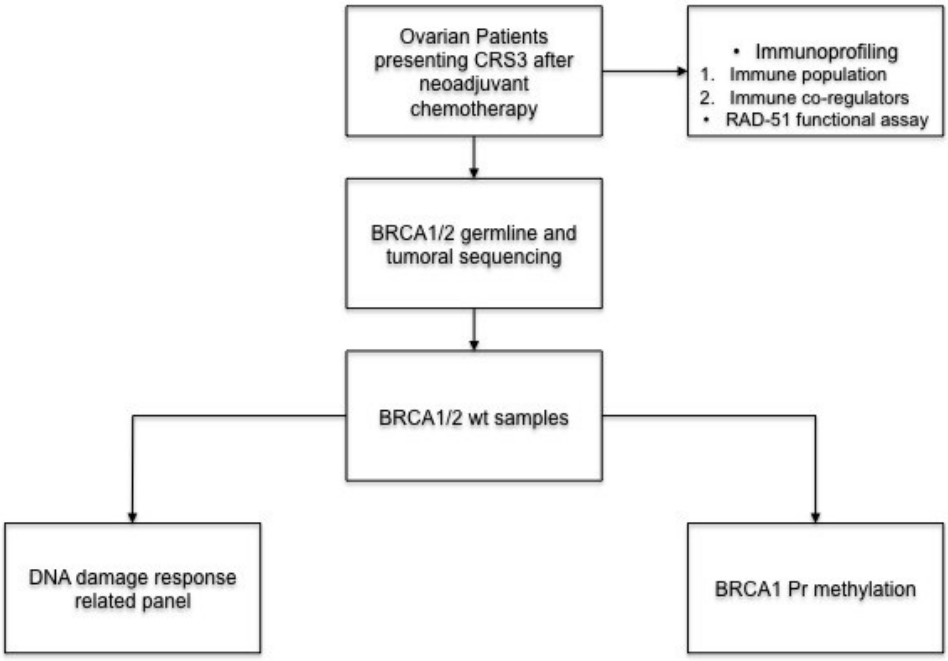


Figure 18 Study flowchart showing a summary of molecular analyzes performed in the cohort of EOC patients presenting complete or near-complete (CRS3) pathological response to neoadjuvant chemotherapy. The DNA damage response (DDR) panel comprises *BRCA1, BRCA2* and 13 additional genes frequently mutated in epithelial ovarian cancer, mainly related to homologous recombination DNA repair pathway, including non-coding regulatory regions of *BRCA1, BRCA2* and *RAD51C* (*BRCA1, BRCA2, ATM, BARD1, BRIP1, CCNE1, CDK12, CHEK2, PALB2, RAD51C, RAD51D, MLH1, MSH2, PMS2, TP53*)

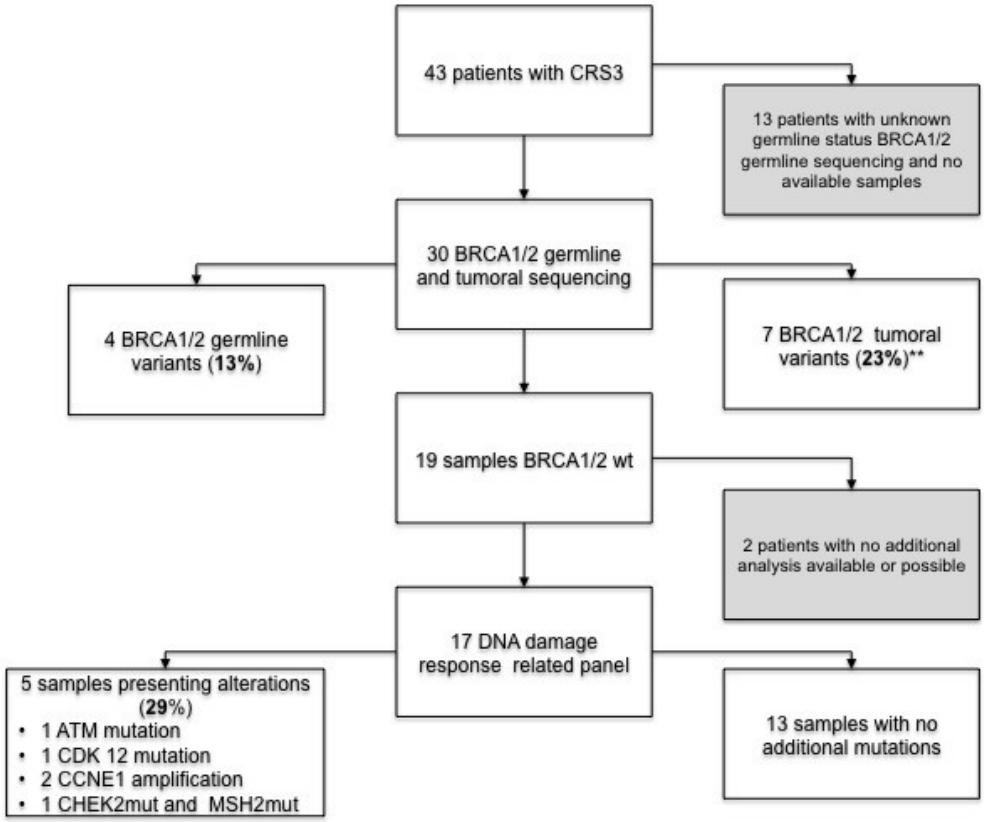


Figure 19 Molecular analysis performed in the cohort of EOC patients presenting complete or near-complete (CRS3) pathological response to neoadjuvant chemotherapy. Tumoral sequencing was performed in pre-treatment biopsies.**4 out of 7 variants are certainly somatic variants pre-treatment biopsies

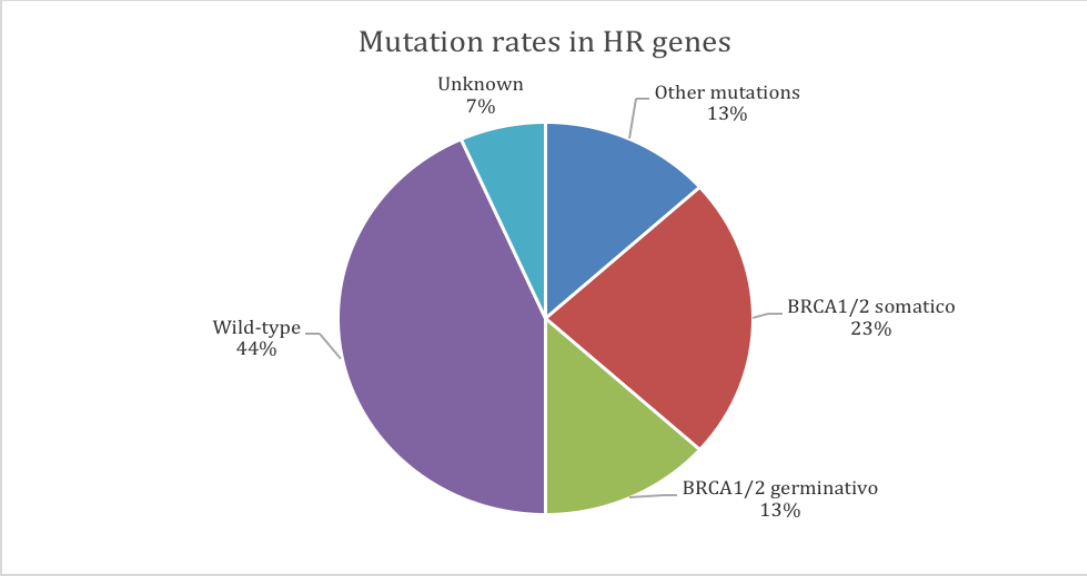
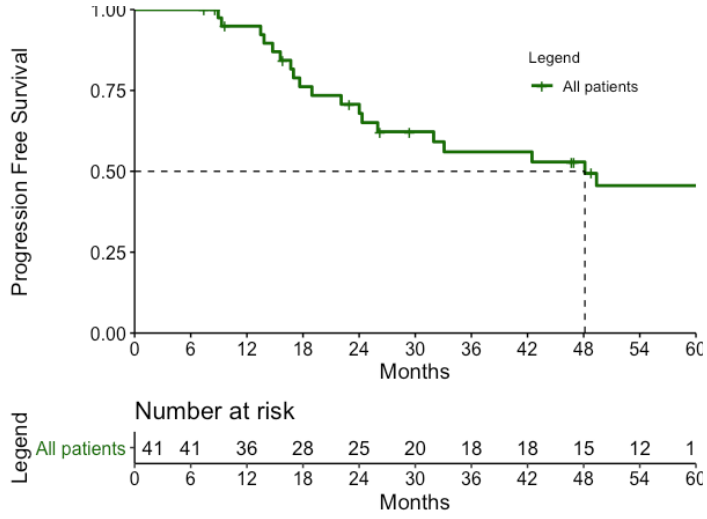


Figure 20 Mutation rates in Homologous recombination genes. Overal 11 out of 30 (36%) patients present BRCA1⁄2 mutations. Five (29%) out of 17 samples screnned with DDR related panel presented a molecular alteration (1 ATM mutation, 1 CDK12 mutation, 1 CHEK2 mutation in association with an MSH2 mutation and 2 samples presented cyclin amplification).

A/



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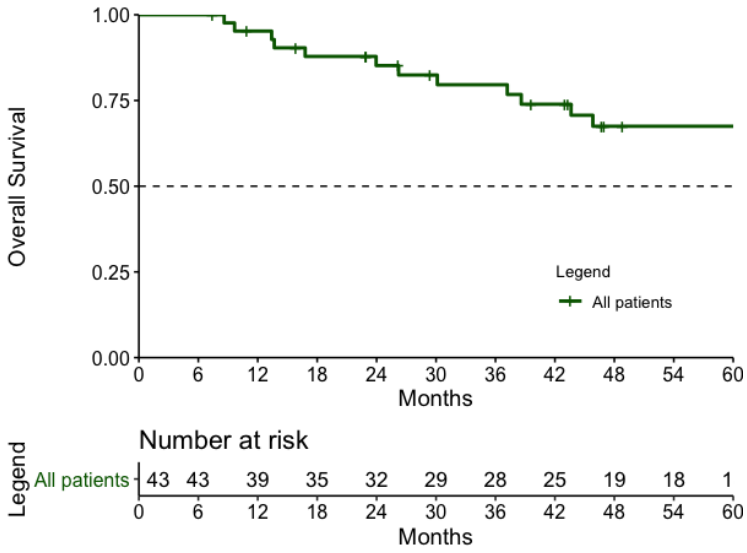
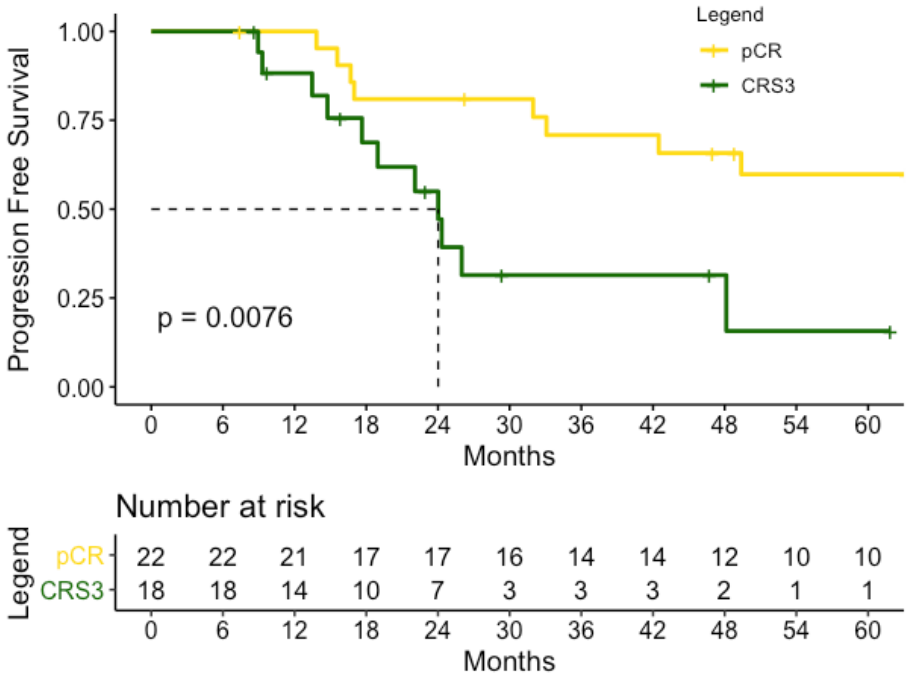


Figure 21 PFS (A) and OS (B) of the entire cohort

A/



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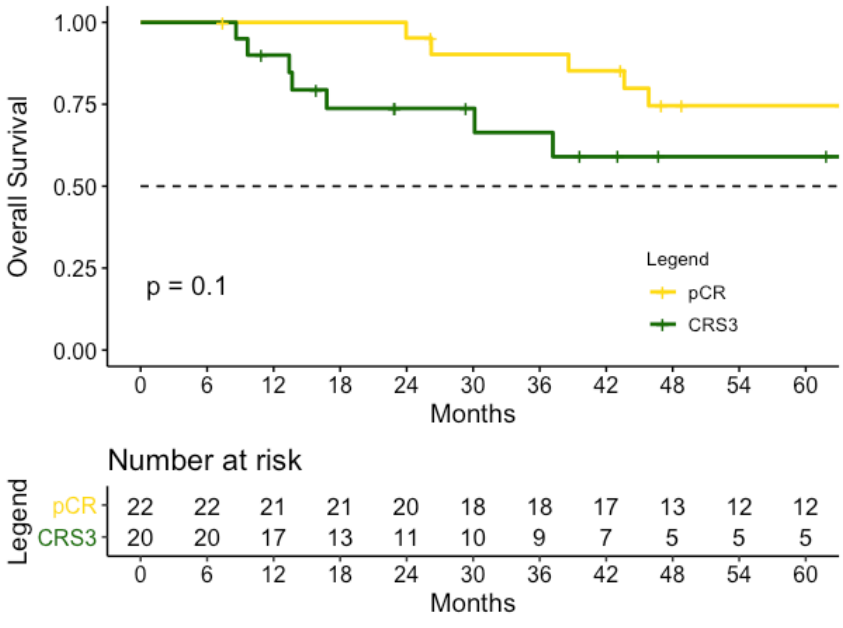
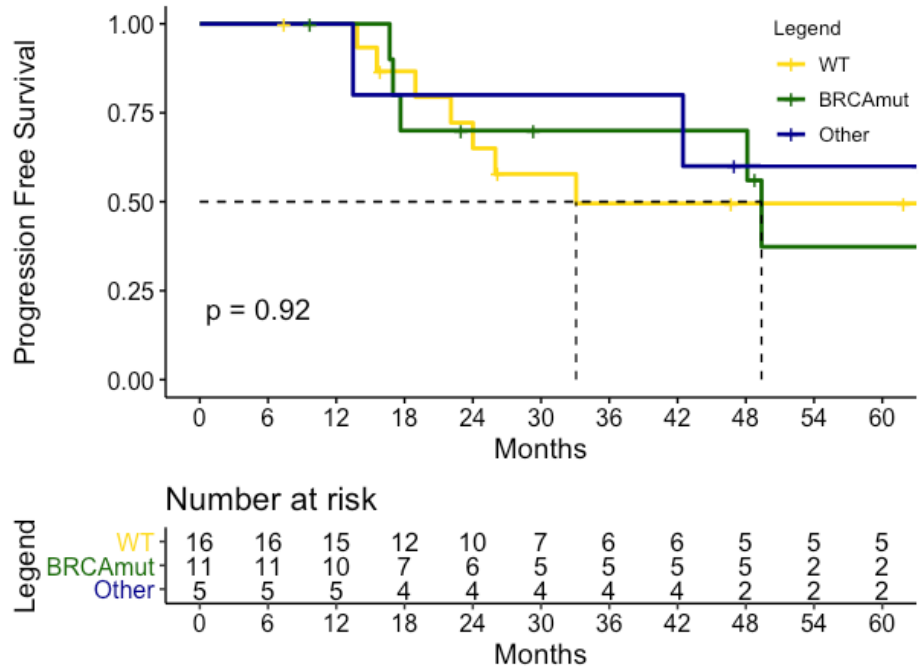


Figure 22 (A) PFS and OS (B) of patients presenting pCR x near-complete pathological response .



B/

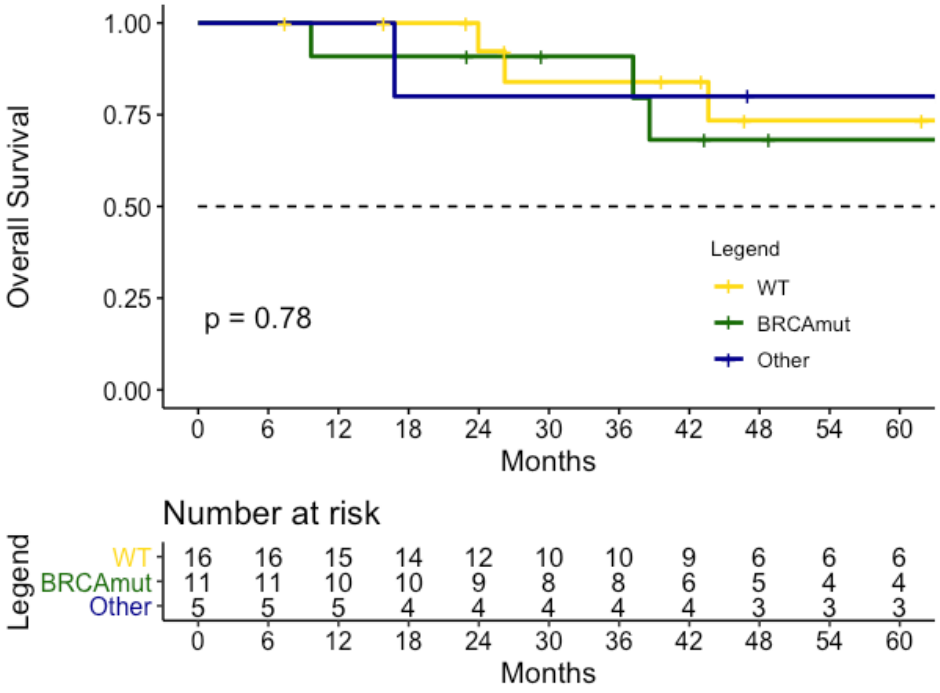


Figure 23 PFS and OS according to molecular staus

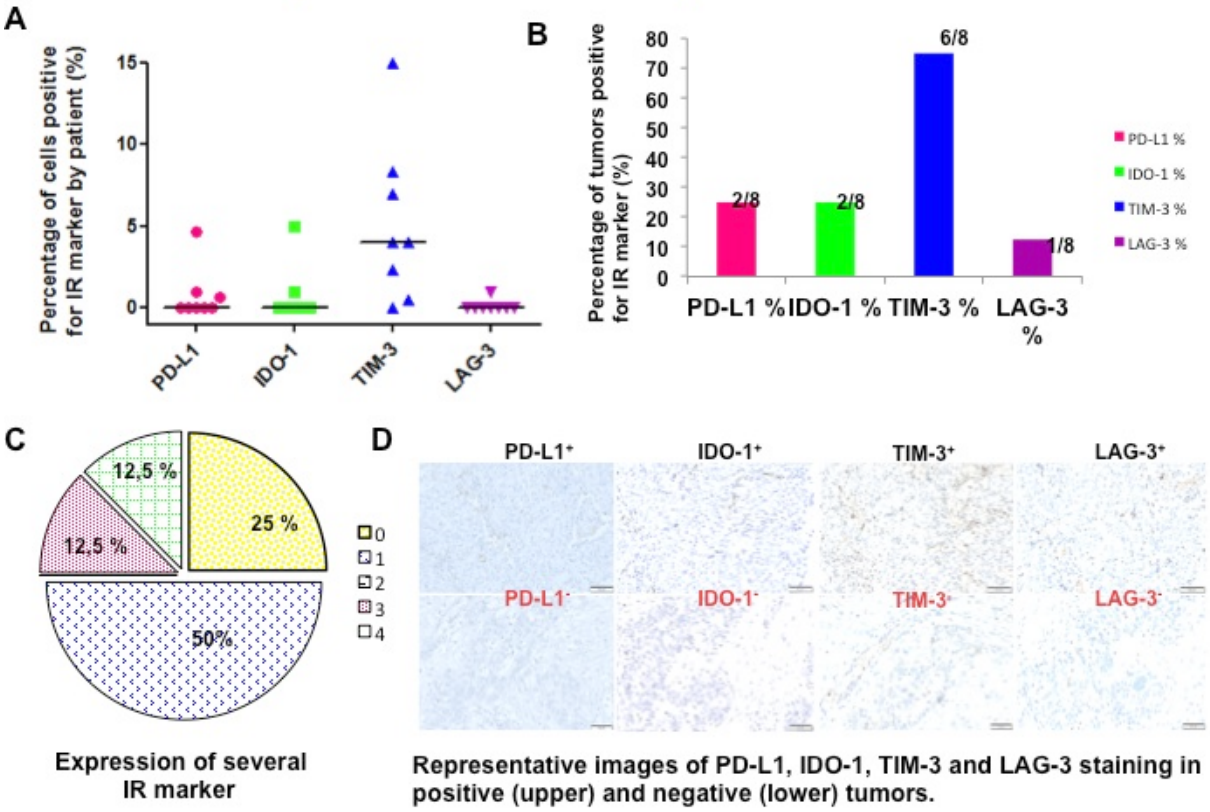
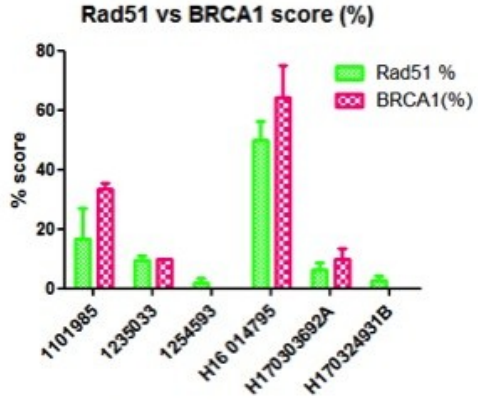
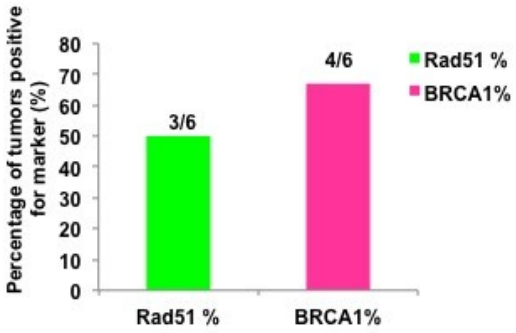
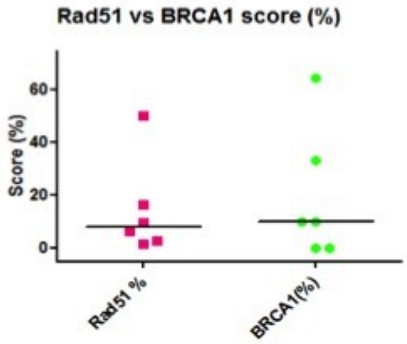


Figure 24: Co-regulator receptor expression in tumors of EOC patients presenting optimal response to neoadjuvant chemotherapy. TIM3 is the more abundant coinhibitory receptor expressed.







	RAD51 score (%)			BRCA1 score (%)		
	Mean±SEM	Median	N	Mean±SEM	Median	N
SCORE (%)	11,5±7,4	8,0	6	19,6±10,2	10	6

Figure 25**:** RAD51 and BRCA1 score in tumors of EOC patients presenting optimal response to neoadjuvant chemotherapy. A tumor is considered as positive if the cut off for *Rad51*$^{+}$ *or BRCA1 is* ≥ *10%.*

Supplementary data

TGTATTTTGAGAGGTTGTTGTTTAGTGGTAGTTTTTTGGTTTTTGTGGTAATGGAAAAGTGTGGGAATTATAGA
TAAATTAAAATTGTGATTGTGTGGTGTGAGTTTGTTGAGATTTTTTGGATG

----------P1NM---------- ---------L1NM---------

----------U1NM----------

ACATAAAACTCTCCAACAACAAATCACCATCAAAAAACCAAAAACACCATTACCTTTTCACACCCTTAATATCT
ATTTAATTTTAACACTAACACACCACACTCAAACAACTCTAAAAAACCTAC

BRCA1_U1NM : GTATTTTGAGAGGTTGTTGTTTAGT (25nt)

BRCA1_L1NM : ACAAACTCACACCACACAATCA (22nt)

BRCA1_P1NM : CTATAATTCCCACACTTTTCCATTACCACA (30nt)

De c.-220 à c.-96 converti méthylé (95nt)

CGTATTTTGAGAGGTTGTTGTTTAGCGGTAGTTTTTTGGTTTTCGTGGTAACGGAAAAGCGCGGGAATTATAG
ATAAATTAAAATTGCGATTGCGCGGCGTGAGTTCGTTGAGATTTTTTGGACG

---------P1M--------- ----------L1M----------

----------U1M----------

GCATAAAACTCTCCAACAACAAATCGCCATCAAAAAACCAAAAGCACCATTGCCTTTTCGCGCCCTTAATATCT
ATTTAATTTTAACGCTAACGCGCCCGCACTCAAGCAACTCTAAAAAACCTGC

BRCA1_U1M : GTATTTTGAGAGGTTGTTGTTTAGC (25nt)

BRCA1_L1M : GCGCAATCGCAATTTTAATTTATCTA (26nt)

BRCA1_P1M : CGCGCTTTTCCGTTACCACGA (21nt)

Supp Figure 26 Primers used for detection of BRCA1 promoter hypermethylation. Experiments were performed on the Naica digital PCR system.

5 4. DISCUSSION AND PERSPECTIVES

4.1 Discussion

In families meeting the clinical criteria for HBOC, there is still a large portion of missing heritability. In a considerable proportion of ovarian cancers and in some breast cancers, a mutation will not be found, even if the HR deficient phenotype is confirmed by signatures. This work aimed to identify alternative mechanisms beyond coding and premature stop in BRCA1/2 and have been extended to the HR pathway inactivation. This information improves genetic counselling and increases the percentage of the population which could benefit from targeted therapies.

For this purpose, germinal and somatic alterations were explored in two populations with high probability of presenting a dysfunction of the HR pathway. The yield is higher for the identification of alterations that could explain the HR deficiency, either because they were at high clinical risk of carrying a pathogenic variant, or because they had presented an exceptional response to platinum-based chemotherapy, a functional *in vivo* test to validate the deficiency in HR repair. We then assumed that alterations found in both cohorts were more likely to be related to the inactivation of DNA repair pathway by homologous recombination.

Initially, the focus was on unexplored regions of the major genes of the pathway, *BRCA1* and *BRCA2*. In this first study, the population had already undergone BRCA screening, with no pathogenic variant identified in the coding regions and intron-exon boundaries. Next, somatic arguments were sought for classifying previously identified variants that remained of uncertain significance. Finally, pathogenic variants in other HR genes were sought, including non-coding regions of these genes and epigenetic mechanisms with potential impact on gene expression as *BRCA1* or *RAD51C* (genes with a relevant and well-defined role in DNA repair by HR).

4.1.1 Second allelic events as a compass to oncogenetic interpretation

An important aspect of this thesis was exploring molecular features of breast and ovarian tumors from *BRCA1/2* variant carriers. The study of the variant's impact on the tumor should better clarify the weight of these somatic characteristics in the classification of variants (LOH, histology, proteins, and BRCAness). Since *BRCA1/2* tumors present typical features, they may help in the understanding whether or not a tumor is linked to a pathogenic variant in

BRCA1/2 genes. Ninety-nine breast and ovarian tumors of 26 different *BRCA1* variants were analyzed. A relatively stable pattern of LOH (67% of wild-type allele) for tumors of the pathogenic variant carriers was observed, while allelic balance or loss of variant allele was seen in 63% and 15% of benign variants carriers tumors, respectively. Also worth pointing out was the successful classification of 2 VUS (c.4963T>C and c.5497G>A) as pathogenic with tumor allele frequency, histopathologic, and co-segregation data. Their LOH analysis was in line with our hypothesis: Loss of wild-type allele was observed for 4 of 5 samples with c.4963T>C, and all 3 samples with c.5497G>A. In this context, we propose to incorporate LOH data into the multifactorial algorithm, combined with the LR pathology. We believe that LOH information is complementary to histology data, which was recently incorporated into the model (Spurdle et al., 2014). This hypothesis will be confirmed with a larger number of samples analyzed and as tumor testing increases, this information will be more readily available.

Our hypothesis arose from the conception that as tumor suppressor genes, the second allele of *BRCA1/2* genes should be inactivated to trigger tumorigenesis. In fact, this leads to a dominant effect of the remaining allele, which is not able to produce any stable or functional protein. However, it should be considered that in a minority of cases, alternative mechanisms of second allele inactivations have been described during the development of *BRCA*-related tumors (VAN HEETVELDE et al., 2018). Adding to the complexity, Van Heetvelde and colleagues demonstrated that different mechanisms inactivating the wild-type allele may be present within the same tumor at various extents. For example, hypermethylation of both *BRCA1* and *BRCA2* promoters have already been described in a few cases (DWORKIN et al., 2009; ESTELLER et al., 2001) but should be taken with caution because of technical limitations, especially for BRCA2 methylation. Inactivation of the second allele with somatic mutations is even rarer, as only one case in ovarian cancer was described by the ovarian TCGA. Potential limitations of the methodology include non-tumor tissue contamination, low tumor cellularity, low quality of tumor DNA, development of more sensitive techniques to detect allelic imbalance, and determination of the ideal cutoff to consider allelic imbalance. We identified alternative possibilities of second allele inactivation for some of the pathogenic variant tumors of our cohort presenting allelic balance. For the remaining, the mechanism remained undefined. Therefore, we conclude that analysis of an isolated sample of a given variant is not sufficient to draw a conclusion about causality. Considering the prevalence of

loss of the wild-type among pathogenic and neutral variants, a minimum number of samples is necessary to drawn a conclusion.

Another point to consider is the fact that there was a difference in LOH pattern according to the type of the variant. LOH was reported for 59% and 74% of pathogenic missense and frameshift/nonsense variants, respectively. Although this difference was not statistically significant, it could be an argument in favor of some dominant negative effect of *BRCA1* missense pathogenic variants, similar to that described for other proteins such as *ATM*, *POLE1*, and *TP53* (CHENEVIX-TRENCH et al., 2002; FERNET et al., 2004; MULLER; VOUSDEN, 2014; SCOTT et al., 2002). Further, it is known that even among missense variants, there may be difference in the magnitude of cancer-associated risks and the potential to predict response to treatment according to the domain where they are located (KUCHENBAECKER et al., 2017). It remains unclear whether all pathogenic *BRCA1/2* variants have similar effects. As an example, while *BRCA1*-BRCT variants seem to increase sensitivity to PARPi (VACLOVÁ et al., 2016), variants located in the RING domain may induce treatment resistance through induced expression of a RING-less BRCA1 protein that mediated resistance to HRD therapies. It seems that RING variants are more easily bypassed with appearance of reversals than *BRCA1* variants. The same is true for variants of *BRCA2* exon11 (DROST et al., 2016). We worked with frameshift and a selection of missense *BRCA1* variants localized in different regions of the gene, which meant the variants had different impacts on the protein. The effect at the protein level as well as the localization of the variant within the gene are issues that should be taken into account. Furthermore, other authors have reported a much more frequent loss of wild-type allele for *BRCA1* than *BRCA2*in both breast and ovarian cancers. As stated before, there are differences in the phenotype and molecular subtypes of *BRCA1*- and *BRCA2*-associated breast cancer, so any difference at some level of tumorigenesis must also exist. All of the issues described above should be considered so that LOH information can finally be integrated into multifactorial model for *BRCA1/2* variant classification.

Also noteworthy was the 15 cases of pathogenic variant tumors that presented an allelic balance, which suggests that genetic instability may be absent despite the presence of a germline pathogenic variant. Although carrying a pathogenic variant in *BRCA1/2* is the best determinant of PARPi response, a significant percentage of *BRCA1/2* patients show primary resistance to these agents. Moreover, the magnitude of benefit is not the same in breast and ovarian cancers. Work is in progress to understand if LOH is also a predictor of greater

sensitivity to PARP, which adds information beyond the presence of the mutation itself. Continuing with the Knudson hypothesis, perhaps in tumors with wild-type allele retention, the *BRCA* mutation would not have a major role in carcinogenesis (JONSSON et al., 2019). For a portion of these samples of pathogenic variants presenting wild-type allele retention, it was possible to evaluate the BRCAness score that showed the absence of base genetic instability.

4.1.2 Non-coding regions: the tip of the iceberg for missing mechanisms

An original feature of this thesis was the exploration of non-coding regions to provide a better understanding of the regulation of *BRCA1/2* genes. As previously stated, current genetic screening is generally limited to *BRCA1*/*2* exons and intron/exon boundaries. However, it is becoming increasingly clear that variants in these regions only account for a small proportion of cancer risk. We and others have screened *BRCA1* and *BRCA2* promoters of predisposed patients with no pathogenic variant identified, in search for potential 5' or 3' UTR mechanisms of gene deregulation (GARCIA et al., 2016). In addition, we explored at the somatic level this as well as other non-coding regions (intronic regions and 3'UTR) of *BRCA1* and the promoter of *RAD51C*, in tumors of patients presenting optimal response to chemotherapy. We described germline and somatic variants in key transcriptional regulatory elements of *BRCA1* and *BRCA2* housed in gene promoters, untranslated regions, and also in introns and long-range elements. The clinical significance of the majority of them is currently unknown and remains a significant clinical challenge. The role of variants in non-coding regions beyond splice donor and acceptor sites, including those that have no qualitative effect on the protein, has not been thoroughly investigated. Among other arguments, the first description of an epigenetic impact of a non-coding variant in *BRCA1* gene launches the necessity to continue the screening of *BRCA1/2* non-coding regions, in parallel with studies to determine their biochemical and clinical significance (EVANS et al., 2018).

It is also worth noting that non-coding screening in a part of the cohort was performed by high resolution melting and the remainder was performed by NGS. Therefore, some technical limitation must have reduced the sensitivity of our screening. Indeed, next generation sequencing techniques are required to improve this approach, especially for the highly duplicated 5' region of *BRCA1*. Current technological sequencing advancements and development of bioinformatics tools has enabled a better exploration of non-coding DNA

regions. Functional elements of the human genome are currently being explored with more advanced tools. Soon, whole genome sequencing data will be produced, providing more information about these regions as well as real frequency data of the variants identified there.Studies in this context of technological advance will help avoid making any misinterpretations. For example, in 2012, the variant *BRCA2* c.6937+594T>G was classified as pathogenic (ANCZUKÓW et al., 2012), but a subsequent study frequency data confirmed that this variant was very common in populations in South America, which is an understudied population (DUTIL et al., 2018). That is why there is still a need to be very prudent in the interpretation of new variants, especially if the locus is poorly explored.

It should also be emphasized that while some non-coding variants were related in reducing promoter activity, others have been associated with increasing gene expression. This latter effect is the opposite of what one would expect from a *BRCA1/2* variant associated with an increased risk of breast/ovarian cancer. Our hypothesis is that these enhancing variants could inhibit some repressor elements localized within *BRCA1* and *BRCA2* promoters, thereby inducing an overexpression of *BRCA1/2*. We have also seen that the *BRCA1/2* expression strongly fluctuates during the cell cycle. *BRCA1/2* expression is very low at the G1 phase when DNA repair by homologous recombination is not the preponderant mechanism, but it increases in the S and G2 phases when BRCA1 activation is favored by cyclin activity. Thus, it can be hypothesized that variants leading to *BRCA1/2* overexpression could still perturb DNA repair mechanisms, thereby inducing genetic alterations that help trigger tumorigenesis. Aditionally, the inconsistent results occasionally observed when different cell-lines were used to evaluate the same variant may reflect the availability of transcription factors or co-factors among the cells and reinforce the utility of performing these tests in more than one cell line (KAO et al., 2009). The correlation between replication cycle and HR expression is a very important point for future therapy. The balance of the different proteins can have an impact. For example, a synthetic lethality has been shown between CCNE1 amplification and BRCA alterations in ovarian cancer (ETEMADMOGHADAM et al., 2013).

Moreover, considering that luciferase activity assay is ultimately indicative of both transcriptional and translational efficiency, it is noteworthy that in functional studies the reduced levels of BRCA1 protein is not always associated with reduced transcript levels (SIGNORI et al., 2001; WANG et al., 2007). The system is highly dynamic and post translational modifications may have an impact (DERIBE; PAWSON; DIKIC, 2010; LEE et al., 2018). Therefore, disruption of post-transcriptional regulation should contribute in some

cases. Using RNAfold secondary structure prediction software, we could demonstrate that a *BRCA1* 5'UTR variant (c.-130del) impacts RNA conformation and probably affects the binding of trans-acting factors, and therefore mRNA translation (DOS SANTOS et al., 2017). This predicted effect was also described for some 3'UTR variants and a 5'UTR polymorphism of *BRCA1*, both with an impact in translational efficiency (GARCIA et al., 2016; WANG et al., 2007).

It should be pointed out that we identified a sensitive region in the *BRCA1* promoter with three functionally active variants, including two with a marked repressor impact on promoter activity. Analysis of the DNA sequence region using the transcription factor database revealed that both variants are located in a putative E2F1 transcription factor binding site. Assays to better characterize protein-DNA interaction, including electrophoretic mobility shift assay (EMSA) and Chromatin immunoprecipitation assays (ChIP), are all useful to investigate the underlying mechanism of variant impact in continuing this project (GARNER; REVZIN, 1981; ORLANDO; STRUTT; PARO, 1997). Our collaborator validated the EMSA to observe the variants impact on these regions (BURKE et al., 2018). Next, we hypothesized that these two variants may then impact the ability of E2F1 to induce BRCA1 transcription. However, a few limitations prevented us from providing our hypothesis. One was the scarcity of Transcription Factor databases. Thus, other transcription factors identified in future studies could therefore increase aou understanding of the biological implication of these variants in TFBSs. A second limitation was that we did not have access to many families carrying the same variant. The last major limitation was the poor availability of co-segregation data and tumor samples for further molecular analysis in families in which variants were identified. We were therefore unable to establish a causality score with the multifactor model. It is now necessary to introduce research partners in order to go further in addressing the new challenges of classifying variants of uncertain significance, whether non-coding or missense.

4.1.3 Other HR genes – much ado about nothing

Finally, the results from the analysis of epithelial ovarian cancer samples emphasize the need to explore other mechanisms of HR inactivation, including those involved in epigenetics regulation. This study allowed us to identify potential predictive biomarkers of response beyond *BRCA1/2* mutations, suggesting that they should also be useful for individualizing treatment and perhaps to explain the missing heritability in a portion of

predisposed families. Also noteworthy is the strategy of exploring populations with a high suspicion of HRD, considering that the alterations identified may have a higher chance of being responsible, at least in part, for the pathway dysfunction. This is also strategic for broadening the understanding of DNA repair pathways and treatment response. Currently, the exploration of those other genes is faced with limited knowledge on the function of those genes and the rarity of those variants.

The next step will be to try to understand what can be done once all the genomic region of the *BRCA1, BRCA2* and other HR genes are explored. Today, different genes implied in the HR reparation pathway are being extensively sequenced. In this thesis, we tried to explore both the non-coding variants and the tumoral information which could be important to correctly interpret a cancer predisposition case.

4.2 Perspectives and conclusions

In view of our findings, even if the large genomic projects are providing more and more data, there are still a few main domains that remain to be explored in order to elucidate the missing heritability and alternative mechanisms of HR pathway inactivation. One can argue in favor to increase the data on RNA and on epigenetic regulation. Those events are certainly more complex in the tumoral background. Other causes should be also adressed as the post-translational regulation factors and their direct alterations.

The exploration of non-coding variants will also open upquestions on moderate risk management and on the consequence of enlarging both the population and the type of alterations.

4.2.1 Splicing isoform

First, the assessment of the splicing form is still very limited. Even if there is some RNA sequencing, very few details are available about the different isoforms expressed, which could explain some specific behaviour. (DAVY et al., 2017; DE LA HOYA et al., 2016). However, a few variants with impact on splicing have been described so far. For instance, in the field of drug resistance, the impact of BRCA1 isoform expression has been studied to explain acquired resistance to PARPi and cisplatin (WANG et al., 2016). The expression of a partial protein lacking the majority of exon 11 (the BRCA1-Δ11q protein) promotes partial PARPi and cisplatin resistance, both *in vitro* and *in vivo*. Four other examples are: (1) a BRCA2 isoform lacking the exon 3 has been shown a low physiological transcript, being associated with an increased risk of breast and ovarian cancer. Interestingly, even if the protein remains stable, variants resulting in complete exon 3 skipping are considered as pathogenic; (2) several isoforms of RAD51D have also been described, as well as their impact on cancer risk (BALDOCK et al., 2019); (3) two isoforms of AR gene (AR –V9 and AR-V7) may explain the escape to therapeutic pressure and the consequent resistance to antiandrogens (KOHLI et al., 2017); and (4) expression of an isoform of BRAF gene (the Δ[3-10] splicing variant) is related to resistance of Vemurafenib during melanoma treatment (MARRANCI et al., 2017).

In view of these findings, it is therefore necessary to increase knowledge about the HR genes isoforms. Even if *BRCA1* and *BRCA2* have been largely assessed, others HR genes should be more susceptible to this inactivation mechanism. All those examples reinforce the

need to better explore the portrait of HR genes isoforms. Since cancer transcriptome is much more diverse, this study will help to identify additional pathogenic isoforms, which could explain the impact of some variants. In contrast, some isoforms related to *BRCA1/2* germline variants have proven to have no effect on cancer predisposition, seenin the example of exon 12 in *BRCA2*, which is now considered as redundant for protein function (BIÈCHE; LIDEREAU, 1999; LI et al., 2009).

4.2.2 Epigenetic alteration

Second, epigenetic events leading to silencing of HR genes is another large domain to be explored. Epigenetic alterations are covalent modifications of DNA and histones which do not affect the sequence of DNA, but rather affect the interpretation of the genome (ALLIS; JENUWEIN, 2016, p. 201). This is a highly dynamic process in which the epigenome cooperates with other regulatory factors, such as transcription factors and noncoding RNAs, to regulate the expression or repression of the genome. It is also influenced by cellular signalling pathways and extracellular stimulation. The main actions in epigenetic regulation are DNA methylation, histone modification, chromatin remodelling, and non-coding RNA regulation. These main aspects of epigenetics present reversible effects on gene silencing and activation via epigenetic enzymes and related proteins. The epigenetic regulation plays a role in the tumorigenesis (CHENG et al., 2019). The diversity of mechanisms is exemplified in Figure 9, sheding light on the complexity of mechanisms that can deregulate the HR pathway.

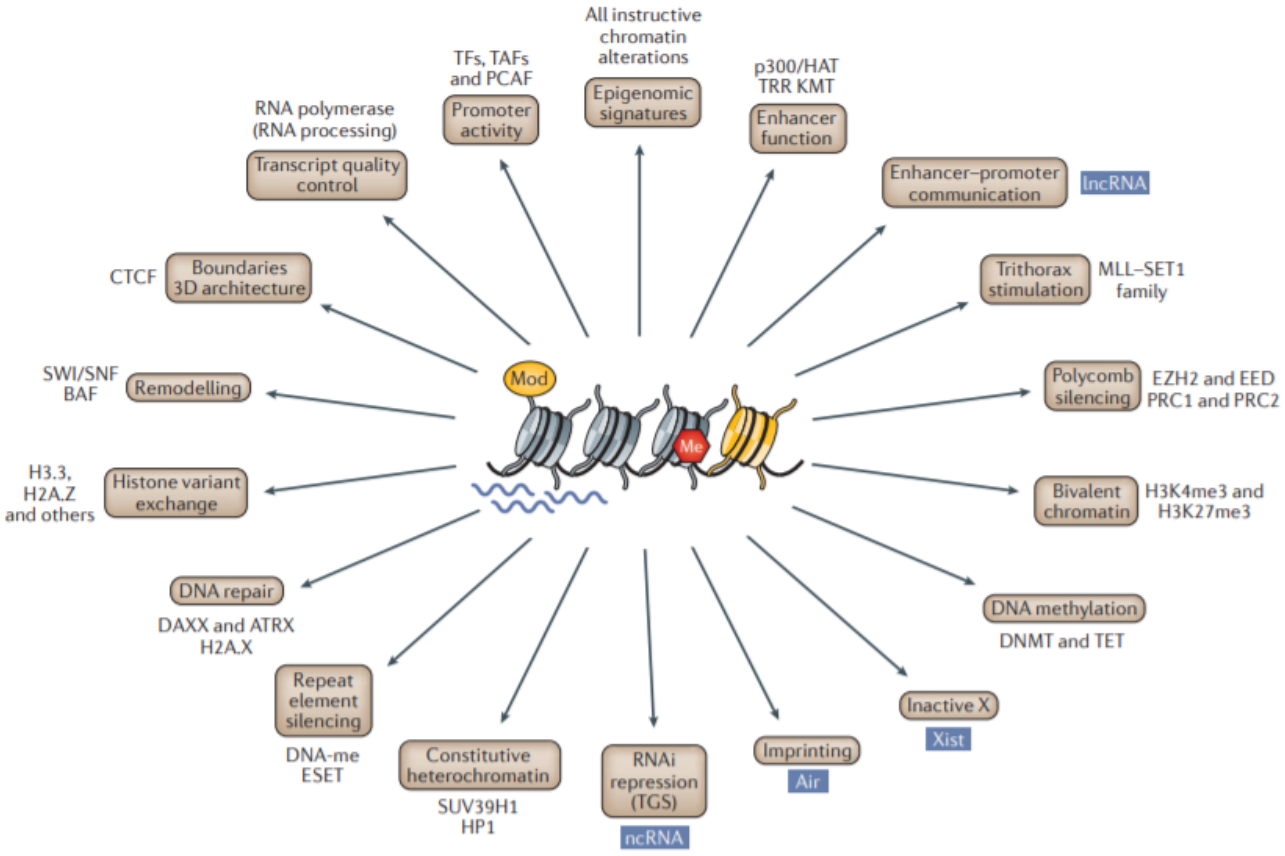


Figure 19 The Molecular Hallmarks of Epigenetic Control. Adapted (ALLIS; JENUWEIN, 2016)

4.2.2.1 Germline disease and epigenetic modifications

Some epigenetic modifications have been related to inherited cancers. The main mechanisms consist in (1) DNA hypermethylation; (2) histone modifications and chromatin remodeling; (3) inheritance of specific mRNAs, long non-coding RNAs (ncRNAs) and siRNAs/miRNAs; (4) feedback loops through which mRNA or protein products of a gene can stimulate its own transcription and enable “heritable states” of gene expression; and (5) the activity of chaperones such as Hsp90 (TREROTOLA et al., 2015).

A recent study explored DNA methylation through deep bisulfite sequencing of CpG islands and known promoter or regulatory regions in DNA extracted from peripheral blood of patients with familial or early-onset breast or ovarian cancer. The same analysis was performed in parallel for unaffected *BRCA* mutation carriers, and unaffected controls. In 9% of patients, altered methylation were identified in the promoter regions of genes known to be involved in cancer, suggesting a role for DNA methylation in HBOC (CHEN et al., 2019).

Note that the imprinting disorders are a group of currently 12 congenital diseases with no cancer predisposition disease. For cancer, only variants on the promoter and hypermethylation have been described. For example, the predisposition to B-cell chronic lymphocytic leukemia (CLL) have described a rare pathogenic variant in the promoter of the *DAPK1* gene (c.-6531A>G) associated with transcriptional repression and promoter methylation owing to enhanced binding of the transcriptional repressor HOXB7 (RAVAL et al., 2007). Constitutional MLH1 epimutations are also characterized by monoallelic methylation of the *MLH1* promoter throughout normal tissues, accompanied by allele-specific silencing. The cause is not identified in all cases (few variants / CNV) (DÁMASO et al., 2018). The same examples have been also identified in *MSH2* and *BRCA1* (KONDRASHOVA et al., 2018; LIGTENBERG et al., 2009).

The environment may also play a role in epimutations. Those events can be transgenerational and non-transgenerational epimutations with an impact on the next generation (MCCARREY, 2014). The inheritance of epigenetic traits is still a domain to be explored more deeply (XAVIER et al., 2019). The influence of epigenetic traits in breast and ovarian cancers should clearly be the next step to be explored.

4.2.2.2 Somatic alterations and the predictive impact

Somatic epigenetic alterations have also been described in the tumors. A study has proposed to use DNA methylation pattern data for assessing *BRCA1* variant pathogenicity, based on the observation that methylation profile can differentiate *BRCA1*-related from *BRCA1* wild-type tumors (FLOWER et al., 2015). The methylation of the promoter in *BRCA1* and *RAD51C* has already been discussed. As another example, *TERT* promoter mutations has been described an early event in bladder cancer development, whereas the methylation happens in parallel (LEÃO et al., 2019). In addition, some long non coding RNAs have been involved directly in the regulation of the HR pathway. Interestingly, TCGA analysis of breast and gynecological cancers revealed a functionally significant estrogen receptor-regulated long non-coding RNAs (lncRNAs) and gene/lncRNA interaction networks were identified (BERGER et al., 2018).

Therefore, epigenetic-targeted therapy is a promising field of drug development for cancer treatment. The first epigenetic targets are Histone deacetylases (HDACs) and DNA methyltransferases (DNMTs). It was then applied for the treatment of hematologic

malignancies, and has shown promising results for the treatment of solid cancers in pre-clinical and clinical scenario (GELATO et al., 2016; MOHAMMAD; BARBASH; CREASY, 2019).

4.2.3 Tumoral variants and predictive effect

An important part of this project was to explore the molecular features of *BRCA1/2*-related breast and/or ovarian tumors. Unlike other diseases such as Lynch syndrome, there are few simple somatic arguments to confirm the pathogenicity of a variant identified in the context of HBOC syndrome. Genetic instability scores, inferring BRCAness phenotype, are useful in this regard, analogous to identifying microsatellite instability in Lynch syndrome-related tumors. However, this information is not readily available in oncogenetics daily life and most often requires access to fresh tissue that is rarely available. So far, LOH data is not taken into account for *BRCA1/2* VUS classification, nor functional tests results. We proposed to incorporate LOH information, which is increasingly available with the widespread of tumoral *BRCA* testing in routine clinical practice, into the mutifactorial score for variant classification. From our perspective, it is indeed an additional argument for *BRCA1* VUS classification.

LOH should be considered in the context of i) potentially confounding factors such as the possibility of other second-hit events ii) other phenomena like the possible negative dominant effect of already described missense variants of other genes and suggested for BRCA1, and iii) haploinsufficiency previously shown for certain BRCA functions. The developpement of HR signatures will complement the LOH information. Discordance should be observed, such as HRD high without LOH if the mecanism is not related to the gene, or HRD low with LOH if the LOH is not related to the tumorigenesis and is indeed a passenger event. In addition, further research is needed in addressing if the retention of the wild-type allele at the tumoral level argues against the role of BRCA pathogenic variants in tumor development, and consequently if this could be a predictor marker of primary resistance to therapies targeting DNA damage repair.

Finally, the tumoral approach can help for the oncogenetic recruitement. Indeed, the predisposition mechanism remains undefined for about two thirds of families meeting the clinical criteria for HBOC. One possibility is a default in the recruitment criteria and many

cases are in fact sporadic cases. The better description of the tumor will certainely help to exclude families with sporadic tumors and stop long-term explorations.

4.2.4 Germline non-coding variant and risk management

In our work, the goal was to detect HR genes rare in pathogenic variants. Most GWAS studies have tried to identify more common variants related to risk of breast cancer in the genome. In fact, they identified many variants in non-coding variants (MICHAILIDOU et al., 2013, 2017). The recent cases were even located in distal regulatory elements more than 50kb from the regulated gene. Few non-coding variants were related to HR genes as *RAD51B* (c.757-98173T>C; c.1037-26520C>T). On the other hand, the genes identified with SNP associated with breast cancer risk had nearly no rare pathogenic variant (DECKER et al., 2019). In terms of risk, those variants are still under an OR of 2. It has been estimated that the susceptibility loci explain 4% of the two-fold familial relative risk of breast cancer.

The role of non-coding variants is very diverse as epigenetics dysregulation or interference of transcription factor binding sites. This possible mechanism remains to be explored. We provided information about new regulatory regions of *BRCA1* and *BRCA2* and some insights about BRCA1⁄2 transcription regulation. We confirmed the enhancing property of 2 intronic regions (in the intron 2 and intron 12) of *BRCA1* and showed a putative TFBS of E2F1 in *BRCA1* promoter, which was probably disrupted by the presence of a variant. However, assays confirming the underlying mechanism of variants impact are pending.

In clinical practice, there is currently no standardized interpretation for the significance of non-coding variants. It remains difficult to determine their clinical significance relying solely on functional tests since they may result in subtle changes in protein quantity, which in some tissues may be decisive in triggering tumorigenesis but not in others. It is expected that with the evolution of techniques and with data generation, the expression reduction threshold in a functional assays will be better defined, for a non-coding variant to be considered pathogenic. Comprehension and control of multiple variables that can influence the experiments is another limitation. For these reasons, functional tests are not sufficient and additional arguments such as tumoral and co-segregation data are necessary. That is why certainly those non-coding variants should be integrated in moderate to low risk factors.

Initially, it appeared that whole genome sequence data on tens of thousands of people would resolve the issue of missing heritability, but now it seems that more sequence data does

not necessarily generate more elucidation. Currently, the major challenge of oncogenetics is to understand the biological impact and clinical significance of the rare genetic variants detected during sequencing. Given the rarity of each variant individually, access to data sources other than genomics remains a limitation. Thus, organizing information in a database through extensive collaboration of researchers and clinicians becomes essential to facilitate data exchange. Moreover, from the knowledge we have so far, it is not possible to quantify the relative- risk of non-coding variants for appropriate genetic counseling. It remains a challenge to understand if they are enough to increase cancer risk individually or whether they should be considered as risk modifiers and taken in consideration in the context of a polygenic risk score (MAVADDAT et al., 2019).

4.2.5 Extension to oncogenetic management

Many articles have argued in favor of extending the test to the entire population in order to identify deleterious mutations related to breast and ovarian predisposition (Tung N et al 2016, Yang S et al 2018, Gabai-Kapara, E et al. 2016, Nordisq et al 2016). The prevalence of BRCA1/2 mutations seems less rare than forecasted in some data of WES analysis. In in non-European populations, an overall prevalence of 1 in 139 was estimated without any familial history screening (Abul-Husn N et al 2019). Many of those variants will not have been detected with the cosegregation criteria. On the other hand, the therapeutic application will certainly lead to extending the screening to all ovarian and breast cancers. The universal genetic testing will raise some new questions in terms of interpretation of variants without any familial history, and also in terms of penetrance of true pathogenic variants (Copur et al 2019).

With the advent of next-generation massively parallel sequencing along with cost reduction for whole-genome sequencing experiments, it is likely that a larger number of individuals will be screened systematically for variation in the complete genomic region spanning *BRCA1* and, *BRCA2*, just like other genes capturing genetic variation beyond the exons and intron– exon boundaries routinely covered by current clinical tests. The extension of the screening will certainly help to better understand the real impact of those variants.

The extension of coding sequence need to be done in conjonction with other data. Looking forward with a broader perspective, in the near future we should be able to integrate all of this data to offer an individualized decision based also in oncogenetics, for both risk

estimation and therapeutic decision. This data includes genome-wide sequencing, variants identified in non-coding regions, variants identified in other genes, clinical information, morphological and molecular tumor pathology features, and non-genetic modifying risk factors.

Currently in oncogenetics, it is already understood that clinical and pathological data are complementary to genomics, and therefore are taken into account in clinical decision making. The effects of polygenic risk scores (PRS) and other risk factors were incorporated on BOADICEA breast cancer (BC) risk prediction model (LEE et al., 2019). It was shown that the highest BC risk stratification is achieved when all genetic and lifestyle/hormonal/reproductive/anthropomorphic factors are considered jointly. Improvement of bioinformatics tools and the contribution of artificial intelligence will be central to this goal. Similar to what is happening in the field of oncology, decisions in oncogenetics should soon be made in the context of personalized medicine based on constitutional and tumor genetics information.

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6. ANNEXES

6.1 Article : "*BRCA1* and *BRCA2* 5' noncoding region variants identified in breast cancer patients alter promoter activity and protein binding." Burke L.J. et al., Human Mutation 2018

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RESEARCH ARTICLE

WILEY HGVS HUMAN GENOME VARIATION SOCIETY

BRCA1 and *BRCA2* 5′ noncoding region variants identified in breast cancer patients alter promoter activity and protein binding

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Abstract

The widespread use of next generation sequencing for clinical testing is detecting an escalating number of variants in noncoding regions of the genome. The clinical significance of the majority of these variants is currently unknown, which presents a significant clinical challenge. We have screened over 6,000 early-onset and/or familial breast cancer (BC) cases collected by the ENIGMA consortium for sequence variants in the 5′ noncoding regions of BC susceptibility genes *BRCA1* and *BRCA2*, and identified 141 rare variants with global minor allele frequency < 0.01, 76 of which have not been reported previously. Bioinformatic analysis identified a set of 21 variants most likely to impact transcriptional regulation, and luciferase reporter assays detected altered promoter activity for four of these variants. Electrophoretic mobility shift assays demonstrated that three of these altered the binding of proteins to the respective *BRCA1* or *BRCA2* promoter regions, including NFYA binding to *BRCA1*:c.-287C>T and PAX5 binding to *BRCA2*:c.-296C>T. Clinical classification of variants affecting promoter activity, using existing prediction models, found no evidence to suggest that these variants confer a high risk of disease. Further studies are required to determine if such variation may be associated with a moderate or low risk of BC.

KEYWORDS
breast cancer, BRCA1, BRCA2, promoter, transcription, variants of unknown clinical significance (VUS)

1 | INTRODUCTION

Genetic susceptibility to breast cancer (BC) is complex. Multiple germline variants have been identified over the past 25 years that are broadly categorized as high, moderate, and low risk. High-risk variants are generally rare, have a major deleterious effect on gene function, are sufficient to confer a high risk of disease, and are highly penetrant within a family. Nonsense, splicing, large deletions, and some missense changes in *BRCA1* and *BRCA2* fall into this category (reviewed in Walsh et al., 2006). There is also evidence that some alleles confer a moderate risk of cancer. These can include hypomorphic variants in known "high-risk" cancer syndrome genes (Shimelis et al., 2017; Spurdle et al., 2012), or clear loss-of-function alleles in other genes such as *CHEK2*, *PALB2*, and *ATM* (Couch et al., 2017). Low-risk variants, largely identified by genome-wide association studies, are usually common and cause subtle functional effects, such as small but significant changes in gene expression due to altered activity of proximal and distal regulatory elements (reviewed in Bogdanova, Helbig, & Dork, 2013; Ghoussaini, Pharoah, & Easton, 2013; Skol, Sasaki, & Onel, 2016). Evidence suggests that combinations of low, moderate, and high-risk variants could confer a clinically significant risk of disease (Ding et al., 2012; Kuchenbaecker et al., 2017; Sawyer et al., 2012). Identification and evaluation of all such variants is therefore crucial for accurately predicting BC risk.

Use of next generation sequence analysis for germline clinical testing of cancer cases is identifying an increasing number of variants in noncoding regions of cancer susceptibility genes, including promoters, untranslated regions (UTRs), and introns. There are currently no firm recommendations for assessing the relevance of noncoding region variants to clinical testing of Mendelian disease genes, and so the vast majority of such variants are deemed of uncertain clinical significance. This adds to the clinical challenge presented by variants of uncertain significance, namely that they complicate test reporting and genetic counseling, limit patient eligibility for intensive surveillance and gene-targeted therapies, and prevent gene testing and guided management of relatives (reviewed in Amendola et al., 2015; Eccles et al., 2013; Plon et al., 2011). It is therefore essential that the functional and clinical significance of variants mapping to noncoding regions of the genome is determined.

Gene expression is controlled at many levels with key regulatory elements being housed in noncoding regions of the genome, such as gene promoters, introns, long-range elements, and 5′ and 3′ UTRs. The *BRCA1* gene is regulated at the transcriptional and posttranscriptional levels, with functional proximal and distal regulatory elements being described in the promoter, introns, and UTRs, by us and others (Brewster et al., 2012; Brown et al., 2002; Santana dos Santos et al., 2017; Saunus et al., 2008; Tan-Wong, French, Proudfoot, & Brown, 2008; Wardrop, Brown, & kConFab, 2005; Wiedemeyer, Beach, & Karlan, 2014). Although less studied, the *BRCA2* promoter has also been mapped and characterized (reviewed in Wiedemeyer et al., 2014).

Common and rare variations in regulatory elements upstream of genes have been shown to alter gene expression and be associated with disease risk (reviewed in Betts, French, Brown, & Edwards, 2013; Diederichs et al., 2016; Millot et al., 2012). We and others have described germline cancer-associated variants in the regulatory regions, including large deletions in the *BRCA1* promoter (Brown et al., 2002), and single nucleotide variants in the promoter and/or

5′ UTR of *BRCA1* and *BRCA2* (Evans et al., 2018; Santana dos Santos et al., 2017), *MLH1* promoter (Hitchins et al., 2011), *POLG* promoter (Popanda et al., 2013), *PTEN* promoter (Heikkinen et al., 2011), *TERT* promoter (Horn et al., 2013), *KLHDC7A* and *PIDD1* promoters (Michailidou et al., 2017), *BRCA1* 3′ UTR (Brewster et al., 2012), and BC-associated Single Nucleotide Polymorphisms (SNPs) in long-range enhancers of *CCND1* (French et al., 2013).

Cancer risk-associated variants within regulatory regions are anticipated to mediate an effect on trans-acting regulatory factors (e.g., transcription factors [TFs] and miRNAs), by disrupting binding of regulatory factors and interactions between regulatory elements, such as promoter–enhancer interactions. For example, a variant in a *Cyclin D1* transcriptional enhancer has been associated with altered binding of the ELK4 TF (French et al., 2013) and a variant within the *BRCA1* 3′UTR has been shown to introduce a functional mir-103 binding site (Brewster et al., 2012). In addition, a dominantly inherited 5′ UTR *BRCA1* variant was recently shown to be associated with *BRCA1* promoter hypermethylation, which is known to impact TF binding, and associated allelic loss of *BRCA1* expression in two families affected by breast and ovarian cancers (Evans et al., 2018).

In this paper, we describe 141 germline variants in the *BRCA1* and *BRCA2* promoter, identified by members of the ENIGMA consortium in early onset or familial BC patients with no known pathogenic variants in the coding region of these genes. Using a combination of bioinformatic and experimental analyses, we have prioritized and analyzed a subset of variants that are most likely to affect the regulation of *BRCA1* and *BRCA2* and thus have the most potential to contribute to BC risk. TF binding site affinity changes resulting from these variants were subsequently analyzed by information theory (IT)-based analyses. In parallel, we have assessed if these variants exhibited the features expected for a high-risk pathogenic *BRCA1* or *BRCA2* variant, on the basis of available clinical and population data.

2 | MATERIALS AND METHODS

2.1 | Study design

An overview of the study design is shown in Figure 1. Collection of variants at all sites enabled an initial catalogue of variants from which variants were prioritized for functional analysis. Additional screening was carried out at three sites, Maastricht (M), Santiago (S), and Prague (Pr), that included additional patients (M, S, and Pr) and controls (Pr) that expanded the list of variants (Pr), the number of patients (M, S, and Pr), and included control subjects (Pr).

2.2 | Clinical and control samples

Clinical and genetic data were collected and analyzed in accordance with local human ethics guidelines of the institutions contributing to this study. All participating individuals provided informed consent for their data to be used for research purposes. An overview of the samples analyzed is shown in Table 1. Clinical samples were collected from nine European sites and were originally selected for *BRCA1* and *BRCA2* testing using ascertainment criteria that included family history

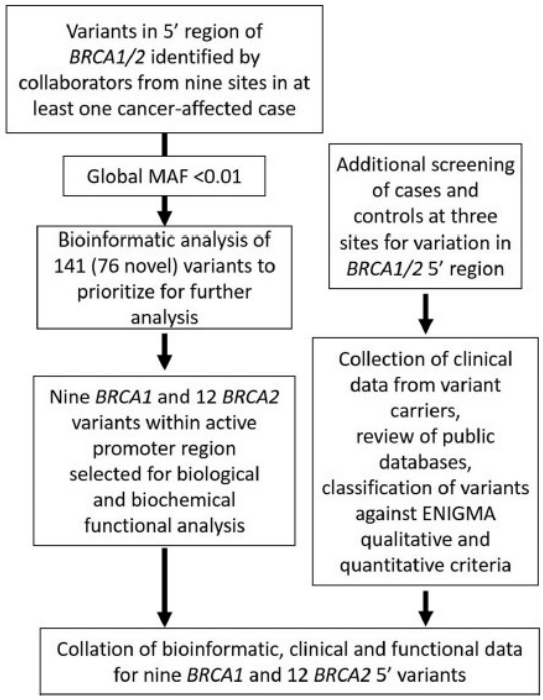

FIGURE 1 Overview of study design. Outline of the workflow of variant collection, prioritization and analysis

and young age of BC diagnosis. Female patients who did not carry a pathogenic variant in *BRCA1* or *BRCA2* coding regions or splice junctions were selected for testing of variation in the *BRCA1* and *BRCA2* 5′ regions. The controls were as follows: 661 healthy female individuals recruited through the Immunohematology and Transfusion Medicine Service of INT and Associazione Volontari Italiani Sangue (AVIS) of Milan; 312 healthy females above 60 years of age and with no malignancy in the first filial generation recruited through First Faculty of Medicine, Charles University in Prague (Lhota et al., 2016; Soukupova, Zemankova, Kleiblova, Janatova, & Kleibl, 2016); and 130 healthy females without cancer diagnosis recruited in Santiago de Compostela.

2.3 | Identification of variants

Regions containing the *BRCA1* and *BRCA2* promoter and 5′ UTR were sequenced using a range of standard DNA sequencing technologies, and bioinformatic filtering pipelines. Variants mapping to the 2,400 bp region (hg19; chr17:41,278,514 – 41,276,114) of *BRCA1* and the 2,000 bp region (hg19; chr13: 32,888,597-32,890,597) of *BRCA2* were considered for further analysis. The identified variants in *BRCA1* and *BRCA2* 5′ noncoding regions are numbered whereby the first translated nucleotide of the translation initiation codon is +1 (https://varnomen.hgvs.org/) using the Mutalyzer website (https://mutalyzer.nl/). *BRCA1* is described using NC_000017.10 (hg19 genomic sequence) and NM_007294.3 (transcript). *BRCA2* is described using NC_000013.10 (hg19 genomic sequence) and NM_000059.3 (transcript).

TABLE 1 Samples used in this study

Location	Institution	Samples	Gene region
Paris	Institut Curie, Saint Cloud	686 cases	*BRCA1* 5′region, *BRCA2* 5′region
Milan	IFOM, Fondazione Instituto FIRC di Oncologia Molecolare	772 cases 661 controls	*BRCA1* 5′region
Pisa	Department of Translational Research and New Technologies in Medicine, University of Pisa	80 cases	*BRCA1* 5′region, *BRCA2* 5′region
Santiago de Compostela	Fundación Pública Galega de Medicina Xenómica-SERGAS, Grupo de Medicina Xenómica-USC, CIBERER, IDIS	270 cases 130 controls	*BRCA1* 5′region, *BRCA2* 5′region
Copenhagen	Center for Genomic Medicine	1157 cases	*BRCA1* 5′region, *BRCA2* 5′region
Ghent	Center for Medical Genetics, Ghent University Hospital	357 cases	*BRCA1* 5′region, *BRCA2* 5′region
Barcelona	Vall d'Hebron Institute of Oncology	192 cases	*BRCA1* 5′region, *BRCA2* 5′region
Prague	CZECANCA – CZEch CAncer panel for Clinical Aplication, Institute of Biochemistry and Experimental Oncology	2961 cases 312 controls	*BRCA1* 5′region, *BRCA2* 5′region
Maastricht	Department of Clinical Genetics, Maastricht University Medical Centre	900 cases	*BRCA2* 5′region

2.4 | Bioinformatic analysis of variants

As an initial screen, each variant submitted for study was assessed for population frequency using intersection of the variants with dbSNP (version 138 or 150, as the study progressed) within the UCSC Genome browser and Variant Effect Predictor at ENSEMBL (https://www.ensembl.org/info/docs/tools/vep/index.html). Variants with a global minor allele frequency (MAF) of < 0.01 were included in subsequent bioinformatic analyses. Further details of bioinformatics analyses to map active regulatory elements and prioritize variants for functional assays are contained in Supporting Information Methods. Variants were considered to be high priority for experimental analysis if they contained all of the following features: (1) resided in DNaseI or formaldehyde-assisted isolation of regulatory elements (FAIRE) peaks, (2) coincided with high scores for DNaseI (Base Overlap Signal > 40) or FAIRE (Base Overlap Signal > 10) in a breast cell line, (3) resided in a region of breast cell specific TF binding, (4) overlapped with a TF consensus motif, and (5) were within an evolutionarily conserved element with a high Phastcons score (>0.75). Medium priority variants lacked one or two of these features, whereas low priority variants had only one or none of these features.

2.4.1 | *In silico* TF binding analysis

All rare variants were analyzed *in silico* using an IT-based method (Caminsky et al., 2016; Mucaki et al., 2016) and a modified version of the Shannon pipeline utilizing TF information models built from ENCODE ChIP-seq datasets (Lu, Mucaki, & Rogan, 2017) to assess potential effects of variants on TF binding. Details of analyses are contained in Supporting Information Methods.

2.5 | Experimental analysis of variants

2.5.1 | Promoter reporter assays

The 499 bp *BRCA1* (chr17:41,277,787-41,277,289) and 750 bp *BRCA2* (chr13:32,889,230-32,889,979) promoter regions were cloned into pCR-Blunt vector (Thermo Fisher, Waltham, MA). Site-directed mutagenesis was used to introduce variants using the primers listed in Supporting Information Table S1. Plasmids were purified using the QIAprep miniprep kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Plasmid preparations were validated using restriction digest and DNA sequencing and inserts were shuttled into pGL3-Basic luciferase reporter vector (Promega, Madison, WI). All plasmids for transfection were analyzed for DNA conformation on a 1% w/v agarose gel and only plasmids possessing a supercoiled conformation were used for transfections. Transfection details are described in Supporting Information Methods.

The luciferase-based reporter assay was performed as described previously (Brewster et al., 2012). Positive controls were B1-Ets, *BRCA1*:c.-330_-329delinsTT, that decreases *BRCA1* promoter activity in MCF7 cells (Atlas, Stramwasser, Whiskin, & Mueller, 2000) and B2-Ets (E2Fmut1: *BRCA2*:c.-282_-281delinsAA), that has been shown to decrease *BRCA2* promoter activity in MCF7 cells (Davis, Miron, Andersen, Iglehart, & Marks, 1999). Statistical analyses were performed in GraphPad Prism using one-way analysis of variance followed by Tukey's post hoc test and values $P < 0.05$ were deemed statistically significant.

2.5.2 | Electrophoretic mobility shift assays

Nuclear proteins were extracted as described in Supporting Information Methods and electrophoretic mobility shift assays (EMSAs) were carried out using a Pierce LightShift Chemiluminescent EMSA Kit (Thermo Fisher, Waltham, MA) with modifications described in Supporting Information Methods. For competition and supershift studies, nuclear extracts were initially incubated with unlabeled double-stranded (ds) competitor probes or antibodies in binding buffer before addition of the biotinylated probe and incubation at room temperature. Positive controls for *BRCA1* and *BRCA2* DNA binding were sequences surrounding the B1-Ets and B2-Ets mutations described above.

2.6 | Qualitative and quantitative classification of variants

Variants were classified according to the ENIGMA classification criteria for variation in *BRCA1* and *BRCA2* (https://enigmaconsortium.org/) to determine whether any of the prioritized variants were associated with a high risk of disease. See Supporting Information Methods for further details.

3 | RESULTS

3.1 | Identification and prioritization of sequence variants in *BRCA1* and *BRCA2* 5′ noncoding regions

The 5′ noncoding regions of *BRCA1* and *BRCA2* in early onset or familial BC patients with no known *BRCA1* or *BRCA2* germline pathogenic variant were sequenced at nine different sites as part of an approved ENIGMA (https://enigmaconsortium.org/) project. For the *BRCA1* 5′ region, 6,475 patients were sequenced at eight different sites along with 1,103 controls. For the *BRCA2* 5′ region, 6,603 patients were sequenced at eight different sites as well as 442 controls.

After excluding variants with global MAF > 0.01 at time of variant identification, a total of 141 unique single nucleotide variants and short insertions/deletions were identified, 81 in *BRCA1* and 60 in *BRCA2* (Supporting Information Tables S2 and S3). Theses variants have been submitted to the LOVD databases, www.lovd.nl/BRCA1 and www.lovd.nl/BRCA2. To evaluate the potential of these rare variants to impact gene regulation, we initially undertook a comprehensive bioinformatic analysis. Promoter regions of *BRCA1* and *BRCA2* were defined by bioinformatic predictors including chromatin marks (Figure 2). These regions show the characteristic histone H3 epigenetic marks, including H3K4me3, H3K27ac, and H3K9ac, as well as occupancy by multiple TFs. Of the variants identified in cases only, 22 *BRCA1* and 23 *BRCA2* variants resided within the minimal promoter regions.

To predict the potential impact of variants on promoter activity, we prioritized variants using breast cell specific data for chromatin accessibility and TF occupancy along with evolutionary conservation. Due to the limited breast cell specific TF ChIP-seq data, we also included ENCODE TF ChIP-seq and TF consensus motif data from all cell lines. A total of nine *BRCA1* and 12 *BRCA2* variants were selected for further functional analysis (Figure 2; Tables 2 and 3).

3.2 | *BRCA1* and *BRCA2* promoter activity is altered by 5′ noncoding sequence variants

To examine the potential effect of the 21 prioritized *BRCA1* and *BRCA2* 5′ noncoding variants on regulatory activity, promoter activity was measured using luciferase assays in MCF7 and MDA-MB-468 BC cell lines. Two of the nine prioritized *BRCA1* variants decreased *BRCA1* promoter activity relative to the wild-type (WT) construct (Figure 3a and 3b). *BRCA1*:c.-315del significantly decreased the *BRCA1* promoter luciferase activity in both cell lines, whereas *BRCA1*:c.-192C decreased luciferase activity in the MCF7 cell line. Furthermore, one variant, *BRCA1*:c.-287T, displayed increased activity relative to the WT construct in the MCF7 cell line. For *BRCA2*, one of the 12 variants, *BRCA2*:c.-296T, decreased *BRCA2* promoter activity relative to the WT construct in the MCF7 cell line (Figure 3c and 3d).

3.3 | *In silico* analyses of *BRCA1* and *BRCA2* 5′ variants predict alterations in TF binding

BRCA1 and *BRCA2* promoters are regulated by a complex array of DNA-binding proteins and transcriptional coactivators and corepressors (reviewed in McCoy, Mueller, & Roskelley, 2003; Mueller & Roskelley, 2003; Wiedemeyer et al., 2014). In silico analysis was carried out to examine whether the *BRCA1* and *BRCA2* promoter variants shown to alter luciferase activity (see above) are likely to affect binding of trans-acting protein factors in breast cells.

Interrogation of ENCODE ChIP-seq datasets derived from breast cell lines show that, although the number of datasets is limited, TFs bind to regions encompassing the prioritized variants (Figure 2 and Supporting Information Figure S1). ENCODE ChIP-seq data from other cell lines indicate that some variants are located within consensus motifs for specific TFs associated with these regions (Tables 2 and 3; Supporting Information Figure S1). *BRCA1*:c.-287C>T overlaps with the consensus binding motif for CCAAT Box binding factors and *BRCA2*:c.-296C>T is located within the consensus motif for PAX5.

IT analysis of the prioritized variants showed that the binding strengths of several TFs are predicted to be altered by the *BRCA1* and *BRCA2* variants (Table 4 and Supporting Information Table S4). All of the variants that altered promoter activity were predicted to have consequences on TF binding. *BRCA1*:c.-287C>T and *BRCA2*:c.-296C>T are predicted to disrupt binding of CCAAT Box binding factors and PAX5, respectively. *BRCA1*:c.-315del is predicted to disrupt the binding of TCF7L2 but creates a POU2F2 (also known as Oct-2) binding site. *BRCA1*:c.-192T>C is predicted to strengthen a RFX5 site and creates an ETS1 site.

3.4 | 5′ variants in *BRCA1* and *BRCA2* alter protein–DNA interactions in EMSA analyses

To examine potential alterations in the binding of nuclear proteins from breast cells by the *BRCA1* and *BRCA2* promoter variants that altered luciferase activity, we carried out EMSA analysis. For *BRCA1*, two of three analyzed variants, c.-315del and c.-287C>T, displayed allele-specific protein binding (Figure 4). For probes containing the region surrounding the *BRCA1*:c.-315del variant, changing the WT sequence to the variant sequence resulted in the enhanced binding of a slower migrating band (Figure 4a and 4b). For probes containing the region surrounding the *BRCA1*:c.-287C>T variant, introduction of the variant sequence resulted in almost complete loss of protein binding to the probe (Figure 4a).

To determine if the DNA-protein interactions were specific, competition experiments were performed. In the case of *BRCA1*:c.-315del, all bands were competed by both the WT and the variant containing probes in two cell lines (Figure 5a and 5b). For *BRCA1*:c.-287C>T, only the WT probe was able to compete for binding (Figure 5c). The nonspecific probe from an unrelated region of the *BRCA1* promoter

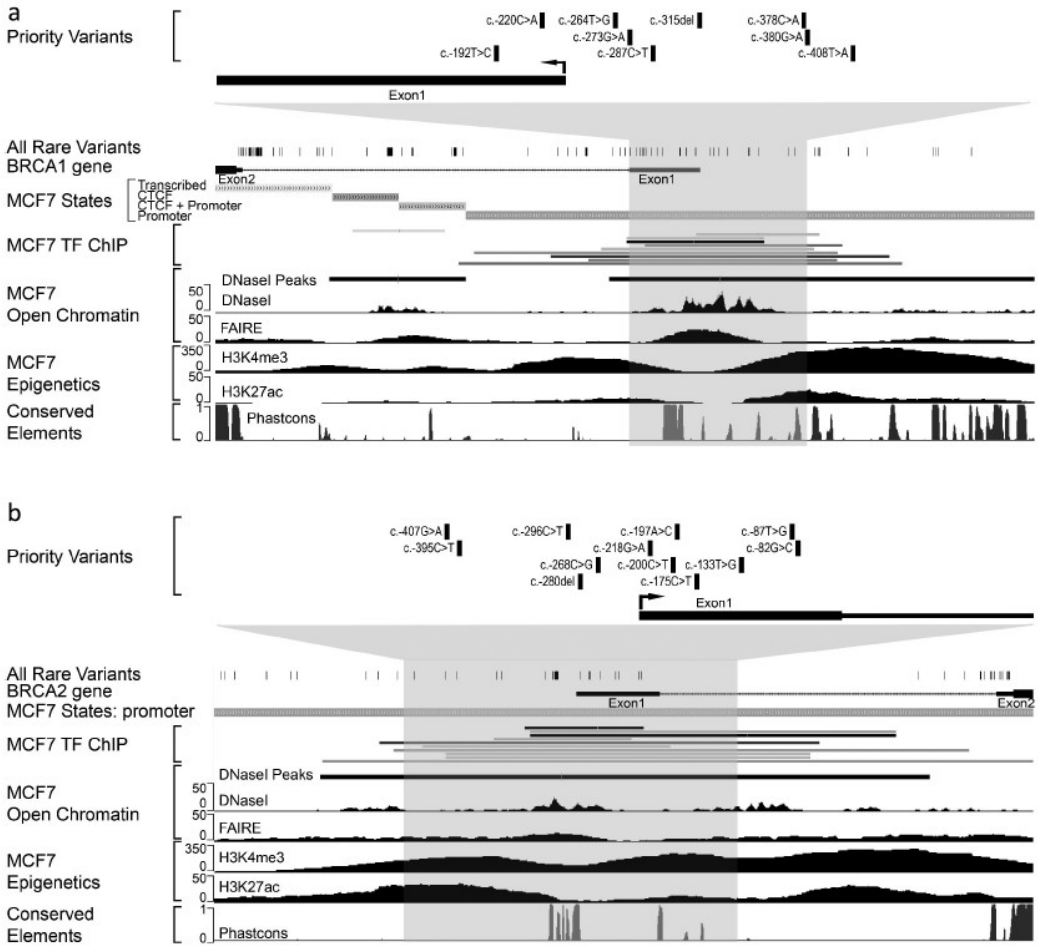

FIGURE 2 Variants identified in the 5′ regions of *BRCA1* and *BRCA2* map to predicted regulatory elements. Snapshots of the UCSC genome browser showing regions of *BRCA1* (a) and *BRCA2* (b) analyzed by targeted sequencing with available ENCODE regulatory marks derived from MCF7 cells. Chromatin segregation states from regulatory region annotation are shown (MCF7 states). The *BRCA1* and *BRCA2* genomic regions used for functional analyses are highlighted in grey. Prioritized variants within these regions are indicated

TABLE 2 *BRCA1* prioritized variants

Gene	hg19 position (chr17)	Variant name[a]	rsID	Global MAF in dbSNP	TF motif (ENCODE)[b]	Bioinformatic priority
BRCA1	g.41277676A>T	c.-408T>A	Novel		CEBPB	High/medium
BRCA1	g.41277648C>T	c.-380G>A	Novel		RXRA	High/medium
BRCA1	g.41277646G>T	c.-378C>A	rs186775935	0.00040	RXRA	High/medium
BRCA1	g.41277583del	c.-315del	rs901029407	0.00003	ATF1,2,3, CREB1[c]	Medium
BRCA1	g.41277555G>A	c.-287C>T	Novel		NFYA, NFYB	High/medium
BRCA1	g.41277541C>T	c.-273G>A	rs112960339	0.00499		Medium
BRCA1	g.41277532A>C	c.-264T>G	rs904148166	0.00003		Medium
BRCA1	g.41277488G>T	c.-220C>A	Novel			Medium
BRCA1	g.41277460A>G	c.-192T>C	rs113323025	0.00519		Medium

TF, transcription factors.
[a]Based on NM_007294.3.
[b]Overlap with TF motif in ENCODE TF-ChIP datasets from all cells.
[c]Variant overlaps this motif, but the deletion does not alter the motif sequence.

TABLE 3 *BRCA2* prioritized variants

Gene	hg19 Position (Chr13)	Variant name[a]	rsID	Global MAF in dbSNP	TF motif (ENCODE)[b]	Bioinformatic priority
BRCA2	g.32889437G>A	c.-407G>A	rs36221751	0.0018		Medium
BRCA2	g.32889449C>T	c.-395C>T	Novel			Medium
BRCA2	g.32889548C>T	c.-296C>T	rs563971900	0.0004	PAX5	High/medium
BRCA2	g.32889564delG	c.-280del	Novel		ELF1, GABPA, ELK1,4	High
BRCA2	g.32889576C>G	c.-268C>G	Novel			High/medium
BRCA2	g.32889626G>A	c.-218G>A	Novel			Medium
BRCA2	g.32889644C>T	c.-200C>T	Novel		MAZ	Medium
BRCA2	g.32889647A>C	c.-197A>C	rs370721506	NA	MAZ	Medium
BRCA2	g.32889669C>T	c.-175C>T	rs55880202	0.0058		Medium
BRCA2	g.32889711T>G	c.-133T>G	Novel			Medium
BRCA2	g.32889757T>G	c.-87T>G	Novel			Medium/low
BRCA2	g.32889762G>C	c.-82G>C	Novel			Medium/low

NA, no data available, TF, transcription factors.
[a]Based on NM_000059.3.
[b]Overlap with TF motif in ENCODE TF-ChIP datasets from all cells.

did not compete any bands showing that the bands seen in the EMSA were specific.

Analysis of the regions of the *BRCA2* promoter using EMSA revealed that region containing the *BRCA2*:c.-296C>T variant bound nuclear proteins from MCF7 nuclear extracts and that this interaction was dramatically reduced by introduction of the variant sequence (Figure 6a). Competition experiments showed that these interactions were specific and not competed by a nonspecific probe from an unrelated region of the *BRCA1* promoter (Figure 6a).

To determine the effect of these variants on the binding of specific TFs, competition and supershift analyses were performed. *BRCA1*:c.-287C>T overlaps with the consensus binding motif for CCAAT Box binding factors, NFYA and NFYB (Table 2 and Supporting Information Figure S1a), and IT analysis predicts that the variant disrupts binding of these TFs (Table 4). Consistent with these predictions, supershift experiments show that *BRCA1*:c.-287C>T disrupts binding of NFYA to this region (Figure. 5d). In addition, we analyzed *BRCA2*:c.-296C>T, which maps within the consensus binding motif for PAX5 (Table 2 and Supporting Information Figure S1b), and is predicted by IT analysis to disrupt binding of PAX5 (Table 4), by cross-competition experiments using known PAX5 binding sites from *hCD19* (Kozmik, Wang, Dorfler, Adams, & Busslinger, 1992) and *hDAO* (Tran et al., 2015) genes. These experiments show that known PAX5 binding sites compete efficiently for binding of nuclear proteins to the *BRCA2* promoter region, indicating that PAX5 binding is reduced as a consequence of the nucleotide sequence change (Figure. 6b). In contrast, supershift experiments for POU2F2 (Oct-2) showed no evidence for *BRCA1*:c.-315del causing a change in binding of POU2F2 in the cell line used (data not shown).

3.5 | Clinical classification of *BRCA1* and *BRCA2* 5′ noncoding sequence variants

Variants were classified according to the ENIGMA guidelines, which are calibrated for classification of variants as high risk, using available population frequency and/or clinical data (Supporting Information Tables S5 and S6). In this context, the term pathogenicity refers to a variant that confers a high risk of disease. Importantly, these classification guidelines do not identify those variants that confer a moderate or low risk of disease.

Of those variants identified in cases only, 26/70 (37%) of BRCA1 variants had been reported in dbSNP at study initiation (maximum global frequency = 0.006; Supporting Information Table S2), and 22/54 (41%) of *BRCA2* variants observed in cases only were identified in dbSNP (maximum global frequency = 0.006; Supporting Information Table S3). Review of variant frequency in public reference groups identified 21 variants that were classifiable, as Not Pathogenic, based on frequency in control groups (Supporting Information Table S5): six *BRCA1* and five *BRCA2* variants were observed at >1% frequency in population subgroups (stand-alone evidence against pathogenicity, when detected in a nonfounder outbred population group); six *BRCA1* and four *BRCA2* variants occurred at frequency 0.001–0.01 (range 0.0014–0.0076) in at least five individuals in the reference set, which combined with a low assumed prior is considered sufficient as evidence against pathogenicity (Supporting Information Table S5). Frequency data from controls screened for this study also supported the frequency-based classifications for eight of these 21 variants (Supporting Information Table S5).

Segregation analysis for seven informative families aided classification for six variants, whereas histopathology likelihood ratios (LRs) derived for 24 tumors altered classification for 10 variants (Supporting Information Table S6). Combining findings from qualitative and quantitative methods, most variants (113/141; 80%) remained Class 3 Uncertain, largely due to a lack of data.

A total of 27/141 (19%) variants were classified as Not Pathogenic or Likely Not Pathogenic. Of the 21 variants prioritized for functional analysis, eight variants (38%) were classified as Not Pathogenic or Likely Not Pathogenic based on frequency information and/or multifactorial analysis (Table 5), including two variants (*BRCA1*:c.-192T>C

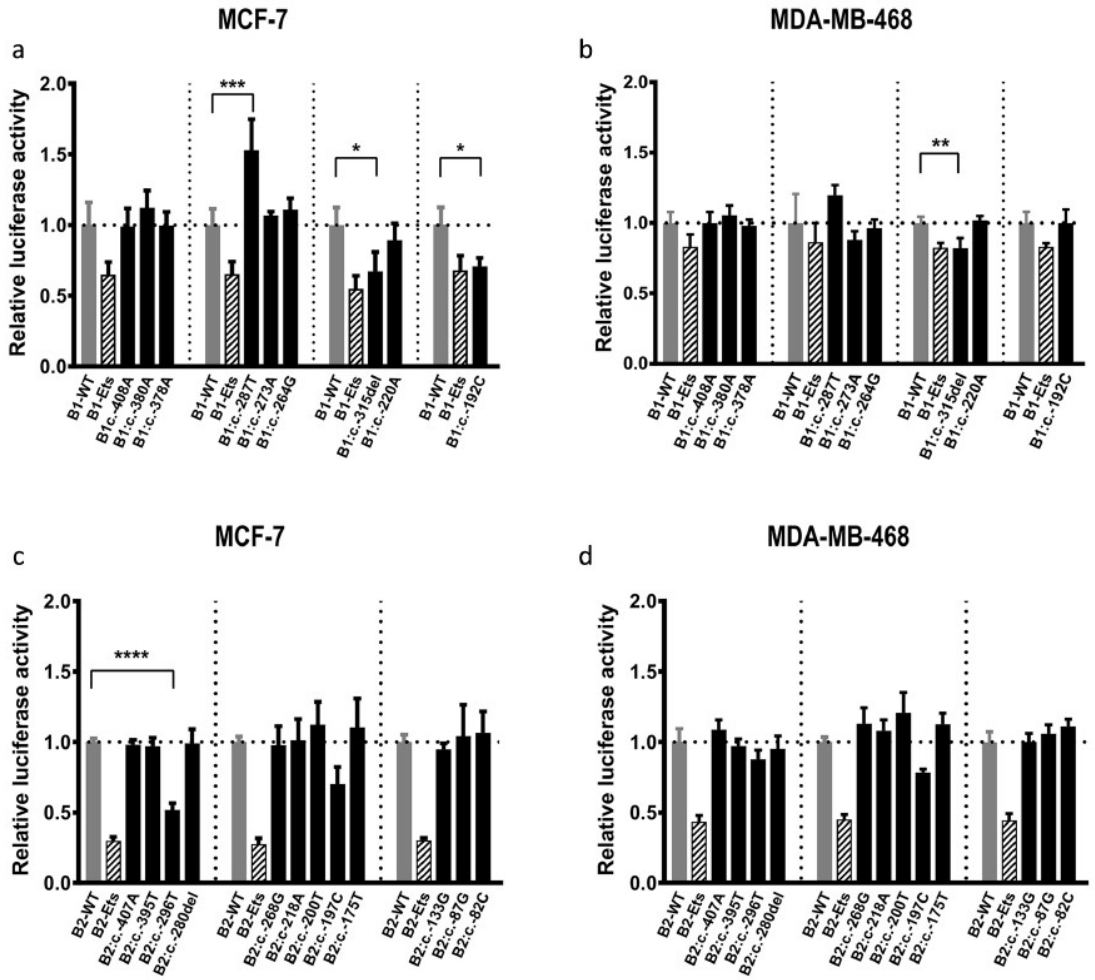

FIGURE 3 Variants mapping to the 5′ regions of *BRCA1* and *BRCA2* alter promoter activity in MCF7 and MDA-MB-468 breast cancer cells. MCF7 (a and c) and MDA-MB-468 cells (b and d) were transfected with pGL3 vectors where luciferase expression is controlled by a portion of the *BRCA1* (B1) (a and b) or *BRCA2* (B2) (c and d) promoter. Cells were transfected with plasmids containing the wild-type (WT) promoter sequence (grey bars), positive control (B1-Ets or B2-Ets; striped bars) or the indicated variants (black bars). Luciferase expression was normalized to a cotransfected pRL-TK plasmid. Data represent the average of three independent biological replicates ± standard deviation (*SD*). The horizontal dotted line represents WT promoter activity set at 1.0-fold. The vertical dotted lines demarcate individual experiments that include WT, positive control, and variant containing plasmids. (* $P < 0.05$; ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$)

and *BRCA2*:c.-296 C > T) that were shown to decrease promoter activity and in the case of *BRCA2*:c.-296 C>T also resulted in perturbed TF binding. Taken together this analysis indicates that none of the variants shown to affect function in this study are associated with a high risk of disease. This analysis is silent, however, on whether these variants may confer a moderate or low risk of disease.

4 | DISCUSSION

Next generation sequencing and gene panel testing enable rapid analysis of gene regions that have previously not been included in standard screening procedures, including promoters, UTRs, introns, and extragenic regions. It is hypothesized that variants in these regions have potential to modulate gene expression (Stranger et al., 2005; Stranger et al., 2007) and impact on relative disease risk, possibly in collaboration with multiple other low-, moderate-, and high-risk variants (Manolio et al., 2009). This extends and validates our previous study (Santana dos Santos et al., 2017) by using a larger number patients analyzed over nine geographical locations, identifying additional BC-associated variants, and showing that a subset of these variants modulate binding of specific TFs. Further, we have compared results from our bioinformatics and functional analysis to variant classifications based on ENIGMA *BRCA1/2* guidelines for high-risk variation in these genes.

Through targeted sequencing of over 6,000 early onset/familial BC patients, we identified 141 single nucleotide variants and small indels mapping to the 5′ noncoding regions of BRCA1 and BRCA2. Of these,

TABLE 4 Information theory analysis of prioritized *BRCA1/2* variants

Variant name	TF motif (ENCODE)	Consequences
BRCA1:c.-408T>A	CEBPB	CEBPB site weakened (did not meet stringent filtering thresholds)
BRCA1:c.-380G>A	RXRA	Weak RXRA and IRF3 sites weakened, HNF4G site weakened.
BRCA1:c.-378C>A	RXRA	RXR unchanged, HSF1 site lost and GR site created
BRCA1:c.-315del	ATF1,2,3, CREB1[a]	TCF7L2 site lost and POU2F2 created
BRCA1:c.-287C>T	NFYA, NFYB	NFYA and NFYB sites lost, weak PBX3 site created
BRCA1:c.-273G>A		Altered TF strength did not fulfill stringent filtering thresholds[b]
BRCA1:c.-264T>G		BHLHE32 and MYC sites created.
BRCA1:c.-220C>A		Altered TF strength did not fulfill stringent filtering thresholds[b]
BRCA1:c.-192T>C		ETS1 site created, weak RFX5 site strengthened.
BRCA2:c.-407G>A		Weak MEF2A site strengthened, GATA2 site lost.
BRCA2:c.-395C>T		TEAD4 site lost.
BRCA2:c.-296C>T	PAX5	PAX5 site weakened.
BRCA2:c.-280del	ELF1, GABPA, ELK1,4	GABPA site unchanged, MXI1 andTCF3 sites lost.
BRCA2:c.-268C>G		Altered TF strength did not meet filtering thresholds[b]
BRCA2:c.-218G>A		Altered TF strength did not meet filtering thresholds[b]
BRCA2:c.-200C>T	MAZ[c]	KLF1 site abolished.
BRCA2:c.-197A>C	MAZ[c]	SP4 weakened, GR site weakened, TCF3 site created
BRCA2:c.-175C>T		Altered TF strength did not fulfill stringent filtering thresholds[b]
BRCA2:c.-133T>G		Altered TF strength did not fulfill stringent filtering thresholds[b]
BRCA2:c.-87T>G		Altered TF strength did not fulfill stringent filtering thresholds[b]
BRCA2:c.-82G>C		Altered TF strength did not fulfill stringent filtering thresholds[b]

[a]Variant overlaps this motif, but the deletion does not alter the motif sequence.
[b]Change in information did not fulfill stringent filtering criteria, where [A] site $R_i < R_{sequence} - 1$ standard deviation of TF model, or [B] where $\Delta R_i < 4$ bits.
[c]No MAZ binding model available.

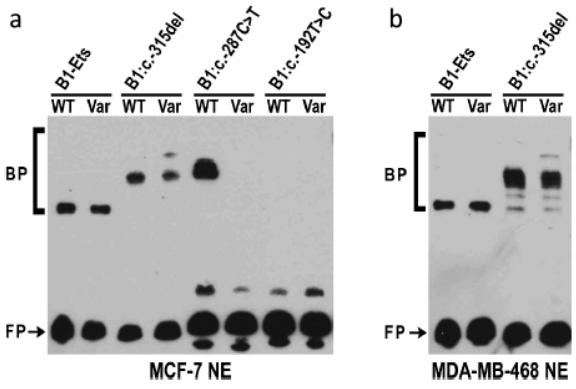

FIGURE 4 Variants in the 5′ regions of *BRCA1* alter DNA:protein complex formation. Electrophoretic mobility shift assay (EMSA) reactions were performed with 3′ biotinylated double-stranded DNA probes from the *BRCA1* 5′ region and nuclear extracts (NE) from (a) MCF7 or (b) MDA-MB-468 cells. DNA probes contained either wild-type (WT) or variant (Var) sequences. Free unbound probe (FP) and probe bound by nuclear proteins (BP) are indicated

four (*BRCA1*:c.-315del, *BRCA1*:c.-287C>T, *BRCA1*:c.-192T>C, and *BRCA2*:c.-296C>T) caused a significant change in promoter activity. The observed alterations in *BRCA1* and *BRCA2* promoter activity are of a similar magnitude to that seen with other germline variants associated with BC risk (Michailidou et al., 2017), including a variant in the *TERT* promoter, which creates a new binding site for Ets factors and results in a 1.2–1.5-fold increase in luciferase activity in a promoter reporter assay (Horn et al., 2013), and variants in the promoters of *KLHDC7A* and *PIDD1* (Michailidou et al., 2017). Although this supports the hypothesis that moderate change in promoter activity can be associated with disease risk, further work is needed to confirm this.

One of the four variants significantly altered luciferase activity in both tested cell lines, whereas the remaining three variants only affected luciferase activity in MCF7 cells. This may reflect the differential availability of crucial TFs in MDA-MB-468 cells (Kao et al., 2009) and highlights the importance of undertaking that assays for functional activity of variants in more than one cell line. Three variants, *BRCA1*:c.-380G>A, *BRCA2*:c.-296C>T, and *BRCA2*:c.-218G>A, were also analyzed in our earlier paper (Santana dos Santos et al., 2017). Although the cell lines used in the two studies were different (MDA-MB-231 in Santana dos Santos et al., 2017 and MCF7 and MDA-MB-468 here), the trends are the same in five out of six analyses. The difference for *BRCA2*:c.-296C>T, which causes a significant decrease in MDA-MB-231 and MCF7 cells, but not MDA-MB-468 cells, may again be indicative of differential gene expression in BC cell lines (Kao et al., 2009). Overall, however, the consistency of results performed in two separate laboratories underscores the robustness of the assay system.

Some variants were associated with a decrease in promoter activity, whereas others were associated with an increase. As TFs can

TABLE 5 Classification of prioritized variants

Gene	Genomic location (hg19)	HGVS c. nomenclature	Ludiferase result	Combined interpretation of frequency data & multifactorial analysis	Highest MAF (population, database)	Prior probability of pathogenicity	Segregation Bayes score (# families)	Tumor histopathology likelihood ratio (# tumors)	Combined odds for causality	Posterior probability of pathogenicity[c]
BRCA1	g.41277676A>T	c.-408T>A	No effect	Uncertain		0.02				
BRCA1	g.41277648C>T	c.-380G>A	No effect	Uncertain		0.02		1.67 (1)	1.67	NA
BRCA1	g.41277646G>T	c.-378C>A	No effect	Uncertain	0.0015 (African, 1,000 Genomes)	0.02				
BRCA1	g.41277583del	c.-315del	Decrease	Uncertain		0.02				
BRCA1	g.41277555G>A	c.-287C>T	Increase	Uncertain		0.02		0.64 (1)	0.64	NA
BRCA1	g.41277541C>T	c.-273G>A	No effect	Not pathogenic[a]	0.0159 (African, 1,000 Genomes)	0.02				
BRCA1	g.41277532A>C	c.-264T>G	No effect	Uncertain		0.02		0.51 (1)	0.51	NA
BRCA1	g.41277488G>T	c.-220C>A	No effect	Uncertain		0.02				
BRCA1	g.41277460A>G	c.-192T>C	Decrease	Not pathogenic[a]	0.0159 (African, 1,000 Genomes)	0.02				
BRCA2	g.32889437G>A	c.-407G>A	No effect	Not pathogenic[b]	0.0080 (Prague, this study)	0.02		0.55 (6)	0.55	NA
BRCA2	g.32889449C>T	c.-395C>T	No effect	Uncertain		0.02				
BRCA2	g.32889548C>T	c.-296C>T	Decrease	Not pathogenic[b]	0.0080 (Prague, this study)	0.02	3.07 (1)	1.91 (8)	5.87	0.1069
BRCA2	g.32889564delG	c.-280del	No effect	Uncertain		0.02		0.69 (1)	0.69	NA
BRCA2	g.32889576C>G	c.-268C>G	No effect	Uncertain		0.02				
BRCA2	g.32889626G>A	c.-218G>A	No effect	Likely not pathogenic		0.02	0.52 (1)	0.72 (1)	0.38	0.0076
BRCA2	g.32889644C>T	c.-200C>T	No effect	Likely not pathogenic		0.02		0.37 (1)	0.37	0.0075
BRCA2	g.32889647A>C	c.-197A>C	No effect	Not pathogenic[b]	0.0014 (African, FLOSSIES)	0.02		1.08 (1)	1.08	NA
BRCA2	g.32889669C>T	c.-175C>T	No effect	Not pathogenic[a]	0.0197 (African, FLOSSIES)	0.02				
BRCA2	g.32889711T>G	c.-133T>G	No effect	Uncertain		0.02				
BRCA2	g.32889757T>G	c.-87T>G	No effect	Uncertain		0.02				
BRCA2	g.32889762G>C	c.-82G>C	No effect	Uncertain		0.02				

NA, not applicable: multifactorial classification not assigned as the combined odds of causality were insufficient (≥0.5 and ≤2) to derive a posterior probability of pathogenicity (Vallee et al., 2016).

[a]Not pathogenic based on frequency > 1% in an outbred sampleset.

[b]Variant allele assigned a low prior probability of pathogenicity of 0.02 assuming conservatively that 2/100 of such variants might be associated with a high risk of cancer and allele frequency ≥0.001 and < 0.01 in outbred sample set.

[c]Posterior probabilities used to assign IARC 5-tier class as described in Plon et al., 2008.

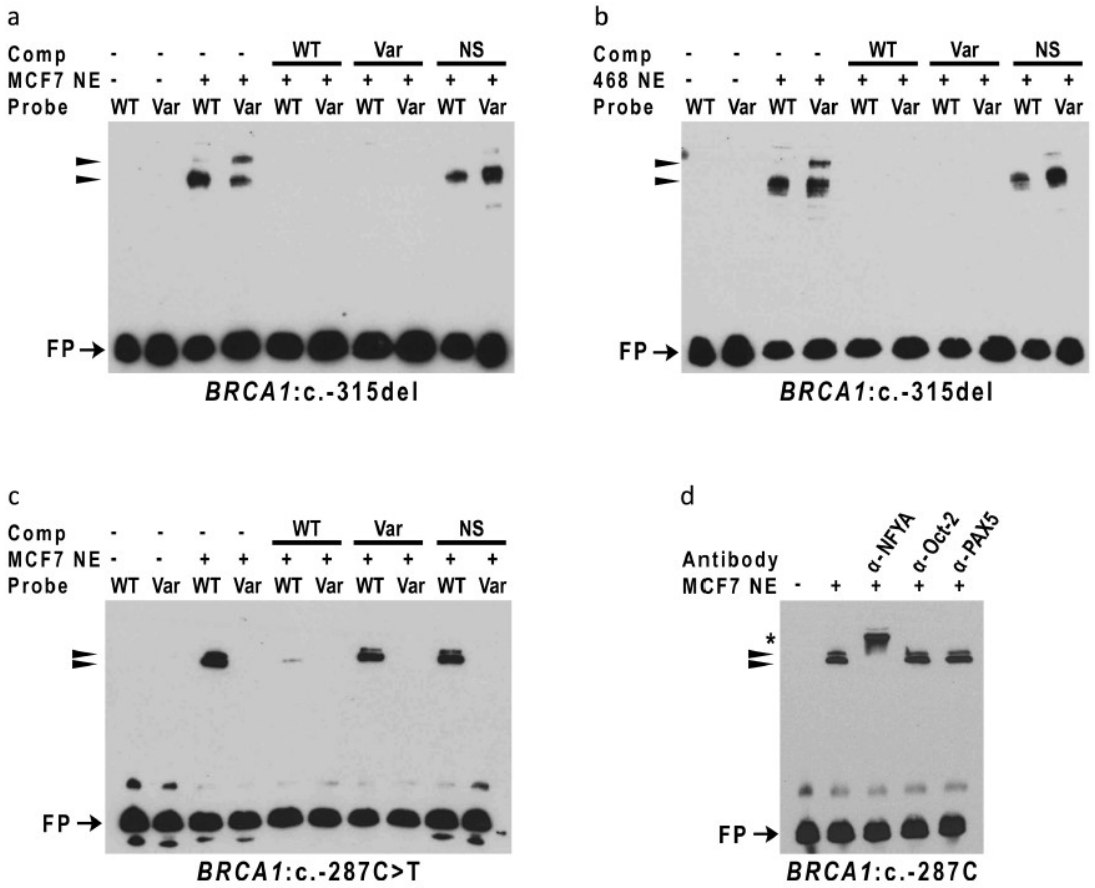

FIGURE 5 Variant sequences in the *BRCA1* 5′ region alter specific DNA:protein complex formation. Competition electrophoretic mobility shift assay (EMSAs) were performed using 3′ biotinylated double-stranded DNA probes containing sequences from the *BRCA1* 5′ region surrounding the B1:c.-315del (a and b) and B1:c.-287C>T (c) variants. DNA probes containing the wild-type (WT) or variant (Var) sequence were incubated with nuclear extracts from MCF7 cells (MCF7 NE) or MDA-MB 468 cells (468 NE) in the presence (+) or absence (–) of unlabeled WT, Var, or nonspecific (NS) competitor (Comp) DNA. Free unbound probe (FP) and specific DNA:protein complexes (arrowheads) are indicated. Supershift experiments (d) were performed with the *BRCA1*:c.-287C (WT) probe and antibodies to NFYA, Oct-2 (POU2F2) and PAX5. The supershifted NFYA complex is indicated by asterisk (*)

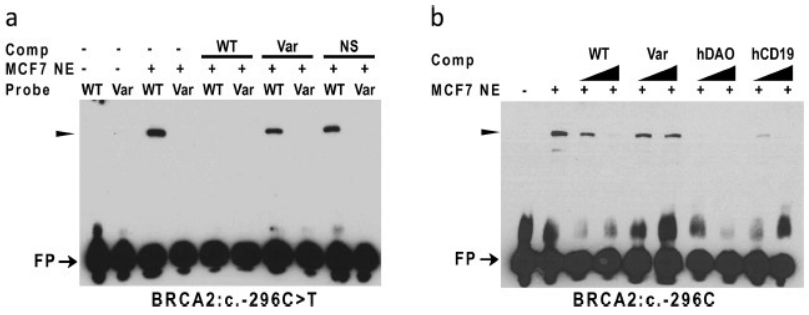

FIGURE 6 Variants in the 5′ region of *BRCA2* alter specific DNA:protein complex formation. Competition electrophoretic mobility shift assay (EMSAs; a) were performed using 3′ biotinylated double-stranded (ds) DNA probes containing sequences from the *BRCA2* 5′ region surrounding the BRCA2:c.-296C>T variant. DNA probes containing the wild-type (WT) or variant (Var) sequence were incubated with nuclear extracts from MCF7 cells (MCF7 NE) in the presence (+) or absence (–) of unlabeled WT, Var, or nonspecific (NS) competitor (Comp) DNA. Cross-competition EMSAs (b) contained *BRCA2* WT sequences and increasing concentrations of ds competitor DNA containing unlabeled WT, Var, or PAX5 binding sites from the *hCD19* gene and D-amino acid oxidase gene (*hDAO*). Free unbound probe (FP) and specific DNA:protein complexes (arrowheads) are indicated

function as activators or repressors, a variant-associated change in TF binding can result in either a decrease or an increase in promoter (or other regulatory element) activity. Differences in the quanta and direction of promoter activity have been reported previously (e.g., Fraile-Bethencourt et al., 2018; Santana dos Santos et al., 2017) and have also been shown to differ between cell lines potentially reflecting the availability of TFs or cofactors (e.g., Zn).

Three of the variants, *BRCA1*:c.-315del, *BRCA1*: c.-287C>T, and *BRCA2*:c.-296C>T, altered protein binding. ENCODE ChIP-seq data from BC cell lines indicate candidate proteins that are bound to the genomic regions containing these variants (Figure 2 and Supporting Information Figure S1). These include E2F1, CEBPB, GATA3, Max, ELF1, GABP, and FOXA1 for *BRCA1* and E2F1, MYC, ELF1, GABP, Max, and PML for *BRCA2*. Interestingly, a number of these factors have previously been implicated in BC.

In addition, ENCODE ChIP-seq data from cell lines derived from tissues other than breast indicate that the variants that affect protein binding are located within consensus motifs for specific TFs associated with these regions (Tables 2 and 3; Supporting Information Figure S1). *BRCA1*:c.-287C>T overlaps with the consensus binding motif for CCAAT Box binding factors, *BRCA1*:c.-315del is located in a consensus motif for CREB/ATF proteins, although the deletion does not modify this motif, and *BRCA2*:c.-296C>T is located within the consensus motif for PAX5. IT analysis also predicts that all these variants alter TF binding (Table 4 and Supporting Information Table S4). We show that *BRCA1*:c.-287C>T disrupts the binding of NFYA to the *BRCA1* promoter region. Furthermore, we present evidence that *BRCA2*:c.-296C>T disrupts the binding of PAX5. *BRCA1*:c.-315del lies in the so-called positive regulator region that has been shown to bind GABPα, CREB, and AP-1 proteins (Atlas et al., 2000; Atlas, Stramwasser, & Mueller, 2001; Graves, Zhou, MacDonald, Mueller, & Roskelley, 2007; Suen & Goss, 1999; Thakur & Croce, 1999). Although these proteins are generally considered activators of transcription, repression of promoter activity by *BRCA1*:c.-315del suggests the recruitment of an additional transcriptional repressor or corepressor to this region. IT analysis predicts creation of a binding site for POU2F2, a known repressor; however, we found no evidence to suggest that this variant increased POU2F2 binding in the cell line used, although it is possible that changes may be observable in other cell lines. Biochemical studies, including mass spectrometry, will be required to validate and discover other alterations in TF binding.

One variant, *BRCA1*:c.-287C>T, increased promoter activity and decreased protein:DNA interactions. This increase in promoter activity was unanticipated because this variant is within a consensus motif for the CCAAT box binding proteins, NFYA and NFYB, and mutation of this CCAAT box has previously been shown to reduce *BRCA1* promoter activity in MCF7 cells (Bindra et al., 2005; Xu, Chambers, & Solomon, 1997). This variant also decreases promoter activity in MDA-MB-231 cells (Santana dos Santos et al., 2017). Here, we show that the *BRCA1*:c.-287C>T variant reduces NFYA binding. Importantly, NFY proteins can function as transcriptional activators or repressors depending on recruitment of corepressors or coactivators (Peng & Jahroudi, 2002; Peng et al., 2007) and recruitment of TFs to neighboring sequences (Zhu et al., 2012) indicating possible mechanisms for divergent activities of NFY proteins at this site.

BRCA1:c.-192T>C, which lies in the 5′UTR, decreased reporter activity but did not bind any proteins from MCF7 nuclear extracts in EMSA analysis. Possibly, EMSA binding conditions are not optimal for binding of factors to this sequence or alternatively, this reduction in promoter activity could be by posttranscriptional mechanisms as seen for *BRCA2*:c.-26G>A (Gochhait et al., 2007).

Using existing prediction models developed for high risk variants, population frequency and clinical information classified 27 variants as "Not Pathogenic" or "likely Not Pathogenic." This included two *BRCA1* and six *BRCA2* variants with functional assay data available, six with no statistically significant effect on promoter activity, and two that decreased promoter activity in vitro. These two variants, *BRCA1*:c.-192T>C and *BRCA2*:c.-296C>T, were observed in population subgroup controls; notably *BRCA1*:c.-192T>C was observed at a frequency of >1%, which is considered stand-alone evidence against pathogenicity (defined as high risk of cancer) for *BRCA1/2* variation. This suggests that promoter region variants, irrespective of bioinformatic prediction or functional assay results, are unlikely to be associated with a high risk of cancer. This is consistent with current evidence from ENIGMA studies (de la Hoya et al., 2016), which suggest that an allele resulting in only ~20–30% expression of *BRCA1* transcript/s encoding functional transcripts is not associated with high risk of BC. The low impact of these variants on risk is likely to reflect the complex interplay of TFs and DNA elements, and possible redundancy in the system. For example, a variant in one TF binding site within a cluster may be buffered by other binding sites and thus insufficient on its own to reduce gene expression markedly (Lu & Rogan, 2018).

Given that moderate- and low-risk variants often occur in >1% of the population, and that the remaining 13 variants had insufficient evidence available to assess clinical significance, we cannot exclude the possibility that *BRCA1/2* promoter region variants, in particular those with proven functional effect, may be associated with a moderate or low risk of cancer. This indicates an urgent need to further develop prediction models to accommodate criteria for moderate- or low-risk variants by extending the *BRCA1/2*-specific criteria developed by ENIGMA (https://www.enigmaconsortium.org/), or even the generic variant classification criteria developed by the American College of Medical Genetics for Mendelian disorders (Richards et al., 2015).

This study has evaluated the significance of single nucleotide variants and small indels mapping to the 5′ noncoding region of *BRCA1* and *BRCA2* using bioinformatic, biological, and biochemical analyses in combination with consideration of clinical data that inform qualitative and quantitative variant classification. We present data to suggest that a subset of these variants have functional effects on gene regulation. We also present evidence that variants mapping to and affecting the function of *BRCA* promoters are not likely to be associated with a high risk of cancer. We propose that studies of differing design, such as very large-scale case-control sequencing studies able to detect rare variation, will be required to address if a low to moderate risk of cancer may be associated with *BRCA1/2* regulatory region variation that has not been captured to date by genome-wide association genotyping platforms. We believe that the bioinformatic and functional analysis

presented will be important to define the design and interpretation of such future sequencing studies. We also believe that this study highlights the challenges associated with classifying variants with respect to low or moderate disease risk, and the need to be cautious in the clinical use of information on individual variants that is likely to be one of many factors contributing to disease risk.

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DISCLOSURE STATEMENT

B.C.S is an employee of and P.K.R is co-founder of CytoGnomix, which has developed algorithms and software for interpretation of variants within transcription factor binding sites.

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SUPPORTING INFORMATION

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6.2 Revue : “Assessment of the functional impact of germline *BRCA1/2* variants located in non-coding regions in families with breast and/or ovarian cancer predisposition. ” Santana dos Santos E. et al., Cancers 2018

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Review

Non-Coding Variants in *BRCA1* and *BRCA2* Genes: Potential Impact on Breast and Ovarian Cancer Predisposition

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Abstract: *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. Most identified pathogenic variants cause the partial or complete loss of function of the protein. However, it is becoming increasingly clear that variants in these regions only account for a small proportion of cancer risk. The role of variants in non-coding regions beyond splice donor and acceptor sites, including those that have no qualitative effect on the protein, has not been thoroughly investigated. The key transcriptional regulatory elements of *BRCA1* and *BRCA2* are housed in gene promoters, untranslated regions, introns, and long-range elements. Within these sequences, germline and somatic variants have been described, but the clinical significance of the majority is currently unknown and it remains a significant clinical challenge. This review summarizes the available data on the impact of variants on non-coding regions of *BRCA1/2* genes and their role on breast and ovarian cancer predisposition.

Keywords: *BRCA1*; *BRCA2*; non-coding variants; promoter; hereditary breast cancer; hereditary ovarian cancer

1. Hereditary Breast and Ovarian Cancer (HBOC) Syndrome

Breast cancer is the most common cancer among women worldwide and ovarian cancer is the deadliest gynecological cancer. Mutations in high risk genes contribute to at least 10% and 15% of breast and ovarian cancer diagnoses, respectively, with cases frequently associated with a strong family history and early onset of disease. Hereditary Breast and Ovarian Cancer (HBOC) Syndrome is an autosomal dominant syndrome, which is caused primarily by germline mutations in two genes: breast cancer susceptibility gene 1 (*BRCA1*), which was first described in 1994, and breast cancer susceptibility gene 2 (*BRCA2*), which was discovered one year later [1,2]. This syndrome is characterized by an

increased risk for female and male breast cancer, ovarian cancer, and to a lesser extent, other cancers, such as prostate cancer, pancreatic cancer, and melanoma. For a heterozygous carrier, it has been reported that the lifetime risk is as high as 70% for breast and 20–40% for ovarian cancer [3–5]. Therefore, prophylactic surgeries, such as bilateral mastectomy and salpingo-oophorectomy, are effective risk reduction strategies.

Beyond the preventive aspects, understanding the mechanism of predisposition can help in the choice of treatment to improve the response and survival of patients. Advances in translational research have confirmed the biological and preclinical evidences making it increasingly apparent that *BRCA1/2* mutations are biomarkers that may predict the clinical response of breast and ovarian cancer patients to platinum salts and poly (ADP-ribose) polymerase (PARP) inhibitors [6–11]. Therefore, mutational status is becoming increasingly important for the management of BRCA related cancers as PARP inhibitors and *BRCA1/2* mutation targeting seem to be a hopeful approach for this group of patients. After three decades of research, several other breast cancer susceptibility genes have been identified, but with lower penetrance and associated risk [12,13].

The *BRCA1* gene encodes a nuclear protein of 1863 amino acids [2]. This protein contains a RING domain in the N-terminal region and two BRCT domains in its C-terminal region, through which it interacts with multiple partners, performing a variety of cellular functions that are particularly related to the DNA damage repair [14–17]. The *BRCA2* gene also encodes a nuclear protein that is composed of 3418 residues [1]. *BRCA2*, like *BRCA1*, is involved in DNA repair by homologous recombination, and it interacts with different partners (such as RAD51 and PALB2) to maintain the stability of the genome [18]. For the moment, three BRCA2 regions have been described as particularly important for homologous recombination function: N-terminal PALB2-binding site, BRC repeats (which correspond to eight consecutive motifs located in the central region of the protein and constitutes the principal RAD51 interaction site), and the C-terminal region (composed of three oligosaccharide binding folds, a helical domain, and a tower domain that together constitute the DNA binding region and contain a RAD51 binding domain) [18,19].

Although sequencing of these high penetrance genes *BRCA1/2* has been available for over 20 years, after two decades of intense research, a pathogenic variant is identified in approximately 10% of tested families [20]. Despite the remarkable advances seen in the past years, for the majority of HBOC families, little is understood about the underlying molecular mechanisms of cancer susceptibility. New technologies are being developed to extensively search in parallel for a pathogenic variant in a panel of other genes related to the syndrome. These high to moderate penetrance variants in known breast cancer related genes, such as *TP53*, *PTEN*, *STK11*, *CDH1*, *ATM*, *BRIP1*, *PALB2*, and *RAD51* isoforms (*RAD51C*, *D*, *B*) may also contribute to hereditary predisposition, but altogether these variants only explain about 5% of the unsolved cases [21]. Additional attempts to identify breast cancer risk genes have uncovered a large number of low risk loci that generally map to gene regulatory regions. The remainder of the risk is therefore likely to be a combination of not yet identified high, moderate, or low risk variants located in the non-coding regions of the aforementioned genes or in currently unidentified breast cancer risk loci. It is noteworthy that current *BRCA1/2* routine screening is limited to the coding region and intron/exon boundaries. However, protein inactivating mutations may not be the only mechanism by which their function is altered. Reduction in gene expression by changes in trans acting factors (TFs) or cis-regulatory regions may achieve the same end as truncating mutations in the gene itself. 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 *BRCA1/2* gene screening remains negative, while introns and proximal untranslated regions remain relatively unexplored. However, evidence of non-coding variants impact on cancer risk and response to treatment begin to emerge [22].

Current technological sequencing advancements and development of bioinformatics tools has enabled the exploration and elucidation of the genome structure and non-coding DNA regions. The description of the functional elements of the human genome by the encyclopedia of DNA elements

provided a better understanding of the human genome expression regulation and how regulatory data is encoded. This effort demonstrated that most of the human genome is involved in gene expression regulation, while the small minority of the nucleotides (1.2%) encodes proteins within humans. The ENCODE project has also described thousands of regulatory active regions and showed that 90% of common variants fall outside the coding regions of the genes [23]. Nevertheless, the majority of the studies to date have focused on the coding regions of the cancer related genes.

This article summarizes current knowledge of non-coding regulatory *BRCA1/2* regions and the variants that are located in these regions.

2. Germline Cancer-Associated Variants in the Regulatory Regions

Until recently, most attention had been focused on the coding regions of the genes that are associated with cancer risk. Exome sequencing of human genome and co-segregation studies have made evident that lots of disease-associated variants play a role in hereditary susceptibility. Since coding changes do not explain all of the predisposition cases, the importance of the non-coding regions (including promoters, introns, intergenic sequences, and non-coding RNAs) in biological functions and hereditary predisposition must be considered.

Gathered evidence indicates that genetic variants in the non-coding but functional elements can contribute to the development of hereditary cancers. The presence of variants in these regions can impact gene transcription by the creation or disruption of transcription factors binding sites, or by interfering with CpG island methylation which leads to an aberrant methylation pattern. In addition, variants may have an impact at the post-transcriptional level, creating or disrupting microRNA 3′ complementary binding sites in 3′UTRs, and interfering with the stability of RNAs and microRNAs. Moreover, the elucidation of three-dimensional (3D) chromatin structure reveals a complex network of interactions within the regulatory regions of the genome that includes long-range interactions between functionally coordinated domains lying hundreds of kilobases upstream or downstream of their target [24,25]. Therefore, non-coding sequence alterations may also influence this model of regulation.

As non-coding sequences correspond to 98% of the genome, the identification of regions with a greater chance of bearing a variant that contributes to disease should be prioritized. Since transcriptional activity is correlated with less condensed chromatin regions, regulatory elements are often located in DNAse I hypersensitive sites. Furthermore, regions that are conserved in mammals, containing multiple binding sites for known transcription factors are most likely to be functional and present a higher probability of containing disease-associated variants [23]. Bioinformatics, experimental, and population-based approaches are complementary in identifying and validating key regulatory regions of the genome.

There is increasing data associating germline non-coding variants with cancer risk. Additionally, most cancer-associated single nucleotide variants (SNVs) that were identified through genome-wide association studies are located in non-coding regions, some of them with a proven role in gene expression regulation [26,27]. As examples: (i) a germline variant in the promoter of *TERT* (telomerase reverse transcriptase) gene (c.-57T>G) significantly increased promoter activity. This variant co-segregated with cancer in a family with 14 melanoma cases who were not carriers of germline mutations in the two known melanoma genes, *CDKN2A* and *CDK4* [28]. The variant increases *TERT* expression, probably by the creation of a new binding site for Ets, Elk1, and Elk4 transcription factors. The increase of *TERT* expression is a fundamental requirement for cell transformation and immortality [29,30]. (ii) Constitutional germline mutations have also been described in *MLH1* and *PTEN* promoters and correlated with the risk of cancer [31–34]. Interestingly, the 5′UTR *MLH1* variant c.-27C>A is an example of a non-coding sequence change associated with an epigenetic modification. The presence of the variant generates aberrant methylation of the promoter and silencing of the affected allele [31,32,34]. (iii) Additionally, it was proved that an enhancer region, which is located in the intergenic sequence on chromosome 8q24, interacts with MYC proto-oncogene, even though it is located 335kb from this gene. A variant located there (rs6983267) is associated with colorectal cancer

risk via the disruption of transcription factor 7-like2 (TCFL2) binding site, a co activator of the Wnt-β catenin pathway [35].

A priority now is to identify the full spectrum of non-coding variants that contribute to disease and then determine their impact of gene function and disease risk. Indeed, as subtle quantitative effects are expected, it is challenging but important to define a threshold of effect that classifies these non-coding variants as "pathogenic variants" to allow for accurate genetic counseling.

3. Regulatory Regions in *BRCA1* and *BRCA2* Genes

BRCA1 and *BRCA2* expression are controlled at the transcriptional and post-transcriptional levels. The key transcriptional regulatory elements are housed in gene promoters, introns, and long-range elements, while the key post-transcriptional control elements are predominantly located in 5′ and 3′ untranslated regions (UTRs). Both genes are expressed in a cell cycle regulated manner, with low levels of proteins being observed in G0 and early G1 phases before entry into S phase, and high levels are maintained through the S and G2 phases of the cell cycle [36,37].

BRCA1 is a tumor suppressor gene that is located on chromosome 17q21 involved in DNA error-free repair by homologous recombination. The core promoter of *BRCA1* includes the non-coding exon 1 and part of intron 1 of *BRCA1*, as well as the exon 1 and part of intron 1 of the neighboring gene *NBR2* (chr17: 43,168,800–43,172,601). BRCA1 expression is complex with its transcription controlled by two different promoters, α and β, located upstream from the alternative first exon 1A (121bp) and 1B (378bp), respectively. These two promoters encode 5′UTR-a and 5′UTR-b [38,39], which share the same translation start codon (located in exon 2). These transcripts differ by the 5′UTR (exon 1) and they are expressed in a tissue specific fashion: exon 1B is only expressed in breast cancer while exon 1A transcripts are present in both normal or tumor tissue. The maintenance of the correct ratio between the two transcripts has the potential to be important for normal regulation and function. In vitro studies show that this structural difference is related to a lower translation efficiency of 5′UTR-a in comparison with 5′UTR-b [40].

The more efficient *BRCA1* promoter (α) consists of a region of 200 base pairs, upstream of the start site, which functions as a bidirectional transcriptional element able to direct expression in either the *BRCA1* or *NBR2* direction. There is some evidence to suggest that these two genes, separated by little more than 200 bp, are reciprocally regulated and present divergent transcription [41]. However, gene expression data from TCGA confirm the co-expression regulation for ovarian serous carcinomas but not in the breast cancer data set [42,43]. *BRCA1* promoter contains: a RIBS element that acts as an activator and possesses multi subunit EtsGA-binding protein binding sites [44], a CREB binding site that is a strong positive transcriptional element [45], a CAAT box [39], and an E2F binding site [46]. Since no estrogen responsive element (ERE) was identified in *BRCA1* promoter α, the stimulation of *BRCA1* expression by estrogen seems to result from an indirect effect of estrogen. In contrast, an ERE was described in *BRCA1* promoter β, so, in this case, the estrogen stimulation effect is due to estrogen bound to the DNA and a subsequently interaction with the transcription machinery to stimulate transcription [39,47]. In addition to promoter elements, upstream repressor elements were also described in regions upstream of the start of transcription and translation [48].

Gene promoter methylation has been proposed as an alternative mechanism for the transcriptional silencing of cancer-associated genes [49]. As a typical example, epigenetic silencing of *MLH1* that is associated with inherited variants leading to promoter methylation was described in familial colorectal cancers [32,50–52]. *BRCA1* promoter methylation appears to be more relevant for sporadic than for hereditary breast and ovarian cancers [53–55]. It is an uncommon event among BRCA mutation carriers. For the *BRCA1* gene, it was detected in about 3%and 11% of breast [56] and ovarian carcinomas [43], respectively.

There is limited information about regulatory elements outside of the *BRCA1* promoter. Suen and Goss localized a 36-bp repressor element in the first intron of *BRCA1* [48]. Wardrop and Brown subsequently described two evolutionarily conserved regions rich of TF binding sites in the second

BRCA1 intron that mediates both the activation and repression of the *BRCA1* gene [57]. In addition, we reported recently the enhancer property of an intronic sequence that is located in the intron 12 of *BRCA1* [58]. The *BRCA1* 3′ untranslated region (3′UTR) has been shown to be important for post-transcriptional regulation and this has been exemplified by a variety of variants located there that negatively regulate mRNA translation, probably by the disruption or creation of complementary MicroRNAs binding sites [59–62].

BRCA2 is also a tumor suppressor gene that is located on chromosome 13ql2.3 [1]. Its core promoter was first described four years after *BRCA2* gene cloning [63]. It is located −66 to +129 from the transcriptional start site, and corresponds to a region rich in CG nucleotides and with several TF binding sites, including E-box, Ets/E2F, and SP1. *BRCA2* promoter is induced by NFκB and Elf1 [63,64], while repressed by P53, PARP1, and SLUG [65–67]. Recently, functional studies that were based on micro deletions mapped other regulatory promoter regions with up and down-regulating elements [68]. Like *BRCA1*, *BRCA2* is expressed in a cell cycle-regulated manner and the estrogen induction is also an indirect effect of mitogenic activity. Low protein levels are observed in G0 and early G1 phases, while peak levels are reached in late G1, S, and G2 phases of the cell cycle. Misra et al. described the bi-directional activity of *BRCA2* promoter, similar to that of *BRCA1*. It was shown that the forward and reverse promoter activity regulates both *BRCA2* and *ZAR2* transcription, respectively. Interestingly, during the G0 and G1 phase of cell cycle, this promoter is 8–20 times more active in the reverse orientation, increasing the production of the ZAR2 protein that binds to the promoter and silencing *BRCA2* expression. Whereas, during the pre-division phases (S/G2), the forward activity is 5–8 times higher and the ZAR2 is trapped in the cytoplasm [37]. Nevertheless, TCGA gene expression data does not confirm this co-expression regulation in the breast cancer data set, while no data is available for ovarian serous carcinomas [42,43].

Evidence suggests that promoter hypermethylation is not an obvious contributor to *BRCA2* related cancers [56]. For now, little information about *BRCA2* non-coding regions is available. A few cis-acting intronic polymorphisms that alter the binding of transcription factors at regulatory sites have been described [69], as well as one 3′UTR variant (*BRCA2*c.*172G>A), but with no clear evidence of pathogenicity [60].

4. Methods to Assess the Pathogenicity of *BRCA1/2* Non-Coding Variants

Variants classification is based on multiple lines of evidence, with population, computational, pathological, functional, and segregation data all being taken into account. Multifactorial prediction models are able to incorporate these different sources of data to calculate the probability of the variant being pathogenic. Currently, virtually all *BRCA1/2* non-coding variants are considered to be variants of uncertain significance (VUS) [70,71], as the prediction models have been built to classify variants that have a significant impact on protein function and they are generally associated with a high risk of disease. Multiple approaches are being applied to evolve in the classification of these variants. Individually, these resources are not enough for variant classification, but taken together, they may allow a better clinical interpretation.

4.1. In Silico Tools and Genetic Data

A variety of prediction software is available to evaluate the impact of variants on splicing and on the structure of the protein. However, since many tools are based on the effect of the protein, it is difficult to extrapolate this analysis to regulatory variants, since, for them, no change in translated protein is expected. Even for deep-intronic variants, it is important to evaluate their effect on splicing before studying their impact in gene expression. For intronic variants, a variety of in silico prediction tools are available, such as Splice Site Finder-like, MaxEntScan, NNSplice, GeneSplicer, and Human Splicing Finder.

Some new tools are beginning to address non-coding sequences [72–74]. As an alternative for non-coding variants, bioinformatics in silico analysis are useful to prioritize the variants that are located

4.2.3. Assays to Measure Gene Expression and Protein Function (Functional Assays)

Functional assays can evaluate the variant's impact on the ability of the protein to perform some key cellular functions, which in the case of non-coding variants, might be related to deficient gene expression.

Luciferase reporter assay is a standard method to evaluate the impact of non-coding variants on gene expression. This assay consists of transfecting cells with a plasmid containing the luciferase gene under the control of DNA regulatory regions (promoter, enhancer, and repressor) with or without the variant of interest. The comparison between luciferase activities of cells transfected with the variant-containing plasmid and cells transfected with the plasmid containing the wild-type sequence, allow for the determination of the variant impact on the biological function of regulatory regions. This assay is also used to evaluate 3'UTR functional regions on gene expression.

It is challenging to integrate calibrated functional assay data into multifactorial models, since pathogenic mutations do not affect the functional endpoints in the same way. Another issue is the low reproducibility between experiments, less prominent for variants with a greater effect. Plasmid DNA is placed in an artificial environment that may fail to reproduce the expression pattern of its endogenous equivalent due to differences on chromatin context. Regarding *BRCA1/2* non-coding variants, although the Luciferase assay is the current standard, the ideal cutoff that abrogates the allele expression has yet to be determined. For Lynch syndrome, it was suggested that 50% reduction of gene expression makes MMR function insufficient [85].

4.2.4. Assays to Investigate the Underlying Mechanism of Variant Impact

Transcription factors (TF) and microRNAs operate via base-paring interactions with DNA and mRNA, respectively. The majority of TF binding sites are located in promoter, enhancer, and repressor elements (some of which overlap with the 5'UTR), while the majority of microRNAs binding sites are placed in 3'UTR. Some in silico tools are available to investigate whether the variant can create or disrupt one of these. For this purpose, microRNA and TF binding site prediction software, ENCODE ChIP-seq data, and information theory analysis can all provide clues that may be confirmed with in vitro experiments.

In vitro experiments are generally the next step to elucidate the underlying mechanism through which the variant can interfere. For 3'UTR variants, the correspondent miRNA vector (synthetic or plasmid) is co-transfected with the Luciferase *BRCA1/2* 3'UTR reporter, with the variant or with the wild-type sequence. The results are then compared to determine if the variant has an impact. For promoter variants, several methods have been used for the characterization of protein-DNA interaction, including electrophoretic mobility shift assay (EMSA) [86] and Chromatin immunoprecipitation assays (ChIP) [87]. EMSA is based on the principle that a protein-DNA complex migrates more slowly through an electrophoresis gel than the corresponding free DNA. Differences in binding patterns between the wild type and mutant DNA sequences that labeled with a radioactive or luminescent tag, are indicative of TFs interacting with the DNA sequence in question. The candidate TF can be then identified by the use of an antibody against itself, using a 'supershift' assay. ChIP assays are an alternative method for directly visualizing an in vivo interaction between a specific protein and a regulatory element. After DNA cleavage by restriction enzymes, protein-DNA complexes are purified by immuno-precipitation with antibodies being directed against the protein of interest. Then, to confirm that the protein was linked to the TF binding site, the bound antibody is neutralized, proteins are digested, and DNA is analyzed for the presence of the regulatory element by PCR. Interacting proteins can also be identified using mass spectrometry.

Finally, promoter methylation has been described as an alternative mechanism of BRCA1 and BRCA2 silencing [56]. This is another mechanism of disrupting transcriptional regulation, which can be evaluated through pyrosequencing or Next Generation Sequencing.

4.3. Tumor Features

Tumors arising in *BRCA1* and *BRCA2* mutation carriers are different from each other and from tumors not associated with these mutations. More than 75% of breast cancers diagnosed in *BRCA1* mutation carriers are high grade and triple negative breast cancers. In contrast, the breast cancers of *BRCA2* pathogenic mutation carriers usually have a Luminal phenotype. A large study proved that histopathological features could predict the *BRCA* mutational status and led to the incorporation of pathological data into the algorithms for variants classification [88,89].

Loss of heterozygosis (LOH) analysis can also be useful. LOH is more frequently found in *BRCA*-mutation positive tumors than in sporadic tumors and it is generally related to the loss of the wild-type chromosome. The loss of the wild type *BRCA* allele usually corresponds to the 'second hit' and thereby adds an additional argument in favor of the variant pathogenicity.

Furthermore, other tumor characteristics such as a high genetic instability score, genome wide tumor methylation profile, evaluation of *PTEN* and *TP53* alterations, and gene expression arrays, could provide useful information. But, for the moment, except for tumor grade, hormonal receptor and HER2 status, this information is not incorporated into multifactorial likelihood models.

5. Impact of *BRCA1/2* Non-Coding Variants on Breast and Ovarian Cancer Predisposition

Because *BRCA1/2* coding mutations only explain 10% of the predisposed families, exhaustive efforts have been undertaken for more than 20 years to identify other loci contributing to breast cancer susceptibility. It remains possible that some of the remaining risk may be related to the main HBOC genes *BRCA1/2*, potentially by variants causing the deregulation of expression. Until now, few studies have analyzed *BRCA1/2* non-coding regions (Figure 1 and Table 1).

Recent data originating from HBOC population screening confirm the presence of variants in *BRCA1/2* regulatory regions. Some of these variants are functionally active, which reinforces their possible link with hereditary predisposition (Table 2). But, for the moment, except for some non-coding variants that were identified in intron and exon boundaries with impact on splicing, all the sequence alterations identified in *BRCA1/2* non-coding regions remain unclassified. The incorporation of next generation sequencing analysis for germline tests should expand the availability of information, including a greater number of sequence variants whose biologic impact remains unknown.

We and others have screened *BRCA1* and *BRCA2* promoters of predisposed patients with no pathogenic variant identified, in search for potential 5'UTR mechanisms of gene deregulation [58,68,90]. The data generated from these studies led to the identification of some variants that demonstrated an impact on transcriptional regulation (Table 2). For some of these, the underlying mechanism of down regulation is related to disruption of interactions between transcription factors and their binding sites. While some variants are related to reduced promoter activity, others have been associated with increased gene expression. This latter effect is the opposite of what one would expect from a *BRCA1/2* variant associated with an increased breast/ovarian cancer risk. Nevertheless, these enhancing variants could inhibit some repressor elements localized within *BRCA1* and *BRCA2* promoters, thereby inducing an over expression of *BRCA1/2* [91]. We have seen that the *BRCA1/2* expression strongly fluctuates during the cell cycle. *BRCA1/2* expression is very low at the G1 phase to prevent DNA repair by homologous recombination at the wrong time. It can be hypothesized that variants leading to *BRCA1/2* overexpression could thus still perturb DNA repair mechanisms, thereby inducing genetic alterations causing cancer. Besides that, the inconsistent results that were occasionally observed when different cell-lines were used to evaluate the same variant may reflect the availability of transcription factors or co-factors among the cells and reinforce the utility of performing these tests in more than one cell line [92].

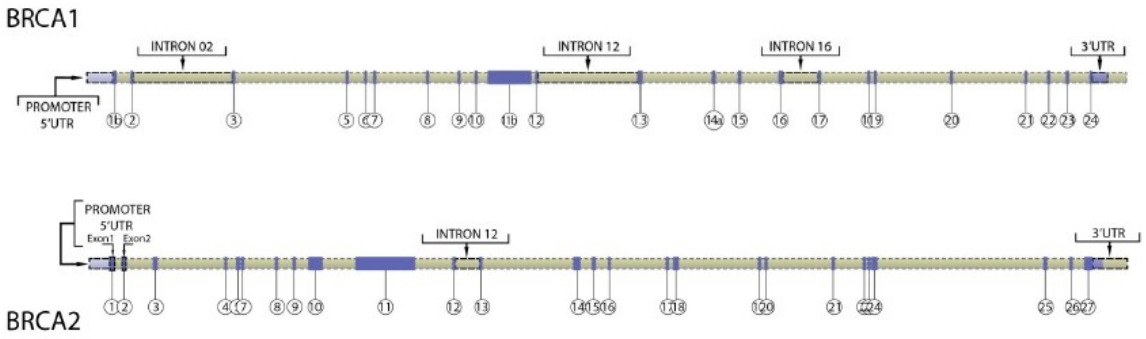

Figure 1. Non-coding regions of *BRCA1/2* genes studied to date.

Table 1. Priority regions of *BRCA1/2* genes for screening.

Region of Interest	Hg19 Coordinates	Length	Comments
BRCA1 promoter	chr17: 41,277,500–41,278,500	1000 bases	Comprises 1 kb upstream on transcription start site
BRCA1 5′UTR (exon 1A)	chr17: 41,277,287–41,277,500	223 bases	Exon 1A
BRCA1 5′UTR (exon 1B)	chr17: 41,277,340–41,277,197	145 bases	Exon 1B
BRCA1 5′UTR + ATG (exon 2 to ATG)	chr17: 41,276,110–41,276,133	22 bases	5′ end of Exon 2
BRCA1 intron 2	chr17: 41,271,250–41,272,100	850 bases	Includes validated enhancer and repressor elements that participate in gene looping and are conserved. Also contains sequences that UCSC/ENCODE indicates this region contains transcription factor binding sites, DnaseHS sites
BRCA1 intron 12 (region 1)	chr17: 41,237,500–41,237,850	350 bases	UCSC/ENCODE indicates this region contains transcription factor binding sites, DnaseHS sites and is conserved.
BRCA1 intron 12 (region 2)	chr17: 41,236,600–41,236,960	360 bases	UCSC/ENCODE indicates this region contains transcription factor binding sites, DnaseHS sites and is conserved.
BRCA1 intron 16	Chr17: 41,220,900–41,221,250	350 bases	UCSC/ENCODE indicates this region contains transcription factor binding sites, DnaseHS sites
BRCA1 3′UTR (exon 24)	chr17: 41,196,311–41,197,698	1387 bases	From and including stop codon
BRCA2 promoter	chr13: 32,888,616–32,889,616	1000 bases	Comprises 1 kb upstream on transcription start site
BRCA2 5′UTR (exon 1)	chr13: 32,889,616–32,889,805	189 bases	Exon 1 (Refseq)
BRCA2 5′UTR (exon 2 to ATG)	chr13: 32,890,558–32,890,600	42 bases	Includes translation start codon
BRCA2 3′UTR	chr13: 32,972,904–32,973,809	905 bases	From and including stop codon

Table 2. Population screening of *BRCA1/2* non-coding regions in patients tested negative for *BRCA1/2* codingmutations. A/Number of samples screened and number of variants identified. B/Variant impact on functional assays.

A

Gene	Region Screened	Population Screened (n)	Number of Samples Presenting a Variant	References
BRCA2	Promoter	95	3	[68]
BRCA1	Promoter (255 bp)	3926	55	[58]
BRCA2	Promoter (380 bp)	3910	21	[58]
BRCA1	Intron 2 (326 bp)	3624	30	[58]
BRCA1	Intron 12 (360 bp)	2973	11	[58]
BRCA1	5′UTR	49	2	[93]
BRCA1	5′UTR	117*	2 (somatic)	[94]
BRCA1	5′UTR	96	1(somatic)	[95]
BRCA1	5′UTR (2400 bp)	6475	81	[90]
BRCA2	5′UTR (2000 bp	6603	60	[90]
BRCA1	3′UTR (1561 bp)	1612	7	[61]
BRCA1	3′UTR (1376 bp)	70	2	[62]
BRCA1	3′UTR (1382 bp)	716	5	[60]
BRCA2	3′UTR (902 bp)	716	1	[60]

B

Gene	Variant	Localization	Functional Test	Effect	References
BRCA1					
BRCA1	c.-395C>T	Promoter	Luciferase assay	NS	[90]
BRCA1	c.-380G>A	Promoter	Luciferase assay	NS	[58,90]
BRCA1	c.-378C>A	Promoter	Luciferase assay	NS	[90]
BRCA1	c.-362T>G	Promoter	Luciferase assay	up regulation	[58]
BRCA1	c.-359G>T	Promoter	Luciferase assay	NS	[58]
BRCA1	c.-315del	Promoter	Luciferase assay	down regulation	[90]
BRCA1	c.-192T>C	Promoter	Luciferase assay	down regulation	[90]
BRCA1	c.-220C>A	Promoter	Luciferase assay	NS	[90]
BRCA1	c.-264T>G	Promoter	Luciferase assay	NS	[90]
BRCA1	c.-273G>A	Promoter	Luciferase assay	NS	[90]
BRCA1	c.-287C>T	Promoter	Luciferase assay	up regulation	[90]
BRCA1	c.-177C>T	Promoter	Luciferase assay	NS	[58]
BRCA1	c.-130del	Promoter	Luciferase assay	down regulation	[58]
BRCA1	c.-125C>T	Promoter	Luciferase assay	down regulation	[58]
BRCA1	c.-121G>C	Promoter	Luciferase assay	up regulation	[58]

Table 2. *Cont.*

B

Gene	Variant	Localization	Functional Test	Effect	References
BRCA1					
BRCA1	c.-107A>T	Exon 1	Promoter methylation assays; RNA analysis by RT-PCR	down regulation	[93]
BRCA1	c.-71G>A	Exon 1	Luciferase assay	NS	[58]
BRCA1	c.-24T>C	Exon 1	Luciferase assay	NS	[58]
BRCA1	c.-3G>C (+117G>C)	Exon2	Luciferase assay; RNA translation assay	down regulation	[95]
BRCA1	c.-2A>T (+118A>T)	Exon2	Luciferase assay; RNA analysis by RT-PCR. Protein analysis by IHC	down regulation	[94]
BRCA1	c.81-3985A>T	Intron 2	Luciferase assay	up-regulation	[58]
BRCA1	c.81-3980A>G	Intron 2	Luciferase assay	down regulation	[58]
BRCA1	c.4186-2022C>T	Intron 12	Luciferase assay	down regulation	[58]
BRCA1	c.*291C>T	3′UTR	Luciferase assay	up-regulation	[61]
BRCA1	c.*528G>C	3′UTR	Luciferase assay	down regulation (MDAMB231) up-regulation (MCF7)	[61]
BRCA1	c.*713C>T	3′UTR	Luciferase assay	up-regulation	[60]
BRCA1	c.*718A>G	3′UTR	Luciferase assay	down regulation	[61]
BRCA1	c.*750A>G	3′UTR	Luciferase assay	NS up-regulation	[60,62]
BRCA1	c.*780C>T	3′UTR	Luciferase assay	up-regulation	[62]
BRCA1	c.*800T>C	3′UTR	Luciferase assay	NS	[61]
BRCA1	c.*1012A>G	3′UTR	Luciferase assay	NS	[62]
BRCA1	c.*1139G>T	3′UTR	Luciferase assay	up-regulation (MDAMB231) down regulation (MCF7)	[61]
BRCA1	c.*1271T>C	3′UTR	Luciferase assay	down regulation	[61]
BRCA1	c.*1286C>A	3′UTR	Luciferase assay	down regulation	[62]
BRCA1	c.*1340_1342del	3′UTR	Luciferase assay	up-regulation	[61]
BRCA2					
BRCA2	c.-492C>T	Promoter	Luciferase assay	NS	[68]
BRCA2	c.-467T>G	Promoter	Luciferase assay	up-regulation	[68]
BRCA2	c.-407G>A	Promoter	Luciferase assay	NS	[90]
BRCA2	c.-408T>A	Promoter	Luciferase assay	NS	[90]

Table 2. *Cont.*

B					
Gene	**Variant**	**Localization**	**Functional Test**	**Effect**	**References**
BRCA2					
BRCA2	c.-296C>T	Promoter	Luciferase assay	down regulation	[58,90]
BRCA2	c.-280_272dup	Promoter	Luciferase assay	up-regulation	[58]
BRCA2	c.-280del	Promoter	Luciferase assay	NS	[90]
BRCA2	c.-273G>T	Promoter	Luciferase assay	NS	[58]
BRCA2	c.-262G>A	Promoter	Luciferase assay	up-regulation	[68]
BRCA2	c.-248G>A	Promoter	Luciferase assay	NS	[68]
BRCA2	c.-220G>T	Exon 1	Luciferase assay	NS	[58]
BRCA2	c.-218G>A	Exon 1	Luciferase assay	NS	[58]
BRCA2	c.-213G>T	Exon 1	Luciferase assay	NS	[58]
BRCA2	c.-200C>T	Exon 1	Luciferase assay	NS	[90]
BRCA2	c.-197A>C	Exon 1	Luciferase assay	down regulation	[90]
BRCA2	c.-188C>T (+46C>T)	Exon 1	Luciferaseassay	NS	[68]
BRCA2	c.-175C>T	Exon 1	Luciferase assay	NS	[90]
BRCA2	c.-175C>T (+59C>T)	Exon 1	Luciferase assay	down regulation	[68]
BRCA2	c.-174G>A	Exon 1	Luciferase assay	up-regulation	[68]
BRCA2	c.-162G>A (+72G>A)	Exon 1	Luciferase assay	up-regulation	[68]
BRCA2	c.-159T>A	Exon 1	Luciferase assay	up-regulation	[68]
BRCA2	c.-133T>G	Exon 1	Luciferase assay	NS	[90]
BRCA2	c.-123G>A	Exon 1	Luciferase assay	up-regulation	[58]
BRCA2	c.-120G>A	Exon 1	Luciferase assay	up-regulation	[68]
BRCA2	c.-119A>G	Exon 1	Luciferase assay	down regulation	[68]
BRCA2	c.-94T>C	Exon 1	Luciferase assay	up-regulation	[68]
BRCA2	c.-87T>G	Exon 1	Luciferase assay	NS	[90]
BRCA2	c.-82G>C	Exon 1	Luciferase assay	NS	[90]
BRCA2	c.-77C>T	Exon 1	Luciferase assay	down regulation	[68]
BRCA2	c.-63C>T	Exon 1	Luciferase assay	NS	[68]
BRCA2	c.-52A>G	Exon 1	Luciferase assay	NS	[58]
BRCA2	c.-52A>G (+182A>G)	Exon 1	Luciferase assay	NS	[68]
BRCA2	c.*172 G>A	3′UTR	Luciferase assay	up-regulation (HBL-100) down regulation (MCF7)	[60]

* Information about BRCA1 coding sequencing not reported.

Promoter variants can also reduce gene expression through interference of CpG islands and consequent methylation-associated epigenetic silencing of the correspondent allele. Recently, this mechanism was described in two families carrying a *BRCA1* promoter variant (c.-107A>T). RNA sequencing revealed that the heterozygous variant that was segregated with the hypermethylated *BRCA1* allele, resulting in the allelic loss of *BRCA1* expression [83]. Similar to Lynch syndrome [90,91], this example raises the question of whether constitutional BRCA1/2 epimutations can represent an alternative mechanism for cancer predisposition. Considering that luciferase activity assay is ultimately indicative of both transcriptional and translational efficiency, it is noteworthy that, in functional studies, the reduced levels of BRCA1 protein is not always associated with reduced transcript levels [94,95]. Therefore, the disruption of post-transcriptional regulation should contribute in some cases. First, using RNAfold secondary structure prediction software, we could demonstrate that a *BRCA1* 5′UTR variant (c.-130del) impacts RNA conformation and it probably affects the binding of trans-acting factors and therefore mRNA translation [58]. This predicted effect was also described for some 3′UTR variants and a 5′UTR polymorphisms of *BRCA1*, both with an impact in translational efficiency [60,94].

A 5′UTR variant may also impact translation efficiency by interfering in the consensus motif for the start of protein translation. Wang et al. described a variant located two bases downstream *BRCA1* start codon that reduced the protein expression in this way. In the presence of the 5′UTR variant (+118A>T, c.-2A>T), luciferase activity was significantly reduced as compared to the wild type, while transcription efficiency and mRNA stability were assured by equal mRNA levels. Immuno-histochemical staining of the tumor could confirm the reduced expression of *BRCA1* protein for the variant carriers. Signori et al. also described a variant at position—3 from the *BRCA1* start codon associated with a significant decrease in mRNA translation through the same mechanism [95].

Germline variants have been described in the 3′UTR region of the *BRCA1/2* genes, some of them with a proven impact on gene expression [59,96,97]. MicroRNA is small non-coding RNA that negatively regulates mRNA translation by recognizing complementary sites, most located in this region. They can induce mRNA degradation or inhibit their translation resulting in gene down regulation. 3′UTR variants can disrupt pre-existing or create new cis-regulatory elements or binding sites for trans acting RNA binding proteins or micro-RNAs. However, there still exists a paucity of data on *BRCA1/2*3′UTRregions. Brewster et al. performed a screening of *BRCA1* 3′UTR in a large population of breast cancer cases with no *BRCA1/2* mutation that put in evidence 15 novel *BRCA1*3′UTR variants. One of them (c.*1340_1342del) related to the creation of a new microRNA binding site: miR-103. Another 3′UTR screening of 716 index cases that tested negative for *BRCA1/2* pathogenic mutations also detected SNPs and six rare variants in these regions, three of which are novel [60].

Though intronic data is even scarcer, a few intronic variants have been described by us and others. Two variants located in regulatory regions in the intron 2 and intron 12 sequences of *BRCA1* (c.81-3980A>G and c.4186-2022C>T, respectively) were able to revert the enhancing impact of these regions over *BRCA1* promoter activity. Although these regions are situated several kilobases downstream of the promoter region, it is hypothesized that they regulate *BRCA1* expression at the transcriptional level, most likely via gene looping [57,58].

For the moment it is difficult to predict the risk attributed to the presence of these variants, given the scarcity of data and the fact that they could have impact in different steps of gene expression, but, contrary to coding mutation, they may not impact protein function. Non-coding variants are expected to have more subtle quantitative effects and may probably be associated with a lower but still important impact on cancer risk. This impact on the relative risk of cancer is likely to occur in collaboration with other low, moderate, or high risk variants.

6. Clinical Practice Recommendations for Non-Coding Variants' Carriers

There is currently no formal recommendation for classifying *BRCA1/2* non-coding variant carriers, nor guidelines for managing patients carrying these variants. As stated before, except for some variants that are located in the intron/exon transition with impact on splicing, the significance of nearly all

variants that were identified in *BRCA1/2* non-coding regions remains uncertain. These sequence changes do not clearly affect the protein but cause subtle changes that are difficult to interpret. As a quantitative effect is expected, it is a major challenge to define a threshold that classifies the variant as causal or to determine their significance and contribution in breast/ovarian cancer susceptibility. So, it is still difficult to reach accurate conclusions that are useful for genetic counseling.

The last American College of Medical Genetics guideline provide no specific recommendation for the reporting and classification of variants that were identified in *BRCA1/2* promoters and intronic and untranslated regions [98]. To date, carriers should be managed exclusively based on their personal and family history which allows for the estimation of cancer risk. BOADICEA [99], BRCAPRO [100] and Tyrer-Cuzick [101] are examples of software-based models that are useful for estimating the risk of a woman developing cancer in the course of her life, regardless of *BRCA* status. Concerning breast cancer prevention, a life time risk >20% justifies intensive surveillance, including annual MRI and discussion of prophylactic surgery. However, available data is inadequate to support the use of chemoprophylaxis with tamoxifen and risk reducing salpingo-oophorectomy in this scenario. Those variants should therefore be included in a specific program for cosegregation and linkage analysis. Once these variants are unlikely to be sufficiently penetrant to co-segregate with disease, case control studies are very useful for assessing their impact.

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

7. Conclusions

BRCA1 and *BRCA2* remain the main candidates for explaining the high risk of cancer in HBOC syndrome. The first description of an epigenetic impact of a non-coding variant in *BRCA1* gene launches the necessity to continue the screening of *BRCA1/2* non-coding regions, in parallel with studies to determine their biochemical and clinical significance.

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