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"*BRCA1* and *BRCA2* mutation detection by a Next Generation Sequencing approach: epidemiological study conducted in Southern Italy and analysis of novel mutations in hereditary breast and ovarian cancer women."

MARIA VALERIA ESPOSITO

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Tutor: Prof. Nadia Tinto

Co-tutor: Prof. Francesco Salvatore

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PUBLICATIONS

1- Izzo V, Pinelli M, Tinto N, **Esposito MV**, Cola A, Sperandeo MP, Tucci F, Cocozza S, Greco L, Sacchetti L.
Improving the Estimation of Celiac Disease Sibling Risk by Non-HLA Genes. PLoS One. 2011;6:(11).

2- D'Argenio V, **Esposito MV**, Gilder JA, Frisso G, Salvatore F.
Should a BRCA2 stop codon human variant, usually considered a polymorphism, be classified as a predisposing mutation?
Cancer. 2014 Feb 5.

Abstract

Hereditary breast and ovarian cancer (HBOCs) accounts for about 10% of all breast cancers and *BRCA1* and *BRCA2* are the most prevalent genes associated to this pathology. They play a role in the maintenance of genome stability, particularly in the homologous recombination (HR) pathway for double-strand DNA breaks repair (DSBR). *BRCA1/BRCA2* germline mutations dramatically escalate the risk of developing HBOCs by up to 20 fold. Therefore, testing for *BRCA* gene mutations is important to improve the clinical management of high-risk patients and of their mutation-carrier family members. Here is reported the *BRCA1/BRCA2* molecular screening of 300 patients with early-onset breast cancer ("under forty"), and/or with positive family history, carried out by using a next-generation sequencing (NGS)-based approach, in order to identify mutation carriers. In particular, all the *BRCA1/BRCA2* coding regions were amplified by multiplex PCRs, using specific sequence tags able to univocally identify each patient. In this way, we were able to simultaneously analyze up to 60 different samples in a single NGS run. After sequence data analysis, 24 known *BRCA1/BRCA2* predisposing mutations were identified in 27 unrelated patients.

About 12.5% of analyzed patients, including some males, carried a causative mutation. Several novel variants were also identified: double mutations, which include 1 nonsense mutation in the *BRCA1* gene thereby causing a premature stop codon; 2 synonymous variants, 1 missense variant predicted not to have clinical significance, and 1 missense variant predicted to be deleterious. In addition, 2 novel possibly pathogenetic variants were also identified and specific functional studies were performed to assess their role. The first is a splice variant for which bioinformatic predictions, performed with both Human Splice Finder and NetGene2 tools, suggested a possible deleterious effect, since it could cause the loss of a canonic donor splice site of a *BRCA1* intron. This was experimentally demonstrated on the HBOC patient cDNA by PCR amplifications and enzymatic digestion. The other is a missense variant located on the BRCA2 DNA-binding site that could impair the *BRCA2* DSBR functions by HR. The role of this variant was tested by cloning the site-directed-mutated *BRCA2* cDNA into a pRc/CMV vector: plasmids were transfected in NIH-GS cells (DR-GFP stable clones) and subsequently transfected with plasmids codifying for I-SceI enzyme. Mutation effects were tested through DSBR assays and the extent of repair by HR was measured by counting GFP-positive cells by FACS: the novel variant resulted unable to bind DNA, probably hampering the normal DSBR pathway. Moreover, 26 variants with unknown clinical significance (VUSs) were also detected. Subsequent analysis of the mutation-carrier families resulted in the identification of at-risk subjects, including healthy male carriers, who were enrolled in surveillance healthcare programs. These results support the inclusion of an NGS-based approach for *BRCA1/BRCA2* mutation detection in a diagnostic workflow and further suggest the use of direct *in vitro* assays to test for functional role of novel mutations found in these genes.

1. BACKGROUND

Breast cancer (BC) is the most common malignancy in females worldwide with highest age-adjusted incidence in developed countries (73%) (Narod 2012). World Health Organization (WHO) report shows that BC incidence increases 2% per year (Parkin 2005). It is reported that 1.38 million of new cases were diagnosed in 2008 (**Fig. 1**) (Ferlay 2010). In Italy, about 38,000 new cases of BC are diagnosed each year, whereas the corresponding figure for Europe is 430,000 (Parkin 2005) (**Fig. 2**). BC, considered a multifactorial disorder, is caused by both non-genetic and genetic factors and, with ovarian cancer, is one of the most common causes of death due to a neoplastic disease affecting women (**Fig. 3**). It is estimated that 1 in 8 women will develop breast cancer in her lifetime in the developed countries (Bray 2004).

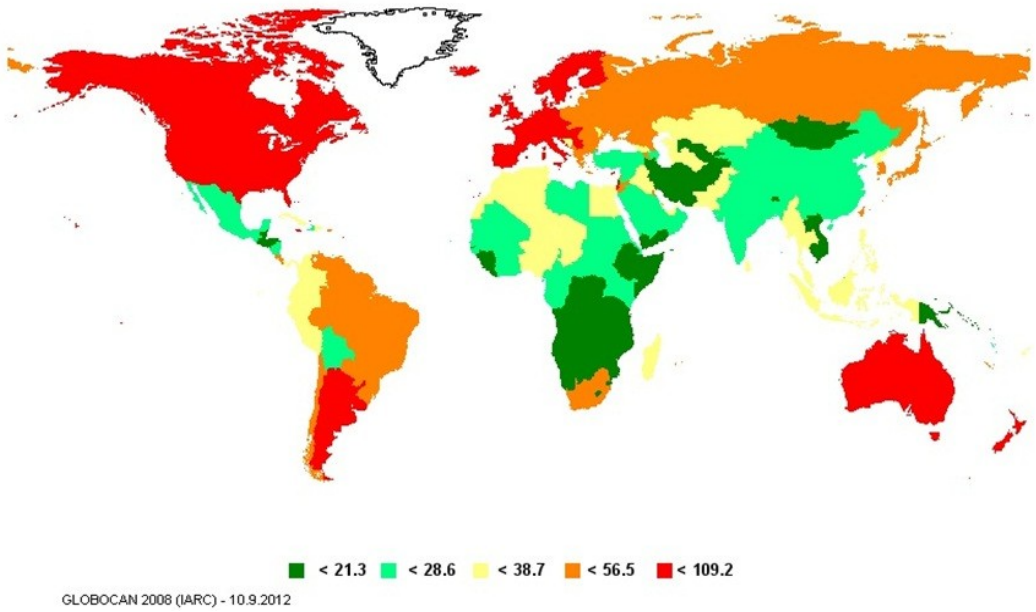


Figure 1. Estimated age-standardized incidence rates per 100,000 (World): breast cancer (Ferlay 2010). BC is now the most common cancer both in developed and developing regions with 690,000 new cases estimated in each region (population ratio 1:4). Incidence rates vary from 19.3 per 100,000 women in Eastern Africa to 89.9 per 100,000 women in Western Europe, and are high (greater than 80 per 100,000) in developed regions of the world (except Japan) and low (less than 40 per 100,000) in most of the developing regions.

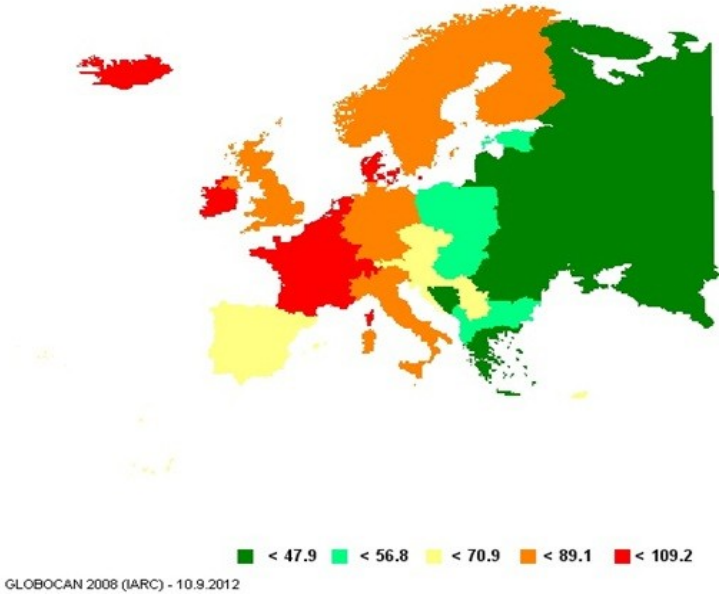


Figure 2. Estimated age-standardized incidence rates per 100,000 (Europe): breast cancer (Ferlay 2010).

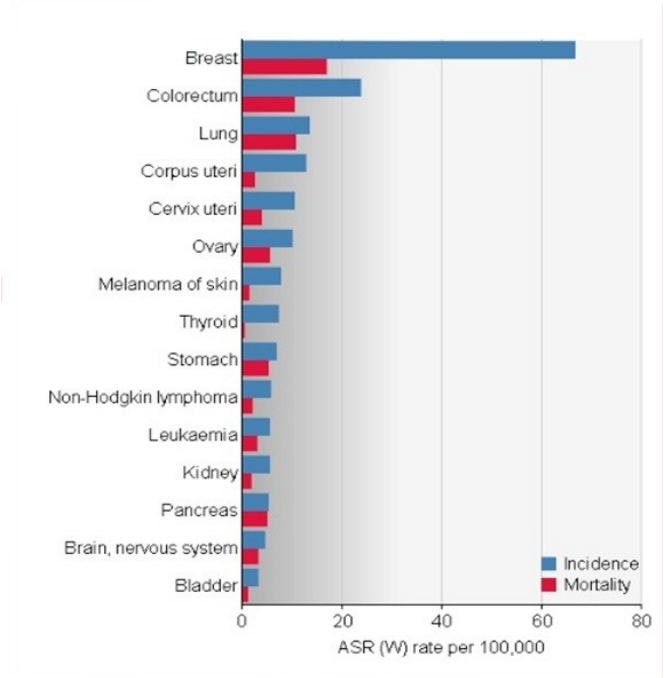


Figure 3. Estimated age-standardized incidence and mortality rates: women (Ferlay 2010). The incidence and the mortality of breast cancer is higher than the other most common cancers in women.

The three most important risk factors for BC, in decreasing order of importance, are gender, aging, and family history (Jatoi 2008). In addition, there are a number of recognized risk factors for BC development including hormonal, reproductive, and menstrual history, lack of exercise, alcohol, radiation, benign breast disease, and obesity (Hilgart 2012). Anyway, individual risk of developing BC increases in people carrying a germline predisposing mutation.
About 90% of BCs are considered sporadic, whereas the remaining account for an inherited disorder, defined as "Hereditary Breast and Ovarian Cancers" (HBOCs). HBOC is an autosomal dominant disease with incomplete penetrance. (Meindl 2011). It is characterized by a young age of onset and the presence in the family of numerous cases of cancer, not only of BC but also ovarian and/or cancer affecting other organs (Metcalfe 2008). Furthermore, although rarely, even men can develop BC, as greatly reported in literature (Bray 2004, Meindl 2011).
The two high-penetrance genes most commonly mutated in HBOCs are the tumor suppressor genes *BRCA1* and *BRCA2* (BReast CAncer, early onset 1 and 2). *BRCA1/BRCA2* germline mutations dramatically escalate the risk of developing HBOCs: women leading heterozygous mutations in *BRCA1* or *BRCA2* gene have, respectively, up to an 80% and a 60% risk of developing breast cancer and about 60% risk of developing ovarian cancer (Parkin 2005). Therefore, is important to recognize predisposing-mutation carriers in high risk families, especially before the onset of the disease.

1.1. Genetic risk of developing BC and non-genetic risk modifiers

The risk for developing BC before the age of 40 is similar throughout the world (Narod 2013). However, the clustering of BC in families has been recognized for many years, suggesting the presence of an inherited component. It has been estimated that about 5-10% of all BCs arise in individuals carrying a germline predisposing-mutation (Musolino 2007). Although approximately 10%-30% of BCs are attributed to hereditary factors, only 5-10% of them have a strong herited component, and just a small fraction (4%-5%) is explained by mutations in high penetrant genes transmitted in an autosomal dominant manner (Narod 2006). Within this group, only around 25% of cancer patients carry a germ line mutation in the two BC high susceptibility genes: *BRCA1* (Miki 1994) and *BRCA2* (Wooster 1994). Indeed, the majority of inherited BCs are attributed to the HBOC-syndrome. Despite that, mutations in other genes causing familial syndromes, characteryzed by highly increased BC incidence, are estimated to cause around 5% of familial breast cancer (FBC). The most investigated are *TP53*, *PTEN*, *STK11/LKB1*, *CDH 1*, *ATM*, and *CHEK2* (Robson 2007, Melchor 2013) as reported below (**Fig. 4**).

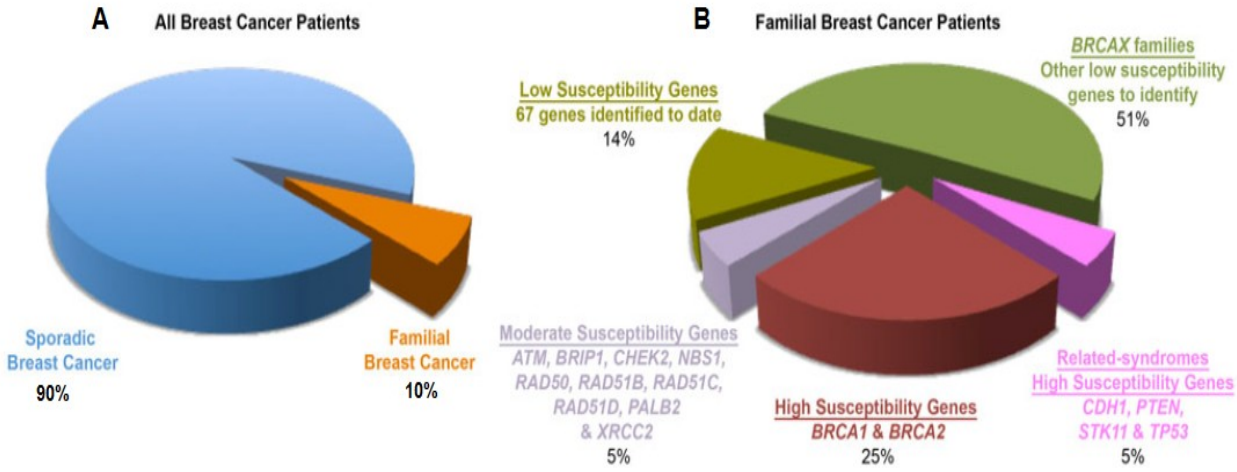


Figure 4. Distribution of breast cancer patients (Melchor 2013). A: Familial breast cancer represents a minor percentage of all breast cancer patients. B: Proportion of familial breast cancer patients due to germ line mutations in high, moderate, and low penetrance cancer genes. *BRCA1* and *BRCA2* explain the vast majority of familial breast cancer attributed to identified cancer-related genes; however, more families carry no mutations in known susceptibility genes and thus, are suggested to be caused by the inheritance of one or many low penetrance cancer genes (*BRCAX* families).

Germ-line mutations in *BRCA1* or *BRCA2* genes confer an average cumulative risk of 65 or 39 % for BC and 39 or 11 % for ovarian cancer by age 70 years, respectively (Borresen 1992, Chen 1998, Pharoah 2001, Evans 2002, Antoniou 2003, Leggett 2003). Fanconi anemia (FA) pathway genes have also been reported in about 5 % of FBC as moderate penetrance genes, given their incomplete segregation in affected families; this is the case of *BRIP1*, *PALB2*, *RAD51C* and *XRCC2* (Seal 2006, Rahman 2007, Meindl 2010, Shamseldin 2012), which present similar penetrance to other non-FA genes, such as *ATM*, *CHEK2*, *NBS1*, *RAD50*, *RAD51D* and *RAD51B* (Meijers-Heijboer 2002, Thompson 2005, Heikkinen 2006, Loveday 2011,Orr 2012,). Lastly, the recent identification of 41 low susceptibility genes, together with up to 26 genes previously identified, may explain around 14% of familial cancer risk (Michailidou 2013). However, there are still about 51% of FBC patients that show no mutation in any of these genes, and are classified into the category of non-*BRCA1/2* or *BRCAX* families. These families may either carry a mutation in a moderate-penetrance BC gene still to be identified or be explained by a truly polygenic model. In the latter, BC susceptibility would be conferred by the joint action of several low-penetrance loci (Easton 2007, Cox 2007, Stacey 2008, Rosa-Rosa 2009, Gracia-Aznarez 2013). Attempts to identify a third breast cancer susceptibility locus (*BRCA3*) have so far been unsuccessful. This is probably

because no single gene can account for the remainder of families with a high incidence of breast cancer not associated with *BRCA1* or *BRCA2* mutations.

Large epidemiological studies have been performed to identify potential modifiers of BC risk in predisposing-mutation carriers (Narod 2002).
Briefly, risk is commonly measured in terms of the lifetime probability of developing breast or ovarian cancer (penetrance), but may also be measured in terms of the probability of developing cancer in a given year. Penetrance is usually defined in terms of a given age (e.g. up to age 70 years) and can be estimated in several ways. Ideally, a cohort of healthy subjects, no carrying mutations in the susceptibility gene of interest, is followed for a defined period of time and incident (new) cases of cancer are recorded. The cancer rate is then calculated from the number of new cases and the person-years of observation. Other methods are based on the reporting of cancers by family members. As reported before, the lifetime risk of BC in women who carry a deleterious *BRCA1* or *BRCA2* mutation is estimated to be as high as 80% - or roughly 10 times greater than that of the general population - but several estimates are lower (**Table 1**) (Risch 2001, Antoniou 2003, Roy 2011).

Table 1. Human cancers arising in *BRCA1* or *BRCA2* mutation carriers (Roy 2011).

Cancer Type	*BRCA1* mutations	*BRCA2* mutations	Features
Breast	70-80% lifetime risk	50-60% lifetime risk	Breast and ovarian cancer is the dominant cancer predisposition in *BRCA1* and *BRCA2* mutation carriers. *BRCA1* mutation carriers develop breast and ovarian cancer at a younger age than *BRCA2* mutation carriers.
Ovarian	50% lifetime risk	30% lifetime risk	
Prostate	Ashkenazi Jewish founder mutations are associated with increased risk	20-fold increased risk	<1% of *BRCA2* mutation carriers have prostate cancer. Prostate cancer is even rarer in *BRCA1* mutation carriers, except in the Ashkenazi Jewish carrier of *BRCA1* mutations.
Pancreatic	Anecdotal evidence and case reports only	10- fold increased risk	<1% of *BRCA2* mutation carriers have pancreatic cancer. No incidence has been clearly documented in *BRCA1* mutation carriers.
Gastric	None reported	Limited reports	It is unclear whether stomach cancer is associated with *BRCA2* mutations.
Fallopian Tube	Observed, but rare	Rare	This is a rare cancer overall and is still uncommon in *BRCA* mutation carriers.

Others	None reported	Brain, medulloblastoma, pharyngeal, CLL and AML	Fanconi anaemia subtype D1 (caused by *BRCA2* mutations) is associated with cancer of the central nervous system.
AML, acute myeloid leukaemia; CLL, chronic lymphocytic leukaemia			

A specific penetrance estimate represents a population average. However, penetrance may vary among populations, within populations and among individuals depending on several potential sources of variation. Allelic variation is due to different mutations in a single gene - for example, the position of the *BRCA* mutation within the coding region of the gene may influence the risk of breast or ovarian cancer. Among *BRCA2* carriers, the risk of ovarian cancer is greatest for women carrying mutations within the ovarian cancer cluster region (defined by nucleotides 4075–6503). The risk of ovarian cancer is increased by 1.9 times for mutations within this region and the risk of BC appears to be decreased (Thompson and Easton 2001). Among *BRCA1* mutation carriers, the relative proportion of ovarian cancer compared with BC seems to be greater for mutations within the two-thirds of the gene (Gayther 1995). So, allelic variation can explain differences in penetrance among families and among countries.

Another potential source of variation is represented by modifier genes. The penetrance of a major gene might be influenced by the presence of one or more variant in one or more minor genes acting as a genetic modifier. Several candidate HBOC modifier genes have been studied; most of them are related to the metabolism of sex hormones and DNA repair. Several published data supported the role of these genes (including androgen receptor, *AIB1*, *HRAS* and progesterone receptor) as potential risk modifiers in *BRCA1* or *BRCA2* mutation carriers (Phelan 1996, Rebbeck 2001).

Environmental and lifestyle factors might also modify the risk of breast and ovarian cancer among *BRCA* mutations carriers. The risk varies from individual to individual, and it is increased in recent generations, underling the weight of environmental factors. To estimate the risk is important to consider also the age of menarche. It is not clear if this implies that neoplastic events take place in women as early as age of 10 years, or if the age of menarche is an indicator of later hormonal events, such as the regularity of established menses or circulating hormone levels. Early menarche is, however, associated with an increased risk of BC in *BRCA1* carriers (Kotsopoulos 2005). Further, spontaneous and therapeutic abortion are considered risk modifiers; indeed, an interruption in pregnancy might be expected to be carcinogenic because the early changes of pregnancy result in increased mitoses, but are not accompanied by the later differentiation of the epithelial cells. On the other hand, is controversial the role of pregnancy on the risk. In the general population, pregnancy offers protection against BC after the age of 40 years, but appears to increase the risk for very early-onset BC. This is consistent with the hypothesis that the ovarian hormones produced during

pregnancy are mitogenic and accelerate the growth of existing tumors. During pregnancy, breast differentiation occurs and thereafter the population of susceptible cells is reduced (Russo 1992). This might explain why pregnancy prevents BCs at a later age. However, the effect of pregnancy on hereditary breast cancer risk appears to differ for *BRCA1* and *BRCA2* carriers. In a large matched case-control study, Cullinane et al. (2005) reported that the risk of breast cancer did not decrease with pregnancy in *BRCA1* carriers until four births were reached, after which there was only a modest protective effect. Furthermore, an early first birth does not appear to be protective for BC risk among *BRCA1* carriers (Kotsopoulos 2006). This is consistent with the theory of Russo et al. (2001) that, in the absence of intact *BRCA1*, pregnancy fails to induce the expected degree of lobular differentiation.

Moreover, a case-control study of breast-feeding and BC in *BRCA* mutation carriers reported a significant protective effect in women with *BRCA1* mutations, but not with *BRCA2* mutations (Jernstrom 2004). In addition, several studies have shown that the incidence of BC declines in women with *BRCA1* or *BRCA2* mutations following an oopohorectomy (Rebbeck 1999, Kauff 2002, Eisen 2005). It is also important to establish whether or not oral contraceptives are hazardous to the breast, because their use has been proposed as a preventive measure against ovarian cancer (Narod 1998). It appears to be a small increase in BC risk associated with pill use among *BRCA1* mutation carriers (Narod 2002). In addition, also drugs represent a BC risk modifier; tamoxifen, an anti-estrogenic drug routinely used in the treatment of estrogen-receptor-positive BCs, has also been demonstrated to reduce the risk of primary invasive and pre-malignant BC in high-risk women in North America (Vogel 2002), and of contralateral BC in unselected women (Early Breast Cancer Trialists' Collaborative Group, 1998). The large National Surgical Adjuvant Breast and Bowel Project (NSABP) reported a highly significant reduction in the incidence of invasive BC among women randomized to tamoxifen, versus placebo (Vogel 2002). These data suggest that tamoxifen may be effective in the primary prevention of *BRCA2* mutation-associated BC, but not of *BRCA1* ones. Moreover, there are little data available so far supporting the hypothesis that hormone replacement therapy (HRT) increases the risk of BC in women at high genetic risk. In the only study published to date by Rebbeck et al. (2005) is reported that the protective effect offered by oophorectomy was not attenuated by HRT. Furthermore, the potential use of antioxidants as chemoprevention in *BRCA* carriers has been discussed elsewhere (Kotsopoulos and Narod, 2005). One of the most investigated retinoid derivative is the Fenretinide. The capacity of this drug to accumulating preferentially in fatty tissue such as the breast, may contribute to the effectiveness against breast cancer (Sabichi, 2003). Moreover, phase III clinical trial data has suggested that fenretinide reduces breast cancer relapse in pre-menopausal women (Veronesi 2006).

Summarizing all the above mentioned factors, the proposed BC risk modifiers in *BRCA1* carriers relate to estrogen exposure and deprivation: the BC

risk declines after menopause; the cancers are largely limited to the breast and ovary, and BC is preventable by tamoxifen and by oophorectomy. The evidence for the importance of hormones in *BRCA2*-related carcinogenesis is much less compelling. The range of cancers seen among *BRCA2* mutation carriers is wide and includes types that are classically associated with exogenous carcinogens, including melanoma and pancreatic cancer (The Breast Cancer Linkage Consortium, 1999). These observations imply that also non-genetic factors may modify the inherited risk. Knowledge of these risk factors is a very useful tool for managing risk and for developing prevention strategies. To address these points, over the last decade, researchers have developed a number of statistical models for predicting risk of harboring mutations in these genes and of subsequently developing breast and ovarian cancer (Vargas 2010).

1.2. Breast cancer genetics

Cancer is considered to originate from the contribution of both inherited and acquired genomic alterations, the first being dispensable, although strongly favorable for cancer induction. These genomic alterations are ultimately the result of the balance between the cell's ability to maintain the integrity of genetic information and the effects of the environmental efforts to alter it. Acquired DNA changes, in addition to inherited predisposition, can push the cell outside the frame which regulates its life, and determinate the timing and shaping of its growth, division, metabolism and death. In particular, genetic alterations such us mutations, deletions and rearrangements can greatly affect the tumor suppressive activity of many genes, while insertions, duplications, translocations and gene fusions can push tumor promoting genes toward gains of function.
Breast cancer susceptibility gene 1 (*BRCA1*) and breast cancer susceptibility gene 2 (*BRCA2*) are the two major genes associated with HBOCs (Hamilton 2009). The *BRCA* genes, discovered approximately 15 years ago, represent a great opportunity to identify high-risk individuals for HBOCs (Pal 2012). As discussed above, germline inherited mutations can increase the risk for BC. *BRCA1* and *BRCA2* mutations account for approximately 5% of all BCs and 10-15% of all ovarian cancers. Specifically, high-penetrance mutations (eg, *BRCA1*, *BRCA2*, *P53*, *PTEN*, *STK11/ LKB1*, *CDH1*) are associated with a high lifetime risk of BC (40%–85%), whereas the low-to-moderate penetrance mutations (eg, in *ATM*, *CHEK 2*) are associated with a lower risk. These mutations linked to a spectrum of syndromes that includes the HBOC-syndrome (*BRCA1* and *BRCA2*), Li-Fraumeni syndrome (*P53*), Cowden's disease (*PTEN*), Peutz-Jeghers syndrome (*STK11/LKB1*), hereditary diffuse gastric carcinoma syndrome (*CDH 1*), ataxia-telangiectasia (*ATM*), and a Li-Fraumeni syndrome (*CHEK 2*) (Jatoi 2008). Because of these evidences is important to identify women who carry predisposing mutations in order to use the latest medical advances in prevention,

early detection, and treatment. In particular, the most important genes which were proposed as highly penetrant for BC genetic susceptibility include *BRCA1* and *BRCA2,* but also *P53*, *PTEN*, *STK11/LKB1*, and *CDH1* genes, while some others like *ATM*, *CHECK2*, *BRIP1*, and *PALB2* are considered as moderate genetic factors. *P53* is involved in Li-Fraumeni syndrome and has about 1400 mutations which almost involve the transactivation domain. BC risk is increased of 4% in women who are carrier of *PTEN* mutations (Melchor 2013). It was described that *PTEN* loss of function not only is involved in tumor formation, but also causes resistance to targeted therapy. *CDH1* gene encodes for E-cadherin which is determined as a primary indicator in addition to estrogen receptor α (ERα) for luminal epithelial tumors of breast (Chen 2000).Women who are carriers of *CDH1* mutations have 39%-52% to be affected with BC in their life. On the other hand, *CDH1* underexpression is associated with more metastasis and poor prognosis in either ER positive or negative BCs (Schrader 2011). Ataxia telangiectasia-mutated (*ATM*) gene encodes for ATM protein which is involved in double-stranded breaks DNA repair and regulation of cell cycle. It was shown that being carrier of heterozygote mutations of this gene is associated with increased risk of BC. However, the greater risk belongs to *BRCA1* and *BRCA2* genes which enhance the risk of BC progression up to 59%-87% and 38%-80% respectively.

BRCA1 and *BRCA2* are tumor-suppressor genes, involved in a common pathway of genome protection (Narod 1994, 2004). These genes play a relevant role at different stages in the DNA damage response and DNA repair, since they code for proteins intimately involved in cellular growth and differentiation (Wooster 1994, Hall 1990, Jatoi 2008). The *BRCA1* and *BRCA2* gene mutations are transmitted in an autosomal dominant manner and therefore may originate from either the maternal or paternal side (Miky 1994, Iau 2001); then, the loss of the second allele (loss of heterozygosity (LOH)) can arise in mutation carriers. The effect of a defective *BRCA1* or *BRCA2* allele in the germ line must cause haploinsufficiency of HR to trigger the subsequent genetic alterations that result in cancer. Each offspring of a *BRCA1* or *BRCA2* mutation carrier has a 50% chance of inheriting that mutation, and a carefully documented family history is essential for the initial assessment of any woman concerned about a hereditary predisposition to BC. Hereditary BC predisposition should be suspected if a woman has: i) multiple close relatives with breast and ovarian cancer diagnosed at an early age (before age 50 years); ii) bilateral breast cancer; iii) male BC in her family; and/or iv) an Ashkenazi Jewish ancestry (Jatoi 2008). Indeed, alterations in the *BRCA1* or *BRCA2* genes are more common in certain ethnic and geographic populations (eg, Ashkenazi Jewish, Norwegian, Dutch, Icelandic), and specific mutations (founder mutations) often are clustered in particular ethnic groups (Ferla 2007). Clearly, women who carry *BRCA* mutations have a markedly increased risk of developing breast and ovarian cancer at an early age. Men who have *BRCA1* or *BRCA2* mutations (particularly *BRCA2*) also are at increased risk

for BC, although that risk is considerably lower than in women. Recently, it was estimated that the cumulative BC risk, by age 70 years, is about 1.2% for male carrying a *BRCA1* mutation and about 6.8% for male carrying a *BRCA2* mutation (Tai 2007). Specifically, *BRCA2* mutations confer a lifetime risk of prostate, breast, and pancreatic cancers of about 20%, 6%, and 3%, respectively (Garber 2005).

1.3. *BRCA1* and *BRCA2*

The *BRCA1*gene, located on chromosome 17q, has a key role in DNA repair, cell-cycle regulation, transcriptional regulation, chromatin remodeling and control of multiple cell cycle checkpoints. This gene has 24 exons, spans approximately 100kb of genomic DNA, and encodes a 1,863 amino acid nuclear phosphoprotein, which acts as a tumour suppressor gene maintaining genomic stability (Rahman 1998). BRCA1 is a versatile protein that links DNA damage sensing and a several effectors of cellular network of signalling events (the DDR) that are triggered in response to genotoxic stress. BRCA1 interacts with tumour suppressors, DNA repair proteins and cell cycle regulators through its various functional domains and thereby has diverse roles in multiple DNA repair pathways (particularly HR, NHEJ and single-strand annealing (SSA)) and in checkpoint regulation (**Fig. 5**) (Deng 2000, Huen 2010).

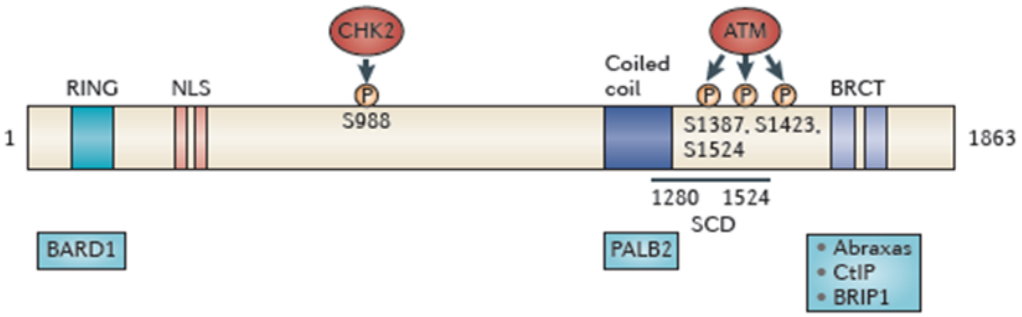


Figure 5. BRCA1 functional domains (Roy et al. 2011).

The BRCA1 amino terminus contains a RING domain which binds BARD1 (BRCA1-associated RING domain protein 1), and a nuclear localization sequence

(NLS). The central region of BRCA1 contains a CHK2 phosphorylation site on S988, necessary to the protein activation during the damage response. The carboxyl terminus of BRCA1 contains: a coiled-coil domain which binds PALB2 (partner and localizer of BRCA2); a SQ/TQ cluster domain (SCD) that contains approximately ten potential ataxia-telangiectasia mutated (ATM) phosphorylation sites and spans amino acid residues 1280-1524; and a BRCT domain that facilitates the binding of phospho-protein such as ATM-phosphorylated abraxas, CtBP-interacting protein (CtIP) and BRCA1-interacting protein C‑terminal helicase 1 (BRIP1). The BRCA1-Abraxas-RAP80 macro-complex is associated with BRCA1 recruitment to sites of DNA damage (Wang 2007, Kim 2007, Liu 2007, Sobhian 2007) through the association with ubiquitylated histones at DNA DSBs and appears to be involved in the G2/M checkpoint in response to ionizing radiation induced DNA damage (Wang 2007). The BRCA1-BRIP1 complex, which also contains DNA topoisomerase 2-binding protein 1 (TOPBP1), is associated with DNA repair during replication (Cantor 2001) and is necessary for the S‑phase checkpoint in response to stalled or collapsed replication forks (Greenberg 2006). The BRCA1-CtIP complex promotes CtIP-mediated 5′-end resection of DSBs (Yun 2009). In addition, the BRCA1-CtIP complex promotes ataxia-telangiectasia and Rad3-related (ATR) activation and homologous recombination (HR) by associating with the MRN complex (which is comprised of MRE11, RAD50 and NBS1) and facilitating DNA double-strand break resection (Yu 1998). The central region of BRCA1, which contains the SCD, is phosphorylated by ATM. This phosphorylation is important for BRCA1-mediated G2/M and S-phase checkpoint activation, as demonstrated by the expression of a *BRCA1* mutant that lacks three of the phosphorylation sites (S1387, S1423 and S1524) and fails to rescue defective checkpoint activation and ionizing radiation hypersensitivity in a BRCA1-deficient cell line (Cortez 1999, Xu 2002). Moreover, BRCA1 contains an amino-terminal RING domain that has E3 ubiquitin ligase activity (which catalyses protein ubiquitylation). Many inherited cancer-associated *BRCA1* mutations have been found within the RING and BRCT domains, indicating that both domains are involved in suppressing breast and ovarian cancer (Friedman 1994, Shattuck-Eidens 1995, Couch 1996). BRCA1 E3 ubiquitin ligase activity is enhanced when associated with the RING domain of its partner protein, BRCA1-associated RING domain protein 1 (BARD1) (Wu 1996). The BRCA1-BARD1 heterodimer generates polyubiquitin chains at unconventional K6 linkages that do not appear to signal for protein degradation, but may instead mediate downstream signaling events through mechanisms that are still unclear. BRCA1 ubiquitylation of CtBP-interacting protein (CtIP; also known as RBBP), may have a role in DSB repair pathway choice, as CtIP-dependent resection promotes HR and inhibits NHEJ. The BRCA1-BARD1 complex is involved in the activation of G1/S, S-phase and G2/M checkpoints. The G1/S-checkpoint requires phosphorylation of BRCA1 by ATM or ATR, which facilitates phosphorylation of p53 on S15, that is necessary for transcriptional induction of the cyclin dependent kinase (CDK) inhibitor p21 and

ionizing radiation induced G1/S checkpoint activation (Siciliano 1997). The BRCT phosphopeptide-binding motif, which is conserved in multiple DDR proteins, is responsible for the association of BRCA1 with proteins phosphorylated on serine in SXXF motifs by ATM. The BRCA1-interacting proteins include abraxas, BRIP1 and CtIP. The binding of these proteins make up separate BRCA1 macro-protein complexes that have distinct and overlapping functions in the DDR. A fourth BRCA1-containing complex mediated through the BRCA1 coiled-coil domain is composed of PALB2 and BRCA2 and is specifically involved in DSB repair by HR (Sy 2009, Zhang 2009 BRCA1 is also required for RAD51 recruitment to the sites of DNA damage through its interactions with PALB2 and BRCA2. This interaction appears to be dependent on CHK2‐mediated phosphorylation of S988 on BRCA1. This protein combines with other tumor suppressors, DNA damage sensors, and signal transducers to form a large multisubunit protein complex, known as the BRCA1-associated genome surveillance complex (BASC) (Wu 1996). However, BRCA1 is directly involved in HR-mediated repair of DSBs (Wang 2007, Kim 2007) (**Fig. 6**).
So, BRCA1 is a gatekeeper of genomic integrity and is implicated at multiple cellular levels including DNA repair by homologous recombination (HR) mechanism, checkpoint control, spindle cell regulation, and transcriptional regulation (Roy 2011).

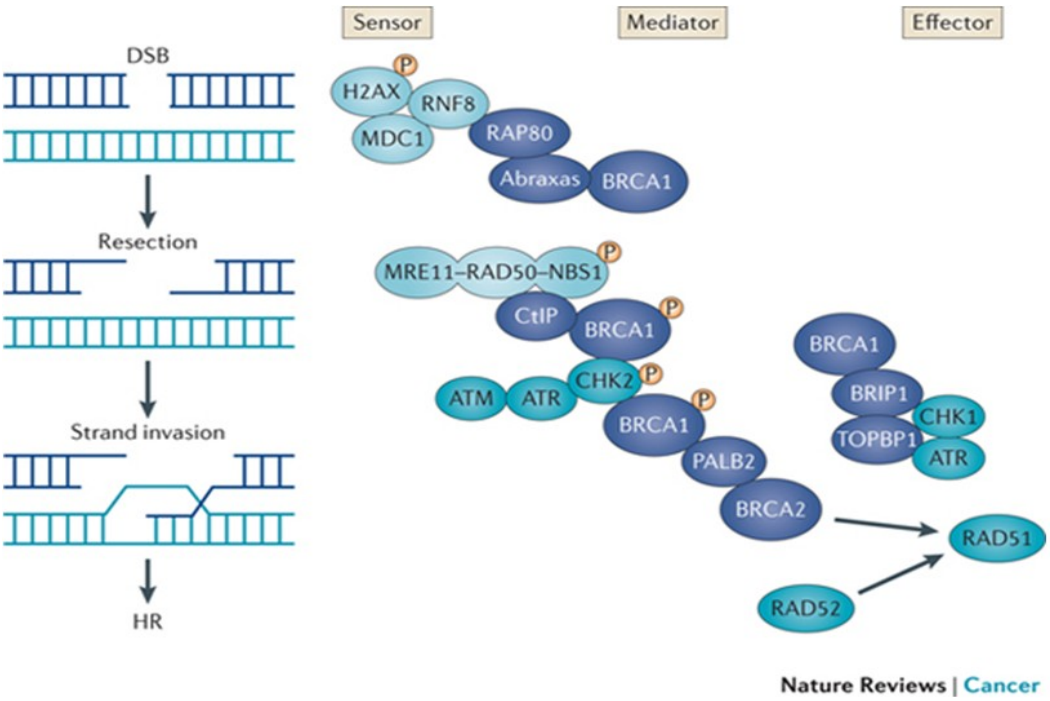


Figure 6. Molecular mechanisms of the DNA damage response (Roy 2011). In response to DNA doublestrand breaks (DSBs) or replication fork collapse (not shown), sensors (light blue) detect the damage, and signalling mediators (blue) recruit or activate effectors (dark blue) that repair the damage and activate cell cycle checkpoints.

BRCA2, located on chromosome 13q, is made up of 27 exons, spans around 70kb and encodes a protein of 3418 amino acids (**Fig. 7**). BRCA2 is crucial as mediator of the HR mechanism by regulating the formation of the RAD51 filament; indeed, functions attributed to BRCA2 are mainly restricted to DNA recombination and DNA repair through a regulating role in RAD51 activity (Jensen 2013). These mechanisms assure the maintenance of genomic stability and more specifically, the correct operation of the homologous recombination (HR) pathway which repairs double-strand DNA breaks.

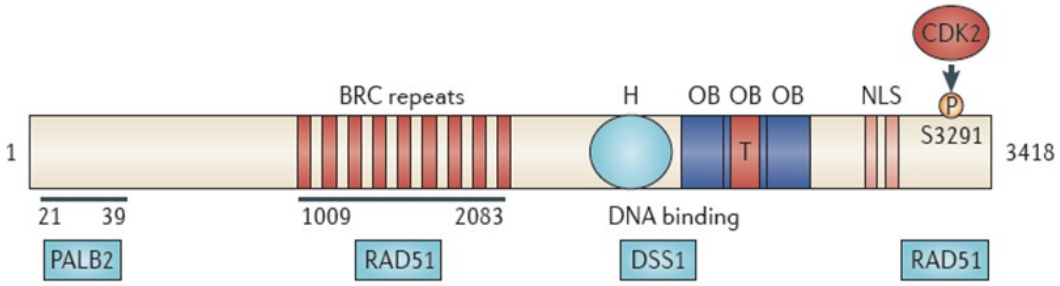


Figure 7. BRCA2 functional domains (Roy 2011).

The N terminus of BRCA2 binds PALB2 at amino acids 21-39 (Roy 2011). BRCA2 contains eight BRC repeats between amino-acidic residues 1,009 and 2,083 that bind RAD51. Then, BRCA2 protein contains a DNA-binding domain (DBD), that binds single-stranded DNA (ssDNA) and doublestranded DNA (dsDNA), and eight BRC repeats that bind RAD51. The DBD contains five components: a 190‑amino-acid α-helical domain, three oligonucleotide binding (OB) folds that are ssDNA-binding modules, and a tower domain (TD) that protrudes from OB2 and binds dsDNA (Yang 2002). The helical domain, OB1 and OB2 also associate with deleted in split-hand/split-foot syndrome (DSS1), which has been linked to BRCA2 protein stabilization (Kojic 2003, Li 2006, Kristensen 2010). Point mutations within BRC repeats that compromise interactions with RAD51, are found in individuals with HBOC syndrome (Venkitaraman 2009). In addition to facilitating the recruitment of RAD51 to ssDNA, the BRC repeats accelerate replication protein A (RPA)-displacement from ssDNA by RAD51, block RAD51 nucleation at dsDNA and facilitate RAD51 filament formation on ssDNA by maintaining the active ATP-bound form of RAD51 on ssDNA (Carreira 2009). The C terminus of BRCA2 contains an

NLS and a cyclin-dependent kinase (CDK) phosphorylation site at S3291 that also binds RAD51. The binding of RAD51 by the C terminus of BRCA2 has been shown to be dependent on CDK activity (Esashi 2005, Ayoub 2009).

In humans, the tumors developed in patients with germline heterozygous mutations in *BRCA1* or *BRCA2* are defective in HR-mediated repair. *BRCA2*-related tumours usually express estrogen and progesterone receptors and tend to have features similar to sporadic BC, unlike *BRCA1*-related cancers (Tessaro 1997, Oei 2006, Hamilton 2007). In general, if a mutation occurs in such genes then the normal controls on cell growth are compromised (Hamilton 2009). Therefore, loss of BRCA1 or BRCA2 leads to a deficiency in the repair of DNA double-strand breaks by homologous recombination (HR), leading to potentially mutagenic repair of DNA lesions by alternative mechanisms such as non-homologous end-joining (NHEJ) and single strand annealing (SSA). Ultimately, genomic instability is developed and contributes to the cancer predisposition generated by loss-of-function mutations in *BRCA1* or *BRCA2* (Venkitaraman 2002). Inactivation of the *BRCA* genes causes ostructural aberrations in chromosomes and aneuploidy, another common feature in cancer cells from *BRCA*-mutation carriers.These observations raise the question of whether BRCA1 or BRCA2 have functions whose inactivation could interfere with chromosome segregation during mitosis, leading to the generation of daughter cells with abnormal numbers of chromosomes (Venkitaraman 2002). Anyway, both proteins, that work in concert in a common pathway to protect the genome during DNA replication, are essentials for the vital DNA repair process that uses the undamaged sister chromatid to carry out high-fidelity repair of predominantly replication-associated DNA double strand breaks (DSBs). HR appears to be the major mechanism for protecting the integrity of the genome in proliferating cells, because other DSB repair pathways are error-prone and generate chromosome deletions and translocations (Schlacher 2011).

Most of *BRCA1/BRCA2* predisposing mutations are frameshifts, and there are a number of missense variations with unclear pathogenicity (variants of unclassified significance-VUS), discussed below.

1.4. *BRCA1* and *BRCA2* mutations

Up to now, more than 3,000 distinct *BRCA1* and *BRCA2* sequence variants have been identified, including deletions, insertions, and many single nucleotide substitutions in coding or non-coding regions. The most common mutations are frameshift mutations due to small insertions/deletions, nonsense mutations, and disruption of splice site leading to entire nonfunctional BRCA proteins (Narod

2010). The higher rate of duplications/deletions in *BRCA1* gene versus *BRCA2* (42% and 20%, resp.) is due to accumulation of Alu sequences (Thompson 2004). Large genomic rearrangements (LGRs) comprise about 1/3 of all mutations occurring in *BRCA1* gene and are typically the result of homologous recombination between *BRCA1* and its pseudogene (Zhang 2010). In general, most of *BRCA2* mutations occur in exons 10 and 11, while most of *BRCA1* mutations usually occur in exon 20. These usually include insertions or deletions which raise the missense alterations and premature stop codon ending in truncated and nonfunctional protein. Some of the *BRCA1/BRCA2* mutations show population specific patterns and some of them have been found in various studies from different populations. All of the most important and frequent *BRCA1* and *BRCA2* mutations which have been discovered so far are collected in several databases, such as Breast Cancer Information Core (BIC) – National Human Genome Research Institute (http://research.nhgri.nih.gov/bic/).

The effects of *BRCA1* and *BRCA2* mutations on histological and pathological features, BC stage, especially the involvement of lymph nodes, are determining factor of BCs prognosis and survival rate. In general, the carriers show higher tumor stage, grade and ER negative tumors, and more metastasis to neighbor vessels relative to those who harbor other gene mutations (Musolino 2007). Fatemeh Karami and ParvinMehdipour (2013) have richly described the mutation spectrum of *BRCA1/BRCA2* mutations around several countries and the the genotype-phenotype correlations that occur in mutation carriers.

1.4.1. VUSs

A major limitation of *BRCA1* and *BRCA2* genetic testing is the number of inconclusive results due to Variants of Unknown Significance (VUSs). About 30-50% of the genetic variants in *BRCA1* and *BRCA2*, respectively, fall under this category (Gomez 2009). VUSs are mainly missense and splice site mutations without a definite role in carcinogenesis, thus representing a real clinical challenge. The interpretation of such variations can be difficult for physicians and problematic for individuals. The approach towards the evaluation of a VUS variant can be multifactorial, involving the *in silico* analysis, where specific softwares are used to predict the phylogenetic conservation and the protein modification caused. Additionally, segregation analysis of the variant with the disease can help in clarify its pathogenicity, even if only functional studies can verify its effect on protein structure and functions.

1.5. Genetic testing: the state of the art and Next Generation Sequencing

The progress made in the discovery of disease causing genes accelerated greatly with the initiation of the worldwide Human Genome Project in 1990. The investigation of the genome composition combined with new diagnostic approaches based on Next-Generation-Sequencing (NGS) methods, allow a better management of genetic diseases. While the number of tests for specific diseases continues to grow, one of the earliest presymptomatic mutation tests was for the HBOCs. Genetic testing for *BRCA1* and *BRCA2* is facilitated by the presence of one or more founder mutations in a population (Narod 2005, Previati 2013).

Several molecular methods have been devised with the aim to detect mutations. They can be classified into methods detecting new mutations or known mutations/polymorphisms in focused genes. Identification of such mutations in cases appropriately preselected, using pedigree and clinical data, is justified in clinical practice, even though such techniques are still complex, time-consuming and expensive (Matyjasic 2008). It is recommended that a subject (the proband) with breast and/or ovarian cancer be the first tested to determine if a mutation is present in either the *BRCA1* or *BRCA2* gene. Therefore, the first person tested for *BRCA* mutations should be a family member most likely to test positive, generally an individual who has developed BC at a young age or an individual who has ovarian cancer. Genetic testing allows the identification of high-risk women; however not all women who have a family history of BC are appropriate candidates for genetic testing. Women must be informed about the potential risks and benefits of genetic testing, and those who are found to carry mutations in the *BRCA1* or *BRCA2* genes should be advised of all management options (Evans 2013).

If a mutation is identified in the proband, other members of the family should be tested for the same mutation in order to manage the risk for cancer development. In a family in which a *BRCA1* or *BRCA2* mutation has been identified, individuals who do not carry the mutation are not at increased risk for breast or ovarian cancer. At worst, their risk is similar to that of the overall population, but it might be even less, because risk estimates of the overall population include women who carry the *BRCA1* and *BRCA2* mutations. According to findings on BC management, to reduce cancer-related mortality, women who have *BRCA1* or *BRCA2* mutations may wish to consider screening, chemoprevention, or prophylactic surgery. Early detection of cancer is beneficial especially for individuals at high risk for developing cancer. If a *BRCA* mutation is not found in a family member who has breast or ovarian cancer, the test is not informative and does not provide useful information to other family members. In such instances, the cluster of BC cases within a family might be attributable to mutations into other genes or to environmental or lifestyle factors.

BRCA1 and *BRCA2* genes are the most commonly mutated genes, but additional genes associated with hereditary BC are emerging (Walsh 2010). New advances in genomic technologies have led to parallel testing of multiple genes. Customized

NGS panels are now providing the simultaneous analysis of BC predisposition genes, from high- to intermediate-penetrant genes. Nonetheless, some of these genes have also been associated with increased risk of other cancers, such as ovarian, pancreatic, and colorectal cancer. *BRCA1* and *BRCA2* negative patients with a personal or family history of hereditary cancer can be eligible for customized gene panels testing.

The low-scale, targeted gene/mutation analysis that currently dominates the clinical genetics field will ultimately be replaced by large-scale sequencing of entire disease gene pathways and networks, especially for the complex disorders like cancers. In addition, the perceived clinical benefit of whole-genome sequencing will outweigh the cost of the procedure, allowing for these tests to be performed on a routine basis for diagnostic purposes, or perhaps in the form of a screening program that could be used to guide personalized medical treatments throughout the lifetime of the individual.

Even for genes extensively studied, as *BRCA1* and *BRCA2*, the most thoroughly sequenced genes in the human genome, previously unseen variants continue to be detected frequently. In the United States, genetic testing of *BRCA1* and *BRCA2* is carried out almost exclusively by a single commercial company, whose protocol is based on PCR amplification of individual exons and Sanger sequencing of the products (Frank 1998). At one reference laboratory alone (Myriad Genetics, Salt Lake City, UT), these two genes have been sequenced completely in over 150,000 people. In the process, upwards of 10,000 deleterious mutations and missense variants of negligible or uncertain clinical significance have been identified and recorded in a database (B. Ward, personal communication).Yet every week, 1% to 2% of patients currently being tested demonstrate missense variants not seen before (B. Ward, personal communication), and each of these must be carefully analyzed in the attempt to assess its likely clinical effect before reporting out the result. While there are a number of deductive and informatics methods for making these assessments (Aymé 2008), in many cases it is simply impossible to draw any conclusion without extensive clinical follow-up of those individuals carrying the variants. Myriad maintains extensive tracking and correlation data, and will sometimes revise the clinical classification of a missense variant years after its first detection. In 2007, a quantitative DNA measurement assay (BART) was added as a supplementary test to detect large exonic deletions and duplications that are not detectable by PCR amplification approaches (BRACAnalysis, http://www.myriadtests.com/provider/doc/BRACAnalysisTechnicalSpecification.pdf). In Europe, genetic testing of *BRCA1* and *BRCA2* is more widely available (Matthijs 2008). Sequencing of the more moderate-risk BC genes is available in various research or commercial diagnostic laboratories (GeneClinics, http://www.ncbi.nlm.nih.gov/sites/GeneTests/?db=GeneTests), but is still not routinely performed.

1.6. *BRCA* analysis

Direct sequencing is considered the gold standard for direct identification of specific sequence alteration, but it is time consuming and costly. Because of that, is important to find a cost effective scanning technique to identify regions containing genetic variants, which can be subsequently subjected to DNA sequencing, and to develop a reliable alternate faster and less expensive method for routine *BRCA1* and *BRCA2* mutation screening program. Some of the techniques which are commonly used are single-strand conformation polymorphism (SSCP), restriction endonuclease fingerprinting (REF)- SSCP, conformation-sensitive gel electrophoresis (CSGE), fluorescence-based conformation-sensitive gel electrophoresis (F-CSGE), two dimensional gene scanning (TDGS), protein truncation test (PTT), and denaturing high performance liquid chromatography (DHPLC). Gerhardus et al. (2007), provide a systematic study of analyzing the accuracy of different scanning methods using for *BRCA1* and *BRCA2* mutation screening. Similarly, K. Somasundaram (2010) used CSGE as a method of scanning to identify the potential exons where the mutations are likely to occur, followed by DNA sequencing to locate and find out the nature of mutations. The speed, accuracy, efficiency, and cost-effectiveness of DNA sequencing have been improving continuously since the initial derivation of the technique by Maxam and Gilbert (1977) and Sanger et al. (1997) until the next generation sequencing techniques (Bosch 2008). Next generation sequencing (NGS), massively parallel or deep sequencing are related terms that describe a DNA sequencing technology which has revolutionized genomic research. Using NGS an entire human genome can be sequenced quickly. In contrast, the previous Sanger sequencing technology required over a decade to deliver the final draft. The spectrum of DNA variations in a human genome comprises single base changes (substitutions), small insertions and deletions, but also large genomic deletions of exons or whole genes, and large rearrangements, such as inversions and translocations. Traditional Sanger sequencing is restricted to the discovery of substitutions and small insertions and deletions. For the remaining mutations dedicated assays are frequently performed, such as fluorescence in situ hybridisation (FISH) for conventional karyotyping, or comparative genomic hybridisation (CGH) microarrays to detect submicroscopic chromosomal copy number changes such as microdeletions. However, these data can also be derived from NGS sequencing data directly, obviating the need for dedicated assays while harvesting the full spectrum of genomic variation in a single experiment. Recent advances in sequencing technologies have dramatically increased the speed and efficiency of DNA testing (Walsh 2010).

NGS technologies involve the isolation of DNA followed by the creation of single stranded DNA libraries. Libraries can be obtained using several different approaches. The key differentiating features specific to each commercial platform are in the subsequent steps. The DNA fragments are modified with the ligation of

an adapter and amplified using a unique adapter chemistry proprietary to each individual commercial platform. These modified DNA library molecules are then amplified either on a bead (emulsion based PCR method-454 and SOLiD) or a glass slide (bridge amplification-Illumina). The amplified single DNA strands on the bead or glass slide are then paired with complementary DNA nucleotides in individual flow cycles of ATGC templates. A complementary match unique to the DNA template strand results in the release of a signal detected by the sequencing instrumentation (Meldrum 2011).

Massively parallel sequencing was demonstrated to be a good strategy for the identification of genes responsible for monogenic diseases or diseases with a high degree of genetic heterogeneity (Gracia-Aznarez 2012), and one of the largest field of the NGS application is cancer. Although capillary-based cancer sequencing has been ongoing for over a decade, these investigations were limited to relatively few samples and small numbers of candidate genes. With the advent of NGS, cancer genomes can be systemically studied in their entirety. This may provide many benefits including a more precise diagnosis and classification of the disease, more accurate prognosis and treatment choice, and potentially the identification of 'drug-able' causal mutations. In addition, individual cancer sequencing may, therefore, provide the basis of personalized cancer management (Behjati 2013). To identify as many mutations as possible that are responsible for inherited predisposition to breast and ovarian cancer, it is useful to analyze multiple genes, especially, at first, *BRCA1* and *BRCA2*.

The advent of next-generation technologies (such as the Roche 454 GS FLX+, llumina Hiseq 2000, Applied Biosystems SOLID and HeliScope single-molecule sequencer machines), which allow a human genome to be sequenced in a single week-long run, has led to a large shift in our understanding of the mutations that drive cancers (Weaver 2011). It is therefore necessary to screen numerous genetic loci to decide on the best course of clinical management for an individual patient and this must be done in a rapid and cost-effective manner. Desmedt et al. (2012) described several NGS technologies applied to BC research. Authors underlined the possibility of integrating NGS in clinical practice in order not only to better understand the BC biology, but especially to create a new molecular classification system for the disease, to refine BC prognosis and to identify predictive markers for response to commonly administrated anticancer treatments. Indeed, they pointed out as tumor-specific DNA rearrangements could be detected in the patient's plasma, suggesting that NGS could be used to personalize the monitoring of the disease. In a recent article, Thompson and colleagues (2011) present a development of a NGS technology - the HeliScope sequencer - that enabled them to detect *BRCA1* mutations, as a model of a clinical diagnostic protocol. Moreover, to evaluate the accuracy of DNA capture followed by massive parallel sequencing, Walsh (2010) and colleagues developed a genomic assay to capture, sequence, and detect all mutations in 21 genes, including *BRCA1* and *BRCA2*, with inherited mutations that predispose to breast or ovarian cancer (Walsh 2010). Another NGS approach was performed through 454 GS Junior (Roche)

technology by Vaca-Paniagua (2012) and colleagues in Mexican women population . Furthermore, Pern (2012) and collaborators, performed a study using several genetic approach, including a NGS method, in order to investigate the genetic basis of the Triple-negative breast cancer (TNBC), an aggressive form of breast carcinoma with a poor prognosis. Microfluidic array PCR and NGS (GS FLX 454 (Roche) pyrosequencing technology) was used for *BRCA1* and *BRCA2* analysis, while conventional high-resolution melting (HRM) and Sanger sequencing was applied to study the coding regions of PALB2 and BRD7, respectively. Further, in order to identify new high susceptibility genes in familial BC, Gracia-Aznarez (2012), performed a NGS (through an Illumina Genome Analyzer II technology) approach to analyze 7 *BRCA1/BRCA2* negative families, each having at least 6 affected women with early-onset BC (Gracia-Aznarez, 2012).
As shown, the recent use of NGS strategies has been revolutionary in the research of BC genetics, like largely demonstrated by the high number of publications about these methodologies (Natrajan 2012, Nik-Zainal 2012, Mavaddat 2013). The ability of NGS technology to deliver information on whole genome sequences of different cancers will be an invaluable tool to the future pathologist and clinician. The data obtained from NGS can provide a comprehensive assessment of the genomic landscape associated with the genesis and evolution of different cancers (Gullapalli 2012). Concerning these great analysis performed on genes causing HBOCs, large consortia such as the Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium, and such companies like Myriad described before, have been formed to sequence thousands of cancers and generate a freely available dataset of DNA sequence changes in different cancer subtypes.

The present study was performed through the Genome Sequencer (GS) FLX System from 454-Roche, present at CEINGE in the High-Throughput Sequencing core-laboratory. The 454 system was the first next-generation sequencing platform available as a commercial product (Rothberg 2008). The first step for samples sequencing through this method is the creation of DNA libraries, by Multiplicom MASTR assays, creating a mixtures of short, adaptor-flanked fragments (**Fig. 8**). The subsequent clonal amplification of the obtained libraries is performed by emulsion PCR (emPCR). This procedure provides the capture of library fragments on the surface of microscopic beads (28-μm), under conditions that favor the linkage of one fragment per bead. The beads are isolated and compartmentalized into the droplets of a PCR-reaction-mixture-oil-emulsion, so that PCR amplification occurs within each droplet resulting in million of different beads, each carrying about ten million copies of a unique DNA template. Subsequently, the emulsions are broken, the DNA strands are denaturated and the beads carrying single-stranded DNA templates are enriched and deposited into the

wells of a fiber-optic slide (PTP). A sequencing primer is hybridized to the universal adaptor for next reactions (**Fig. 9-11**).
Sequencing is performed by the pyrosequencing chemistry. At each of several hundred cycles, a single species of unlabeled nucleotide is introduced. On templates where this results in an incorporation event, pyrophosphate is released. Via ATP sulfurylase and luciferase, the incorporation events immediately drive the generation of a burst of light, which is detected by the CCD camera as corresponding to the PTP coordinates of specific wells. Across multiple cycles, the pattern of detected incorporation events reveals the sequence of templates represented by individual beads. The 454 FLX instrument generates ~400,000 reads per instrument-run at lengths of about 450 bp (**Fig. 12**).

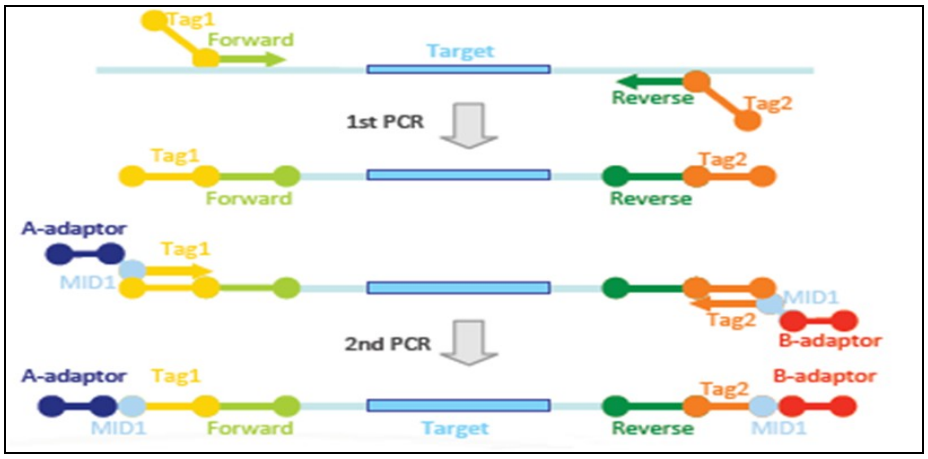


Figure 8. Creation of DNA libraries through Multiplicom MASTR assays. In the 1st PCRs each library was tagged with Tag sequences and A and B adaptors, that allowed the binding of the specific "multiple identifiers" (MID) in the 2nd PCR reactions.

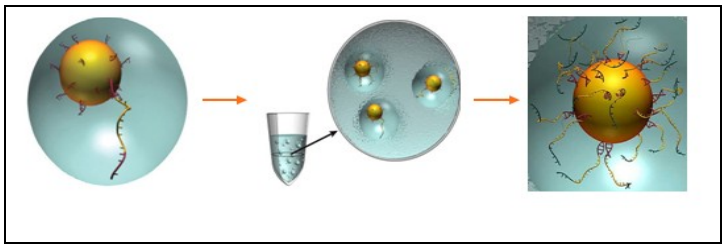


Figure 9. Em-PCR. Single-strand DNA is annealed to Capture beads and emulsified in a water-in-oil emulsion with PCR reagents, in order to allow a clonal amplification in each microreactor.

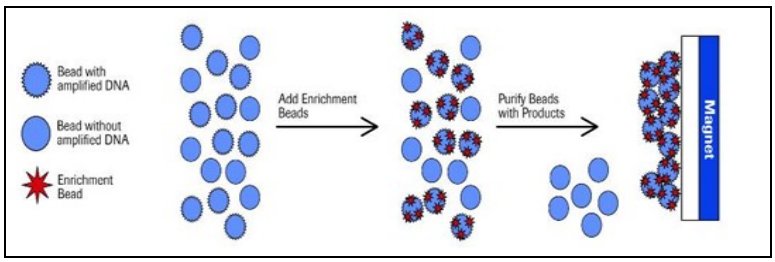


Figure 10. Enrichment of the reaction beads. After the amplification reaction, the emulsions are broken and the beads with amplified DNA are purified using magnetic enrichment beads.

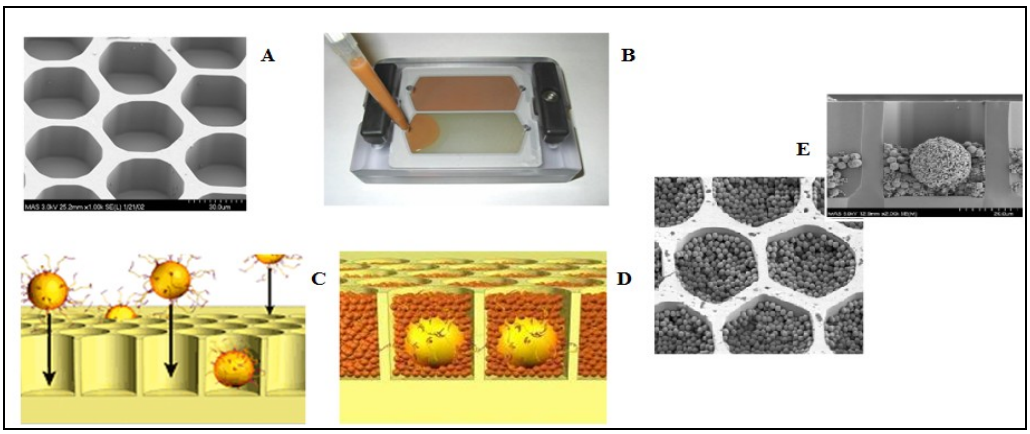


Figure 11. DNA beads are loaded into the PTP device. A: Micro wells of the PTP. B: Loading of DNA beads. C-D-E: DNA beads packed into wells with surrounding beads and sequencing enzymes.

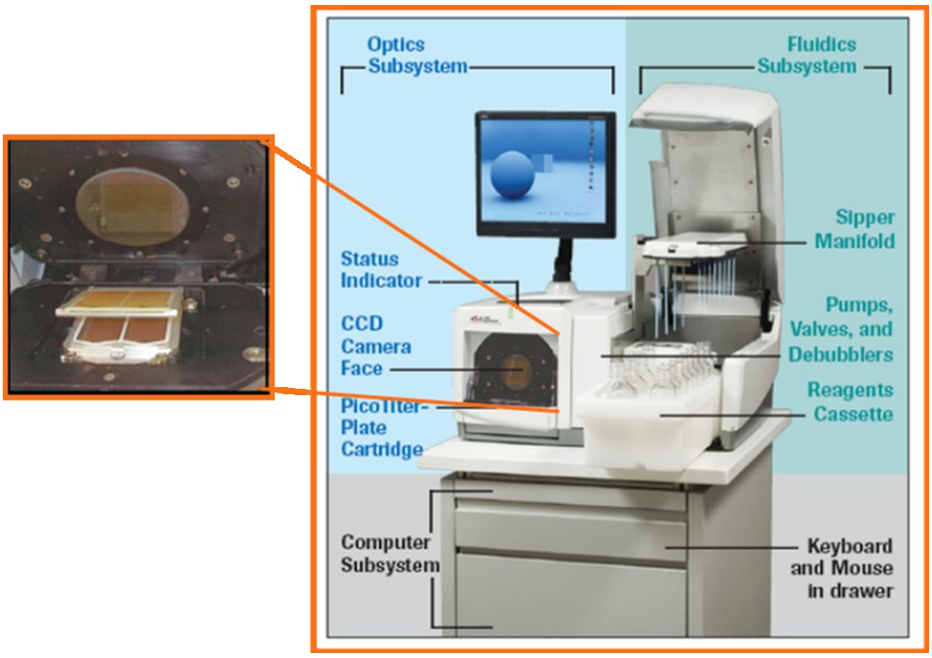


Figure 12. High Throughput Sequencing Open view of the GS FLX System (Roche).

2. AIMS OF THE STUDY

The first aim of the present project was to set-up and validate a NGS-based approach to characterize each *BRCA1/BRCA2* mutation in DNA of patients affected by HBOCs. The validated procedure was subsequently used to perform a large population-based study to assess the presence of *BRCA1/BRCA2* germline predisposing mutations in HBOC at risk subjects. Once a pathogenetic mutation has been identified in the affected women, it was investigated also in the healthy people composing their high-risk families, in order to identify mutation carriers before the disease onset. Indeed, one of the principal targets of this study was to underline the importance of breast cancer early prevention, especially in young women, and accordingly the improvement of clinical management, pharmacological treatments, surgery options, and of the patient's care in order to direct the treatments ever more toward a personalized medicine.

Since novel variants, predicted to be potentially pathogenetic, were identified, the second aim of the project was to verify their effects on disease onset and development, through *in silico* gene function predictions, functional studies and *in vitro* cellular assays.

3. MATERIALS AND METHODS

3.1. Patient samples and ethics

Samples and clinical data were obtained from a total of about 300 women attending the Senology Unit of the "Istituto Nazionale dei Tumori - Fondazione G. Pascale" of Naples. All participants were fully informed about the study and provided written informed consent prior to samples collection. The protocol was approved by the Ethics Committee of the School of Medicine Federico II, Naples, Italy, and carried out in accordance with the Declaration of Helsinki, good clinical practices, and local ethical and legal requirement.

In particular, the women included in this study should have at least one of specific selection criteria, such as early disease onset (especially "*under forty*"), tumors positive family history, advanced tumor staging, etc., discussed below. All patients were clinically approached, for everyone were collected extensive family information: a three-generation genealogy of each family was made in order to value the possible family history of breast cancer and other malignancies.

3.1.1. Patients selection criteria

Patients enrolled for the *BRCA1/2* screening must have at least one of the following specific selection criteria:

- Young age (<40 years) of onset of BC;
- Invasive and/or bilateral BC (any age) and/or multiple organ cancers;
- Family history of breast or ovarian cancer;
- Invasive ovarian cancer (any age);
- One first degree female relative with BC at <40 years of age;
- One first degree male relative with BC at any age;
- One first degree relative with bilateral BC where the first primary was diagnosed at <50 years of age;
- Two first degree relatives, or one first degree plus one second degree relative, with BC at any age;
- One first degree or second degree relative with BC at any age plus one first degree or second degree relative with ovarian cancer at any age (one of these should be a first degree relative);
- Three first degree or second degree relatives on the same side of the family with BC at any age;

- If more than one relative is involved, they should be on the same side of the family.

3.2. DNA Isolation

Genomic DNA was isolated from peripheral blood of the patients using the Nucleon BACC3 Genomic DNA Extraction Kit (GE Healthcare, Life Sciences) according to the manufacturer's instructions. Then, samples quantification was done through the NanoDrop 2000c Spectrophotometer (Thermo Scientific).

3.3. 454-Pyrosequencing

The creation of the DNA library of amplicons covering all the coding regions of *BRCA1* and *BRCA2* genes was performed using the BRCA MASTR v2.1 Assay kit (Multiplicom), following the manufacturer's instructions (**Fig. 8**). In brief, for each patient a template of 50ng of gDNA were used to perform a five reactions multiplex PCR covering the exons and about 50bp of the flanking sites of the intronic genetic regions. After the amplification, a 1:1000 dilution of the purified multiplex PCR products were re-amplified using specific molecular identification (MID) adaptors (Multiplicom) for each patient. Amplicons from the second PCR were cleaned and purified using the Agencourt AMPure XP Beads (Beckman Coulter), according to the manufacturer's recommendations. Then, amplicons, having a length ranging from 350 to 500 bp, were subjected of quality and quantity controls using the Experion DNA 1k Analysis kit (Bio-Rad). An equimolar concentration of the five PCR products were pooled together to create a *BRCA* amplicon library of each sample in order to generate the Sequencing Master library. The subsequent clonal amplification of the obtained libraries was performed by emulsion PCR (emPCR), using the GS FLX Titanium emPCR kit-LibA MV (Roche), according to manufacturer's instructions (**Fig. 9-11**). Next, a total of 60 patients were pooled together into 2 regions of the wells of a fiber-optic slide (PTP) and were sequenced in each single sequencing run. Pyrosequencing of the Master libraries were performed using the 454 GS FLX Tianium Series (Roche) technology (**Fig. 12**).

3.4. Bioinformatics and sequencing results validation

The downstream data analysis was carried out through the SeqNext tool (JSI Medical Systems) SeqPilot software version 3.5.2 (JSI Medical Systems,

www.jsi-medisys.de) using to the reference sequences *BRCA1* (NG_005905.2; Isoform NM_007294.3) and *BRCA2* (NG_012772.1; Isoform NM_000059.3) given in the NCBI-database (http://www.ncbi.nlm.nih.gov) (**Fig. 13**). According to the JSI Medisys approach, was considered a minimum absolute coverage of 40X per exon. Only the distinct mutations present in both sequencing directions, and with a minimum coverage of 10% default, excluding homopolymers, were considered in the genetic analysis. All sequence variants were named according to the nomenclature used by Human Genome Variation Society, HGVS (http://www.hgvs.org). The variants found were characterized using the Breast Cancer Information Core (BIC) Database, the Ensemble Database (http://www.ensembl.org) and the Human Genome Mutation Database, HGM (www.hgmd.cf.ac.uk). The *BRCA1/BRCA2* identified variants were classified according to theirs *in silico* gene function predictions and biological significance. The results were validated by standard Sanger sequencing of the patient's DNA. The *BRCA* exons PCR primers amplification were designed to prime the intron sequences flanking the corresponding exon; in this way, all coding sequence alterations could be detected. Sanger sequencing allowed to verify the presence of found variations in patient's DNA and provides the basis for a simplified test for at-risk relatives.

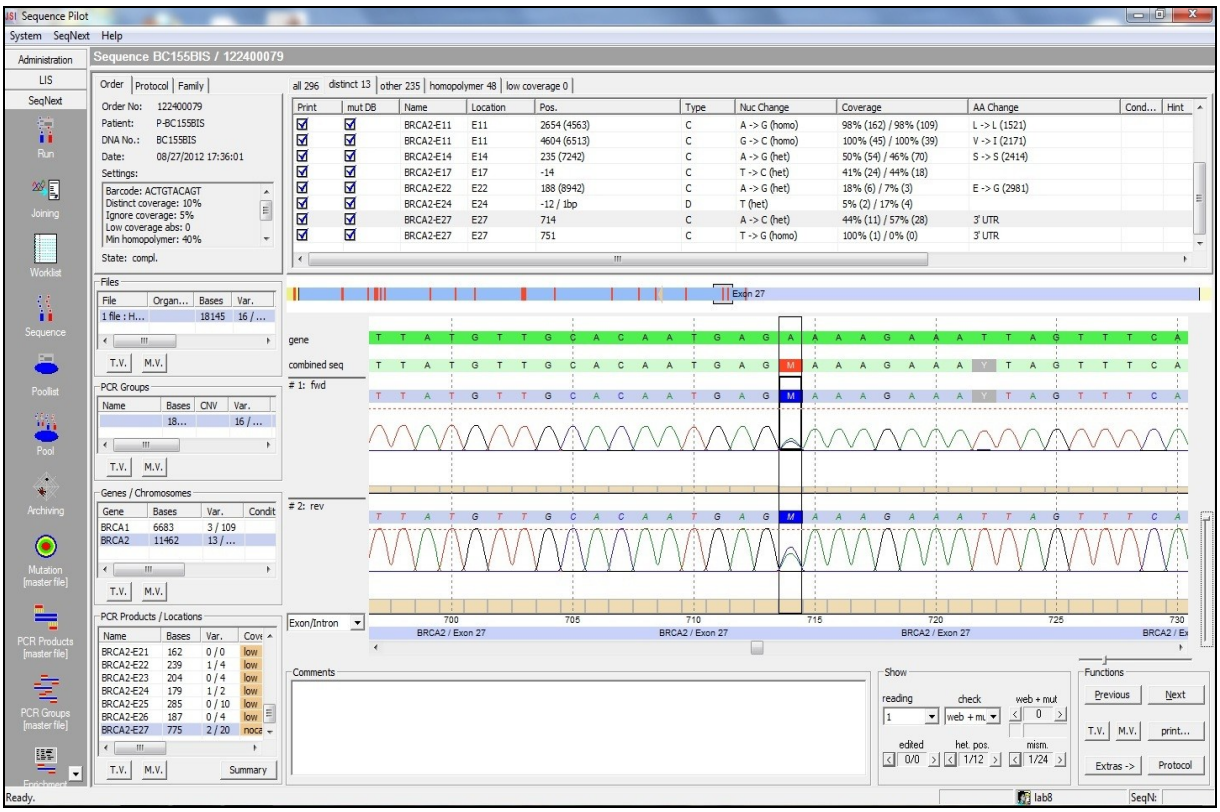


Figure 13. SeqNext tool (JSI Medical Systems) SeqPilot software: example analysis page.

3.5. Functional Assays

Each novel variant identified was analyzed by *in silico* predictions, using many bioinformatic programs described below. Variants resulted "deleterious" or "probably pathogenetic" or that showed particular alterations into protein folding or translation, were subsequently tested through functional studies and/or *in vitro* cellular assays.

3.5.1. *BRCA1* splice variant

To test the splice variant effects several bioinformatic predictions were performed through Human Splice Finder (http://www.umd.be/HSF/) and NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/). Patient RNA was isolated from peripheral blood using TRIzol® protocol (Ambion, Life Technologies). Retrotranscription reactions of patient and controls RNAs were performed by High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) according to the manufacturer's instructions.
CDNAs were amplified using different primers pairs designed *ad hoc* (primers list shows in **table 2**). To demonstrate the presence of the target *BRCA1* cDNA and to avoid the amplification of gDNA contamination, a pair of primers was designed on flanking cDNA exons. Another pair was designed to cover about 400bp of the retained intron, and then was performed another PCR reaction using a forward primer complementary to a cDNA region and a reverse primer annealing to the retained intronic region. PCR reactions were performed using *Pfu*Ultra High-Fidelity DNA Polymerase (Agilent Technologies), following a touch-down amplification protocol (**Table 3**). Results of PCR amplifications were analyzed through Sanger sequencing.

In addition, a restriction map was performed on the sequence of the retained intron and another one was performed on the portion of the cDNA sequence amplified by designed primers described, using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/) and Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/rest_map.html) bioinformatic tools. The NcoI (BioLabs) restriction enzyme was chosen for the enzymatic digestion of patient and controls amplified cDNAs, and of a gDNA used as control of enzyme functionality. The reaction was performed using 0,5 µl of NcoI enzyme for 1 µg of DNA to digest, added to 5 µl of enzyme buffer, without BSA. Then, DNA fragments were analyzed by DNA Chip 1K (BioRad).

Table 2. Primers list to validate the splice variant on *BRCA1* cDNA.

	Primer sequence	Product Length (bp)
BRCA1_cDNA_fw	CAACATGCCCACAGATCAAC	885
BRCA1_cDNA_rw	AATTTCCTCCCCAATGTTCC	885
BRCA1_intr21_fw	CCCACCCCTGTAATCACAAC	472
BRCA1_intr21_rw	GATCCCCAGGAAGGAAAGAG	472
BRCA1_intr21_ctr_rw	TCCCTCCCCCTCCTCTCTGT	1417

Table 3. *Pfu*Ultra touch-down amplification protocol.

Temperature	Cycles
95° x 5’	1
95° x 30’’ 55° x 60’’ – 0,5°/cycle 72° x 65’’	14
95° x 5’	1
95° x 30’’ 55° x 60’’ 72° x 65’’	25
72° x 5’	1
4°	∞

3.5.2. *BRCA2* missense variant

The impact of protein alteration was evaluated using SIFT (http://sift.jcvi.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) computational approaches. The bioinformatic evaluation of the variants was performed also using "Database of human missense variants mapped to 3D protein structures" (http://decrypthon.igbmc.fr/msv3d/cgi-bin/analyse).
The last 2500bp of *wt BRCA2*-10921bp-cDNA, including the DNA binding sites, the nuclear localization sequences (NLS) and one of the RAD51 binding regions, were cloned into a pRc/CMV vector (Life Technologies). Cloning experiments were performed into the Apa I/Not I restriction enzymes sites, and fragments were ligated by T4 DNA Ligase (New England BioLabs). The constructs were cloned in frame under the vector CMV promoter and using an initial ATG with the complete translation started sequence, including the Kozak consensus sequence, to assure the translation of the mutant proteins. Clones were purified from Cloning Competent DH5a™ Cells (Life Technologies) through GenElute Plasmid Miniprep (Sigma-Aldrich) procedures. Two single-bases mutagenesis reactions on the *wt* construct were performed, through QuikChange Lightning Mutagenesis Kit (Agilent Technologies): into one clone was introduced the investigate variant and into another one was introduced a reported deleterious mutation used as positive control for the next assays. Mutants were amplified and purified from XL10-Gold Ultracompetent Cells (Agilent Technologies) using QIAGEN plasmids Maxi kit.
To test the BRCA2 mutant proteins expression, the *wt* fragment and the mutants were transfected in NIH-3T3 cells, using Lipofectamine 2000 (Invitrogen). Whole-cell extracts and nuclear fractions were used for Western blot analysis. For the preparation of the cellular extracts, monolayer cultures were harvested in cold phosphate-buffered saline after 36 hours and sonicated in lysis buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 0.4 mM EDTA, 50 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride) added to a protease inhibitor cocktail (2 mM PMSF, 5 ug/mL leupeptin, 5 ug/mL pepstatin). Nuclear proteins were isolated from whole-cell lysates through 3 minutes of pulse Bioruptor (Diagenode) sonication. The extracts were clarified by centrifugation at 16,000 × g at 4 °C, and the protein concentration was determined by the Bio-Rad protein assay according to manufacturer's instructions. Proteins (50µg) were separated on 8% and 12% low bis acrylamide gels, subjected to SDS-PAGE, transferred onto a polyvinylidene difluoride Immobilon P membrane (Millipore), and blocked with 5% milk-TBST (50mMTris [pH 7.5], 150mMNaCl, 0.05% Tween 20) for 1 h at room temperature. Immunodetection of the BRCA2 mutant proteins was performed using the C-terminal BRCA2 ((T-18) sc-21230; Santa Cruz Biotechnology, 1:1000) goat primary antibody. Actin, used to normalize the amount of protein in different samples, was revealed using anti-β-actin (Sigma-Aldrich, 1:1000) mouse primary antibody. Goat polyclonal anti-goat and anti-mouse IgG conjugated to horseradish peroxidase were used for secondary antibodies (GE Healthcare). Bands were detected using ECL

chemiluminescence detection methods (PerkinElmer) and exposure to X-ray film (Molecular Technologies).

Transient transfections of plasmids were done with the DNAs described before, by using Lipofectamine 2000 (Invitrogen), following the instructions of the manufacturer, into NIH-GS cells, stable containing the pDR-GFP plasmid, and subsequently trasfected with I-SceI-ER plasmids, described in reference (Gunn 2012 and Stante 2009). PDR-GFP and I-SceI-ER plasmids were kindly provided by prof. Giuseppina Minopoli. To obtain the NIH-GS clones, NIH-3T3 cells were, at first, transfected with pDR-GFP plasmid (Stante 2009). 48 hours after transfection, cells were selected with puromycin (3 μg/ml) (Sigma-Aldrich) for 5 days. Puromycin-resistant colonies were pooled and amplified under puromycin selection to obtain NIH3T3-G stable clones. Resistant colonies were pooled and amplified (Stante 2009).

NIH-3T3 fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich), 2mML-Ultraglutamine 1 (Lonza), and 1% penicillin and streptomycin (100μg/ml streptomycin, and 100 units/ml penicillin) (Sigma-Aldrich).

After the transfection of I-SceI-ER into NIH3T3-G stable clones, cells became NIH-GS. 24 hours from transfection NIH-GS cells were exposed to 1 μM 4-OH-tamoxifen (Calbiochem) to allow cells growth upon I-SceI-ER induction. 48 hours after I-Sce-I-ER induction, cells were harvested, resuspended in PBS at 500,000 cells/ml and GFP positive cells were counted with FACScanto (BD Biosciences, San Jose, CA) instrument. Each experiment was performed at least in triplicate by counting 30,000 events per sample.

The extent of repair, into NIH-GS cells, was measured by counting GFP positive cells by FACS, the Annexin V Apoptosis detection was performed using the Annexin V-APC Staining Protocol (BD Biosciences) according to manufacturer's instructions; GFP emission in transfected cells was also observed through fluorescence microscopy (Leica DMS 4000B).

4. RESULTS AND DISCUSSION

Over 2,000 different mutations have been reported in *BRCA1/BRCA2* genes including deletions, insertions, and many single nucleotide substitutions in coding or noncoding sequences. The most common mutations are attributed to frameshift due to small insertions/deletions, nonsense protein-truncation, and disruption of splice site leading to entire nonfunctional BRCA proteins. According to geographic areas, there are several predominant founder mutations in the same populations, different from the others, as greatly reported by Fatemeh Karami and Parvin Mehdipour (2013).

About 12.5% of the analyzed patients, including several men, carried a mutation in the *BRCA1/BRCA2* genes mostly known to be causative (**Table 4**). Mutations were found in patients affected by HBOC principally, but also in healthy carrier with strong familial history of cancer. The analyzed population, derived mainly from Southern Italy, showed the presence of predominant mutations that occurred many times with an allelic frequency ranging from 0.16%, for mutations detected once, to 0.5% for more frequent mutations such as c.6037A>T p.K2013X in *BRCA2* gene (occurred twice) and c.5263_5264insC p.Q1756fs*74 in *BRCA1*, discussed below. Therefore, the most frequent *BRCA1* gene mutation is c.5263_5264insC (c.5266dupC or 5382insC) which was also found roughly in all of the populations. The maximum likelihood method considering any mutation or crossing-over occurrence has shown that this insertion at first came from Scandinavia, probably Denmark as it includes the founder mutation in Danish population around 200 AC. However, Russia is second candidate for occupying the primary origin of it, and after that it was disseminated to other areas including Ashkenazi Jews. It was also proposed that 5382insC has entered into the Ashkenazi Jewish through affecting Polish population about 400 yrs earlier. 5382insC is the most important and prevalent *BRCA1* mutation in European countries while Asian and American BC individuals rarely demonstrate it (Hamel 2011). In our population was found in 3 patients and confirmed in many relatives of their high-risk families. One representative genealogy of an analyzed patient shows the presence of the variant in the family (**Fig. 14**).

Table 4. Causative *BRCA1* and *BRCA2* mutations found into analyzed population.

Gene	Exon	HGVS[1] cDNA	BIC Designation	HGVS[1] Protein	Mutation Type	Clinically Important (BIC)	Ref. number NCBI	Allelic frequency on 600 alleles
BRCA1	5	c.181T>G	C61G	p.Cys61Gly	Missense	Deleterious	rs28897672	0.16%
	11	c.2761C>T	Q921X	p.Gln921Ter	Nonsense	Deleterious	rs80357377	**0.3%**
	11	c.3351_3352insT	3470insT (Q1118SfsX*4)	p.Val1117_Gln1118?fs	Frameshift	Deleterious	rs80357785	0.16%
	11	c.3403C>T	Q1135X	p.Gln1135Ter	Nonsense	Deleterious	rs80357136	0.16%
	11	c.3419G>T	S1164I	p.Ser1164Ile	Nonsense	Deleterious	n.r.[2]	0.16%
	14	c.4484G>T	R1495M	p.Arg1495Met	Missense	Deleterious	rs80357389	0.16%
	16	c.4964_4982del	5083del19 (S1655Yfs*16)	p.Ser1655_Glu1661?fs	Frameshift	Deleterious	rs80359876	0.16%
	18	c.5123C>A	A1708E	p.Ala1708Glu	Missense	Deleterious	rs28897696	0.16%
	18	c.5153-1G>C	IVS18-1G>C	-	Intervening Sequence	Deleterious	rs80358137	0.16%
	19	c.5153G>A	W1718X	p.Trp1718Ter	Nonsense	Deleterious	rs41293461	0.16%
	20	c.5263_5264insC	5382insC (Q1756fs*74)	p.Ser1755?fs	Frameshift	Deleterious	rs80357906	**0.5%**
BRCA2	6	c.1238delT	1466delT (L413Hfs*16)	p.Leu413Hisfs	Frameshift	Deleterious	rs80359271	0.16%
	7	c.631G>A	V211I	p.Val211Ile	Missense	Deleterious	rs80358871	0.16%
	10	c.1496_1497delAG	1724delAG (Q499Rfs*14)	p.Gln499Argfs	Frameshift	Deleterious	rs80359285	0.16%
	11	c.2808_2811delACAA	3036delACAA (K936Qfs*21)	p.Lys936_Gln937?fs	Frameshift	Deleterious	rs80359352	0.16%
	11	c.4131_4132insTGAGA	1377insXG	p.Asn1377_Thr1378?	In Frame Insertion	Deleterious	rs80359429	0.16%
	11	c.5722_5723delCT	5950delCT (L1908RfsX*2)	p.Leu1908Argfs	Frameshift	Deleterious	rs80359531	0.16%
	11	c. 6037A>T	K2013X	p.Lys2013Ter	Nonsense	Deleterious	rs80358840	**0.3%**

	14	c.7008-2A>T	IVS13-2A>T	-	Intervening Sequence	Deleterious	n.r.[2]	0.16%
	22	c.6486_6489delACAA	6714del4 (K2162fx)	p.Lys2162_Gln2163?fs	Frameshift	Deleterious	rs80359598	0.16%

Cont. table 3: Causative *BRCA1* and *BRCA2* mutations found into analyzed population. [1]Human genome variation society nomenclature, [2]n.r. as not reported into NCBI databases.

Table 5. *BRCA1* and *BRCA2* unreported new variants.

Gene	Exon	HGVS[1] cDNA	BIC Designation	HGVS[1] Protein	Mutation Type	Clinically important (BIC)	Ref. number NCBI	Time Observed and allelic frequency	Bioinformatic predicted effect
BRCA1	11	c.2811G>A	K397K	p.Lys397Lys	Synonymous	n.r.[1]	n.r.[1]	1 – 0.16%	-
	11	c.3514C>T	E1172X	p.Glu1172Ter	Nonsense	n.r.[1]	n.r.[1]	2 – 0.3%	Deleterious
	14	c.4481A>G	E1494G	p.Glu1494Gly	Missense	n.r.[1]	n.r.[1]	1 – 0.16%	Benign
	19	c.5237A>C	H1746P	p.His1746Pro	Missense	n.r.[1]	n.r.[1]	1 – 0.16%	Deleterious
	21	**c.5406+2T>C**	**IVS22+2T>C**	**-**	**Intervening Sequence**	**n.r.[1]**	**n.r.[1]**	**1 – 0.16%**	**Deleterious**
BRCA2	11	c.6567C>T	N218N	p.Asn218Asn	Synonymous	n.r.[1]	n.r.[1]	1 – 0.16%	-
	18	**c.8299C>T**	**P2767S**	**p.Pro2767Ser**	**Missense**	**n.r.[1]**	**n.r.[1]**	**1 – 0.16%**	**Deleterious**

Highlighted variants are confirmed by functional studies or *in vitro* assays.

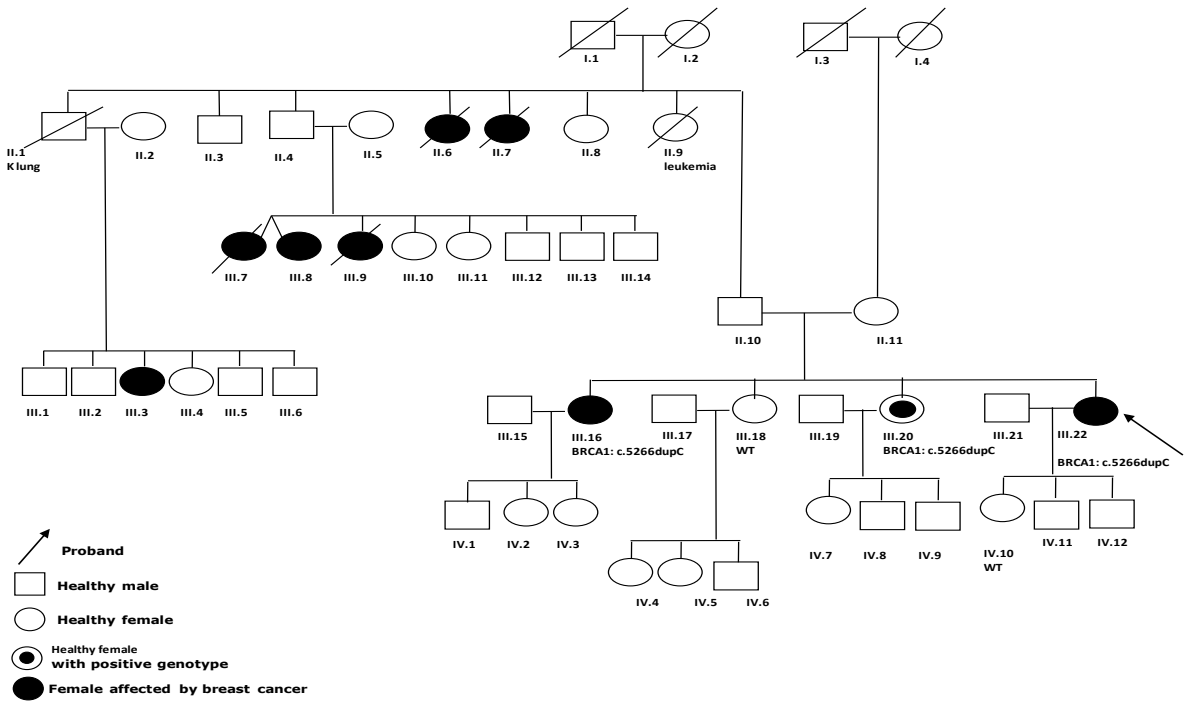


Figure 14. Genealogy of the family carrier of the c.5266dupC *BRCA1* deleterious mutation. Genetic testing allowed the identification of a healthy mutation carrier. Starting from these findings, the carrier and also her children can start a prevention program designed *ad hoc* to prevent the onset of the disease or to fight against cancer with personalized medicine, in order to perform a better management of surgery and of drug treatments.

4.1. Synonymous and missense non deleterious variants

Moreover, several detected variants are still unknown and not previously described in public databases (**Table 5**). All these novel variants were not found in all other alleles analyzed into our population and are on going to insertion into the BIC database. Among these variants there are: 2 synonymous variants, c.6567C>T N2189N and c.2811G>A K937K, and 1 missense variant, the c.4481A>G E1494G. All of them were predicted by SIFT as benign and without clinical significance. Synonymous variants were found in women with positive familial history but affected by benign breast diseases. Instead, the not deleterious variant was found in a woman affected by early-onset BC. Despite these variants seem to be harmless from the first bioinformatic predictions, other insights are needed to understand their role into disease, also because they were not found into

healthy alleles. In this regard, several synonymous variants that were previously annotated in BIC like polymorphisms, were lately classified as VUSs. These evidences strongly suggest that also synonymous variants need more investigations, and that cannot be considered merely with no clinical significance.

4.2. Double and triple mutations

One nonsense mutation, c.3514C>T p.E1172X, not previously reported and predicted deleterious, causing a premature stop codon, was found in 2 women affected by BC, together with the mutation c.3491G>T p.S1164I localized on *BRCA1* gene and previously described (Di Cecco 2009), even if annoted yet into databases. In addition, one of these patients resulted carrier also of the *BRCA2* stop codon c.9976A>T K3326X (largely described below). This is a very interesting case of triple mutation, detected for the first time into our population. The effect of the E1172X *BRCA1* mutation could cover the effect of the S1164I missense one in the same exon, causing anyway the production of a truncated and/or non functional BRCA1 protein; nevertheless, the K3326X *BRCA2* variant, causes a further stop codon that probably alters the BRCA2 function too. This cumulative effect could be dramatic for the functionality of BRCA1-BRCA2 pathway leading the severe phenotypic effects shown by the patient.

Furthermore, one more woman was found to carry a double mutation: c.631G>A p.V211I and c.7008-2 A>T IVS13-2 A>T, both localized on *BRCA2* gene. In this case, each mutation found in the patient was previously reported but never both in the same woman. Nevertheless, was not possible to build the genealogy of this patient's family, neither of the triple mutation carrier's family, to confirm the mutation into other subjects given that their relatives did not accept to undergo genetic testing.

Every woman carrier of double mutations was affected by early-onset and bilateral BC and had a positive family history for BC and other cancers. The triple mutation carrier woman was affected by ovarian cancer too. These very aggressive phenotypes probably depend of the cumulative effect of two mutations that could severely reduce the protein function. These evidences strongly suggest the pathogenetic effects of the new variant added to the reported ones. The same mutations were confirmed by Sanger sequencing in several relatives of patient's family. These data allowed us to build, when it was possible, the genealogical three and to perform the linkage analysis of mutations (**Fig. 15**).

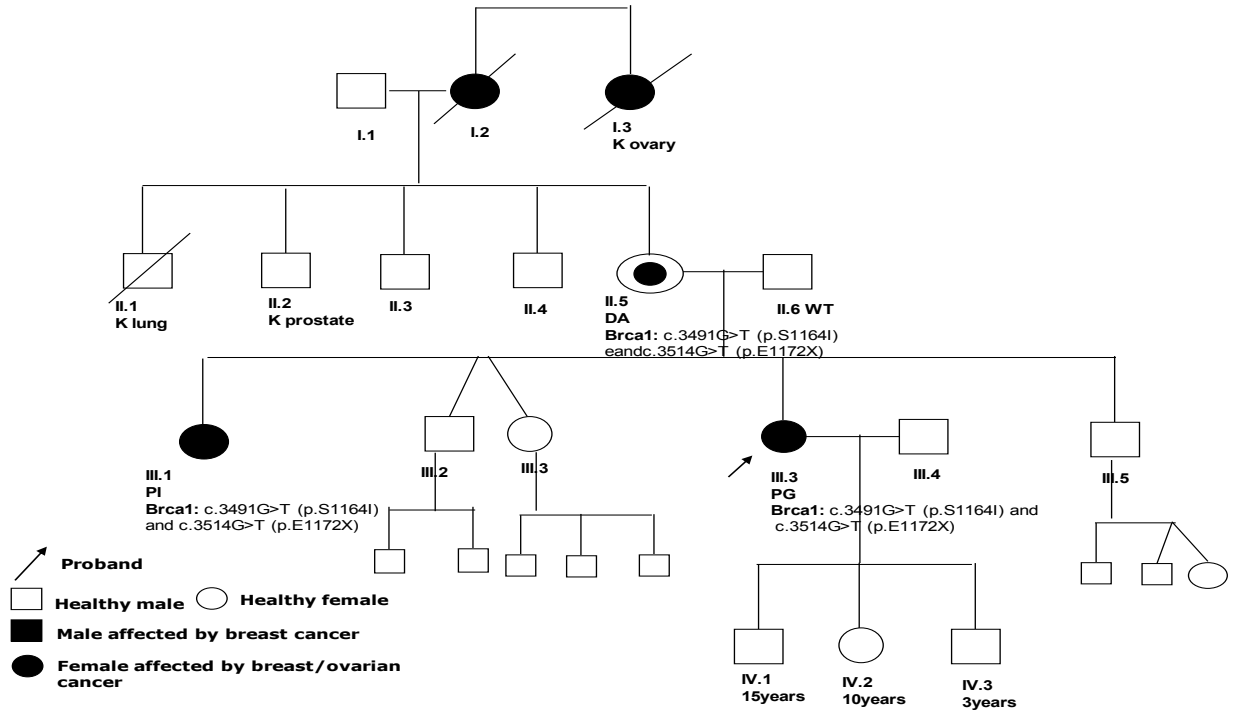


Figure 15. Genealogy of the family carrier of the double deleterious mutations c.3491G>T p.S1164I and c.3514C>T p.E1172X. These double mutations were found for the first time both localized on the same gene, following the inheritance showed in the analyzed family.

In the future, will be interesting to test the biological effect of these double and triple mutations through functional and *in vitro* assays, to better understand the *BRCA1/BRCA2* mutations cumulative effect on disease onset and development. One more future aim will be to collect more informations about the family through the relatives genotyping, in order to build genealogical trees and to perform a detailed linkage analysis of mutations.

4.3. The c.5237A>C p.H1746P missense variant

The c.5237A>C p.H1746P missense variant, was identified in a healthy patient belonging to a family with several cases of oncological diseases and, in particular, the mother was affected by BC. The variant is localized in the BRCA1 C-terminal region, at the BRCT domain level; this domain is important for the binding of BRCA2 and other proteins, like Abraxas, CtIP and BRIP1, during the

DSBR pathway activation. The tandem BRCT domains of BRCA1 and MDC1 facilitate protein signaling at DNA damage foci through specific interactions with serine-phosphorylated protein partners. Starting from these findings, the amino acidic change caused by the variant probably hampers the BRCA1/BRCA2 complex functions.
The first bioinformatic predictions through SIFT and PolyPhen-2 tools, describe the variant as deleterious. In particular, this mutation is predicted by PolyPhen-2 to be possibly damaging with a score of 0.834 (sensitivity 0.84, specificity 0.93). This result derives from HumanDiv index, that is the preferred model for evaluating rare alleles, dense mapping of regions identified by genome-wide association studies and analysis of natural selection. Instead, the same variant resulted benign according to HumanVar index (score 0.177, based on sensitivity of 0.89 and specificity of 0.73), that is a model preferred for diagnostics of Mendelian diseases. This model requires distinguishing mutations with drastic effects from all the remaining human variations, including abundant mildly deleterious alleles (**Fig 16**). Results from multiple sequence alignment, showed that the amino acidic residue changed by variation is not much conserved among species; this could justify the prediction of benignity made from the software (**Fig.17**).

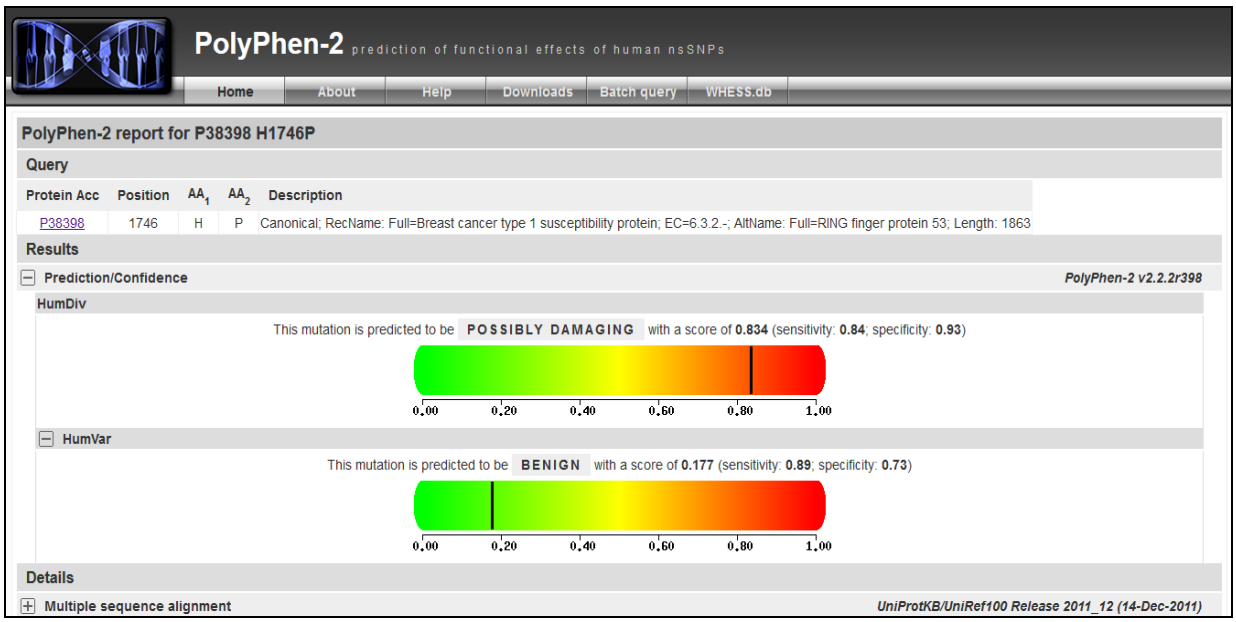


Figure 16. Prediction of pathogenicity of the c.5237A>C p.H1746P missense variant, performed by PolyPhen-2 tool.

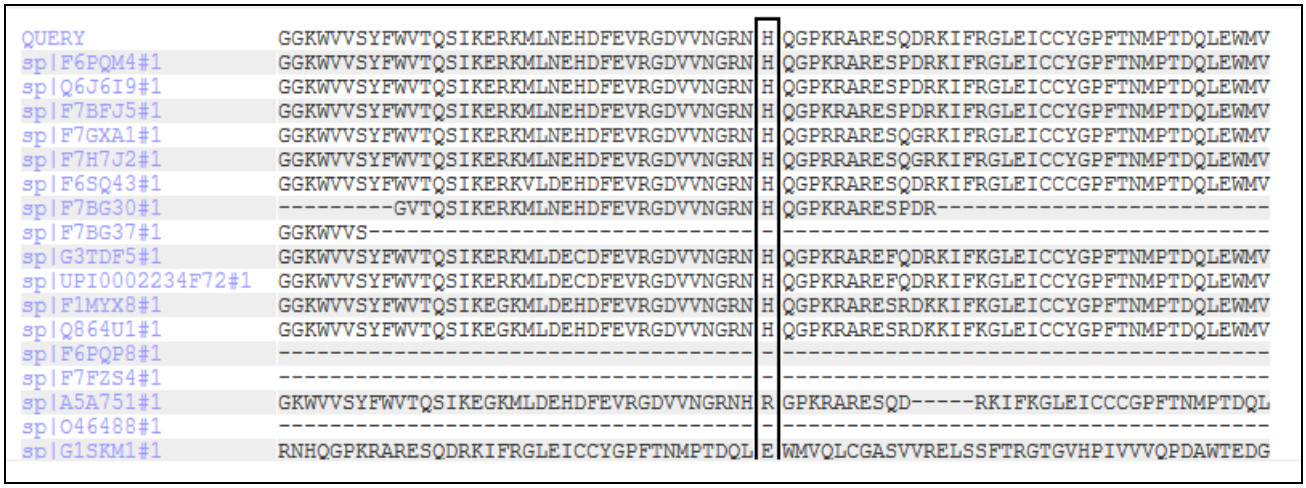


Figure 17. Multiple sequence alignment variant derived from PolyPhen-2 software. In this picture are shown only the first alignments derived from the software: when the alignment proceeds among species becomes clear that the residue is not much conserved.

In addition, the analysis performed by SIFT, predicted the variant as damaging (**Fig. 18**).

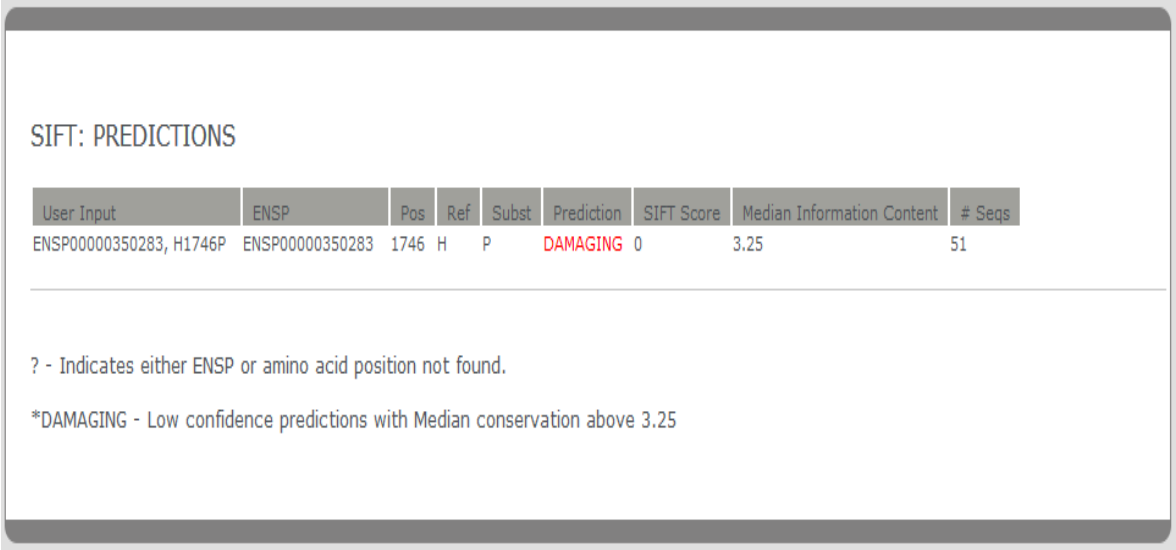


Figure 18. Prediction of pathogenicity of the c.5237A>C p.H1746P missense variant, using SIFT computational approach.

Further predictions were performed by Database of human missense variants mapped to 3D protein structures (MSV3d) bioinformatic tool. The analysis showed that the variant causes protein size and charge decreases, plus polarity and hydrophobicity reductions, strongly supporting the hypothesis that the mutation can alter the protein folding and interaction with other protein complexes. However, is still not clear if these alterations can really hamper the DBSR processes.
Moreover, this variant was analyzed also using PMut software (http://mmb2.pcb.ub.es:8080/PMut/) that classified the variation as pathological with a score of 0.976 on 1, which corresponds to the closest pathogenicity score. Starting from these assumptions, other studies are needed to confirm the missense variant's role, to understand if it could be considered as deleterious or merely as a "polymorphism".

4.4. The Pro2767Ser BRCA2 DNA-binding site missense variant

The Pro2767Ser (c.8299C>T p.P2767S) missense variant, falling in the exon 18 of the *BRCA2* gene, was identified in a young woman (28 years at the time of diagnosis) affected by breast fibrocystic dysplasia. In her family, at the time of the analysis, were not present other relatives affected by BC or by other cancers. Thus, it seems to be a sporadic breast disease.
The BRCA2 DNA-binding domain contains a helical domain (H), three oligonucleotide binding (OB) folds and a tower domain (T), which may facilitate BRCA2 binding to both single-stranded DNA and double-stranded DNA (Roy 2011). The unreported variant is localized in the oligonucleotide binding domain 2 (OB2), into the tower domain. The substitution of the Proline residue with the Serine one generates polarity and hydrophobicity increases, suggesting the occurrence of folding changes into protein structure and in particular into the DNA binding domain. The mutant residue, located at the DNA binding site of BRCA2 protein as described before, probably interferes with the DNA binding, altering the HR and the DSBR processes in which this protein is involved. In particular, conformational changes caused by mutation in this important domain, could prevent the RAD51 and other repair proteins complexes binding, strongly hampering the damage response processes.

Bioinformatic evaluations were performed using SIFT and PolyPhen-2 computational approaches and confirmed by "Database of human missense variants mapped to 3D protein structures"too. Therefore, the first bioinformatic predictions of the variants showed a strong probability of pathogenicity, classifying that as deleterious (**Fig. 19-23**).

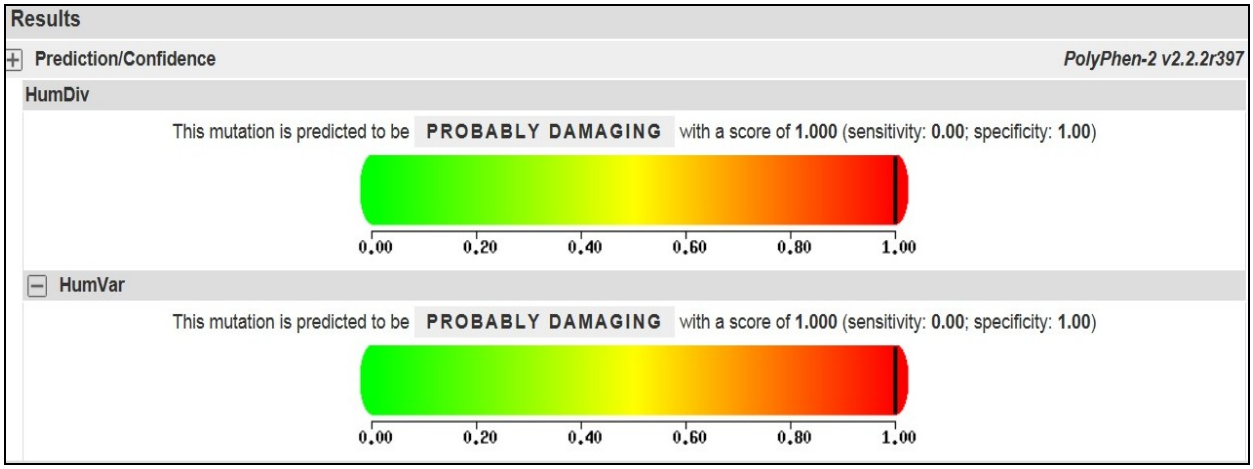


Figure 19. Prediction of pathogenicity of the Pro2767Sser missense variant, performed using PolyPhen-2 tool.

Moreover, the amino 2767 acidic residue changed by variation resulted highly conserved among species, strongly supporting the hypothesis of a harmful mutation effect (**Fig. 20**).

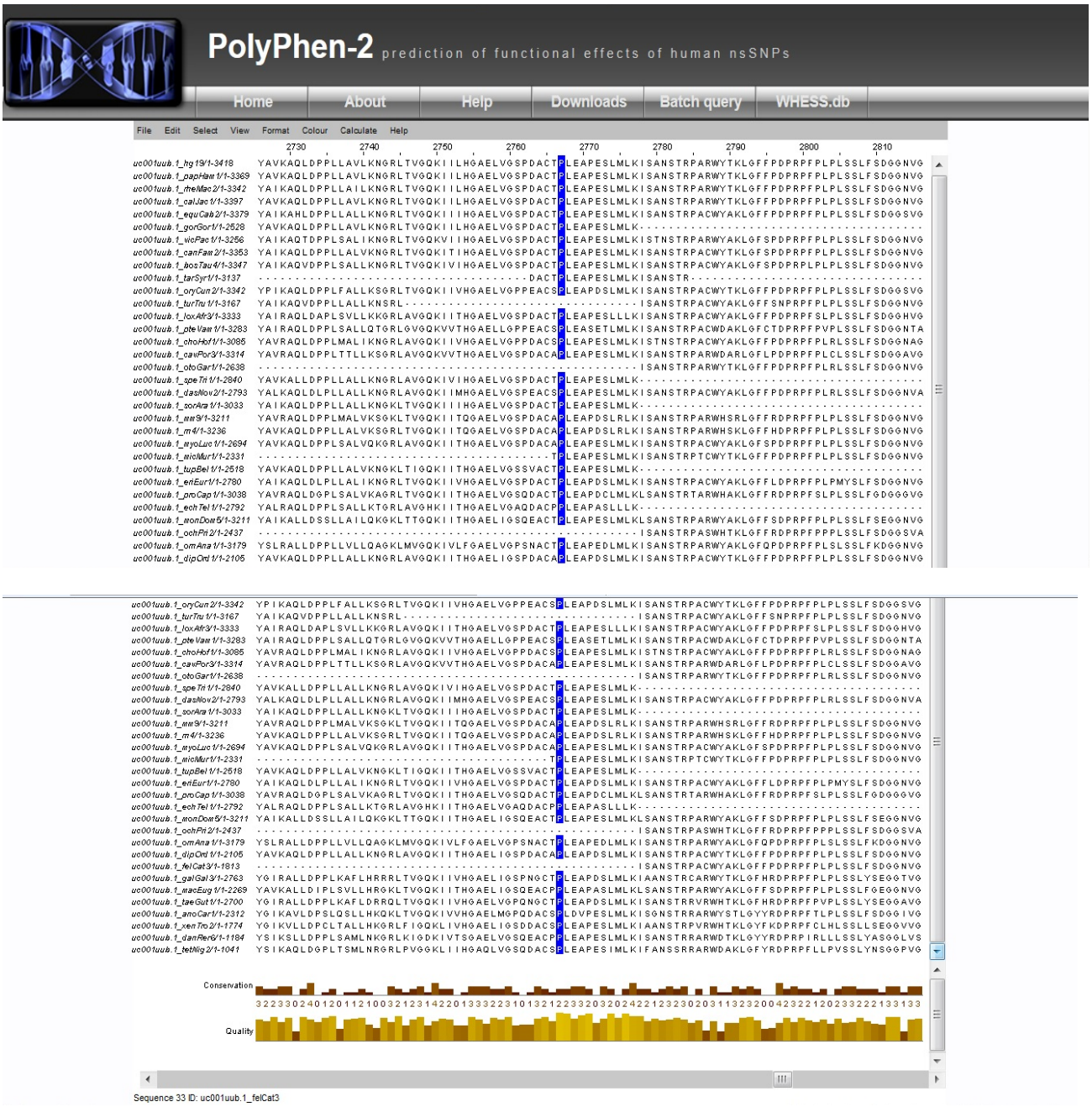


Figure 20. Multiple sequence alignment derived from PolyPhen-2 software.
Software results show that Proline residue interested by variation is highly conserved among species.

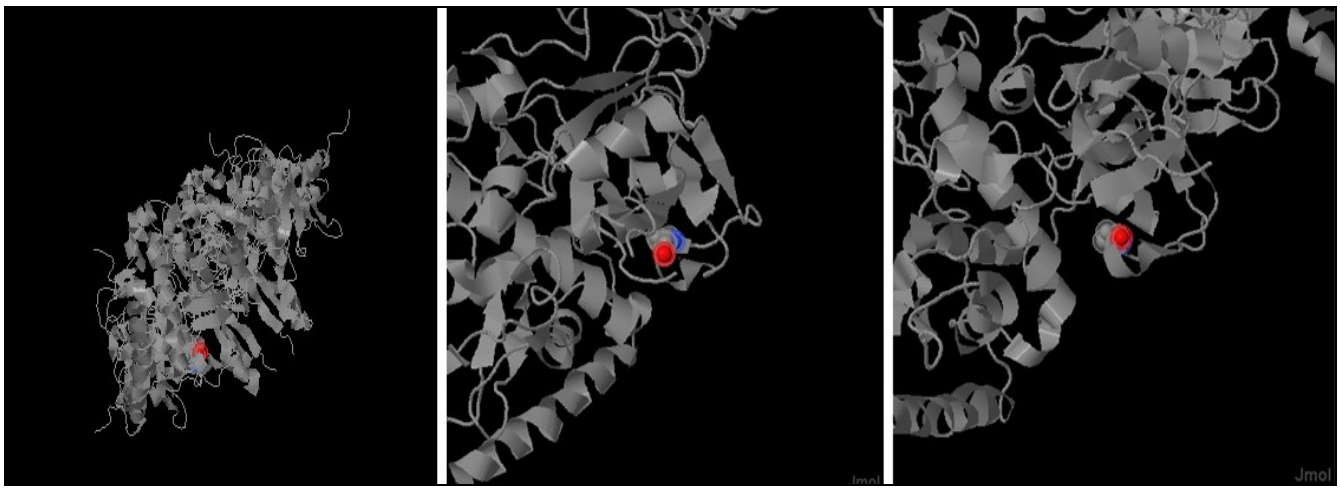


Figure 21. 3D Visualization of residue Pro2767 interested by variation, resulting from PolyPhen-2 software.

SIFT: PREDICTIONS

User Input	ENSP	Pos	Ref	Subst	Prediction	SIFT Score	Median Information Content	# Seqs
ENSP00000369497, P2767S	ENSP00000369497	2767	P	S	DAMAGING	0	2.48	71

? - Indicates either ENSP or amino acid position not found.

*DAMAGING - Low confidence predictions with Median conservation above 3.25

Figure 22. Prediction of pathogenicity of the Pro2767Ser missense variant, using SIFT computational approach.

GENERAL INFORMATION			
Protein ID	Protein name		Gene name
P51587	Breast cancer type 2 susceptibility protein		BRCA2_HUMAN
Substitution	SNP ID	Allele frequency	Database origin
p.Pro2767Ser	-	-	user-defined
Phenotype			
-			
PHYSICO-CHEMICAL PROPERTIES			
Size	Charge	Polarity	Hydrophobicity
decrease	unchanged	increase	increase
Disulfid Bond	Gly or Pro	Modification Score	
unchanged	disparition	23	

Figure 23. Prediction of pathogenicity of the Pro2767Ser missense variant derived from MSV3d tool.

Starting from these evidences, the last 2500 nucleotides of BRCA2-10921bp-cDNA were cloned into a pRc/CMV vector (**Fig.24**), under the complete translation started sequence to assure the production of the mutant proteins. This DNA fragment contained the DNA binding sites, the nuclear localization sequence and one of the RAD51 binding regions. The Not I and Apa I enzymes were chosen because of their capability to recognize polylinker sequence sites and because they do not cut into *BRCA2* selected sequence. In this way, into cloning primers were inserted Not I in forward and Apa I in reverse sequence in order to, first, amplify the target 2500bp *BRCA2* sequence and, second, to create a fragment that was made up of restriction enzyme sites on its 5' and 3' ends. Through enzymatic digestions of amplicons were created blunt ends DNA fragments that were subsequently ligated into the pRc/CMV vector. Plasmids were transformed into bacterial cells and, after propagation, were purified through midi-prep plasmids preparation procedures.

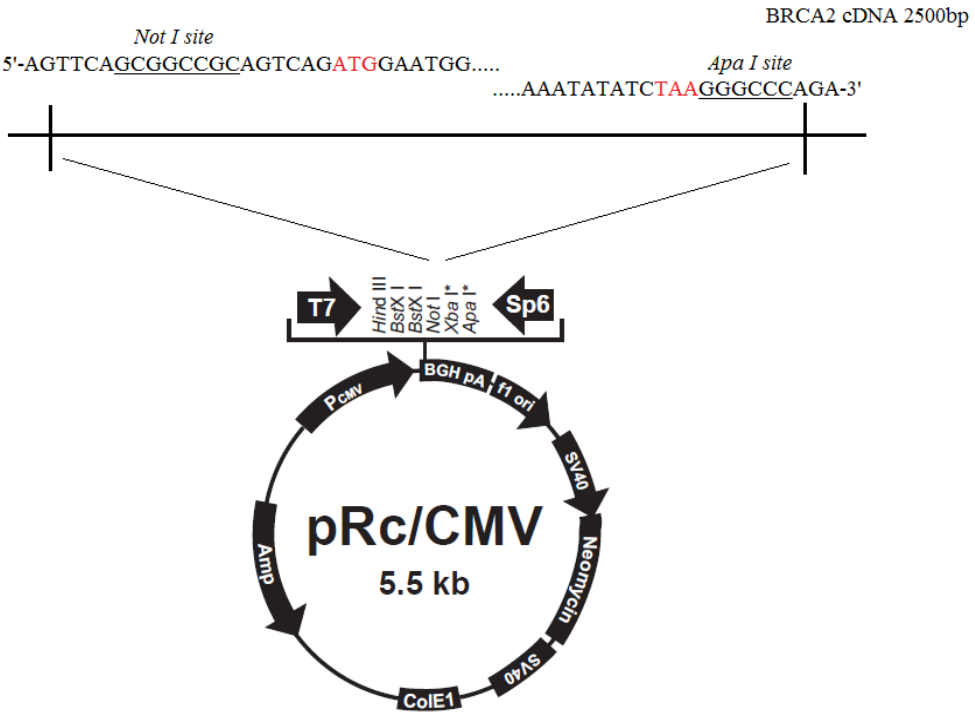


Figure 24. PRc/CMV vector used for *BRCA2* cDNA cloning. Between T7 and Sp6 sequences, fragments were cloned into the Apa I/Not I restriction enzymes sites. The constructs were cloned in frame under the vector CMV promoter and using an initial ATG with the complete translation started sequence, including the Kozak consensus sequence, to assure the translation of the mutant proteins.

On the *wt* construct were performed two single-bases mutagenesis reactions, in order to obtain two different clones: one containing the analyzed missense mutation, and the second one, used as a positive control for the next assay, containing a known deleterious mutation. The c.8297delC Thr2766Fs mutation was used as control of missed DBSR. The single base deletion causes the loss of the translation frame, followed by the sequence shifting and the production of a truncated protein, characterized by the loss of about 180 amino acids. Since the cloning sequence started to p.2633 amino acid residue, the derived truncated and non functional protein was made up of only 133 amino acids (about 15kDa), showing loss of the C-terminus domain. Therefore, after several *in silico* translation predictions performed to test the expressions of proteins and to verify wheter or not the inserted translation sequence was correct, the *wt* fragment and the mutants were transfected in NIH-3T3 cells. BRCA2 mutant proteins expression levels were tested through Western Blot analysis. Nuclear proteins were isolated through performing 3 minutes of pulse sonication, as described

above, in order to break DNA-proteins bindings, to avoid the loss of protein linked to DNA and assuring their separation from chromatin. Lysates were quantized by Bradford assay and 50µg of protein were loaded on 8% and 12% polyacrylamide gels. The 390kDa endogenous BRCA2 protein was too large to be detected into performed gels and was not considered in these experiments. As shown in the pictures (**Fig. 25a-b**), a 95kDa mutant BRCA2 protein was expressed in cells contained *wt* and Pro2767Ser constructs, but Thr2766Fs deleted protein showed no signal. This because truncated protein was too short to be identified using an 8% but also a 12% gel. Furthermore, the used antibody recognizes the C-terminus domain, that was not present in the mutant of deletion. Transfections and Western Blot analysis were performed at least five times and in duplicate, showing the same results. These findings demonstrated the translation of cloning sequences and the proteins production.

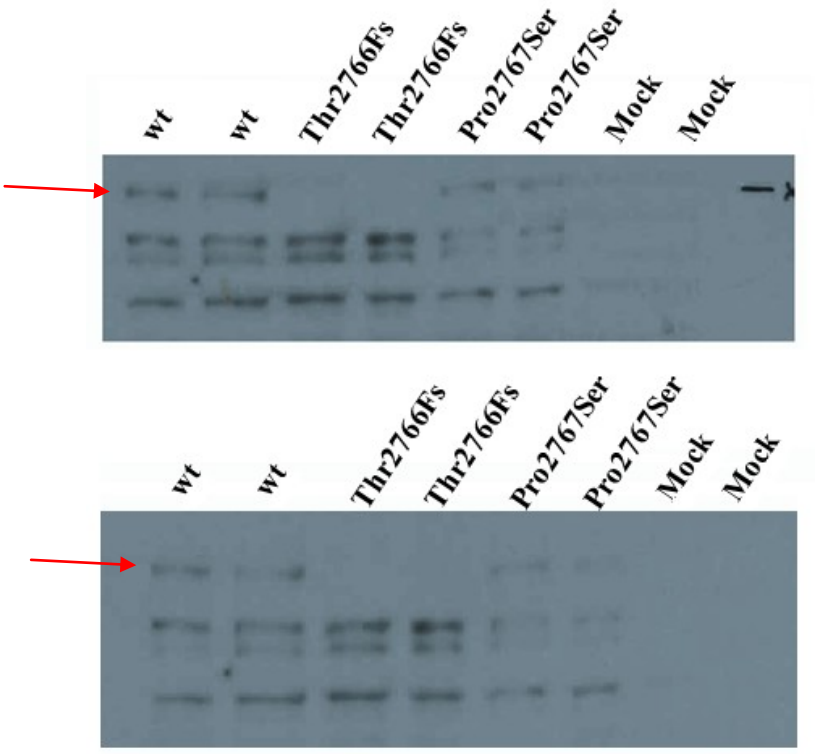


Figure 25a. Western Blot analysis of BRCA2 mutant proteins. Pictures show the results of two 8% polyacrylamide gels performed at same conditions and in duplicate. The arrows show the bands strictly under 100kDa, corresponding to BRCA2 mutants. Only *wt* and Pro2767Ser proteins were detected, but no signals for Thr2766Fs were found. Moreover, were detected several nonspecific signals belonging to shorter proteins.

Since they were not revealed into Mock lanes, probably represented BRCA2 mutant proteins degradation products. This protein instability is probably due to the structure of mutants, cloned without N-terminus domain that maybe causes translation problems or post-translational changes, leading to several shorter and likely non functional products. Actin (not shown) was used to normalize the amount of protein in different samples and as a control of loading. The presence of proteins, especially into Mock lanes, was verified using the Ponceau S staining (not shown) that demonstrated the presence of proteins into all lanes.

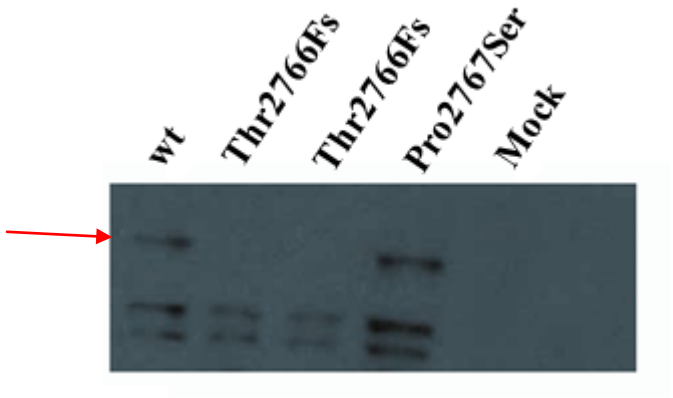


Figure 25b. Western Blot analysis of BRCA2 mutant proteins. Results of 12% polyacrylamide gels show the detection of *wt* and Pro2767Ser proteins. Despite the gel conditions were more stringent, no signals Thr2766Fs were revealed. The arrow indicates the bands strictly under 100kDa, corresponding to BRCA2 mutants. Also in this case, were present bands of lower molecular weight not detected into Mock lanes.

Subsequently, these mutant DNA sequences were trasfected into NIH-GS cells, containing constitutively the pDR-GFP plasmid, and previously trasfected with I-SceI-ER plasmids too (**Fig. 26**).

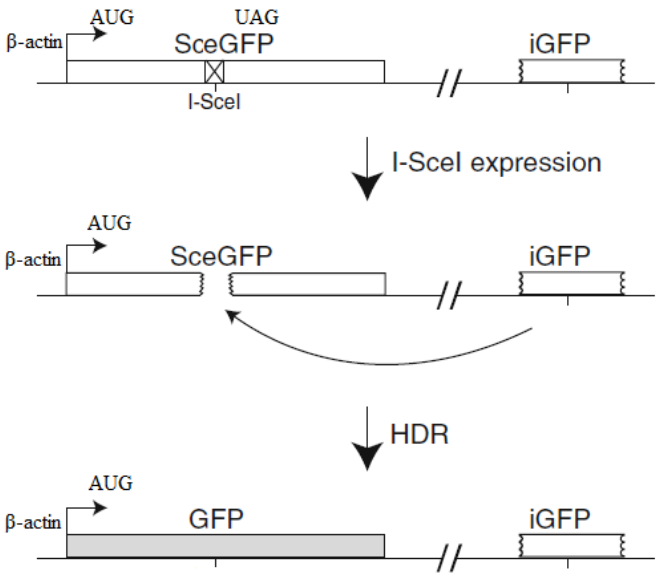


Figure 26. DR-GFP/I-SceI system used for DSBR assay. The expression of I-SceI induces DSB. Gene conversion events are able to repair the double strand break induced by I-SceI by using the downstream 3′ GFP as donor by homologous recombination processes that allow GFP expression.

Then, was evaluated the capability of mutation to interfere with the DSBR processes in cells using the DR-GFP/I-SceI experimental system (Richardson 1999). Briefly, this system was capable to mimic the DNA double strand breaks and the damage repair by homologous recombination (HR). To this aim, were generated clones of NIH-3T3 cells stably transfected with a DNA construct (DR-GFP) containing two non-functional GFPs. The upstream (5') GFP is under the control of the β-actin gene promoter and contains a single recognition site for the I-SceI endonuclease. Considering that no I-SceI sites are present in mammalian genomes, the expression of this enzyme results in generation of single DNA DSB only at DR-GFP sites. Gene conversion events are able to repair the double strand break induced by I-SceI by using the downstream 3'GFP as donor. The upstream 5' GFP contains 2 in-frame stop codons that cause the end of translation, thereby inactivating the gene. The downstream (3′) GFP is inactivated by upstream and downstream truncations, leaving only about 500 bp of the GFP sequence. The recombination event leads to the reactivation of the GFP gene (**Fig. 26**). Thus, when NIH-GS cells were transfected with the I-SceI plasmid, nuclear translocation results in DSBs generation. Homologous recombination repair in this system induces intra chromosomal gene conversion leading to the reactivation of the 5' GFP gene. Indeed when the DSB is correctly repaired the 5' GFP gene's

frame is restored, allowing GFP expression. In this way, the cells where a successful repair takes place, express a functional GFP. The extent of repair can be measured by counting GFP positive cells by FACS.

For this work an I-SceI-ER expression vector was generated, in which the I-SceI cDNA is fused in frame with the cDNA fragment encoding the hormone-binding site of the estrogen receptor alpha (ERα) (Stante 2009). The stable clones NIH-GS bearing DR-GFP were trasfected with I-SceI-ER and treated with 4-OH tamoxifen. When NIH-GS cells were treated with tamoxifen, the I-SceI-ER nuclear translocation results in DSBs generation with consequent DSBR and GFP emission.

Based on concept that BRCA2 is involved in HR repair processes, in the proposed system, because mutant proteins were cloned without N-terminal portion and cannot operated normally, had to act in a dominant negative manner. The constructs, cloned as described above, led the production of a truncated and non-functional BRCA2 proteins. Assuming that the tested mutations fall in the DNA-binding site, performing the experiments only the capability of mutant proteins to bind DNA and to compete with endogenous BRCA2 protein was considered. Therefore, DNA repair efficiency in NIH-GS cells was measured by counting the percentage of GFP-positive cells after the transfection of I-SceI-ER plasmid into cells, previously transfected with *BRCA2* clones, and 48 hours after their exposure to tamoxifen, which activates I-SceI ER.

FACS results showed that cells transfected with positive control mutation (Thr2766Fs) and with investigated mutation (Pro2767Ser) appeared able to repair DNA damage, not avoiding the DNA binding of the endogenous BRCA2 proteins (**Fig. 27a-e**). On the other hand, in cells trated with the *wt* construct the DNA binding of the endogenous BRCA2 should have been avoided though a competition for the DNA-binding sites, preventing the GFP emission. In this case GFP emission was comparable among samples: in NIH-GS cells treated with Thr2766Fs and Pro2767Ser the number of GFP positive cells was 2.0% and 1.7% respectively, showing that the pathogenetic effect of new mutation was comparable to the mutant of deletion, probably because Pro2767Ser makes BRCA2 incapable to bind DNA in the same way of Thr2766Fs. Similarly, cells transfected with Mock (empty vector without *BRCA2* construct) showed about the same pattern of GFP emission (2.0%) and, accordingly, the same DSBR by HR in the cells where I-SceI-ER was activated by tamoxifen. NIH-GS cells treated with *wt BRCA2*, instead, showed a massive apoptotic effect. All cells were counted before the FACS analysis: *wt* cells, after 48h of tamoxifen induction, were only 180,000/ml while Thr2766Fs, Pro2767Ser and Mock cells were counted over 800,000/ml (exemplifying numbers deriving from one experiment, starting to the same number of cells plated before transfections). Whereby, GFP emission revealed in *wt* cells (0.9-1.2%) was due only to the percentage of cells that did not acquired exogenous *BRCA2* gene and did not expressed BRCA2 mutated proteins. These results were confirmed by fluorescence microscopy analysis that showed GFP emission particularly in Thr2766Fs, Pro2767Ser and Mock cells but

relatively revealed in *w*t cells too (**Fig. 28a-d**). All cells with a *wt* BRCA2 successful transfection, expressing mutant proteins, were conducted to apoptotic processes, probably caused by the effects of several degradation products derived from mutants (and because all the different-length-truncated BRCA2 proteins could compete with endogenous BRCA2 for DNA binding) added to the loss of repair efficiency. However, GFP emission revealed in *wt* cells seems wrongly the same of the other samples if is not considered the percentage of cells that survive to DNA double strand breaks and that repair DNA damage only because they do not express mutant *wt* BRCA2.

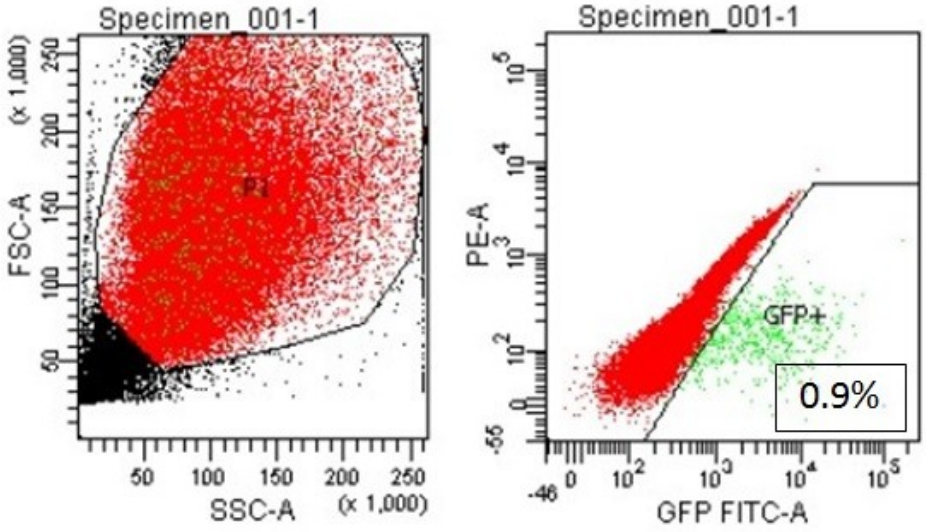


Figure 27a. Measurement of repair efficiency in NIH-GS cells by FACS analysis. The figure shows the GFP emission of NIH-GS cells treated with *wt BRCA2* construct. As largely described above, the percentage of GFP emission seems to be similar, or slightly lower then the other samples. Whereby, GFP emission revealed in *wt* cells was due only to percentage of cells that did not acquired exogenous *BRCA2* gene and that survive correctly repairing DNA, as shown by GFP emission. Conversely, the remaining percentage of cells shows a massive apoptosis probably due to the several degradation products effects derived from mutants added to the loss of repair efficiency. This phenomenon could be explained by the great reduction in the number of cells (180,000/ml *vs* 800,000/ml).

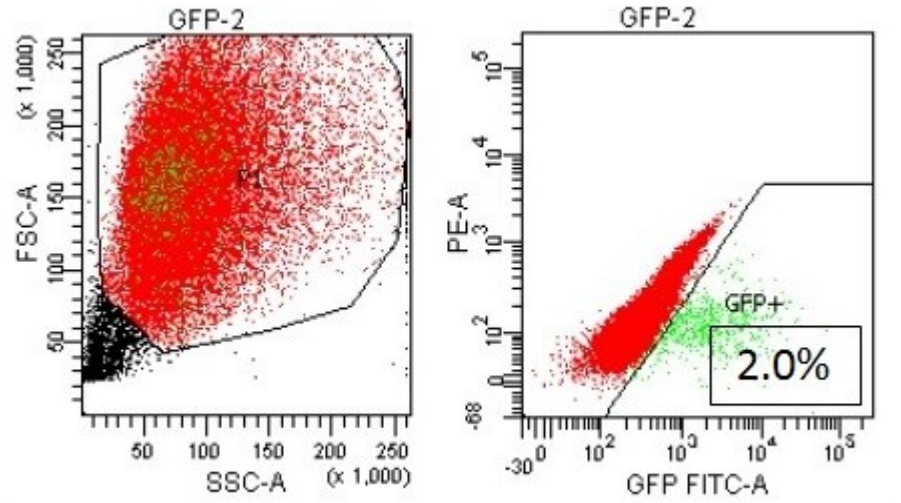


Figure 27b. Measurement of repair efficiency in NIH-GS cells by FACS analysis. GFP emission of NIH-GS cells treated with Thr2766Fs *BRCA2* deleterious mutation. The extent of repair is demonstrated by the percentage of GFP emission, suggesting that mutant BRCA2 protein cannot bind DNA and cannot compete with endogenous BRCA2 that allow the normal repair efficiency.

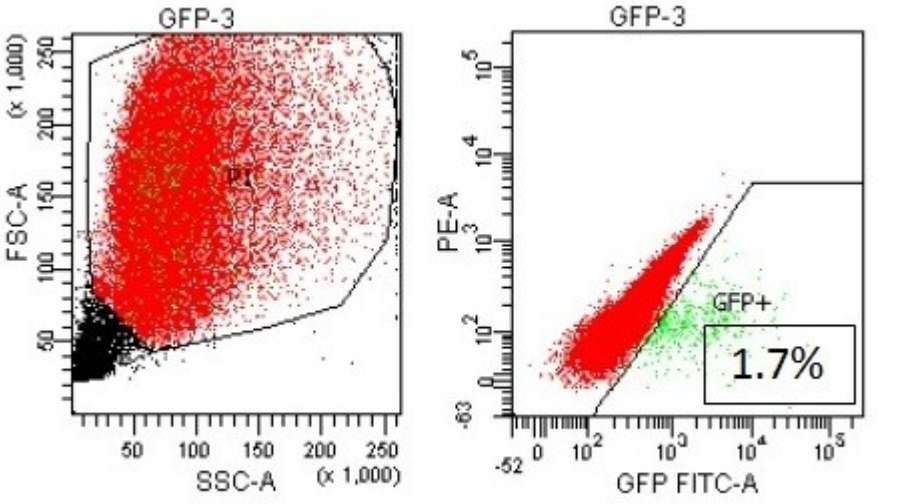


Figure 27c. Measurement of repair efficiency in NIH-GS cells by FACS analysis. GFP emission of NIH-GS cells treated with Pro2767Ser *BRCA2* novel variant. The percentage of GFP emission suggests that the mutant BRCA2 protein cannot bind DNA

and cannot compete with endogenous BRCA2, allowing the normal repair efficiency. The effect of this variant appears comparable to the deletion reported as surely pathogenetic.

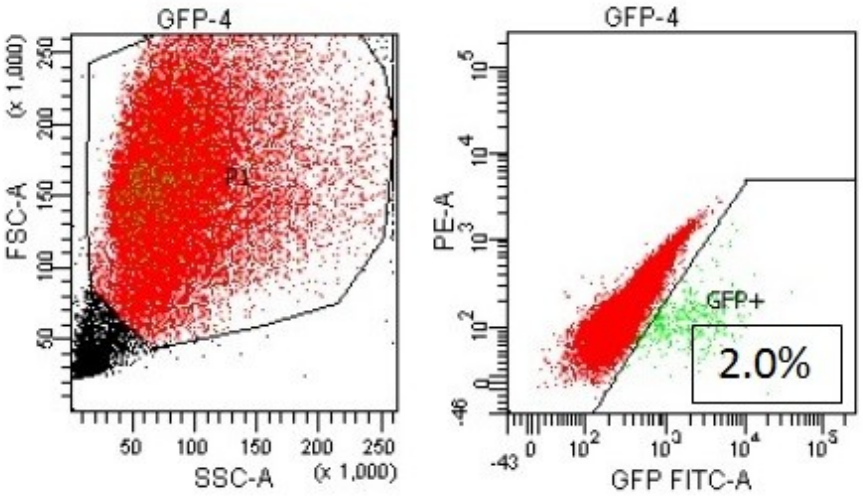


Figure 27d. Measurement of repair efficiency in NIH-GS cells by FACS analysis. GFP emission of NIH-GS cells treated only with empty vector, used as control. The extent of repair is revealed by the percentage of GFP emission, demonstrating that the system DR-GFP/I-SceI-ER works correctly after tamoxifen induction and allows the normal DSBR processes managed by endogenous BRCA2 protein.

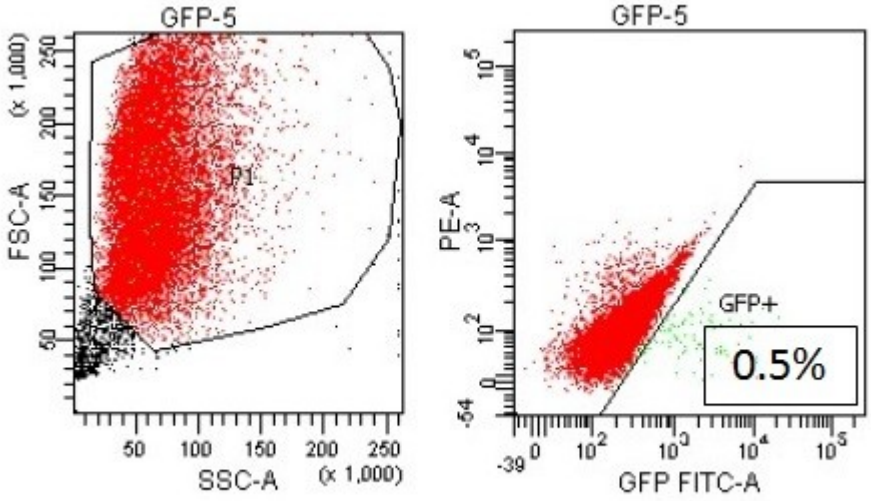


Figure 27e. Measurement of repair efficiency in NIH-GS cells by FACS analysis. GFP emission of NIH-GS cells treated with vehicle and not with tamoxifen. In this case, the system DR-GFP/I-SceI-ER was not activated, and was used as negative control. The

percentage of GFP emission represents the fluorescence background and does not originate from inducted biological effects.

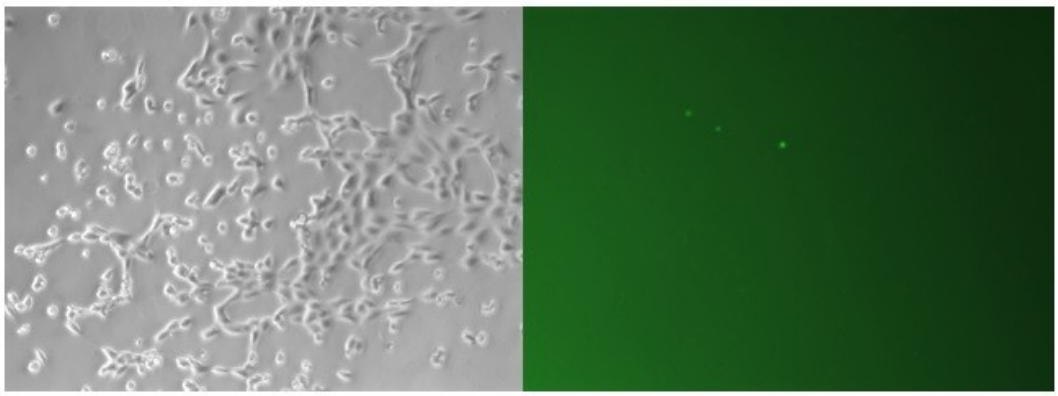


Figure 28a. Visualization of repair efficiency in NIH-GS cells by fluorescence microscopy. GFP emission of NIH-GS cells treated with *wt* BRCA2 construct. On the left is shown the image on bright field (10X): the cell morphology and the great number of in suspension cells show the massive apoptotic effect described before. On the right the image on GFP fuorescence: the GFP emission is very low and present only for cells that survive to apoptosis.

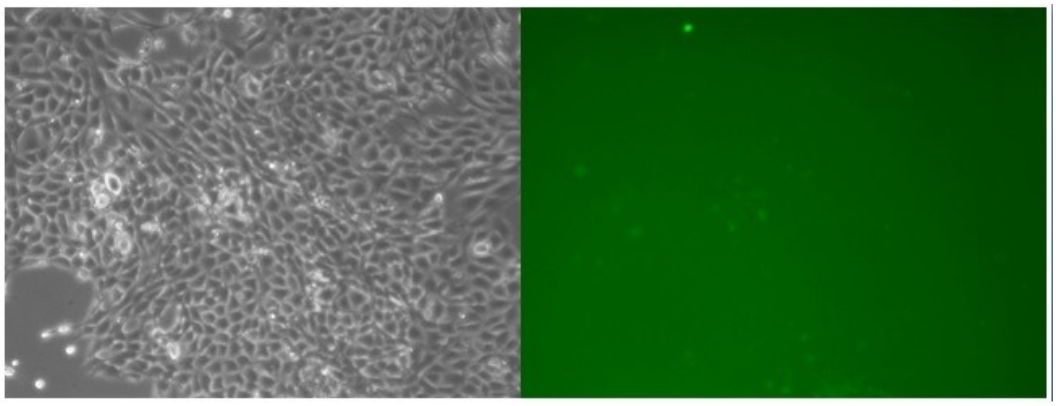


Figure 28b. Visualization of repair efficiency in NIH-GS cells by fluorescence microscopy. GFP emission of NIH-GS cells treated with Thr2766Fs BRCA2 deleterious mutation. In the image on bright field (10X) cells appear more confluent than *wt* and more vital, the numer of in suspension cells was lower and comparable to cells trated with Pro2767Ser BRCA2 novel variant and empty vector (Mock). The percentage of GFP emission, in the right side of the picture, seems grater than *wt* cells.

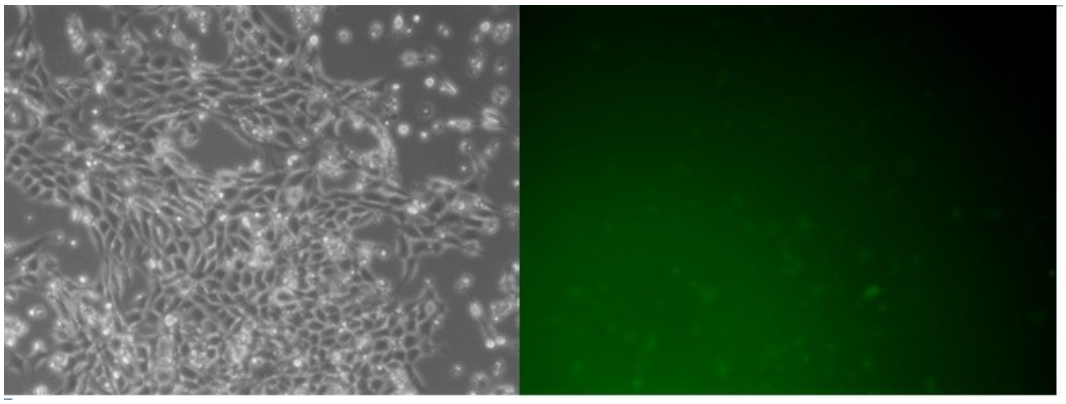


Figure 28c. Visualization of repair efficiency in NIH-GS cells by fluorescence microscopy. GFP emission of NIH-GS cells treated with Pro2767Ser BRCA2 novel variant. Despite the GFP emission appeared low in all samples, the GFP revealed into these cells suggested that mutant BRCA2 protein does not compete with endogenous BRCA2. The effect appears comparable to the mutant Thr2766Fs.

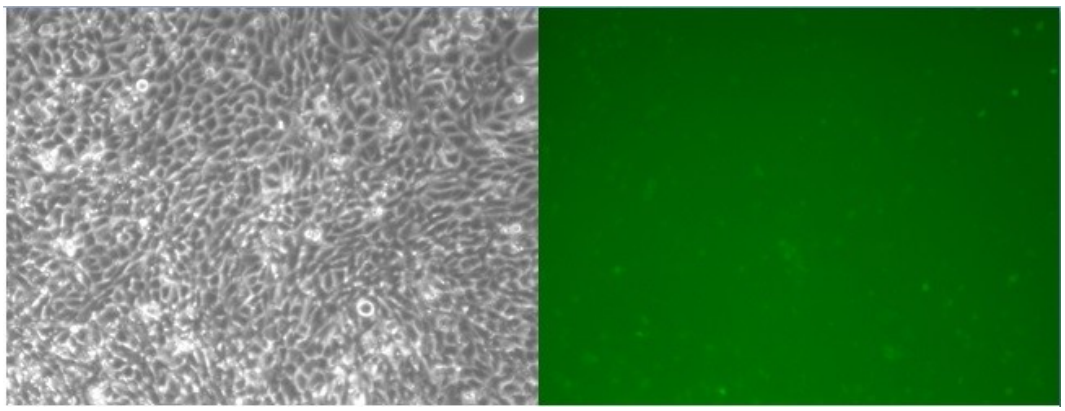


Figure 28d. Visualization of repair efficiency in NIH-GS cells by fluorescence microscopy. GFP emission of NIH-GS cells treated only with empty vector, used as control. The image on bright field (10X) demonstrates the cell viability and confluence. The percentage of GFP emission, on the right side of the picture, demonstrates that the system DR-GFP/I-SceI-ER after tamoxifen induction allows the normal DSBR processes managed only by endogenous BRCA2 protein.

The massive apoptotic effect in *w*t BRCA2 cells has been demonstrated using Apoptosis Assay Annexin V protocol, performed by flow-cytometry (**Fig. 29a-b** and **Fig. 30**).
Briefly, Annexin V is a member of a calcium and phospholipid binding family of proteins with vascular anticoagulant activity. Results from *in vitro* experiments indicate that it may play a role in the inhibition of blood coagulation by competing for phosphatidylserine (PS) binding sites with prothrombin. In healthy cells, PS is usually kept in the cytosolic side of the cell membrane. When a cell undergoes apoptosis, one of the earliest detectable indicators is the loss of membrane asymmetry. No longer restricted to the cytosolic part of the membrane, PS is translocated to the outer-leaf and becomes exposed on the surface of the cell (Van Engeland 1998). Using Annexin V-APC and Propidium Iodide (PI) reagents was possible to distinguishing two populations of dying cells from viable cells. Cells in the early stages of apoptosis with intact cell membranes and surface-exposed PS will stain positive for Annexin V-APC. PI is used to identify late apoptotic and necrotic cells, which have lost plasma membrane integrity. These cells are labeled with Annexin V-APC and Propidium Iodide. Live cells with intact plasma membranes exclude PI and remain unstained by the Annexin V-APC probe.

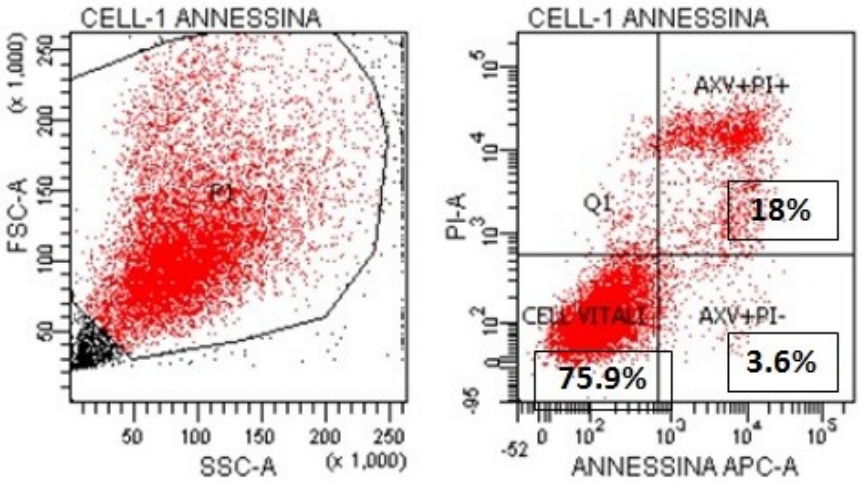


Figure 29a. Measurement of Annexin V in NIH-GS cells by FACS analysis. Apoptosis found in *wt* BRCA2 cells at 24h after tamoxifen induction. In order to differentiate late to early apoptotic stages, PI+ indicates necrotic or late apoptotic cells, while PI- are cells that still maintain plasma membrane integrity but that have started the apoptotic processes. 24h after induction of DBS, cells started to die slowly probably because they cannot support replicative processes or doing HR without the binding of endogenous BRCA2 to DNA.

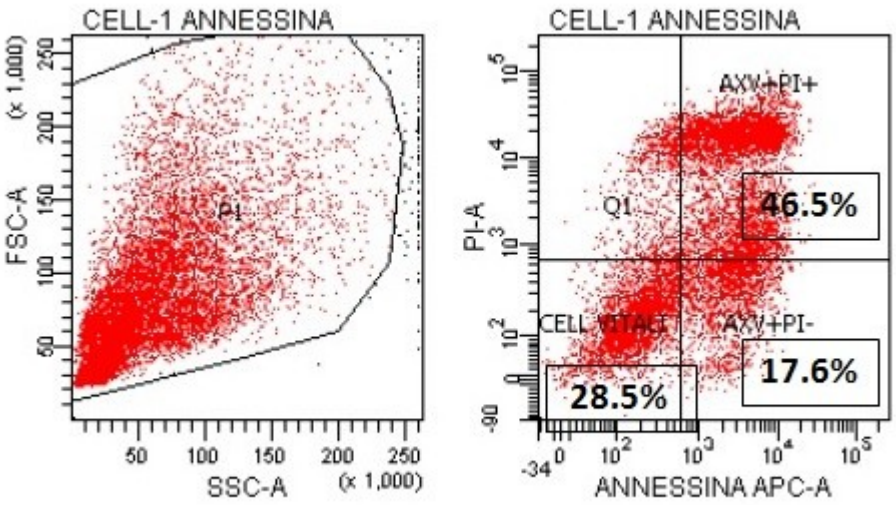


Figure 29b. Measurement of Annexin V in NIH-GS cells by FACS analysis. The figure shows the apoptosis in cells treated with *wt* BRCA2 protein 48h after tamoxifen induction: cells demonstrated a large apoptotic effect, and only few viable cells were detected.

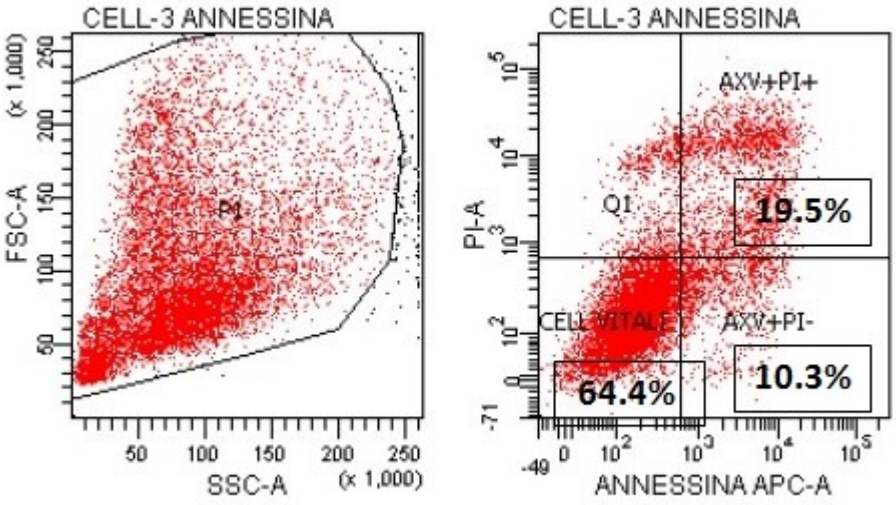


Figure 30. Measurement of Annexin V in NIH-GS cells by FACS analysis. Apoptosis in cells trated with Pro2767Ser BRCA2 novel variant, 48h after tamoxifen induction. Percentages of PI internalization showed that the apoptotic effect of the variant was lower than the *wt* and comparable to fisiological effects of several transfections on cells viability.

Considering these very preliminary results, Pro2767Ser novel mutation seems to be pathogenetic: the single nucleotide substitution allows an aminoacidic change that can strongly compromise the DNA binding of BRCA2 protein and, consecutively, the repair efficiency. These data reflected and confirmed all bioinformatic predictions.

Despite that, other investigations are necessary to better understand the role of this variant. In particular, is important to verify wheter or not BRCA2 mutant proteins really do not bind DNA, or if the repair is compromised by other mechanisms, still not clear. For this reason, on this variant other experiments are ongoing: in the near future, on one hand, several chromatin immunoprecipitation (ChIP) experiments will be performed to confirm the missed DNA binding. On the other hand, will be demonstrated, at first, how *wt* BRCA2 mutant proteins determine a toxic apoptotic effect on cells that expressed the exogenous construct, and second, will be explored if Pro2767Ser variant can hamper the formation of nuclear *foci* because of the RAD51 and related proteins complexes recruitment failure.

In conclusion, further analysis are needed to understand the Pro2767Ser variant effect on cancer. However, patient's disease that seemed to derive from sporadic and non-genetic causes should be connected to a *BRCA2* germline mutation and the search of the Pro2767Ser variant should be performed in the patient's relatives before the onset of probable futures oncologic diseases.

4.5. The *BRCA1* c.5406+2T>C IVS22+2T>C splice variant

A new splice variant, namely c.5406+2T>C IVS22+2T>C, was identified in heterozygous status. Predicted as deleterious by Human Splice Finder and NetGene2 that showed the loss of a canonic donor splice site localized at position +2 in the intron 21 of *BRCA1* gene, this splice variant was evaluated by the functional studies reported below. The variant was found in a 60 years old woman, affected by breast cancer, showing the presence of several cases of cancer into her families (**Fig. 31**). The patient's mother and one sister are the only relatives still alive and to date they are not affected by BC or other cancers. Despite that, they did not undergo to genetic testing. The presence of the variant into the family cannot be confirmed because all affected relatives are died. This evidence could suggest the probable role of the variant.

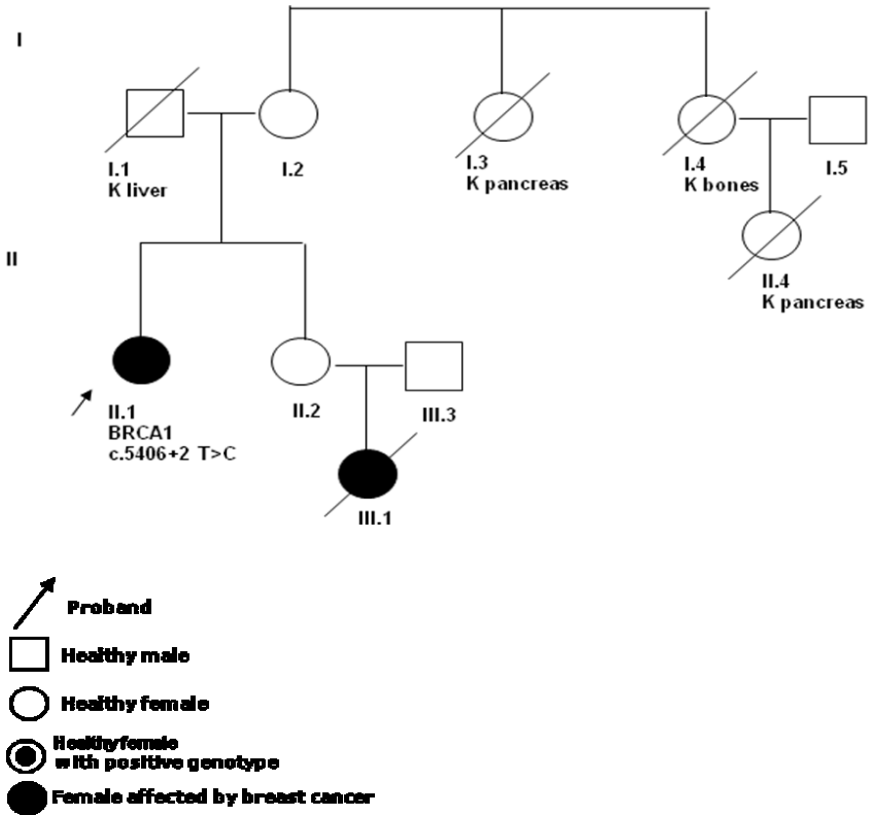


Figure 31. Genealogy of the patient's family carrying the novel c.5406+2T>C splice mutation in *BRCA1* gene. A patient's nephew (III.1) died for early-onset breast cancer and her sister (not shown) was, to date, affected by BC.

Splicing is a process in which mRNA is modified after transcription. It allows the introns removal and the union of exons to form mature mRNA, ready for translation into protein. The gene splicing can be easily affected by mutations in the sequence surrounding the splice site junction, leading to alternate splicing and thus adversely affecting the translated protein. Indeed, when a mutation occurs into a splice site and contributes to the loss or to the gain of a donor or an acceptor splice site, the normal splice mechanism is altered and leads to exon skipping or intron retention events.
Bioinformatic predictions suggest that the novel splice variant IVS22+2T>C produces the retention of the intron in which is localized (**Fig. 32**), with probably consequences on mRNA maturation.

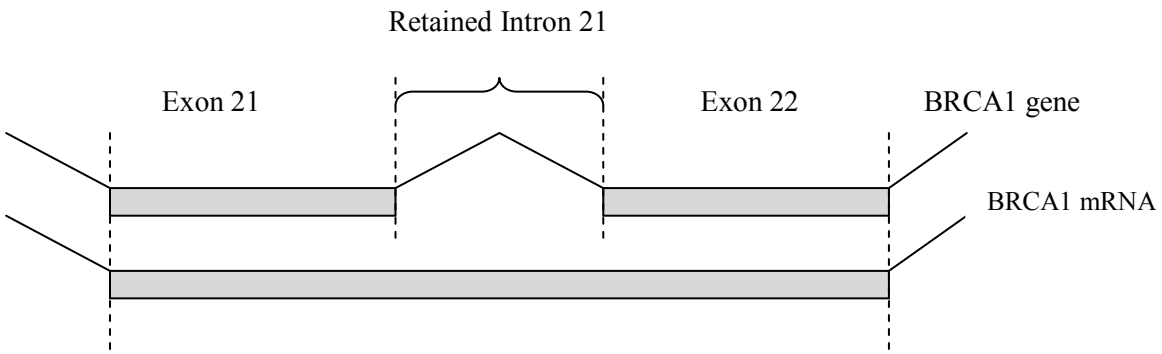


Figure 32. Definition of scored intron retention events. Gray rectangles represent exons of *BRCA1* gene, and mRNA. Exon/intron boundaries are marked by dotted lines.

To test the pathogenicity of this splice variant and to confirm the bioinformatic predictions, patient RNA was retrotranscripted together with two control RNAs: a woman affected by BC and a woman with strong familiar history of cancer but not affected, totally screened for *BRCA1/2* mutations and resulted negatives. The resulted cDNAs were amplified using different pairs of primers designed *ad hoc*. To demonstrate the presence of the target *BRCA1* cDNA and to avoid the amplification of gDNA contamination, a pair of primers was designed to anneal on flanking cDNA exons. One more was designed to cover about 470bp of the retained intron, to show the presence of this one only in mutated cDNA. Finally, to demonstrate that the result of the latter amplification do not derive from gDNA, another PCR reaction using a forward primer annealing a cDNA region and a reverse primer complementary to the retained intronic region was performed: only the mutated cDNA has given positive amplifications (**Fig. 33 a-b**).

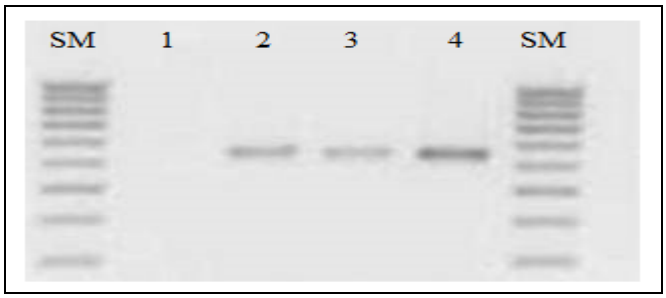


Figure 33a. PCR amplification confirms of splice variant, agarose 2%. The amplifications on *BRCA1* cDNA with primers that give a product of about 870bp, showed the same profile for mutated cDNA and for controls (not shown). The retained intron produced, for mutated allele, two amplicons: one of 870bp and one of more than 1400bp, not amplifiable through used PCR conditions and not visible in the first gel. Therefore, on mutated and controls cDNAs was performed a second amplification using a pair of primers covering a 470bp intronic region. In the picture are shown the amplification results: in the lane 1 was loaded a control cDNA in which there was no intronic retention with consequential no amplification; in the lane 2 was loaded the mutated cDNA with intronic retention, in the lane 3 was loaded an duplicate of sample loaded into lane 2, in the lane 4 was loaded a gDNA used as positive control of amplification.

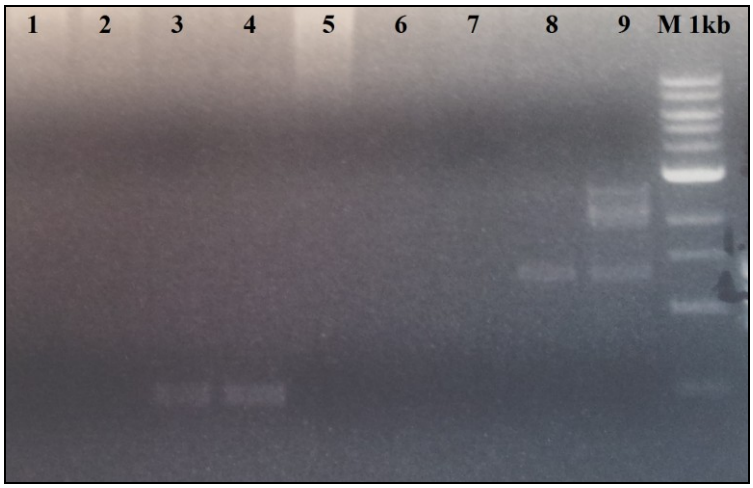


Figure 33b. PCR amplification confirms of splice variant, agarose 1%. In the lane 1-4 the results of amplification of the 470bp retained intron (also described above): in the lanes 1 and 2 were loaded the controls cDNA with no retention, in the lane 3 was loaded the mutated cDNA with the intronic sequence that gave positive amplification, and in the lane 4 was loaded the gDNA used as positive control of amplification. The amplification performed using a forward primer covering a cDNA region and a reverse primer covering the retained intronic region produced a 1417bp amplicon only on mutated cDNA. In the lanes 6 and 7 were loaded the control cDNAs that gave no products, in the lane 8 was

loaded the patient mutated cDNA and in the lane 9 was loaded a gDNA that gave only aspecific amplicons. The aspecifics bands were analyzed by Sanger sequencing after extraction and purification from gel. In the lane 10 were loaded 7µl of Ladder 1Kb (BioLabs).

In addition, two restriction maps were performed: one on the retained intron sequence and another one on a portion of the cDNA sequence amplified by designed primers described before. From the analysis and the comparison of these restriction maps, the NcoI restriction enzyme was chosen because of its capability to recognize the cleavage site C|CATGG present only in the intronic sequence and not in the the *BRCA1* cDNA amplified region. The enzyme, when recognizes its cutting sequence, produces two fragments on mutant cDNA: one of about 1100bp and another of about 280bp. Given that the patient's variant is in heterozygosity, the two alleles show different digestion patterns in the same lane. After the enzymatic digestion of the in exam cDNA and of controls, DNA fragments were analyzed by DNA Chip 1K (BioRad). The analysis showed different digestion patterns among samples: only the mutated cDNA was cutted by NcoI, while the controls cDNAs profiles appeared the same of the uncut cDNA using as ulterior control. In this way, was ultimately demonstrated the intronic retention (**Fig. 34-39**).

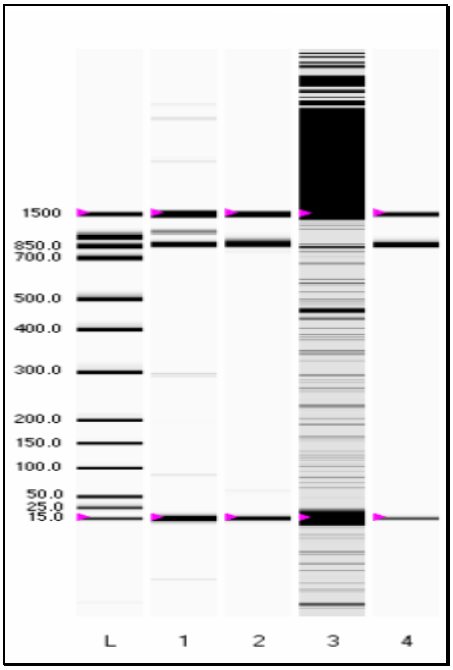


Figure 34. Virtual gel report from 1K DNA Assay. In the lane L was loaded the DNA molecular weights ladder, from 15bp to 1500bp; in the lane 1 was loaded the the amplified cDNA digested by NcoI and carrier of the mutation; in the lane 2 was loaded an amplified control cDNA digested by NcoI; in the lane 3 was loaded a gDNA digested by NcoI using as positive control of the enzyme cutting; in the lane 4 was loaded the amplified not cutted cDNA.

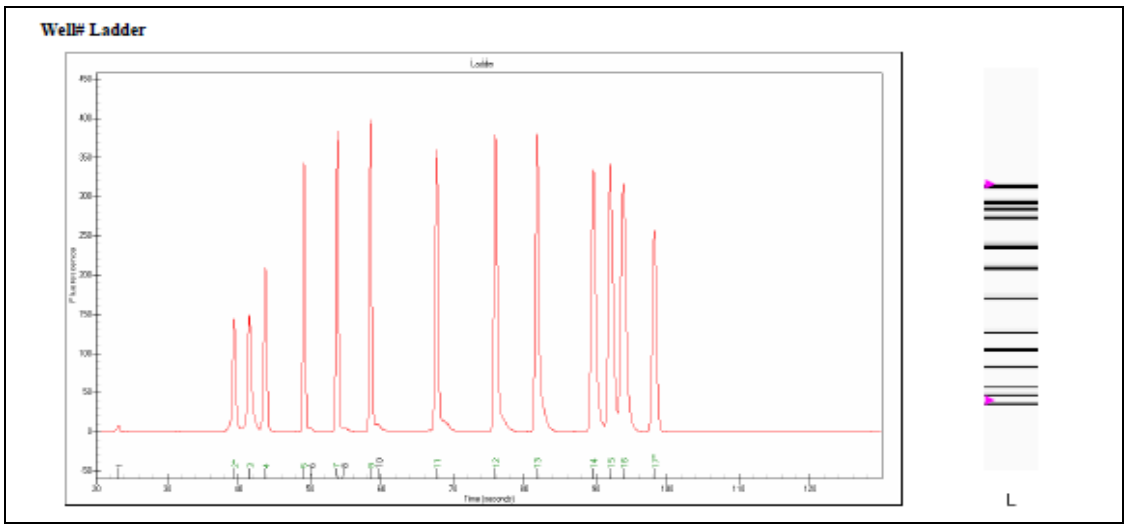
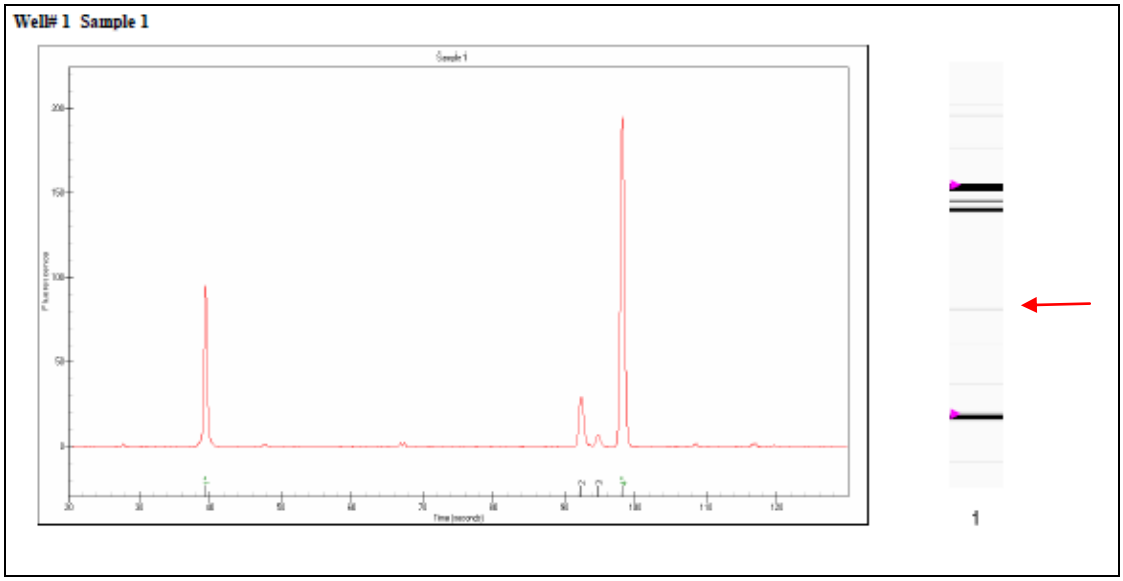


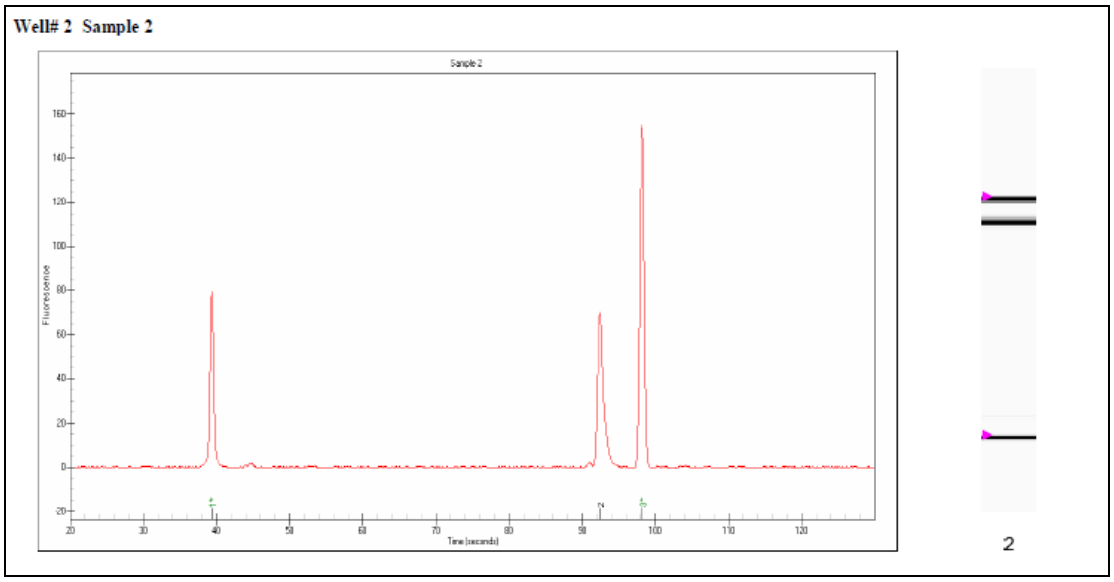
Figure 35. Ladder peaks and ladder lane from 1K DNA Assay.
Area under each peak corresponds to specific quantity of ladder and is useful for the comparison of fluorescence of other samples in order to quantize them.



Well# 1 Sample 1

Peak State	Peak Number	Mig. Time (secs)	Size (bp)	Corrected Area	Area Ratio	Concentration (ng/µl)	Molarity (nmole/l)	% Total	Observation
	1	39.35	15	141.70					Lower Marker
	2	92.39	878	25.12	0.1812	0.47	0.81	82.11	
	3	94.76	1,105	5.58	0.0403	0.10	0.14	17.89	
	4	98.20	1,500	138.66					Upper Marker

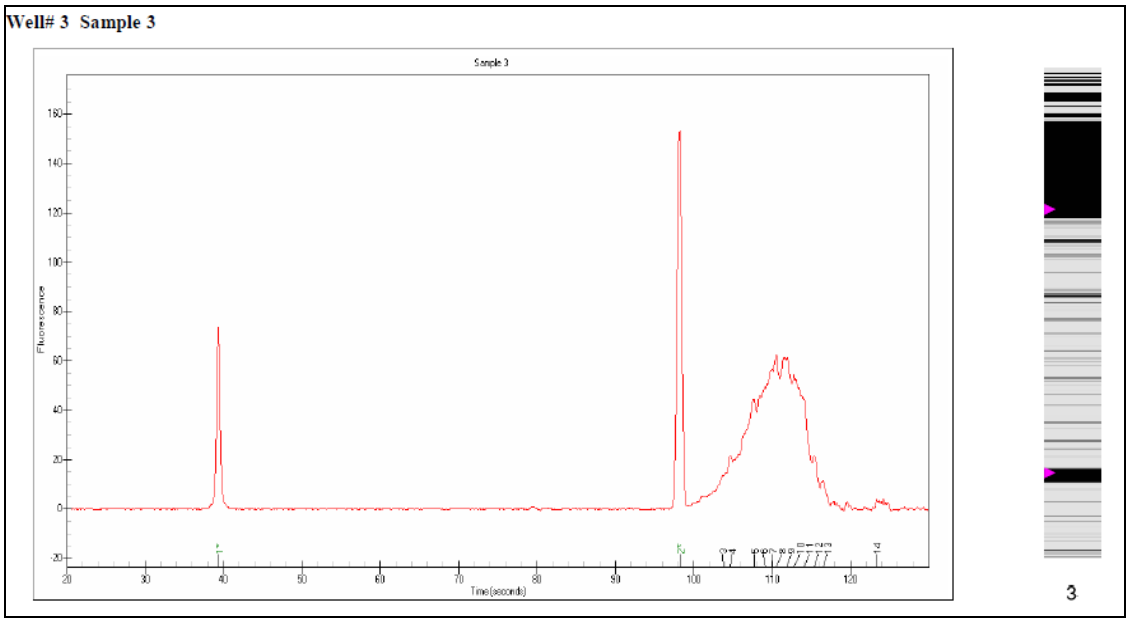
Figure 36. Sample 1 lane from 1K DNA Assay.
The portion of 800bp cDNA carrying the mutation, was digested with NcoI enzyme and the product of the digestion was loaded into lane 1. In the picture are shown the lower marker and the upper marker that are the first and the last peaks, useful to normalize fluorescence as internal control of quantification. The peaks resulting from the sample are one of 878bp, corresponding to uncut cDNA derived from the wt allele, and another one of 1105 corresponding to mutated allele cut by the enzyme. The peak showing the 280bp fragment was too low to be detected in fluorescence (according to the detection limits of the used assay) if compared to the ladders fluorescence, but it can be revealed in the virtual gel (indicated by red arrow). The patient's variant is in heterozygosity and two alleles show different digestion patterns in the same lane.



Well# 2 Sample 2

Peak State	Peak Number	Mig. Time (secs)	Size (bp)	Corrected Area	Area Ratio	Concentration (ng/µl)	Molarity (nmole/l)	% Total	Observation
	1	39.35	15	118.71					Lower Marker
	2	92.45	884	72.92	0.6715	1.73	2.97	100.00	
	3	98.20	1,500	108.59					Upper Marker

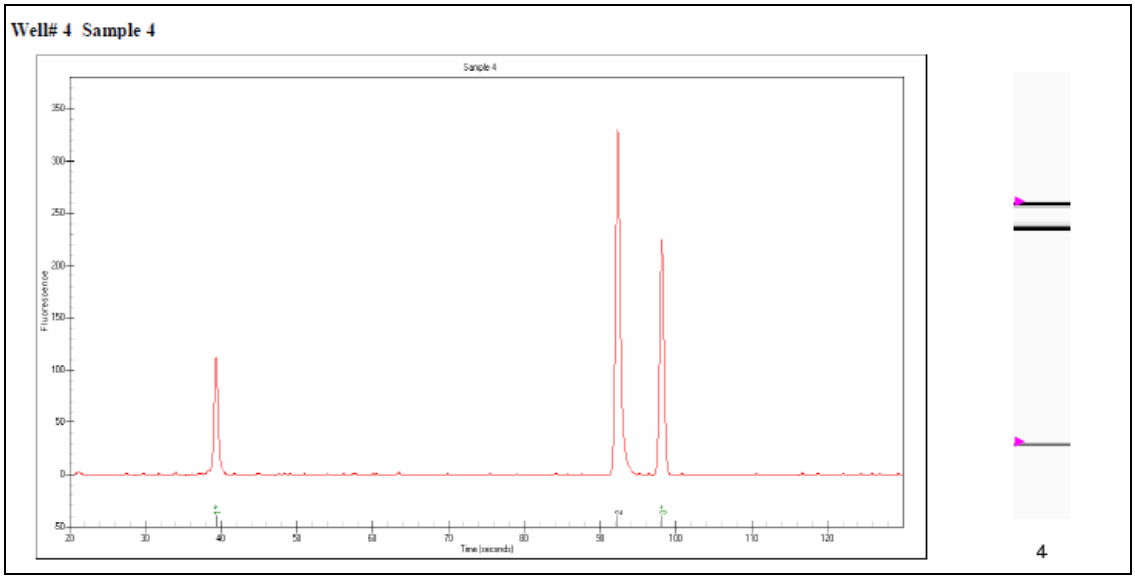
Figure 37. Sample 2 lane from 1K DNA Assay.
The control *wt* cDNA amplified with primers that produce an amplicon of about 800bp, digested with NcoI enzyme: the product of the digestion was loaded into lane 2. Only the peak of 884bp, corresponding to uncut *wt* cDNA appeared into this lane.



Well# 3 Sample 3

Peak State	Peak Number	Mig. Time (secs)	Size (bp)	Corrected Area	Area Ratio	Concentration (ng/µl)	Molarity (nmole/l)	% Total	Observation
	1	39.35	15	108.65					Lower Marker
	2	98.20	1,500	105.67					Upper Marker
	3	103.77	0	11.22					
	4	104.66	0	14.08					
	5	107.68	0	45.40					
	6	109.14	0	27.21					
	7	109.92	0	42.69					
	8	110.54	0	43.45					
	9	111.90	0	34.16					
	10	112.78	0	48.88					
	11	114.08	0	29.10					

Figure 38. Sample 3 lane from 1K DNA Assay.
A gDNA was digested by NcoI as positive control of enzymatic cleavage, and the digestion product was loaded into lane 3. Several fragments of different molecular weights were presents, and appeared after the upper marker, showing many enzymatic cuts and demonstrating the enzyme functionality.



Well# 4 Sample 4

Peak State	Peak Number	Mig. Time (secs)	Size (bp)	Corrected Area	Area Ratio	Concentration (ng/µl)	Molarity (nmole/l)	% Total	Observation
	1	39.35	15	170.42					Lower Marker
	2	92.37	876	279.36	1.7343	4.48	7.74	100.00	
	3	98.20	1,500	161.07					Upper Marker

Figure 39. Sample 4 lane from 1K DNA Assay.
The control *wt* cDNA amplified with primers that produce an amplicon of about 800bp, was loaded into lane 4, without enzymatic digestion. Only the peak of 876bp, appeared into this lane in a concentration more higher than upper ladder. This was the same profile of the *wt* digested cDNA.

Despite other studies are needed to confirm the role of this splice variant, the positive amplifications present only in the cDNA carrying the mutation and not into the controls, and the restriction enzyme digestion patterns have demonstrated the bioinformatic predictions and the very probable pathogenicity of the found variant.

4.6. Male breast cancer mutation carriers

Furthermore, 2 men were found to be carrier of a *BRCA1/BRCA2* causative mutation. One, affected by early-onset breast cancer, carries the c.2808_2811delACAA p.K936Qfs*21 frameshift mutation in *BRCA2* gene: men affected by BC and carrying a causative *BRCA2* mutation are very rare if compared to man carrying a causative *BRCA1* one, and the risk for these carriers to develop oncologic diseases is very high. In the patient's family there are a lot of cases of cancers, especially ovarian, normally characterized by *BRCA1* mutations; in addition, is interesting to underline that the inheritance of mutation seems to derive from paternal side (**Fig. 40**).

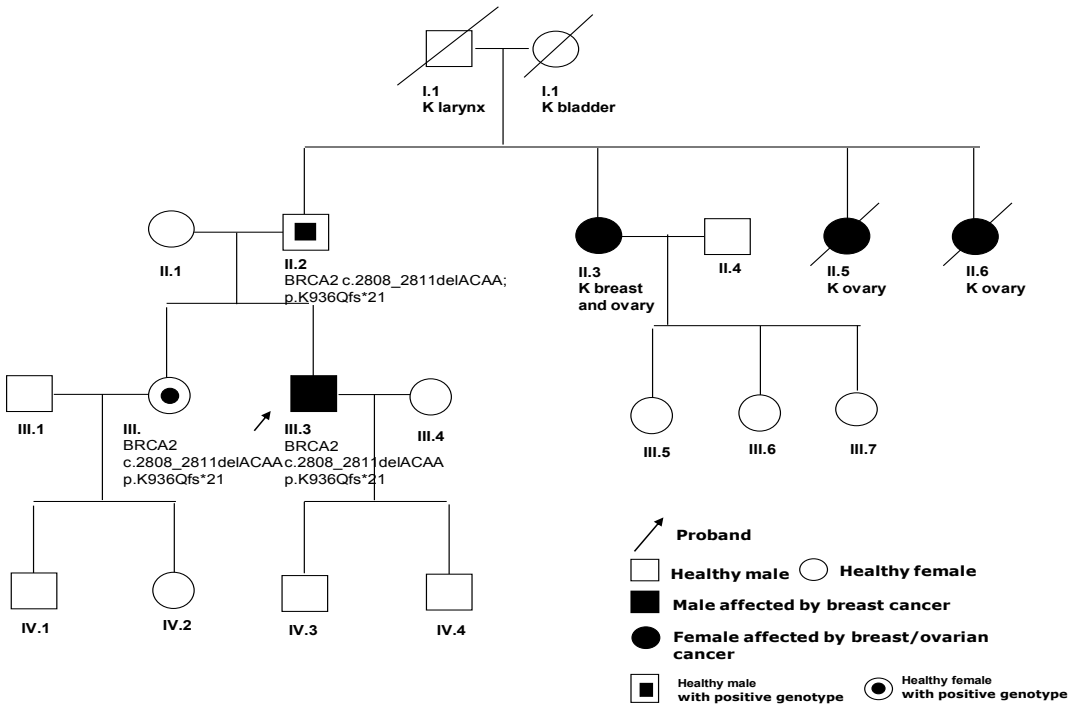


Figure 40. Genealogy of the male patient's family carrying the c.2808_2811delACAA p.K936Qfs*21 frameshift mutation in *BRCA2* gene. Genetic testing allowed the identification of two healthy carriers, one of them was a man. The transmission of the variant seems to derive from the paternal side.

A causative *BRCA1* mutation was found into a healthy carrier belonging to a high-risk family that carries the c.5431C>T p.Q1811X nonsense mutation. In this family, the analysis started from the aunt, that submitted the analysis in the past,

she was carrier of the same mutation, and died for ovarian cancer. The patient's mother died for colon cancer and no genetic information about her were available (**Fig. 41**).

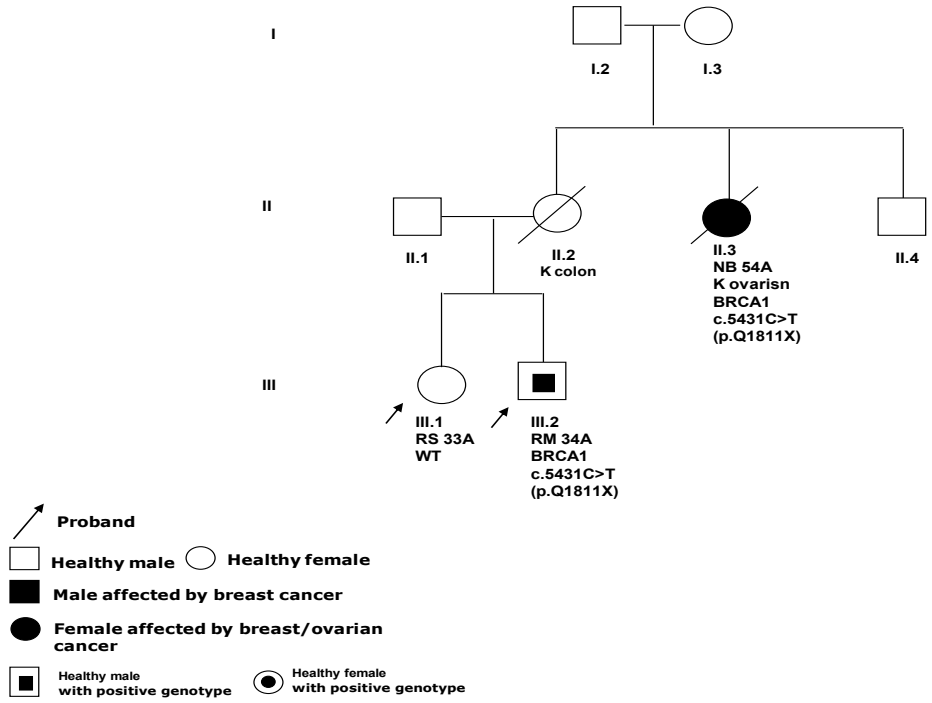


Figure 41. Genealogy of the male patient's family carrying the c.5431C>T p.Q1811X nonsense mutation in *BRCA1* gene. The mutation was searched into two brothers and only the man resulted carrier.

It is not rare to find a man affected by BC carrying a germline mutation, especially with strong familiarity for BC and/or other cancers. Indeed, further 4 men were identified to be carrier of mutations in several high-risk families. All these men were (to date) healthy carriers, not affected by BC and/or others disease.

Male carriers of mutations in *BRCA1* or *BRCA2* are susceptible to cancer; however, their risks remain poorly understood and their optimal clinical management has not yet been defined. Male *BRCA1* mutation carriers show an increased risk of prostate and breast cancer. Evidence supporting increased susceptibility to colon cancer is limited to women (as demonstrated in our

patient's *BRCA1* carrier family, in which the mother of proband died for colon cancer). In contrast to women, who have a greater lifetime risk of cancer with mutations of the *BRCA1* gene, *BRCA2* is the more important gene for men. The *BRCA2* spectrum of cancers is wide and some studies have reported that the overall cancer risk for male *BRCA2* carriers exceeds the risk for female carriers. In particular, the relative risk for male *BRCA2* mutation carriers is higher before age of 65 years, largely attributable to breast, prostate, and pancreatic cancers. *BRCA2* mutation carriers are also at risk of stomach cancer and melanoma (of the skin and eye). Male breast cancer is a characteristic element of the *BRCA2* phenotype (Wooster 1994, Tonin 1995). Breast cancer accounts for less than 1% of all cancers in men (The U.S. Cancer Statistics Working Group 2002). The lifetime risk of male breast cancer in *BRCA2* mutation carriers is approximately 80 to 100 times higher than in the general population (Thompson 2001), and *BRCA2* mutations account for roughly 15% of all male breast cancers. For *BRCA2* mutation carriers, the risk of male breast cancer before age 80 years was recently estimated to be 6.9% (Thompson 2001). Generally, men with breast cancer present at a more advanced stage than women and have a poorer prognosis (SEER Database 2003). Knowledge of mutation status for men in *BRCA2* families may be useful for risk assessment and for prevention, as greatly reported by Liede (2004).

4.7. The *BRCA2* stop codon c.9976A>T K3326X

Furthermore, the *BRCA2* stop codon human variant c.9976A>T K3326X, was found into an affected woman, with a two primary breast cancers history, belonging to a family characterized by many cases of cancers (**Fig. 42**).

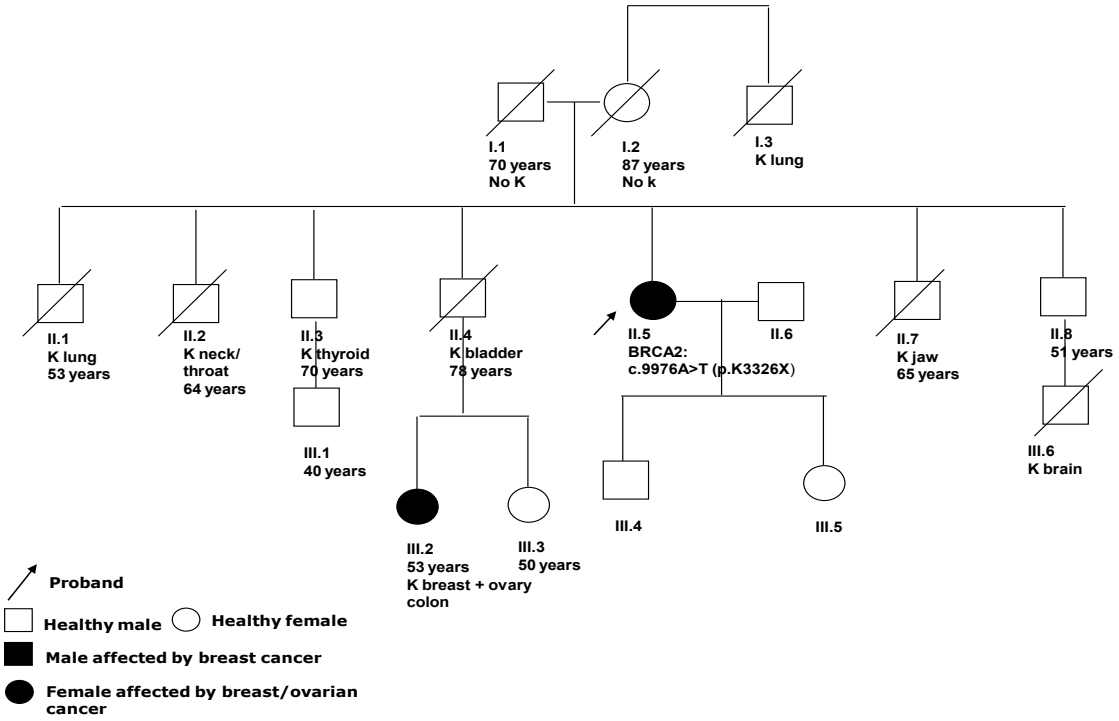


Figure 42. Genealogy of family carrying the K3326X variant.
The family shows a cluster of tumors. In particular, the patient had six brothers. Three of them were affected by a single neoplasia: one had a lung cancer at age 53 years, one had a bladder cancer at 78 years, and the third a throat cancer at 64 years. Two other brothers had been affected by two primary cancers each: one had oesophagus and lung cancers at 65 and 71 years respectively (still alive), and the other jaw and bladder cancers at the age of 52 and 65 respectively. The other four brothers died as a consequence of their disease. The sixth brother (currently 60 years old) has a negative history for cancer, however his daughter died from brain cancer at 4 years. Moreover, an uncle of the patient died from lung cancer. Finally, the daughter of the brother affected by bladder cancer has a history of ovarian cancer diagnosed at 35 years and has recently been diagnosed with rectal cancer (56 years) with hepatic lesions.

This variant was previously described as a polymorphism, also in data banks of breast cancer-predisposing mutations (Mazoyer 1996). Despite several functional studies about this variant appeared inconclusive, and it seemed unrelated to BC, a number of studies have suggested that it may increase the risk of other oncological diseases (Morimatsu 1998, Howlett 2002, Martin 2005, Rudd 2006, Akbari 2008). Although our patient's history strongly suggests the presence of familial neoplasias, the molecular analysis of *BRCA1* and *BRCA2* did not identify any causative mutation previously described. Instead, it revealed the *BRCA2* K3326X variant, which causes a premature stop codon and the loss of 93 amino acids at the protein's C-terminus. The K3326X variant was identified in only 1 allele of 200 BC analyzed patients. This corresponds to a frequency of 0.25% (calculate at the time of the patient's genetic test).

Furthermore, the variant was lately found into another patient of 39 years old, affected by BC and belonging to a family with several cases of breast, ovarian and other types of malignancies, in which no deleterious reported mutations were identified. Moreover, as described above, this variant was found into an HBOC patient already carrier of other mutations in *BRCA1* gene.

Given the above-mentioned reports (referred to manuscript attached to the thesis, D'Argenio 2014), caution should be exerted in considering *BRCA2* K3326X merely a benign polymorphism, as codified in the BIC database; rather we open the debate that it may be considered a pathogenetic variant.

4.8. VUSs and polimorphisms

Moreover, several polimorphisms and 26 (15 localized in *BRCA1* and 11 in *BRCA2*) variants with unknown clinical significance (VUSs) were also totally detected (**Tables 6-9**).

VUSs are mostly missense sequence variations without a definite role in carcinogenesis, thus representing a real clinical challenging. Indeed, in many cases they were found in women resulted negatives for *BRCA1/2* causative mutations but affected by HBOC. These findings induce to pay more attention to these variants, and to not consider lightly such as benign polimorphisms. In general, a VUS is considered "benign" only when is found together with a causative mutation.

Table 6. *BRCA1* polimorphisms.

Gene	Exon	HGVS[1] cDNA	BIC Designation	HGVS[1] Protein	Mutation Type	Clinically important (BIC)	Reference number NCBI
BRCA1	11	c.2077G>A	D693N	p.Asp693Asn	Missence	No	rs4986850
	11	c.3113A>G	E1038G	p.Glu1038Gly	Missense	No	rs16941
	11	c.3548A>G	K1183R	p.Lys1183Arg	Missense	No	rs16942
	16	c.4837A>G	S1613G	p.Ser1613Gly	Missense	No	rs1799966

Table 7. *BRCA1* VUSs.

Gene	Exon	HGVS[1] cDNA	BIC Designation	HGVS[1] Protein	Mutation Type	Clinically important (BIC)	Reference number NCBI
BRCA1	8	c.442-3_442-3delT	IVS7-3delT	-	Intervening Sequence	Unknown	n.r.[1]
	9	c.591C>T	C197C	p.Cys197Cys	Synonymous	Unknown	rs1799965
	10	c.671-12_671+12delT	IVS10+12delT	-	Intervening Sequence	Unknown	n.r.[1]
	11	c.1067A>G	Q356R	p.Gln356Arg	Missense	Unknown	rs1799950
	11	c.2082C>T	S694S	p.Ser694Ser	Synonymous	Unknown	rs1799949
	11	c.2311T>C	L771L	p.Leu771Leu	Synonymous	Unknown	rs1694011
	11	c.2612C>A	P871L	p.Pro871Gln	Missense		rs799917
	11	c.3119G>A	S1040N	p.Ser1040Asn	Missense	Unknown	rs4986852
	11	c.1911T>C	T637T	p.Thr637Thr	Synonymous	Unknown	n.r.[1]
	11	c.3418A>G	S1140G	p.Ser1140Gly	Missense	Unknown	rs2227945
	11	c.3711A>G	I1237M	p.Ile1237Met	Missense	Unknown	rs80357388
	13	c.4308T>C	S1436S	p.Ser1436Ser	Synonymous	Unknown	rs1060915
	16	c.4837A>T	S1613C	p.Ser1613Cys	Missense	Unknown	rs1799966

	16	c.4843G>A	A1615T	p.Ala1615Thr	Missense	Unknown	rs80356987
	16	c.4956G>A	M1652I	p.Met1652Ile	Missense	Unknown	rs1799967

Cont. table 6: *BRCA1* VUSs

Table 8. *BRCA2* polimorphisms.

Gene	Exon	HGVS[1] cDNA	BIC Designation	HGVS[1] Protein	Mutation Type	Clinically important (BIC)	Reference number NCBI
BRCA2	10	c.1114C>A	H372N	p.Asn372Asn	Synonymous	No	n.r.[1]
	10	c.1365A>G	S455S	p.Ser455Ser	Synonymous	No	rs1801439
	10	c.1151C>T	S384F	p.Ser384Phe	Missense	No	rs41293475
	10	c.865A>C	N289H	p.Asn289His	Missense	No	rs766173
	11	c.3396A>G	K1132K	p.Lys1132Lys	Synonymous	No	rs1801406
	11	c.4563G>A	L1521L	p.Lys1521Lys	Synonymous	No	n.r.[1]
	11	c.3807T>C	V1269V	p.Val1269Val	Synonymous	No	rs543304
	11	c.5199C>T	S1733S	p.Ser1733Ser	Synonymous	No	rs28897734
	11	c.4585G>A	G1529R	p.Gly1529Arg	Missense	No	rs28897728
	11	c.5312G>A	G1771D	p.Gly1771Asp	Missense	No	rs80358755
	11	c.6513C>G	V2171V	p.Val2171Val	Synonymous	No	n.r.[1]
	14	c.7242A>G	S2414S	p.Ser2414Ser	Synonymous	No	rs1799955
	27	**c.9976A>T**	**K3326X**	**p.Lys3326Ter**	**Nonsense**	**No**	**rs11571833**

Highlighted variant is annotated as SNP but we open the debate that it may be considered a pathogenetic variant; I found that in 3 patients affected by BC and belonging to a family with several cases of oncologic diseases. On 600 alleles of our population, this particular SNP shows an allelic frequency of 0.5%.

Table 9. *BRCA2* VUSs.

Gene	Exon	HGVS[1] cDNA	BIC Designation	HGVS[1] Protein	Mutation Type	Clinically important (BIC)	Reference number NCBI
BRCA2	10	c.865A>G	N289D	p.Asn289Asp	Missense	Unknown	rs766173
	10	c.1124C>T	P375L	p.Pro375Leu	Missense	Unknown	rs80358409
	10	c.1909+12_1909+12delT	IVS10+12delT	-	Intervening Sequence	Unknown	n.r.[1]
	11	c.2229T>C	H743H	p.His743His	Synonymous	Unknown	rs1801499
	11	c.2971A>G	N991D	p.Asn991Asp	Missense	Unknown	rs1799944
	11	c.3515C>G	S1172W	p.Ser1172Trp	Missense	Unknown	rs80358600
	11	c.3824T>C	I1275T	p.Ile1275Thr	Missense	Unknown	rs80358625
	11	c.5744C>T	T1915M	p.Thr1915Met	Missense	Unknown	n.r.[1]
	14	c.7008-20A>G	IVS13-20A>G		Intervening Sequence	Unknown	rs81002903
	16	c.7397C>T	A2466V	p.Ala2466Val	Missense	Unknown	rs169547
	16	c.7806-14T>C	IVS16-14 T>C	-	Intervening Sequence	Unknown	rs9534262

Starting from the finding of a causative mutation into an at-risk family, relatives are subsequently subjected to the note mutation research. The subsequent analysis of the families of the mutation carriers, allowed the identification of the at-risk subjects that have been involved in surveillance programs of preventing health care, especially before the disease onset. Here is shown one representative family in which was found the causative mutation in a large number of healthy carriers starting only from the criteria that in the family there were many cases of cancers (**Fig. 43**). The first proband was tested by NGS screening and, when causative mutation was found, the same mutation was searched into the relatives. Identification of mutation carriers before the onset of the disease is a very important weapon for the prevention and the fight against HBOC.

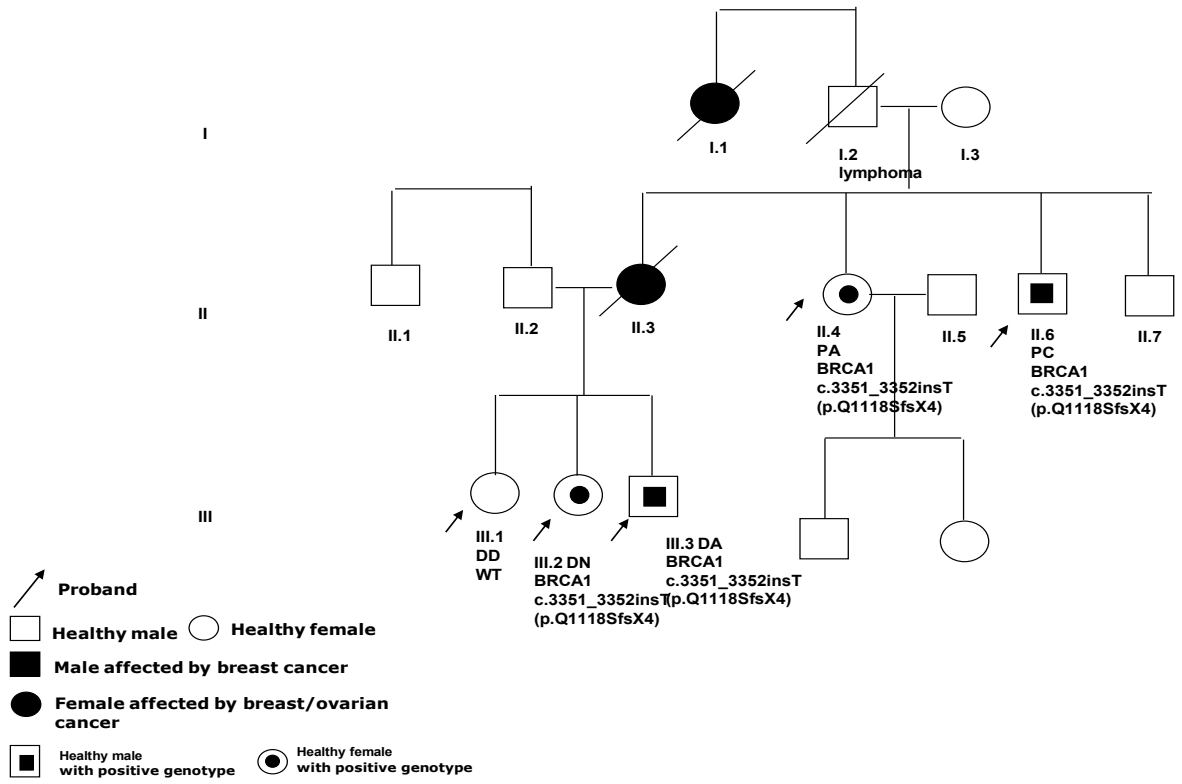


Figure 43. Genealogy of a family carrying the c.3351_3352insT Q1118SfsX*4 causative mutation in *BRCA1* gene. The analysis started from the concept that the family had a strong positive history of cancers; indeed two women died for BC and a man for lymphoma. Causative mutation was searched by the NGS screening in one healthy relative (II.4) and was confirmed into one brother and in two nephews. Also in this family is possible to note the presence of male mutation carriers.

Therefore, results of genetic testing have a great decision-making role for what concern the surgery and the treatments for BC patient especially, but also for healthy carriers of causative mutations, considering that this is a very difficult medical aspect.

Indeed, the relatively poor prognosis of young women with very-early onset breast cancer raises a critical question: should women diagnosed before the age of 40 be treated differently than older women? For example, should all very-young women with invasive breast cancer receive chemotherapy, regardless of tumour size, grade or nodal status? A second question is whether or not women with very-early onset breast cancer are candidates for breast-conserving surgery. Given that no compelling data from randomized trials have shown that there is an inferior outcome for young women treated with breast-conserving surgery, compared to mastectomy, the arguments in favour of mastectomy are indirect: first, the risk of local recurrence is much higher for young women than for older women; second, the mortality rate for young women after a local recurrence is very high. This risk of contralateral breast cancer is also relevant, in particular if contralateral preventive mastectomy is being considered. Risk factors for contralateral breast cancer include young age at diagnosis, and the presence of *BRCA1* or *BRCA2* mutation.

However, for a 30-year-old woman with breast cancer, the 10-year cumulative risk of contralateral breast cancer is only about 5%. It has been shown that contralateral mastectomy reduces the incidence of contralateral breast cancer in women with a hereditary predisposition and in women at high risk, but there are few data on mortality benefit (Narod 2013). A third question is whether or not there is benefit from ovarian ablation that goes beyond that of chemotherapy and hormone therapy. In *BRCA1* and *BRCA2* carriers, oophorectomy is recommended to prevent cancer recurrence, contralateral breast cancer and new primary ovarian cancer, but in non-carriers there is less evidence in support of the procedure.

The Early Breast Cancer Trialists Collaborative Group study confirmed the benefit of chemotherapy in treating breast cancer in women diagnosed before the age of 50. Overall, the mortality rate was reduced by 38% with adjuvant chemotherapy (EBCTCG 2012). They also reported that the benefit of chemotherapy was present for very-young women with node-negative breast cancer and with small breast cancers. Young age was a risk factor for recurrence, but the adverse effect of age on survival was greatly attenuated in women treated with chemotherapy. Interestingly, this finding is analogous to the situation in *BRCA1*-associated cancers, wherein the adverse effect of a *BRCA1* mutation is present only in women not treated with chemotherapy. The situation is easier for hormone receptor-negative cases, where there are fewer treatment options. For young women with receptor-positive cancer, alternates to cytotoxic chemotherapy include tamoxifen, ovarian ablation (surgical or chemical suppression) and in some cases, the addition of an aromatase inhibitor. Several studies suggest that for women with low-risk hormone receptor-positive disease, endocrine therapy might be as effective as cytotoxic chemotherapy. Accordingly, there is much interest in

preventing breast cancer. In this regard, recently, Fenretinide (4-hydroxy(phenyl)retinamide; 4-HPR), a synthetic retinoid derivative, is emerging as a protective drug in *BRCA1/2* mutation carriers women. Our group participates to a collaboration with IEO of Milan to test this drug on mutation carriers healthy relatives of BC patients, in order to understand the role of this "natural" drug on the disease onset during the next few years.

Further, the argument for breast cancer awareness is more compelling. Currently, routine mammographic breast screening is not recommended for women under the age of 40. Nevertheless, if is accepted that BC should be diagnosed at the smallest possible size, and that tumor size is a good predictor of mortality, then reducing mean tumor size will have a beneficial effect on mortality. Breast self-examination is promoted throughout the world as an inexpensive alternative to mammography, if added to periodic preventive echographies.

However, as greatly reported in medical literature, is still difficult to manage treatments of an HBOC mutation carrier patient.

In this regard, lately, a little breast tumor with a good response to chemiopreventive treatment was found into a young patient (44 years old). Starting from these evidences, the patient was candidate only for a quadrantectomy. Meanwhile, she underwent genetic testing and resulted carrier of a *BRCA1* causative mutation, in particular of c.3403C>T p.Q1135X. The rapidity of genetic result has leaded medical decisions to a total mastectomy and to a different and more aggressive pharmacological treatments, in order to better remove cancer and to avoid recurrence of pathology.

These results assess the feasibility of a next generation sequencing approach for *BRCA1/BRCA2* mutation detection to be included in a routine diagnostic workflow, because of speed and cost-sparing features of this recently developed methodology.

Nevertheless, when genetic informations about patients are collected, management of genetic counseling is a very sensitive and difficult aspect. Women who carry a *BRCA* mutation are candidate for options of early and intensive surveillance, chemoprevention and prophylactic surgery. Oncologists and geneticists help women understand the risk that their *BRCA* status represents for them and their relatives. They also identify and address biopsychosocial factors that may foster women's adherence to preventive and/or risk-reducing strategies, in order to reduce the morbidity and mortality associated with hereditary or familial cancers.

Since the initial application of *BRCA* testing in research first, and subsequently also in oncology practices, scientists, physicians and bioethicists have consistently cared about the medical and psychosocial well-being of mutation carriers, and have cautioned the public about the limited predictive power of genetic testing, especially outside high-risk families, due to the relatively low gene penetrance, the possibility of new mutations with different significance that are still to be identified, and the role of environmental factors in cancerogenesis and tumor

progression. Furthermore, preventive and interventional measures are still being developed and genetic testing carries potentially negative psychosocial repercussions for individual carriers and their families. Knowledge about the ethical and juridical implications of genetic testing is becoming essential for oncologists, who are being asked with increasing frequency to counsel their patients with respect to the medical, psychological and social repercussions of genetic information, even when obtained outside the context of an established patient-doctor relationship. Many articles and reviews have addressed the main ethical and social implications of *BRCA* testing (Surbone 2011), including informed consent, privacy and confidentiality, a person's right wheter or not to know their genetic information, carriers' responsibility to share genetic information with relatives at risk, reproductive choices based on preimplantation diagnosis, appropriate testing of children and adolescents, equitable access to testing, disposition of biological samples, and genetic discrimination.

Indeed, genetic information refers to genetic testing for patients and/or for family members up to fourth-degree relatives. A genetic test is any analysis to detect genotypes, genetic mutations or chromosomal changes, not including analysis of proteins or metabolites directly related to a manifested disease. Genetic information also refers to any manifestation of disease or disorder in a family member, and/or to the participation of a person or family member in research that involves genetic testing, counseling or education.

Genetic information thus has unique aspects with respect to other medical information, as it carries potential value, but also danger, for individuals other than the person tested. Beyond the ethical and legal implications of genetic testing, experts and the public have expressed different views on the value of *BRCA* testing, from stressing the importance of genetic knowledge for high-risk women as a means to enhance control of their lives, to worrying about the potentially negative repercussions of genetic information.

5. CONCLUSIONS

In recent years the spectrum of cancer-related genes has widened considerably. The potential of new sequencing technologies, from whole genome to exome sequencing, can accelerate the discovery of new susceptibility genes, not only for breast cancer, but also for other types of cancer. Targeted capture and massively parallel sequencing of specific genes can successfully identify families at risk for developing breast and/or ovarian cancer; now it seems that this technique is ready to be applied in a clinical setting. Knowing the genetic defect responsible for cancer-predisposition in at risk families can pave the way to customized, targeted therapies with extremely beneficial results. Increasing evidences indicate that knowledge of the *BRCA1* and *BRCA2* mutation status will enable a patient and her/his health care provider to make informed decisions about cancer prevention, screening and treatment. To date, there is a compelling evidence of the efficancy of the surgical prevention of hereditary breast and ovarian cancer, through mastectomy and oophorectomy; however, further studies need to be performed on chemoprevention and on individualized therapy for women with BC and a *BRCA1* or *BRCA2* mutation. Nevertheless, genetic counseling for these genes can be complicated.

The above NGS mentioned method allowed the discovery of *BRCA1/BRCA2* mutations not only in BC affected subjects but also in their relatives, in order to improve prevention and treatment of *BRCA*-mutation carriers. In addition, functional studies performed on novel variants, and ones that will be performed in the future, will contribute to improve the knowledge about HBOC genetics and to make medical decisions: indeed, a variant considered deleterious will be treated differently, and more safety, from a VUS that remains of uncertain significance and difficult to approach.

Therefore, based on these findings the future aims of this work are to enlarge the number of women enrolled for *BRCA1/BRCA2* screening in order to improve and increase the prevention of BC. This increase of prevention must be done especially in young women and before the disease onset, but also in men who in the past were wrongly considered not at risk to develop BC or other malignancies connected to *BRCA* mutations. The study proposes, on one hand, to include known causative mutation-carriers in clinical studies, and on the other hand to build panels of disease-related genes for NGS. Indeed, one of the future aims of this work will be to screen negative *BRCA* breast cancer patients with a strong family history of cancers, using large panels of cancer-related genes, through Illumina sequencing technologies. This second level screening will be useful to find other BC-related genes and to improve the knowledge about HBOC genetics.

Finally, the methods described in this study will open new aspects of cancer knowledge and management, not only to improve early BC diagnosis but especially to direct therapies to “personalized” medicine.

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