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PHENOTYPIC AND GENOTYPIC HETEROGENEITY OF HEREDITARY BREAST AND OVARIAN CANCER

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Dissertation for applying to a Doctoral degree in Biomedical Sciences submitted to the Institute of Biomedical Sciences Abel Salazar of the University of Porto.

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SUMMARY

Inherited predisposition to breast cancer is estimated to account for about 5-10% of all cases and is characterized by an autosomal dominant pattern of inheritance, young age at presentation, and association with bilateral breast cancer and ovarian cancer. Germline pathogenic mutations in the *BRCA1* and *BRCA2* genes are responsible for the Hereditary Breast and Ovarian Cancer (HBOC) syndrome. Mutations in the *BRCA1*/*BRCA2* genes have also been associated with inherited predisposition to other cancers in HBOC families, like those of the prostate, pancreas, male breast, peritoneum, and fallopian tube. Molecular analyses of the *BRCA1* and *BRCA2* genes have shown that most populations exhibit a wide spectrum of mutations throughout both genes and several founder mutations have been identified in individuals of different ancestries. In the Portuguese population, the *BRCA2* c.156_157insAlu and the *BRCA1* c.3331_3334del account for about 43% of the total deleterious mutations in these genes. Multiple other genes, besides *BRCA1* and *BRCA2*, have been described as conferring an increased risk for the development of breast or ovarian cancer when mutated and many of these genes are involved in homologous DNA recombination.

The aims of this thesis were to characterize the phenotypic heterogeneity associated with *BRCA1* and *BRCA2* mutations and the genetic heterogeneity of hereditary breast and ovarian cancer. Specifically, the objectives of this thesis were: a) To develop a method to detect the founder mutations *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del in formalin-fixed paraffin-embedded archival tissue; b) To quantify the contribution of the founder mutations *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del for cancer etiology in unselected hospital-based cohorts of patients diagnosed with rarer cancers associated with HBOC, namely, cancer of the pancreas, male breast, peritoneum, and fallopian tube; c) To compare the sensitivity and specificity of next-generation sequencing (NGS) and those of Sanger sequencing for the detection of point mutations in the *BRCA1* and *BRCA2* genes; d) To evaluate the genetic heterogeneity of hereditary breast and ovarian cancer by analyzing a panel of 17 genes associated with predisposition to these diseases in a consecutive series of high-risk breast/ovarian cancer families.

The *BRCA2* c.156_157insAlu mutation was observed with a frequency of 7.8% in male breast cancers, 3.0% in peritoneal/fallopian tube cancers, and 1.6% in pancreatic cancers, with estimated total contributions of germline *BRCA2* mutations of 14.3%, 5.5%, and 2.8%, respectively. No carriers of the *BRCA1* c.3331_3334del mutation were identified. During our study, a patient with an ampulla of Vater carcinoma was incidentally found to carry the *BRCA2* c.156_157insAlu mutation, so we decided to test a consecutive series of additional 15 ampullary carcinomas for *BRCA1/BRCA2* mutations using a combination of direct founder mutation testing and full gene analysis with NGS. *BRCA2* mutations were observed with a frequency of 14.3% in ampulla of Vater carcinomas. In suspected HBOC families, the frequency of deleterious mutations identified was 22.3% for *BRCA2,* 10.6% for *BRCA1*, 5% for *PALB2*, 2.5% for *ATM*, and 1.3% for both *CHEK2* and *TP53*. In addition, the efficiency of NGS for the detection of *BRCA1/BRCA2* point mutations was validated with a 100% sensitivity and specificity obtained when compared to the gold standard Sanger sequencing.

The main conclusions of this thesis are: a) The detection of germline founder mutations and full *BRCA1*/*BRCA2* gene analysis are possible in archival tissue, making it an alternative for the molecular diagnosis of inherited predisposition; b) *BRCA2* germline mutations are estimated to occur in 14.3% of male breast cancers, 5.5% of peritoneal/fallopian tube cancers, and 2.8% of pancreatic cancers; c) *BRCA2* germline mutations were observed recurrently for the first time in patients with ampulla of Vater carcinomas, with a frequency of 14.3%; d) The sensitivity and specificity of NGS are as high as those of the gold-standard Sanger sequencing for the detection of *BRCA1*/*BRCA2* germline point mutations, when a validated bioinformatic pipeline is used; e) Hereditary breast and ovarian cancer is genetically heterogeneous, with 20.5% of the germline deleterious mutations being found in genes other than *BRCA1*/*BRCA2*.

RESUMO

A predisposição hereditária para cancro da mama é responsável por cerca de 5-10% de todos os casos e é caracterizada por um padrão de transmissão autossómico dominante, idade precoce de diagnóstico e associação com cancro da mama bilateral e cancro do ovário. Mutações germinativas patogénicas nos genes *BRCA1* e *BRCA2* predispõem para a síndrome de cancro da mama/ovário hereditário (Hereditary Breast and Ovarian Cancer – HBOC). Mutações nestes genes estão também associadas com predisposição para outros tumores em famílias HBOC, nomeadamente, tumores da próstata, pâncreas, peritoneu, trompa do Falópio e tumores da mama em homens. A análise molecular dos genes *BRCA1* e *BRCA2* mostra que a maioria das populações apresenta um padrão de mutações heterogéneo, havendo várias mutações fundadoras identificadas em diferentes populações. Na população portuguesa, as mutações *BRCA2* c.156_157insAlu e *BRCA1* c.3331_3334del representam cerca de 43% de todas as mutações patogénicas nestes genes. Múltiplos outros genes, para além dos genes *BRCA1* e *BRCA2*, estão descritos como conferindo um risco aumentado para o desenvolvimento de cancro da mama ou do ovário quando mutados, estando estes normalmente envolvidos na recombinação homóloga do DNA.

O presente trabalho teve como objetivos a caracterização da heterogeneidade fenotípica associada a mutações nos genes *BRCA1* e *BRCA2* e da heterogeneidade genética do cancro hereditário da mama e do ovário. Mais especificamente, os objetivos foram: a) Desenvolver um método para a deteção das mutações fundadoras *BRCA2* c.156_157insAlu e *BRCA1* c.3331_3334del em tecido fixado em formalina e incluído em parafina; b) Quantificar a contribuição das mutações fundadoras *BRCA2* c.156_157insAlu e *BRCA1* c.3331_3334del para a etiologia de cancro em pacientes diagnosticados com tumores mais raros associados a HBOC, nomeadamente, carcinomas do pâncreas, peritoneu, trompa do Falópio e da mama masculino; c) Comparar a sensibilidade e especificidade da sequenciação de nova-geração (Next-generation sequencing – NGS) e da sequenciação de Sanger para a deteção de mutações pontuais nos genes *BRCA1* e *BRCA2*; d) Avaliar a heterogeneidade genética do cancro hereditário da mama e do ovário, analisando um painel de 17

genes associados a predisposição para estes tumores numa série consecutiva de famílias com alto risco para cancro da mama/ovário.

A mutação *BRCA2* c.156_157insAlu foi observada com uma frequência de 7.8% em homens com cancro da mama, 3.0% em carcinomas peritoneais/trompa do Falópio, e 1.6% em carcinomas do pâncreas, com estimativas de mutações germinativas no gene *BRCA2* de 14.3%, 5.5% e 2.8%, respetivamente. Não foram identificados portadores da mutação *BRCA1* c.3331_3334del. Durante o estudo, um paciente com carcinoma da ampola de Vater foi identificado como sendo portador da mutação *BRCA2* c.156_157insAlu, pelo que analisamos uma série adicional consecutiva de 15 tumores da ampola de Vater para a presença de mutações nos genes *BRCA1/BRCA2* usando uma combinação de pesquisa de mutações fundadoras com análise completa destes genes por NGS. Mutações no gene *BRCA2* foram observadas com uma frequência de 14.3% em carcinomas da ampola de Vater. Em famílias suspeitas de HBOC, a frequência de mutações patogénicas identificada foi de 22.3% no gene *BRCA2,* 10.6% no *BRCA1*, 5% no *PALB2*, 2.5% no *ATM*, e 1.3% nos genes *CHEK2* e *TP53*. Adicionalmente, a eficiência de NGS para a deteção de mutações pontuais nos genes *BRCA1/BRCA2* foi validada, tendo sido obtida uma sensibilidade e especificidade de 100% comparada com a sequenciação de Sanger.

As principais conclusões desta tese são: a) A deteção de mutações fundadoras germinativas e a análise completa dos genes *BRCA1/BRCA2* é possível em tecido de arquivo, sendo uma alternativa para o diagnóstico molecular de predisposição hereditária; b) Mutações germinativas no gene *BRCA2* estimaram-se ocorrer em 14.3% dos homens com cancro da mama, 5.5% dos carcinomas peritoneais/trompa do Falópio e 2.8% em carcinomas do pâncreas; c) Mutações germinativas no gene *BRCA2* foram observadas recorrentemente pela primeira vez em pacientes com carcinoma ampular, com uma frequência de 14.3%; d) A sensibilidade e especificidade da NGS são tão elevadas como as da sequenciação de Sanger para a deteção de mutações pontuais nos genes *BRCA1/BRCA2*, quando uma “pipeline” bioinformática validada é utilizada; e) O cancro hereditário da mama e do ovário é geneticamente heterogéneo, sendo que 20.5% de todas as mutações patogénicas identificadas são em outros genes que não os genes *BRCA1/BRCA2*.

PUBLICATIONS

Ao abrigo do nº2, alínea a, do artigo 31º do Decreto-Lei n.º 230/2009 de 14 de Setembro, fazem parte integrante desta tese de doutoramento os seguintes manuscritos aceites para publicação:

PAPER I

Analysis of founder mutations in rare tumors associated with hereditary breast/ovarian cancer reveals a novel association of *BRCA2* mutations with ampulla of Vater carcinomas

Pedro Pinto, Ana Peixoto, Catarina Santos, Patrícia Rocha, Carla Pinto, Manuela Pinheiro, Luís Leça, Ana Teresa Martins, Verónica Ferreira, Carla Bartosch, Manuel R. Teixeira

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PAPER II

Implementation of next-generation sequencing for molecular diagnosis of hereditary breast and ovarian cancer highlights its genetic heterogeneity

Pedro Pinto, Paula Paulo, Catarina Santos, Patrícia Rocha, Carla Pinto, Isabel Veiga, Manuela Pinheiro, Ana Peixoto, Manuel R. Teixeira

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LIST OF ABBREVIATIONS

ASR	Age-standardized rate
BER	Base excision repair
BRCT	BRCA1 C-terminal
CI	Confidence interval
DBD	DNA binding domain
DCIS	Ductal carcinoma *in situ*
DDR	DNA damage response
DSBs	Double-stranded DNA breaks
dsDNA	Double-strand DNA
FANC-J	Fanconi anemia complementation group J
FFPE	Formalin-fixed, paraffin-embedded
GWAS	Genome-wide association studies
HBOC	Hereditary breast and ovarian cancer
HDGC	Hereditary diffuse gastric carcinoma
HER2	Human epidermal growth factor receptor 2
HR	Homologous recombination
IARC	International Agency for Research on Cancer
LFS	Li-Fraumeni syndrome
LGRs	Large gene rearrangements
MLPA	Multiplex ligation-dependent probe amplification
MMR	Mismatch repair genes
MRI	Magnetic resonance imaging
MRN	MRE11-RAD50-NBS1
NCCN	National Comprehensive Cancer Network
NGS	Next-generation sequencing
NHEJ	Nonhomologous end joining
NLS	Nuclear localization signals

NST	No special type
OB	Oligonucleotide binding
PARP	Poly (ADP-ribose) polymerase
PIKK	PI3K-related protein kinases
RING	Really interesting new gene
RPA	Replication protein A
RR	Relative risk
RRBSO	Risk-reducing bilateral salpingo-oophorectomy
RRM	Risk-reducing mastectomy
SNPs	Single nucleotide polymorphisms
SSA	Single-strand annealing
ssDNA	Single-strand DNA
USA	United States of America
VUS	Variants of uncertain significance

INTRODUCTION

1. Cancer epidemiology

Cancer is a major concern in public health and despite the efforts to improve prevention, diagnosis and treatment, the incidence is expected to grow, mostly due to the growth and aging of the world population and the increasing prevalence of established risk factors worldwide [Torre *et al*, 2015]. In 2012, 14.1 million new cases and 8.2 million cancer-related deaths were estimated by GLOBOCAN, through the International Agency for Research on Cancer (IARC) [Ferlay *et al*, 2015]. Cancer epidemiology is different between developed and developing countries, with the most incident and leading cancer death being lung cancer among males and breast cancer among females in less developed countries. In developed countries, although lung cancer leads mortality rates, prostate and breast cancer are the most incident, respectively, among males and females. In Europe, breast, colorectal, prostate and lung cancers are the most frequently diagnosed cancers, together representing half of the overall cancer burden [Ferlay *et al*, 2013].

1.1. Breast cancer epidemiology and risk factors

Breast cancer is the most frequent cancer and the leading cause of cancer death among females worldwide, with estimates of 1.7 million cases and 522,000 deaths in 2012, representing 25% and 15% of all cancer cases and deaths, respectively [Torre *et al*, 2015]. In males, breast cancer is a rare disease. Incidence rates are higher in more developed countries when compared with less developed countries. In most of the developed countries incidence rates have been stable recently, with mortality rates decreasing. In contrast, in less developed countries both the incidence and mortality rates are increasing [DeSantis *et al*, 2015]. In the United States of America (USA), the 5-year survival rate has increased from 60% in the 1950s to about 90% in the 2000s [Ban and Godellas, 2014]. In Portugal, breast cancer is the most frequent cancer among women, representing about 30% of the cancers diagnosed, with an estimated age-standardized rate (ASR) incidence of 85.6 per 100,000. Breast cancer is the main cause of death by cancer in Portuguese women, with an estimated ASR of 18.4 in 2012 [Ferlay *et al*, 2013].

Similar to most cancers, age is an established risk factor for breast cancer. The incidence of breast cancer increases rapidly with age during the reproductive years, increasing at a slower rate after 50 years old, the average age at menopause [Key *et al*, 2001]. Gender is probably the most important risk factor of breast cancer, being at least 100 times more common in women than in men. A higher exposure of the breast tissue to endogenous and exogenous hormones (progesterone and, especially, estrogen) also increases the risk of breast cancer. Reproductive hormones stimulate cell division, thereby increasing the likelihood of DNA damage and the risk of cancer. Hence, factors such as an early menarche, late menopause, use of oral contraceptives, hormone replacement therapy and higher serum concentration of endogenous hormones all contribute to an increase in breast cancer risk [Hsieh *et al*, 1990, Collaborative Group on Hormonal Factors in Breast Cancer, 1997, Key and Verkasalo, 1999, Hunter *et al*, 2010]. Childbearing has a dual effect on breast cancer risk; immediately after pregnancy the risk is higher, but it diminishes gradually and in the long term there is a protective effect [Lambe *et al*, 1994]. Women with a personal and/or family history of breast cancer have an increased risk for developing breast cancer, being about double of the general population if the affected member is a first degree relative [Pharoah *et al*, 1997]. Familial aggregation is present in about 20% of the cases and can be attributed to genetic, environmental, and lifestyle factors. Pathogenic mutations in the *BRCA1* and *BRCA2* genes account for about 5-10% of all breast cancers and are responsible for the Hereditary Breast and Ovarian Cancer (HBOC) syndrome. The cumulative risk for developing breast cancer at 70 years old is 60% for *BRCA1* mutation carriers and 55% for *BRCA2* carriers [Mavaddat *et al*, 2013]. Other breast cancer risk factors include race, ethnicity, breast density, breast benign lesions, breastfeeding, alcohol use, diet, physical activity and exposure to radiation [Ban and Godellas, 2014].

1.2. Ovarian cancer epidemiology and risk factors

Ovarian cancer is the seventh most common cancer worldwide among women, with 238,700 new cancer cases diagnosed in 2012 and the eight most lethal cancer

with 151,900 deaths estimated [Torre *et al*, 2015]. In Portugal, it accounts for about 3% of the cancers diagnosed in women, with an estimated ASR incidence of 8.2 and is the sixth cause of cancer death in Portuguese females with an estimated ASR of 4.4 [Ferlay *et al*, 2013].

The risk of ovarian cancer increases with age, whereas the use of oral contraceptives confers long-term protection [Tsilidis *et al*, 2011, Doufekas and Olaitan, 2014]. Factors that interrupt ovulation, such as pregnancy and breastfeeding, are also associated with a reduced risk of developing ovarian cancer [Whittemore *et al*, 1992, Adami *et al*, 1994]. Women with endometriosis have an increased risk of ovarian cancer and the use of hormone replacement therapy is also associated with a small increase in risk [Modugno *et al*, 2004, Beral *et al*, 2007]. Women with a personal history of breast cancer have a two-fold increase in ovarian cancer risk, increasing to four-fold if it was diagnosed before 40 years old and even more if they also have a family history of breast and/or ovarian cancer [Bergfeldt *et al*, 2002]. Pathogenic mutations in *BRCA1* or *BRCA2* confer a cumulative risk for developing ovarian cancer at age 70 of 59% and 17%, respectively [Mavaddat *et al*, 2013]. Other risk factors such as age at menarche and menopause or infertility have been studied, but without a clear association demonstrated [Whittemore *et al*, 1992, Venn *et al*, 1995].

2. Inherited predisposition to breast and ovarian cancer

Descriptions of families with multiple cases of breast cancer date back to ancient Greek physicians. In 1866, Paul Broca was the first to report in detail a family with multiple generations affected with breast cancer. At the time, he hypothesized that breast cancer in this family was hereditary, present in a “latent state” until later in life, when it presented and progressed to a malignant disease. In the 1920s, Janet Elizabeth Lane-Claypon demonstrated that women whose mothers had died of breast cancer had an increased mortality due to breast cancer when compared with women whose mothers had died of other causes. By the 1970s, multiple families with two or more first-degree relatives affected with breast cancer in association with ovarian cancer and other cancers were described together with epidemiological studies

showing that the risk of breast cancer was increased in first-degree relatives of affected women [Anderson, 1972, Lynch *et al*, 1972]. In 1988, Newman and colleagues evaluated a total of 1579 families and demonstrated that familial clustering of breast cancer was fully explained by an autosomal dominant, highly penetrant susceptibility gene. Using a mathematical model, they predicted that in 4% of the families breast cancer could be explained by the presence of a susceptibility gene and that in these, the risk of breast cancer by age 70 was 82% [Newman *et al*, 1988]. By that time, “the race” to find a high susceptibility gene to breast cancer was ongoing and in 1990, Hall and coworkers [1990] mapped a hypothetical gene to chromosome 17q21, which was immediately confirmed by Narod and colleagues [1991], who mapped predisposition to breast and ovarian cancer on the same location in different families. It was only four years later that the *BRCA1* gene was positionally cloned by Miki et al [1994] and subsequently confirmed in an independent study [Friedman *et al*, 1994]. These two studies together presented 15 families with truncating mutations cosegregating with breast and ovarian cancer. One year later, a second breast cancer gene, *BRCA2*, located on chromosome 13q12-13, was identified with germline mutations present in six different families [Wooster *et al*, 1995].

Inherited predisposition to breast cancer is estimated to account for about 5-10% of all cases. Breast cancer susceptibility genes can be divided in three classes: rare high penetrance genes, rare moderate penetrance genes and common low penetrance alleles. High penetrance genes are those who confer a risk of breast cancer, defined in terms of disease incidence, more than four times as high as that in the general population and they were mostly identified through linkage analysis. Moderate penetrance genes are those who confer a risk between two to four times higher than the general population and most have been identified by mutational screening of candidate genes. Genome-wide association studies (GWAS) have been used to identify low penetrance variants and they all confer risks that are less than 1.5 times as high as those in general population [Turnbull and Rahman, 2008, Easton *et al*, 2015]. Ovarian cancer susceptibility genes include *BRCA1*, *BRCA2*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *BRIP1*, *RAD51C* and *RAD51D* [ten Broeke *et al*, 2015, Norquist *et al*, 2016].

Pathogenic mutations in the *BRCA1* and *BRCA2* genes account for about 15% of familial breast cancers and 30% of high-risk breast cancer families, with mutations in other high or moderate penetrance genes accounting for about 7% of familial breast cancer (Figure 1) [Couch *et al*, 2014]. These families are often characterized by an autosomal dominant pattern of inheritance, young age at presentation, and association with bilateral breast cancer and ovarian cancer.

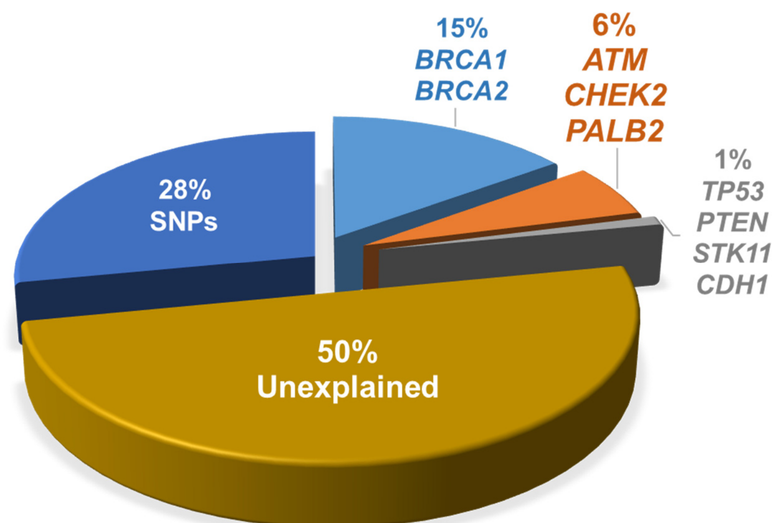

Figure 1 – Estimated percentage contribution of genetic variants that predispose to familial breast cancer, namely, *BRCA1* and *BRCA2* genes, other high penetrance genes (*TP53, PTEN, STK11* and *CDH1)*, moderate penetrance genes (*ATM*, *CHEK2* and *PALB2*), and common low penetrance alleles (Single Nucleotide Polymorphisms, SNPs) [adapted from Couch *et al*, 2014].

2.1. Hereditary breast and ovarian cancer syndrome

2.1.1. *BRCA1*

BRCA1 is a large gene with 23 exons (22 of them coding) encoding a protein with 1863 aminoacids and a predicted molecular mass of 207kDa. Exon 11 is unusually large and encodes almost 60% of the full length BRCA1 protein. The *BRCA1* gene is ubiquitously expressed and plays a role in multiple DNA repair pathways, namely, homologous recombination (HR), nonhomologous end joining (NHEJ) and

single-strand annealing (SSA) and in checkpoint regulation [Roy *et al*, 2012]. This gene contains two highly conserved domains in the N- and C-terminal regions of the protein (Figure 2). The N-terminal region of BRCA1 has the RING (Really Interesting New Gene) domain (aminoacids 1-109) with a conserved pattern of cysteine and histidine residues that is found in a large number of proteins and functions as an E3 ligase enzyme involved in ubiquitination [Clark *et al*, 2012]. It also encompasses sequences responsible for the interaction and formation of a heterodimer with BARD1, which enhances BRCA1 ubiquitin ligase activity [Wu *et al*, 1996]. At the C-terminal end lie two tandem repeat globular domains (aminoacids 1650-1863), termed BRCA1 C-terminal (BRCT), a common feature of proteins involved in the DNA damage repair and cell cycle control [Clark *et al*, 2012]. This domain is responsible for interactions with other proteins involved in DNA damage repair (Abraxas, BRIP1 and CtIP) that are phosphorylated by DNA damage-activated kinases, such as ATM [Huen *et al*, 2010].

Figure 2 – BRCA1 functional domains. At the N-terminus lies a RING domain (encoded by exons 2-7, aminoacids 1-109) and two NLS within the large central exon 11 (aminoacids 503-508 and 607-614). The C-terminus of BRCA1 contains a coiled-coil domain spanning exons 11-13 (aminoacids 1364-1437) that associates with PALB2, and a BRCT domain (exons 16-24, aminoacids 1650-1863) that binds to Abraxas, CtIP and BRIP1 [Narod and Foulkes, 2004, Clark *et al*, 2012, Roy *et al*, 2012].

Other BRCA1 functional domains include two central nuclear localization signals (NLS) within exon 11 (aminoacids 503-508 and 607-614) and a coiled-coil domain spanning exons 11-13 (aminoacids 1364-1437). NLS domains are highly important for BRCA1 localization mediating BRCA1 transport from the cytosol to the nucleus, whereas the coiled-coil domain mediates protein-protein interactions and contains the binding site for PALB2 protein [Clark *et al*, 2012]. Mutations in this domain inhibit the interaction between BRCA1 and PALB2 [Sy *et al*, 2009].

The prevalence of *BRCA1* mutations in the general population is estimated to be about 0.1%, 3.7% in women diagnosed with breast cancer and 9.5% in women diagnosed with ovarian cancer [Lalloo and Evans, 2012, Norquist *et al*, 2016, Tung *et al*, 2016]. More than 1800 rare variants have been reported, most of them only once. Mutations are found throughout the coding sequence of the gene, with the majority of pathogenic mutations being either frameshift or nonsense mutations that result in truncated proteins. Missense mutations account for approximately 2% of pathogenic mutations in *BRCA1,* usually in either the RING or BRCT domains [Lalloo and Evans, 2012]. Large gene rearrangements (LGRs), including deletions and duplications of one or more exons represent 10-15% of all deleterious germline mutations in *BRCA1* [Mazoyer, 2005, Sluiter and van Rensburg, 2011]. Mutations in either the 5' or 3' end of the gene are more associated with breast cancer, whereas mutations in the central part of *BRCA1* (approximately exon 11) are associated with the development of ovarian cancer [Rebbeck *et al*, 2015].

2.1.2. *BRCA2*

BRCA2 is a large gene with 27 exons, 26 of them coding, encoding a 3418 aminoacid protein with a predicted molecular mass of 384kDa. Like in *BRCA1*, exon 11 is the largest. BRCA2 primary function is to facilitate HR but it is also involved in protection of the DNA replication fork [Schlacher *et al*, 2011]. It can be divided into three regions: the N-terminal region, the BRC repeat region, and the C-terminal region containing a DNA Binding Domain (DBD) and an NLS domain (Figure 3). The N-terminal region contains a conserved sequence (aminoacids 21-39) that provides a binding site for PALB2 protein [Oliver *et al*, 2009]. In the central region of the BRCA2 protein there are eight copies of the BRC repeat motifs of ~40 residues each (aminoacids 900-2000), which play a central role in mediating binding to RAD51 [Bork *et al*, 1996, Chen *et al*, 1998]. The C-terminal region (aminoacids 2459-3190) contains a DBD, which comprises a 190 aminoacid helical domain, three oligonucleotide binding (OB) folds that are single-strand DNA-binding modules, and a tower domain that mediates BRCA2 binding to single-strand DNA (ssDNA) and double-strand DNA

(dsDNA). The helical domain, OB1 and OB2 also associate with DSS1, a small acidic protein that has been linked to BRCA2 protein stabilization [Yang *et al*, 2002]. In the C-terminus region there is another RAD51 binding site (aminoacids 3265-3330) and two NLS (aminoacids 3263-3269 and 3381-3385) that are important for the translocation of BRCA2 to the nucleus [Spain *et al*, 1999].

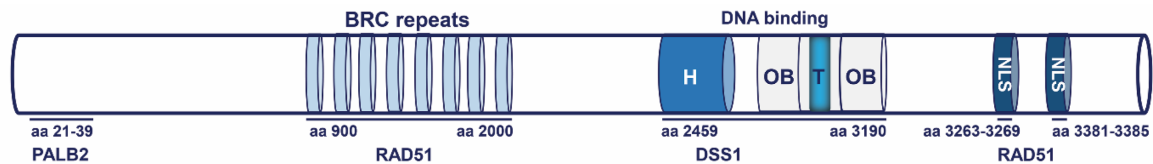

Figure 3 – Functional domains of the *BRCA2* gene. The N-terminus binds to PALB2 at aminoacids 21-39. The central region (within exon 11) contains eight copies of the BRC repeat motifs (aminoacids 900-2000), which mediates binding to the RAD51 recombinase. The C-terminal region (aminoacids 2459-3190) contains a DBD, which includes a helical domain (H), three OB folds, and a tower domain (T). This domain also associates with DSS1. The C-terminus of BRCA2 contains another RAD51 binding site (aminoacids 3265-3330) and two NLS (aminoacids 3263-3269 and 3381-3385) [Venkitaraman, 2009, Roy *et al*, 2012, Guidugli *et al*, 2014].

BRCA2 mutations are present in about 0.1% of the general population, in 2.5% of women with breast cancer and in 5.1% of women diagnosed with ovarian cancer [Lalloo and Evans, 2012, Norquist *et al*, 2016, Tung *et al*, 2016]. More than 2000 individual variants have been described and, similar to *BRCA1*, there are no hotspots for mutations and most of the pathogenic mutations are either frameshift or nonsense. Pathogenic missense mutations are usually found within the DBD domain [Guidugli *et al*, 2013]. The frequency of LGRs is lower, accounting for 1-7% of all deleterious mutations [Mazoyer, 2005, Sluiter and van Rensburg, 2011]. Biallelic mutations in *BRCA2* have been shown to cause Fanconi anemia, a condition characterized by multiple congenital abnormalities including short stature and microcephaly, and predisposition to childhood solid tumors and hematological malignancies [Reid *et al*, 2005]. As in *BRCA1,* breast cancer cluster regions are found in the 5' and 3' end of the gene with ovarian cancer cluster regions located in the central region of *BRCA2* [Rebbeck *et al*, 2015].

2.1.3. Cancers associated with *BRCA1* and *BRCA2* mutations

HBOC syndrome is an autosomal dominant disease with incomplete penetrance. The most common cancers associated with this syndrome are breast and ovarian cancer. Women carrying germline *BRCA1* mutations have a cumulative risk at 70 years of 60% for breast cancer and 59% for ovarian cancer, whereas *BRCA2* mutations appear to confer a similar risk of breast cancer in females (55%), but a lower risk (17%) for ovarian cancer [Mavaddat *et al*, 2013].

Most *BRCA1* breast tumors are high grade, invasive breast carcinomas of no special type (NST), with a high incidence of triple negative tumors: negative staining for estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) [Mavaddat *et al*, 2012]. There is also an increased frequency of medullary features like pushing margins, high degree of nuclear pleomorphism and mitotic frequency. They have a similar immunohistological profile to sporadic basal carcinomas, expressing basal markers such as cytokeratins 5/6 and cytokeratin 14 [Lakhani *et al*, 2005]. Ovarian tumors associated with *BRCA1* mutations are usually high-grade serous epithelial carcinomas with endometrioid, clear cell and mucinous carcinomas occurring less frequently [Mavaddat *et al*, 2012].

In contrast to tumors in *BRCA1* carriers, *BRCA2* associated breast tumors appear to be more heterogeneous. The most common histological type in *BRCA2* tumors is invasive breast carcinoma NST with a higher frequency of lobular and tubular carcinomas described [Mavaddat *et al*, 2012]. Ductal carcinoma *in situ* (DCIS) is also more common in *BRCA2* carriers. Overall, these tumors are similar to sporadic tumors regarding expression of estrogen and progesterone receptors and rarely overexpress HER2 [Lakhani *et al*, 2002, Mavaddat *et al*, 2012]. Ovarian tumors associated with *BRCA2* have similar features to those associated with *BRCA1* mutations [Lakhani *et al*, 2004].

Mutations in the *BRCA1/BRCA2* genes have also been associated with inherited predisposition to other cancers in HBOC families, like those of the prostate, pancreas, male breast, peritoneum, fallopian tube and melanoma. The lifetime risk of male breast cancer has been estimated to be 5-10% for *BRCA2*, and 1-5% for *BRCA1* mutation carriers, compared with a risk of 0.1% in the general population [Thompson

et al, 2002, van Asperen *et al*, 2005, Tai *et al*, 2007]. The frequency of *BRCA2* mutations in male breast cancer has been reported as ranging between 7-16% [Chodick *et al*, 2008, Ottini *et al*, 2009, Ding *et al*, 2011]. BRCA-associated male breast tumors have distinct pathologic characteristics compared with BRCA-associated female breast tumors, being usually of a higher stage and more likely to be estrogen and progesterone receptor positive [Silvestri *et al*, 2016]. Similar to male breast cancer, pancreatic and prostate cancers are also more commonly associated with *BRCA2* mutations. Estimates of the cumulative prostate cancer risk are around 9% for *BRCA1* and 15% for *BRCA2* mutation carriers at age 65, with *BRCA1/BRCA2* mutations accounting for about 2% of prostate cancer cases [Kote-Jarai *et al*, 2011, Leongamornlert *et al*, 2012]. Prostate tumors of *BRCA1/BRCA2* mutation carriers are also associated with a more aggressive phenotype [Castro *et al*, 2013]. A large study with *BRCA2* mutation carriers described that the occurrence of pancreatic cancer in males and females was 22 times greater than expected in the study population [Mersch *et al*, 2015]. The prevalence of *BRCA1/BRCA2* mutations in pancreatic cancer varies according to the selection criteria used. In unselected series was reported to be about 5%, ranging from 13% to 19% in patients with a strong family history of the disease or in individuals with Ashkenazi Jewish ancestry [Lal *et al*, 2000, Murphy *et al*, 2002, Hahn *et al*, 2003, Stadler *et al*, 2012, Holter *et al*, 2015].

Peritoneal and fallopian tube cancer are more associated with mutations in the *BRCA1* gene, although there is limited data available. Only a few studies have analyzed the frequency of *BRCA1/BRCA2* mutations in fallopian tube and peritoneal cancer independently of ovarian cancer, with frequencies observed ranging from 16% to 30% [Vicus *et al*, 2010, Walsh *et al*, 2011, Alsop *et al*, 2012]. An increased incidence of melanoma has been reported in both *BRCA1* and *BRCA2* mutation carriers [Moran *et al*, 2012, Mersch *et al*, 2015].

2.1.4. ***BRCA1*** **and** ***BRCA2*** **mutation pattern in Portuguese HBOC families**

A large characterization of the mutational spectrum of germline *BRCA1*/*BRCA2* mutations in 1050 Portuguese breast/ovarian cancer families has recently been

performed [Peixoto *et al*, 2015]. In 524 families, screening of the entire coding regions of *BRCA1/BRCA2* was performed, with the remaining 526 families screened for the two most prevalent founder mutations in Portuguese HBOC families, the *BRCA2* c.156_157insAlu and the *BRCA1* c.3331_3334del mutation. Inherited cancer predisposition could be linked to *BRCA1* or *BRCA2* mutations in 21.4% of the 524 fully screened probands, a proportion that reaches 28.9% of the families with an *a priori* BRCAPRO mutation probability >10%. Seven additional pathogenic mutations were detected in the 526 families with BRCAPRO mutation probability <10% that were screened only for the two most frequent mutations. A total of 119 pathogenic mutations were detected, 41.2% in *BRCA1* and 58.8% in *BRCA2*. The *BRCA2* c.156_157insAlu mutation was present in 32% of all Portuguese HBOC families and represented 55% of the *BRCA2* mutations, whereas the *BRCA1* c.3331_3334del mutation was present in 11% of all families and 26% of the families with a *BRCA1* mutation, together representing a large proportion of the mutations identified in Portuguese HBOC families. The *BRCA2* c.156_157insAlu mutation has only been reported in families of Portuguese ancestry [Teugels *et al*, 2005, Machado *et al*, 2007, Peixoto *et al*, 2009, Peixoto *et al*, 2011, Moreira *et al*, 2012, Peixoto *et al*, 2015], whereas the *BRCA1* c.3331_3334del mutation has been reported in several populations, including Spanish, Canadian and Colombian [Durocher *et al*, 1996, Blesa *et al*, 2000, Torres *et al*, 2007].

2.2. Other breast cancer predisposition genes

2.2.1. Genes associated with other hereditary cancer syndromes

2.2.1.1. *TP53*

TP53 is a tumor suppressor gene, located on chromosome 17, consisting of 11 exons with the core DNA binding domain encoded by exons 4-8. It has been called the “guardian of the genome” and plays an essential role in cell-cycle control and apoptosis [Lane, 1992, Levine, 1997]. Somatic mutations in the *TP53* gene are

common in solid tumors. Germline mutations are rare and responsible for the Li-Fraumeni syndrome (LFS), a cancer predisposition syndrome affecting children and adults [Li *et al*, 1988]. It is associated with soft tissue sarcomas, osteosarcomas, early onset breast cancer, acute leukemia, colon cancer, adrenocortical carcinoma, and brain tumors [Li *et al*, 1988, Malkin *et al*, 1990, Varley *et al*, 1997, Krutilkova *et al*, 2005, Gonzalez *et al*, 2009]. Sarcoma, breast cancer, adrenocortical tumors, and certain brain tumors are considered the “core” cancers of LFS, since they account for the majority of cancers observed in individuals with germline mutations in the *TP53* gene [Gonzalez *et al*, 2009]. Carriers of *TP53* mutations have a risk of developing cancer estimated to be approximately 60% and 95% by 45 and 70 years, respectively [Lustbader *et al*, 1992]. Patients with germline *TP53* mutations have an abnormal response to low-dose radiation, hence radiotherapy is not recommended in these patients because of the increased risk of developing a second primary tumor [Evans *et al*, 2006].

Although LFS only accounts for about 0.1% of breast cancer cases and 1% of hereditary breast cancer cases, mutations in *TP53* confer a 105 estimated relative risk (RR) (90% confidence interval (CI), 62 to 165) of developing early onset breast cancer [Sidransky *et al*, 1992, Lalloo and Evans, 2012, Easton *et al*, 2015]. In patients with early onset breast cancer (<30 years) the frequency of *TP53* mutations ranges from 3 to 8% [Lalloo *et al*, 2006, Gonzalez *et al*, 2009, Mouchawar *et al*, 2010, McCuaig *et al*, 2012, Bougeard *et al*, 2015]. More recently, a very high frequency of HER2-positive breast tumors (67-83%) was observed in patients with germline *TP53* mutations, which can be helpful for directing *TP53* mutation testing and for targeted treatment [Wilson *et al*, 2010, Melhem-Bertrandt *et al*, 2012].

2.2.1.2. *PTEN*

Germline mutations in the tumor suppressor gene *PTEN* are responsible for the Cowden syndrome, a multiple hamartoma syndrome that includes increased risk of benign and malignant tumors of the breast, thyroid and endometrium [Pilarski, 2009]. Other features associated with this syndrome are mucocutaneous lesions,

macrocephaly and hamartomatous intestinal polyps. The *PTEN* gene is located on chromosome 10q, contains 9 exons, and encodes a lipid phosphatase that functions as a tumor suppressor through negative regulation of a cell-survival signaling pathway [Cully *et al*, 2006]. Over 90% of individuals with Cowden syndrome will express some clinical manifestation in their lifetime [Hobert and Eng, 2009].

Several studies have projected lifetime estimates of cancer risk and determined cumulative risks of 77-85% for female breast cancer, 21-38% for thyroid cancer and 19-28% for endometrial cancer [Riegert-Johnson *et al*, 2010, Tan *et al*, 2012, Bubien *et al*, 2013]. Other studies have estimated that women diagnosed with Cowden syndrome have a lifetime risk of breast cancer between 25-50%, with the average age of diagnosis ranging from 38 to 50 years old [Brownstein *et al*, 1978, Starink *et al*, 1986, Pilarski *et al*, 2013]. Although *PTEN* is usually considered a high penetrance breast cancer gene, the selection of patients for studies evaluating *PTEN* penetrance was based on the presence of features associated with the syndrome, suffering from ascertainment bias, therefore not making possible to estimate reliable RR for the development of breast cancer in mutation carriers [Easton *et al*, 2015].

2.2.1.3. ***STK11***

Peutz-Jeghers syndrome is an autosomal dominant disorder, characterized by hamartomatous intestinal polyps, mucocutaneous pigmentation, and elevated risk for gastrointestinal cancers as well as breast, ovarian, small bowel or pancreatic cancers [Hearle *et al*, 2006]. Mutations in the tumor suppressor gene *STK11*, located on chromosome 19p, were identified by studying patterns of loss of heterozygosity in polyps of affected individuals from 17 Peutz-Jeghers families [Hemminki *et al*, 1998, Jenne *et al*, 1998]. STK11 is a serine/threonine kinase that inhibits cellular proliferation, controls cell polarity and interacts with the TOR pathway. Carriers of *STK11* mutations have a cumulative risk of 85% to develop any cancer by 70 years, with breast cancer risk estimated to be 45% at the same age [Hearle *et al*, 2006].

2.2.1.4. *CDH1*

Germline mutations in *CDH1* are associated with the development of hereditary diffuse gastric carcinoma (HDGC), often with signet ring cells histology. The cumulative risk for developing HDGC in male and female carriers is 67% and 83%, respectively [Pharoah *et al*, 2001]. This gene consists of 16 exons, is located on chromosome 16q, and encodes the E-cadherin protein, a calcium-dependent cell-cell adhesion molecule important for the maintenance of cell polarity [Graziano *et al*, 2003]. A high frequency of lobular breast cancer is also observed in carriers of *CDH1* pathogenic mutations [Pharoah *et al*, 2001], with the occasional observation of families with lobular breast cancer without gastric cancer [Masciari *et al*, 2007]. The cumulative risk for developing breast cancer is estimated to be 53% with a reported RR of 6.6 (90% CI, 2.2 to 19.9) but, similar to *PTEN* and *STK11*, studies performed on *CDH1* carriers are subject to ascertainment bias and reliable RR for the development of breast cancer are not possible to determine [Pharoah *et al*, 2001, Easton *et al*, 2015].

2.2.2. Moderate penetrance breast cancer predisposition genes

2.2.2.1. *PALB2*

PALB2 was originally identified as interacting with the BRCA2 protein by precipitation of BRCA2-containing complexes, showing that this protein was important for the localization and stability of BRCA2, facilitating BRCA2-mediated DNA repair [Xia *et al*, 2006]. Biallelic truncating mutations were afterwards detected in Fanconi anemia families with phenotypes very similar to those of Fanconi anemia caused by mutations in *BRCA2* [Reid et al, 2007, Xia et al, 2007]. These findings provided sufficient evidence to consider *PALB2* as an attractive candidate for breast cancer predisposition. Mutation analysis in 923 breast cancer families negative for *BRCA1/BRCA2* mutations identified 10 carriers of truncating mutations (1%) [Rahman *et al*, 2007]. Two different founder mutations, one in Canada and another in Finland, were identified in 0.5% and 1%, respectively, of women with breast cancer not

selected on the basis of a positive family history [Foulkes *et al*, 2007, Erkko *et al*, 2008]. In families with a family history of breast cancer, pathogenic mutations are found in 0.6% to 3.9% of patients, depending on the population [Antoniou *et al*, 2014].

The cumulative risk by 70 years of age for developing breast cancer in a large cohort of *PALB2* mutation carriers has been reported to range from 33% without family history taken into account to 58% in those with a strong family history (being 44% and 67%, respectively, at age 80), which is similar to the risks described for *BRCA2* [Antoniou *et al*, 2014]. A meta-analysis of published case-control and family studies estimated the RR for developing breast cancer to be 5.3 (90% CI, 3.0 to 9.4) [Easton *et al*, 2015]. Although the estimated RR points towards *PALB2* being a high penetrance gene, the lower CI is below four, with larger studies required for a reclassification of the penetrance of this gene. Similar to *BRCA2*, an increased risk to male breast cancer and pancreatic cancer has also been associated with carriers of *PALB2* loss-of-function mutations [Jones *et al*, 2009, Slater *et al*, 2010, Ding *et al*, 2011, Blanco *et al*, 2012].

2.2.2.2. *ATM*

Ataxia-telangiectasia is an autosomal recessive disease caused by homozygous or compound heterozygote mutations in the *ATM* gene. This condition is characterized by progressive cerebellar ataxia, oculomotor apraxia, immunodeficiency, and cancer predisposition. Individuals with ataxia-telangiectasia are estimated to have a 100-fold increased risk of cancer compared with the general population. Lymphoid cancers predominate in childhood, and epithelial cancers, including breast cancer, are seen in adults [Ahmed and Rahman, 2006]. The *ATM* gene is located at 11q and consists of 66 exons, 62 of which encode a protein of 3056 aminoacids. The first observation of *ATM* as a possible breast cancer predisposition gene came in 1976, when an excess of breast cancer in female relatives of patients with ataxia-telangiectasia was observed in an epidemiological study [Swift *et al*, 1976]. When the function of this protein started to be uncovered, the initial suspicions increased; ATM belongs to a family of proteins known as the PI3K-related protein

kinases (PIKK) and plays a central role in the response to double-stranded DNA breaks (DSBs) by initiating a pathway that includes other proteins, such as p53, BRCA1 and CHK2 [Ahmed and Rahman, 2006].

After some inconclusive studies regarding its involvement in breast cancer susceptibility, in 2006 one study found heterozygous mutations in 12/443 familial cases negative for mutations in *BRCA1* and *BRCA2* and only 2 in 521 controls [Renwick *et al*, 2006]. Many breast cancer predisposing *ATM* variants have been identified, including not only truncating variants but also a variety of missense ones. In fact, some missense *ATM* variants have been described as conferring a higher risk for breast cancer than truncating variants [Goldgar *et al*, 2011]. The prevalence of these variants varies greatly among populations from different geographical areas or ethnicity. The cumulative risk for developing breast cancer at age 80 is estimated to be 27% and two different meta-analyses have identified similar RR, 2.8 (90% CI, 2.2 to 3.7) and 3.2 (95% CI, 2.04 to 5.04) [Aloraifi *et al*, 2015, Easton *et al*, 2015]. Loss-of-function variants in *ATM* were also recently associated with an increased risk for the development of gastric, pancreatic, prostate and colorectal cancer [Helgason *et al*, 2015].

2.2.2.3. *CHEK2*

The checkpoint kinase gene *CHEK2*, a tumor suppressor gene, encodes CHK2, a serine/threonine kinase that is activated in response to DNA damage and phosphorylates both p53 and BRCA1 to regulate repair of DSBs [Stracker *et al*, 2009]. Most of the data about the involvement of *CHEK2* mutations in predisposition to breast cancer comes from the c.1100delC mutation that is found fairly frequently in Northern European populations. This mutation was identified with a frequency of 4.2% (30/718) in breast cancer families and with a frequency of 1.9% (201/10860) in population-based breast cancer cases compared to 0.7% (64/9065) in controls [Meijers-Heijboer *et al*, 2002, Chek Breast Cancer Case-Control Consortium, 2004]. In other populations, this mutation is much less frequent [Cybulski *et al*, 2009]. The RR for the development of breast cancer in carriers of this mutation is estimated to be 3.0 (90%

CI, 2.6 to 3.5) with an absolute risk of 29% at age 80 [Easton *et al*, 2015]. *CHEK2* c.1100delC carriers have an increased risk of bilateral breast cancer and, more recently, homozygous carriers were identified with a 6-fold higher risk of breast cancer when compared to heterozygotes [Mellemkjaer *et al*, 2008, Adank *et al*, 2011].

A summary of the genes conferring an increased risk for the development of breast cancer can be found on Table 1.

Table 1 – Genes associated with predisposition to breast cancer

Gene	Population frequency (%)	Proportion of familial breast cancer risk (%)	Estimated relative risk (90% CI)	Cumulative risk by age 80 (%)
BRCA1	0.1	5-10	11.4	75
BRCA2	0.1	5-10	11.7	76
TP53	<0.1	0.1	105 (62-165)	80-90
CDH1	<0.1	0.1	6.6 (2.2-19.9)	53
PTEN	<0.1	0.02	No reliable estimate	25-50
STK11	<0.1	0.04	No reliable estimate	45
ATM	0.5	2	2.8 (2.2-3.7)	27
CHEK2	0.5	2	3.0 (2.6-3.5)	29
PALB2	0.1	2.4	5.3 (3.0-9.4)	44

2.2.3. Low penetrance breast cancer predisposition alleles

Until now, common genetic variants in 94 loci associated with breast cancer risk have been identified [Couch *et al*, 2014, Michailidou *et al*, 2015]. The majority of these variants have been identified through GWAS of large numbers of breast cancer patients from the general population along with healthy controls and large-scale replication studies. Some are associated with a slightly increased risk, whereas others confer a small decrease in breast cancer risk. They can follow a polygenic risk model, or can act synergistically with environmental factors or lifestyle, to account for a

fraction of familial breast cancer cases. The 94 loci identified so far explain 16% of the two-fold risk of breast cancer in the first-degree relatives of women with the disease, with another 12% estimated to be explained by currently unknown loci (Figure 1). Some of these variants are associated with overall breast cancer risk, while others are associated with a specific molecular subtype of breast cancer: estrogen receptor positive, estrogen receptor negative or triple-negative breast cancer. The clinical utility of these low-penetrant common variants, either alone or in combination, remains debatable, although there are reports that, for instance, a combination of five common variants in *BRCA2* carriers can vary the lifetime risk of breast cancer from 45% to 95% [Antoniou *et al*, 2010]. A recent study showed that combining 77 common genetic variants into a polygenic risk score can be useful to stratify breast cancer risk in women without family history and to refine genetic risk in women with a family history of breast cancer [Mavaddat *et al*, 2015].

2.3. Other ovarian cancer predisposition genes

2.3.1. *BRIP1*

BRIP1, also known as *BACH1*, encodes a protein that was identified as a binding partner of BRCA1 and has BRCA1-dependent roles in DNA repair and checkpoint control [Cantor *et al*, 2001]. Biallelic mutations in *BRIP1* result in Fanconi anemia complementation group J (FANC-J), which is phenotypically different from that associated with *BRCA2*. In 2006, truncating mutations in this gene were identified in families negative for *BRCA1* and *BRCA2* mutations with estimated RR of 2.0 for breast cancer [Seal *et al*, 2006]. However, a recent study in a large cohort of 48,144 cases and 43,607 controls found no association of truncating variants with breast cancer risk [Easton *et al*, 2016].

The association of *BRIP1* mutations and ovarian cancer risk is more consistent. Three independent large studies conducted in women diagnosed with ovarian carcinoma found 0.9-1.4% frequencies of deleterious mutations in this gene with RR

estimated to be 8.1-11.2 for the development of this disease [Rafnar *et al*, 2011, Ramus *et al*, 2015, Norquist *et al*, 2016].

2.3.2. *RAD51C* and *RAD51D*

Genes of the RAD51 protein family are involved in HR and DNA repair. The initial report of *RAD51C* involvement in cancer predisposition was done in families with breast and ovarian cancer [Meindl *et al*, 2010], but subsequent analyses only revealed an association of *RAD51C* or *RAD51D* mutations to the development of ovarian cancer [Loveday *et al*, 2011, Pelttari *et al*, 2011, Loveday *et al*, 2012, Pelttari *et al*, 2012, Song *et al*, 2015, Norquist *et al*, 2016]. Overall, mutations in these two genes together seem to account to about 1% of ovarian cancer cases, with estimates of RR varying from 5.2 to 6.3 for *RAD51C* and 6.3 to 12.0 for *RAD51D*.

2.3.3. *MLH1, MSH2, MSH6* and *PMS2*

Lynch syndrome is a hereditary disease caused by germline mutations in one of the DNA mismatch repair genes (MMR), *MLH1, MSH2, MSH6* or *PMS2*. Colorectal cancer is the most common cancer associated with this syndrome, with mutations in these genes accounting for 2-4% of all cases [Hampel *et al*, 2005, Hampel *et al*, 2008]. The lifetime risk for developing colorectal cancer in carriers of MMR mutations is estimated to be up to 80% and microsatellite instability is a common feature of these tumors, occurring in up to 90% of them [Aaltonen *et al*, 1994, Vasen *et al*, 1996]. Other tumors associated with this syndrome, in women, include endometrial and ovarian cancer, with risks estimated to be up to 54% and 24%, respectively [Bonadona *et al*, 2011]. Ovarian cancers in Lynch syndrome are usually diagnosed at a younger age (average 42-48) compared to the general population with a predominance of endometrioid/clear cells histology [Lu and Daniels, 2013, Helder-Woolderink *et al*, 2016]. A summary of the contribution of genetic variants to ovarian cancer can be found on Figure 4.

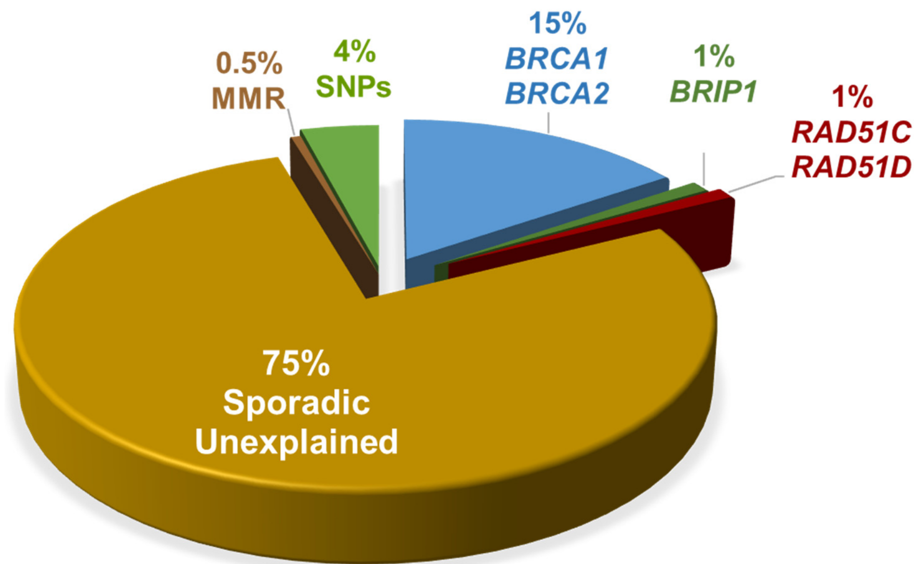

Figure 4 – Estimated percentage contribution of genetic variants in consecutive series of ovarian cancer, namely, *BRCA1* and *BRCA2* genes, *RAD51C*, *RAD51D*, *BRIP1*, MMR genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*) and SNPs [Walsh *et al*, 2011, Kuchenbaecker *et al*, 2015, Song *et al*, 2015, Norquist *et al*, 2016].

2.3.4. Low penetrance ovarian cancer predisposition alleles

Similar to breast cancer, several common genetic variants associated with an increased risk for the development of ovarian cancer have been described. In total, 18 different loci have been identified, explaining approximately 3.9% of the excess familial relative risk of ovarian cancer in the general population [Kuchenbaecker *et al*, 2015]. The majority of the identified loci displayed associations in *BRCA1* and *BRCA2* mutation carriers similar with the associations observed in cases from the general population, suggesting a general model of susceptibility whereby *BRCA1* and *BRCA2* mutations and common alleles interact multiplicatively on the relative risk for ovarian cancer [Wacholder *et al*, 2011]. Hence, the incorporation of ovarian cancer susceptibility variants for risk assessment might be particularly useful for *BRCA1/BRCA2* mutation carriers.

2.4. Homologous recombination and predisposition to breast and ovarian cancer

During chromosome replication, errors can occur that ultimately result in a stall of DNA replication forks. Stalled forks can be cleaved to generate DSBs and these can be repaired by HR, an error-free DNA repair mechanism that uses an undamaged sister chromatid as a template. In the absence of HR, DSBs can be repaired by error-prone mechanisms, such as NHEJ, that generate chromosome deletions and translocations causing genomic instability [Schlacher *et al*, 2011]. BRCA1 and BRCA2 are individually essential for efficient HR in mammalian cells, but there are other proteins involved in this pathway [Moynahan *et al*, 1999, Moynahan *et al*, 2001].

The DNA damage response (DDR) to DSBs involves sensors for the detection of broken ends, effectors that execute repair and mediators that facilitate interactions between sensors and effectors. It also includes the activation of checkpoints that allow time for DNA repair to be executed, by delaying the cell cycle before or during replication or before cell division (Figure 5) [Roy *et al*, 2012]. BRCA1 binds to DSBs through its association with the abraxas-RAP80 complex that is activated by ubiquitylated histones at DSBs [Wang *et al*, 2007]. BRCA1 is also involved in processing DSBs, forming a complex with CtIP that associates with the MRN complex (MRE11-RAD50-NBS1), a DNA damage sensor, and promotes resection of 5´ends of the broken DSB ends. After resection of DSBs, long stretches of 3' ssDNA are produced on either side of the DSB. Replication protein A (RPA) binds to the ssDNA preventing the formation of secondary DNA structures. Another BRCA1 complex (BRCA1/PALB2/BRCA2) promotes the exchange of RPA for RAD51. Phosphorylation of BRCA1 by CHK2 seems to be required for the formation of this BRCA1/PALB2/BRCA2 effector complex [Roy *et al*, 2012]. BRCA2 is an important mediator in the recruitment of RAD51 and on its function as an effector of HR. RAD51 must form a helical nucleoprotein filament on ssDNA but, under normal conditions, RAD51 preferentially forms stable complexes on dsDNA. BRCA2 binds directly to RAD51, through its BRC repeats, stabilizing RAD51 filament formation on ssDNA while inhibiting RAD51-dsDNA binding. The RAD51-ssDNA filament subsequently

mediates sister chromatid strand invasion, promoting DNA pairing between homologous sequences resulting in an error-free repair [Venkitaraman, 2014].

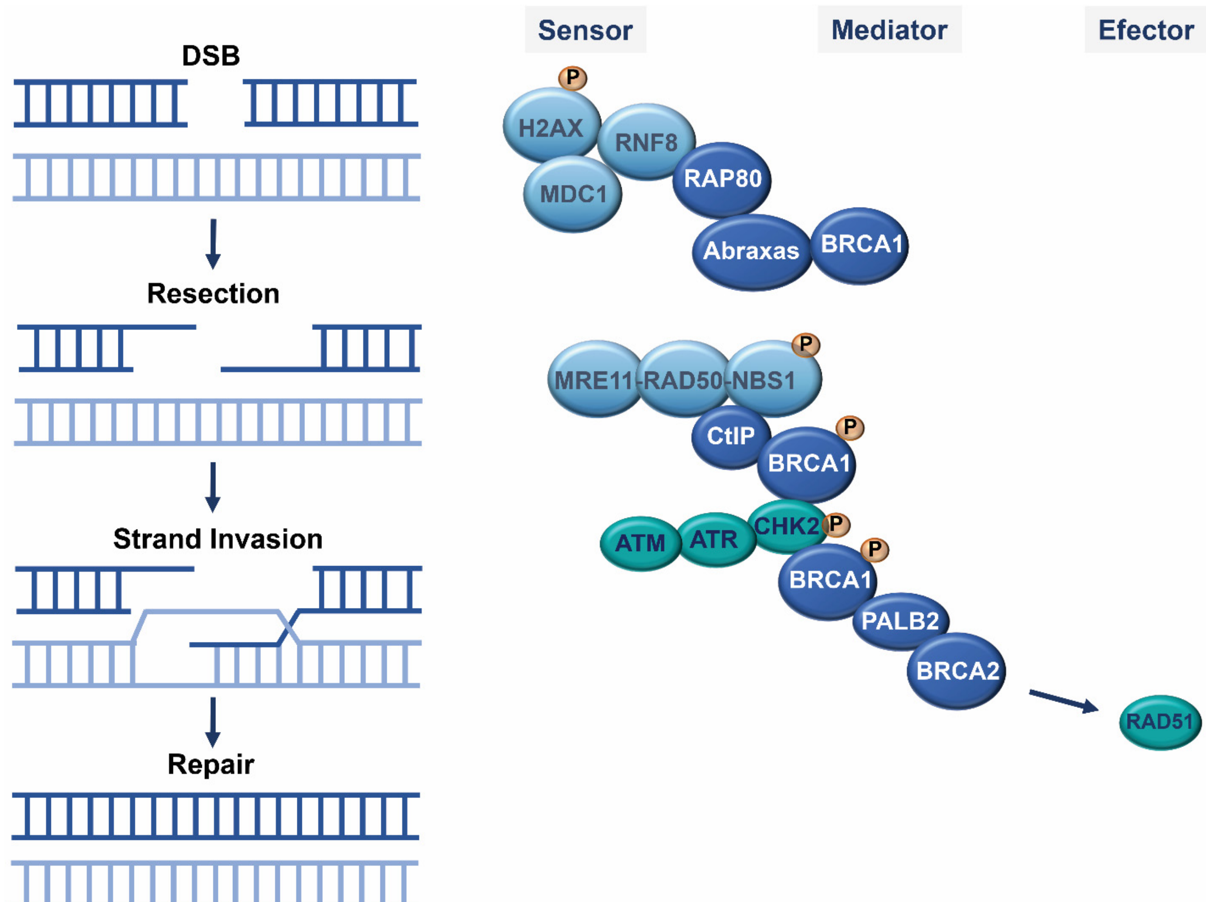

Figure 5 – Homologous recombination. In response to DNA DSBs, sensors (light blue) detect the damage, and signaling mediators recruit or activate effectors that repair the damage and activate cell cycle checkpoints. BRCA1-containing macro-complexes (dark blue) are crucial mediators of the DNA damage response. The BRCA1–abraxas–RAP80 complex associates with ubiquitylated histones near the sites of DNA damage, that is dependent on phosphorylation of histone H2AX. The BRCA1-CtIP complex associates with the MRN complex (MRE11-RAD50-NBS1), which senses DSBs and is responsible for DSB resection. The BRCA1-PALB2-BRCA2 complex is important in mediating RAD51-dependent HR. CHK2-dependent phosphorylation of S988 in BRCA1 appears to be required for the BRCA1–PALB2–BRCA2 effector complex, which is important in RAD51-mediated HR. DNA damage is also recognized by ATM and ATR kinases, which phosphorylate BRCA1, BRCA1-associated proteins and p53 and mediate signaling to form macro-complexes and activate cell cycle checkpoints [adapted from Roy *et al*, 2012].

Germline mutations in many of the genes involving this common HR pathway are associated with predisposition to breast and/or ovarian cancer, which suggests that this pathway is crucial in the suppression of tumorigenesis.

3. Hereditary breast and ovarian cancer diagnosis and management

3.1. Risk assessment

Assessment of the *a priori* probability of finding a *BRCA1* or *BRCA2* mutation is essential to select patients who are eligible for genetic testing. In general, this risk increases with increasing number of personal and family history of associated cancers and decreasing age at which those cancers were diagnosed. The National Comprehensive Cancer Network (NCCN) panel has recommendations on the criteria for referral of patients to genetic counseling for a personalized risk assessment [NCCN, 2016]. Briefly, they include any breast cancer diagnosed before 45 years old, a breast cancer diagnosed before 50 years old plus another primary breast cancer or family history of breast, pancreatic or prostate, or a breast cancer diagnosed at any age plus one of the following: one close blood relative with breast cancer before 50 years, two relatives with breast cancer, one family member affected with ovarian cancer, one case of male breast cancer in the family or two cases of prostate and/or pancreatic cancer. Individuals with ovarian cancer or male breast cancer diagnosed at any age should also be referred to genetic counseling. An individual diagnosed with prostate or pancreatic cancer also fulfills criteria for genetic counseling if they have a family history of other tumors (breast, ovarian, pancreatic or prostate).

3.2. Genetic testing

The criteria for genetic testing might vary between countries based on mutation prevalence and the existence of founder mutations. Several methods to determine the likelihood of detecting a *BRCA1* or *BRCA2* mutation exist, including computer models

such as BRCAPRO or BOADICEA [Parmigiani *et al*, 1998, Antoniou *et al*, 2008]. A common threshold to perform genetic testing in several countries is 10% [NICE guidelines [CG164], 2015]. Genetic testing should be performed in adults after they have received genetic counseling and given informed consent and, whenever possible, in the affected family member with the highest likelihood of carrying a *BRCA1* or *BRCA2* mutation.

Until recently, genetic testing of hereditary breast and ovarian cancer had been based on the identification of mutations in *BRCA1/BRCA2* by Sanger sequencing or alternative screening methods that are labor-intensive, have low throughput, and high turnaround time. With the advent of next-generation sequencing (NGS), a multi-gene testing approach is now possible, including other genes of high or moderate penetrance to breast and ovarian cancer, which can explain a fraction of *BRCA1/BRCA2* negative families. The detection of a deleterious germline mutation in an established breast or ovarian cancer predisposition gene has the potential to alter clinical management [Desmond *et al*, 2015]. However, knowledge on the penetrance and the clinical utility of germline mutations in many of the genes included in commercial panels is still incomplete and, for some, the information from testing does not change risk management compared to that based of family history alone [Easton *et al*, 2015]. Furthermore, the probability of finding variants of uncertain significance (VUS) increases when genetic testing is performed for multiple genes.

3.3. Surveillance and prevention

Breast cancer surveillance in carriers of a *BRCA1* or *BRCA2* mutation includes breast self-examination, clinical breast examination, mammography and magnetic resonance imaging (MRI). Mammography has been the standard screening method for detection of breast cancer in the last decades, but recently has been under great scrutiny because decreasing breast cancer mortality rates have been more attributed to improvements in treatment than mammography. Furthermore, a lower sensitivity for detection of breast cancers in high-risk women was observed due to a variety of factors, including an increased density of breast tissue and the presence of more

aggressive and rapidly growing tumors, both of which are common in younger women [Tilanus-Linthorst *et al*, 2002]. MRI has greater sensitivity, although with a lower specificity, and studies have demonstrated that a combination of MRI and mammography detects 70-100% of tumors in high-risk women [Kriege *et al*, 2004, Warner *et al*, 2004, Leach *et al*, 2005].

Current guidelines for female carriers recommend monthly breast self-examination, starting at age 18, and semiannual clinical breast examination beginning at age 25 years [NCCN, 2016]. MRI screening should be performed between the ages of 25 and 29 years with both annual mammography and MRI recommended between 30-75 years. After age 75, management should be considered on an individual basis. Current surveillance methods available for ovarian cancer (transvaginal ultrasounds and CA-125 serum levels) should only be considered for women who have not opted to perform ovarian cancer risk-reducing surgery, as they have not been shown to be effective [Evans *et al*, 2009].

Male carriers of a *BRCA1/BRCA2* mutation are recommended to perform monthly breast self-examination and annual clinical breast examination starting at age 35 years. Screening for prostate cancer after age 40 is recommended for *BRCA2* carriers and should be considered for *BRCA1* carriers. For both male and female carriers, a full body skin and eye exam for melanoma screening and investigational screening protocols for pancreatic cancer should be considered [NCCN, 2016].

Risk-reduction surgeries are one of the options for women at high risk of breast and ovarian cancer. These include risk-reducing mastectomy (RRM) and risk-reducing bilateral salpingo-oophorectomy (RRBSO). RRM decreases the risk of developing breast cancer by at least 90% [Hartmann *et al*, 2001]. Complete removal of breast tissue is not obtained and, therefore, there is a small residual risk of breast cancer [Rebbeck *et al*, 2004]. RRBSO has been shown to reduce the risk of ovarian cancer by about 80% and breast cancer risk by approximately 50%, if performed before 40-45 years old, although a recent study suggests that estimates of breast cancer risk after RRBSO may be overestimated due to several types of bias [Rebbeck *et al*, 2009, Heemskerk-Gerritsen *et al*, 2015]. Current guidelines from the NCCN panel support discussing the option to perform RRM for women on a case-by-case basis, taking into

account the potential psychosocial effects of RRM, and recommend the performance of RRBSO due to the absence of reliable screening methods for ovarian cancer and its poor prognosis. As ovarian cancer is more common and has a younger age of onset in *BRCA1* carriers, the recommendation to perform RRBSO in these is between ages 35 and 40 years and between 40-45 years for *BRCA2* carriers, in both cases after completion of childbearing [NCCN, 2016].

The most recent NCCN guidelines already recommend breast MRI screening for carriers of *ATM*, *CHEK2* and *PALB2* mutations (in addition to previously known breast cancer genes *BRCA1*, *BRCA2*, *TP53*, *CDH1*, *STK11* and *PTEN*), and that the possibility of RRM should be discussed with *PALB2* carriers. Carriers of mutations in ovarian cancer susceptibility genes (*BRIP1*, *RAD51C* and *RAD51D)*, on the other hand, should consider the option of performing RRBSO in line with what is recommended for *BRCA1/BRCA2* and Lynch syndrome carriers [NCCN, 2016].

Tamoxifen, a selective estrogen receptor modulator, works by binding to the estrogen receptor, blocking the proliferative effect of estrogen on breast tissue. This agent has been shown to be effective in reducing the risk of breast cancer by about 50% in high-risk women [Fisher *et al*, 1998]. It has also been associated with a reduction in risk for contralateral breast cancer in *BRCA1/BRCA2* carriers [Gronwald *et al*, 2006]. In unaffected individuals, the association with risk reduction was seen only in *BRCA2* mutation carriers, but the available data are too limited for statistical significance [King *et al*, 2001]. The use of oral contraceptives has been shown to reduce risk for ovarian cancer by about 50% in both *BRCA1* and *BRCA2* mutation carriers [Iodice *et al*, 2010]. There are conflicting reports regarding its effect on breast cancer risk, but no association seems to exist between the use of oral contraceptives and risk for breast cancer in *BRCA1/BRCA2* mutation carriers [Moorman *et al*, 2013].

3.4. Targeted therapy

BRCA1 and *BRCA2* mutation carriers can also benefit from targeted therapy. BRCA1 and BRCA2 are critical proteins in the process of HR repair of DSBs. The absence of HR, which is a characteristic of BRCA1/BRCA2 deficient cancer cells,

activates error-prone DSB mechanisms like NHEJ and results in genomic instability [Bryant *et al*, 2005]. BRCA1/BRCA2-deficient cancers are now recognized as the target for a class of drugs known as PARP (poly (ADP-ribose) polymerase) inhibitors. PARP inhibition, by blocking Base Excision Repair (BER), prevents single-strand break repair and leads to the formation of DSBs, which cannot be accurately repaired in HR-deficient cells and may result in cell death [Ashworth, 2008]. This synthetic lethality in BRCA-deficient tumors is the basis for the improved response in patients treated with PARP inhibitors. So far, PARP inhibitors have been approved in Europe and in the USA for the treatment of ovarian cancer in *BRCA1/BRCA2* mutation carriers [Ledermann *et al*, 2014]. They are also currently being evaluated for the treatment of other BRCA-associated tumors and for the treatment of patients with mutations in other genes that could impair HR [Kaufman *et al*, 2015, Mateo *et al*, 2015].

AIMS

The aims of this thesis were to characterize the phenotypic heterogeneity associated with *BRCA1* and *BRCA2* mutations and the genetic heterogeneity of hereditary breast and ovarian cancer. Specifically, the objectives of this thesis were:

1. To develop a method to detect the founder mutations *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del in formalin-fixed paraffin-embedded archival tissue.

2. To quantify the contribution of the founder mutations *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del for cancer etiology in unselected hospital-based cohorts of patients diagnosed with rarer cancers associated with hereditary breast and ovarian cancer syndrome, namely, cancer of the pancreas, male breast, peritoneum, and fallopian tube.

3. To compare the sensitivity and specificity of next-generation sequencing and Sanger sequencing for the detection of point mutations in the *BRCA1* and *BRCA2* genes.

4. To evaluate the genetic heterogeneity of hereditary breast and ovarian cancer by analyzing a panel of 17 genes associated with predisposition to these diseases in a consecutive series of high-risk breast/ovarian cancer families.

PAPER I

Analysis of founder mutations in rare tumors associated with hereditary breast/ovarian cancer reveals a novel association of *BRCA2* mutations with ampulla of Vater carcinomas

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Abstract

BRCA1 and *BRCA2* mutations are responsible for hereditary breast and ovarian cancer, but they also confer an increased risk for the development of rarer cancers associated with this syndrome, namely, cancer of the pancreas, male breast, peritoneum, and fallopian tube. The objective of this work was to quantify the contribution of the founder mutations *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del for cancer etiology in unselected hospital-based cohorts of Portuguese patients diagnosed with these rarer cancers, by using a strategy that included testing of archival tumor tissue. A total of 102 male breast, 68 pancreatic and 33 peritoneal/fallopian tube carcinoma cases were included in the study. The *BRCA2* c.156_157insAlu mutation was observed with a frequency of 7.8% in male breast cancers, 3.0% in peritoneal/fallopian tube cancers, and 1.6% in pancreatic cancers, with estimated total contributions of germline *BRCA2* mutations of 14.3%, 5.5%, and 2.8%, respectively. No carriers of the *BRCA1* c.3331_3334del mutation were identified. During our study, a patient with an ampulla of Vater carcinoma was incidentally found to carry the *BRCA2* c.156_157insAlu mutation, so we decided to test a consecutive series of additional 15 ampullary carcinomas for *BRCA1/BRCA2* mutations using a combination of direct founder mutation testing and full gene analysis with next generation sequencing. *BRCA2* mutations were observed with a frequency of 14.3% in ampulla of Vater carcinomas. In conclusion, taking into account the implications for both the individuals and their family members, we recommend that patients with these neoplasias should be offered *BRCA1/BRCA2* genetic testing and we here show that it is feasible to test for founder mutations in archival tumor tissue. Furthermore, we identified for the first time a high frequency of germline *BRCA2* mutations in ampullary cancers.

Introduction

Inherited predisposition to breast cancer is estimated to account for about 5-10% of all cases and is characterized by an autosomal dominant pattern of inheritance, young age at presentation, and association with bilateral breast cancer and ovarian cancer [1, 2]. It has been estimated that up to 1 in 300 and 1 in 800 individuals of the general population carry a *BRCA1* or *BRCA2* mutation, respectively, two genes that are responsible for hereditary breast and ovarian cancer (HBOC). Women carrying germline *BRCA1* mutations have a cumulative risk at 70 years of 60% for breast cancer and 59% for ovarian cancer, whereas *BRCA2* mutations appear to confer a similar risk of breast cancer in females (55%), but a lower risk (17%) for ovarian cancer [3]. Mutation analysis is required to confirm the clinical suspicion of HBOC and to allow appropriate screening and prophylactic measures to carriers in the family [2].

Molecular analyses of the *BRCA1* and *BRCA2* genes have shown that most populations exhibit a wide spectrum of mutations throughout both genes and several founder mutations have been identified in individuals of different ancestries [4]. We have recently characterized the mutational spectrum of the *BRCA1* and *BRCA2* genes in Portuguese HBOC families [5], showing that it is indeed heterogeneous, including two prevalent founder mutations, the *BRCA2* c.156_157insAlu mutation and the *BRCA1* c.3331_3334del mutation. The *BRCA2* c.156_157insAlu mutation was present in 32% of all Portuguese HBOC families and represented 55% of the *BRCA2* mutations, whereas the *BRCA1* c.3331_3334del mutation was present in 11% of all families and 26% of the families with a *BRCA1* mutation, together representing a large proportion of the mutations identified in Portuguese HBOC families. The *BRCA2* c.156_157insAlu mutation has only been reported in families of Portuguese ancestry [5-10], whereas the *BRCA1* c.3331_3334del mutation has been reported in several populations, including Spanish, Canadian and Colombian [11-13].

Mutations in the *BRCA1*/*BRCA2* genes have also been associated with inherited predisposition to other cancers in HBOC families, like those of the prostate,

pancreas, male breast, peritoneum, and fallopian tube [14, 15]. We have recently evaluated the contribution of the germline *BRCA1*/*BRCA2* founder mutations for early-onset and/or familial prostate cancer in Portugal [16]. Mutations in *BRCA2* confer a higher risk for developing cancers of the pancreas and male breast, and *BRCA1* mutations seem to be predominantly associated with a higher risk for developing peritoneal and fallopian tube cancer. The objective of this work was to quantify the contribution of the founder mutations *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del for cancer etiology in unselected hospital-based cohorts of patients diagnosed with these rarer cancers in Portugal.

Materials and Methods

Ethics Statement

This study was approved by the Institutional Ethics Committee of the Portuguese Oncology Institute of Porto (IPO-Porto) (approval number CES 019/08 regarding the use of archival samples for research) and written informed consent was obtained for all patients referred for genetic counselling.

Subjects

A consecutive series of patients diagnosed at IPO-Porto with any of the cancers strongly associated with HBOC besides female breast, ovarian, and prostate cancer (pancreatic, male breast, peritoneal and fallopian tube) from 1997 to 2013, and from which formalin-fixed, paraffin-embedded (FFPE) tissue was available, was identified. A total of 68 patients with pancreatic tumors (65 ductal adenocarcinomas, 1 mixed ductal-neuroendocrine carcinoma, 1 intraductal papillary mucinous neoplasm with an associated invasive carcinoma and 1 mucinous cystic neoplasm with low grade dysplasia), 27 with male breast invasive ductal carcinomas of no special type and 33 with peritoneal/fallopian tube high-grade serous carcinomas were included in the study with FFPE tissue. Given the large retrospective period of time covered,

peritoneal/fallopian tube carcinomas included in the study were limited to those that involved the peritoneum and/or fallopian tube without or only with superficial (<5mm) involvement of the ovary. Furthermore, a consecutive series of 16 patients diagnosed at IPO-Porto with carcinomas of the ampullary region (7 pancreato-biliary type and 9 intestinal type adenocarcinomas), from 1997 to 2013, and from which FFPE tissue was available, were subsequently included. Hematoxylin and eosin-stained slides were carefully reviewed by a pathologist, who delimited tumor and surrounding non-tumoral areas. Family history was not available from any of the patients from whom FFPE tissue was collected. Patients where a mutation was identified during this study were subsequently contacted to provide genetic counselling and to offer their family history.

Additionally, 75 male breast cancer (MBC) patients (39 previously reported by Peixoto et al. [5]) that were referred to the Genetics Department of IPO-Porto for genetic testing of *BRCA1/BRCA2* mutations, not selected for family history of cancer, were also included and peripheral blood samples were collected, giving a total of 102 MBC patients.

Founder Mutation Screening

In FFPE samples, DNA extraction was performed from both tumor and surrounding non-tumoral tissue, whenever available, with the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol and DNA quality was evaluated with the NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA). The *BRCA2* c.156_157insAlu mutation was detected by amplification of exon 3 followed by a nested PCR specific for the Alu rearrangement. *BRCA2* exon 3 amplification was performed with the following primers: forward 5`-CTGAACCTGCAGAAGAATCTGAA-3`; reverse 5`-GAAGCCAGCTGATTATAAGATGGTT-3`. The cycling conditions were 94ºC for 1 min, 35 cycles of 94ºC for 1 min, 52ºC for 1 min, and 72ºC for 4 min, and a final extension of 72ºC for 10 min. In the nested PCR, specific primers for the c.156_157insAlu mutation were used (forward 5`-GACACCATCCCGGCTGAAA-3`;

reverse 5`-GAAGCCAGCTGATTATAAGATGGTT-3`) and the cycling conditions were 95ºC for 10 min, 25 cycles of 95ºC for 45 sec, 62ºC for 45 sec, and 72ºC for 45 sec, and a final extension of 72ºC for 7 min. In the first PCR, due to preferential amplification of the shorter allele, only one amplicon of 111 bp corresponding to the wild-type allele is visible. In the nested PCR, a second amplicon (in positive samples) of about 343 bp corresponding to the allele with the c.156_157insAlu mutation is expected (Fig 1A). Sequence analysis of genomic fragments with the insertion was carried out on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Carlsbad, CA), using the dye terminator method.

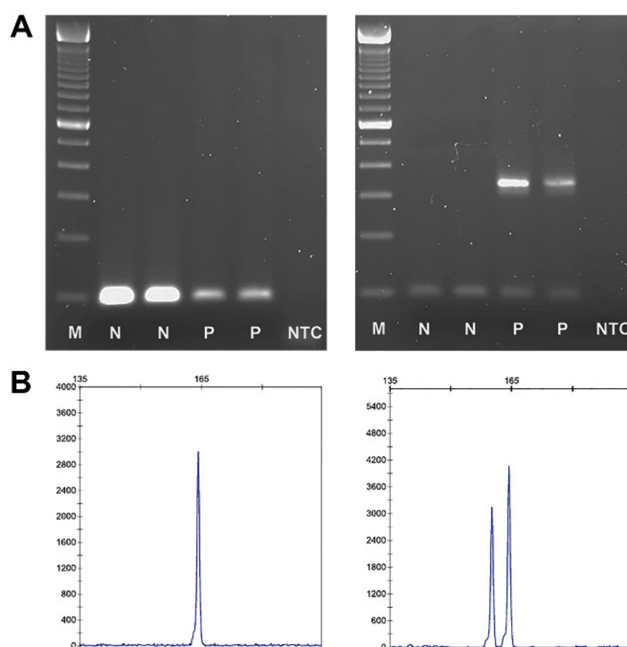

Figure 1 – Detection of the *BRCA2* c.156_157insAlu mutation and the *BRCA1* c.3331_3334del mutation in FFPE tissue. (A) Gel electrophoresis pattern of amplification of *BRCA2* exon 3 (left panel) and nested PCR specific for the *BRCA2* c.156_157insAlu mutation (right panel). In non-carriers of the mutation (N) only one amplicon is expected, whereas in carriers (P) a second amplicon is visible in the nested PCR. Non template control (NTC) and 100 bp DNA standard (M) also shown. (B) Capillary electrophoresis pattern from a negative sample (left panel) and a positive control of the *BRCA1* c.3331_3334del mutation (right panel) showing one peak (wild-type alleles) and two peaks (wild-type and mutant allele with 4 bp deletion), respectively.

The c.3331_3334del mutation located in *BRCA1* exon 11 was screened using the labelled primers forward 5`-TTAAAGAAGCCAGCTCAAGC-3` and reverse

5`HEX-CTGAAATCAGATATGGAGAG-3`, with the following cycling conditions: 95ºC for 10 min, 35 cycles of 95ºC for 45 sec, 58ºC for 45 sec, and 72ºC for 45 sec, and a final extension of 72ºC for 10 min. Each sample was run on an ABI PRISM 310 Genetic Analyser together with a fluorescence labeled DNA fragment size standard. The c.3331_3334del mutation status was determined by the presence of one or two peaks corresponding to the wild type and mutated samples, respectively (Fig 1B). All mutations were confirmed by Sanger DNA sequencing.

In patients from whom DNA was extracted from peripheral blood samples, both the *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del mutations were screened as previously described [5].

Next-Generation Sequencing

Next-generation sequencing (NGS) was performed in 12 ampullary tumors in which no founder mutations had been found (in two tumors DNA did not have enough quality). Library preparation was performed using the BRCA Tumor MASTR™ Plus Dx (Multiplicom, Niel, Belgium), which targets the full coding sequence and adjacent intronic regions of the *BRCA1/BRCA2* genes and is optimized for FFPE tissue, following the manufacturer's protocol. Sequencing was carried out using a standard flow cell in the MiSeq platform (Illumina, Inc., San Diego, CA, USA) in a 2x250 bp paired end run. Sequencing alignment and variant analysis was performed using the software Sophia DDM® version 3.5 (Sophia Genetics, Saint-Sulpice, Switzerland). All variants with an alternative variant frequency ≤5%, minor allele frequency (MAF) >1% and/or intronic variants at more than 12bp away from exon-intron boundaries were excluded. For MAF filtering, data was obtained from the 1000 Genomes Project (1000G; Based on Project Phase III Data), Exome Variant Server (from NHLBI Exome Sequencing Project) and Exome Aggregation Consortium (ExAC) databases.

Results

A total of 102 MBC patients were analyzed for both the *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del mutations. Of the total samples analyzed, eight (7.8%) were positive for the *BRCA2* c.156_157insAlu mutation (three detected in FFPE and five in peripheral blood samples, of which two had previously been reported by us [5]) and the *BRCA1* c.3331_3334del mutation was not identified in any case (Table 1). Of the three patients where the mutation was identified in FFPE tissue, in one the mutation was confirmed to be germline in peripheral blood, another was deceased but belonged to a family that had already been identified in our institution, and in the third it was not possible to test the germline. The age of diagnosis of breast cancer in the *BRCA2* carriers ranged from 47 to 78 years old with a median age of 65 years. It was possible to obtain family history information for seven patients and all of them had a family history of cancers associated with HBOC. One of the patients, besides breast cancer at the age of 47, was also diagnosed with prostate cancer at the age of 55 and four women in his family were diagnosed with breast cancer. Four patients had only family history of female breast cancer, two with one family member (Fig 2A), one with three family members, and the other with five women affected with breast cancer. One patient had three family members affected with female breast cancer and one with ovary cancer. The last patient belongs to a large family with 12 cases of female breast cancer, five cases of prostate cancer and one case with pancreatic cancer.

Table 1 – Samples analyzed and mutation frequencies observed in tumors associated with HBOC.

Cancer	Samples	*BRCA2* c.156_157insAlu	*BRCA1* c.3331_3334del	% Positive	Estimated *BRCA2* (%)[a]	*BRCA1 / BRCA2* (%)
Male Breast	102	8	0	7.8	14.3	NA
Peritoneal / Fallopian Tube	33	1	0	3.0	5.5	NA
Pancreatic	64	1	0	1.6	2.8	NA
Ampullary	16	2	0	12.5	NA	14.3[b]

NA – Not available/not applicable

[a] *BRCA2* c.156_157insAlu represents 55% of the total *BRCA2* mutations identified in Portuguese HBOC families that performed screening of the entire *BRCA1/BRCA2* coding regions [5].

[b] Frequency of *BRCA1/BRCA2* mutations observed in the 14 samples in which screening of the entire *BRCA1/BRCA2* coding regions was performed.

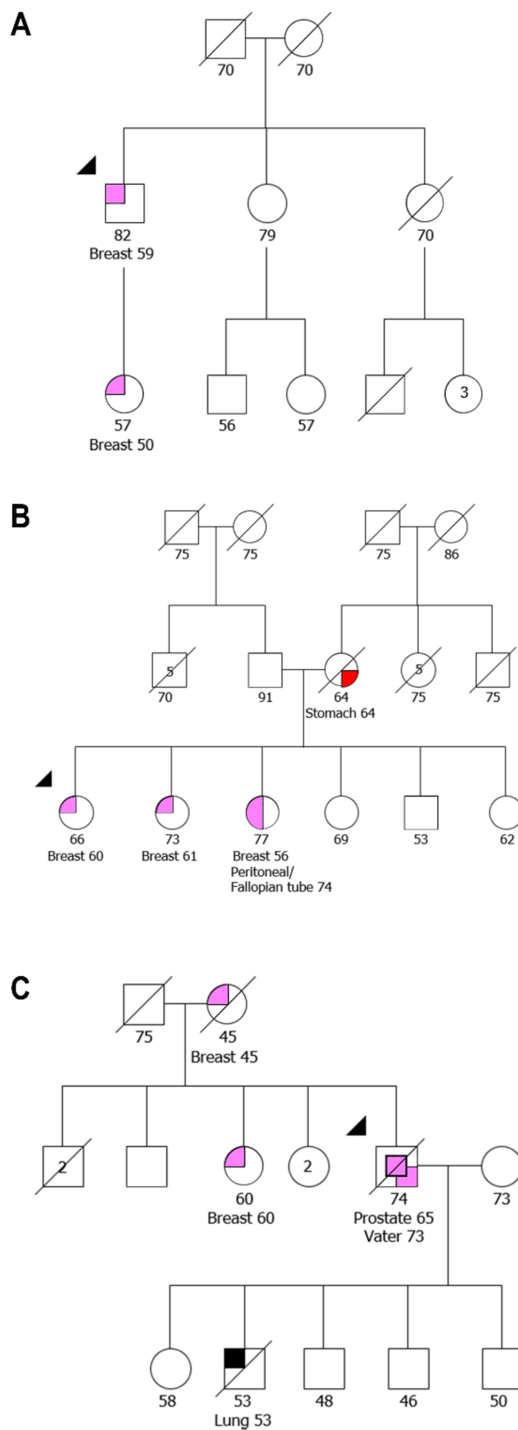

Figure 2 – Pedigrees of individuals with the *BRCA2* c.156_157insAlu mutation detected in FFPE tissue. Family of an individual with male breast cancer (A), an individual with peritoneal/fallopian tube cancer (B), and one individual with an ampulla of Vater carcinoma (C). The index case is indicated by an arrow.

In the 33 patients with peritoneal/fallopian tube cancer analyzed, none was carrier of the *BRCA1* c.3331_3334del mutation and one patient (3.0%) was a carrier of the *BRCA2* c.156_157insAlu mutation (Table 1). This patient was diagnosed at 74 years old with a high-grade serous carcinoma of the fallopian tube with extensive involvement of the peritoneum. The mutation was confirmed to be germline in peripheral blood and the patient belonged to a family that had already been identified in our institution. She was also diagnosed with breast cancer at 56 years of age and had two sisters with breast cancer (Fig 2B).

An initial series of 69 consecutive cases of putative pancreatic carcinoma was analyzed for the Portuguese founder mutations. Of these, four samples did not have good quality DNA and it was not possible to obtain a result. The *BRCA2* c.156_157insAlu mutation was identified in two samples and no carriers of the *BRCA1* c.3331_3334del mutation were found. When the histopathology material was reviewed it was shown that one of the patients carrying the *BRCA2* c.156_157insAlu mutation had a pancreato-biliary type adenocarcinoma that originated in the ampulla of Vater and not in the pancreas. Hence, a consecutive series of 15 carcinomas of the ampulla of Vater were collected in order to evaluate the contribution of the founder mutations for the pathogenesis of these tumors. One more patient carrying the *BRCA2* c.156_157insAlu mutation was identified in this series, giving a total of two (12.5%) positive samples in the 16 cases of ampullary cancer analyzed for founder mutations (Table 1). The first carrier identified was diagnosed with an adenocarcinoma of the ampulla of Vater at the age of 73 and had been previously diagnosed with prostate cancer at 65 years old. The mutation was confirmed to be germline in peripheral blood and his family history included his mother and one sister diagnosed with breast cancer at the ages of 45 and 60, respectively (Fig 2C). The other patient was diagnosed at 68 years also with a pancreato-biliary type adenocarcinoma of the ampullary region and had no family history of tumors associated with HBOC, only one sister diagnosed with colorectal cancer.

Given the high frequency of the *BRCA2* c.156_157insAlu mutation observed in the ampullary tumors analyzed, we decided to perform screening of the entire *BRCA1/BRCA2* coding regions by NGS. In two of the fourteen negative samples for

founder mutations it was not possible to obtain DNA of sufficient quality to perform the analysis. A median coverage of 5100 was obtained for *BRCA1* and of 3770 for *BRCA2* with a minimum coverage of 150 obtained in all samples and only 4.3% of the exons analyzed with a minimum coverage below 500 (data not shown). No additional *BRCA1/BRCA2* deleterious mutations were identified in the 12 samples analyzed by NGS (Table 1).

Of the 64 pancreatic cancer samples where it was possible to obtain a result, one (1.6%) individual carrying the *BRCA2* c.156_157insAlu mutation was identified and none was a carrier of the *BRCA1* c.3331_3334del mutation (Table 1). This patient was diagnosed with an intraductal papillary mucinous neoplasm with an associated invasive carcinoma (ductal adenocarcinoma) of the pancreas at the age of 72 and he had one cousin diagnosed with ovary cancer and another with breast cancer.

Discussion

The aim of this study was to quantify the contribution of the founder mutations prevalent in Portugal (*BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del) for cancers associated with HBOC other than the common female breast, ovarian, and prostate cancer, more specifically, the rarer pancreatic, male breast, peritoneal, and fallopian tube cancers. In the 102 MBC patients screened for these mutations, we identified eight (7.8%) carriers of the *BRCA2* c.156_157insAlu mutation. Although these patients were not selected for family history of cancer, all the seven carriers from whom it was possible to obtain information about family history had at least one more family member affected with breast cancer. *BRCA2* mutations are considered the major genetic risk factor for male breast cancer, conferring a lifetime cumulative risk to develop the disease of about 9% [17], but the frequency of these mutations varies considerably between different populations. A study in Southern California detected *BRCA2* mutations in 4% of MBC patients [18], whereas another study in Iceland found mutations in the *BRCA2* gene in 40% of the cases [19]. More recent and larger studies in Israel, Italy and USA described prevalences of 8%, 7%, and 16%, respectively, of *BRCA2* mutations in male breast cancer patients [20-22]. These

differences in the frequency of *BRCA2* mutations across different studies can be caused by small sample sizes, mutation screening methods with different sensitivities, mutation screening strategy (entire gene *vs* founder mutations only), presence/absence of family history of tumors associated with HBOC or different classifications of missense mutations. In our study, only the c.156_157insAlu mutation was tested, which accounts for about 55% of all families with pathogenic *BRCA2* mutations in the Portuguese population [5]. Hence, we could expect an overall frequency of about 14.3% of *BRCA2* germline mutations in Portuguese male breast cancer patients in an unselected hospital-based cohort. On the other hand, our data shows that germline *BRCA1* mutations have a limited contribution to the pathogenesis of male breast cancer, which is in accordance with the literature [22, 23].

In the series of 33 peritoneal/fallopian tube cancers analyzed, we identified only one patient (3.0%) carrying the *BRCA2* c.156_157insAlu mutation (estimated total contribution of *BRCA2* mutations of 5.5%) and no carriers of the *BRCA1* c.3331_3334del mutation. There are only a few studies that have analyzed the frequency of *BRCA1/BRCA2* mutations in fallopian tube and peritoneal cancer independently of ovarian cancer. Alsop and colleagues [24] analyzed a series of 152 patients with peritoneal cancer and 40 with fallopian tube cancer and identified a total of 15.8% and 20% patients carrying a *BRCA1/BRCA2* mutation, respectively. Another study performed on 108 patients with fallopian tube cancer identified 21% of patients with a mutation in *BRCA1* and 9% in *BRCA2*, whereas one study performed on 79 patients with peritoneal/fallopian tube cancer identified mutations in *BRCA1/BRCA2* in 23% of the patients [25, 26]. Our low frequency of mutations (3.0%) identified compared to these studies can be explained by the fact that only founder mutations were analyzed and the *BRCA1* founder mutation, which is the gene more commonly associated with these tumors, only represents 11% of all families and 26% of the families identified with a *BRCA1* mutation in Portuguese HBOC families. Whereas our estimation of the contribution of *BRCA2* germline mutations for peritoneal/fallopian tube cancers in hospital-based cohorts is likely to be reliable, the evaluation of the contribution of *BRCA1* mutations may require additional larger studies that include full gene analysis.

We have also evaluated the contribution of *BRCA1/BRCA2* founder mutations in a consecutive series of pancreatic cancers diagnosed at a tertiary cancer center. One of the 64 tumors analyzed (1.6%) had the *BRCA2* c.156_157insAlu mutation. Since this mutation represents 55% of all *BRCA2* germline mutations in our population, it can be estimated that the total contribution of mutations in this gene for pancreatic cancer is about 2.8%. Most of the previous studies conducted for the detection of *BRCA1/BRCA2* mutations in pancreatic cancer were performed in patients with a strong family history of the disease or in individuals with Ashkenazi Jewish ancestry and the reported prevalence of BRCA mutations is variable, ranging from 13% to 19% [27-30]. A recent study was carried out on an unselected, consecutive series of 306 patients from Canada with pancreatic ductal adenocarcinoma and mutations in *BRCA2* were identified in 3.6% of the patients, with a total of 4.6% *BRCA1/BRCA2* carriers identified [31], which does not differ significantly from our estimate for unselected Portuguese patients.

Perhaps the most interesting aspect of our study was the recurrent finding of germline *BRCA2* mutations in carcinomas of the ampullary region. Two of 16 cases of this rare tumor (12.5%) were shown to have the *BRCA2* Portuguese founder mutation, with a 14.3% (2/14) frequency observed when considering only the samples with mutations and those in which all *BRCA1/BRCA2* coding regions were analyzed. Ampullary carcinomas are very rare, accounting for about 0.5% of all gastrointestinal cancers, being often included in the group of pancreato-biliary tumors, but usually have a good prognosis when compared to pancreatic carcinomas [32]. Familial adenomatous polyposis (FAP) patients often develop ampullary adenomas that may progress to ampullary cancer, with a cumulative risk of 10% at the age 60 [33]. Until now, only one study has identified a *BRCA2* mutation in one patient with a carcinoma of the ampulla of Vater, but it was identified in an individual with a family history of breast cancer where this mutation had previously been identified in other family members [34]. To our knowledge, this is the first study that has performed full analysis of the *BRCA1/BRCA2* genes in a consecutive series of ampullary carcinomas. Although the mutation frequency observed is high, our sample size is relatively small

and further studies are warranted to confirm the association of *BRCA1/BRCA2* mutations with this rare neoplasia.

The identification of BRCA mutation carriers has implications for both the individuals and their family members, allowing reliable genetic counseling and predictive genetic testing. Female carriers of BRCA mutations can decide whether they want to participate in surveillance protocols and/or perform risk-reducing surgical interventions such as prophylactic bilateral mastectomy and bilateral salpingo-oophorectomy, whereas mutation positive males can engage in breast and/or prostate cancer screening [15]. Moreover, BRCA mutation carriers can also benefit from targeted therapy. BRCA1 and BRCA2 are critical proteins in the process of homologous recombination (HR) repair of double-strand DNA breaks (DSBs). The absence of HR, which is a characteristic of BRCA1/BRCA2 deficient cancer cells, activates error-prone DSB mechanisms like non-homologous end joining (NHEJ) and results in genomic instability [35]. BRCA1/BRCA2-deficient cancers are now recognized as the target for a class of drugs known as PARP (poly (ADP-ribose) polymerase) inhibitors. PARP inhibition, by blocking Base Excision Repair (BER), prevents single-strand break repair and leads to the formation of DSBs, which cannot be accurately repaired in HR-deficient cells and may result in cell death [36]. This synthetic lethality in BRCA-deficient tumors is the basis for the improved response in patients treated with PARP inhibitors [37, 38]. We here show that rarer cancers besides female breast, ovarian, and prostate cancer may be sentinel features that allow the diagnosis of HBOC families and these patients may be included in clinical trials with PARP inhibitors.

In conclusion, we report the contribution of founder mutations to rarer cancers associated with HBOC in Portugal and an optimized method for the detection of these mutations in FFPE tissue (applicable both in neoplastic cells or in the surrounding normal tissue). This optimized method for FFPE tissue is especially important for the detection of the *BRCA2* c.156_157insAlu mutation in patients with Portuguese ancestry, as this prevalent mutation is not readily detectable by standard sequencing technologies [5, 10], therefore allowing its detection even in deceased patients diagnosed with poor prognosis cancers like that of the pancreas. The *BRCA2*

c.156_157insAlu mutation was observed with a frequency of 7.8% in male breast cancers, 3.0% in peritoneal/fallopian tube cancers, and 1.6% in pancreatic cancers, with estimated total contributions of germline *BRCA2* mutations of 14.3%, 5.5%, and 2.8%, respectively. In ampullary cancers, we here show for the first time a frequency of 14.3% *BRCA1/BRCA2* mutations after a combination of direct founder mutation testing and full gene analysis in archival tissue with NGS. Taking into account the implications for both the individuals and their family members, we recommend that patients with these neoplasias may be offered *BRCA1/BRCA2* genetic testing and we here show that it is feasible to reliably perform this analysis in FFPE tissue.

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PAPER II

Implementation of next-generation sequencing for molecular diagnosis of hereditary breast and ovarian cancer highlights its genetic heterogeneity

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Abstract

Molecular diagnosis of hereditary breast and ovarian cancer (HBOC) by standard methodologies has been limited to the *BRCA1* and *BRCA2* genes. With the recent development of new sequencing methodologies, the speed and efficiency of DNA testing has dramatically improved. The aim of this work was to validate the use of next-generation sequencing (NGS) for the detection of *BRCA1/BRCA2* point mutations in a diagnostic setting and to study the role of other genes associated with HBOC in Portuguese families. A cohort of 94 high-risk families was included in the study and they were initially screened for the two common founder mutations with variant-specific methods. Fourteen index patients were shown to carry the Portuguese founder mutation *BRCA2* c.156_157insAlu and the remaining 80 were analyzed in parallel by Sanger sequencing for the *BRCA1*/*BRCA2* genes and by NGS for a panel of 17 genes that have been described as involved in predisposition to breast and/or ovarian cancer. A total of 506 variants in the *BRCA1/BRCA2* genes were detected by both methodologies, with a 100% concordance between them. This strategy allowed the detection of a total of 39 deleterious mutations in the 94 index patients, namely, 10 in *BRCA1* (25.6%), 21 in *BRCA2* (53.8%), four in *PALB2* (10.3%), two in *ATM* (5.1%), one in *CHEK2* (2.6%), and one in *TP53* (2.6%), with 20.5% of the deleterious mutations being found in genes other than *BRCA1/BRCA2*. These results demonstrate the efficiency of NGS for the detection of *BRCA1/BRCA2* point mutations and highlight the genetic heterogeneity of HBOC.

Introduction

More than 20 years have passed since the identification of the two major breast cancer susceptibility genes, *BRCA1* and *BRCA2* [1,2]. The identification of pathogenic mutations in these two genes in families with multiple cases of early onset breast cancer was at the time a major breakthrough in hereditary cancer genetics. In *BRCA1* and *BRCA2* mutation carriers, the cumulative risk at 70 years of developing breast cancer is estimated to be 60% and 55%, respectively, whereas for ovarian cancer is estimated to be 59% and 17%, respectively [3]. Genetic testing of *BRCA1/BRCA2* has several clinical implications, especially for female carriers, who should be offered the option to undergo annual MRI screening and mammography, prophylactic mastectomy and/or salpingo-oophorectomy [4]. In addition, *BRCA1/BRCA2* mutation carriers can now benefit from the use of targeted therapy with the recent approval of PARP inhibitors for the treatment of ovarian cancer [5]. However, the contribution of *BRCA1/BRCA2* pathogenic mutations to high-risk breast cancer families is only around 30%, and can vary according to the population and the criteria for selection of patients with predisposition to breast and/or ovarian cancer [6]. In a recent study from our group, 28.9% of the families with an *a priori* BRCAPRO mutation probability >10% harbored deleterious mutations in these genes [7].

Until now, molecular diagnosis of hereditary breast and/or ovarian cancer (HBOC) has been based on the identification of mutations in *BRCA1/BRCA2* and is usually performed by Sanger sequencing or alternative screening methods that are labor-intensive, have low throughput, and high turnaround time. With the recent development of next-generation sequencing (NGS), the speed and efficiency of DNA testing has dramatically improved. At the same time, NGS allows the possibility to analyze not only *BRCA1/BRCA2* but multiple other genes that have been described as conferring an increased risk for the development of breast or ovarian cancer and that can explain a fraction of *BRCA1/BRCA2* negative families. Germline mutations in *TP53* (Li-Fraumeni syndrome) [8], *CDH1* (Hereditary diffuse gastric cancer) [9], *STK11* (Peutz-Jeghers syndrome) [10], and *PTEN* (Cowden syndrome) [11] predispose to a variety of different cancers, but have in common the fact that they

confer a high risk of breast cancer. Additionally, *PALB2*, *ATM*, *CHEK2* and *NBN* are considered moderate risk breast cancer genes [12-15]. On the other hand, mutations in Lynch syndrome genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*), together with those in *BRIP1*, *RAD51C* and *RAD51D*, are associated with an increased risk for the development of ovarian cancer [16-19]. However, knowledge on the penetrance and the clinical utility of germline mutations in many of these genes is still incomplete [20]. The aim of this work was to validate the use of NGS for the detection of mutations in the *BRCA1* and *BRCA2* genes in a diagnostic setting by performing parallel analysis by Sanger sequencing and NGS in a consecutive series of high-risk breast/ovarian cancer families, as well as to evaluate the genetic heterogeneity in this setting by analyzing a panel of 17 genes associated with predisposition to those diseases.

Methods

Patients

The study included a consecutive series of 94 patients referred to the Genetics Department of the Portuguese Oncology Institute of Porto (IPO Porto) with a family history of breast and/or ovarian cancer and with either an *a priori* >20% probability of finding a *BRCA1/BRCA2* mutation using the BRCAPRO software or a high-risk familial history for which BRCAPRO could underestimate the mutation probability. Samples for genetic testing were obtained after genetic counseling according to institutional review board approved guidelines and standard clinical practice. DNA was extracted from peripheral blood leucocytes and its quality was evaluated using Qubit® Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

BRCA1/BRCA2 analysis

Screening of the Portuguese founder mutations (*BRCA1* c.3331_3334del and *BRCA2* c.156_157insAlu) was initially performed in all cases using a methodology we previously described [7]. In the 80 samples in which no founder mutations were

identified, Sanger sequencing of the entire coding regions and adjacent intronic regions of *BRCA1* and *BRCA2* was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit in a 3500 Genetic Analyzer (Thermo Fisher Scientific), according to the manufacturer's instructions. Sanger sequencing was also performed for confirmation of all the deleterious variants identified by NGS. Multiplex Ligation-dependent Probe Amplification (MLPA) (MRC-Holland, Amsterdam, Netherlands) was used to detect *BRCA1/BRCA2* large genomic rearrangements (LGRs) in the 80 samples negative for founder mutations, according to the manufacturer's instructions.

Next-generation sequencing

Panel gene testing with NGS was used in the 80 samples in which no founder mutations were found after the initial screening. Library preparation was performed using the TruSight Cancer kit (Illumina, Inc., San Diego, CA, USA), which targets the full coding sequence of 94 genes involved in hereditary predisposition to cancer, following the manufacturer's protocol. Sequencing was carried out using a standard flow cell in the MiSeq platform (Illumina, Inc.) in 2x150 bp paired end runs of 24 samples. Sequencing alignment and variant analysis was performed using a bioinformatics pipeline previously validated by us for 23 different genes (Paulo et al., submitted). In brief, alignment and variant calling was done using three different software programs, namely, Isaac Enrichment (v2.1, Illumina, Inc.), BWA Enrichment (v2.1, Illumina, Inc.) and NextGENe (v2.3.4.4, Softgenetics, State College, PA, USA), with *.vcf* files being imported into GeneticistAssistant™ (Softgenetics) for variant annotation. For the purpose of this study, a virtual panel of 17 genes associated with predisposition to breast and/or ovarian cancer was created for variant analysis (Table 1). Variants were retained according to the following criteria: ≤10% frequency in our in-house database, coverage ≥15x, alternative variant frequency ≥15% and minor allele frequency (MAF) <1%, excluding intronic variants more than 12bp away from exon-intron boundaries. For MAF filtering, data was obtained from the 1000 Genomes Project (1000G; Phase III Data), Exome Variant Server (ESP6500) and Exome Aggregation Consortium (ExAC) databases.

Table 1 – Genes included in the NGS panel associated with predisposition to breast/ovarian cancer.

Gene	Reference sequence	Cancer risk	Median coverage
ATM	NM_000051.3	Breast	420
BRCA1	NM_007294.3	Breast/Ovarian	285
BRCA2	NM_000059.3	Breast/Ovarian	367
BRIP1	NM_032043.2	Ovarian	363
CDH1	NM_004360.3	Breast	315
CHEK2	NM_007194.3	Breast	303
MLH1	NM_000249.3	Ovarian	320
MSH2	NM_000251.2	Ovarian	380
MSH6	NM_000179.2	Ovarian	327
NBN	NM_002485.4	Breast	383
PALB2	NM_024675.3	Breast	324
PMS2	NM_000535.5	Ovarian	383
PTEN	NM_000314.4	Breast	370
RAD51C	NM_058216.2	Ovarian	339
RAD51D	NM_002878.3	Ovarian	255
STK11	NM_000455.4	Breast	161
TP53	NM_000546.5	Breast	242

Variant classification

Variants were classified as deleterious if they were predicted to originate a premature codon stop, if they were located in canonical splice sites or if there was literature and/or own evidence to support their classification as pathogenic/likely pathogenic. The potential pathogenicity of the remaining variants, after variant filtering settings were applied, was evaluated depending on the type of mutation. Missense variants were evaluated using MetaSVM and MetaLR scores, which combine 10

different *in silico* prediction tools (SIFT, PolyPhen-2 HDIV, PolyPhen-2 HVAR, GERP++, MutationTaster, Mutation Assessor, FATHMM, LRT, SiPhy and PhyloP) and the maximum frequency observed in 1000G, having a higher predictive power than any of the prediction tools alone [21]. They were also evaluated using the Combined Annotation–Dependent Depletion (CADD) method, which integrates many diverse annotations into a single measure (C-Score) [22]. Missense variants were retained as variants of uncertain significance (VUS) only if they were predicted to be damaging by MetaSVM (rankscore>0.834), MetaLR (rankscore>0.823) and CADD (C-Score>15). Synonymous and intronic variants were retained only if they were predicted to have an impact on splicing by having at least a 15% decrease in MaxEntScan and a 5% decrease of the SpliceSiteFinder score, which was shown to have a 96% sensitivity and 83% specificity for the prediction of *BRCA1/BRCA2* VUS that result in a splicing defect when compared with transcript analysis [23]. Ada and RF scores (dbscSNV), two ensemble learning methods integrating several *in silico* prediction tools, were also evaluated with a cutoff value of 0.6 used [24]. In-frame deletions and insertions were also retained.

Results

Deleterious mutations in *BRCA1* and *BRCA2*

The two most common *BRCA1/BRCA2* mutations in the Portuguese population were screened in the 94 index patients under study and 14 (14.9%) were shown to be carriers of the *BRCA2* c.156_157insAlu (no *BRCA1* c.3331_334del carriers were identified). In the 80 samples negative for founder mutations, *BRCA1/BRCA2* screening of the entire coding regions was performed by Sanger sequencing. A total of 10 pathogenic mutations in *BRCA1* and seven in *BRCA2* were additionally detected, corresponding to a total of 31 (33%) *BRCA1/BRCA2* pathogenic mutations identified in the 94 index cases analyzed. Personal and family cancer history of all *BRCA1/BRCA2* carriers is detailed on Table 2.

Table 2 – Deleterious mutations identified in the 80 index patients by NGS

Sample	Gene	HGVSc	Predicted Protein	Personal History	Family History[a]
S25	***BRCA1***	c.211A>G	r.(spl?)	BC (34)	4x PrCa
S76	***BRCA1***	c.470_471del	p.(Ser157Ter)	OC (46)	2x BC
S75	***BRCA1***	c.2037delinsCC	p.(Lys679AsnfsTer4)	BC (47)	1x BBC, 1x PrCa
S63	***BRCA1***	c.2309C>A	p.(Ser770Ter)	BBC (34,34)	1x BC
S41	***BRCA1***	c.2418del	p.(Ala807HisfsTer8)	OC (46)	4x BC
S32	***BRCA1***	c.3477_3480del	p.(Ile1159MetfsTer50)	OC (41), BC (52)	-
S21	***BRCA1***	c.3817C>T	p.(Gln1273Ter)	BC (38)	1xBC
S44	***BRCA1***	c.3817C>T	p.(Gln1273Ter)	BC (40)	2x BC
S58	***BRCA1***	c.4165_4166del	p.(Ser1389Ter)	BBC (32,47)	3x BC, 1x PrCa
S49	***BRCA1***	c.5266dup	p.(Gln1756ProfsTer74)	BC (37)	3x BC
S54	***BRCA2***	c.2T>G	p.Met1?	BC (41)	4x BC
S61	***BRCA2***	c.793+1G>A	r.spl?	BC (49)	3x BC, 1x OC
S34	***BRCA2***	c.5934dup	p.(Ser1979Ter)	BC (52)	1x MBC
S52	***BRCA2***	c.6656C>G	p.(Ser2219Ter)	BC (60)	3x BC, 1x MBC
S55	***BRCA2***	c.7738C>T	p.(Gln2580Ter)	BC (50)	2x BC, 1x OC
S61	***BRCA2***	c.9097dup	p.(Thr3033AsnfsTer11)	BC (43)	1x BBC, 3x BC, 1x OC
S57	***BRCA2***	c.9453del	p.(Glu3152ArgfsTer11)	BC (50)	3x BC, 1x PrCa
S66	***PALB2***	c.1192del	p.(Val398CysfsTer26)	BC (52)	5x BC
S49	***PALB2***	c.1240C>T	p.(Arg414Ter)	BC (37)	3x BC
S67	***PALB2***	c.1633G>T	p.(Glu545Ter)	BC (47)	5x BC
S56	***PALB2***	c.2257C>T	p.(Arg753Ter)	BC (49)	1x BBC, 2x BC
S5	***ATM***	c.652C>T	p.(Gln218Ter)	BBC (36,48)	3x BC
S28	***ATM***	c.8264_8268del	p.(Tyr2755CysfsTer12)	CRC (57), BC (79)	1x BBC, 4x BC
S1	***CHEK2***	c.349A>G	p.(Arg117Gly)	BC (79)	1x BBC, 1x BC, 1x OC
S13	***TP53***	c.388C>T	p.(Leu130Phe)	CRC (17)	8x BC

[a] Only tumors associated with HBOC included: Breast, Ovarian, Prostate and Pancreatic cancer.
Legend: BC – breast cancer; BBC – bilateral breast cancer; OC – ovarian cancer; PrCa – prostate cancer; MBC – male breast cancer; CRC – colorectal cancer

In order to compare the efficiency of NGS for the detection of *BRCA1/BRCA2* point mutations, we analyzed the same 80 samples that were fully screened by Sanger sequencing using the TruSight Cancer panel. The comparison between NGS and Sanger sequencing was extended to all single nucleotide variants (SNVs) and indels identified. Analysis was restricted to all the variants detected in the coding regions and 12 bp flanking the exons. All the variants detected by NGS with coverage ≤15x and alternative variant frequency ≤15% were filtered out. A total of 506 variants (495 SNVs, 11 indels) were detected by NGS, giving a 100% concordance with Sanger sequencing for detecting *BRCA1/BRCA2* point mutations (data not shown). A median coverage of 285 was obtained for *BRCA1* and of 367 for *BRCA2* (Table 1). Overall, 3840 regions were analyzed in both genes considering all samples, with only 33 (0.86%) having at least one nucleotide with a coverage below 30 and 10 (0.26%) with a coverage below 20 (data not shown).

Deleterious mutations in other genes

In the 80 samples where NGS was performed, we evaluated 15 other genes besides *BRCA1/BRCA2* that have been associated with increased risk of developing either breast or ovarian cancer. The median coverage ranged from 161 in *STK11* to 420 in *ATM* (Table 1). Deleterious mutations were detected in eight different families (10%), four in *PALB2* (three nonsense and one frame-shift mutation) (Fig. 1), two in *ATM* (one nonsense and one frame-shift) (Fig. 2), one missense mutation in *CHEK2* (Fig. 3a) and one missense mutation in *TP53* (Fig. 3b). The *CHEK2* missense mutation c.349A>G (p.Arg117Gly) has been reported in ClinVar as likely pathogenic, with functional studies showing that this variant results in a CHEK2 protein with impaired function due to reduced kinase activity, reduced protein stability, and incomplete phosphorylation [25-27]. The c.388C>T (p.Leu130Phe) missense mutation in *TP53* has been previously described as deleterious [28,29]. Personal and family cancer history of all carriers is detailed on Table 2.

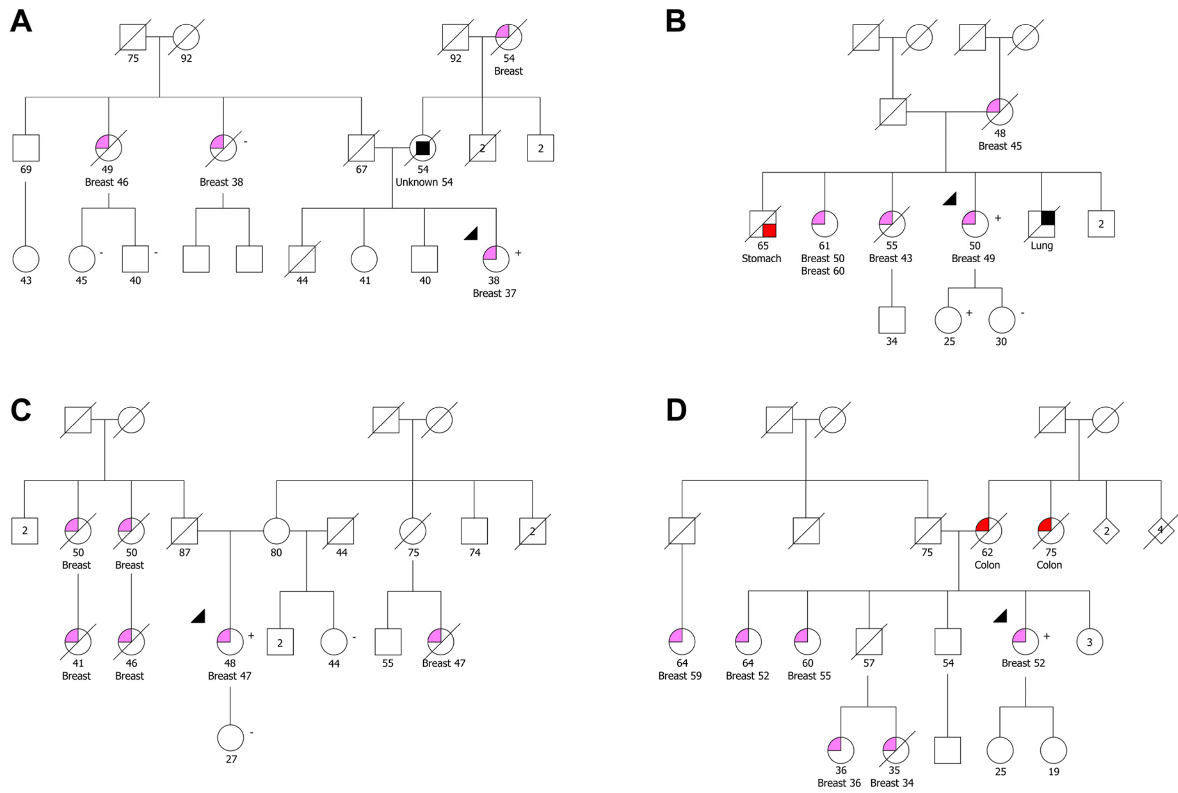

Figure 1 – Pedigrees of individuals with *PALB2* deleterious mutations detected. Family of the individual with both the *BRCA1* c.5266dup and the *PALB2* c.1240C>T mutation (A), the individual with the *PALB2* c.1633G>T mutation (B), the individual with the *PALB2* c.1192del mutation (C) and the individual with the *PALB2* c.2257C>T mutation (D). The index case is indicated by an arrow.

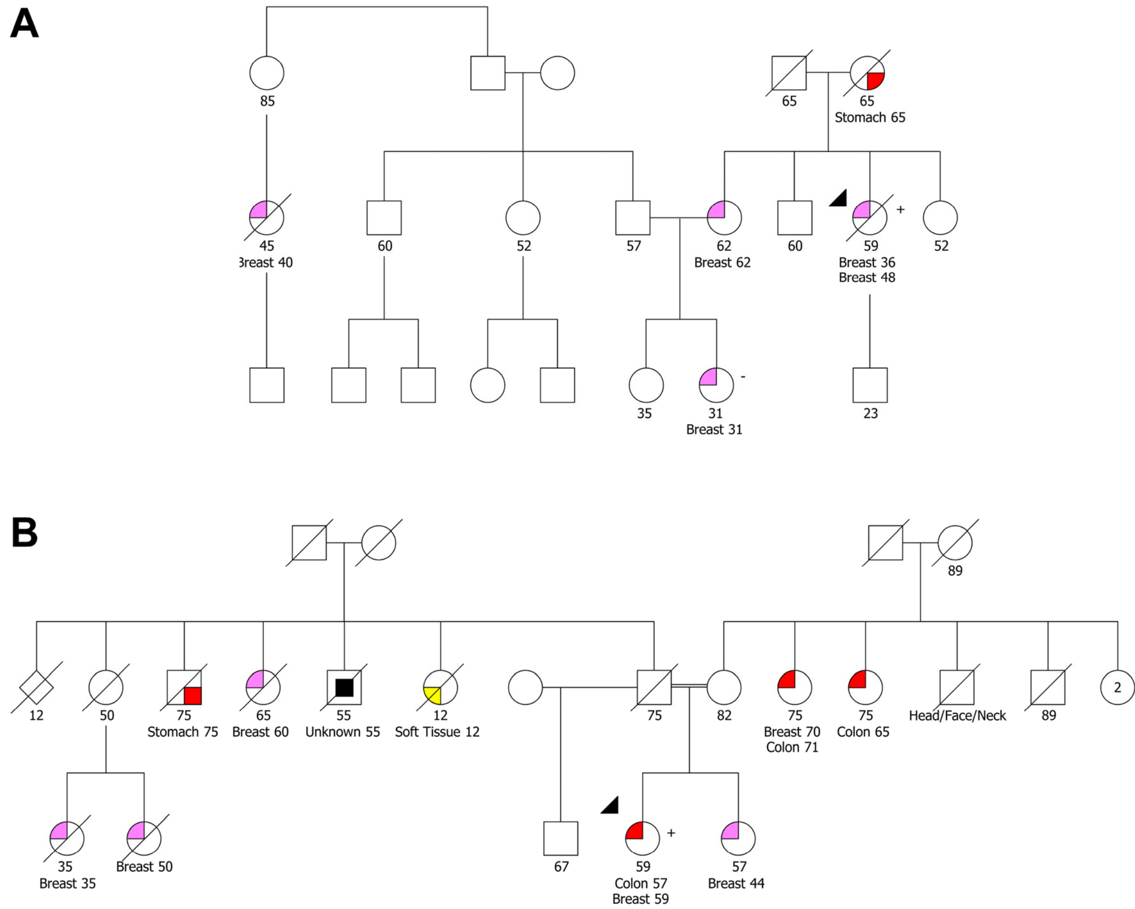

Figure 2 – Pedigrees of individuals with *ATM* deleterious mutations detected. Family of the individual with the *ATM* c.652C>T mutation (A) and the individual with the *ATM* c.8264_8268del mutation (B). The index case is indicated by an arrow.

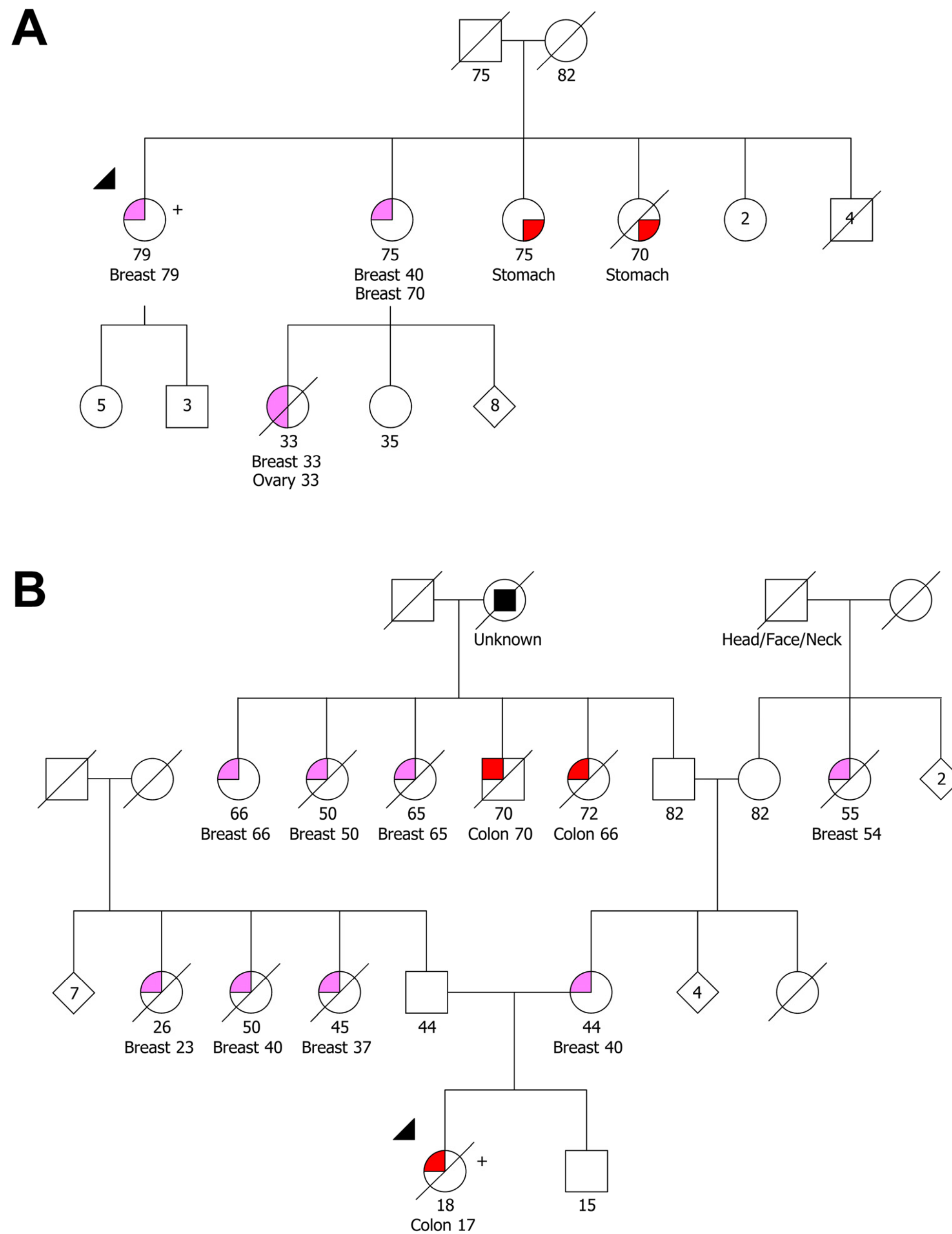

Figure 3 – Pedigrees of individuals with *CHEK2* and *TP53* deleterious mutations detected. Family of the individual with the *CHEK2* c.349A>G mutation (A) and the individual with the *TP53* c.388C>T mutation (B). The index case is indicated by an arrow.

Incidental findings

We detected an in-frame deletion of 15 bp in the *MSH6* gene (c.3848_3862del, p.Ile1283_Tyr1287del) in a patient diagnosed with breast cancer at the age of 32 years. This variant had been previously identified in two Lynch syndrome families in our laboratory with loss of MSH6 expression in the tumor (unpublished data) and it is also described as a causal mutation in the UMD database (www.umd.be) in a patient with colorectal cancer and loss of MSH6 expression in the tumor, hence we consider it to be likely pathogenic. However, we did not observe loss of MSH6 expression in the breast tumor of our index patient (data not shown). Her family history includes an uncle diagnosed with male breast cancer at 60 years and both the maternal and paternal grandmother diagnosed with colorectal cancer at 72 years (Online Resources 1).

Variants of uncertain significance

Applying the thresholds for missense and potential splicing mutations described earlier (see variant classification) after variant filtering, 10 missense variants were predicted to be deleterious, one variant was predicted to induce a splicing defect and one in-frame deletion was retained (Table 3). Of these, eight variants (66.7%) were observed in families where no clearly deleterious mutations were identified.

Table 3 – Variants of uncertain significance identified in the 80 index patients by NGS

Sample	Gene	HGVSc	Predicted Protein	dbSNP ID	1000G_AF	ExAC_AF	ESP6500_AF	MetaSVM[a]	MetaLR[a]	CADD (C-Score)[a]	MaxEntScan (% decrease)[b]	SpliceSiteFinder (% decrease)[b]	Ada Score[b]	RF Score[b]
S67	***ATM***	c.1049C>T	p.Ala350Val	rs375049090	N/A	N/A	0.008	0.853	0.845	27.8	N/A	N/A	N/A	N/A
S80	***BRCA1***	c.80+5G>C	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-48.8%	-13.9%	0.998	0.876
S36	***BRCA1***	c.190T>A	p.Cys64Ser	N/A	N/A	N/A	N/A	0.968	0.998	25.1	N/A	N/A	N/A	N/A
S21	***BRCA2***	c.4933_4935del	p.Lys1645del	N/A	N/A	0.001	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S77	***BRCA2***	c.7975A>G	p.Arg2659Gly	rs80359026	N/A	N/A	N/A	0.960	0.958	27.7	N/A	N/A	N/A	N/A
S79	***BRCA2***	c.9004G>A	p.Glu3002Lys	rs80359152	N/A	N/A	N/A	0.910	0.903	22.4	N/A	N/A	N/A	N/A
S9, S49	***BRIP1***	c.139C>G	p.Pro47Ala	rs28903098	N/A	0.024	0.023	0.836	0.829	24.1	N/A	N/A	N/A	N/A
S39	***CHEK2***	c.757A>G	p.Lys253Glu	N/A	N/A	N/A	N/A	0.912	0.899	17.1	N/A	N/A	N/A	N/A
S60	***CHEK2***	c.1169A>C	p.Tyr390Ser	rs200928781	N/A	0.004	N/A	0.944	0.915	28.7	N/A	N/A	N/A	N/A
S3	***MLH1***	c.649C>T	p.Arg217Cys	rs4986984	0.060	0.032	N/A	0.952	0.943	22.4	N/A	N/A	N/A	N/A
S43	***MLH1***	c.2066A>G	p.Gln689Arg	rs63750702	N/A	0.028	0.023	0.840	0.877	22.2	N/A	N/A	N/A	N/A
S63	***MSH6***	c.3478G>A	p.Val1160Ile	rs376799914	N/A	0.005	0.008	0.864	0.866	22.1	N/A	N/A	N/A	N/A

N/A – Not available/Not applicable

[a] Missense variants were retained as VUS if they were predicted to be damaging by MetaSVM (rankscore>0.834), MetaLR (rankscore>0.823) and CADD (C-Score>15) [21,22].

[b] Synonymous and intronic variants were retained if they had at least a 15% decrease in MaxEntScan, a 5% decrease of the SpliceSiteFinder score and an Ada and RF score higher than 0.6 [23,24].

Discussion

NGS is increasingly being adopted in diagnostic laboratories because it offers higher throughput, faster turnaround time and the possibility to expand the molecular diagnosis to rarer causative mutations, all without an increase in the cost of the analysis when compared to conventional methodologies. Nevertheless, before integration of NGS in a clinical setting, the efficiency of the methodology needs to be validated by individual laboratories, considering the different library preparation methods, the different sequencing chemistries and especially the different bioinformatics algorithms for alignment, variant calling and variant filtering available. We have recently established a bioinformatics NGS pipeline validated on a series of 32 samples with various types of mutations in 23 different genes involved in hereditary predisposition to cancer (Paulo et al., submitted). Here, we wanted to validate this previously established pipeline for the detection of *BRCA1/BRCA2* point mutations in a large series of high-risk HBOC patients and to take advantage of the higher throughput offered by NGS to characterize the involvement of other genes associated with an increased risk for developing breast and/or ovarian cancer.

We obtained 100% sensitivity and specificity (total of 506 variants) for the detection of *BRCA1/BRCA2* point mutations with our bioinformatics pipeline using a targeted enrichment approach when compared to the gold standard Sanger sequencing. Although the majority of the variants were SNVs, 11 indels were present in the samples analyzed, which are known to be particularly sensitive to false negatives by NGS (Paulo et al., submitted) [30,31]. Other studies have reported the validation of NGS for the detection of *BRCA1/BRCA2* mutations using different workflows and platforms. All achieved a sensitivity of 100% with false positives ranging from 1-1.8% in Illumina platforms [32,33] to 7.5-8.8% on the Ion Torrent [31,34]. In a diagnostic setting, low coverage regions require Sanger sequencing to ensure that a putative mutation is not missed because there were not enough reads covering that nucleotide. In our series, only 0.41 (33/80) or 0.13 (10/80) sequencing reactions per sample would be required if the minimum coverage threshold used was 30 or 20, respectively. Currently, molecular diagnosis of *BRCA1/BRCA2* needs to be completed

by other methodologies, such as MLPA, for the detection of LGRs, but it is expected that in the future these will also be reliably detected by NGS with the validation of specific algorithms for detection of copy number variations, such as CONTRA, CNV-seq or ExomeCNV [35-37].

A frequency of 33% pathogenic *BRCA1/BRCA2* mutations was observed in our 94 patients, which is slightly higher than the frequency of 28.9% that we previously observed in a larger series of HBOC patients [7], a difference that may be explained by the more stringent criteria used for cohort selection in the current study. The *BRCA2* c.156_157insAlu rearrangement remains the most frequent *BRCA1/BRCA2* mutation in our population (45%) and this Alu insertion is not detectable using regular NGS bioinformatic algorithms designed for the detection of SNVs and indels [32] or by standard Sanger sequencing. Although its high frequency in our population warrants initial screening of this mutation before *BRCA1/BRCA2* full screening, in other populations patients with Portuguese ancestry should be offered specific testing for this mutation somewhere in the genetic testing algorithm [38]. Of all the other deleterious mutations identified in this study, the *BRCA2* c.2T>G deserves some attention, as it had been previously identified by our group and classified as a VUS due to nonsegregation in an affected relative in the initial family [39]. However, recent evidence suggests that mutations disrupting *BRCA2* initiation codon induce exon 2 skipping, with translation being initiated mostly at an out-of-frame ATG, leading to loss of protein function [40].

The other objective of this work was to characterize the spectrum of mutations in other genes predisposing to breast/ovarian cancer in high-risk families. We found deleterious mutations in eight families (10% of the families analyzed by NGS and 8.5% of all families), corresponding to 20.5% of all deleterious mutations identified (8/39) (Fig. 4). In families negative for *BRCA1/BRCA2* mutations, the frequency of deleterious mutations was 11.1% (7/63), which highlights the genetic heterogeneity underlying inherited predisposition to breast/ovarian cancer. Mutations were observed in *PALB2* (4), *ATM* (2), *CHEK2* (1) and *TP53* (1). *PALB2* mutations have been consistently described in familial and early-onset breast cancer and the cumulative risk until age 70y for developing breast cancer in a large cohort of *PALB2* mutation

carriers has been reported to range from 33% without family history taken into account to 58% in those with a strong family history (being 44% and 67%, respectively, at age 80y), which is similar to the risks described for *BRCA2* [12]. In our study, mutations in this gene were found in 5% of the families analyzed by NGS. In one of the families, a *BRCA1* pathogenic mutation was also identified, but they could have arisen from different branches of the family as both have relatives affected with breast cancer, with segregation studies required to confirm this possibility (Fig. 1a). Truncating variants in *ATM* also confer an increased risk to breast cancer (relative risk=2.8), which seems to be similar to *CHEK2* (relative risk=3.0) but lower than *PALB2* (relative risk=5.3) [20]. Both the probands with *ATM* and *CHEK2* deleterious mutations had a family history of breast and/or ovarian cancer, but other tumors, such as colorectal, stomach and soft tissue, were also present (Fig. 2, 3a). We also detected a missense mutation in *TP53* in a proband diagnosed with colorectal cancer at age 17 years and a significant family history of breast and colon cancer (Lynch syndrome had been excluded). Interestingly, this family did not fulfill the Chompret (or other) criteria for *TP53* mutation testing to diagnose Li-Fraumeni syndrome [29], being a good example of the potential of NGS to increase the molecular diagnosis yield in situations in which different syndromes have overlapping clinical features and in which genetic testing criteria do not have a 100% sensitivity. Although the index patient had early-onset colorectal cancer, which is not part of the most typical tumor spectrum of either HBOC or Li-Fraumeni syndrome, this family had been selected because of very strong family history of early-onset breast cancer (especially from the paternal side, Fig. 3b) and indeed recent data shows that *TP53* mutations are found in 6% of females with breast cancer diagnosed before the age of 31 years in the absence of other features indicative of Li-Fraumeni syndrome, especially if their tumors are HER2-positive [41]. Some of the other genes included in our study and in many commercial NGS panels for HBOC still require further evidence from larger studies to confirm the relative risks for developing cancer, which will be helpful in determining their clinical utility. One example is *BRIP1*, which was initially described as conferring an increased risk for breast cancer [42], but a recent study in a large cohort of patients found no association of truncating variants with breast cancer risk [43]. Having said that, the most recent

NCCN guidelines already recommend breast MRI screening for carriers of *ATM*, *CHEK2* and *PALB2* mutations (in addition to previously known breast cancer high-risk genes *BRCA1*, *BRCA2*, *TP53*, *CDH1*, *STK11* and *PTEN*), and that the possibility of risk-reducing mastectomy should be discussed with *PALB2* carriers. Carriers of *BRIP1*, *RAD51C* and *RAD51D* mutations, on the other hand, should consider the option of performing risk-reducing salpingo-oophorectomy according to the latest NCCN guidelines, in line with what was already recommended for *BRCA1/BRCA2* and Lynch syndrome carriers [4].

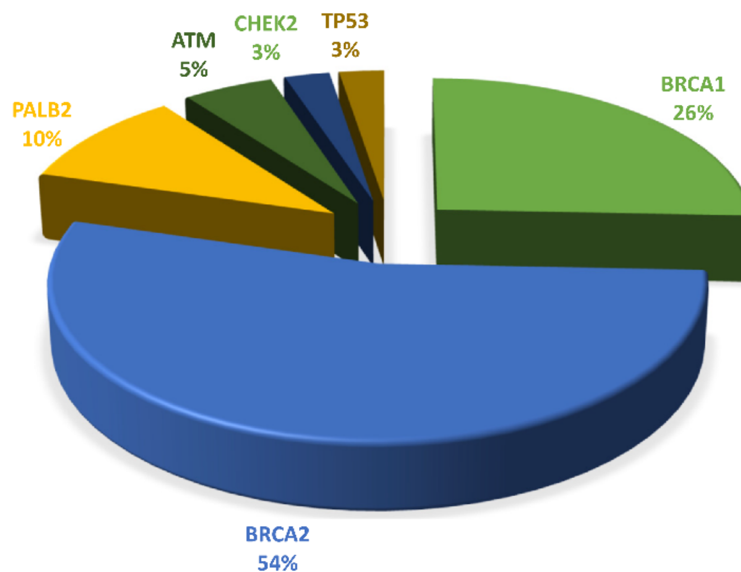

Figure 4 – Deleterious mutations identified per gene (%) in the 94 index patients.

With the adoption of NGS there is some concern about the identification of incidental findings, disease-causing variants in high-penetrance genes in patients without the associated phenotype. Here, we detected a likely pathogenic mutation in *MSH6* (c.3848_3862del, p.Ile1283_Tyr1287del) in a patient with breast cancer without loss of MSH6 expression in the tumor, indicating that her breast carcinoma was not related with the *MSH6* germline mutation, contrarily to the existent evidence for its involvement in the pathogenesis of colorectal cancer in typical Lynch syndrome families. Taking into account the family history of the patient, there was no indication

to perform genetic testing of mismatch repair (MMR) genes (Online Resources 1), but the carriers of this mutation in this family are still at risk of developing Lynch syndrome-associated neoplasias and adequate surveillance has been offered to the patient and her relatives after genetic counselling.

The use of bioinformatic tools is mandatory in order to compensate for the increased risk of finding VUS when one increases the number of genes analyzed by NGS, especially in whole-genome and whole-exome studies [21,44,45]. Here, we report the use of a panel of 94 genes with analysis restricted to the genes of interest taking into account the clinical phenotype together with the use of *in silico* prediction tools for stratification of VUS. Although these tools cannot be used for classification of variants *per se*, they are useful for prioritization of VUS for further segregation and functional studies [23,46]. We identified 12 VUS predicted to be deleterious *in silico*, eight of them in families where no clearly deleterious mutations were found, and these are the variants that we will prioritize for segregation studies (Table 3). The *BRCA1* c.190T>A (p.Cys64Ser) is located in the highly conserved RING domain of this gene and there are already various missense mutations in this domain described as pathogenic [47,48]. Other VUS were identified in *ATM, BRCA1, BRCA2, BRIP1, CHEK2, MLH1* and *MSH6*, but the data available for these variants is scarce. Most of these variants may in the future be reclassified as deleterious or benign, but in the meantime they cannot be used to make clinical decisions.

There are some limitations in our study. Our sample size is relatively small and we selected families with high-risk to breast/ovarian cancer, which may increase the likelihood of identifying a deleterious mutation in breast/ovarian cancer predisposing genes. Nonetheless, the frequency of *BRCA1/BRCA2* mutations identified is only slightly higher compared to a previous study where less stringent criteria were used and it is not certain that mutations in moderate penetrance genes are more likely to be found in high-risk families. Furthermore, the gene panel used in our study did not include the *RECQL* gene, recently reported to be associated with the risk of breast cancer in populations from Canada and Poland [49].

In conclusion, we have validated the use of NGS for the detection of *BRCA1/BRCA2* point mutations in a large series of patients, offering a higher

throughput and higher molecular diagnostic yield in the study of inherited predisposition to breast/ovarian cancer and making possible to address its extensive genetic heterogeneity. This strategy allowed the identification of 39 deleterious mutations in 40% of the families (38/94). The detection of deleterious mutations in some of these genes already has a significant impact in the clinical management of carriers, although further studies are necessary to make reliable estimates of cancer risk for many of the other genes included in current multigene panel testing to allow appropriate genetic counseling of these patients and their relatives.

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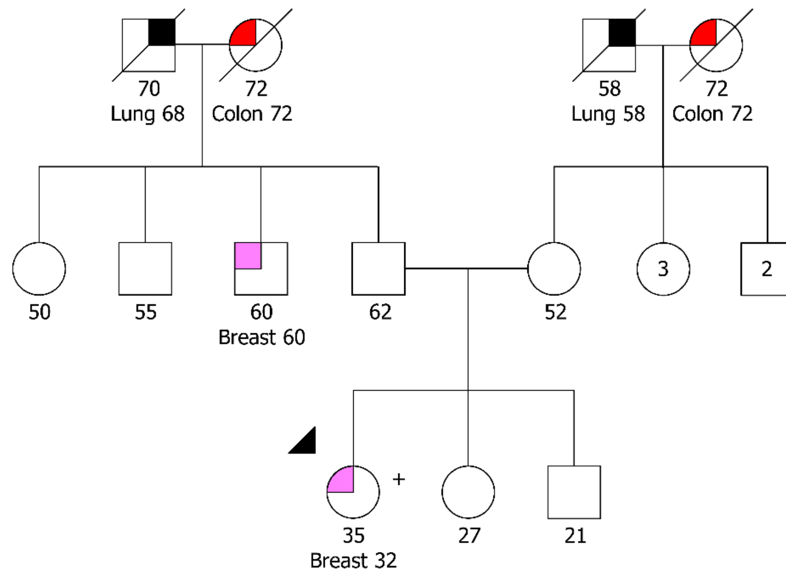
Ethical standards

This study was performed according to institutional review board approved guidelines and standard clinical practice and informed consent was obtained from all individual participants included in the study.

Conflicts of interest

The authors declare no conflicts of interest.

Supplementary Information



Online Resources 1 – Pedigree of the individual with the *MSH6* c.3848_3862del mutation. The index case is indicated by an arrow.

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GENERAL DISCUSSION

1. Diagnosis of inherited cancer predisposition in archival tissue

Molecular testing of *BRCA1/BRCA2* is usually performed on genomic DNA extracted from peripheral blood leucocytes. Nevertheless, families with a strong family history of tumors associated with *BRCA1* and *BRCA2* mutations without an affected member available for genetic testing are not uncommon. This occurs because either all affected relatives are already deceased or they are living in other cities or countries. Genetic testing in unaffected individuals in such families is not ideal and is often uninformative. Hence, the possibility of identifying *BRCA1/BRCA2* mutations in archival tissue of affected relatives is helpful, being also useful for retrospective studies of rarer cancers. Furthermore, it may allow the identification of both somatic and germline mutations. However, DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue is usually of low quantity and quality, making harder the analysis of large genes such as *BRCA1* and *BRCA2*.

We developed a method that allows the identification of the *BRCA2* c.156_157insAlu and the *BRCA1* c.3331_3334del mutations in FFPE tissue (applicable both in neoplastic cells or in the surrounding normal tissue) (Paper I). This optimized method for FFPE tissue is especially important for the detection of the *BRCA2* c.156_157insAlu mutation in patients with Portuguese ancestry, as this prevalent mutation is not readily detectable by standard or next-generation sequencing technologies [De Brakeleer *et al*, 2013, Peixoto *et al*, 2015]. The preferential amplification of the shorter allele makes detection of the c.156_157insAlu mutation unviable with the standard method used for genomic DNA extracted from peripheral leucocytes [Peixoto *et al*, 2015]. Therefore, our method for the detection of this mutation in FFPE tissue consists in the amplification of exon 3 followed by a nested PCR specific for the Alu rearrangement. For the detection of the *BRCA1* c.3331_3334del mutation, a shorter amplicon was designed with the presence of the mutation being determined by fragment analysis. We have also performed screening of the entire coding regions of *BRCA1* and *BRCA2* in a consecutive series of ampullary tumors using a commercial kit optimized for FFPE tissue on a MiSeq, showing that it is now feasible to perform full analysis of *BRCA1/BRCA2* in archival tissue (Paper I). With the approval of a PARP inhibitor for the treatment of BRCA-mutated (germline or

somatic) high-grade serous ovarian tumors [Ledermann *et al*, 2014], and with clinical trials ongoing on other tumors associated with HBOC syndrome, it is expected that the identification of *BRCA1/BRCA2* mutations in archival tissue will soon become crucial for several cancers associated with this pathogenetic mechanism.

2. Contribution of the founder mutations *BRCA2* c.156_157insAlu and *BRCA1* c.3331_3334del for cancer etiology in unselected hospital-based cohorts of patients diagnosed with rarer cancers associated with HBOC syndrome

Germline mutations in the *BRCA1* and *BRCA2* genes are responsible for the HBOC syndrome, which is characterized by an increased risk to breast and ovarian cancer, as well as other tumors like those of the prostate, pancreas, male breast, peritoneum and fallopian tube. Recently, a large characterization of the mutational spectrum of germline *BRCA1*/*BRCA2* mutations in 1050 Portuguese breast/ovarian cancer families was reported [Peixoto *et al*, 2015]. A total of 119 pathogenic mutations were detected, 41.2% in *BRCA1* and 58.8% in *BRCA2*. The *BRCA2* c.156_157insAlu mutation was present in 32% of all Portuguese HBOC families and represented 55% of the *BRCA2* mutations, whereas the *BRCA1* c.3331_3334del mutation was present in 11% of all families and 26% of the families with a *BRCA1* mutation. Hence, the two most common *BRCA1/BRCA2* mutations in the Portuguese population account for about 43% of the total deleterious mutations in these genes. One of the applications of the analysis of frequent and founder *BRCA1/BRCA2* mutations is in the study of other tumors that are part of the HBOC syndrome spectrum. We have recently evaluated the contribution of the two most frequent *BRCA1*/*BRCA2* founder mutations for early-onset and/or familial prostate cancer in Portugal [Maia *et al*, 2016].

In this work we analyzed a consecutive series of patients diagnosed with rarer tumors associated with the HBOC syndrome, namely, cancer of the pancreas, male breast, peritoneum, and fallopian tube (Paper I). A total of 102 male breast, 68 pancreatic and 33 peritoneal/fallopian tube carcinoma cases were included in the

study. The *BRCA2* c.156_157insAlu mutation was observed with a frequency of 7.8% in male breast cancers, 3.0% in peritoneal/fallopian tube cancers, and 1.6% in pancreatic cancers, with estimated total contributions of germline *BRCA2* mutations of 14.3%, 5.5%, and 2.8%, respectively. No carriers of the *BRCA1* c.3331_3334del mutation were identified. The frequencies of *BRCA1/BRCA2* mutations we observed in the different tumors analyzed are generally in accordance with the literature, although a higher frequency has been reported in other studies of peritoneal and fallopian tube cancers, usually similar to frequencies observed in consecutive series of ovarian cancer (~15%) [Vicus *et al*, 2010, Walsh *et al*, 2011, Alsop *et al*, 2012]. In fact, it is currently accepted that most high grade serous ovarian carcinomas arise from a precursor lesion in the fallopian tube, which progress to an invasive, high-grade tumor eventually involving the ovary itself [Crum *et al*, 2007, Kindelberger *et al*, 2007]. Therefore, a similar frequency of *BRCA1/BRCA2* mutations is expected in ovarian and fallopian tube cancers if they have the same origin. The lower frequency we observed can be explained by the fact that only founder mutations were analyzed and the *BRCA1* founder mutation, which is the gene more commonly associated with these tumors, only represents 11% of all families and 26% of the families identified with a *BRCA1* mutation in Portuguese HBOC families.

The identification of *BRCA1/BRCA2* mutation carriers has implications for both the individuals and their family members, allowing reliable genetic counseling and predictive genetic testing. Female carriers can decide whether they want to participate in surveillance protocols and/or perform risk-reducing surgical interventions such as RRM and RRBSO, whereas mutation positive males can engage in breast and/or prostate cancer screening [NCCN, 2016]. Furthermore, *BRCA1/BRCA2* mutation carriers can also benefit from targeted therapy agents, such as olaparib [Ledermann *et al*, 2014, Kaufman *et al*, 2015]. Taking into account the implications of the identification of *BRCA1/BRCA2* mutations and the results we obtained, we recommend that patients with the neoplasias studied (pancreas, male breast, peritoneum and fallopian tube) may be offered *BRCA1/BRCA2* genetic testing, or at least, testing of founder mutations in the Portuguese population (Paper I).

3. Germline *BRCA2* mutations in patients with ampullary carcinomas

In the course of our study, a patient with an ampulla of Vater carcinoma was incidentally found to carry the *BRCA2* c.156_157insAlu mutation, so we decided to test a consecutive series of additional 15 ampullary carcinomas for *BRCA1/BRCA2* mutations using a combination of direct founder mutation testing and full gene analysis with NGS. *BRCA2* mutations were observed in two patients with ampulla of vater carcinoma, representing a frequency of 14.3% in these tumors (Paper I). In one of the patients, the mutation was confirmed to be germline in peripheral blood and he had been previously diagnosed with prostate cancer and had two close blood relatives affected with female breast cancer. The other patient had no family history of tumors associated with HBOC (only one sister diagnosed with colorectal cancer), highlighting the fact that in some cases *BRCA1/BRCA2* mutations can be identified in families without familial aggregation of breast and/or ovarian cancer. Ampullary cancer is currently not part of the HBOC syndrome tumor spectrum, although a *BRCA2* mutation carrier with a carcinoma of the ampulla of Vater has been previously identified during predictive genetic testing [Aburjania *et al*, 2014]. Our study is the first to perform full analysis of the *BRCA1/BRCA2* genes in a consecutive series of ampullary carcinomas. Considering the small sample size of our study, larger independent studies are warranted to confirm the association of *BRCA1/BRCA2* mutations with ampullary cancer and its eventual inclusion in the tumor spectrum of HBOC syndrome.

4. Sensitivity and specificity of next-generation sequencing for the detection of point mutations in the *BRCA1* and *BRCA2* genes compared with Sanger sequencing

The identification of *BRCA1/BRCA2* mutations has been traditionally performed by Sanger sequencing or alternative screening methods that are labor-intensive and have low throughput and high turnaround time. High-throughput NGS technologies, which allow the simultaneous analysis of thousands to millions of DNA sequence fragments, have unwrapped a new paradigm in the search for the molecular

causes of genetic disorders, such as HBOC. Nonetheless, before the implementation of a new methodology in a clinical laboratory, a validation is required to ensure that quality standards, such as sensitivity and specificity, are maintained.

We performed a validation of NGS for the detection of *BRCA1/BRCA2* point mutations by analyzing a total of 80 samples, negative for the two common Portuguese founder mutations, in parallel by Sanger sequencing and NGS (Paper II). The analysis by NGS was performed using a commercially available kit (TruSight Cancer, Illumina) and a previously validated bioinformatics pipeline (Paulo et al., submitted). A total of 506 variants (495 SNVs, 11 indels) were detected by both methodologies, giving 100% sensitivity and specificity of NGS for the detection of *BRCA1/BRCA2* point mutations. A median coverage of 285 was obtained for *BRCA1* and of 367 for *BRCA2*. Overall, 3840 regions were analyzed in both genes considering all samples, with only 33 regions (0.86%) having at least one nucleotide with a coverage below 30 and 10 (0.26%) with a coverage below 20 (Paper II). Our bioinformatics pipeline consists of three different software programs for alignment and variant calling. Although in this study all the mutations were identified by the three different software programs, we have previously observed that they do not have the same sensitivity for the detection of mutations, especially for the detection of deletions or insertions of more than one base pair (Paulo et al., submitted). Hence, a combination of different algorithms and its proper validation is recommended before the implementation of NGS in a clinical laboratory. The maintenance of sensitivity and specificity, the faster turnaround time, the possibility in the near future to replace other technologies (such as MLPA, for the detection of LGRs, in the same analysis), and the higher throughput (allowing the analysis of other genes besides *BRCA1* and *BRCA2*), all without an increase in the cost of the analysis, are reasons to recommend the implementation of NGS in diagnostic laboratories.

5. Genetic heterogeneity of hereditary breast and ovarian cancer

One of the major advantages of NGS is its higher throughput, allowing the expansion of the molecular diagnosis of HBOC to other genes not commonly screened

due to methodology limitations. In order to evaluate the genetic heterogeneity of HBOC, we analyzed a panel of 17 genes (*ATM, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, MLH1, MSH2, MSH6, NBN, PALB2, PMS2, PTEN, RAD51C, RAD51D, STK11* and *TP53*) that have been described as conferring an increased risk to the development of breast and/or ovarian cancer in a consecutive series of 94 high-risk families (Paper II). The two most common *BRCA1*/*BRCA2* Portuguese founder mutations were initially screened in all samples, with the negative samples being analyzed by NGS for the 17 genes. A total of 39 deleterious mutations in the 94 index patients were detected, namely, 10 in *BRCA1* (25.6%), 21 in *BRCA2* (53.8%), four in *PALB2* (10.3%), two in *ATM* (5.1%), one in *CHEK2* (2.6%), and one in *TP53* (2.6%), with 20.5% of the deleterious mutations being found in genes other than *BRCA1/BRCA2*. The *BRCA2* c.156_157insAlu mutation was the most common mutation identified, being present in 14 (15%) index patients. *BRCA1/BRCA2* mutations were detected in 33% of the index cases tested, a slightly higher frequency than we previously observed in a larger series of HBOC patients (29%) [Peixoto *et al*, 2015], probably due to the more stringent criteria used for cohort selection.

The use of panel gene testing for the molecular diagnosis of HBOC has advantages but also brings some concerns. The diagnostic yield can be improved, as exemplified by the fact that 20.5% of the mutations we identified are in genes other than *BRCA1* or *BRCA2*. A 11.1% frequency of deleterious mutations was found in families negative for *BRCA1/BRCA2* mutations, representing an overall increase of 7% in the detection of families with deleterious mutations (from 33% to 40%; Paper II). It also provides the opportunity to identify deleterious mutations when different syndromes have overlapping clinical features and in which genetic testing criteria do not have a 100% sensitivity, as illustrated by the identification of a pathogenic *TP53* missense mutation in a family with a significant family history of breast and colorectal cancer but not fulfilling the Chompret (or other) criteria for *TP53* mutation testing to diagnose LFS. On the other hand, we have identified one index patient with a deleterious *BRCA1* mutation that also harbored a *PALB2* deleterious mutation, showing that mutations in different genes can occur in the same family. During predictive genetic testing in a family with a deleterious *BRCA1* or *BRCA2* mutation, it

is common to communicate to family members that have not inherited the mutation that their risk of breast and/or ovarian is similar to that of the general population. However, this may not apply to this family, as a relative of the index patient may not be a carrier of the *BRCA1* mutation but still be at increased risk for the development of breast cancer if it has inherited the *PALB2* mutation.

The major concerns regarding the use of an extended panel of genes are the identification of incidental findings (disease-causing variants in high-penetrance genes in patients that do not have the associated phenotype) and VUS (variants with an unclear clinical significance). We detected a likely pathogenic mutation in *MSH6* in a patient with breast cancer without loss of MSH6 expression in the tumor, indicating that her breast carcinoma was not related with the *MSH6* germline mutation. There was no indication to perform genetic testing of the MMR genes considering the pedigree of the family but carriers of this mutation in this family are still at risk of developing Lynch syndrome-associated neoplasias and genetic counseling should be offered in such cases. The identification of VUS increases largely with the increase in the number of genes being analyzed. This can only be compensated with the use of bioinformatic tools to predict the impact of the mutations *in silico* combined with curated settings for variant filtering. Although these tools cannot be used for classification of variants *per se*, they are useful for prioritization of VUS for further segregation and functional studies [Houdayer *et al*, 2012, Vallee *et al*, 2016]. We have identified a total of 12 VUS predicted to be deleterious by algorithms that combine a variety of different *in silico* prediction tools and the population frequency of these variants, as the combination of different prediction tools increases the predictive power compared to their use individually (Paper II). Until the development of better *in silico* prediction tools or segregation or functional studies that allow reclassification of VUS into either pathogenic or benign mutations, these variants cannot be used to make clinical decisions. Other concern regarding the use of multigene panel testing is the fact that there are many genes that have been described as predisposing to breast and/or ovarian cancer, but the relative and cumulative risks for carriers of mutations in those genes have not been reliably estimated, which is important to ascertain their clinical utility [Easton *et al*, 2015]. We have analyzed in our study a total of 17 genes

associated with HBOC but many more have been described in the literature and others will be identified in the future with the increasing adoption of whole-exome and whole-genome sequencing studies. The majority of the other genes associated with predisposition to breast and/or ovarian cancer are involved in HR or in the Fanconi anemia pathway [Ghimenti *et al*, 2002, Heikkinen *et al*, 2003, Kiiski *et al*, 2014, Cybulski *et al*, 2015, Ellingson *et al*, 2015]. They can and should be used in research projects in order to evaluate their contribution to HBOC, but only those in which their clinical utility has been reliably estimated should be used to engage patients in surveillance and/or prevention protocols.

The *BRCA1* and *BRCA2* genes are usually the only genes that are recognized as the cause of the HBOC syndrome. This is probably because they are the main genes that predispose to breast and/or ovarian cancer, and for the most part of the last 20 years they were indeed the only ones that were feasible to test routinely in familial breast and/or ovarian cancer. Although mutations in these genes mainly predispose to breast and ovarian cancer, other tumors are included in the spectrum of the HBOC syndrome (Paper I). With the advances introduced by NGS, other genes can now easily be included in genetic testing of families with a significant family history of breast and/or ovarian cancer (Paper II). Some of these genes, such as *PTEN, TP53, CDH1* and *STK11*, have other distinct features associated with germline mutations and predisposition to breast cancer is not the main feature of their respective syndromes. Other genes such as *ATM*, *CHEK2* and, especially, *PALB2*, are more similar to *BRCA1* and *BRCA2* with regard to having breast cancer as the core feature. However, it is presently unclear whether germline mutation carriers in these genes have clinically significant risks for other cancers (namely, ovarian cancer) or indeed what is the name of the cancer predisposition disease they carry and what its relationship is with HBOC.

CONCLUSIONS

The main conclusions of this thesis are:

1. The detection of the germline founder mutations *BRCA2* c.156_157insAlu and the *BRCA1* c.3331_3334del, and eventually full gene analysis, is possible in archival tissue, making it an alternative for molecular diagnosis of inherited predisposition.

2. The *BRCA2* c.156_157insAlu mutation was observed with a frequency of 7.8% in male breast cancers, 3.0% in peritoneal/fallopian tube cancers, and 1.6% in pancreatic cancers, with estimated total contributions of germline *BRCA2* mutations of 14.3%, 5.5%, and 2.8%, respectively.

3. *BRCA2* germline mutations were observed recurrently for the first time in patients with ampulla of Vater carcinomas, with a frequency of 14.3%, raising the possibility of ampullary cancer being part of the cancer spectrum of the HBOC syndrome.

4. The sensitivity and specificity of NGS are as high as those of the gold-standard Sanger sequencing for the detection of *BRCA1/BRCA2* germline point mutations, when a validated bioinformatic pipeline is used.

5. Hereditary breast and ovarian cancer is genetically heterogeneous, with 20.5% of the germline deleterious mutations being found in genes other than *BRCA1/BRCA2*.

FUTURE PERSPECTIVES

The following points will be addressed in future studies:

1. A lower than expected frequency of *BRCA1/BRCA2* mutations was observed in peritoneal/fallopian tube cancers, but only the two most common founder mutations in Portugal were tested. We will perform screening of the entire coding regions of *BRCA1/BRCA2* in all samples of our series of peritoneal/fallopian tube cancer to ascertain the contribution of both somatic and germline *BRCA1/BRCA2* mutations for the pathogenesis of these tumors.

2. Considering that the frequency of *BRCA2* mutations in ampullary carcinomas we observed was obtained in a small series of tumors, we will attempt to perform *BRCA1/BRCA2* screening in a larger series of cases to confirm the association of *BRCA1/BRCA2* mutations with this rare neoplasia.

3. We aim to perform segregation studies in families where VUS were identified, starting with those predicted to be deleterious *in silico*, to evaluate the potential pathogenicity of these variants.

4. We identified deleterious mutations in 40% of high-risk HBOC families, using a panel of genes associated with HBOC. In selected families with a strong family history of breast and/or ovarian cancer and with multiple patients available for study, we will perform whole-exome sequencing to identify new genes predisposing to breast and/or ovarian cancer.

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