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Functional Characterization of Variants of Unknown Significance in Familial Breast Cancer

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Professora Doutora Susana Nunes da Silva,
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Dissertação para a obtenção do Grau de Mestre em Genética Molecular e Biomedicina pela Faculdade de Ciências e Tecnologia da Universidade NOVA de Lisboa

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RESUMO

O cancro de mama familiar representa cerca de 5-10 % dos casos de cancro de mama, devendo-se maioritariamente a mutações herdadas nos genes *BRCA1* e *BRCA2*. Outros genes foram já associados ao seu desenvolvimento encontrando-se na sua maioria relacionados com o sistema de recombinação homologa, um dos principais sistemas envolvidos na reparação de quebras da dupla cadeia de DNA. A realização de testes genéticos para o cancro de mama tem-se tornado padrão, contudo, para além dos resultados positivos e negativos, também se tem detetado variantes de significado desconhecido (VUS - do inglês *variants of unknown significance*), que não podem ser definidas com benignas ou patogénicas. Na tentativa de clarificar o impacto de VUS foram realizados estudos funcionais *in vitro* em linfócitos periféricos de duas pacientes com cancro de mama e uma mutação patogénica no gene *ATM*, duas portadoras de VUS no gene *BRCA1* e duas mulheres controlos. Diversas metodologias foram selecionadas para avaliar a resposta celular às lesões induzidas por radiação-γ (2Gy): técnica de aberrações cromossómicas (AC), de micronúcleos (MN) e ensaio do cometa. Na técnica de AC, não foi observada diferença estatística entre amostras. Na técnica de MN, os portadores de alterações apresentam menor quantidade de células binucleadas com MN que as amostras controlo, possivelmente devido a morte celular. Já os resultados do ensaio do cometa mostram um claro aumento da sensibilidade à radiação ionizante, possivelmente associado à deficiência na reparação, das amostras portadoras da mutação patogénica no gene da *ATM* e dos portadores de VUS no gene *BRCA1*. No geral, com exceção da técnica de AC, os resultados mostram um aumento de suscetibilidade à radiação ionizante nos portadores de alterações genéticas, com e sem doença. No entanto, mais estudos terão de ser realizados para compreender completamente os resultados obtidos e o impacto das alterações no risco de cancro.

PALAVRAS-CHAVE: Cancro de Mama Familiar; Variantes de Significado Desconhecido; VUS; Recombinação Homologa; Radiação Ionizante; Estudos Funcionais

ABSTRACT

Familial breast cancer (BC) cases account for 5-10 % of all BC cases and are mainly associated with inherited mutations in *BRCA1* and *BRCA2* genes. Many other genes related with BC development have already been identified and are mostly related with Homologous Recombination (HR) repair system, one of the main pathways that repair DNA double-strand breaks (DSBs). Genetic testing for BC has become standard and with more widespread genetic testing, an increased detection of variants of unknown significance (VUS) as either benign or pathogenic will occur. Functional analyses on VUS may identify pathogenicity, and clearly categorize their mutational status. We carried-out a proof-of concept *in vitro* functional analysis in peripheral blood lymphocytes of VUS-harboring individuals and controls assessing the cellular response to γ-radiation. Six samples were collected, two BC patient with a pathogenic *ATM* mutation, two *BRCA1* VUS carriers, and two controls. Several methodologies were selected to evaluate the cellular response to genetic lesions induced by γ-radiation (2Gy): chromosomal aberrations (CA), micronuclei (MN) and comet assay. The CA assay results present no statistical difference between samples. In the MN assay the carriers show lower amount of binucleated cells with MN when compared to control samples, which is possibly due to cellular death events. The comet assay results show a clear increase in sensitivity to ionizing radiation, possibly associated with deficiency in repair, of samples from carrying a pathogenic mutation in the *ATM* gene and those with the *BRCA1* VUS. Overall, except for the CA assay, the results show an increased susceptibility to ionizing radiation in pathogenic *ATM* mutation carriers and *BRCA1* VUS carriers. However, some additional studies should be performed to completely understand the results obtained, and the impact of alterations in cancer risk.

KEYWORDS: Familial Breast Cancer; Variants of Unknown Significance; VUS; Homologous Recombination; Ionizing Radiation; Functional Assays.

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LIST OF ABBREVIATIONS, GENES AND PROTEINS

% ACEG - Percentage of binucleated cells with micronuclei per thousand

% DNA in tail - Percentage of DNA in tail

‰ MNBN - Percentage of binucleated cells with micronuclei per thousand

‰ TMN - Percentage of total micronuclei

ACE - Acentric fragment

Akt - Protein Kinase B (PKB)

AMPK - AMP-activated protein Kinase

AT - Ataxia Telangiectasia

ATM - Ataxia Telangiectasia Mutated

BC - Breast Cancer

BCDX2 - RAD51B, RAD51C, RAD51D and XRCC2

BER - Base-excision Repair

BIR - Break-induced Replication

BRCA1 - Breast Cancer 1

BRCA2 - Breast Cancer 2

BRCT - BRCA1 C terminus

BRIP1 - BRCA1 Interacting Protein C-Terminal Helicase 1

CA - Chromosomal Aberrations

CDC25 - Cell Division Cycle 25

CDH1 - Cadherin 1

CDKs - Cyclin-dependent kinases

CHB - Chromosome with a break

CHEK - Checkpoint kinase

CHG - Chromosome with a gap

CS - Cowden Syndrome

CTB - Chromatid with a break

CTG - Chromatid with a gap

CX3 - RAD51C and XRCC3

DBD - DNA-binding domain

DDR - DNA Damage Response

d-HJ - double-Holliday Junction

DIC - Dicentric Chromosome

D-loop - Displacement loop

DNA - Deoxyribonucleic Acid

DSB - Double-strand Break

DSBR - Double-strand break repair

H2AX - Histone H2AX

HDGC - Hereditary Diffuse Gastric Cancer

HR - Homologous Recombination

IR - Ionizing Radiation

IRIS - Ionizing Radiation Installations

LBC - Lobular Breast Cancer

LFS - Li-Fraumeni syndrome

MN - Micronuclei

MRE11 - Meiotic Recombination 11 homolog

MRN - MRE11, RAD50 and NBS1

NBS1 - Nijmegen Breakage Syndrome 1

NDI - Nuclear Division Index

NER - Nucleotide-excision Repair

NGS - Next Generation Sequencing

NHEJ - Non-homologous Endjoining

OC - Ovarian Cancer

PALB2 - Partner and Localizer Of BRCA2

PI3K - Phosphatidylinositol-3-kinasea

PIKKs - Phosphoinositide-3-kinase-related Kinases

PIP3 - Phosphatidylinosito-3,4,5-triphosphate

PJS - Peutz-Jeghers syndrome

PTEN - Phosphate and tensin homolog deleted on chromosome ten

RAD50 - DNA repair protein RAD50

RAD51 - DNA repair protein RAD51

ROS - Reactive Oxygen Species

RPA - Replication protein A

SCGE - Single Cell Gel Electrophoresis

SD - Standard deviation

SDSA - Synthesis-dependent Strand-annealing

SSA - Strand Annealing Pathway

SSD - Single-strand Break

ssDNA - Single-stranded DNA

STK11 - Serine/Threonine Kinase 11

TP53 - Tumor protein 53

VUS - Variants of Unknown Significance

XRCC - X-ray Repair Cross-Complementing Protein

1. INTRODUCTION

Cancer is generally described as a group of diseases that can affect any part of the body. It involves an uncontrollable cell growth with the probability of invading other parts of the body (National Cancer Institute, 2018; World Health Organization, 2018). In normal circumstances, cells grow and divide to form new cells as the body needs them. When the cells grow old or become damaged, they may enter in programmed cell death, also known as apoptosis, and new cells take their place. However, in tumor development this process breaks down. Cells become more abnormal or old, damaged cells survive when they should die, and new cells are formed when they were not supposed to. These cells can then divide without stopping and may form lumps or growths called tumours, in the case of solid tumors (National Cancer Institute, 2018).

Breast Cancer (BC) is the most common type of cancer in women and the third leading cause of cancer death (Slavin et al., 2017). Its development is associated to several risk factors, such as age, hormonal and environmental, like dietary patterns and other lifestyle factors. But one major factor is the genetic predisposition, or family history (Slavin et al., 2017). Familial BC cases account for approximately 5-10% of all breast cancer cases and are due to mutations in inherited cancer susceptibility genes (Augusto et al., 2018). Many of these genes have already been identified, with *BRCA1* and *BRCA2* being the most relevant ones (Antoniou & Easton, 2006). These genes, and most of the other ones associated with BC development, are related with cell response to DNA damage, including repair systems.

DNA damage occurs with surprising frequency. DNA lesions can cause mutations, block transcription and replication, and trigger the DNA damage response (DDR). The DDR arrests cell cycle progression and activates signalling pathways that impact cell fate: repair, apoptosis, or cellular senescence (Niedernhofer et al., 2018).

DNA repair pathways are among the mechanisms most frequently deregulated in cancer. These mechanisms allow cells to repair their DNA after specific damage or to induce apoptosis if repair is not possible. Protecting against uncontrolled proliferation of damaged cells (Amir et al., 2010). Disruption of these pathways produces an increase in chromosome breaks and mutagenesis, that may lead to loss of genomic integrity, which predisposes the organism to cancer and other disorders (Amir et al., 2010; Hakem, 2008).

DNA damage result from either endogenous sources (cellular metabolic processes) or exogenous sources (environmental factors). Endogenous sources of DNA damage include hydrolysis, oxidation, alkylation, and mismatch of DNA bases, while sources for exogenous damage of DNA include ultraviolet radiation, ionizing radiation (IR), and various chemicals agents (Hakem, 2008). In order to respond to all these injuries, cells have evolved several refined, interlinked DNA repair systems that, as a whole, cover most of the injuries inflicted on cells genetic information (Hoeijmakers, 2001). Inherited defects in any of these systems in general predisposes to malignancy. Due to the occurrence of DNA damage since the beginning of life, all known repair pathways are highly conserved through evolutionary borders and in mammals there are at least four main ones (figure 1.1) (Hoeijmakers, 2001).

One of these main pathways is nucleotide-excision repair (NER), which deals with helix-distorting DNA lesions that can interfere with base pairing and obstruct normal replication and transcription. Most of these lesions arise from exogenous sources, including ultraviolet-induced photoproducts and numerous chemical adducts (Hoeijmakers, 2001; Schärer, 2013). On the other hand, base-excision repair (BER) focuses on more subtle types of base damage, such as small chemical alterations due to reactive oxygen species, methylation, deamination and hydroxylation. Therefore, in contrast with NER, BER is mainly concerned with damage of endogenous origin (Hoeijmakers, 2001; Whitaker et al., 2017). Homologous recombination (HR) and non-homologous endjoining (NHEJ) are the two major pathways that fix DNA double-strand breaks (DSB) (Hoeijmakers, 2001).

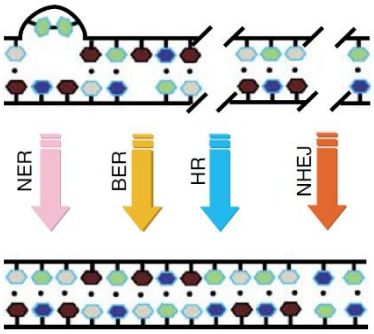


Figure 1. 1 – DNA damages and DNA repair pathways. Different repair pathways repair different DNA damages: (1) NER pathway and helix distorting lesions; (2) BER pathway and subtle types of base damage; (3) HR and (4) NHEJ pathway and DSB. (Acquired from Hakem, 2008)

DNA lesions, such as DSB, threaten the integrity of the genome. They are highly toxic and can be generated by endogenous sources, including collapsed replication forks and repair intermediates (e.g. single-strand breaks (SSB)) or exogenous sources like genotoxic agents, such as chemicals or ionizing radiation (Krejci et al., 2012). If left unrepaired, only a few DSBs can lead to aneuploidy, genetic aberrations or cell death. Therefore, proper repair of these DSBs is crucial for cellular integrity and survival (Krejci et al., 2012). As referred before, cells are equipped with two fundamentally different pathways to repair DSB. NHEJ can be performed throughout the cell cycle and promotes direct ligation of the DSB ends in a non-conservative fashion, possibly introducing mutations during repair (Lieber, 2010). HR, in contrast, is a template dependent process, typically involving the presence of an identical or nearly identical DNA template (usually sister chromatids), what restricts this type of repair to S and G_2 phases of the cell cycle (Heyer et al., 2010; Krajewska et al., 2015; Ma et al., 2018). This type of mechanism is conservative and non-mutagenic. In BC, impairment of HR is well described in hereditary tumours caused by mutations in *BRCA1* and *BRCA2*, as well as in other BC susceptibility genes.

Genetic testing for hereditary cancer risk assessment has become standard for BC. Recent advances in next generation sequencing (NGS) have allowed entire panels of genes to be sequenced, or simultaneous testing for mutations in multiple genes (Lumish et al., 2017). With the increasing prescription rates of genetic tests comes an increase in detection of pathogenic variants in genes with moderate penetrance, without established clinical guidelines, and of variants of unknown significance

(VUS). These VUS are variants, mainly rare missense variants that lead to single amino acid changes, which have an uncertain effect on protein function. They are unknown to be either benign or disease-causing and as a result, clinicians cannot give an exact answer to VUS carriers. Functional analysis on VUS may identify those alterations as pathogenic or not and clarify their mutational status.

1.1. HOMOLOGOUS RECOMBINATION REPAIR SYSTEM

HR repair system is a key pathway and a universal mechanism to maintain genomic integrity between generations and during oncogenic development in a single organism (Heyer et al., 2010). It is a system that repairs a variety of complex DNA damages, involving DSB.

HR repair uses a DNA template for repair with significant sequence homology (most frequently, sister chromatids), restricting this type of repair to S and G_2 phases of the cell cycle, after DNA replication has occurred (Krajewska et al., 2015; Ma et al., 2018). The highly regulated process of HR can be divided in three main phases - presynapsis, synapsis and post-synapsis (Heyer et al., 2010; Krajewska et al., 2015). In presynapsis, the DSB lesion is processed by nucleolytic degradation of the 5'-strands, to form 3'-single-stranded DNA (ssDNA) (figure 1.2). This first step is catalysed by endonucleases, including the MRN complex (consisting of MRE11, RAD50 and NBS1 proteins). The ssDNA-ends are then coated by the major eukaryotic ssDNA binding protein, replication protein A (RPA). Binding of RPA eliminates secondary structures in ssDNA, which is needed for competent RAD51 filaments to assemble. RAD51 is then recruited to ssDNA, replacing RPA through its displacement by the breast cancer-associated protein BRCA2 in partnership with PALB2 (Heyer et al., 2010; Krajewska et al., 2015; Trego et al., 2016). The breast cancer-associated protein BRCA1 functions early in HR repair to regulate end resection and to recruit BRCA2 trough interaction with PALB2 (Trego et al., 2016). During synapsis, the RAD51 nucleoprotein filament performs homology search between the ssDNA end and the intact sister chromatid, invading the duplex DNA, generating a displacement loop (D-loop) in a process that also requires PALB2 (figure 1.2). Within this loop, RAD51 dissociates from dsDNA to expose the 3'-OH required for DNA synthesis. During post-synapsis, DNA is synthesized using the invading 3'-end as a primer (figure 1.2) (Heyer et al., 2010; Trego et al., 2016).

Once DNA synthesis is initiated, there are at least three different routes that can be pursued (figure 1.2) (Heyer et al., 2010; Krejci et al., 2012). In the double-strand break repair (DSBR) model, the other end of the DSB that did not invaded the duplex DNA, can be captured to the duplex DNA, stabilizing the D-loop structure and leading to the formation of a double-Holliday Junction (d-HJ). The d-HJ are converted into recombination products by restriction endonucleases that cuts only one DNA strand (figure 1.2). The DSBR pathway commonly results in crossover, though it can sometimes result in non-crossover. What determines its fate is how the d-HJ is cut or resolved. If one HJ is cut on the crossing strand and the other HJ is cut on the non-crossing strand, it will result in a chromosomal crossover. However, if the two HJ are cut on the crossing strands, a non-crossover chromosome is produced. In another model, the invading 3' strand is extended along the recipient DNA duplex and then

displaced from the D-loop. The newly synthesized end of the invading strand anneals to the other DNA end. This represents the synthesis-dependent strand-annealing (SDSA) mode of HR (figure 1.2). This pathway inherently avoids crossovers, which reduces the potential for genomic rearrangements. For last, the D-loop structure may become a fully developed replication fork and copy the entire chromosome arm in a process called break-induced replication (BIR) (figure 1.2). This mechanism is induced more often in the absence of a second end. Although this process restores the integrity of the chromosome, it can lead to loss of heterozygosity of all genetic information distal to the DSB (Heyer et al., 2010; Krejci et al., 2012; Sung & Klein, 2006).

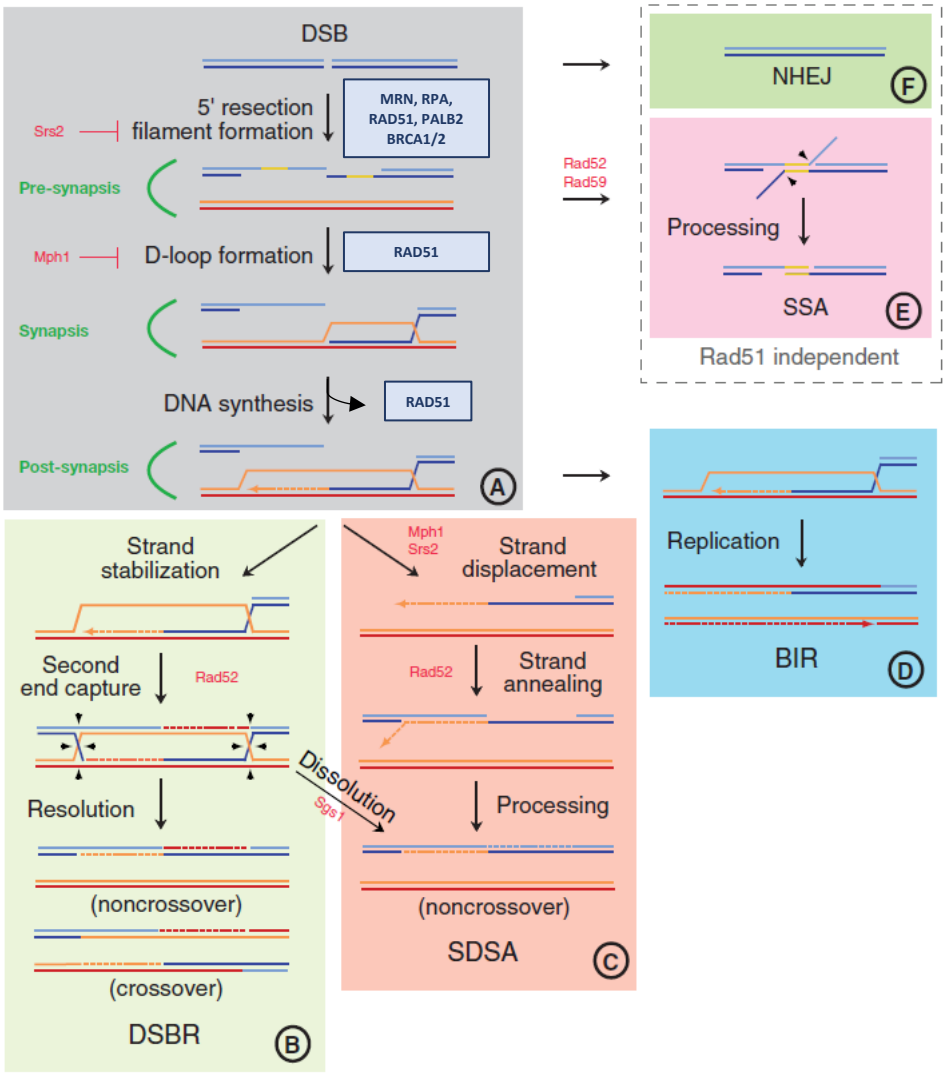


Figure 1. 2 – Resumed representation of DSB repair. DSB repair can be done by HR. (A) After resection of DSB generating 3'-ssDNA follows the pre-synapsis phases where ssDNA is coated with RPA. Subsequently, this protein is replaced by RAD51 forming a filament. In synapsis RAD51 performs homology search in the sister chromatid generating the D-loop. In post-synapsis DNA is synthesised. There are three routes that can be pursued: (B) Double strand break repair (DSBR) where dHJ is formed and commonly results in cross-over but sometimes results in non-crossover; (C) Synthesis-dependent strand-annealing (SDSA) that mainly results in non-crossovers and (D) Break-induced replication (BIR) that is induced more often in the absence of a second end. DSBs can also be sealed by two other pathways: (E) SSA and (F) NHEJ, RAD51 independent. (Adapt from Krejci et al., 2012)

Basically, all these pathways require RAD51. However, DSBs can also be sealed by pathways independent of RAD51. One of these pathways is the NHEJ, already referred. Another one is the single-strand annealing pathway (SSA), in which ssDNA ends generated during nucleolytic degradation contain regions of homology at both sides of the DSB and can be annealed and ligated. SSA does not require RAD51 but involves other HR proteins that mediate annealing (figure 1.2) (Bhargava et al., 2016; Krejci et al., 2012).

DDR is the main pathway controlling HR. It consists of multiple kinase and ubiquitin ligases working in parallel to coordinate a cell cycle arrest with DNA repair and/or induction of apoptosis. DDR work in signalling layers that coordinate the cellular response to DNA damage. The first step is the detection of the lesions by DNA damage sensor proteins. These complexes are required for recruiting several factors to the site of lesion such as DNA repair factors, but also transmit a signal to transducer proteins. These transducers diversify and amplify the damage signal to the third layer, which are the effectors. Effector proteins control the activity of several cellular processes and pathways, such as cell cycle arrest and apoptosis. Sensor and transducer signalling primarily relies on protein interactions and alterations in protein activity, but several effectors are transcription factors, such as TP53 (Derks et al., 2014). In the context of DNA breaks, the MRN complex acts as the sensor of DNA DSBs. It recruits and activates the upstream DDR kinase ataxia telangiectasia mutated (ATM), which is a member of the family of phosphoinositide-3-kinase-related kinases (PIKKs). The activation of ATM leads to phosphorylation of various targets that contribute to the overall DDR. Therefore, just after of DSB formation, active ATM phosphorylates different substrates that are essential for DNA-damage response and repair, such as BRCA1, CHEK2 and TP53. These are mediators of ATM on DNA repair, cell-cycle arrest, apoptosis and other downstream events. Phosphorylation of histone H2AX at the site of DNA damage by ATM is also necessary for recruitment of other proteins and chromatin-remodelling, essential for the DNA damage repair (Lee & Paull, 2007; Maréchal & Zou, 2013; Paull, 2015).

ATM is essential for the G1/S, intra-S-phase and G2/M DNA-damage checkpoints (Hakem, 2008). Phosphorylation and subsequent activation of CHEK2 leads to phosphorylation of nuclear proteins involved in different points of the DDR. In the presence of DSB, CHEK2 arrests the cell cycle at G1/S and G2/M. In the arrest of G2/M, necessary for HR, CHEK2 phosphorylates CDC25C, which results in translocation of this phosphatase to the cytoplasm and in there it can no longer activate cyclin-dependent kinases (CDK), necessary for G2/M transition (Samadder et al., 2016; Zannini et al., 2014). In addition, TP53 is phosphorylated and activated by ATM and CHEK2 promoting p21 accumulation and sustain G2/M arrest (Samadder et al., 2016; Zannini et al., 2014).

DDR members, besides regulating the recruitment of HR factors to site of DNA DSBs, also regulates the recombination phase of HR. ATM regulates the post-translational modification and assembly of RAD51 filaments (G. Chen et al., 1999). ATM activates CHEK2 which in turn phosphorylates BRCA1 and BRCA2 promoting HR over NHEJ. (Zannini et al., 2014; Zhang et al.,

2004). On the other hand, CHEK2 phosphorylation of BRCA1 facilitates recruitment of RAD51 to the lesion (Zannini et al., 2014).

The most relevant genes associated with BC development are mostly if not all related with HR repair system. In fact, genetic testing for BC has been done with the resort to gene panels sequencing for the detection of mutations and these panels are mostly made up of genes involved in the HR repair system (Couch et al., 2017; Easton et al., 2015; Lumish et al., 2017).

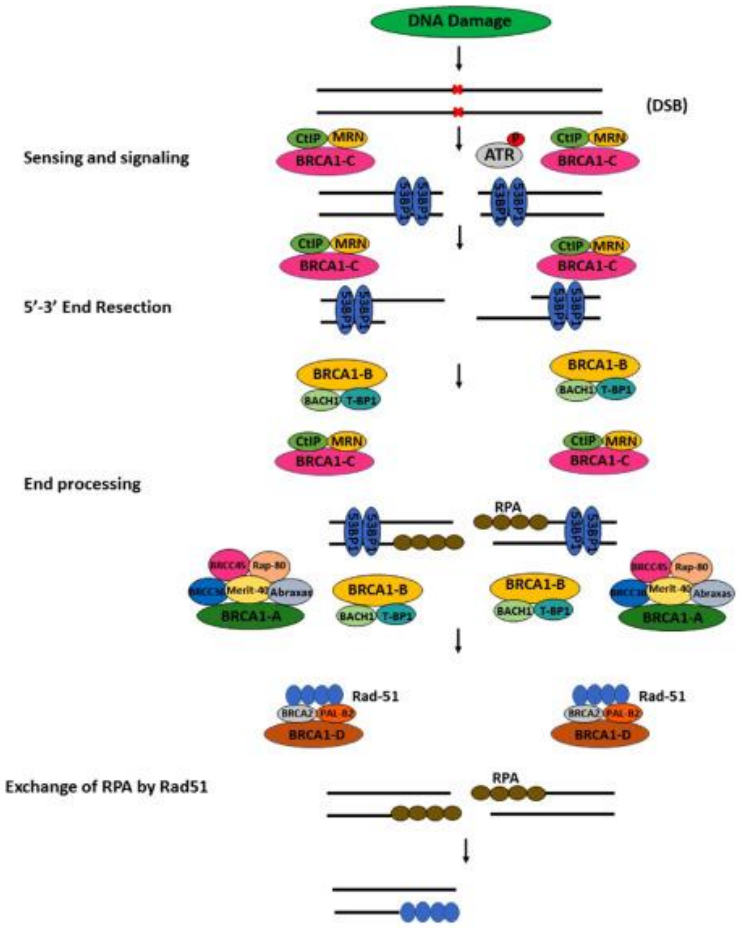
1.2. BREAST CANCER GENETICS

All genes are subjected to mutations, which might result in protein function impairment. When proteins involved in DDR and HR repair are defective, repair of DNA DSB might be compromised and defective DSB repair is a major driver in cancer (Chapman et al., 2012). Mutations in *BRCA1* and *BRCA2* genes confer a high risk of breast cancer, and genetic testing for both genes has been routinely offered to individuals with an apparent predisposition to breast cancer (Lerner-Ellis et al., 2015). In addition to *BRCA1/2*, other susceptibility genes have also been implicated in hereditary breast cancer. These susceptibility genes are categorized based on their penetrance, which is the proportion of individuals with the mutation who exhibit the disease. Besides *BRCA1/2* genes, a number of other genes are now considered as high penetrant genes too, such as *TP53, PTEN, STK11* and *CDH1* (Economopoulou et al., 2015; Santonocito et al., 2017). On the other hand, some genes are associated with moderate penetrance, for example, *CHEK2, ATM, PALB2* and *BRIP1* and for last, low penetrant genes such as *RAD51C* and *RAD51D* (Economopoulou et al., 2015). All these genes are somehow related with DDR to DSB and are involved in HR pathway.

1.2.1. HIGH PENETRANCE GENES

The first major gene associated with BC was *BRCA1* (Shiovitz & Korde, 2015). Pathogenic mutations in this gene accounts for about 7-10% of familial BC and confer a lifetime risk of BC between 60% and 85%, with increased relative risk at younger ages. *BRCA1* is a large gene with 24 exons located on chromosome 17q that produces a multidomain protein (Lalloo F & Evans D G, 2012; Sharma et al., 2018). Mutations are found throughout the coding sequence of the gene, with the majority being frameshift mutations and nonsense mutations resulting in truncated proteins. Missense mutations account for approximately 2% of pathogenic mutations in *BRCA1* but may be difficult to interpret or distinguish from polymorphisms. Between 15% and 27% of mutations may be due to large rearrangements, including large deletions (whole exon) and insertion/duplications (Lalloo F & Evans D G, 2012; Sharma et al., 2018). Different proteins interact with BRCA1 domains to carry out crucial cellular processes, such as cell cycle control and DNA damage repair, acting as a tumour suppressor (Sharma et al., 2018). It has 4 major domains, RING domain, the BRCA1 serine domain and two BRCT (BRCA1 C terminus) domains. The BRCT domain appears to be crucial for HR repair of DSB. It forms at least four different complexes called A, B, C and D depending on the different proteins they interact

with, and each one plays a role in HR at various levels (figure 1.3). BRCA1-A complex gets recruited at DSB sites and helps in DNA damage signalling and also prevents over resection of DNA. BRCA1-B complex and -C complex are crucial for DNA end resection that leads to ssDNA ends formation. For last, BRCA1-D complex helps in exchange of RPA protein by RAD51 dependent of BRCA2 and PALB2



(Sharma et al., 2018).

Figure 1. 3 - BRCA1 complexes involvement in homologous recombination repair system. MRN complex senses DNA DSB and recruits and activates the ATM. BRCA1-B complex and -C complex are crucial for DNA end resection that leads to ssDNA ends formation. Further, BRCA1-A complex gets recruited at DSB sites and helps in DNA damage signaling and also prevents over resection of DNA. These overhangs are covered by RPA. For last, BRCA1-D complex helps in exchange of RPA protein by RAD51 in BRCA2 and PALB2 dependent manner (Acquired from Sharma et al., 2018)

BRCA2 mutations account for about 10% of families with breast cancer. Mutations in this gene confer a BC lifetime risk of around 40-85%. As *BRCA1*, *BRCA2* is a large gene with 27 exons. Mutations occur throughout the gene, again the majority being frameshifts. There are many missense mutations found within *BRCA2*, but the pathogenicity of these may be difficult to establish. Large gene rearrangements also occur in *BRCA2* but are less frequent than in *BRCA1*. *BRCA2* is a tumour suppressor gene and is involved in DSB repair by HR system. It interacts directly with RAD51, forming a complex and holding it in an inactive state and mediates its recruitment to DSB (Lalloo F & Evans D G, 2012; Roy et al., 2011). BRCA2 also contains a DNA-binding domain (DBD) that binds ssDNA and dsDNA

which might be involved in facilitating RAD51 filament formation and accelerate RPA-displacement (Roy et al., 2011).

The tumour protein 53 (TP53) is a tumour suppressor gene that plays a major role in the regulation of cell growth. It is located on chromosome 17p and known to be the most frequently altered gene in human tumours. Inherited *TP53* germline mutations are rare but known to give rise to Li-Fraumeni syndrome (LFS). LFS is a rare predisposition cancer syndrome that causes childhood tumours and very early onset BC, being associated with approximately 1% of BC cases (Economopoulou et al., 2015). Among female *TP53* mutation carries, BC is the most frequent malignancy. *TP53* gene consists of 11 exons and mutations in this gene are most commonly missense, but deletions of the coding or promoter region of *TP53* can also occur. Even though LFS is responsible for a minor fraction of BC cases, a woman with LFS has a breast cancer risk of 56% by the age of 45 and more than 90% by the age of 60. TP53 is crucial for cell-cycle control, leading to either a delay in cell-cycle progression or apoptosis (Economopoulou et al., 2015; Lalloo F & Evans D G, 2012). Its major functions include controlling the arrest of cell-cycle in G2-M phase, activated by DNA strand breaks and mediation of apoptosis when necessary, in response to DNA damage. It is also involved in HR repair system because of its direct interaction with RAD51, regulating the extent and timing of homologous recombination (Stürzbecher et al., 1996).

The tumour suppressor gene *PTEN* (phosphate and tensin homolog deleted on chromosome ten) is present in chromosome 10q and is one of the most frequently mutated tumour suppressor genes in human cancer, with a frequency closer to the one of *TP53*. The protein encoded by *PTEN* gene is a 403-amino-acid phosphatase, member of the large protein tyrosine phosphatase family (Kechagioglou et al., 2014). Due to its phosphatidylinositol-3-kinase (PI3K) phosphatase activity is a major break for carcinogenesis. Its precise function is not clear, but impairment of PTEN leads to inability to activate cell cycle arrest and apoptosis, what leads to abnormal cell survival (Economopoulou et al., 2015). Phosphatidylinosito-3,4,5-triphosphate (PIP3) that is formed by PI3K action, is an important lipid second messenger in tumourigenesis that activates Akt and other signalling molecules involved in a several cellular processes, such as survival, proliferation, cell motility and invasion. By acting on PIP3 with its phosphatase activity, PTEN has the ability to reduce PIP3 quantity, inhibiting growth and survival signals (Economopoulou et al., 2015; Kechagioglou et al., 2014). Germline mutations in *PTEN* are responsible for the Cowden Syndrome (CS) or PTEN hamartoma tumour syndrome, an autosomal dominant disorder with incomplete penetrance. This syndrome is characterized by the formation of multiple hamartomas throughout the body with an increased risk of malignancy. Of the affected individuals, approximately 80% have a detectable mutation in the *PTEN* gene, which might be a missense, point, deletion, insertion, frame shift or nonsense mutation. Although being responsible for only <1% of BC cases, the most common malignancy associated with CS is breast cancer. It is associated with a lifetime risk of BC of 50% in affected females, with an younger age at diagnosis compared to sporadic (Economopoulou et al., 2015).

The *STK11* is a tumour suppressor gene located on chromosome 19p and encodes for serine-threonine protein kinase 11 (STK11) (J. Chen & Lindblom, 2000; Economopoulou et al., 2015). It has several roles in the cell, both in the nucleus and in the cytoplasm, regulating many cellular processes, such as cell cycle arrest, apoptosis, cell polarity and energy metabolism by activating other kinases with AMPK being the most relevant (Alkaf et al., 2017). Germline mutations in *STK11* gene are the cause of the autosomal dominant disorder Peutz-Jeghers syndrome (PJS), characterized by multiple gastrointestinal hamartomatous polyps, pigmentation of the lips, buccal mucosa and digits and an increased risk for several neoplasms, including colorectal, gastrointestinal, ovarian and breast cancer. The risk of developing breast cancer in women with PJS is 8% at the age of 40 and 30-50% by the age of 70 (Alkaf et al., 2017; Campeau et al., 2008; J. Chen & Lindblom, 2000; Economopoulou et al., 2015). Most of the mutations in the *STK11* gene have been shown to be either frameshift or nonsense, which result in a truncated protein and consequently the loss of kinase activity. Nevertheless, some *STK11* missense mutations have been identified in PJS (Li et al., 2018).

The cadherin 1 (*CDH1*) gene produces a protein called epithelial cadherin or E-cadherin, a calcium dependent cell-cell adhesion molecule found within the membrane of epithelial cells (Carneiro et al., 2012; Economopoulou et al., 2015). It is a tumour suppressor, being a key factor in tumour progression and invasion in epithelial cancer, supressing cell invasion (Carneiro et al., 2012; Corso et al., 2016). *CDH1* gene is located on chromosome 16q, and germline mutations in this gene have been mainly associated with hereditary diffuse gastric cancer (HDGC) (Corso et al., 2016). Of those families with HDGC due to *CDH1*, 30% also had women with lobular breast cancer (LBC). In fact, novel *CDH1* germline variations were found in women with LBC, mainly early-onset, but with no family history of HDGC. Therefore, *CDH1* has been considered as a BC susceptibility gene (Corso et al., 2016; Economopoulou et al., 2015). The most frequent alterations are missense mutations, in contrast to HDGC. These account for 60% of the cases described so far, with the truncating mutations accounting for the remaining 40% (Economopoulou et al., 2015).

1.2.2. MODERATE PENETRANCE GENES

The checkpoint kinase 2 (*CHEK2*) gene encodes for a serine threonine kinase (CHEK2), a tumour suppressor protein that regulates cell division and is a signalling component in the cellular response to DNA damage (Economopoulou et al., 2015; Lalloo F & Evans D G, 2012). It is activated by the ATM in response to DNA strand breaks. Once activated, CHEK2 phosphorylates downstream targets including CDC25 phosphatases, responsible for dephosphorylation and activation of CDKs. Phosphorylation of CDC25C leads to subsequent cell-cycle arrest in G2/M phase (Hakem, 2008; Samadder et al., 2016; Zannini et al., 2014). Furthermore, the CHEK2 protein interacts with several other proteins including TP53, leading to cell-cycle arrest in G2 phase (Hakem, 2008; Samadder et al., 2016; Zannini et al., 2014). CHEK2 is also involved in regulation of recombination phase of HR (Zannini et al., 2014). Mutations in *CHEK2* have been associated with BC development, being detected

in approximately 5% of BC patients, although it varies between populations (Economopoulou et al., 2015; Lalloo F & Evans D G, 2012).

The *ATM* gene is localized in chromosome 11q and encodes for a multifunctional tumour suppressor protein. The ATM is a protein kinase that is manly located in the nucleus of cells, where it helps controlling cell growth and division. This protein also assists cells in recognizing DNA damage, playing a major role in the response to DSB (Economopoulou et al., 2015; Hall, 2005). Upon DNA breaks ATM is activated subsequently activating proteins such as TP53, CHEK2, BRCA1 and others, resulting in activation of cell-cycle checkpoints, DNA repair or the induction of apoptosis (Hall, 2005). Homozygous mutations in *ATM* are associated with an autosomal recessive condition called ataxia telangiectasia (AT). This disorder is characterized by cerebral ataxia, immunodeficiency and increased risk of malignancy, including BC. Furthermore, *ATM* heterozygotes have an increased risk of developing BC. Mutations described in *ATM* include truncating mutations, splice site and missense mutations. With the predominant type of *ATM* mutation resulting in a truncated and unstable ATM (Economopoulou et al., 2015; Hall, 2005; Lalloo F & Evans D G, 2012).

The partner and localizer of BRCA2 (*PALB2*) gene is localized on chromosome 16p and encodes for a protein that interacts with BRCA1 and BRCA2 during HR repair, contributing to DNA repair (Buisson & Masson, 2012; Economopoulou et al., 2015; Lalloo F & Evans D G, 2012). Biallelic mutations in this gene might be one cause of Fanconi Anaemia, similar to that caused by biallelic *BRCA2* mutations. On the other side, monoallelic mutations increase the risk of BC, such as *BRCA1* and *BRCA2* (Foo et al., 2017). The emerging of *PALB2* as a BC susceptibility gene was based on the fact that mutations in this gene were present in 1% of BC families negative for mutations in *BRCA1/2*. It is although characterized as a rare, intermediate-risk gene with regards to inherited genetic susceptibility to BC (Economopoulou et al., 2015; Lalloo F & Evans D G, 2012). Mutations in *PALB2* gene are mainly truncating, but some missense mutations were also associated with BC (Park et al., 2014).

In contrast with *PALB2*, BRCA1 interacting protein C-terminal helicase 1 (*BRIP1*) gene encodes a protein that was identified as the binding partner of BRCA1. This gene is localized on chromosome 17q near *BRCA1* locus, and the protein encoded is a DNA helicase required for the maintenance of chromosomal stability. As with *PALB2*, truncating mutations of the gene were identified in BC families negative for *BRCA1/2* (De Nicolo et al., 2008; Economopoulou et al., 2015; Lalloo F & Evans D G, 2012). Interestingly, *BRIP1* missense mutations have been found in high risk women who are *BRCA1/2* negative (Economopoulou et al., 2015). Also as with *PALB2*, biallelic mutations cause Fanconi anaemia, but differently from the previous gene, this is different to that of biallelic mutations in *BRCA1* (Economopoulou et al., 2015; Lalloo F & Evans D G, 2012).

1.2.3. LOW PENETRANCE GENES

RAD51C (RAD51 homolog C) gene is a member of the RAD51 family and is localized on chromosome 17q. It encodes for RAD51C protein that plays an important role in DSB repair through

HR. Homozygous mutations in *RAD51C* are related with the Fanconi anaemia-like syndrome, while heterozygous mutations have been identified in BC and ovarian cancer (OC) families. However, germline mutations in *RAD51C* occur in low frequency. Pathogenic alterations in this gene were found in approximately 1% of non-*BRCA1/2* BC/OC and OC-only families (Clague et al., 2011; Economopoulou et al., 2015; Neidhardt et al., 2017). This protein interacts with other RAD51 paralogs in two distinct complexes that bind ssDNA. In BCDX2 complex, RAD51C associates with RAD51B, RAD51D and XRCC2, and in CX3 complex, it associates only with XRCC3. The BCDX2 complex is responsible for binding single- and double-stranded DNA, assisting in RAD51 loading onto DNA ends, and hydrolyse adenosine triphosphate, while CX3 complex is responsible for homologous pairing of DNA strands, what suggest the role of RAD51C in the initial stages of the HR repair system (Economopoulou et al., 2015; Prakash et al., 2015; Somyajit et al., 2010).

RAD51D (RAD51 homolog D) is also a member of RAD51 family and is located in chromosome 17q. RAD51D protein is part of BCDX2 complex which, as said before, assists in RAD51 loading onto DNA ends, by binding to single- and double-stranded DNA and plays an important role in response to DSB in HR. Although in rare cases, mutations in *RAD51D* has been associated with BC development (Baker et al., 2015; Economopoulou et al., 2015; Prakash et al., 2015).

1.3. VARIANTS OF UNKNOWN SIGNIFICANCE (VUS)

Over the last years, genetic testing for hereditary cancer risk assessment has become widely used for BC. Recent advances in next generation sequencing (NGS) have allowed entire panels of genes to be sequenced, or simultaneous testing for mutations in multiple genes (Lumish et al., 2017). With the availability of NGS panels in cancer genetic testing, comes an increase in speed and cost effectiveness and also an improved clinical sensitivity (LaDuca et al., 2014). However, with increasing rates of genetic testing comes an increased detection of pathogenic variants in genes with moderate penetrance, without established clinical guidelines, and of variants of unknown significance (VUS). These VUS are genetic variants, mainly rare missense variants that lead to single amino acid changes, but can also be in-frame small deletions or insertions that have impact in just a few amino acids, or even alterations that may influence splicing or translations (Lindor et al., 2012). They may or may not have clinical consequence and cannot be classified as either benign or pathogenic. Because of the lack of knowledge, clinicians cannot give an exact answer and carriers of VUS and their family members cannot take advantage of the risk assessment, prevention, and therapeutic measures that are available to carriers of known pathogenic mutations. Moreover, the ambiguity of VUS results have been shown to have negative psychological impact on patients, causing high levels of anxiety and distress compared to those with definite benign or pathogenic variants (Lumish et al., 2017; Welsh et al., 2017). Although individual VUS are rare, finding one is not an unusual event (Lindor et al., 2012). Thus, it is of great importance to determine the clinical significance of VUS present in genes susceptible to breast cancer.

Numerous *in silico* methods can predict the influence of variants on protein function, structure or splicing mechanisms based on sequence information. Though, some of these prediction models mainly rely on assumptions, and due to that VUS can be misclassified (Ernst et al., 2018; Lindor et al., 2012). Therefore, other methods are needed to fully characterize these gene variants influence in protein function and their possible effect on cancer risk. Functional assays have been intensively used in order to clarify and provide important information for VUS classification.

1.4. FUNCTIONAL ANALYSIS

Functional analysis is used to assess, directly or indirectly, the influence of gene alterations on protein function producing information that can be combined with available genetic and epidemiological data (Millot et al., 2012). Ionizing radiation is a clastogenic agent, i.e. induces DNA breaks, which allows to determine the impact of alterations in genes associated with breaks repair. For such, three techniques can be used to assess the cells capacity to repair the damage inflicted: CA assay, MN assay and single cell gel electrophoresis (SCGE), also known as comet assay.

1.4.1. BIOLOGICAL EFFECTS OF RADIATION

Ionizing radiation can be classified as natural or artificial, depending of its source. It is an environmental factor that is dispersed in nature and also a clinically important diagnostic and treatment tool. CT scans are widely used in diagnostics and about half of cancer patients receive treatment through radiotherapy. Furthermore, exposure can occur in occupational settings or as a consequence of nuclear accidents (Miousse et al., 2017; Wrixon et al., 2004).

IR is a high-energy radiation type that removes an electron from an atom and/or molecule, producing ions that can break covalent bonds. It can be divided into five types: X-rays, gamma (γ)-rays, alpha (α) and Beta (β) particles and neutrons. γ-radiation is a very high energy photon emitted from an unstable nucleus, that ionizes atoms when passing through matter, mainly due to interactions with electrons (Borrego-Soto et al., 2015; Wrixon et al., 2004).

Radiation directly affects DNA structure causing a wide range of lesions, such as DNA breaks, particularly, DSBs. It can also indirectly affect DNA by generating reactive oxygen species (ROS). ROS are formed when radiation is absorbed by the water in the organism, leading to excitation and ionization of water molecules producing the reactive species. These will then oxidize proteins and lipids, and induce several damages to DNA, like generation of abasic sites and SSB, the last ones may also evolve to DSB. Both direct and indirect effects of radiation will lead to DNA damage, particularly DSB, triggering the DDR pathways and resulting in repair of damage or, when repair is unsuccessful, apoptosis or damage accumulation through the subsequent generations (Borrego-Soto et al., 2015; IAEA, 2010; Santivasi & Xia, 2014).

1.4.2. CHROMOSOMAL ABERRATIONS ASSAY

Chromosomal Aberrations are an important consequence of exposure to genotoxic agents, such as ionizing radiation, mainly due to the fact that the presence of high frequencies of CA in peripheral blood lymphocytes have been associated with elevated risk of cancer development. The principle lesion associated with CA formation is DNA DSB, which can arise spontaneously or directly induced by genotoxic agents such as ionizing radiation. If DSB are left unrepaired they may accumulate and lead to broken chromosomes, and if repaired improperly, may lead to mutations, chromosome rearrangements, and oncogenic transformation (Obe et al., 2002). CA consist in chromosome number and structure alteration. Structural alterations may affect only one chromatid or both. The most frequent CA directly correlated to ionizing radiation is the dicentric chromosome, which is the result of the merging of two damaged chromosomes, with the formation of an acentric fragment. The ring chromosome and acentric fragments are also consequential structures of ionizing radiation (Ballarini & Carante, 2016). The CA assay is considered the "Gold Standard" for radiation biodosimetry mainly due to dicentric analysis, because these structures exist in very low levels in healthy general population and are specific of the response to ionizing radiation(Antunes et al., 2014; Martins et al., 2013). This technique allows the analysis of metaphases containing 46 chromosomes, obtained through a mitotic spindle inhibitor (for example, colcemid) for the presence of abnormal chromosome structures (Martins et al., 2013). The criteria followed for the identification of the different types of CA are described in Rueff et al., 1993.

1.4.3. MICRONUCLEI ASSAY

Micronuclei are smaller nuclei formed from fragments or whole centric chromosomes or chromatids which are not included in the daughter cells nuclei after cell division. Chromosomal fragments result from non-repaired or misrepaired DSBs and are formed after cells incubation with a clastogenic agent, such as ionizing radiation (Doherty, 2012; Garaj-Vrhovac et al., 1992; Qian et al., 2016). The MN assay, or the cytokinesis-block micronucleus (CBMN) assay is the preferred method for the assessment of MN. In this technique, cells that have completed one or more nuclear divisions have their cytokinesis blocked, with the addition of cytochalasin B, producing binucleated (BN) or polynucleated cells. The scoring is focused only on BN cells, excluding non-proliferative cells or cells that passed successive divisions (Doherty, 2012; Pinto et al., 2010). The criteria for BN selection and MN scoring were described by Fenech, 2000.

1.4.4. SINGLE CELL GEL ELECTROPHORESIS (COMET ASSAY)

The Single Cell Gel Electrophoresis or the Comet assay has become one of the standard methods for assessing DNA damage, with applications in several areas including fundamental research in DNA damage and repair. It is a simple, fast, versatile and highly sensitive assay that has the ability to detect low levels of DNA damage, such as DNA strand breaks and alkali-labile sites (Azqueta et al., 2014; Collins, 2004; Gunasekarana et al., 2015).

The basic principle of the Comet assay is the movement of DNA fragments of single cell nuclei, that are negatively charged, in the direction of the positive electrode, during electrophoresis. This leaves a trail giving a comet-like image, with the intensity of the tail reflecting the extent of DNA damage, or frequency of breaks (Azqueta et al., 2014; Gunasekarana et al., 2015). The alkaline variant of the Comet assay, in its standard version, detects DNA single- and double-strand breaks and alkali-labile sites (abasic sites) (Azqueta et al., 2014; Focke et al., 2010). Briefly, after cells being suspended in a thin agarose gel on a microscope slide they are treated with a hypertonic lysis solution and non-ionic detergent, removing the cell membrane, cytoplasm and nucleoplasm, including nucleosomes. In the case of alkaline variant of Comet assay, cells are then subjected to an alkaline treatment followed by the electrophoresis and stained with fluorescent DNA binding dye (Collins, 2004; Gunasekarana et al., 2015).

The Comet assay is mainly used to measure DNA damage, but it has also been widely used for measuring DNA repair (Azqueta et al., 2014). This technique was first referred by Östling and Johanson in 1984, where they used the neutral version of the Comet assay, where DNA is not denatured (Ostling & Johanson, 1984). But a few years later, Singh et al. published the use of this assay with treatment at high pH (pH>13) which facilitates denaturing and unwinding, maximizing sensitivity for measurement of low numbers of strand breaks (Singh et al., 1988). Both used this technique to study the re-joining of DNA breaks, after irradiation (γ- and X-radiation, respectively).

Therefore, the Comet assay is a technique that appeals to researchers due to its advantages, such as: its high sensitivity to detect low levels of DNA damage; it is simple, fast and cheap to perform; it can be applied to a wide range of cell types; it can give us the ability of the cells to repair the DNA damage (challenge assay); it is performed and analysed at the level of a single cell and it can differentiate between viable, apoptotic and necrotic cells. However, it cannot detect damage resulting from small deletions. Even so, it is a versatile and potential tool to assess the DNA damage and repair capacity (Azqueta et al., 2014; Collins, 2004; Gunasekarana et al., 2015).

1.5. GOALS

In this work we carried-out a proof-of concept *in vitro* functional analysis in peripheral blood lymphocytes of two BC patients carrying a pathogenic mutation in the *ATM* gene, two *BRCA1* VUS-harbouring individuals and two controls assessing the cellular response to γ-radiation, a clastogenic agent. In order to achieve this goal, we performed three endpoints: Micronucleus assay, Chromosomal Aberrations assay and the Single Cell Gel Electrophoresis assay. By analysing structures associated with DNA breaks, these techniques may give us an answer about these alterations impact in protein function and cancer risk.

2. MATERIALS AND METHODS

2.1. POPULATION

The population enrolled in this study is composed by six Portuguese individuals with two negative controls, no alteration and no disease (V4 and V6), two BC patients, mother and daughter, both with a pathogenic mutation in the *ATM* gene (V5 and V1, respectively) and two healthy VUS-carriers individuals in the *BRCA1* gene (V2 and V3). Peripheral blood samples of 20 mL collection by venous puncture was accompanied by an informed consent which was signed agreeing its use for research, as well as questionnaire (Appendix 1) and the detailed family history of oncological disease for each patient (Appendix 2). The consent document for the use of samples for research have the approval of the National Commission of Data Protection.

The population enrolled was sequenced for a panel of twelve genes: *BRCA1, BRCA2, PTEN, TP53, STK11, ATM, PALB2, CHEK2, RAD51C, RAD51D AND BRIP1*. The NGS methodology was performed through Ion Torrent technology, by AmpliSeq method in IonPGM apparatus. Whenever the variants frequencies found were in exonic region and higher than 10%, it was confirmed by Sanger sequencing (ABI3100 Avant). This part of the project was performed in collaboration with José Pereira Leal of Ophiomics, who has extensive experience in the field (sequencing and *in silico* analysis results in Appendix 3).

2.2. *IN VITRO* IRRADIATION

The blood samples were irradiated *in vitro* with a ^{60}Co radiation source from Precisa 22 irradiator at the Ionizing Radiation Installations (IRIS) in C^{2}TN-IST. For each donor, a dose of 2 Gy was given and a non-irradiated control (0 Gy) was included.

For the irradiation, approximately 4 mL of whole blood from each donor were distributed in glass tubes of 4 mL for the chromosomal aberrations assay and the micronuclei assay. For the comet assay lymphocytes were previously isolated and then distributed in the glass tubes of 4 mL.

2.3. CHROMOSOMAL ABERRATIONS ASSAY

After irradiation, blood samples were cultured, and triplicates or quadruplicates were performed for each donor.

To each tube, previously containing 4.5 mL of RPMI-1640 medium with L-Glutamine (SIGMA), supplemented with 25% fetal bovine serum (SIGMA), 1.5% of penicillin-streptomycin (10000 U/mL + 10 mg/mL; SIGMA), 0.5% of sodic heparin (5000 U.I./mL; B. Braun) and 2.5% of phytohemagglutinin (Gibco), was added 0.5 mL of irradiated and non-irradiated whole blood. Cultures were kept in an incubator at 37ºC, 5% CO_2 and in an angle of 40º for 48h. By the 24thh, it was added colcemid (0.08 µg/mL; Gibco). At the end of the 48h, cultures were centrifuged at 400 xg for 5 min at room temperature. The *pellet* was then resuspended with mild stirring and 10 mL of KCl solution [0.56 % (p/v); MERCK] previously heated at 37ºC was added and homogenised by inversion. The tubes were then placed in a water bath 37ºC for 20 min, to promote hypotonic shock, and after that time they were

centrifuged as described before. Cells were fixed, under stirring, with 5 mL of fixative mixture of methanol: acetic acid [3:1 (v/v); both from Panreac], previously cooled at -20ºC, and then centrifuged as described before. These two steps of fixation and subsequent centrifugation were repeated two to three times, until the supernatant was clean. At the end, 10 mL of fixative mixture was added to each tube and at this point the samples can be kept at -20ºC for long periods of time.

To prepare the slides, the samples that were kept at -20ºC were centrifuged at 400 xg for 5 min at room temperature, supernatant discharged (leaving about 500 µL of mixture) and suspension homogenised. The slides were previously wash and submersed in distilled water at 4ºC. With the help of a glass Pasteur pipette, three drops of suspension were spread in each slide, from a height of 15 cm. Once well dried, at room temperature for 24h, at least, the slides were stained with Giemsa's solution (MERCK), previously filtrated, 4% (v/v) in phosphate buffer 0.01 M (pH 6.8; VWR, BDH Prolabo), for 10 min. The excess dye was withdrawn under running water. Once well dried, coverslips were placed over the slides with three drops of mounting medium (Entellan®) (MERCK).

The slides were then analysed with a 1000x amplification with an optical microscope. For each donor and each dose 200 metaphases with 46 chromosomes were scored by two independent operators (100 each) for the different types of aberrations, according to the criteria described by Rueff et al., 1993 and following the recommendations of the IAEA, 2011.

2.4. MICRONUCLEUS ASSAY

As with the chromosomal aberrations assay, after irradiation, blood samples were cultured, and triplicates or quadruplicates were performed for each donor.

To each tube, previously containing 4.5 mL of RPMI-1640 medium with L-Glutamine (SIGMA), supplemented with 25% fetal bovine serum (SIGMA), 1.5% of penicillin-streptomycin (10000 U/mL + 10 mg/mL; SIGMA), 0.5% of sodic heparin (B. Braun) and 2.5% of phytohemagglutinin (Gibco), was added 0.5 mL of irradiated whole blood. Cultures were kept at 37ºC, 5% CO_2 and in an angle of 40º, approximately, for 72h. By the 44thh, it was added cytochalasin-B (6 µg/mL; SIGMA). At the end of the 72h, cultures were centrifuged at 110 xg for 10 min, at room temperature. After discarding the supernatant, cells were washed two times with 5 mL of washing solution [RPMI-1640 medium with L-Glutamine (pH=7.2; SIGMA) and $NaHCO_3$ (0.1 g/L; B. Braun)], supplemented with 2% fetal bovine serum (SIGMA)] and centrifuged at 110 xg for 7 min, at room temperature. Next, it was performed a mild hypotonic shock by adding 5 mL of shock solution [4:1 of distilled water + RPMI-1640 medium with L-Glutamine (pH=7.2; SIGMA) and $NaHCO_3$ (0.1 g/L; B. Braun), supplemented with 2% fetal bovine serum (SIGMA)] followed by centrifugation at 110 xg for 5 min, at room temperature. After pellet concentration, by discarding most of the supernatant, a drop of cell suspension was placed in each slide and the smear was performed.

By the next day, and once the slides were dried (24h at room temperature, at least), they were fixed with 5 mL of fixing mix of methanol: acetic acid [3:1 (v/v); both from Panreac], previously cooled,

for 20 min at -20 ºC. After that time, and the slides were dry, they were stained with Giemsa's solution (MERCK), previously filtrated, 4% (v/v) in phosphate buffer 0,01 M (pH 6.8; VWR, BDH Prolabo), for 8 min. The excess dye was withdrawn under running water. Once well dried, coverslips were placed over the slides with three drops of mounting medium (Entellan®).

The slides were then analysed with a 400x amplification with an optical microscope. For each donor and dose 2000 binucleated cells were scored by two independent operators (1000 each), according to the criteria of IAEA, 2011.

2.5. SINGLE-CELL GEL ELECTROPHORESIS (COMET ASSAY)

2.5.1. LYMPHOCYTES SEPARATION

For lymphocyte separation it was used Histopaque – 1077 (Polysucrose 57 g/L and sodium diatrizoate 90 g/L; SIGMA), a ready-to-use separation medium, that simplifies the process of rapid recovery of viable lymphocytes and other mononuclear cells from whole blood.

To a conical centrifuge tube, previously containing 3.5 mL of Histopaque – 1077, was carefully added 6.5 mL of whole blood that was then centrifuged at 700 xg for 30 min at room temperature. At the end of that time, an opaque layer was observed, and the upper layer was aspirated with a Pasteur pipette to a fresh conical tube. The cells were then washed with 10 mL of PBS (isotonic phosphate buffered saline; 10x, pH 6.8) solution and centrifuged again at 200 xg for 10 min. The pellet was suspended in RPMI-1640 medium with L-Glutamine and part of the cell suspension (approximately 4 mL) was used for irradiation while the rest was used as control. Cell suspension was kept at 4º C until the next point.

2.5.2. COMET ASSAY

After irradiation, 30 µL of the cell suspension was taken dissolved in 70 µL of 0.5% low melting point agarose (SIGMA). Next, the suspensions were spread on microscope slides that were previously coated with 1% normal melting point agarose (BIOLINE) and kept at 4ºC for 20min. After that, slides were left over-night in a cold lysis buffer (2.5M NaCl, 10mM Tris, 100mM EDTA, 1% Triton, pH 10; SIGMA).

By the next day, the slides were washed with double-distilled water, previously cooled, and kept in it for 10 min at 4ºC. Then the slides were immersed in cold electrophoresis buffer (10M NaOH, 200mM EDTA, pH>13) and left for 20min at 4ºC. Electrophoresis was conducted for 20 min at 25V (400mA) and, afterwards, the slides were neutralized three times with neutralization buffer (0.4M Tris, pH 7.5), 5min each, dried with ethanol (50%, 75%, 100%, 5 min each) and stained with GelRed (3X).

The slides were then analysed with a 400x amplification with a fluorescent microscope (Leica DMLB) and were selected and captured about 20-30 fields. The cells images captured were then analysed by the CometScore software, that returned the percent of DNA in tail (% DNA in tail) of comets.

2.6. STATISTICAL ANALYSIS

All graphs were plotted using GraphPad Prism 5 software and represented with mean expression ± standard deviation (SD). The statistical analysis was performed with IBM SPSS Statistics 25. For the CA assay and MN assay a Chi-square, Fisher's exact test was applied, and the p-value was considered significant when less than 0.05. For the SCGE or comet assay a Kolmogorov-Smirnov normality test was performed to examine if samples followed a Gaussian distribution and since it was not observed, non-parametric tests were used to analyse samples. To compare between control (0 Gy) and irradiated (2 Gy) in each sample, a Wilcoxon Signed Ranks test was applied, and the p-value was considered significant when less than 0.05. To compare between the different groups of samples, the non-parametric Mann-Whitney test was performed, and the p-value was considered significant when less than 0.05.

3. RESULTS

3.1. CHROMOSOMAL ABERRATIONS ASSAY

The Chromosomal Aberrations assay results were analysed with an optical microscope with 1000x amplification. For each donor, 100 metaphases containing 46 chromosomes were count and analysed by two independent individuals, giving a total of 1200 metaphases. Each metaphase was investigated for the presence of chromosomal aberrations, namely chromatid with gaps or breaks (CTG/CTB), chromosomes with gaps or breaks (CHG/CHB), excess acentric fragments (excess ACE), dicentric chromosomes (DIC) and rings. Every metaphase containing at least one chromosome aberration except gaps was accounted for the frequency of aberrant cells excluding gaps (% ACEG). All these results are represented in table 3.1. Where is possible to see an increase in % ACEG in all samples when radiation is applied. The most frequent aberrations present in metaphases after irradiation are the acentric fragments and dicentric chromosomes that, as well as the presence of rings, are the main chromosomal aberrations related to γ-radiation exposure (Ballarini & Carante, 2016; Martins et al., 2013). These structures are represented in figure 3.1.

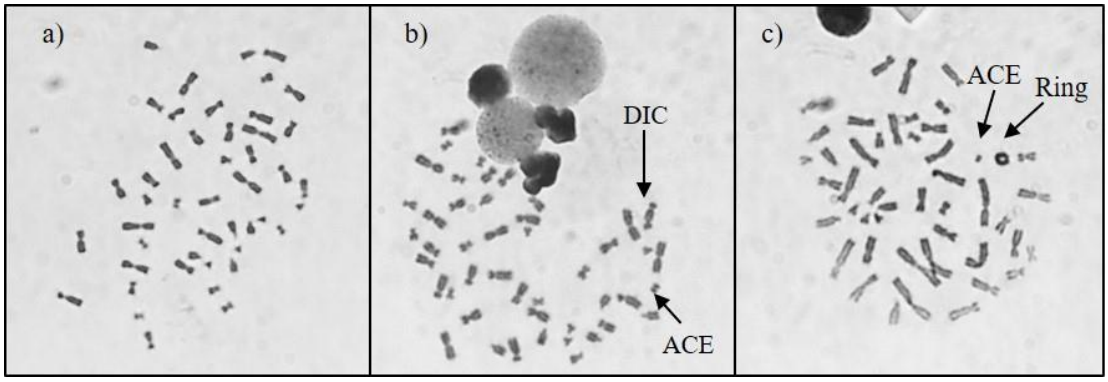


Figure 3. 1 – Representative images of metaphases obtained during analysis, with the characteristic structures associated with γ-radiation exposure. a) representation of a normal metaphase with 46 chromosomes (0 Gy dose); b) representation of a metaphase with 46 chromosomes, containing one dicentric chromosome (DIC) and one acentric fragment (ACE); c) representation of a metaphase with 46 chromosomes, containing one ring and one acentric fragment.

Table 3. 1 – The number of chromosomal aberrations and their distribution in cells, as well as the frequency of cells with at least one chromosomal aberration except gaps (% ACEG). Observation through an optical microscope with 1000x amplification of 100 metaphases containing 46 chromosomes in which were counted the number of chromosomal aberrations by two independent individuals. A total of 1200 metaphases were counted for the all six donors and both doses. An increase in the percentage of cells containing at least one chromosomal aberration excluding gaps is observed with radiation for all samples.

Sample Name	Dose (Gy)	Total Cells	CTG	CHG	CTB	CHB	Excess ACE	DIC	DIC Distribution					Ring	% ACEG
									0	1	2	3	4		
V1	**0 Gy**	200	3	0	5	1	0	0	200	0	0	0	0	1	3.5
	2 Gy	200	2	1	3	1	39	33	167	21	6	0	0	2	29
V5	**0 Gy**	200	0	0	1	1	9	0	200	0	0	0	0	0	5
	2 Gy	200	2	0	2	3	31	27	173	27	0	0	0	2	28
V2	**0 Gy**	200	5	0	6	1	3	0	200	0	0	0	0	0	4.5
	2 Gy	200	0	1	6	0	26	22	178	22	0	0	0	4	25
V3	**0 Gy**	200	0	0	4	1	0	1	199	1	0	0	0	0	3
	2 Gy	200	2	0	7	3	30	30	170	22	4	0	0	3	30.5
V4	**0 Gy**	200	0	0	5	0	2	1	199	1	0	0	0	0	3.5
	2 Gy	200	0	0	2	2	34	21	179	21	0	0	0	2	25.5
V6	**0 Gy**	200	0	0	5	1	3	3	197	3	0	0	0	1	6
	2 Gy	200	1	0	3	3	45	28	172	22	3	0	0	1	32.5

CTG – Chromatid with a gap; CHG – chromosome with a gap; CTB – chromatid with a break; CHB – Chromosome with a break; ACE – Acentric fragment; DIC – Dicentric fragment; % ACEG – Aberrant cells excluding gaps.

The distribution of % ACEG is represented in figure 3.2 with mean expression ± SD. The Chi-square, Fisher's exact test was used to compare the number of aberrant cells between doses in each sample, and between samples only for 2 Gy dose values. All samples showed statistical significance between % ACEG in control and the 2 Gy dose (p-value < 0.05). When comparing between samples after the 2 Gy dose irradiation, no difference statistically significant was observed. The distribution of % AECG and statistical significances are represented in figure 3.2.

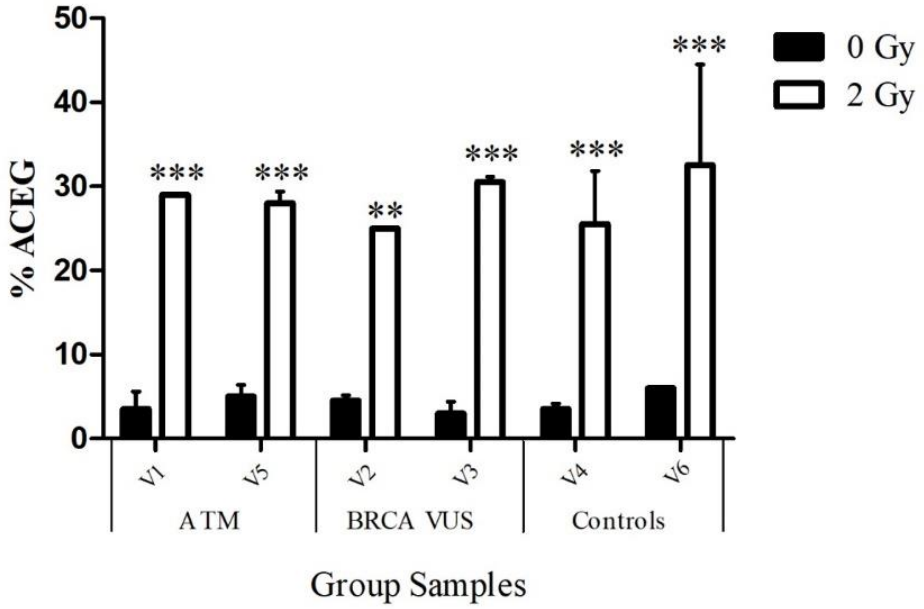


Figure 3. 2 – Frequency of aberrant cells excluding gap (% ACEG). Results are expressed as mean values ± SD from two independent readings. Statistical analysis was performed with IBM SPSS Statistic 25, using Chi-square, Fisher's exact test. The difference obtained was considered statistically significant when p-value < 0.05. All samples showed statistically significant difference between control (0 Gy) and irradiated (2 Gy) (p-value < 0.05), represented with * (the amount of * represents how relevant is the statistical difference). No statistical difference was observed between samples.

3.2. MICRONUCLEI ASSAY

Micronuclei assay results were analysed with an optical microscope with 400x amplification. For each donor and dose, 1000 binucleated cells were scored and analysed by two independent individuals, which gives a total of 24000 binucleated cells. A total of 1791 micronucleus were count and a total of 1562 binucleated cells had at least one micronucleus (Appendix 4). These structures are illustrated in figure 3.3.

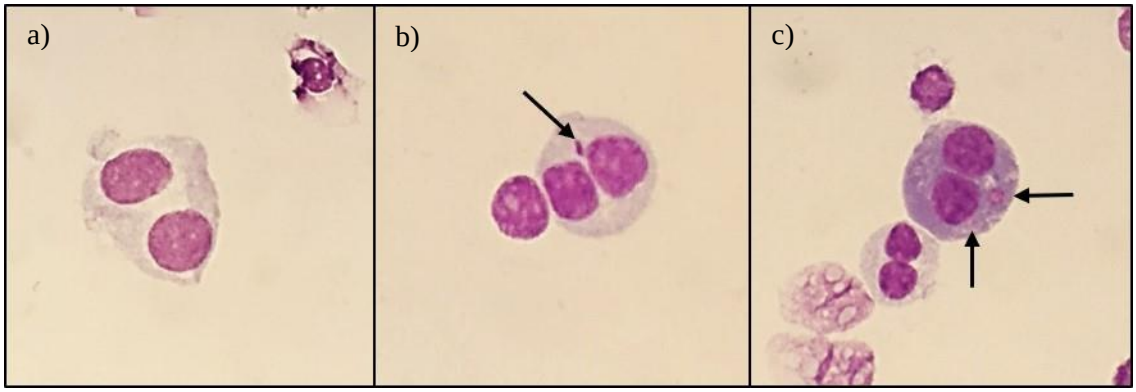


Figure 3. 3 – Representative images of binucleated cells obtained during analysis, with the micronuclei structures, associated with γ-radiation exposure. a) representation of a normal binucleated cell, obtained with cytochalasin-B treatment; b) and c) representation of binucleated cells containing one and two micronuclei, respectively.

In a paralleled analysis, the amount of mononuclear (M_1), binuclear (M_2), trinuclear (M_3) and tetranuclear (M_4) cells in 1000 total cells were count by two independent individuals (Appendix 4). The nuclear division index (NDI) was calculated according to the formula: $NDI = (M_1 + 2M_2 + 3M_3 + 4M_4)/N$, where N is the total number of cells scored according to Fenech, 2007. These results and the distribution of micronuclei in binucleated cells after irradiation with γ-rays at a 2 Gy dose, are presented in table 3.2. An increase in the percentage of binucleated cells with micronuclei (‰ MNBN) and total micronuclei (‰ TMN) is observed when cells are exposed to a dose of 2 Gy comparing to control (0 Gy) (table 3.2 and figure 3.4). Furthermore, the presence of two or more micronuclei is also observed, almost exclusively, when radiation is applied (table 3.2). In all samples but V1, the NDI values decrease from control to 2 Gy in each donor (table 3.2).

Table 3. 2 - Number of micronuclei and their distribution in binucleated cells, frequency of binucleated cells with micronuclei, total micronuclei and nuclear division index for each donor and doses. Observation through an optical microscope with 400x amplification of 1000 binucleated cells in which were counted the number of micronuclei by two independent individuals A total of 12000 binucleated cells were counted for all 6 donors and of those 1562 cells had at least one micronuclei. An increase in micronuclei with radiation was observed for all samples, whereas a decrease in NDI values from control to 2 Gy was observed in all samples except V1.

Sample Name	Dose (Gy)	Total BN	MN Distribution					MNBN (‰)	TMN (‰)	NDI
			0 MN	1 MN	2 MN	3 MN	4 MN			
V1	**0 Gy**	2000	1992	8	0	0	0	4	4	1.71
	2 Gy	2000	1834	126	20	0	0	73	83	1.77
V5	**0 Gy**	2000	1980	18	1	0	0	9.5	10	1.23
	2 Gy	2000	1745	192	27	3	0	111	127.5	1.17
V2	**0 Gy**	2000	1984	16	0	0	0	8	8	1.84
	2 Gy	2000	1753	176	29	3	1	104.5	123.5	1.81
V3	**0 Gy**	2000	1991	9	0	0	0	4.5	4.5	1.82
	2 Gy	2000	1733	209	26	2	0	118.5	133.5	1.71
V4	**0 Gy**	2000	1981	15	2	0	0	8.5	9.5	1.58
	2 Gy	2000	1626	263	43	7	1	157	187	1.54
V6	**0 Gy**	2000	1961	35	2	0	0	18.5	19.5	1.72
	2 Gy	2000	1629	288	37	3	0	164	185.5	1.60

The distribution of ‰ MNBN is represented in figure 3.4 with mean expression ± SD. The Chi-square, Fisher's exact test was used to compare the frequency of binucleated cells with at least one micronuclei (‰ MNBN) between the control (0 Gy) and irradiated (2 Gy) for each sample, and between samples for only after irradiation of 2 Gy dose values. All samples presented statistical significant difference between ‰ MNBN in control and the 2 Gy dose (p-value < 0.05) (figure 3.4 (a)). When comparing between samples, both V2 and V3 showed a difference statistically significant with both control samples (V4 and V6) and with sample V1 (figure 3.4 (b) and (c)). V1 and V5, both showed a statistically significant difference with control samples too (figure 3.4 (d)), but V5 instead did not showed statistical significant difference with V2 and V3 (figure 3.4 (b)).

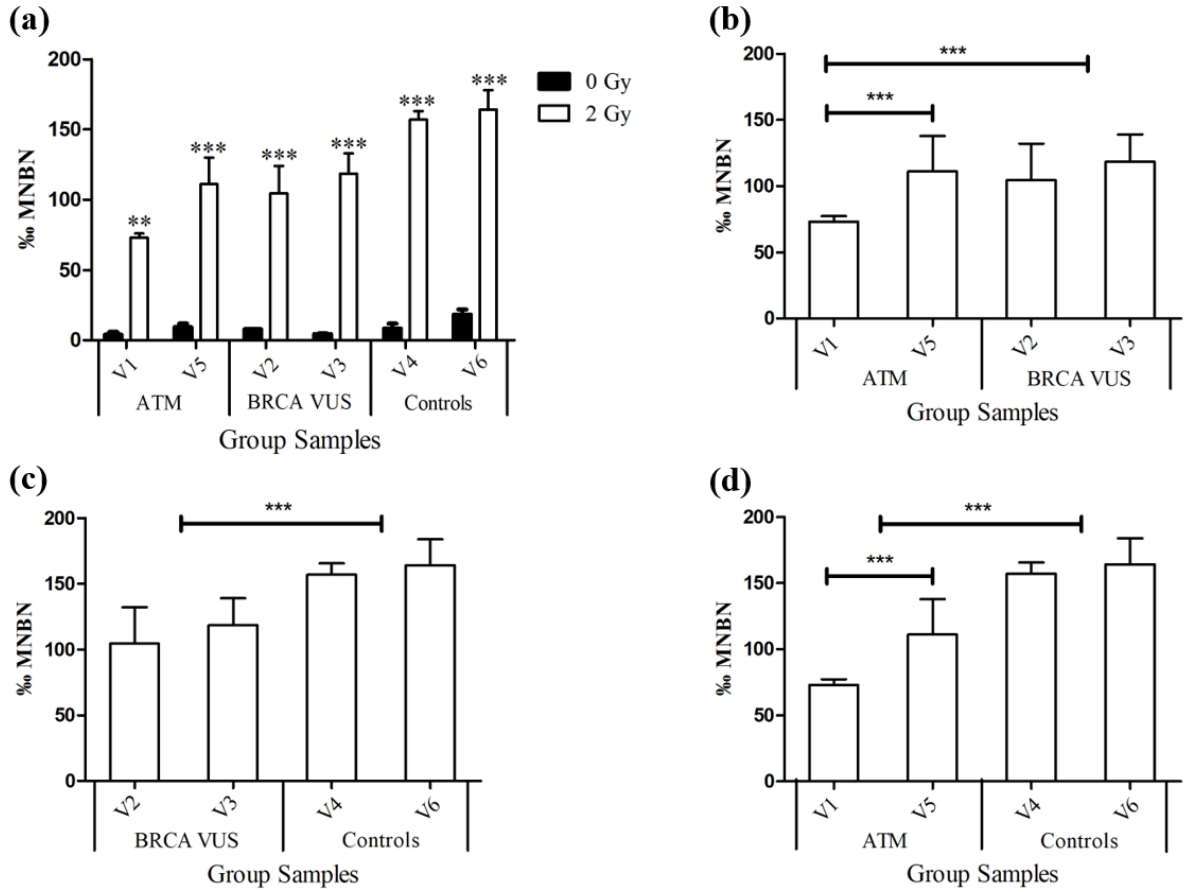


Figure 3. 4 – Frequency of binucleated cells with micronuclei in 1000 binucleated cells (‰ MNBN). Results are expressed as mean values ± SD from two independent readings. Statistical analysis was performed with IBM SPSS Statistic 25, using Chi-square, Fisher's exact test to compare between doses, represented in **(a),** and between samples for the 2 Gy dose, represented in **(b)**, **(c)** and **(d)**. Difference was considered statistically significant when p-value < 0.05. **(a)** All samples sowed statistically significant difference between control (0 Gy) and irradiated (2 Gy) (p-value < 0.05). **(b)** V1 showed statistically significant difference with V5 and with both samples V2 and V3 (p-value < 0.05). **(c)** Both V2 and V3 showed a statistically significant difference with both control samples, V4 and V6 (p-value < 0.05). **(d)** Both V1 and V5 showed statistically significant difference with both control samples, V4 and V6 (p-value < 0.05).

3.3. SINGLE CELL GEL ELECTROPHORESIS (COMET ASSAY)

The results from comet assay were analysed through the use of a fluorescent microscope with 400x amplification. For each slide, 20-30 fields were captured and 200 cells on each dose and sample were randomly chosen (example of fields captured in figure 3.5 (a) and (b)). In some cases, the number of cells was not reached, but the maximum limit was kept at 200 cells. They were then analysed by the CometScore software that returned the percentage of DNA in tail of comets. Each sample distribution of % of DNA in tail was then plotted in a graph represented in figure 3.6, with mean percentage ± SD. The Wilcoxon Signed Ranks test was applied to see if the difference between control (0 Gy) and irradiated (2 Gy) was statistically significant (p-value < 0.05), which was only detected in samples V1, V2 and V3.

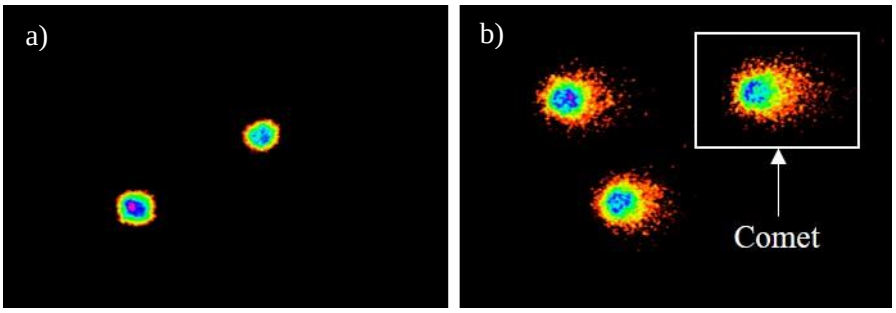


Figure 3. 5 – Representative images of fields captured for the comet assay. (a) example of field with only normal cells with all DNA in nucleus; (b) example of field with comets.

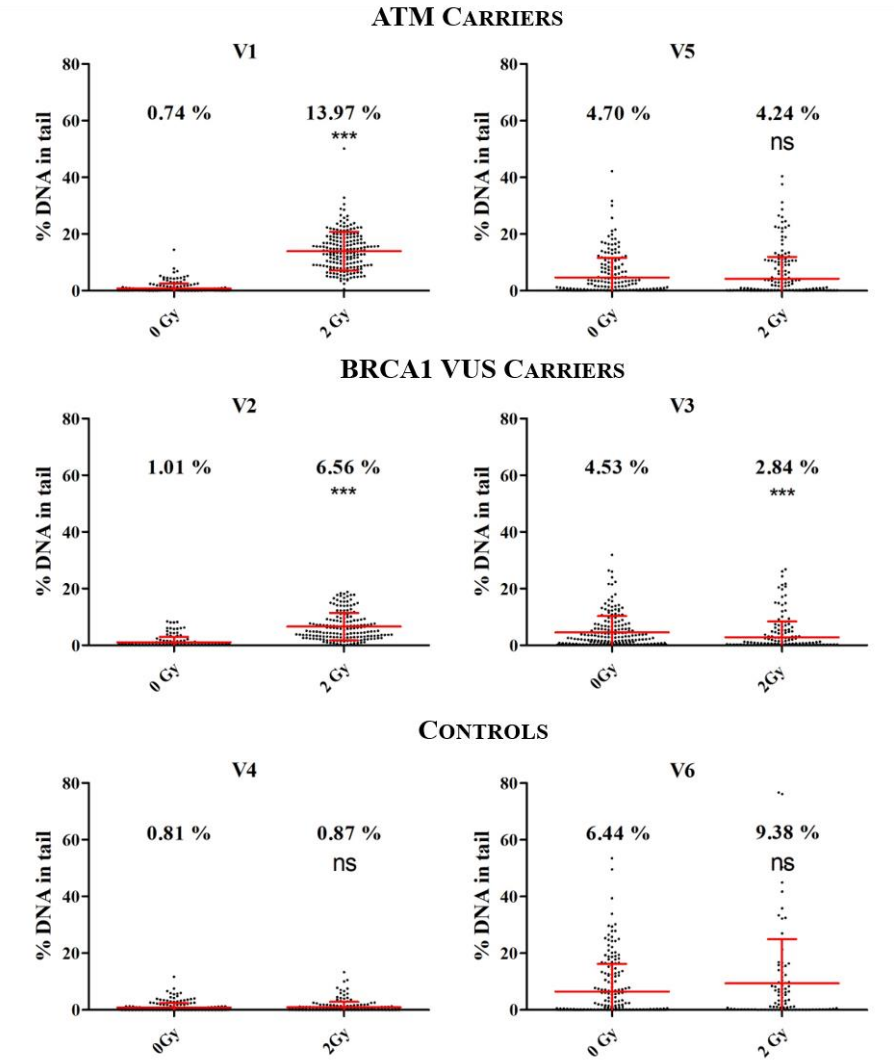


Figure 3. 6 – Distribution of % of DNA in tail for each donor and dose. % of DNA in tail distribution was plotted for each sample and dose using GraphPad Prism 5 software. Mean values ± SD are represented by the red lines. Statistical analyse with Wilcoxon Signed Ranks test was performed by IBM SPSS Statistics 25 and a p-value < 0.05 was considered significant. Only V1, V2 and V3 showed statistically significant difference between control (0 Gy) and irradiated (2 Gy), represented with * (the amount of * represents how relevant is the statistical difference).

The plot for the 2 Gy data obtained for the six samples is represented in figure 3.7 (a), (b) and (c), with mean percentage ± SD. To evaluate if the difference between the different groups of samples was statistically significant, the non-parametric Mann-Whitney test was performed, and only samples V5 and V3 showed no statistical significant difference (figure 3.7 (a)). Both V4 and V6 showed statistically significant difference with both V2 and V3 (figure 3.7 (b)) and with both V1 and V5 (figure 3.7 (c)).

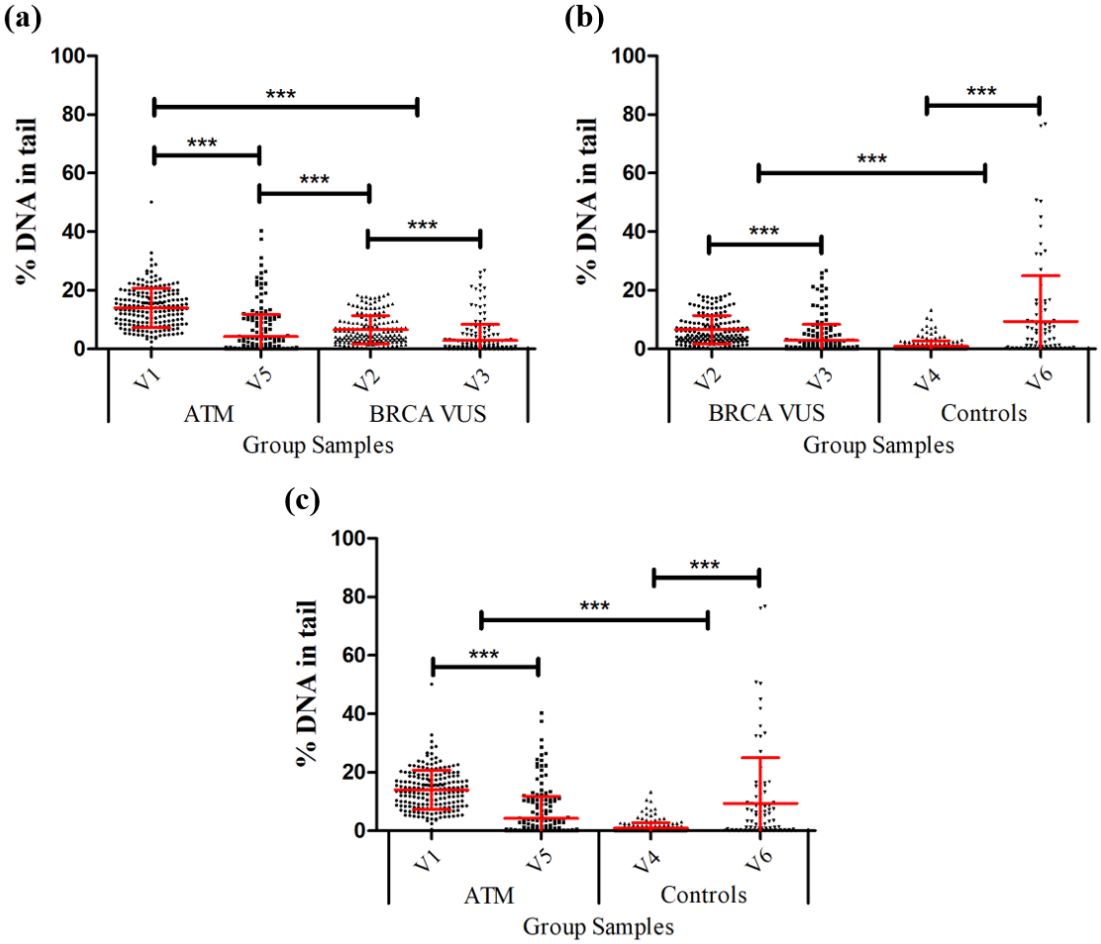


Figure 3. 7 - Distribution of % of DNA in tail for the 2 Gy dose for each donor. % of DNA in tail for the 2 Gy dose for each sample was plotted with GraphPad Prism 5 software. Mean values ± SD are represented by the red lines. Statistical analysis with non-parametric Mann-Whitney test was performed using IBM SPSS Statistics 25 and a p-value < 0.05 was considered significant. (**a)** Only V5 and V3 showed no difference statistically significant. (**b)** Both V2 and V3 showed statistically significant difference with both controls, V4 and V6 (p-value < 0.05). (**c)** Both V1 and V5 showed statistically significant difference with both controls, V4 and V6 (p-value < 0.05).

3.4. ON GOING RESULTS

In order to analyse the influence of radiation dose in our samples, we decided to add additional doses to our study (1 Gy and 5 Gy). At the end of the study we should be able to understand the radiation effect in our samples based on a dose-response curve (0 Gy, 1 Gy, 2 Gy and 5 Gy). However, at this time we only can do it for samples V2 and V6. Data shown represents the results obtained for the comet assay (figure 3.8). The results were plot in the two graphs represented in figure 3.8 (a) and (b). The mean percentage of DNA in tail ± SD is represented for each sample. The pattern observed between samples is very different. In sample V6, an increase in mean % of DNA in tail with radiation dose is observed, with the highest mean % present in dose 5 Gy (3.438 %) (figure 3.8(a)). However, difference between percentages is very low. On the other hand, in V2 the highest mean % is for the 1 Gy dose (5.190 %), decreasing with dose increase, with lowest being for the 5 Gy (2.535 %) and the difference between doses is higher (figure 3.8 (b)).

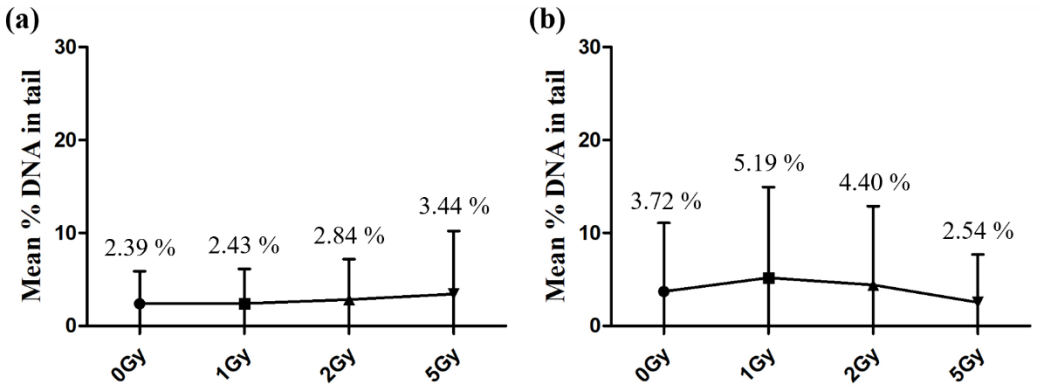


Figure 3. 8 - Mean % of DNA in tail for no radiation, 1 Gy dose, 2 Gy dose and 5 Gy dose for donors V6 (a) and V2 (b). Mean % of DNA in tail for both samples in four different doses was plotted with GraphPad Prism 5 software. In V6 there's an increase in mean % of DNA in tail with radiation dose, while in V2 the highest mean % is for the 1 Gy dose, and a decrease with radiation increase.

4. DISCUSSION

DNA molecules are recurrently exposed to damage, due to chemical or physical insults of diverse origin. Damage, such as DSB, is highly toxic and if not repaired or misrepaired it can lead to aneuploidy, mutations, chromosomal aberrations or uncontrolled cell death. Therefore, proper repair of these breaks is critical for cellular integrity (Ahmed et al., 2018; Krejci et al., 2012). In mammalian cells, DSB can be repaired by two main repair mechanisms, the HR and NHEJ repair systems (Hoeijmakers, 2001). Any defect on these mechanisms, may lead to improper DSB repair, leading to loss of cell integrity, and possibly tumourigenesis (Krejci et al., 2012). HR impairment has been highly associated with BC development, in fact the gene panels used for BC genetic testing include mostly genes related to DSB repair by HR (Slavin et al., 2017). With entire panels of genes sequencing through NGS comes an increased detection of VUS. These alterations cannot be classified as benign or pathogenic and *in silico* prediction tools only give the theoretical effect of the variant in protein structure and function. So, the role of the gene variants and their effect on cancer risk is still not clarified. With that in mind we performed a functional analysis in peripheral blood lymphocytes of two *BRCA1* VUS carriers (V2 and V3), as well as of two BC patients with a pathogenic mutation in the *ATM* gene (V1 and V5) and two controls (V4 and V6), without any variant detected after sequencing analysis. The peripheral blood lymphocytes were irradiated with γ-radiation, a clastogenic agent, inducing DSB, and the lymphocytes response to the damage was assessed with three different endpoints: CA, MN and comet assay.

CA are microscopically visible result of DNA damage, such as DSB which are the principal lesion related to CA formation. (Obe et al., 2002). This technique is known as the "Gold Standard" method for radiation biodosimetry, allowing the detection of CA associated with radiation, such as the dicentric chromosomes (figure 3.1) (Antunes et al., 2014; Martins et al., 2013). It has also been shown that high frequencies of CA in peripheral blood lymphocytes have a significantly elevated risk of cancer development (Obe et al., 2002). As expected, our results showed a clear increased of CA after irradiation (table 3.1) showing statistical significance of % ACEG (figure 3.2) in all samples. These results are consistent with the literature related to this technique applicability in biodosimetry (Martins et al., 2013). However, when comparing between samples they showed no significant difference (figure 3.2). It is known that cells with the *ATM* gene mutated in both alleles are hypersensitive to ionizing radiation, associated with a deficiency in repair (Parshad & Sanford, 2001). On the other side high frequencies of CA is related to elevated risk of cancer development (Obe et al., 2002; Terzoudi & Pantelias, 2006). Samples V1 and V5 are BC patients with a monoallelic pathogenic mutation in the *ATM* gene, in which would be expected to see an increased % ACEG comparing to control individuals (samples V4 and V6), but this expectation is not seen in these results. A possible explanation is the fact that both tumours (mother and daughter) might present loss of heterozygosity (LOH), in contrast to the peripheral blood lymphocytes that we used (Maxwell et al., 2017; Polak et al., 2017). Furthermore, the same situation might be associated with the *BRCA1* VUS carriers and a second hit mutation would be necessary for this variation to have an impact in cancer development. However, a few BC cases of carriers of

pathogenic germline *BRCA1/2* variants did not exhibited LOH and in those cases *BRCA1/2* haploinsufficiency might be triggering some other events (Polak et al., 2017). Concerning such fact, this technique might not be sensitive enough to discriminate and assess the impact of these specific alterations in protein function and cancer risk.

The micronuclei assay is an alternative method to the chromosomal aberrations assay, allowing the detection and evaluation of the damage induced by ionizing radiation (Antunes et al., 2014). This assay requires lower time consuming to read each slide sample when compare to chromosomal aberrations assay and also does not require highly trained scorers. However, it has less specificity for ionizing radiation, due to the high impact of other factors in the frequency of MN. Even though, it has been widely used to evaluate ionizing radiation exposure (Antunes et al., 2014). As expected, our results showed an increase in binucleated cells with micronuclei (‰ MNBN) and total micronuclei (‰ TMN) when a dose of 2 Gy of ionizing radiation was applied (table 3.2 and figure 3.4 (a)). This goes according to the MN assay application in biodosimetry (Antunes et al., 2014). The NDI gives a measure of the proliferative status of the viable cell fraction (Fenech, 2007). Our results revealed a decrease from control to radiated in each sample, except for sample V1, but the difference between values is minor and so not relevant (table 3.2). So, we can conclude that radiation had no influence in viable cells proliferation.

When observing the graphs in figure 3.4, it is possible to see an increase in ‰ MNBN from sample V1 to V6. Previous studies have already associated higher frequencies of MN with increased risk of cancer (Cardinale et al., 2012; Murgia et al., 2008; Scott et al., 1996) so it would be expected that samples V1 and V5, BC patients with the pathogenic mutation in the *ATM* gene, had higher frequencies of MNBN than the control samples. However, V1 and V5 have lower amount of binucleated cells with MN when compared with V4 and V6 controls (figure 3.4 (d)). The same is observed in the case of those carrying a VUS in *BRCA1* gene, that have lower frequency of MNBN comparing with controls but higher than V1 (figure 3.4 (b) and (c)). So, a non-linear relationship of binucleated cells with micronuclei frequency and the risk of cancer is a possible explanation. Due to the acute exposure of radiation employed in blood cells, cells lacking the ability to repair the damage inflicted might have accumulate an excessive amount of genome damage. And, cells with that excessive genome damage may be eliminated by apoptosis (Bonassi et al., 2007). To see if this will be the case, cell viability or apoptosis assays should be performed in the future.

The comet assay has been used to evaluate both DNA damage and repair and is considered a well-known biomarker for assessing DNA damage due to radiation exposure (Kaur et al., 2017). When comparing to CA and MN assay, the comet assay shows some advantages. It represent less time consuming and high sensibility, detecting low levels of DNA damage (Azqueta et al., 2014; Collins, 2004; Gunasekarana et al., 2015). When analysing the influence of a 2 Gy dose of radiation, only samples V1, V2 and V3 showed a statistical difference with control (0 Gy) (figure 3.6). However, sample V3 show the double of % of DNA in tail in the non-radiated than the radiated case, possible due to intra-

individual differences resulting from two difference end times experiments. Sample V6 on the other hand, has no significant difference between non-irradiated and irradiated even showing a slight difference between them. This is due to the high SD observed in the irradiated sample (figure 3.6) and is possibly related to the small number of cells counted, only 90 in 200 (data not shown) in the irradiated sample. One limitation of using peripheral blood lymphocytes is a limited cell supply. Furthermore, the recurrent collection of patients' blood samples is an invasive methodology and is a burden on patients and relatives. Nevertheless, the fact that V4, a control sample, shows no statistical significance after radiation (figure 3.6), might be associated with the fact that cells have less sensitivity to radiation, associated with a deficiency in repair. However, for sample V5 a carrier of a pathogenic mutation in the *ATM* gene and a BC patient, we would expect to have more damage after radiation, just like V1. The presence of different results between samples with the same alteration might be associated with inter-individuality, and not directly related to the mutation.

When comparing between samples the % of DNA in tail after radiation, is possible to see a decrease from V1 to V6, excluding V6 and V3 (figure 3.7 (a), (b) and (c)). This is consistent with the fact that V1 have more sensitivity to ionizing radiation than V4, which might be associated with a deficiency in repair. V2 is a carrier of a VUS in the *BRCA1* gene, the impact of this alteration in repair ability is not known, but in fact this sample shows significantly more DNA damage (% of DNA in tail) than the negative control V4 (figure 3.7 (b)), what may indicate an increased sensitiveness to ionizing radiation. When comparing to V1, V2 shows significantly less damage (figure 3.7 (a)). So, we might infer that V2 has increased sensitiveness to radiation, possibly related to repair deficiency, but not as much as V1, a BC patient with a pathogenic mutation in the *ATM* gene.

The difference between the results obtained in the three techniques is probably related to the difference in sensitivity of each one. Micronuclei assay is usually used to complement the chromosomal aberrations assay. It might be more sensitive, but the MN structures are not specific of γ-radiation, and many other factors might influence the formation of these structures (Pinto et al., 2010). The comet assay is a more sensitive, however its analysis measure directly the presence of DSB and not consequences of these lesions (such as CA and MN) which might be influencing the results between techniques. Overall, except for the CA assay, the results show an increased susceptibility to ionizing radiation in pathogenic *ATM* mutation carriers and *BRCA1* VUS carriers. However, some additional assays should be performed to support these results.

In the ongoing results we decide to evaluate the effects of radiation dose through the establishment of a dose-response curve. For that and using samples V2 and V6 additional dose points were added, 1 Gy and 5 Gy besides the 2 Gy and the non-irradiated (figure 3.8 (a) and (b)). With the results obtained is possible to see a different pattern between the two samples. V6 shows a direct correlation between mean % DNA in tail and radiation, the amount of DNA lesion increase with dose increasing, as expected (Wang et al., 2013) (figure 3.8 (a)). On the other hand, in V2 the correlation between mean % DNA in tail and dose is inverse, which means the peak of higher % of DNA lesion

was obtained to 1 Gy assuming the lower value at 5 Gy of radiation dose (figure 3.8 (b)). A possible explanation is that the VUS present in in V2 sample might be increasing cells susceptibility to ionizing radiation due to deficiency in repair, and only 1 Gy of dose is enough to increase DNA damage. Once cells repair capacity might be compromised, the damage inflicted was too much and cells with excessive genome damage may have been eliminated by apoptosis, similar to the results seen for the micronuclei assay (figure 3.4) (Bonassi et al., 2007). Nevertheless, more studies will have to be developed to confirm the results obtained.

5. CONCLUSIONS

Over the last years, genetic testing for hereditary cancer risk assessment has become standard for BC. Recent advances in NGS have allowed entire panels of genes to be sequenced. The high throughput development of genetic testing platforms increased the detection of variants which significance remains unknown, or VUS. These alterations have no clinical significance established and cannot be classified as benign or pathogenic. Due to that, clinicians cannot give an exact answer to patients and carriers of VUS and their family members cannot take advantage of the risk assessment, prevention, and therapeutic measures that are available to carriers of known pathogenic mutations. Therefore, it is critical to determine the impact of these alterations in protein function and cancer risk, reason why the functional studies are an important tool in clinical classification of VUS.

The methodologies used in this study showed controversial results. CA assay has been described as the "Gold Standard" end point to evaluate the effects of radiation, however the results obtained did not reveal statistical difference between samples. It is well known that the need of a second hit mutation and the loss of heterozygosity in tumours might be crucial to have an impact in cancer development, what could be applied to our donors with the pathogenic mutation or VUS carriers. Concerning such fact, this technique might not be sensitive enough to discriminate and assess the impact of these specific alterations in protein function and cancer risk. However, in the MN assay the samples in study show lower amount of binucleated cells with MN when compared to control samples. A non-linear relationship of binucleated cells with micronuclei frequency and the risk of cancer might be associated with the fact that cells lacking the ability to repair the damage inflicted might have accumulate an excessive amount of genome damage. And, cells with that excessive genome damage may be eliminated by apoptosis. Yet, to assess this possibility cell viability or apoptosis assays should be performed. Nevertheless, the comet assay results showed a clear increased sensitivity to ionizing radiation, that might be correlated with deficiency in repair, of samples from donors carrying a pathogenic mutation in the *ATM* gene and those with the *BRCA1* VUS.

Overall, except for the CA assay, the results showed an increased susceptibility to ionizing radiation in pathogenic *ATM* mutation carriers and *BRCA1* VUS carriers. However, some additional studies should be performed to completely understand the results obtained, and the impact of alterations in cancer risk. The ongoing results so far are indicative of a possible increased susceptibility to ionizing radiation due to deficiency in repair, and only 1 Gy of dose is enough to increase DNA damage, in *BRCA1* VUS carrier cells. Still, these results will be complemented with those from the CA and MN assay, and see if the same response is observed. Additionally, cell viability or apoptosis assays will be performed to assess if cell death events are influencing our results and the γ-H2AX assay a highly sensitive assay for DSB damage will be established. Furthermore, one limitation of using peripheral blood lymphocytes is a limited cell supply, and the recurrent collection of patients' blood samples is an invasive methodology and is a burden on patients and relatives. To overcome these problems, we intend to establish patient-derived lymphoblastoid cell lines which have several advantages such as cost effectiveness, no limitation to sample size, easy to manipulate, allows follow-up and replication studies.

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APPENDIX

ADDITIONAL INFORMATION OF POPULATION

APPENDIX 1

Informed consent and questionnaire filled and signed by donors.

Exma. Senhora,

O estudo que pretendemos desenvolver será realizado no Departamento de Genética da Faculdade de Ciências Médicas da Universidade Nova de Lisboa e tem como título **"Cancro da Mama: a relevância clínica de variantes de significado desconhecido (VUS) em genes de reparação por recombinação homóloga – análise funcional em doentes com cancro de mama familiar"**. Pretende-se estudar a relevância clínica de alterações em genes associados com o aumento de risco para o cancro de mama familiar.

Este estudo está inserido numa bolsa financiada pela Liga Portuguesa Contra o Cancro (LPCC-NRS) – Terry Fox 2017 - sob responsabilidade da Doutora Susana Silva, investigadora da NMS|FCM - UNL em parceria com a Ophiomics na pessoa do Doutor José Leal, e ainda com a Doutora Octávia Monteiro Gil (CTN-IST-UL). O estudo consistirá numa avaliação da resposta celular a exposições genotóxicas induzidas por agentes lesivos para o ADN.

Durante este estudo será recolhida uma amostra de sangue total a partir da qual se realizarão todos os ensaios *in-vitro* associados ao estudo. Será necessário recolher alguma informação pessoal que será tratada de forma confidencial e anónima. Este estudo não acarreta benefícios diretos para si, nem quaisquer riscos. É garantida a total confidencialidade e anonimato dos dados colhidos, nomeadamente a sua identificação só será conhecida pelo investigador responsável e não estará associada diretamente à manipulação da amostra.

Este estudo será realizado sempre com o acompanhamento do investigador responsável e colaboradores que manipularão amostras já codificadas e totalmente anónimas.

Agradecendo antecipadamente a sua colaboração,

Doutora Susana Nunes da Silva
Investigadora / Professora Auxiliar Convidada
Faculdade de Ciências Médicas da UNL

CAMPO DOS MÁRTIRES DA PÁTRIA, 130 · 1169-056 LISBOA · PORTUGAL · T.+351 218 803 000 · F.+351 218 851 920 · www.nms.unl.pt

ID AMOSTRA: _____

DECLARAÇÃO

Eu,_____, declaro ter sido devidamente esclarecida sobre a natureza e objetivos do estudo "*Cancro da Mama: a relevância clínica de variantes de significado desconhecido (VUS) em genes de reparação por recombinação homóloga – análise funcional em doentes com cancro de mama familiar",* tendo decidido colaborar voluntariamente neste estudo.

Assinatura do Participante

_____

Assinatura Investigador responsável

_____

Lisboa, ____ de _____ de ____

ID AMOSTRA: _____

INQUÉRITO

Género: Feminino ☐ Masculino ☐

Data de Nascimento: _____

Gestações: _____ **Amamentação:**_____

Fumador: Sim ☐ Não ☐ Ex-Fumador ☐ _____

Consumo de Álcool: Regular/Refeições ☐ Social ☐ Nunca☐

Restrições Alimentares: Não ☐ Sim ☐ Quais:_____

Data Diagnóstico Patologia Oncológica: _____

Antecedentes Familiares doença oncológica: _____

Outras patologias: _____

Toma medicação regularmente: Não ☐ Sim ☐ Qual:_____

Métodos Complementares de Diagnóstico: _____

Data dos últimos exames radiológicos (e.g. Raios-X, ortopantomografias):

_____

Notas Relevantes: _____

_____

_____

_____

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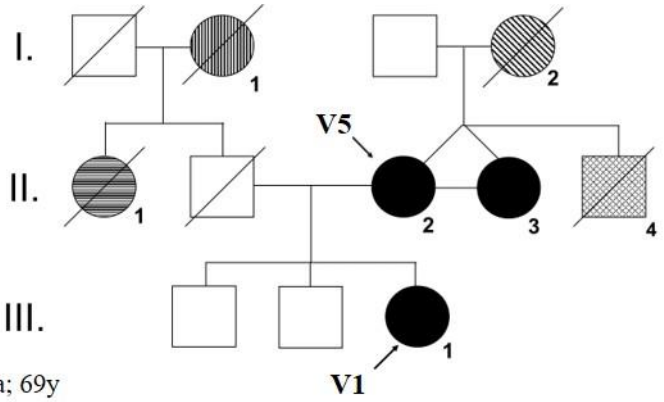
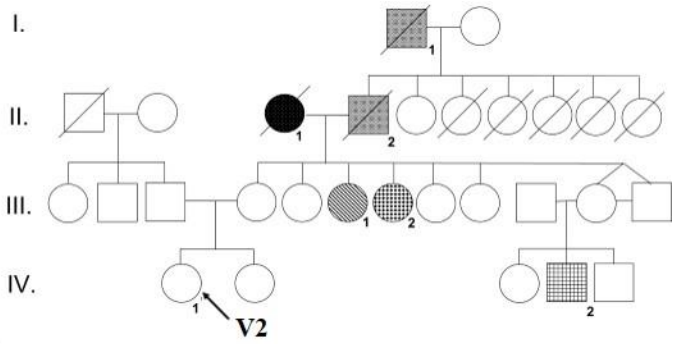
APPENDIX 2

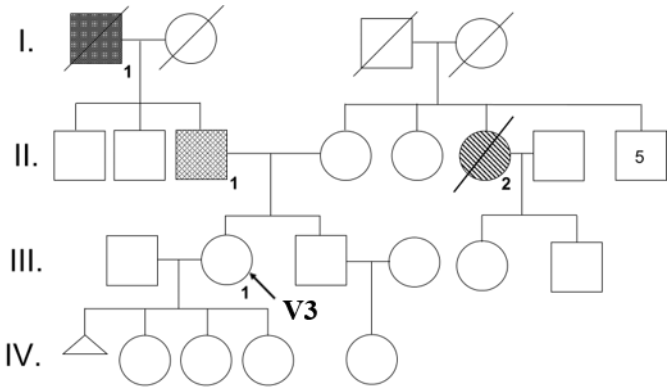
Pedigree Diagrams and family history of samples V1 and V5, V2 and V3.



I.1. Intestinal Cancer; 97y
I.2. Ovarian Cancer; 60y
II.1. Breast Cancer and Sarcoma; 69y
II.2. Breast Cancer; 52y
II.3. Breast Cancer; 52y
II.4. Melanoma and Hepatocellular Carcinoma; 47y
III.1. Breast Cancer; 36y



I.1. Prostate Cancer; ?
II.1. Esophageal Cancer; 75y
II.2. Prostate Cancer; 80y
III.1. Hypophysis Adenoma; 50y
III.2. Tumoral Mass Intraperitoneal; 65y
IV.1. Healthy; Mutation Carrier; 25y
IV.2. Thyroid Carcinoma; 20y



I.1. Unknown Cancer; ?
II.1. Chronic Thrombocytopenia; 69y
II.2. Ovarian Cancer; >50<60y
III.1. Healthy; Mutation Carrier; 39y

APPENDIX 3

Results obtained from sequencing and *in silico* analysis at Ophiomics with the alteration present in each donor and probable outcomes predicted.

ID	V1		V5	V2		V3
Age	36		52	25		39
Cancer	Breast		Breast	Healthy		Healthy
Family History	Yes	Yes	Yes	Yes		Yes
Gene	ATM	BRCA2	ATM	BRCA1		BRCA1
VUS	NM_000051.3:c.4394T>C	NM_000059.3:c.865A>G	NM_000059.3:c.865A>C	NM_000051.3:c.4394T>C	NM_007297.3:c.926A>G	NM_007294.3:c.1067A>G
EBI amino/ genomic	ENSP00000278616.4:p.Leu1465Pro NC_000011.10:g.108289759T>C	ENSP00000369497.3:p.Asn289His NC_000013.11:g.32332343A>C		ENSP00000278616.4:p.Leu1465Pro NC_000011.10:g.108289759T>C	ENSP00000350283.3:p.Gln356Arg 17:g.43094464T>C	ENSP00000418960.2:p.Gln356Arg 17:g.43094464T>C
rs	rs730881391	rs766173		rs730881391	rs1799950	rs1799950
Cons. Type	Missense	Missense		Missense	Missense	Missense
PolyPhen	Probably damaging (0.974)	Benign (0.052)		Probably damaging (0.974)	Probably damaging (0.969)	Possibly Damaging (0.795)
Clin. Signif.	Likely Pathogenic	Benign		Likely Pathogenic	Benign	Benign
Diagnosis	Pathogenic	Benign		Pathogenic	Benign	Benign

ADDITIONAL RESULTS FOR MICRONUCLEI ASSAY

APPENDIX 4

Additional tables with the amount of binucleated cells with micronuclei and total micronuclei and the amount of mononucleated (MONO), binucleated (BN), trinucleated (TRI) and tetranucleated (TETRA).

Sample Name	Dose (Gy)	Total BN	MN Distribution					MNBN	TMN
			0 MN	1 MN	2 MN	3 MN	4 MN		
V1	0 Gy	2000	1992	8	0	0	0	8	8
	2 Gy	2000	1834	126	20	0	0	146	166
V5	0 Gy	2000	1980	18	1	0	0	19	20
	2 Gy	2000	1745	192	27	3	0	222	255
V2	0 Gy	2000	1984	16	0	0	0	16	16
	2 Gy	2000	1753	176	29	3	1	209	247
V3	0 Gy	2000	1991	9	0	0	0	9	9
	2 Gy	2000	1733	209	26	2	0	237	267
V4	0 Gy	2000	1981	15	2	0	0	17	19
	2 Gy	2000	1626	263	43	7	1	314	374
V6	0 Gy	2000	1961	35	2	0	0	37	39
	2 Gy	2000	1629	288	37	3	0	328	371
								1562	1791

Sample Name	Dose (Gy)	Total Cells	MONO	BN	TRI	TETRA	NDI
V1	0 Gy	2000	827	1015	72	86	1.71
	2 Gy	2000	807	973	98	122	1.77
V5	0 Gy	2000	1571	409	9	11	1.23
	2 Gy	2000	1705	266	16	13	1.17
V2	0 Gy	2000	694	1065	104	137	1.84
	2 Gy	2024	744	1043	122	115	1.81
V3	0 Gy	2000	703	1087	79	131	1.82
	2 Gy	2061	924	915	114	108	1.71
V4	0 Gy	2005	1073	774	80	78	1.58
	2 Gy	2000	1180	653	77	90	1.54
V6	0 Gy	2000	764	1105	61	70	1.72
	2 Gy	2000	947	962	38	53	1.60