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RAD51C germline mutation analysis in families with clinical criteria of Li-Fraumeni syndrome

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Dissertação de candidatura ao grau de Mestre em Oncologia – especialização em Oncologia Molecular – submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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ABSTRACT

Li-Fraumeni syndrome was first described in 1969 as a familial syndrome that predisposes to the development of soft tissue sarcomas, breast cancer, leukemia, and other cancers. Germline alterations in the *TP53* gene are, so far, the only known cause of the phenotype of these families. About 70% of the families that meet the classic criteria for Li-Fraumeni syndrome and about 20-40% of the families that meet the Chompret criteria have germline pathogenic variants in the *TP53* gene, and for these families there are specific surveillance programs for early cancer detection. However, there are families that meet the criteria for Li-Fraumeni syndrome that do not present any alterations in the *TP53* gene, so it is important to identify other genes that may be associated with this syndrome. The *RAD51C* gene, which encodes a protein that plays different roles in several phases of homologous recombination, Fanconi anemia and cell cycle arrest, emerged as a possible candidate after the identification of a pathogenic variant in a family that meet the classic criteria for Li-Fraumeni.

The aims of this work were identification of germline variants in the *RAD51C* gene in families that complied with the classic or Chompret criteria for Li-Fraumeni syndrome (negative for non-benign *TP53* variants), identification of other candidate genes, and evaluation of the possible association of these variants with the phenotype of the families.

A total of 111 genomic DNA samples from patients whose families met the criteria for *TP53* mutation testing for molecular diagnosis of LFS were evaluated. Gastric cancer was included in the tumor spectrum of this syndrome due to the high incidence of this cancer in Portugal. Screening for *RAD51C* germline variants was performed by next generation sequencing (NGS) in 61 samples and by Sanger sequencing in 50.

Of the 111 families analyzed, three presented a heterozygous germline pathogenic variant in the *RAD51C* gene: one family with the c.709C>T, p.(Arg237Ter), variant, and two families with the c.890_899del, p.(Leu297HisfsTer2), variant. The prevalence of germline pathogenic variants in this gene in our series was 2.7%. *RAD51C* pathogenic variants were identified in four patients with soft tissue sarcoma in the three families. Interestingly, two of the families were only included in this study due to the addition of gastric cancer as a tumor of the Li-Fraumeni spectrum, as we had also previously seen for germline *TP53* mutations. These data corroborate the importance of including this type of cancer in the spectrum of this syndrome, mostly in countries with high incidence of gastric cancer, as is the case of Portugal. Pathogenic germline variants in the *RAD51C* gene confer an increased risk for the development of ovarian cancer, but the risk for other

neoplasms is not yet well established. Further studies will be needed to better define the risk for other cancers and thus allow referring these patients to specific surveillance programs and, possibly, to new therapeutic options.

Additionally, four of 61 samples (59 without *RAD51C* variants) analyzed by NGS presented potentially deleterious variants in other genes, namely, *FANCA*, *CHEK2*, *BUB1B* and *FANCM*. Additional studies will be required to determine the clinical relevance of these variants.

RESUMO

A síndrome de Li-Fraumeni foi inicialmente descrita em 1969 como uma síndrome familiar que predispõe para o desenvolvimento de sarcomas das partes moles, cancro da mama, leucemia e outros tipos de cancro. Alterações germinativas no gene *TP53* são, até agora, a única causa conhecida do fenótipo apresentado por estas famílias. Cerca de 70% das famílias que cumprem os critérios clássicos de Li-Fraumeni e cerca de 20-40% das famílias que cumprem os critérios de Chompret apresentam variantes germinativas no gene *TP53*, havendo programas específicos de vigilância e diagnóstico precoce para estas famílias. No entanto, existem famílias que cumprem os critérios clínicos para a síndrome de Li-Fraumeni que não apresentam qualquer alteração no gene *TP53*, sendo assim importante a identificação de outros genes que poderão estar associados com esta síndrome. O gene *RAD51C*, que codifica uma proteína com papéis importantes nas diversas fases da recombinação homóloga, na Anemia de Fanconi e na regulação do ciclo celular, surgiu como um possível candidato após a identificação de uma variante patogénica neste gene numa família que cumpria os critérios clássicos de Li-Fraumeni.

Os objetivos deste trabalho foram a identificação de variantes germinativas no gene *RAD51C* em famílias que cumprem os critérios clínicos para Li-Fraumeni (sem variantes germinativas no gene *TP53*), a identificação de variantes germinativas noutros genes e a avaliação da possível associação destas variantes com o fenótipo destas famílias.

Foram testadas 111 amostras de DNA genómico de indivíduos cujas famílias cumprem os critérios para teste do gene *TP53* para diagnóstico molecular da síndrome de Li-Fraumeni, sendo que o cancro gástrico foi também incluído no espectro de tumores desta síndrome devido à elevada incidência desta neoplasia em Portugal, conforme mostramos anteriormente para mutações germinativas do gene *TP53*. A pesquisa de variantes germinativas no gene *RAD51C* foi realizada por sequenciação de nova geração (NGS) em 61 amostras e por sequenciação de Sanger em 50.

Das 111 famílias analisadas, três são portadoras de variantes germinativas patogénicas em heterozigotia no gene *RAD51C*: uma família com a variante c.709C>T, p.(Arg237Ter), e duas famílias com a variante c.890_899del, p.(Leu297HisfsTer2). A prevalência de variantes patogénicas germinativas neste gene na nossa série foi de 2,7%. Foram identificadas variantes patogénicas em quatro pacientes com sarcomas das partes moles nas três famílias. Duas destas três famílias só foram inseridas neste estudo devido à inclusão do cancro gástrico como um tumor do espectro de Li-Fraumeni. Este

resultado corrobora a importância de incluir esta neoplasia no espectro desta síndrome em países com alta incidência de cancro gástrico, como é o caso de Portugal. Variantes germinativas patogénicas no gene *RAD51C* conferem risco aumentado para o desenvolvimento de cancro do ovário, mas o risco para outras neoplasias não está ainda bem estabelecido. Serão necessários mais estudos para melhor definir o risco para outros cancros e assim poder encaminhar os portadores destas alterações para programas específicos de vigilância e, possivelmente, para novas opções terapêuticas.

Adicionalmente, quatro das 61 amostras (59 sem variantes no gene *RAD51C*) analisadas por NGS apresentaram variantes provavelmente deletérias nos genes *FANCA*, *CHEK2, BUB1B* e *FANCM*. Serão necessários estudos adicionais para determinar a relevância clínica destas variantes.

LIST OF ABBREVIATIONS

ATM – Serine-protein kinase ATM

ATP – Adenosine triphosphate

ATR – Serine/threonine kinase ATR

BARD1 – BRCA1 associated RING domain 1

BIR – Break-induced replication

BLM – Bloom syndrome RecQ like helicase

CDK – Cyclin-dependent kinases

cDNA – Complementary DNA

CHK1 – Serine/threonine-protein kinase Chk1

CHK2 – Serine/threonine-protein kinase Chk2

CNS – Central nervous system

CtIP – DNA endonuclease RBBP8

ddNTP – Dideoxynucleotide

DEB – Diepoxybutane

DNA – Deoxyribonucleic acid

DNA2 – DNA replication ATP-dependent helicase

dNTP – Deoxynucleoside triphosphate

DSB – Double strand break

dsDNA – Double stranded DNA

DSS1 – 26S proteasome complex subunit

dHJ – double Holliday Junction

EXO1 – Exonuclease 1

FA – Fanconi anemia

FAAP20 – Fanconi anemia core complex-associated protein 20

FAAP100 – Fanconi anemia core complex-associated protein 100

FDA – Food and Drugs Administration

gDNA – Genomic DNA

GEN1 – Flap endonuclease GEN homolog 1

HR – Homologous recombination

IARC – International Agency for Research on Cancer

ICL – Interstrand cross-link

IFAR – International Fanconi Anemia Registration

IGV – Integrative Genomics Viewer

IPO – Instituto Português de Oncologia

IR – Ionizing radiation

LFL – Li-Fraumeni like

LFS – Li-Fraumeni syndrome

LOH – Loss of heterozygosity

MLPA – Multiplex ligation-dependent probe amplification

MMC – Mitomycin C

MMS22L – Protein MMS22-like

MRE11 – Double-strand break repair protein MRE11

MRI – Magnetic resonance imaging

MRN – MRN complex-interacting protein

MUS81 – MUS81 structure-specific endonuclease subunit

MVA – Mosaic variegated aneuploidy

NBN – Nibrin

NCCN – National Comprehensive Cancer Network

NER – Nucleotide excision repair

NGS – Next-generation sequencing

p53 – Tumor suppressor protein p53

PARP – Poly (ADP-ribose) polymerase family

PCR – Polymerase chain reaction

RAD50 – DNA repair protein RAD50

RAD54L – DNA repair and recombination protein RAD54-like

RECQ1 – ATP-dependent DNA helicase Q1

RETL1 – Regulator of telomerase elongation helicase 1

RPA – Replicated protein A

SAC – Spindle assembly checkpoint

SDSA – Synthesis-dependent strand annealing

SLX1 – SLX1 homolog A, structure-specific endonuclease subunit

SPRI – Solid Phase Reversible Immobilization

SSA – Single strand annealing

SSB – Single strand break

ssDNA – Single stranded DNA

TONSL – Tonsoku-like protein

VUS – Variants of uncertain significance

GENES

AIP – Aryl hydrocarbon receptor interacting protein

ALK – ALK receptor tyrosine kinase

APC – APC, WNT signaling pathway regulator

ATM – ATM serine/threonine kinase

BAP1 – BRCA1 associated protein 1

BAX – BCL2 associated X, apoptosis regulator

BCL10 – B cell CLL/ lymphoma 10

BLM – Bloom syndrome RecQ like helicase

BMPR1A – Bone morphogenetic protein receptor type 1A

BRCA1 – BRCA1, DNA repair associated

BRCA2 – BRCA2, DNA repair associated

BRIP1 – BRCA1 interacting protein C-terminal helicase 1

BUB1B – BUB1 mitotic checkpoint serine/threonine kinase B

CDC73 – Cell division cycle 73

CDH1 – Cadherin 1

CDK4 – Cyclin dependent kinase 4

CDKN1C – Cyclin dependent kinase inhibitor 1C

CDKN2A – Cyclin dependent kinase inhibitor 2A

CEBPA – CCAAT enhancer binding protein alpha

CEP57 – Centrosomal protein 57

CHEK1 – Checkpoint kinase 1

CHEK2 – Checkpoint kinase 2

CYLD – CYLD lysine 63 deubiquitinase

DDB2 – Damage specific DNA binding protein 2

DICER1 – Dicer 1, ribonuclease III

DIS3L2 – DIS3 like 3'-5' exoribonuclease 2

EGFR – Epidermal growth factor receptor

EPCAM – Epithelial cell adhesion molecule

ERCC2 – ERCC excision repair 2, TFIIH core complex helicase subunit

ERCC3 – ERCC excision repair 3, TFIIH core complex helicase subunit

ERCC4 – ERCC excision repair 4, endonuclease catalytic subunit

ERCC5 – ERCC excision repair 5, endonuclease

EXT1 – Exostosin glycosyltransferase 1

EXT2 – Exostosin glycosyltransferase 2

EZH2 – Enhancer of zeste 2 polycomb repressive complex 2 subunit

FANCA – FA complementation group A

FANCB – FA complementation group B

FANCC – FA complementation group C

FANCD1 – FA complementation group D1

FANCD2 – FA complementation group D2

FANCE – FA complementation group E

FANCF – FA complementation group F

FANCG – FA complementation group G

FANCI – FA complementation group I

FANCJ – FA complementation group J

FANCL – FA complementation group L

FANCM – FA complementation group M

FANCN – FA complementation group N

FANCO – FA complementation group O

FANCP – FA complementation group P

FANCQ – FA complementation group Q

FANCR – FA complementation group R

FANCS – FA complementation group S

FANCT – FA complementation group T

FANCU – FA complementation group U

FANCV – FA complementation group V

FANCW – FA complementation group W

FH – Fumarate hydratase

FLCN – Folliculin

GATA2 – GATA binding protein 2

GPC3 – Glypican 3

HNF1A – HNF1 homeobox A

HRAS – HRas proto-oncogene, GTPase

KIT – KIT proto-oncogene receptor tyrosine kinase

MAX – MYC associated factor X

MEN1 – Menin 1

MET – MET proto-oncogene, receptor tyrosine kinase

MLH1 – MutL homolog 1

MSH2 – MutS homolog 2

MSH6 – MutS homolog 6

MUTYH –MutY DNA glycosylase

NBN – Nibrin

NF1 – Neurofibromin 1

NF2 – Neurofibromin 2

NSD1 – Nuclear receptor binding SET domain protein 1

PALB2 – Partner and localizer of BRCA2

PHOX2B – Paired like homeobox 2b

PMS1 – PMS1 homolog 1, mismatch repair system component

PMS2 – PMS1 homolog 2, mismatch repair system component

POT1 – Protection of telomeres 1

PRF1 – Perforin 1

PRKAR1A – Protein kinase cAMP-dependent type I regulatory subunit alpha

PTCH1 – Patched 1

PTEN – Phosphatase and tensin homolog

RAD51 – RAD51 recombinase

RAD51B – RAD51 paralog B

RAD51C – RAD51 paralog C

RAD51D – RAD51 paralog D

RB1 – RB transcriptional corepressor 1

RECQL4 – RecQ like helicase 4

RET – Ret proto-oncogene

RHBDF2 – Rhomboid 5 homolog 2

RUNX1 – Runt related transcription factor 1

SBDS – SBDS, ribosome maturation factor

SDHAF2 – Succinate dehydrogenase complex assembly factor 2

SDHB – Succinate dehydrogenase complex iron sulfur subunit B

SDHC – Succinate dehydrogenase complex subunit C

SDHD – succinate dehydrogenase complex subunit D

SLX4 – SLX4 structure-specific endonuclease subunit

SMAD4 – SMAD family member 4

SMARCB1 – SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1

STK11 – Serine/threonine kinase 11

SUFU – SUFU negative regulator of hedgehog signaling

TMEM127 – Transmembrane protein 127

TP53 – Tumor protein p53

TP63 – Tumor protein p63

TSC1 – TSC complex subunit 1

TSC2 – TSC complex subunit 2

VHL – Von Hippel-Lindau tumor suppressor

WRN – Werner syndrome RecQ like helicase

WT1 – Wilms tumor 1

XPA – XPA, DNA damage recognition and repair factor

XPC – XPC complex subunit, DNA damage recognition and repair factor

XRCC2 – X-ray repair cross complementing 2

XRCC3 – X-ray repair cross complementing 3

I. INTRODUCTION

Since Knudson's hypothesis was proposed in retinoblastoma in 1971 (Knudson, 1971), many other germline mutations have been associated with cancer susceptibility. Knudson proposed that a mutation in both alleles (the "two-hit hypothesis") in a tumor suppressor gene, as is the case of the *RB1* gene in retinoblastoma, is required to induce the carcinogenesis process. According to this theory, an individual with an heterozygotic germline mutation (*de novo* or inherited from one of the parents) in a tumor suppressor gene, has a high probability to acquire a second hit in the other allele either by mutation or epigenetic event, which will lead to its inactivation and consequently to the development of cancer (Balmain *et al.*, 2003). Although less than 10% of all cancers are attributed to deleterious germline variants, this knowledge contributed to the development of different programs of screening and surveillance and to specific treatments in patients with cancer susceptibility (Balmain *et al.*, 2003).

I.1 Li-Fraumeni syndrome

A familial syndrome characterized by the occurrence of soft-tissue sarcomas, breast cancer, leukemia, and other cancers was first proposed by Drs. Frederick Li and Joseph Fraumeni in 1969, after retrospective evaluation of medical reports of several children with rhabdomyosarcoma from 17 institutions, some of them presenting families with high penetrance of malignancies including soft tissue sarcomas, premenopausal breast cancer, leukemia, and brain tumors. At that time they mentioned that this transmission seemed to be associated with a pleiotropic autosomal dominant gene, without ruling out the possibility of some environmental influence on these families like the possibility of a viral infection (Li and Fraumeni, 1969).

The classic criteria for families being classified with Li-Fraumeni Syndrome (LFS), comprise a proband with a sarcoma that has been diagnosed before 45 years of age, with a first-degree relative with any cancer before the age of 45, and another first- or second-degree relative with either any cancer before the age of 45 or a sarcoma at any age (Li *et al.*, 1988).

Malkin and co-workers, in 1990, revealed the presence of mutations in the tumor suppressor gene *TP53* in the families initially reported by Li & Fraumeni (Malkin *et al.*, 1990). At the same time, another report revealed that an inherited *TP53* mutation in a new

LFS family may predispose to increased susceptibility to cancer, reinforcing the role of the *TP53* gene in carcinogenesis (Srivastava *et al.*, 1990).

I.1.1 *TP53* gene and the association with LFS

TP53, also known as the guardian of the genome, is a tumor suppressor gene located in the shorter arm of chromosome 17 (Lane, 1992). As a transcription factor, when activated, the protein p53 stimulates the transcription of many genes involved in several pathways (figure 1; Vogelstein *et al.*, 2000; Blattner *et al.*, 2002).

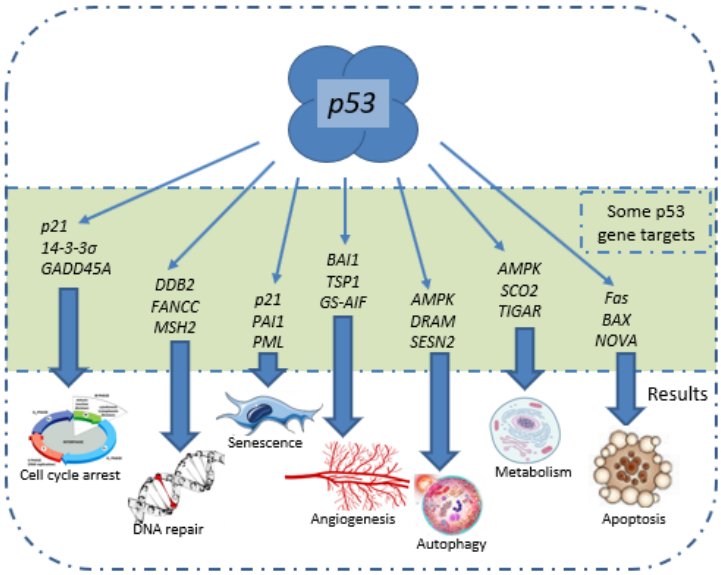

Figure 1 – Cell pathways mediated by p53. The p53 activation will lead to the transcription of different genes (indicated in the green zone of this figure) involved in processes that prevent the development of tumors, for example, cell-cycle arrest, DNA repair, apoptosis, senescence, and modulation of autophagy.

Germline mutations in *TP53* are, so far, the only alteration definitively associated with LFS. A total of 1229 germline variants are described in the International Agency for Research on Cancer (IARC) *TP53* database, gathered from about 347 reports up until the end of June of 2018 (http://p53.iarc.fr/GermlineGrowthStats.aspx).

Although the initial reports from Malkin *et al.* and Srivastava *et al.* identified a complete correlation between the families with the classic criteria for LFS and germline mutations in *TP53* (Malkin *et al.*, 1990; Srivastava *et al.*, 1990), currently only about 70% of the families with classic LFS criteria present a germline mutation in this gene (Olivier *et al.*, 2002; Varley, 2003; Mai *et al.*, 2012). The lifetime risk of cancer in LFS is estimated to

be 73% for males and nearly 100% for females, the latter mainly due to the increased risk for breast cancer (Chompret *et al*. 2000).

I.1.2 LFS tumor distribution

The tumor spectrum of *TP53* mutation carriers is clinically heterogeneous, and table 1 describes the most predominant cancers.

Table 1 – Prevalence of the most frequent tumors observed in *TP53* mutation carriers.

Core Cancer Type	Prevalence in LFS (IARC *TP53* DATABASE)	Prevalence in LFS (cohort studies)
Breast cancer	29.5%	27-31% (Gonzalez *et al.*, 2009b; Id Said *et al.*, 2016)
Soft tissue sarcoma	12.8%	17.8-27% (Gonzalez *et al.*, 2009b; Bougeard *et al.*, 2015; Id Said *et al.*, 2016)
CNS tumor	12.1%	9-13% (Gonzalez *et al.*, 2009b; Bougeard *et al.*, 2015; Id Said *et al.*, 2016)
Adrenocortical carcinoma	10.4%	6-13% (Gonzalez *et al.*, 2009b; Bougeard *et al.*, 2015; Wasserman *et al.*, 2015)
Osteosarcoma	9.6%	13-16% (Gonzalez *et al.*, 2009b; Bougeard *et al.*, 2015; Id Said *et al.*, 2016)
Leukemia	4.3%	2-4% (Gonzalez *et al.*, 2009b; Bougeard *et al.*, 2015)

LFS, Li-Fraumeni syndrome; IARC, International Agency for Research on Cancer; CNS, Central Nervous System.

According to age and gender, the tumor type distribution is variable (see figure 2). The childhood phase, which accounts for 22% of all cancers, is mostly characterized by osteosarcomas and adrenocortical carcinomas. The adrenocortical carcinoma is considered to be a diagnosis signature for LFS since 50-80% of children with sporadic adrenocortical carcinoma present a germline mutation in the *TP53* gene (Libe and Bertherat, 2005; Wasserman *et al.*, 2015). The most prevalent tumors of the CNS at this age (and between the 20-40 years of age) are choroid plexus carcinoma and medulloblastoma. There is a strong association between the occurrence of choroid plexus carcinoma and LFS, as almost 100% of children with choroid plexus carcinoma harbor an alteration in the *TP53* gene (Krutilkova *et al.*, 2005; Gonzalez *et al.*, 2009b). The most prevalent soft tissue sarcoma is rhabdomyosarcoma, which is usually diagnosed before the age of five (Amadou *et al.*, 2018).

The early adulthood phase accounts for 51% of all diagnoses and this percentage is strongly associated with breast cancer in women. About 45-79% of female *TP53* mutation carriers develop breast cancer with the median age of 33 years (Bougeard *et al.*, 2015; Amadou *et al.*, 2018). In this age interval, a series of cancers may be also diagnosed, including soft tissue sarcomas, osteosarcoma, leukemia, CNS tumors, colorectal cancer and lung cancer, all of which are diagnosed at earlier ages than sporadic cancers (Amadou *et al.*, 2018).

In late adulthood, pancreatic and prostate cancer are the most frequently detected cancers. In these cases, the median age of diagnosis is slightly earlier that in sporadic cancers. It seems that in this phase of life the contribution of *TP53* mutations to cancer predisposition is minimal, and that there is a mechanism that may protect cells from the effects of *TP53* inactivation (Amadou *et al.*, 2018).

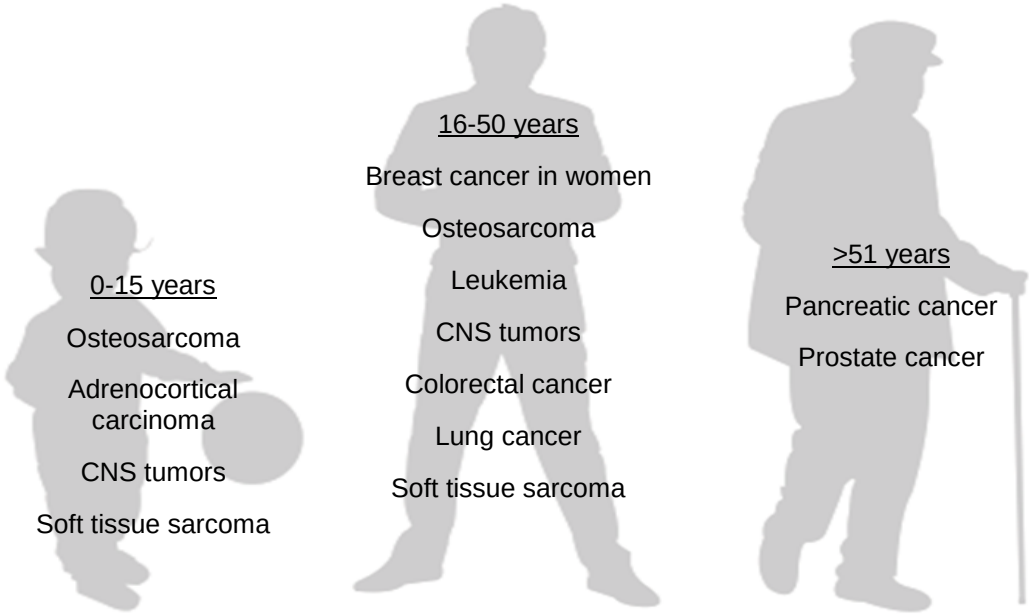

Figure 2 – Tumor spectrum in Li-Fraumeni syndrome according to age.
CNS, Central Nervous System.

Beyond this spectrum, more malignancies were described in several reports, most of them occurring earlier than in sporadic cases, including stomach, renal, head and neck, ovarian cancer, melanoma and lymphoma (Chompret *et al.*, 2000; Varley, 2003; Gonzalez *et al.*, 2009b; Amadou *et al.*, 2018). Gastric cancer has been observed quite frequently in the context of LFS, especially in countries with high incidence of gastric cancer (as is the case of Portugal) and many authors proposed the inclusion of gastric cancer in the LFS tumor spectrum (Keller *et al.*, 2004; Kim *et al.*, 2004; Pinto *et al.*, 2009).

The occurrence of several primary tumors is also observed in LFS patients. The risk of developing a second tumor is three to five times greater than in the general population (Hisada *et al.*, 1998), and it is estimated that the cumulative risk of a second tumor ten years after the first cancer is around 50% in both sexes (Mai *et al.*, 2016). However, it is important to note that carcinogenic effects of previous therapies can also cause some secondary tumors, as radiotherapy and/or chemotherapy that often cause hematopoietic malignancies (Felix *et al.*, 1996; Churpek *et al.*, 2016).

I.1.3 Genetic testing for germline *TP53* variants

To facilitate the identification of individuals or families with LFS and enable *TP53* gene testing and counselling, new criteria were developed by different authors (Eeles, 1995; Birch *et al.*, 1998). In 2009, considering the spectrum of cancers associated with LFS and in order to increase the families tested for *TP53* mutations, the Chompret criteria emerged (Tinat *et al.*, 2009). These new criteria take into account the familial presentation, namely when a proband has, under the age of 46 years, one of the LFS spectrum tumors (premenopausal breast cancer, soft tissue sarcoma, osteosarcoma, CNS tumor, leukemia, lung bronchoalveolar cancer or adrenocortical carcinoma) and also has at least a first or second degree relative with a tumor belonging to the narrow LFS spectrum (except breast cancer if the proband has breast cancer) before the age of 56 years or with multiple tumors. Furthermore, it includes a proband with multiple primary tumors (except in the case of multiple breast cancer), two of them belonging to the LFS tumor spectrum and the first occurring before 46 years of age. The rare tumors are also covered by these criteria, if the patient has an adrenocortical carcinoma or choroid plexus tumor, regardless of age and family background (Tinat *et al.*, 2009). In 2015, these criteria were revised and early-onset breast cancer (before the age of 31 years) was also included (Bougeard *et al.*, 2015). These criteria and the classic LFS criteria are summarized in table 2.

Nowadays, the revised Chompret criteria are the ones used for *TP53* mutation testing, and it is estimated that the sensitivity of these criteria is around 82-95% and that 20-40% of the families fulfilling these criteria present a *TP53* germline mutation (Gonzalez *et al.*, 2009b; Tinat *et al.*, 2009; Mai *et al.*, 2012; Mai *et al.*, 2016).

Table 2 – Classic and Chompret criteria for LFS.

Classic criteria (Li *et al.*, 1988)	• Proband with a sarcoma diagnosed before 45 years old AND a first-degree relative with any cancer before the age of 45 AND a first- or second-degree relative with either any cancer before the age of 45 OR a sarcoma at any age
Revised Chompret criteria (Bougeard *et al.*, 2015)	• Proband with a LFS spectrum tumor* before 46 years old AND a first- or second-degree relative with a LFS spectrum tumor** before the age of 56 OR with multiple tumors • Proband with multiple primary tumors (except multiple breast cancer), two of them belonging to the LFS spectrum and the first occurring before 46 years old. • Proband with adrenocortical carcinoma or choroid plexus tumor regardless family history • Proband with early-onset breast cancer (before the age of 31 years old)

* including premenopausal breast cancer, soft tissue sarcoma, osteosarcoma, central nervous system tumor, leukemia, lung bronchoalveolar cancer or adrenocortical carcinoma
** except breast cancer if proband have breast cancer
LFS, Li-Fraumeni syndrome.

I.1.4 Cancer surveillance in LFS

Early cancer detection greatly increases the chances of successful treatment and the overall survival of patients. Additionally, in hereditary syndromes, the identification of high-risk mutation carriers gives an even greater importance to early detection. The broad spectrum of tumors associated with LFS makes the implementation of a consensual program of screening very challenging, existing several methods approved by different institutes. According to National Comprehensive Cancer Network (NCCN) Guidelines, if a person or family meets classic or Chompret criteria a risk evaluation appointment and germline *TP53* testing must be offered. In the case of a positive result for a pathogenic *TP53* variant, a precise plan of exams is recommended in order to facilitate early detection of cancer. The exams recommended by NCCN Guidelines for individuals with pathogenic *TP53* germline mutations are synthesized in table 3.

Table 3 – NCCN Guidelines for screening of *TP53* mutation carriers.

Breast Cancer	**Other cancers**

• Breast awareness from 18 years old
• Clinical breast examination every 6-12 months, starting at age 20y
  • **20-29y:** breast MRI with contrast
  • **30-75y:** breast MRI with contrast (may consider mammogram or breast tomosynthesis)
  • **>75:** Individual recommendation
	• Comprehensive physical exam including neurologic exam every 6-12 months
	• Colonoscopy and upper endoscopy every 2-5 years starting at 25 years of age or 5 years before the earliest known colon cancer in the family
	• Annual dermatologic exam from 18 years of age
	• Annual whole-body MRI, examining the brain as part of exam or as a separate exam

NCCN; National Comprehensive Cancer Network; MRI, Magnetic Resonance Imaging

It is important to point out that this plan of incessant visits to the clinic also creates psychological, social, and emotional alterations in these patients, promoting in some patients states of anxiety and a reduction in the quality of life (McBride *et al.*, 2014).

In addition to undergoing exams, it is important to discuss with women the option of resorting to prophylactic mastectomy, including its potential benefits but also the psychosocial effects and how it may interfere in the quality of life. For women who have already had a breast cancer but didn't have a bilateral mastectomy, the continuation of the annual breast magnetic resonance imaging (MRI) and mammogram is important, because there is the possibility of a second tumor. Furthermore, in carriers at a reproductive age, it is important to discuss prenatal diagnosis and/or the possibility of assisted reproduction.

In 2016, Villani *et al.* observed a significantly improved survival rate in a group of LFS patients who carried out a rigorous program of screening, observing a five years overall survival of 88.8% *versus* 59.6% in the non-surveillance group (Villani *et al.*, 2016).

I.1.5 Other genes associated with LFS

The lack of molecular explanation for families who fulfilled the LFS clinical criteria but do not have a *TP53* germline mutation, often called Li-Fraumeni like (LFL) families, triggered several studies which suggested that other genes could explain this phenotype.

The *CHEK2* gene has in the past been associated to LFS. In 1999, Bell *et al.* identified germline heterozygous mutations in the *CHEK2* gene in three unrelated families with criteria for LFS and without *TP53* germline mutations. In 2001, Vahteristo *et al.* analyzed 44 Finish families with criteria for LFS or similar phenotypes and found a heterozygous mutation in the *CHEK2* gene in only two families, being this mutation the same previously found by Bell *et al.* in a family with classic LFS criteria (Bell *et al.*, 1999; Vahteristo *et al.*, 2001). However, Bougeard *et al.* did not find any *CHEK2* germline mutation in French LFS families (Bougeard *et al.*, 2001).

More recently, other mutations in genes like *POT1* and *CDKN2A* appear to be associated with the development of cancers that are part of LFS tumor spectrum in patients without *TP53* mutations. *POT1* was associated with the development of cardiac angiosarcomas, while some *CDKN2A* mutations contribute to genetic determinism of sarcomas (Calvete *et al.*, 2015; Jouenne *et al.*, 2017).

Other genes involved in the p53 pathways have been studied in LFL families without *TP53* mutations, namely *BAX, TP63, CHEK1, BCL10,* and *PTEN*. However, so

far, no association has been made between these genes and this syndrome (Stone *et al.*, 1999; Brown *et al.*, 2000; Bougeard *et al.*, 2001; Barlow *et al.*, 2004).

I.1.6 Gene-panel analysis and new candidates

The development of next-generation sequencing (NGS) techniques has greatly accelerated the identification of new genes associated with different syndromes. The possibility of analyzing a gene panel associated with cancer predisposition has allowed the discovery of alterations in genes that were not expected in some types of cancer.

In 2015, a 59-years-old patient with a soft tissue sarcoma was treated at the Portuguese Institute of Oncology of Porto (IPO-Porto) and, after genetic counseling, it was noted that her family fulfilled the classic criteria for LFS, considering her paternal uncle as a proband who had been diagnosed with a sarcoma at 42 years of age, his brother a first-degree relative who had lung cancer at 35 years of age, and also two second-degree relatives with sarcomas, one of them being the patient in question. We screened for *TP53* germline mutations analyzing a panel of genes associated with cancer predisposition by NGS, and no mutations were found in the *TP53* gene. Instead, a pathogenic variant was detected in the *RAD51C* gene. This incidental finding raised the hypothesis that this gene could explain the family phenotype and that could be a new candidate gene predisposing to LFS.

I.2 The *RAD51C* gene

Breast cancer is the most common cancer in women and accounts for approximately 25-30% of all cancers in LFS families (Gonzalez *et al.*, 2009a; Id Said *et al.*, 2016). The major cause of breast cancer predisposition are germline mutations in *BRCA1* and *BRCA2* genes, associated with around 5-10% of all breast cancers (Bonadona *et al.*, 2005). Those genes are associated with the regulation of homologous recombination (HR) pathways and some other genes implicated in this pathway have already been described as linked to breast cancer, such as *PALB2*, *ATM*, *CHEK2* and *BRIP1* (Meijers-Heijboer *et al.*, 2002; Renwick *et al.*, 2006; Seal *et al.*, 2006; Erkko *et al.*, 2007). Meindl *et al.* identified *RAD51C* as another cancer susceptibility gene for breast and ovarian cancer, although the function of RAD51C was not well understood at the date of the publication (Meindl *et al.*, 2010). Nowadays, NCCN Guidelines consider the

RAD51C gene as a moderate-to-high risk susceptibility gene just for ovarian cancer, since several studies have found pathogenic variants in this gene in families with history of ovarian cancer (Pelttari *et al.*, 2011; Loveday *et al.*, 2012; Sopik *et al.*, 2015).

RAD51C is a protein encoded by a 48 Mb gene composed by nine exons located in the long arm of chromosome 17 (17q22). The *RAD51C* gene has a conserved sequence in the Walker A and B domains that confer the ATPase activity (Figure 3) (French *et al.*, 2003).

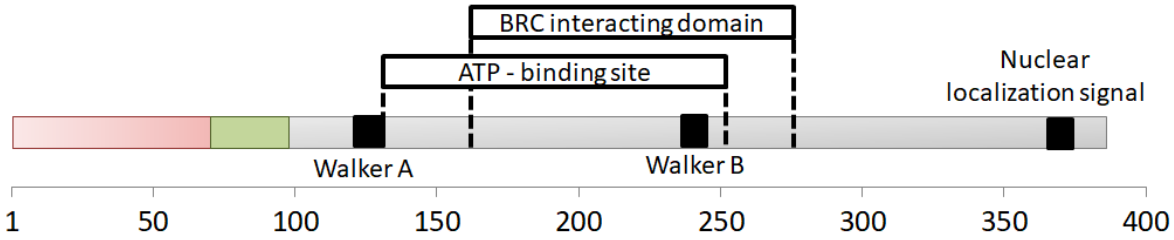

Figure 3 – Schematic representation of the RAD51C protein. Colored in red are represented the N-terminal domain, in green the linker region and in silver the C-terminal domain. The Walker A and B domain are indicated in the figure by a black rectangle, as the nuclear localization signal. The ATP binding site and the BRC interacting domains are also indicated.

RAD51C was identified as part of a family of five proteins (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) known as RAD51 paralogs (Masson *et al.*, 2001). They share approximately 20-30% identity at the amino acid level with RAD51 and it is presumed that the genes encoding these factors have derived from *RAD51* by gene duplication events and those proteins have acquired new functions (Lin *et al.*, 2006). Those five paralogs form two major complexes: the BCDX2 complex (RAD51B-RAD51C-RAD51D-XRCC2) and the CX3 complex (RAD51C-XRCC3) (Thacker, 2005). All paralogs, except XRCC2, have a linker region between the C-terminal and N-terminal domains that allow the interaction between them and the formation of those complexes (Miller *et al.*, 2004). The paralog C is part of both complexes and several studies show that RAD51C participates in the initial and in the late stages of HR (Somyajit *et al.*, 2010).

I.2.1 Homologous recombination

HR is an important cellular process in all organisms, which allows the maintenance of the integrity of the genome by repairing double-strand breaks (DSB) (Takata *et al.*, 1998). The DSB can be triggered by endogenous process or exogenous agents such as ionizing radiation (IR) (Ward, 1988). Some processes like meiosis or meiotic chromosome segregation can lead to a broken DNA replication fork usually resolved by HR (Michel *et al.*, 2004). HR is a slow and mechanistically complex process that involves a large number of enzymes (Kowalczykowski, 2015). This process uses the sister chromatid as a repair template, which allows the restoring of any missing genetic information. HR occurs only in S and G2 phase of the cell cycle due to the availability of the sister chromatids (Orthwein *et al.*, 2015). The HR pathway can be subdivided into different sub-pathways, which significantly differ in terms of mechanisms and enzymes required: the single strand annealing (SSA), the break-induced replication (BIR), the synthesis-dependent strand annealing (SDSA), and the canonical HR (figure 4).

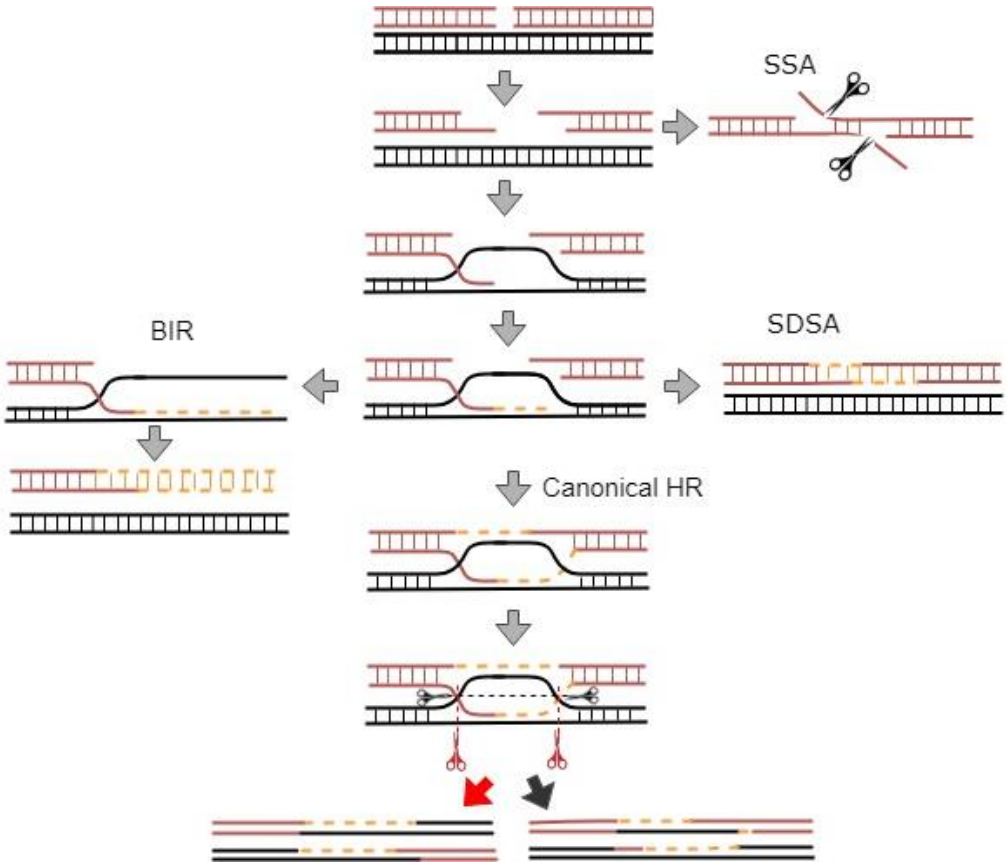

Figure 4 – Overview of homologous recombination. A schematic representation of single strand annealing (SSA), break-induced replication (BIR), synthesis-dependent strand annealing (SDSA), and canonical HR.

I.2.1.1 The mechanism of HR

The first step of HR consists in the processing of the DNA ends, the degradation of the 5'-terminal DNA strand from the break site to generate a long 3'-ssDNA overhang. This process begins with the recognition of DSB by the MRN (MRE11-RAD50-NBN) sensor complex that recognizes and binds to the structure (Uziel *et al.*, 2003). The resection is initiated by endonucleolytic cleavage of the 5'-terminated DNA in the vicinity of DNA end by the MRE11 that require the ATPase activity from RAD50, with NBN and CtIP working as cofactors (Neale *et al.*, 2005; Garcia *et al.*, 2011; Cannavo and Cejka, 2014). This cleavage allows the entry sites for the long-range resection enzymes, EXO1 or DNA2 (Bonetti *et al.*, 2010; Cejka, 2015). While in the formation of long 3'-ssDNA overhang, the single strand will be coated by the RPA protein (figure 5a) (Pinto *et al.*, 2016). RPA protects ssDNA from the action of nucleases and blocks the formation of secondary structures (Wold, 1997).

In SSA, or RAD51-independent HR, the DNA end resection occurs until revealing a repetitive DNA sequence, followed by the annealing of the two resected strands generating a stable complex between them (Ivanov *et al.*, 1996; Shinohara *et al.*, 1998). This sub-pathway is restricted to cases in which the DSB is flanked by two repeats sequences and it is considered a mutagenic repair process since it causes the deletion of the DNA sequence between the two repeats (Ranjha *et al.*, 2018).

In the remaining sub-pathways, the key recombination protein RAD51 replaces the RPA that was initially coating 3'-ssDNA (figure 5). The nucleoprotein filament formed by RAD51 and ssDNA is also called presynaptic filament (Benson *et al.*, 1994; Sugiyama *et al.*, 1997). RPA has more affinity to ssDNA than RAD51 and this replacement has to be moderated by a recombination moderator protein and in high eukaryotes, including humans, the main mediator is the BRCA2 (Yang *et al.*, 2002; Jensen *et al.*, 2010). The BRCA2 human protein has eight conserved motifs of about 35 amino acids (BRC repeats) that have the ability to bind directly to RAD51. Those different BRC motifs present distinctive functions as recruitment of RAD51 by directly binding, promotion of the ssDNA binding of RAD51, stabilizing the ligation between them and inhibition of the RAD51 ligation to the dsDNA (figure 5b). Also, the initial displacement of RPA is performed by BRCA2 and DSS1, a direct partner of BRCA2 (Jensen *et al.*, 2010). PALB2 also interacts with BRCA2 and BRCA1, localizes the BRCA2 in the DSB and promotes the formation of the RAD51 filament (Sy *et al.*, 2009; Buisson *et al.*, 2010). Additional proteins interact in this process facilitating and promoting the RAD51 nucleoprotein filament assembly, like

RAD54L, the MMS22L-TONSL complex and the RAD51 paralogs (Wolner and Peterson, 2005; Qing *et al.*, 2011; Piwko *et al.*, 2016).

When the presynaptic filament is formed (figure 5c), the next step is to look for the homologous sequence in the sister chromatid that will serve as template. The homology search mechanism is still not yet well defined, but it is proposed that indiscriminately the presynaptic filament will make multiple contacts with different DNA duplexes creating a relatively quick search in the genome (Forget and Kowalczykowski, 2012; Renkawitz *et al.*, 2014). After the recognition of the sequence, the presynaptic filament invades the duplex DNA and, recently, the complex BRCA1-BARD1 was associated with this invasion by interacting directly with RAD51 (figure 5d) (Zhao *et al.*, 2017).

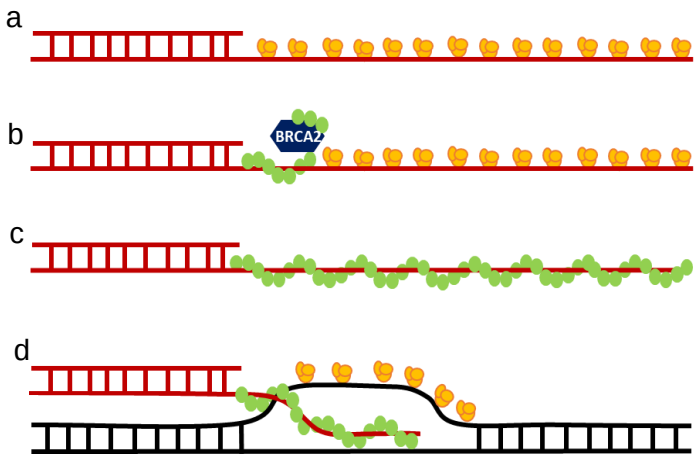

Figure 5 – Presynaptic filament formation and the invasion of template dsDNA. a) 3'-ssDNA coated by RPA; b) BRCA2 exchanges RPA for RAD51; c) nucleoprotein filament or presynaptic filament; d) invasion of template dsDNA.

The strand invasion forms a D-loop structure that will be stabilized by RPA preventing the formation of other additional structures and the RAD51 will be removed from dsDNA by RAD54 (Solinger *et al.*, 2002). The synthesis of DNA during recombination is catalyzed by polymerase δ and polymerase ε (Li *et al.*, 2009; Wilson *et al.*, 2013). The process downstream to this pathway can have different outcomes according to the sub-pathways used: BIR, SDSA or canonical HR (figure 4).

In the BIR pathway, the synthesis of DNA proceeds throughout the chromosome arm, copying the sequence since the end of the chromosome (Llorente *et al.*, 2008). This sub-pathway occurs at an elevated cost of mutagenesis and its activated only when the cell has no possible alternative, such as in the absence of the second DNA end. The BIR

pathway is important in the telomere region maintenance in the absence of telomerase (Sakofsky and Malkova, 2017).

In SDSA, the D-loop structure is disrupted and the annealing occurs between the two broken DNA strands, while in the canonical HR the D-loop is stabilized and proceeds to the arrest of the second DNA end, leading to a “double” or “complement-stabilized” D-loop (Ranjha *et al.*, 2018). The balance between these two pathways will be determined by the stability of the D-loop and by the proteins that are available and that will bind to the structure. BLM, RECQ1, and RETL1 are some of the proteins involved in the disruption of the D-loop promoting the SDSA (Bugreev *et al.*, 2007; Barber *et al.*, 2008; Daley *et al.*, 2013).

The regulation between SDSA and canonical HR is important because the final genomic result varies according to the different sub-pathways. The canonical HR is the only sub-pathway that can generate a crossover recombination. A crossover is the exchange of DNA between the two homologous *loci* of sister chromatids. While in terms of genetic evolution this process allows the increase of the species variability, in case of cancer this process is sometimes associated with the loss of heterozygosity (LOH) (Matos and West, 2014).

In canonical HR, following the capture of the second end, the DNA synthesis takes place and ligation forms an intermediated structure called double Holliday junctions (dHJs) (Duckett *et al.*, 1988). Several enzymes and nucleases are involved in the resolution of the dHJ, like MUS81, SLX1/4 complex and the GEN1 nuclease. This resolution can lead to crossover or non-crossover recombination products (Ranjha *et al.*, 2018).

I.2.1.2 RAD51C in the early and late stages of HR

Although a precise molecular mechanism of RAD51 paralogs action is not well established, the important role in HR is already reported by different experiments (Liu *et al.*, 1998; Takata *et al.*, 2001; French *et al.*, 2002; Godthelp *et al.*, 2002). Takata *et al*. in 2001 was one of the first to report the role of RAD51 paralogs in HR using chicken DT40 B-lymphocytes mutants for one of each RAD51 paralogs. They obtained cell lines sensitive for DNA damaging agents, like IR, mitomycin C (MMC), cisplatin and camptothecin. After the contact with these agents, the cell lines developed spontaneous chromosome aberrations, abnormal centromere numbers and had also a reduction of sister chromatid exchanges and defective RAD51 foci formation (Takata *et al.*, 2001). The

authors compared these phenotypes with other experiments in which *BRCA2* was mutated and reported similar phenotype alterations, suggesting a role of RAD51 paralogs in assisting the RAD51 assembly at sites of DSB DNA (Patel *et al.*, 1998; Yuan *et al.*, 1999; Yu *et al.*, 2000; Rodrigue *et al.*, 2006).

In other reports, the BCDX2 complex (RAD51B-RAD51C-RAD51D-XRCC2) is shown to be able to bind single and double DNA strands and has the ability to hydrolyze ATP (Braybrooke *et al.*, 2000; Sigurdsson *et al.*, 2001). The RAD51C and RAD51B also form a stable complex capable of interacting with RPA and RAD51, functioning as a mediator in RPA exchange for RAD51 during the formation of the nucleoprotein filament (Sigurdsson *et al.*, 2001; Rodrigue *et al.*, 2006). Moreover the CX3 complex (RAD51C-XRCC3) and the XRCC2-RAD51D are associated with the early phase of HR, exhibiting the capacity of homologous pairing (Kurumizaka *et al.*, 2001; Kurumizaka *et al.*, 2002).

Although none of the RAD51 paralogs has nuclease activity, the CX3 complex was for many years associated with the ability of binding specifically with HR double junctions promoting the HJ branch migration and resolution (Yokoyama *et al.*, 2003; Liu *et al.*, 2004; Yokoyama *et al.*, 2004; Sharan and Kuznetsov, 2007). However, the identification of GEN1 as the major nuclease responsible for resolution of dHJs raises some questions about the role of RAD51C in this process. Further studies are needed to improve our knowledge about the RAD51C in all sub-pathways of the HR (Ip *et al.*, 2008).

I.2.2 DNA interstrand cross-link (ICL)

In the reports of Takata *et al.* and Liu *et al.*, the lack of RAD51 paralogs leads to chromosome aberrations, defective gene targeting and genome instability in presence of MMC, diepoxybutane (DEB) and cisplatin (Liu *et al.*, 1998; Takata *et al.*, 2001). This kind of phenotype is usually observed in Fanconi Anemia (FA) and is caused by errors in resolving the DNA ICLs induced by these agents (Joenje and Patel, 2001). DNA ICLs are the most deleterious DNA lesions since they block the replication and the transcription of DNA.

FA is a genetic disorder that, so far, has 22 genetic subtypes identified caused by mutations in different genes (see table 4).

Table 4 – Fanconi Anemia genes.

FA GENE	ALTERNATIVE NAME	ESTABLISHED MOLECULAR FUNCTION
FANCA	*FANCH*	Belongs to the multisubunit FA complex
FANCB	*FAAP95*	Belongs to the multisubunit FA complex
FANCC	-	Belongs to the multisubunit FA complex
FANCD1	*BRCA2*	Effector Recruitment in DNA repair
FANCD2	-	Forms a heterodimer with FANCI Is monoubiquitylated by the multisubunit FA complex Recruits the DNA repair proteins
FANCE	-	Belongs to the multisubunit FA complex
FANCF	-	Belongs to the multisubunit FA complex
FANCG	*XRCC9*	Belongs to the multisubunit FA complex
FANCI	*KIAA1794*	Forms a heterodimer with FANCD1 Is monoubiquitylated by the multisubunit FA complex Recruits the DNA repair proteins
FANCJ	*BRIP1, BACH1*	DNA helicase essential for BRCA1 dependent DNA repair
FANCL	*PHF9*	Ubiquitin ligase protein that mediates monoubiquitination of FANCD2 and FANCI
FANCM	-	Belongs to the multisubunit FA complex DNA helicase involved in repair Hollyday junctions
FANCN	*PALB2*	Ability to recruit BRCA2 and RAD51 to DNA breaks
FANCO	***RAD51C, RAD51L2***	**Essential for the HR pathway of DNA repair**
FANCP	*SLX4*	Resolution of DNA secondary structures generated during DNA repair and recombination like Holliday junctions Interact with several nucleases, including ERCC4
FANCQ	*ERCC4, XPF*	DNA repair endonuclease
FANCR	*RAD51*	Essential for the HR pathway of DNA repair
FANCS	*BRCA1*	Essential for the HR pathway of DNA repair
FANCT	*UBE2T*	E2 ubiquitin ligase that in association with FANCL catalyze the monoubiquitination of FANCD2 and FANCI
FANCU	*XRCC2*	Essential for the HR pathway of DNA repair
FANCV	*MAD2L2, REV7*	Translesion DNA synthesis
FANCW	*RFWD3*	Promotes ATR activation and HR

FA, Fanconi anemia; HR, homologous recombination

This disease is characterized by bone marrow failure, developmental anomalies and susceptibility to cancer (Lobitz and Velleuer, 2006). The diagnosis of FA is made by a cytogenetic analysis of chromosome breakage of lymphocytes cells from the patients in the presence of ICL agents, like MMC or DEB (Shimamura *et al.*, 2002).

In cells during the G1 phase of cell cycle, the nucleotide excision repair (NER) can remove the ICLs (Deans and West, 2011). However, in S and G2 phase these errors lead to blockage of the replication fork and the initial steps of DNA ICLs resolution are the key function of the FA pathway, being the monoubiquitination of FANCD2 and FANCI heterodimer the process that regulates the DNA damage response. After DNA damage, by post-translational modifications of FA proteins, the core complex is formed through the assembly of at least nine FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCT, FANCM and FANCL) and two accessory proteins (FAAP20 and FAAP100) (Garcia-Higuera *et al.*, 2001; Smogorzewska *et al.*, 2007). This core complex will be able to monoubiquitylate the FANCD2-FANCI heterodimer (Nalepa and Clapp, 2018). The modified FANCD2-FANCI complex will be withheld in the chromatin and will make the recruitment of nucleases and polymerases that are required for the repair process (see figure 6) (Yamamoto *et al.*, 2011; Lachaud *et al.*, 2014). In this process the proteins from HR, namely the RAD51C, mediate the stabilization of the replication fork (Sobeck *et al.*, 2006; Schwab *et al.*, 2015; Nalepa and Clapp, 2018).

FANCO (or *RAD51C*) is one of the FA genes participating in early HR and mutations in this gene were associated in a family with a characteristic phenotype of FA (Vaz *et al.*, 2010). Other studies identified monoallelic mutations in *RAD51C* causing ovarian and breast cancer, although no hematological anomalies were observed (Meindl *et al.*, 2010; Somyajit *et al.*, 2010; Somyajit *et al.*, 2012).

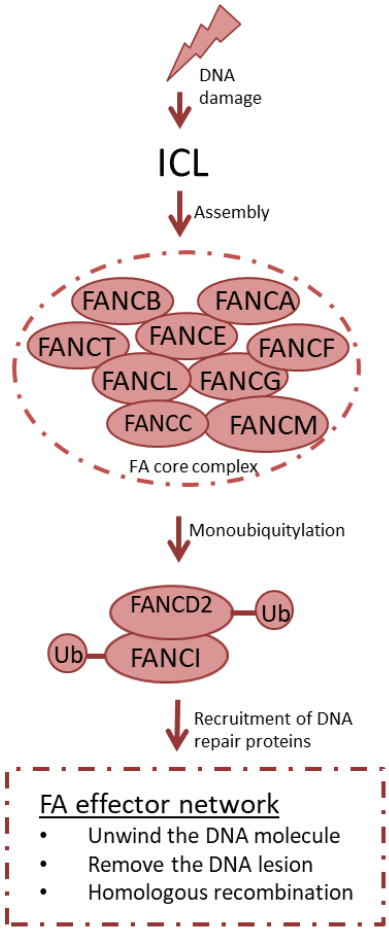

Figure 6 – The Fanconi Anemia pathway in response of DNA damage. In phase S and G2 after the DNA damage and formation of an ICL occurs the assembly of the FA core complex that will trigger the monoubiquitylation of FANCD2-FANCI complex. This complex will coordinate the DNA damage response.

I.2.3 DNA damage response and cell cycle

The response to DNA damage involves a complex network that coordinates the DNA damage repair by recruiting the machinery to sites of damage and coordinates the progression of cell in the cell cycle, activating the checkpoints and inducing cell arrest or apoptosis.

The ATM and the ATR kinases are principal players in the DNA damage signaling mechanism, phosphorylating the subtracts required for cell cycle control and activation of DNA repair pathways (Shiloh, 2003; Bartek and Lukas, 2007). During the interphase, the recruitment of MRN complex triggers ATM phosphorylation (Petrini and Stracker, 2003). This activation will initiate a phospho-signaling cascade, leading to phosphorylation of the checkpoint effector kinase CHK2 (Matsuoka *et al.*, 1998; Buscemi *et al.*, 2001). In parallel,

the MRN complex will recruit the machinery to generate the 3'-ssDNA overhangs that will be coated by RPA. The RPA-coated ssDNA tails trigger the ATR-dependent signaling, activating the CHK1 checkpoint kinase (Zou and Elledge, 2003; Garcia-Muse and Boulton, 2005; Adams *et al.*, 2006). The activation of CHK1 and CHK2 will inhibit the cyclin-dependent kinases (CDK), and consequently impede the progression of the cell cycle for mitosis (West, 2003).

Badie *et al.* observed the accumulation of RAD51C into sites of DNA damage before and independently of the assembly of RAD51 into ssDNA. They proved that RAD51C was involved in the activation of CHK2, promoting its phosphorylation in phase S and G2 (see figure 7). Although the mechanism is not totally understood, the authors thought that RAD51C could act as a recruitment mediator of checkpoint kinases or like a transduction or amplifier of CHK2 phosphorylation (Badie *et al.*, 2009). Badie *et al.* showed that the depletion of CHK2 causes the progression of cells into phase G2/M when exposed to IR similar to the results of RAD51C depletion. After the induction of DSB, by IR, they observed an increase of cells entering mitosis, the accumulation of endogenous DNA damage and mitosis with unrepaired DSBs (Badie *et al.*, 2009; Somyajit *et al.*, 2010).

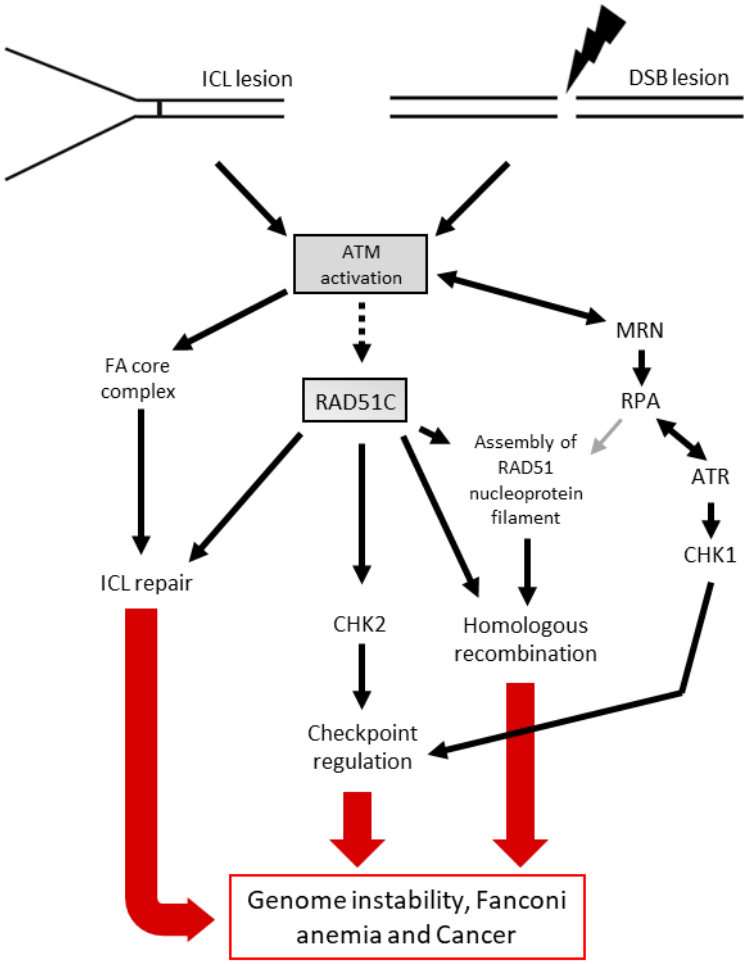

Figure 7 – The role of *RAD51C* in damage response. After an ICL or a DSB, ATM activation occurs that will activate the FA pathway and HR. Besides the intervention in both pathways, RAD51C is also implicated in the activation of CHK2 and in the regulation of checkpoints. Alterations on RAD51C, represented in red arrows, will lead to genome instability, Fanconi Anemia and cancer.

The participation of the C paralog of RAD51 in several steps of HR, in the FA pathway and in regulation of DNA damage response, along with the identification of a pathogenic variant in a LFS family, lead us to hypothesize that germline pathogenic variants in *RAD51C* (or even in other genes involved in these pathways) may be responsible for increased susceptibility for developing cancer in families that comply with the clinical LFS criteria but do not have germline pathogenic *TP53* variants.

II. AIMS

II. AIMS

The general aim of this study was to search for germline variants in the *RAD51C* gene in families with clinical criteria for LFS molecular testing without *TP53* germline variants.

The specific aims of this study were:

- ✓ To identify germline variants in the *RAD51C* gene in a series of families that complied with the classic criteria or the Chompret criteria for LFS molecular testing and that were negative for non-benign *TP53* variants;
- ✓ To identify other candidate genes involved in HR or FA pathways;
- ✓ To discuss possible associations of gene variants with the phenotype.

III. MATERIALS AND METHODS

III.1 Patients and sample collection

A consecutive series of DNA samples was collected from 61 patients that met the classic or Chompret criteria for analysis by gene-panel NGS. Those patients had been referred to the Genetics Department of IPO-Porto between October 2014 and October 2016 for *TP53* germline variant analysis. All patients with non-benign *TP53* germline variants were excluded from this study. In this series, only one family had the classical criteria for LFS, whereas the remaining 60 families studied met the Chompret criteria (the criteria were applied to each family as a whole and not necessarily to the proband). Furthermore, gastric cancer was included in the LFS tumor spectrum due to the high incidence of this disease in Portugal, as discussed previously by our group (Pinto *et al.*, 2009).

Another series of DNA samples from 50 patients that met the classic and Chompret criteria were retrospectively selected for analysis by Sanger sequencing. Those patients had been referred to the Genetics Department of IPO-Porto between April 2000 and September 2014 for *TP53* germline variant analysis. The criteria used for this selection was the same as that of the series analyzed by NGS and one family met the classic criteria for LFS and the remaining 49 met the Chompret criteria.

Whenever possible, the samples from family members of the index patients were also studied.

III.2 Gene-panel analysis by NGS

A total of 61 DNA samples were analyzed by NGS using TruSight Cancer [Illumina, San Diego, CA, USA] that includes a panel of 94 genes associated with cancer predisposition (table 5) for screening of germline variants. For this purpose, the Nextera DNA transposome [Illumina] was used to convert genomic DNA (gDNA) into adapter-tagged indexed libraries. Approximately 50 ng of gDNA were used in the tagmentation process, which involves simultaneous fragmentation and adapter tagging of gDNA followed by adapter ligation.

Table 5 – TruSight Cancer gene list.

Genes					
AIP	*CEBPA*	*FANCA*	*KIT*	*PRF1*	*SLX4*
ALK	*CEP57*	*FANCB*	*MAX*	*PRKAR1A*	*SMAD4*
APC	*CHEK2*	*FANCC*	*MEN1*	*PTCH1*	*SMARCB1*
ATM	*CYLD*	*FANCD2*	*MET*	*PTEN*	*STK11*
BAP1	*DDB2*	*FANCE*	*MLH1*	***RAD51C***	*SUFU*
BLM	*DICER1*	*FANCF*	*MSH2*	*RAD51D*	*TMEM127*
BMPR1A	*DIS3L2*	*FANCG*	*MSH6*	*RB1*	*TP53*
BRCA1	*EGFR*	*FANCI*	*MUTYH*	*RECQL4*	*TSC1*
BRCA2	*EPCAM*	*FANCL*	*NBN*	*RET*	*TSC2*
BRIP1	*ERCC2*	*FANCM*	*NF1*	*RHBDF2*	*VHL*
BUB1B	*ERCC3*	*FH*	*NF2*	*RUNX1*	*WRN*
CDC73	*ERCC4*	*FLCN*	*NSD1*	*SBDS*	*WT1*
CDH1	*ERCC5*	*GATA2*	*PALB2*	*SDHAF2*	*XPA*
CDK4	*EXT1*	*GPC3*	*PHOX2B*	*SDHB*	*XPC*
CDKN1C	*EXT2*	*HNF1A*	*PMS1*	*SDHC*	
CDKN2A	*EZH2*	*HRAS*	*PMS2*	*SDHD*	

The tagmented DNA was purified using Solid Phase Reversible Immobilization (SPRI) beads, and then it was analyzed by high-resolution capillary electrophoresis in a QIAxcel Advanced system [QIAGEN, Hilden, Germany]. The electrophoresis results were analyzed using the QIAxcel ScreenGel software [QIAGEN].

The purification reaction was followed by the first PCR amplification, in which the purified tagmented DNA was amplified and index adapters required for cluster generation and sequencing were added. The tagmented DNA was amplified in a solution containing 20 µL of Nextera Library Ampification Mix [Illumina], 5 µL of Index 1 [Illumina] and 5 µL of Index 2 [Illumina]. PCR reaction was performed in a thermocycler [Veriti™ Thermal Cycler, Applied Biosystems] according to the conditions of table 6.

Table 6 – PCR program used in the first PCR amplification.

Step	Temp	Time	
Initial Denaturation	72ºC	3 min	
Initial Denaturation	98ºC	30 sec	
Denaturation	98ºC	10 sec	
Annealing	60ºC	30 sec	10 cycles
Extension	72ºC	30 sec	
Final Extension	72ºC	5 min	
Pause	10ºC	∞	

The PCR products were purified using the SPRI beads and then quantified using a Qubit 2.0 Fluorometer [Invitrogen, Carlsbad, CA, USA]. The quality of the library was assessed using high-resolution capillary electrophoresis in a QIAxcel Advanced system [QIAGEN].

Approximately 500 ng of individual libraries were pooled in batches of 12 samples, followed by a first hybridization. The reaction consisted on mixing 40 µL of DNA library sample, 50 µL of Enrichment Hybridization Buffer [Illumina] and 10 µL of TruSight Content Set CSO [Illumina]. This step mixes the DNA library with capture probes to targeted regions of interest and it was performed according to the conditions of table 7.

Table 7 – PCR program used in the first hybridization.

Step	Temp	Time	
Initial step	95ºC	3 min	
Hybridization	94ºC (-2ºC per cycle)	30 sec	10 cycles
Pause	58ºC	For at least 90 minutes and up to a maximum of 24 hours	

The first hybridization was followed by capture of the probes hybridized to the target regions of interest using streptavidin beads. The biotinylated gDNA fragments bound to the streptavidin beads were magnetically pulled down from the solution. The partly enriched gDNA fragments were then eluted from the beads and subjected to a second round of hybridization and second capture.

The capture library was purified with SPRI beads, which was followed by a second PCR amplification. The capture library was amplified in a solution containing 5 µL of PCR Primer Cocktail [Illumina] and 20 µL of Nextera Enrichment Amplification Mix [Illumina]. PCR reaction was performed in a thermocycler [Veriti™ Thermal Cycler, Applied Biosystems] according to the conditions of table 8.

Table 8 – PCR program used in the second PCR amplification.

Step	Temp	Time	
Initial Denaturation	72ºC	3 min	
Initial Denaturation	98ºC	30 sec	
Denaturation	98ºC	10 sec	
Annealing	60ºC	30 sec	12 cycles
Extension	72ºC	30 sec	
Final Extension	72ºC	5 min	
Pause	10ºC	∞	

The PCR products were purified using SPRI beads. The tagged and amplified sample libraries were quantified using a Qubit 2.0 Fluorometer [Invitrogen] and the quality of the library was checked using high-resolution capillary electrophoresis in a QIAxcel Advanced system [QIAGEN]. The pools were diluted to a final concentration of 12 pM and loaded for sequencing on the MiSeq platform [Illumina], according to the manufacturer's instructions.

The trimmed FASTQ files were generated using MiSeq Reporter [Illumina]. Alignment and variant calling were performed using NextGENe (v.2.4.2) [SoftGenetics, State College, PA, USA] with .vcf files being imported into Geneticist Assistant™ [SoftGenetics] for variant annotation and filtering. All variants detected by NGS were confirmed by Sanger sequencing.

III.3 *RAD51C* germline variant analysis by Sanger sequencing

RAD51C germline variant analysis was performed in 50 DNA samples by Sanger sequencing of all entire coding regions (exons 1-9) and flanking splice junctions. For this purpose, DNA was amplified in a solution containing 10x Taq reaction buffer [Thermo Fisher Scientific, Waltham, Massachusetts, USA] (75mM Tris-HCl, 20 mM $(NH_4)_2SO_4$), 1.5 mM of $MgCl_2$ [Thermo Fisher Scientific], 0.5 mM dNTP mix [Thermo Fisher Scientific], 0.33 mM of each primer (reverse and forward) [Frilabo, Portugal], 1 U of Taq DNA polymerase [Thermo Fisher Scientific] and bidestilled sterile water [B. Braun, Foster City, CA, USA] in a final reaction volume of 25µL. The sequence of each primer used for the amplification of *RAD51C* exons are represented in table 9. PCR reaction was performed in a thermocycler [Perkin-Elmer, Gene Amp PCR Systern 9700, Waltham, Massachusetts, USA] according to the following conditions: an initial denaturation step at 94ºC for 10

minutes, followed by 35 cycles of denaturation, annealing and extension step as represented in table 10, and a final extension step at a 72ºC for 10 min.

Table 9 – Primers used for PCR.

	Primer	Nucleotide sequence
RAD51C	1F	5'-TCC GCT TTA CGT CTG ACG TC-3'
	1R	5'-AGG CGA GAG AAC GAA GAC TG-3'
	2F	5'-TCC ACT CCT AGC ATC ACT GTT GT-3'
	2R	5'-ACC CAC CCT TAA AAG GAG AAC ACT T-3'
	3F	5'-ACA TTT CTG TTG CCT TGG GGA GT-3'
	3R	5'-TGC TGA GGT CTC AGA TGG GCA C-3'
	4F	5'-ACA ATT GCC AAT ACA TCC AAA CAG GT-3'
	4R	5'-AGA GAT TTT CTC AAT TGG CTT TGA CTT TG-3'
	5F	5'-AGA AGG TCC CTG CTC TCT TGG A-3'
	5R	5'-TGT CAG GCA AAC GCT ATT TTG ACA T-3'
	6F	5'-CAA AGA GAC TCA CCT AAT TTT CTT ACA TTT TGT-3'
	6R	5'-ACC AGT GAA CAA GAC AAA TAC AGT CTG C-3'
	7F	5'-TGA TCA GAG GCG TTC TGA GAA ATG T-3'
	7R	5'- AGT GTC ACT TCA TGG GTC ACT GT-3'
	8F	5'-ACA TAC GGG TAA TTT GAA GGG TGT ATT T-3'
	8R	5'-TGC TTG CTG CCT ACA GAA GTT GAC A-3'
	9F	5'-CAC AGT GGT TGA TAA ATT TCT ATC TCA AG-3'
	9R	5'-TGG ATT CAT TCA TGC CAT AGT GTG T-3'

F: Forward; R: Reverse.

Table 10 – PCR program used for amplification of all exons of the *RAD51C* gene.

Step	Temp	Time
Denaturation	94ºC	1 min
Annealing	58ºC	1 min
Extension	72ºC	1 min

5 cycles

Step	Temp	Time
Denaturation	94ºC	1 min
Annealing	56ºC	1 min
Extension	72ºC	1 min

5 cycles

Step	Temp	Time
Denaturation	94ºC	1 min
Annealing	54ºC	1 min
Extension	72ºC	1 min

25 cycles

Amplified PCR products were then analyzed by high-resolution capillary electrophoresis in a QIAxcel Advanced system [QIAGEN] and the electrophoresis results were analyzed using the QIAxcel ScreenGel software [QIAGEN].

The ExoSAP-IT method was used to remove excess of primers, enzymes, salts and dNTPs from the PCR amplification products. Briefly, to 5 µL of the PCR products were added 2 µL of ExoSAP solution (Exonuclease I [Thermo Fisher Scientific] (20 U/µL) and Fast Thermosensitive Alkaline Phosphatase [Thermo Fisher Scientific] (1 U/µL), in a proportion of 1:2, followed by incubation at 37ºC for 50 minutes, and enzyme inactivation at 85ºC for 15 minutes.

The purification was followed by the sequencing reaction using the BigDye® Terminator v3.1 Cycle Sequencing Kit [Applied Biosystems]. The reaction consisted on mixing 3.4 µL of sequencing buffer, 0.5 µL of BigDye® Terminator v3.1 (containing dNTPs, ddNTPs-fluorocromes, $MgCl_2$ and Tris-HCl buffer), 0.32 µL of one of the corresponding primer (forward or reverse), bidestilled sterile water [B. Braun] and 1.0 µL of the purified DNA to reach a final reaction volume of 10 µL. The sequencing reaction was performed according to the conditions of table 11.

Table 11 – PCR program of sequencing reaction.

Step	Temp	Time	
Initial Denaturation	95ºC	4 min	
Denaturation	95ºC	10 sec	
Annealing	50ºC	10 sec	35 cycles
Extension	60ºC	2 min	
Final Extension	60ºC	10 min	

To remove excess of dNTPs, labelled ddNTPs, and non-incorporated primers, the sequencing products were purified with Illustra Sephadex® G-50 fine [GE Healthcare, Life Sciences, Cleveland, USA], according to standard procedures. After purification, 15 µL of Hi-Di™ Formamide [Applied Biosystems] were added to the sequencing products to help stabilize the single stranded DNA. The products were then analyzed in a 3500 Genetic Analyzer [Applied Biosystems] by capillary electrophoresis. The electropherograms of each sample were analyzed with the Sequencing Analysis Software v5.4 [Applied Biosystems]. All of them were examined at least twice, reviewed manually and with the Mutation Surveyor® DNA Variant Analysis Software v4.0.8 [Softgenetics] by two independent observers.

IV. RESULTS

IV.1 *RAD51C* germline variants

IV.1.1 NGS cohort

We analyzed using NGS 61 DNA samples from patients with clinical criteria for molecular testing for LFS. Two heterozygous variants were found in the *RAD51C* gene, including the one who triggered this project, corresponding to a frequency of 3.3% of *RAD51C* germline variants (see table 12).

Table 12 – Germline variants found in the *RAD51C* gene in the NGS cohort.

Sample number	cDNA description	Exon number	Protein description	Effect	Biological significance
#22	c.709C>T	5	p.(Arg237Ter)	Nonsense	Pathogenic
#25	c.890_899del	6	p.(Leu297HisfsTer2)	Frameshift	Pathogenic

IV.1.2 Retrospective cohort

In the retrospective cohort we analyzed 50 DNA samples using Sanger sequencing from patients with clinical criteria for molecular testing for LFS. We found a heterozygous variant (table 13) in one patient, corresponding to a frequency of 2% of *RAD51C* germline variants.

Table 13 – Germline variants found in the *RAD51C* gene in the retrospective cohort.

Sample number	cDNA description	Exon number	Protein description	Effect	Biological significance
#6	c.890_899del	6	p.(Leu297HisfsTer2)	Frameshift	Pathogenic

IV.2 Description of *RAD51C* variants

The variant found in sample #22 (figure 8) consists of a nonsynonymous substitution of a cytosine for a thymine (transversion, c.709C>T) in the first position of codon 237 (CGA → TGA) that creates a premature translational stop signal in this codon. This alteration is expected to result in an absent or disrupted protein product. This variant was reported in the literature in a patient with thyroid and ovarian cancer (Blanco *et al.*, 2014), in a patient with gastric cancer (Sahasrabudhe *et al.*, 2017), and in a patient with family history of breast and/or ovarian cancer (Tavera-Tapia *et al.*, 2017).

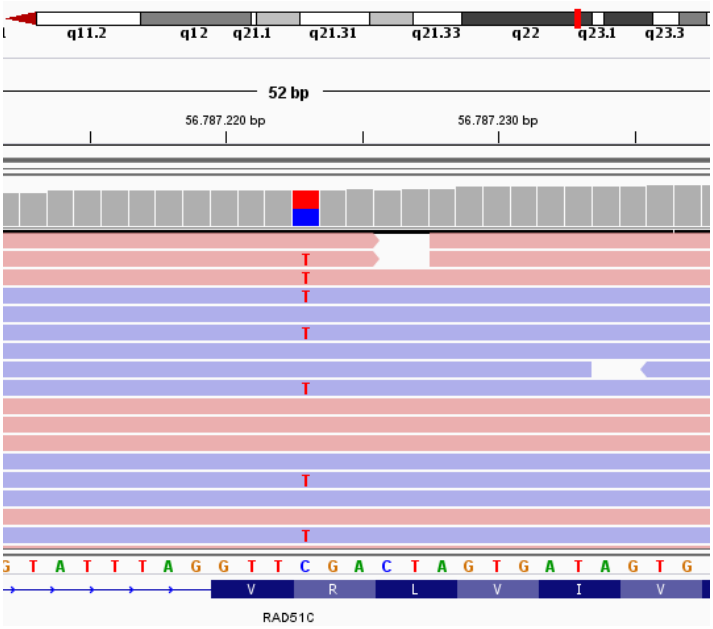

Figure 8 – Visualization of *RAD51C* reads using the Integrative Genomics Viewer (IGV) in patient #22

The second variant consists of a deletion of 10 base pairs (TTGTTCCTGC) that starts in the nucleotide 890 in exon 6 (see figure 9). This variant leads to an alteration in the reading frame and was found in the two cohorts: samples #25 and #6. This genetic variation was described in the literature in a patient with colorectal cancer (Yurgelun *et al.*, 2015) and in a patient with bladder and early-onset prostate cancer (Paulo *et al.*, 2018).

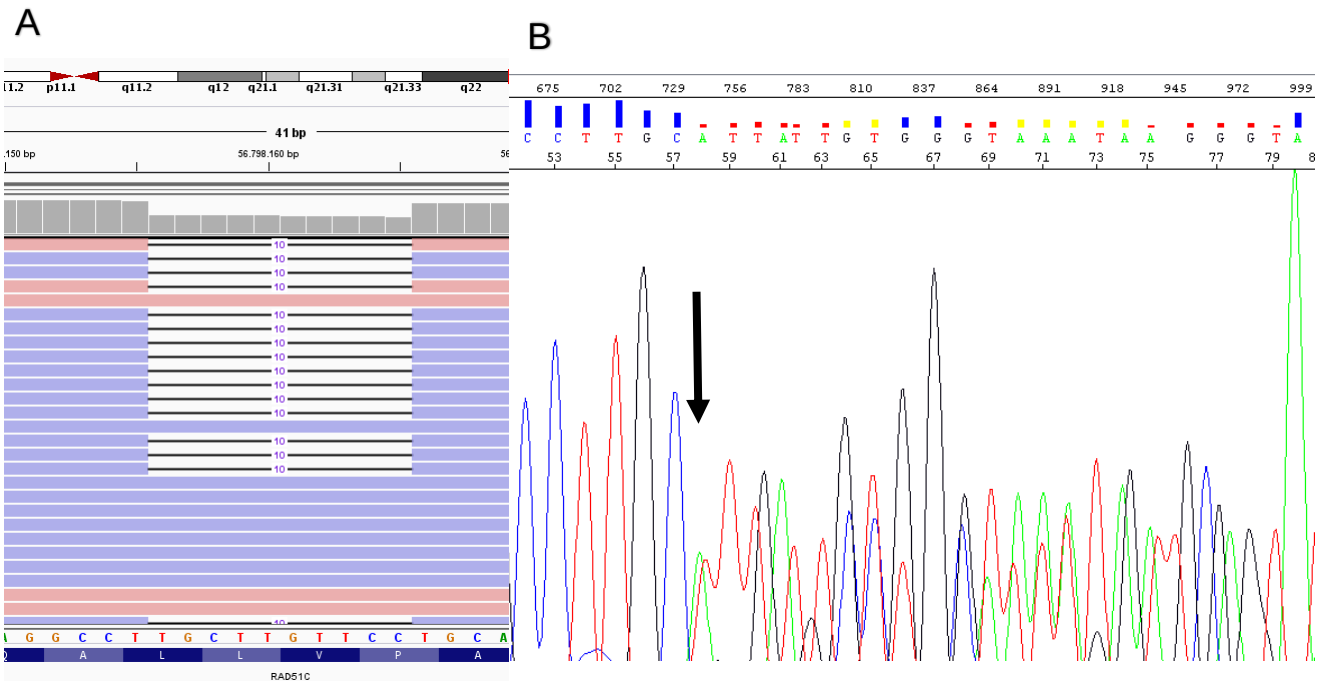

Figure 9 – Variant c.890_899del by NGS and Sanger sequencing: A. Alignment of the *RAD51C* gene by Integrative Genomics Viewer (IGV) obtained by analysis of sample #25; **B.** Sanger sequence electropherogram obtained from sample #6.

IV.3 Clinical characteristics of *RAD51C* variant carriers

Sample #22 is from a family with classic criteria for LFS. The index patient is a woman who was diagnosed with a pleomorphic undifferentiated liposarcoma at the age of 59. We were able to perform, after genetic counselling, screening of the pathogenic *RAD51C* variant in 11 family members. Seven relatives are carriers of the variant, including a sister with a gastrointestinal stromal tumor, a type of sarcoma (figure 10).

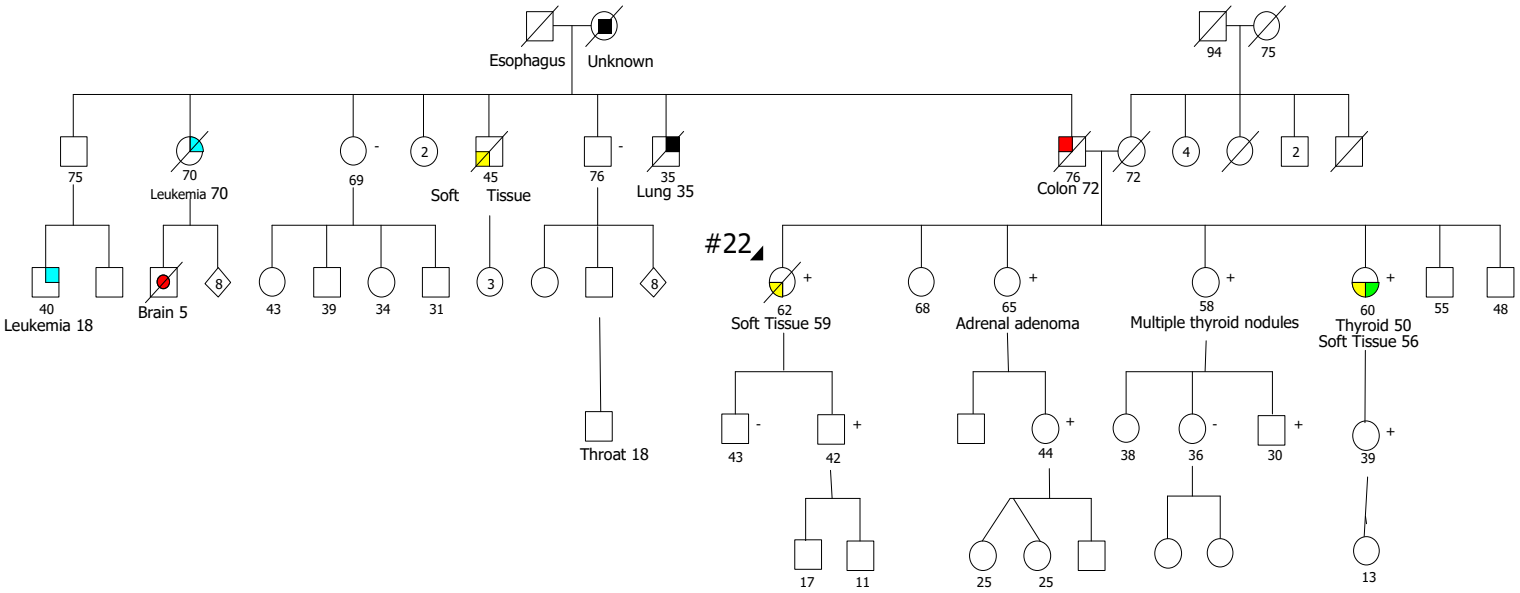

Figure 10 – Pedigree of patient #22, indicating the relatives that were tested for the *RAD51C* variant (**+** for carriers and **-** for non-carriers).

The patient corresponding to sample #25 is a man who was diagnosed with a spindle cell liposarcoma on the anterolateral region of the left thigh at 50 years old. The pedigree is shown in figure 11.

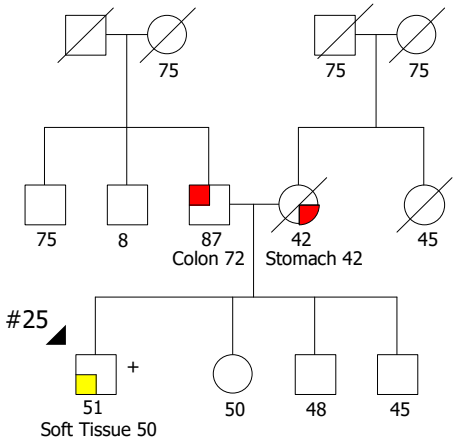

Figure 11 – Pedigree of patient #25.

Patient #6 was diagnosed with a gastric carcinoma at 39 years of age. Two years later he was diagnosed with a lymphoma and, six years later, with a low grade leiomyosarcoma in the fourth finger of the left hand (figure 12). His 38 years old son was also shown to be a carrier.

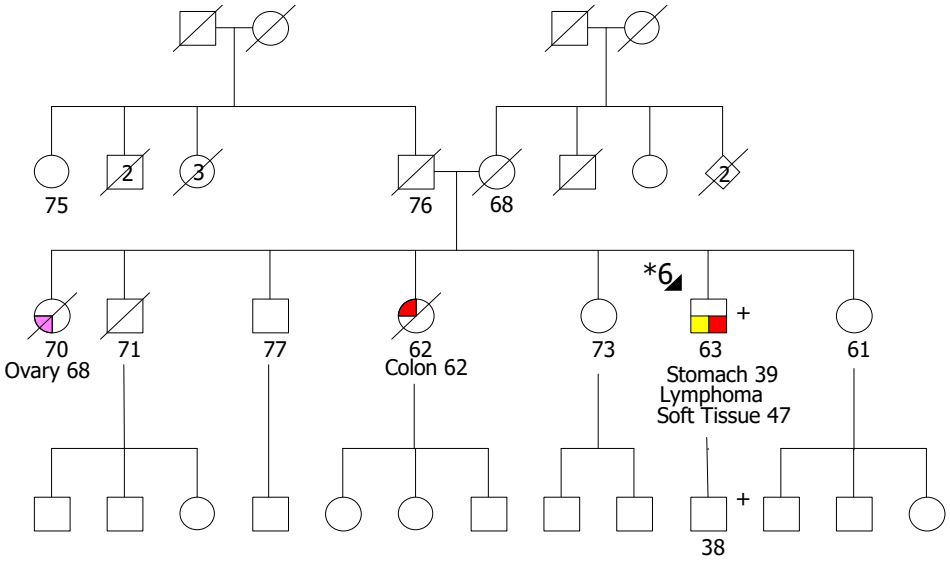

Figure 12 – Pedigree of patient #6.

IV.4 Other germline variants in the NGS cohort

In the 61 samples analyzed by NGS, we identified four potentially deleterious germline variants in other genes (table 14).

Table 14 – Other germline variants found in the NGS cohort.

Sample number	Mutated gene	cDNA description	Exon number	Protein description	Effect	Biological significance
#19	*FANCA*	c.295C>T	4	p.(Gln99Ter)	Nonsense	Pathogenic in homozygosity
#40	*CHEK2*	c.1169A>C	12	p.(Tyr390Ser)	Missense	Likely pathogenic
#57	*BUB1B*	c.709_712del	6	p.(Thr237GlnfsTer8)	Frameshift	Not described
#61	*FANCM*	c.1972C>T	11	p.(Arg658Ter)	Nonsense	VUS

VUS: Variants of uncertain significance.

Patient #19 presents a germline variant that consists of a substitution of a cytosine for a thymine (transversion, c.295C>T) in nucleotide 295 of the *FANCA* gene, leading to a

premature translational stop signal in codon 99. In the literature, this variant was classified as pathogenic in homozygosity causing Fanconi Anemia (Callen *et al.*, 2005).

Patient #40 presents a missense variant in *CHEK2* that substitutes an adenine for a cytosine in nucleotide 1169. This variant was described in a series of non-BRCA breast/ ovarian cancer families and was showed to have a deleterious effect in the *CHK2* protein (Desrichard *et al.*, 2011).

Patient #57 presented a four base pair deletion (ACAG) that starts in nucleotide 709 of *BUB1B* exon 6. This variant results in a modification in the reading frame and has not been previously described.

The alteration found in sample #61 was a germline variant that consists in a substitution of a cytosine for a thymine (transversion, c.1972C>T) on the *FANCM* gene, leading to the formation of a premature translational stop signal in codon 658. This variant was found in several studies in non-BRCA breast/ ovarian cancer families, but the significance of this alteration remains uncertain (Renwick *et al.*, 2006; Nguyen-Dumont *et al.*, 2018; Silvestri *et al.*, 2018).

V. DISCUSSION

Since the initial report in 1969 describing LFS as an inherited disease with high predisposition to develop rhabdomyosarcomas, breast cancer and several other cancers, many authors have been studying this syndrome in order to better understand its molecular basis and to implement appropriate surveillance programs to improve the overall survival of the affected families. After more than four decades of research to identify the gene or genes associated with this syndrome, *TP53* is still the only gene consistently associated with the predisposition to develop the heterogeneous spectrum of malignancies typical of LFS. Although many pathogenic germline variants have already been described in *TP53*, there are still several families that comply with the LFS genetic testing criteria who do not have variants in this gene. Only about 20-40% of the families with the Chompret criteria and 70% of those with the classic LFS criteria present a pathogenic *TP53* variant, therefore further studies are required to explain cancer predisposition in the remaining families (Gonzalez *et al.*, 2009b; Tinat *et al.*, 2009).

The present study includes 111 families that were tested in the Genetics Department of IPO-Porto and were negative for non-benign *TP53* variants. These families were in this study tested for *RAD51C* variants by different methodologies and we found three families with a *RAD51C* pathogenic germline variant: one family with variant c.709C>T, p.(Arg237Ter), and two families with variant c.890_899del, p.(Leu297HisfsTer2), corresponding to a prevalence of 2.7% (3/111). Considering all families with Chompret or classic criteria studied in the Genetics Department of IPO-Porto between 2000-2016 (n=127), the prevalence of *RAD51C* pathogenic variants was 2.4% (3/127) and the prevalence of *TP53* pathogenic variants was 12.6% (16/127) (data not shown). If we consider all families identified with a pathogenic variant either in the *TP53* gene or in *RAD51C* gene, we observe that the contribution of *RAD51C* pathogenic variants is about 15.8% (3/19).

The variant *RAD51C* c.709C>T was identified by NGS in a family with classic criteria for LFS. The initial index patient was diagnosed with a soft tissue sarcoma at the age of 59. Although the origin of the variant (maternal or parental side) is still not known, it should be noted that the paternal side of the family presents several individuals affected with different types of cancer belonging to the LFS tumor spectrum, namely, soft tissue sarcoma, lung cancer, brain cancer, and leukemia. We were able to study 11 relatives, of which seven are carriers of the variant identified in the index patient. Of all carriers, two developed soft tissue sarcomas, one had an adrenal adenoma, another had multiple

thyroid nodules and the remaining carriers are under 44 years old and healthy. This variant is classified in the ClinVar Database as pathogenic, as the substitution of a cytosine for a thymine creates a premature translational stop signal in the exon five. This variant has been reported in the literature in three individuals, one with hereditary diffuse gastric cancer, one with thyroid and ovarian cancer, and another with family history of breast and/or ovarian cancer (Blanco *et al.*, 2014; Sahasrabudhe *et al.*, 2017; Tavera-Tapia *et al.*, 2017). The tumor from one of these patients reported in the literature presented an enriched mutation signature indicative of HR defects, reinforcing the evidence for the causality and pathogenicity of this mutation (Sahasrabudhe *et al.*, 2017).

The *RAD51C* c.890_899del variant was identified in two index cases, one detected by NGS and the other by Sanger sequencing in the retrospective series, and no evidence was found of the two families being related. The first patient is a man diagnosed with a soft tissue sarcoma and with both parents affected with different cancers, the mother with gastric cancer at 42 years old and the father with colon cancer at 72 years old. Unfortunately, it was not possible to determine the origin of the variant (maternal or paternal) or if it arose *de novo*. The second patient presents multiple tumors: gastric cancer diagnosed at 39 years of age, lymphoma diagnosed at 41 years of age and a soft tissue sarcoma diagnosed at 47 years of age. This patient had six siblings, of which one sister was affected with ovarian cancer at 68 years of age and another sister with colon cancer at 62 years old, but it was not possible to carry out segregation studies in this family. This variant leads to an alteration of the reading frame causing a premature translational stop signal, which results in the loss of 80 amino acids at the protein C-terminus, including the nuclear localization motif, being therefore classified in ClinVar Database as pathogenic. In the literature, this variant was described in a patient with colon cancer (Yurgelun *et al.*, 2015) and in an individual with bladder cancer and early-onset prostate cancer (Paulo *et al.*, 2018). In our work, the identification of this variant in two families was only possible due to the incorporation of gastric cancer in the tumor spectrum of LFS to comply with the Chompret criteria. These results reinforce the importance of including gastric cancer in the LFS tumor spectrum in countries with high incidence for gastric carcinoma, as is the case of Portugal, as we have previously shown regarding the identification of *TP53* variants (Keller *et al.*, 2004; Kim *et al.*, 2004; Pinto *et al.*, 2009).

Germline variants in the *RAD51C* gene have been identified in patients with breast and ovarian cancer without BRCA mutations, although more predominantly in families with history of ovarian cancer, so *RAD51C* is primarily considered an ovarian cancer susceptibility gene (Sopik *et al.*, 2015). However, *RAD51C* pathogenic variants have been

reported in patients with other types of neoplasias, such as lung, kidney, colorectal, pancreatic, thyroid, prostate, gastric, head and neck cancer, as well as with leukemia and lymphoma (Meindl *et al.*, 2010; Vuorela *et al.*, 2011; Blanco *et al.*, 2014; Scheckenbach *et al.*, 2014; Yurgelun *et al.*, 2015; Sahasrabudhe *et al.*, 2017; Paulo *et al.*, 2018). Our study is the first to identify *RAD51C* pathogenic germline variants in families with classic LFS criteria and in patients with the Chompret criteria for *TP53* mutation testing. The fact that we found *RAD51C* variants in four patients with soft tissue sarcomas in three different families, one of them with classic LFS criteria, is a strong argument for its role as an alternative cause of LFS besides *TP53*. However, given the phenotypic diversity of LFS, we cannot exclude the possibility that *RAD51C* mutations increase the risk for several different cancers, with some families complying with the clinical criteria for LFS by chance. The identification of *TP53* pathogenic germline variants in LFS families allows predictive tests and specific surveillance programs to variant carriers. According to the NCCN Guidelines, pathogenic germline variants in the *RAD51C* gene should be offered risk-reducing salpingo-oophorectomy due to the high risk of development of ovarian cancer. More studies are needed to determine the risk for other cancers in *RAD51C* variant carriers to allow development of specific surveillance programs, but for now surveillance programs for other cancers must be based on family history.

Recent reports have proved the efficacy of PARP inhibitors as targeted therapy for patients with deficient HR. PARP enzymes are key components in the activation and recruitment of repair enzymes at sites of a single strand breaks (SSBs)(Fong *et al.*, 2009). PARP inhibitors block these enzymes and lead to the accumulation of this type of errors, which will lead to the collapse of DNA replication forks and formation and accumulation of DSBs (Walsh, 2015). In cancers with deficient HR, DSBs will not be repaired and cause cell death, a concept known as synthetic lethality (Farmer *et al.*, 2005). The Food and Drugs Administration (FDA) have already approved the use of PARP inhibitors for the treatment of recurrent ovarian, fallopian tube, or primary peritoneal cancer in adult patients with deleterious *BRCA* variants and in *BRCA*-mutated metastatic breast cancer, and several clinical trials are ongoing in other cancers. Some studies have shown that tumors deficient in other genes involved in HR may respond also to these inhibitors (McCabe *et al.*, 2006). Since *RAD51C* is involved in HR, it is likely that in the future LFS patients with pathogenic variants in this gene might be eligible for this targeted therapy.

In our cohort, we also found potentially deleterious germline variants in the genes *BUB1B, CHEK2, FANCA* and *FANCM* in four patients. The *BUB1B* gene encodes a protein called BUBR1, a multidomain protein kinase that has an important role in the mitotic spindle assembly checkpoint (SAC) (Kapanidou *et al.*, 2015). This checkpoint

certifies the correct chromosome segregation during anaphase, ensuring that each sister chromatid is attached to a spindle microtubule. Biallelic pathogenic variants in *BUB1B* were identified in families with mosaic variegated aneuploidy (MVA), an autosomal recessive disorder characterized by mosaic aneuploidies involving multiple chromosomes (Hanks *et al.*, 2004; Matsuura *et al.*, 2006). Individuals affected with MVA present growth retardation, profound developmental delay, severe microcephaly, and other disabilities, having also a high risk of developing some specific neoplasms such as rhabdomyosarcoma, Wilms tumor and leukemia (Kajii *et al.*, 2001). Homozygous variants in the *BUB1B* gene were also identified in a patient with gastrointestinal early-onset cancer (Rio Frio *et al.*, 2010). The truncated *BUB1B* variant described in patient #57 has so far not been described in the literature and was found in heterozygosity. The significance of this alteration and its association to cancer susceptibility remains to be clarified.

The *CHEK2* gene encodes the CHK2 protein that, once activated, inhibits the CDKs and consequently blocks the progression of the cell cycle for mitosis after DNA damage. The identification of germline heterozygous mutations in the *CHEK2* gene in some families with clinical criteria for LFS suggested that this gene would be a possible candidate for the cause of the LFS phenotype (Bell *et al.*, 1999; Vahteristo *et al.*, 2001). Although this hypothesis was not supported by other studies, *CHEK2* remains as a breast cancer susceptibility gene, the most common cancer in the adult phase in *TP53* carriers (Amadou *et al.*, 2018). For some specific pathogenic variants in *CHEK2,* the cumulative risk for breast cancer in women with familial breast cancer was estimated to be around 28 to 37% (Weischer *et al.*, 2008; Cybulski *et al.*, 2011). Some variants were also associated with high risk for prostate cancer (Walsh *et al.*, 2006; Wang *et al.*, 2015) and some heterozygous germline variants were found in patients with pancreatic and colorectal cancers (Yurgelun *et al.*, 2017; Chaffee *et al.*, 2018; Stoffel *et al.*, 2018). The missense variant detected in our work is classified as likely pathogenic in the ClinVar Database, detected in a patient diagnosed with a breast cancer at 36 years old with two relatives diagnosed with leukemia at young ages. Segregation studies will be required to better evaluate the pathogenicity of this variant and its correlation with the phenotype presented by the family.

FANCA and *FANCM* are involved in the FA pathway and biallelic alterations in these genes are known to lead to FA and consequently susceptibility to cancer (Nalepa and Clapp, 2018). However, heterozygous variants in other FA genes within the HR pathway are known to cause predisposition to breast and/or ovarian cancer, namely, *BRCA1*, *BRCA2*, *BRIP1*, *PALB2*, and *RAD51C* (Wooster *et al.*, 1995; Bonadona *et al.*,

2005; Levran *et al.*, 2005; Xia *et al.*, 2006; Meindl *et al.*, 2010). Using the International Fanconi Anemia Registration (IFAR) data, Berwick and co-workers analyzed 312 families of probands diagnosed with FA and identified more than 404 heterozygous FA carriers in the most known genes involved in the FA pathway (*FANCA/B/C/D1/D2/E/F/G/J*). According to their results in general, excluding variants in *FANCD1*/*BRCA2*, there is not a striking or significant increase in cancer incidence among the FA heterozygotes, with only some evidence that *FANCC* variants may slightly increase the susceptibility to breast cancer (Berwick *et al.*, 2007). However, more recently some studies have made associations between heterozygous variants in these FA genes and an increased risk to develop cancer. Solyom and co-workers, identified a heterozygous deletion in the *FANCA* gene that predispose to breast cancer, and Kiiski and colleagues identified *FANCM* variants in patients with triple-negative breast cancers and proposed this gene as a breast cancer susceptibility gene (Solyom *et al.*, 2011; Kiiski *et al.*, 2014). The identification in the present work of two truncating variants in these two genes (*FANCA* and *FANCM*) in patients with clinical criteria for LFS reinforces the hypothesis that heterozygous alterations in these FA genes might be involved in predisposition to cancer. However, more studies are needed to clarify the effect of these alterations in cancer predisposition and, consequently, to better understand their clinical significance and their potential role in LFS.

VI. CONCLUSIONS

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This study allows us to conclude that:

- Pathogenic germline variants in *RAD51C* are present in about 2.7% of families with clinical criteria for LFS molecular testing that were negative for non-benign *TP53* variants;
- Although *RAD51C* variants are mainly known to be associated with increased risk for ovarian cancer, this study found that families carrying them have frequently soft tissue sarcomas and gastric cancers;
- The identification of pathogenic germline variants in LFS families allows carriers to be referred to surveillance programs, potentially improving prognosis and increasing the overall survival, but further studies are required to define the risk of cancer associated with *RAD51C* pathogenic variants;
- Other genes involved in the FA pathway and/or cell cycle regulation might play a role in families with a tumor spectrum associated with LFS.

VII. FUTURE PERSPECTIVES

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This study may benefit from further analysis to support our conclusions and to allow a more specific evaluation of the *RAD51C* germline variant carriers and the association with the phenotype of LFS. Thus, we plan:

- To find out if there is loss of heterozygosity in the tumors of germline variant carriers;
- To complete the segregation analysis in the families identified, including the study of archival tissue of deceased relatives, in order to better understand the correlation between these variants and the phenotype;
- To perform haplotype studies in the two families sharing the c.890_899del variant to find out if they are related and thereby extend the co-segregation data. If possible, the haplotype study might be extended to other cancer families carriers of the two *RAD51C* variants here reported in LFS-like families;
- Look for exonic rearrangements in *RAD51C* using the available NGS data and MLPA.

VIII. BIBLIOGRAPHY

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