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Inherited predisposition of early-onset HER2-positive breast cancer

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SUMMARY

SUMMARY

Breast cancer is the single most frequent event in Li-Fraumeni syndrome, accounting for more than 25% of all tumors in affected families. This syndrome is a rare inherited cancer susceptibility disease associated with germline mutations in the *TP53* gene. Recent studies have shown that breast cancers in women with Li-Fraumeni syndrome are commonly hormone receptor and HER2-positive, the latter being rare in *BRCA1/2* mutation carriers, suggesting that *HER2* amplification or over-expression in a young woman may be a useful criterion for identifying carriers of germline *TP53* mutations. However, only a minority of early-onset HER2-positive breast cancers are explained by the existence of germline mutations in the *TP53* gene.

The aims of this work were to identify the contribution of germline *TP53* mutations for early-onset HER2-positive breast cancer, to identify other genes with germline mutations in patients with early-onset HER2-positive breast cancer, to define the genetic testing criteria to complement established recommendations and to compare the *HER2* amplification pattern in carcinomas from women with pathogenic germline mutations in the *TP53* gene compared with women with other germline variants. We therefore performed Sanger sequencing in peripheral blood samples from 88 women and gene-panel next-generation sequencing (NGS) in additional 36 women, all with HER2-positive breast cancer diagnosed until the age of 40. *HER2* amplification analyses was performed in seven tumors from patients with germline mutations.

Of the 124 patients, five were shown to carry heterozygous variants in the *TP53* gene, namely, c.642T>G, p.(His214Gln); c.524G>A, p.(Arg175His); c.559+19_559+34del, p.?; c.383delC, p.(Pro128LeufsTer42); and c.935C>G, p.(Thr312Ser). Two of the five *TP53* variants are classified as deleterious mutations and the remaining three are variants of uncertain significance (VUS), with the probands carrying the pathogenic mutations showing pedigrees with various early-onset cancers that are not among the most typical in Li-Fraumeni syndrome. Additionally, two of the 36 patients tested by NGS were shown to have deleterious mutations in other genes, namely, the mutation c.9105T>A, p.(Tyr3035Ter), in the *BRCA2* gene and the mutation c.295C>T, p.(Gln99Ter), in the *FANCA* gene. All tumors were confirmed as *HER2*-amplified (HER2/CEP17 ≥ 2.0), with the tumors of the patients with *TP53* pathogenic mutations showing the highest degree of *HER2* amplification.

We conclude that, although most breast cancers in women with Li-Fraumeni syndrome are HER2-positive, germline *TP53* mutations account for only a small proportion of early-onset HER2-positive breast cancer and other genes, like *BRCA2* and *FANCA*, may also contribute to the pathogenesis of this breast cancer subtype. Taking into account the potential clinical impact, women diagnosed with early-onset HER2-positive breast cancer,

especially those having first-degree relatives with any cancer until the age of 45 years, as well as all cases of breast cancer diagnosed before age 30 irrespective of family history, should receive genetic counseling and genetic testing that includes *TP53*. Furthermore, we show evidence that breast carcinomas from patients with deleterious *TP53* germline mutations might show higher *HER2* amplification ratios than those from patients with other germline variants.

RESUMO

O cancro da mama é o evento mais frequente na síndrome de Li-Fraumeni, correspondendo a mais de 25% de todos os tumores diagnosticados em famílias afetadas. Esta síndrome é uma doença hereditária rara de suscetibilidade para o desenvolvimento de cancro associada a mutações germinativas no gene *TP53*. Estudos recentes têm mostrado que os cancros da mama em mulheres com síndrome de Li-Fraumeni são frequentemente positivos para os recetores hormonais e para o gene *HER2*. Cancros da mama HER2-positivos são raros em portadores de mutações nos genes *BRCA1/2*, o que sugere que a amplificação ou a sobre-expressão de *HER2* numa mulher jovem pode ser um critério útil para a identificação de portadores de mutações da linha germinativa no gene *TP53*. No entanto, apenas uma minoria dos cancros da mama HER2-positivos de início precoce são explicados pela existência de mutações germinativas no gene *TP53*.

Os objetivos deste trabalho foram identificar a contribuição das mutações germinativas no gene *TP53* para cancro da mama HER2-positivo em idade jovem; identificar outros genes com mutações germinativas em pacientes com cancro da mama HER2-positivo em idade jovem; definir os critérios de testes genéticos para complementar as recomendações estabelecidas; e ainda comparar o padrão de amplificação do gene *HER2* em carcinomas de mulheres com mutações patogénicas germinativas no gene *TP53* com o de mulheres com outras variantes germinativas. Foi realizada sequenciação de Sanger em amostras de sangue periférico de 88 mulheres e testado um painel de genes por sequenciação de nova geração (NGS) em 36 mulheres, todas com cancro da mama HER2-positivo diagnosticado até a idade de 40 anos. A análise da amplificação do gene *HER2* foi realizada em sete tumores de doentes com variantes na linha germinativa.

Dos 124 doentes analisados, cinco eram portadores de variantes heterozigóticas no gene *TP53*, nomeadamente, c.642T>G, p.(His214Gln); c.524G>A, p.(Arg175His); c.559+19_559+34del, p.?; c.383delC, p.(Pro128LeufsTer42); e c.935C>G, p.(Thr312Ser). Duas das cinco variantes foram classificadas como mutações deletérias e as três restantes como variantes de significado desconhecido, com os probandos com mutação patogénica no gene *TP53* a mostrarem pedigrees com vários cancros de início precoce fora do espectro mais típico da síndrome de Li-Fraumeni. Além disso, dois dos 36 doentes testados por NGS mostraram ter mutações deletérias em outros genes, nomeadamente, a mutação c.9105T> A, p. (Tyr3035Ter), no gene *BRCA2* e a mutação c.295C> T, p. (Gln99Ter), no gene *FANCA*. Todos os tumores foram confirmados como tendo amplificação do gene *HER2* (HER2/CEP17 ≥ 2,0), sendo que os tumores dos doentes com mutação patogénica no gene *TP53* apresentaram maior grau de amplificação.

Concluímos que, embora a maioria dos cancros da mama em mulheres com síndrome de Li-Fraumeni sejam HER2-positivo, as mutações germinativas no gene *TP53* representam apenas uma pequena proporção do cancro da mama HER2-positivo de início precoce, sendo que outros genes, como o *BRCA2* e o *FANCA*, podem também contribuir para a patogénese deste subtipo de cancro da mama. No entanto, considerando o potencial impacto clínico, as mulheres diagnosticadas com cancro da mama HER2-positivo em idade jovem, especialmente aquelas que têm parentes em primeiro grau afetados com qualquer tipo de cancro até à idade de 45 anos, bem como todos os casos de cancro da mama diagnosticados antes dos 30 anos, devem receber aconselhamento genético e ser submetidas a testes genéticos que incluam a análise ao gene *TP53*. Concluímos, ainda, que os carcinomas da mama de doentes com mutações patogénicas no *TP53* podem apresentar rácios de amplificação do gene *HER2* superiores aos de doentes com outras variantes na linha germinativa.

LIST OF ABBREVIATIONS

AR – Amphiregulin

ASCO – American Society of Clinical Oncology

ASR - Age-standardized rate

BTC – Betacellulin

CAP – College of American Pathologists

CISH – Chromogenic *In Situ* Hybridization

COSMIC – Catalogue of Somatic Mutations in Cancer

CTRD – C-terminal regulatory domain

DAPI – 4', 6-diamidino-2-phenylindole

DBD – DNA-binding domain

DCIS – Ductal Carcinoma *in situ*

ddNTP – Dideoxynucleotide

DNA – Deoxyribonucleic acid

dNTP – Deoxynucleoside triphosphate

EPG – Epigen

EPR – Epiregulin

EZH2 – Enhancer of zeste homolog 2

FISH – Fluorescence *In Situ* Hybridization

gDNA – Genomic DNA

HB-EFG – Heparin-binding EGF-like ligand

HBOC – Hereditary Breast and Ovarian Cancer

IARC – International Agency for Research on Cancer

IHC – Immunohistochemistry

ISH – *In Situ* Hybridization

LCIS – Lobular Carcinoma *in situ*

LOVD – Leiden Open Variation Database

NaSCN – Sodium thiocyanate

NGS – Next Generation Sequencing

NRG – Neuregulin

p53 – Tumor suppressor protein p53

PCR – Polymerase chain reaction

PI3K – Phosphoinositide 3-kinase

PRD – Proline-rich domain

Rb – Retinoblastoma-associated protein

RNA – Ribonucleic acid

SISH – Silver *in situ* hybridization

SV40 – Simian vacuolating virus 40

TAD – Transactivation domain

TD – Tetramerization domain

TNM – Tumor, Node, Metastasis

VUS – Variants of uncertain significance

GENES

AIP – Aryl hydrocarbon receptor interacting protein

ALK – Anaplastic lymphoma receptor tyrosine kinase

APC – APC, WNT signaling pathway regulator

ATM – ATM serine/threonine kinase

BAP1 – BRCA1 associated protein 1

BCL2 – BCL2, apoptosis regulator

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

CDH3 – Cadherin 3

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

CHEK2 – Checkpoint kinase 2

CYLD – CYLD lysine 63 deubiquitinase

CYP24A1 – Cytochrome P450 family 24 subfamily A member 1

DDB2 – Damage specific DNA binding protein 2

DHCR24 – 24-dehydrocholesterol reductase

DICER1 – Dicer 1, ribonuclease III

DIS3L2 – DIS3 like 3'-5' exoribonuclease 2

EGFR – Epidermal growth factor receptor

EGR1 – Early growth response 1

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 – Fanconi anemia complementation group A
FANCB – Fanconi anemia complementation group B
FANCC – Fanconi anemia complementation group C
FANCD2 – Fanconi anemia complementation group D2
FANCE – Fanconi anemia complementation group E
FANCF – Fanconi anemia complementation group F
FANCG – Fanconi anemia complementation group G
FANCI – Fanconi anemia complementation group I
FANCL – Fanconi anemia complementation group L
FANCM – Fanconi anemia complementation group M
FEN1 – Flap structure-specific endonuclease 1
FH – Fumarate hydratase
FLCN – Folliculin
GATA2 – GATA binding protein 2
GPC3 – Glypican 3
HER2 – Erb-b2 receptor tyrosine kinase 2 (*ERBB2*)
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
MMP13 – Matrix metallopeptidase 13
MMP3 – Matrix metallopeptidase 3
MSH2 – MutS homolog 2
MSH3 – MutS homolog 3
MSH6 – MutS homolog 6
MUTYH –MutY DNA glycosylase

MYC – V-myc avian myelocytomatosis viral oncogene homolog

NAT1 – N-acetyltransferase 1

NBS1 – Nibrin (*NBN*)

NF1 – Neurofibromin 1

NF2 – Neurofibromin 2

NFKB2 – Nuclear factor kappa B subunit 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

PRF1 – Perforin 1

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

PTCH1 – Patched 1

PTEN – Phosphatase and tensin homolog

RAD51 – RAD51 recombinase

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 assembly guanine nucleotide exchange 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

TSC1 – Tuberous sclerosis 1

TSC2 – Tuberous sclerosis 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

I. INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled cell division and dissemination of abnormal cells (Chambers et al., 2002; Preston-Martin et al., 1990). If cancer is detected after the spread of cancer cells, treatments are much less successful and it can result in death (Chambers et al., 2002). Cancer is caused by extrinsic factors (tobacco, infectious organisms, drugs) and/or intrinsic factors (hormones, inherited genetic mutations, immune conditions) (Torre et al., 2015). Surgery, radiation, chemotherapy, hormone therapy, immune therapy, and targeted therapy are the most common treatments used in cancer (American Cancer Society, 2015).

Cancer is currently the major public health problem, with 14.1 million of new cases and 8.2 million deaths in the world, during the year 2012 (Ferlay et al., 2015). In 2025 are estimated 19 million new cases per year in the world and 24 million of new cases will be diagnosed by 2035 (Coleman, 2015). In the year 2012, the most commonly diagnosed cancers were lung (1.82 million), breast (1.67 million), and colorectal (1.36 million) (Ferlay et al., 2015).

I.1 Breast Cancer

I.1.1 Breast Cancer Epidemiology

Considering both sexes, breast cancer was the second most frequent cancer in the world in 2012 and the most common invasive tumor diagnosed among women, both in developed and developing countries (Figure I.1) (Ferlay et al., 2015).

Excluding skin cancers, breast cancer is the most common cancer diagnosed among women in Europe, with an incidence of 69.9 per 100 000 in 2012. This cancer represents the leading cause of cancer mortality, with a mortality of 16.1 per 100 000 (Figure I.2a). In Portugal, as in Europe, breast cancer is the most common cancer diagnosed among women, with an incidence of 67.6 per 100 000 and a mortality of 13.1 per 100 000 in 2012 (Figure I.2b) (IARC, GLOBOCAN 2012).

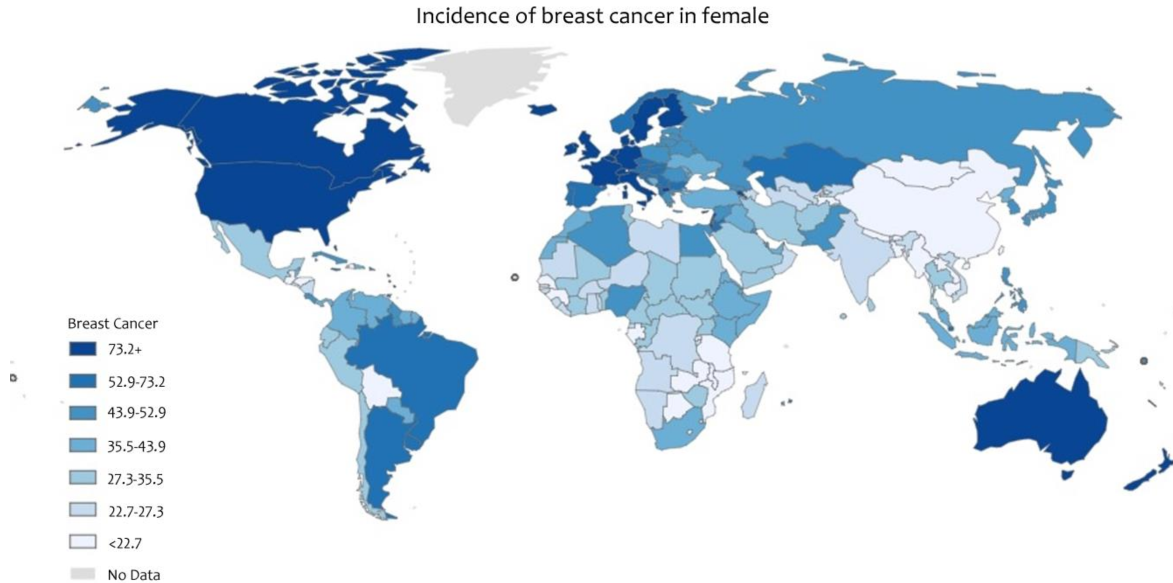


Figure I.1. Estimated age-standardised incidence rates of breast cancer in the world in women [Adapted from GLOBOCAN 2012 (IARC)]

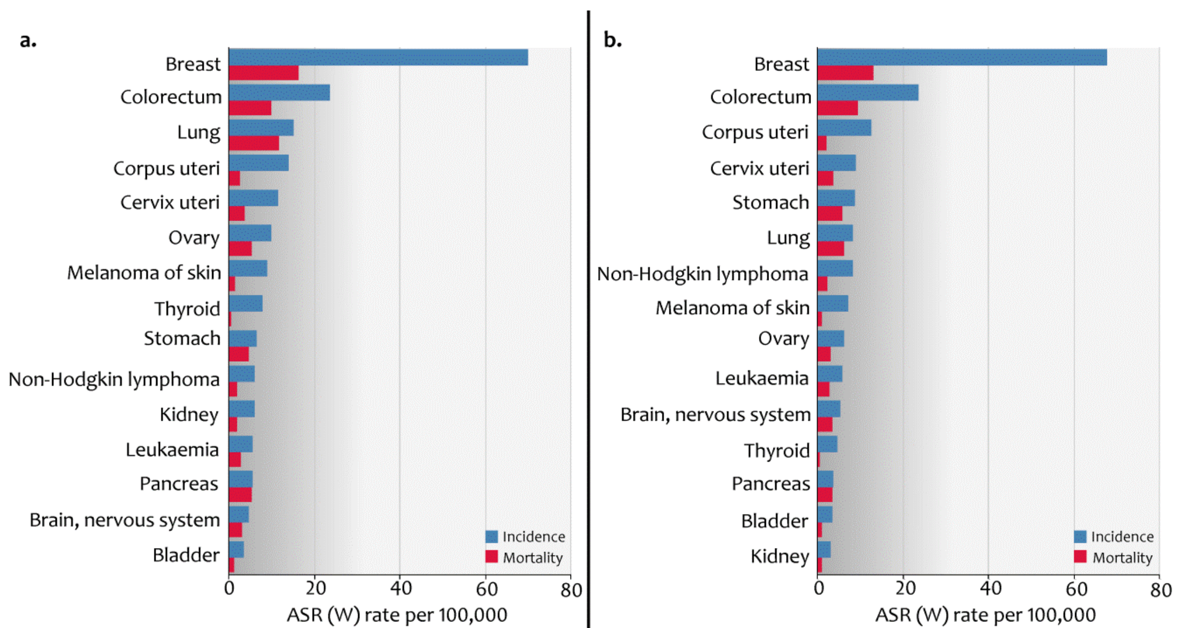


Figure I.2. Estimated age-standardized incidence and mortality rates among women: **a.** in Europe and **b.** in Portugal. **ASR**: age-standardized rates [adapted from GLOBOCAN 2012 (IARC)]

In northern Portugal, according to the Northern Region Cancer Registry (RORENO) data from 2010, breast cancer was the most commonly diagnosed cancer among women, accounting for over a quarter of all tumors diagnosed (29.2%) (RORENO, 2015).

I.1.2 Etiology and Risk Factors

Breast cancer has been attributed to a combination of genetic susceptibility and other patient factors, including age and reproductive, hormonal and lifestyle features (diet, smoking, alcohol, weight). Although the specific causes are not yet fully understood, gender is the greatest risk factor, as breast cancer occurs 100 times more frequently in women than men (Richie and Swanson, 2003). Another well-documented risk factor for breast cancer (and for many other cancers) is age. The incidence of breast cancer is low before the age of 35 (less than 5%), after which it increases linearly with age (Senkus et al., 2013; Singletary, 2003). Early menarche, late menopause and null parity also are associated with an increased risk of developing breast cancer (Hulka and Moorman, 2001; Singletary, 2003).

Furthermore, alcohol consumption and overweight have also been reported quite consistently as breast cancer risk factors. Alcohol consumption at a level of one to two drinks per day modestly increases breast-cancer risk (Singletary, 2003). In premenopausal women, body mass index is not a risk factor for breast cancer incidence, but in postmenopausal women the relative risk of breast cancer incidence is higher in overweight women (Hulka and Moorman, 2001; Singletary, 2003).

Family history is one of the most well-established breast cancer risk factors. A woman with a first-degree relative with breast cancer has an approximately two- to threefold excess risk of developing the disease (Hulka and Moorman, 2001). In turn, if a woman has multiple relatives affected, the risk of developing breast cancer further increases. It is estimated that approximately 7% of all breast cancers are due to inherited gene alterations (Hulka and Moorman, 2001).

I.1.3 Diagnosis and Treatment

Breast cancer may be diagnosed as the result of a screening program. The underlying principle for breast cancer screening is that it allows the detection of tumors at a pre-clinical stage, in order to improve the chance of survival. The European Union recommends biannual mammography screening in the age group of 50 to 69 years, since in this age group the regular examination has resulted in reduction of breast cancer mortality (Senkus et al., 2013). A mammography is an X-ray picture of the breast often used as a screening tool, but one should take into account the false-positive findings that require additional imaging or histopathological assessment (Nounou et al., 2015). The diagnosis of breast cancer is based on clinical examination in combination with imaging, and confirmed by pathological assessment (Senkus et al., 2013).

Breast cancer can be classified into *in situ* carcinoma or invasive carcinoma. *In situ* carcinoma may be further distinguished in lobular (LCIS) or ductal (DCIS), both having no

invasion of the underlying basement membrane. As would be expected with such localized and confined malignancy, there is little potential for metastases formation (Richie and Swanson, 2003). When there is infiltration beyond the basement membrane, the malignancy is considered invasive (or infiltrating) and it comprises several histological subtypes: invasive carcinoma of no special type, invasive lobular carcinoma, tubular carcinoma, cribriform carcinoma, mucinous carcinoma, carcinoma with medullary features, carcinoma with apocrine differentiation, invasive micropapillary carcinoma and metaplastic carcinoma of no special type (Lakhani et al., 2012).

Breast cancer has been described as a heterogeneous disease that displays a variety of subtypes with different expression profiles that have substantial implications for prognosis and survival rates (Carraro et al., 2013). The molecular classification of breast cancer by gene-expression patterns established four major breast cancer subtypes: the Luminal A, Luminal B, Basal-like and the HER2-positive (Table I.1) (Ades et al., 2014; Perou et al., 2000; Skibinski and Kuperwasser, 2015; Sorlie et al., 2001; Sorlie et al., 2003). These subtypes differ in genomic complexity, key genetic alterations and clinical prognosis (Banerji et al., 2012).

Table I.1. Breast cancer molecular subtypes [Adapted from Ades et al., 2014; Perou et al., 2000; Skibinski and Kuperwasser, 2015; Sorlie et al., 2001 and Sorlie et al., 2003]

Subtypes	Characteristics
Luminal A	Estrogen receptor positive, HER2-negative, Ki-67 protein low, and progesterone receptor high.
Luminal B	Estrogen receptor positive, HER2-positive or negative, and Ki-67 protein high or progesterone receptor low.
Basal-like	Typically lacks expression of the molecular targets that confer responsiveness to highly effective targeted therapies, such as tamoxifen and aromatase inhibitors or trastuzumab.
HER2-positive	Overexpression of HER2 protein, which is highly associated with *HER2* gene amplification. This subtype is more heterogeneous with the majority being negative for hormone receptors but a sizeable minority showing expression of estrogen or progesterone receptor.

Breast cancer is usually treated through combinations of different therapies, such as surgery, radiation therapy, chemotherapy, hormone therapy, targeted therapy and bisphosphonates. Breast conserving surgery is the most common approach in the treatment of localized breast cancer, being possible in about 60 to 80% of cancers diagnosed in Central Europe. However, in some patients, mastectomy is still carried out because of tumor

size (relative to breast size), tumor multicentricity, inability to obtain negative surgical margins after multiple resections, prior radiation to the chest wall or breast, inability to perform radiation therapy, or even by patient choice. Radiation therapy is a process in which cancer cells are directly exposed to high levels of radiation. Whole breast radiation therapy alone reduces the risk of local recurrence by two-thirds (Nounou et al., 2015; Senkus et al., 2013).

The decision on systemic adjuvant therapies should be based on the molecular classification (Figure I.3). All luminal cancers should be treated with endocrine therapy. Most luminal A tumors require no chemotherapy, except those with highest risk of relapse. Similarly, indications for chemotherapy within luminal B HER2-negative tumors depend on the risk of relapse (taking into account the tumor extent, your grade, cell proliferation and vascular invasion), presumed responsiveness to endocrine therapy and patient choice. Luminal B HER2-positive tumors are treated with chemotherapy, endocrine therapy and anti-HER2 therapies. The triple-negative tumors are treated with chemotherapy, and HER2-positive tumors are also treated with chemotherapy coupled with anti-HER2 therapies (Nounou et al., 2015).

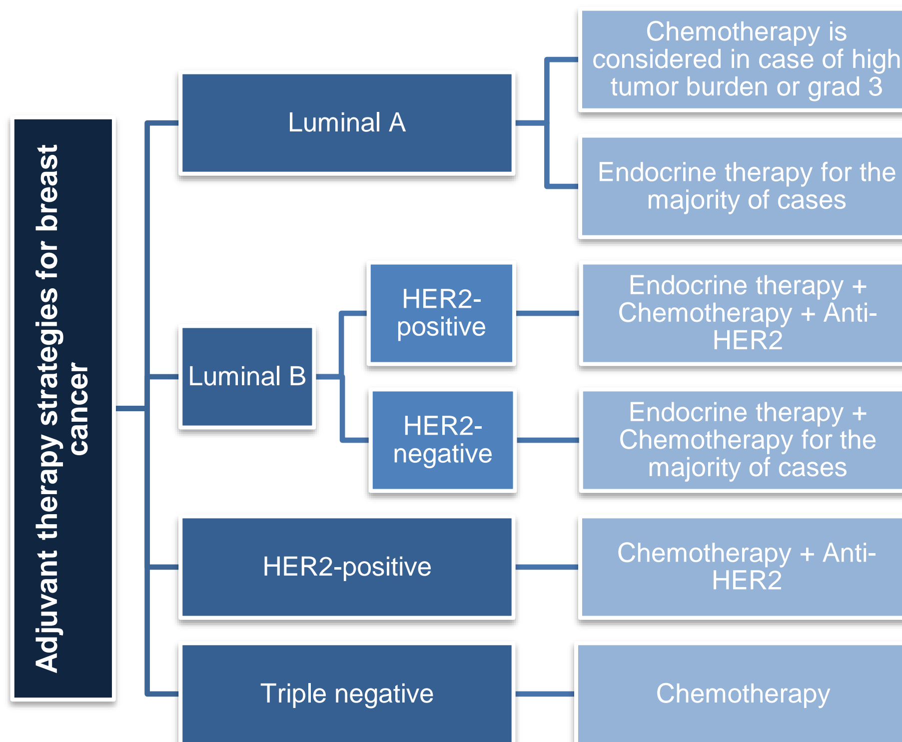


Figure I.3. The adjuvant therapy options according to the molecular subtypes [Adapted from Nounou et al., 2015]

I.1.4 Breast Carcinogenesis

The chronic and often uncontrolled cell proliferation, which is the essence of the neoplastic disease, involves not only deregulation in the control of cell proliferation, but also adjustments on the energy metabolism in order to boost the growth and division of cells. Currently, it is widely accepted that normal cells evolve progressively to a neoplastic state by acquiring hallmark capabilities: 1) sustaining proliferative signaling; 2) resisting cell death; 3) evading growth suppressors; 4) enabling replicative immortality; 5) inducing angiogenesis; 6) activating invasion and metastasis; 7) deregulating cellular energetics; and 8) avoiding immune destruction. Moreover, the acquisition of these hallmarks is possible due to two essential characteristics, genomic instability that lead to increased variability (mutability), and inflammation (Hanahan and Weinberg, 2011).

Cancer cells may acquire the ability to sustain a proliferative signal by different approaches: 1) generating their growth factors, resulting in autocrine proliferative stimulation; 2) sending signals to stimulate normal cells to produce various growth factors; 3) elevating the levels of protein receptors at the cancer cell surface; 4) through structural alterations in receptor molecules that facilitate activation independent of ligand receptor; or 5) constitutive activation of elements of signaling pathways operating downstream of these receptors (Hanahan and Weinberg, 2011).

In breast cancer, some of these mechanisms have been demonstrated. For example, *HER2* amplification with resultant HER2 protein overexpression has been shown to play a role in sustaining multiple cancer pathways, as self-sufficiency in growth signals. *HER2* amplification is also involved in sustaining angiogenesis, increasing cell division and enhancing invasion and metastization (Slamon et al., 2011). Other mechanisms that contribute for the ability of cancer cells to sustain a proliferative signal are mutations in the phosphoinositide 3-kinase (PI3K) pathway. These mutations cause resistance to HER2 targeted therapies by leading to constitutive activation of elements of signaling pathways operating downstream of growth factor receptors (Arteaga et al., 2012). In addition to the ability to control cellular proliferation signals, cancer cells can also avoid the pathways that negatively regulate cell growth. This occurs by inactivation of tumor suppressors such as tumor suppressor protein p53 (p53) and retinoblastoma-associated protein (Rb) (Hanahan and Weinberg, 2011). Somatic mutations in the *TP53* gene are frequent in sporadic basal breast cancer subgroups (Rath et al., 2013).

Genetic mutations are considered the major causes for the development of cancer. However, this paradigm has been expanded to incorporate epigenetic regulatory mechanisms. The key processes responsible for epigenetic regulation are DNA methylation (including global hypomethylation and *locus* specific hypermethylation), histone modifications, chromatin remodeling and post-transcriptional gene regulation by noncoding

RNA (microRNAs) (You and Jones, 2012). DNA hypomethylation can be associated with gene reactivation and might lead to the upregulation or overexpression of proto-oncogenes, while DNA hypermethylation is frequently associated with silencing of suppression of tumor suppressor genes and compaction of chromatin. Although breast carcinomas are frequently hypomethylated on a genome-wide scale, the number of genes described as hypomethylated in breast cancer is relatively small. The endonucleases *FEN1*, the N-acetyltransferase *NAT1* and the cadherin *CDH3* are examples of genes that are hypomethylated in primary breast tumors (Jovanovic et al., 2010). On the other hand, more than one hundred genes have been reported to be hypermethylated in breast tumors or breast cancer cell lines. Many of these genes play important roles in cell-cycle regulation, DNA repair, apoptosis, tissue invasion and metastasis, angiogenesis and hormone signaling. *BRCA1*, *APC* and *BCL2* are examples of genes that may be silenced by hypermethylated in breast cancer (Jovanovic et al., 2010; Stefansson and Esteller, 2013). Another epigenetic alteration in breast cancer is the silencing of *RAD51* induced by the histone methyltransferase EZH2 (Stefansson and Esteller, 2013).

I.1.5 Hereditary Breast Cancer

Cancer usually occurs in one of three patterns: inherited, familial or sporadic. It is estimate that 5 to 10% of human tumors occur in individuals with an inborn cancer susceptibility (Bakry and Malkin, 2013). Some cancer predisposition syndromes that comprise breast cancer have been described, and include hereditary breast and ovarian cancer (HBOC), hereditary diffuse gastric cancer, and the Cowden, Peutz-Jeghers, and Li-Fraumeni syndromes (Table I.2). These syndromes are caused by germline mutations in the *BRCA1* and *BRCA2*, *CDH1*, *PTEN*, *STK11*, and *TP53* genes, respectively (Apostolou and Fostira, 2013; Easton et al., 2015; van der Groep et al., 2011).

Table I.2. List of the main susceptibility genes for breast cancer development and associated syndromes [Adapted from Apostolou and Fostira, 2013; Easton et al., 2015; van der Groep et al., 2011]

Gene involved	Cytoband	Breast cancer risk	Syndrome	Clinical features
BRCA1	17q21	High	Hereditary breast cancer and ovarian syndrome	Breast cancer, ovarian cancer
BRCA2	13q12.3	High		Breast cancer, ovarian cancer, prostate cancer, pancreatic cancer, melanoma
TP53	17p13.1	High	Li-Fraumeni syndrome	Breast cancer, sarcomas, brain tumors, adrenocortical cancer, leukemia
ATM	11q22.3	Intermediate	Ataxia telangiectasia syndrome	Lymphoma, cerebellar ataxia, immune deficiency, glioma, medulloblastoma (in homozygosity); breast cancer (in heterozygosity)
CDH1	16q22.1	Intermediate	Hereditary diffuse gastric cancer syndrome	Diffuse gastric cancer, lobular breast cancer
PTEN	10q23.31	Intermediate	Cowden syndrome	Breast, thyroid, and endometrial carcinomas, hamartomatous polyps of the gastrointestinal tract
STK11	19p13.3	Intermediate	Peutz-Jeghers syndrome	Melanocytic macules of the lips, oral mucosa and perioral region, multiple gastrointestinal hamartomatous polyps, increased risk of breast, testis, pancreas and ovarian cancer
NBS1	8q21	Intermediate	Nijmegen breakage syndrome	Microcephaly, growth retardation, immunodeficiency and a marked susceptibility to cancer (in homozygosity); moderate risk of breast cancer (in heterozygosity)
CHEK2	22q12.1	Intermediate	CHEK2- related	Breast, colorectal, ovarian, bladder cancers
PALB2/FANCN	16p12	Intermediate	Fanconi anemia	Aplastic anemia, acute myeloid leukemia and squamous cell carcinoma (in homozygosity); breast cancer (in heterozygosity)
FANCA	16q24.3	Low		
FANCE	6p22-p21	Low		

Breast cancers developing in individuals with specific hereditary predisposition syndromes have been shown to have preferential disease subtypes. For example, the majority of BRCA1-associated breast cancers shares the gene expression profile of sporadic basal-like tumors (estrogen receptor negative, progesterone receptor negative, and HER2-negative) (Masciari et al., 2012). On the other hand, BRCA2-associated breast cancers are often estrogen and progesterone receptor positive and HER2-negative (Masciari et al., 2012). Furthermore, several recent studies proposed that breast cancers in women with Li-Fraumeni syndrome are hormone receptor and HER2-positive (Lee et al.,

2012; Masciari et al., 2012; Melhem-Bertrandt et al., 2012; Rath et al., 2013; Wilson et al., 2010).

Whereas germline mutations in the *BRCA1* and *BRCA2* genes are clearly the most frequent known cause of hereditary breast cancer predisposition (Lalloo et al., 2003; Lee et al., 2012; Rapakko et al., 2001; Walsh et al., 2006), some studies demonstrated that *TP53* germline mutations occur at a comparable frequency with *BRCA1* and *BRCA2* germline mutations among very early-onset breast cancer patients (Lee et al., 2012; McCuaig et al., 2012). However, given the phenotypic differences mentioned above, the breast cancer subtype may help to identify and be taken into consideration in the genetic testing criteria.

I.1.6 Germline Mutations in the *TP53* gene

In 1979 p53 was discovered as a protein interacting with the oncogenic T antigen from SV40 virus (DeLeo et al., 1979; Kress et al., 1979; Lane and Crawford, 1979). The human *TP53* gene is located at the short arm of chromosome 17 (17p13.1) and encodes for a ubiquitous transcription factor involved in multiple cellular processes, including cell proliferation, differentiation, apoptosis, senescence, metabolism, angiogenesis and genomic stability (Ganguly and Chen, 2016; Gonzalez et al., 2009; Sorrell et al., 2013). The ability to activate transcription is the critical biochemical function of p53 that is intimately linked to its tumor suppressor activity (Bakry and Malkin, 2013). This gene contains 11 exons (first exon is not translated) and encodes a 393 amino acid protein of 53kDa. The p53 protein is composed of an acidic N-terminal transactivation domain (amino acids 1-42), a proline-rich domain (amino acids 40-92), a centralized DNA-binding domain (amino acids 101-306), an oligomerization domain (amino acids 307-355) and a basic C-terminal regulatory domain (Freed-Pastor and Prives, 2012). The central DNA-binding domain is essential to the protein-DNA interaction (Ganguly and Chen, 2016).

Somatic mutations in the tumor suppressor gene *TP53* are frequently observed in most types of human cancers (Baker et al., 1989; Nigro et al., 1989), namely in sporadic basal and HER2-positive breast cancer subgroups (Rath et al., 2013). On the other hand, germline mutations in the *TP53* gene are associated with a the phenotypically heterogeneous Li-Fraumeni syndrome (Bakry and Malkin, 2013; Lacroix et al., 2006). This genetic disease is a rare inherited cancer susceptibility syndrome described in 1969 (Li and Fraumeni, 1969), with an autosomal dominant mode of inheritance, and characterized by a diversity of early onset tumors (typically sarcomas, brain tumors, adrenocortical cancers, leukemias and breast cancers) (Gonzalez et al., 2009). Surprisingly, the tumor spectrum observed in this syndrome is quite different from the tumors usually associated with somatic *TP53* mutations. In contrast to epithelial cancers where somatic mutations are prevalent, patients with Li-Fraumeni syndrome present more commonly tumors of embryonal,

neuroectodermal, and mesenchymal cell lineage, with the exceptions of carcinomas of the breast, choroid plexus epithelium, and adrenal gland (Bakry and Malkin, 2013). The lifetime risk of cancer in Li-Fraumeni syndrome 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).

The vast majority of cancer-associated mutations in *TP53* are missense alterations, which consist in substitutions of one amino acid for another leading to alterations in the protein conformation or functionality. This type of mutations is usually transcriptionally inactive (Inoue et al., 2012; Sorrell et al., 2013). Other alterations include splice site mutations (10.6%), nonsense mutations (7.7%), frameshift insertions and deletions (6.6%), as well as other infrequent alterations such as large deletion and deep intronic mutations (Figure I.4) (Ganguly and Chen, 2016; Petitjean et al., 2007).

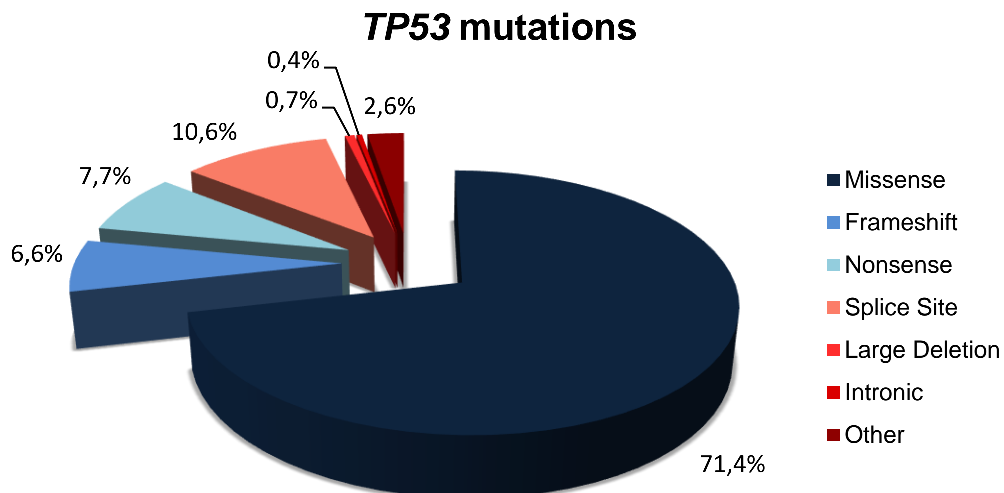


Figure I.4. Type of germline mutations in the *TP53* gene [Adapted from Ganguly and Chen, 2016]

The great majority of the germline mutations (83.7%) in the *TP53* gene occur within exons 5-8 (amino acids 126-306), the DNA binding domain, and there are six hotspot mutational hotspots at residues Arg175, Arg213, Gly245, Arg248, Arg273 and Arg337 (Figure I.5) (Ganguly and Chen, 2016). These mutations frequently interfere with DNA binding or disrupt the structure of the binding surface, interfering with the protein ability to regulate transcription of target genes and consequently losing the ability to mediate most if not all of p53's multiple functions (Bakry and Malkin, 2013; Ganguly and Chen, 2016). Germline mutations in this domain cause highly penetrant disease with very early onset cancers, while mutations outside the DNA binding domain are associated with slower rates of tumor development (Sorrell et al., 2013).

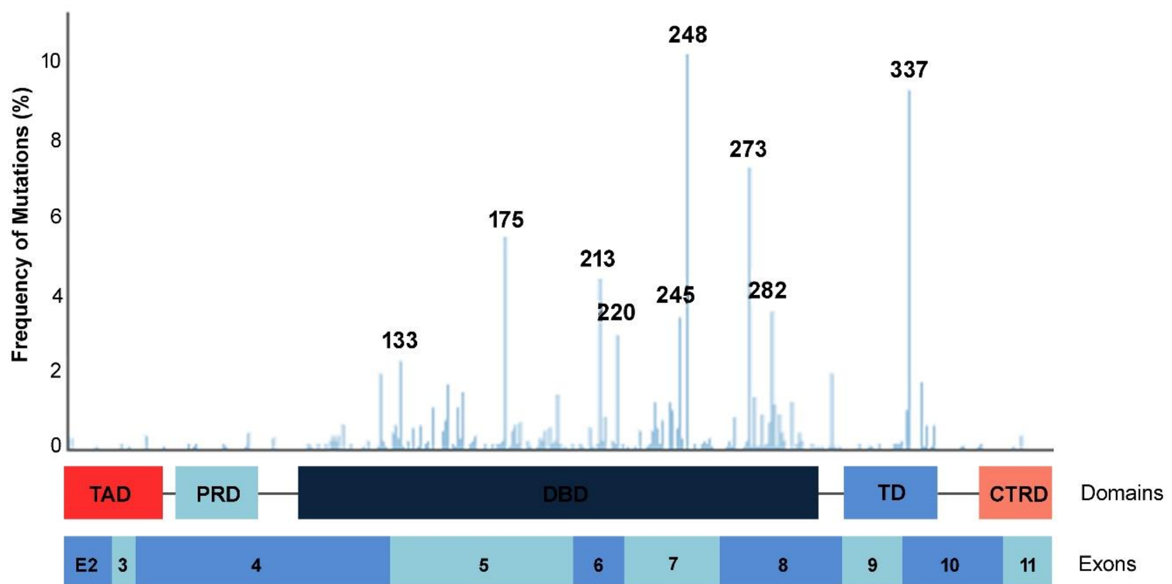


Figure I.5. Distribution of germline mutations in the *TP53* gene Abbreviations: TAD, transactivation domain; PRD, proline-rich domain; DBD, DNA-binding domain; TD, tetramerization domain; CTRD, C-terminal regulatory domain. [Adapted from the International Association for Research on Cancer databases (R18, April 2016)]

Mutations in *TP53* can generally be classified as either “DNA contact” (class I) or “conformational” (class II) mutants. The first are missense mutations in the amino acid residues that normally make direct contact with target DNA sequences, whereas the conformational mutants are missense mutations that disrupt the structure of the p53 protein on either a local or global level (Freed-Pastor and Prives, 2012; Liu et al., 2010).

I.1.7 HER2-positive Breast Cancer

The *HER2* gene (*ERBB2* is the official name provided by the HUGO Gene Nomenclature Committee) is located at the long arm of chromosome 17 (17q12) and encodes a transmembrane receptor with tyrosine kinase activity but without a known ligand (Mano et al., 2007). HER2 belongs to a family of four receptors (EGFR/HER1, HER2, HER3, HER4) and it is capable of homodimerization and heterodimerization with any of the other three HER proteins. These receptors are involved in regulating cell growth, survival and differentiation through activation of the PI3K/Akt and the Ras/Raf/MEK/MAPK pathways (Figure I.6) (Alotaibi et al., 2015; Arteaga et al., 2012).

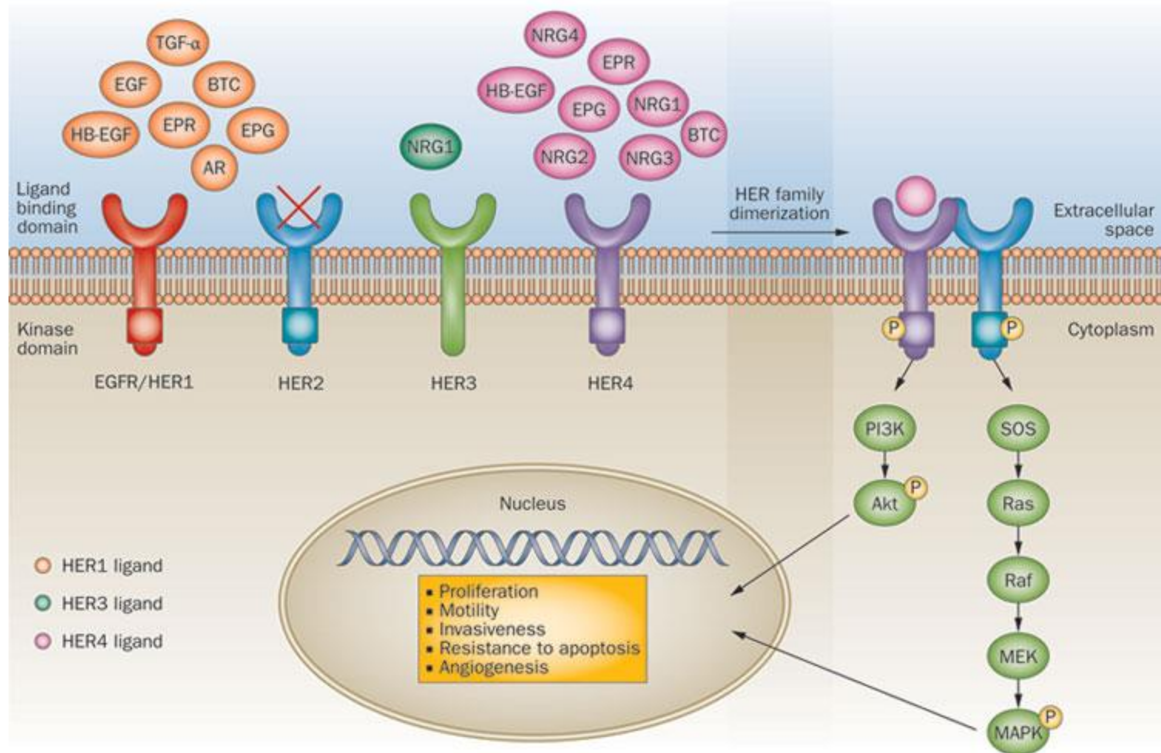


Figure I.6. Heterodimer formation of members of the HER family and downstream signaling. Abbreviations: AR, amphiregulin; BTC, betacellulin; EPG, epigen; EPR, epiregulin; HB-EFG, heparin-binding EGF-like ligand; NRG, neuregulin [Arteaga et al., 2012]

In young women with breast cancer, 20 to 25% have HER2-positive tumors (Rath et al., 2013). The DNA amplification of the *HER2* gene can be in tandem arrays, as head-to-tail or head-to-head repeats, within a chromosome or may occur in extrachromosomal entities called double minutes. Double minutes do not contain centromeres and so do not bind the mitotic spindle, being likely not distributed equally between daughter cells. Vicario and co-workers (2015) showed, in metaphases and nuclei, that the *HER2* gene is amplified in double minutes or in tandem arrays regions in approximately 30 and 60% of HER2-positive breast tumors, respectively (Vicario et al., 2015).

HER2-positive tumors have been associated with poor histological features and aggressive behavior, including poorly differentiated and high-grade tumors, high rates of cell proliferation, lymph-node involvement, and resistance to certain types of hormonal and chemotherapies (Mano et al., 2007; Masciari et al., 2012). The American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines recommend that HER2 status should be determined for all invasive breast cancers (Wolff et al., 2013). The most popular genetic test for assessing *HER2* amplification is fluorescence *in situ* hybridization (FISH). However, other techniques can also be used, for example, chromogenic *in situ* hybridization (CISH) and silver *in situ* hybridization (SISH) (Papouchado et al., 2010; Wolff et al., 2007). In clinical diagnosis the most common approach is to screen

for protein overexpression with immunohistochemistry (IHC) and confirm the presence of gene amplification only in equivocal cases (scores of 2+). Despite the regular use of IHC, FISH has been considered a more reproductive test (Mano et al., 2007). According to the ASCO/CAP guidelines, positive HER2 status is defined as a score of 3+ in IHC and/or a HER2/CEP17 ratio of 2.0 or more. A ratio below 2.0 with an average HER2 copy number of ≥ 6.0 signals per cell is also considered positive. Negative HER2 status is defined as a score of 0 or 1+ in IHC and/or a ratio below 2.0 with an average HER2 copy number below 4.0 signals per cell in dual-probe FISH. Equivocal HER2 status is defined as a score of 2+ in IHC and/or a ratio below 2.0 with an average HER2 copy number ≥ 4.0 and < 6.0 signals/cell (Wolff et al., 2013).

The outcome for women with HER2-positive tumors has improved markedly after the introduction of HER2 targeted therapies. Trastuzumab (Herceptin), a humanized monoclonal antibody, was initially shown to improve response rates, time to progression, and even survival, with an acceptable safety record, when used alone or added to chemotherapy in advanced HER2-positive disease (Pegram et al., 2004; Robert et al., 2006; Tripathy et al., 2004). Furthermore, it has been approved as adjuvant treatment for patients with HER2-positive early stage breast cancer (Gianni et al., 2011; Smith et al., 2007). Lapatinib is a small molecule tyrosine kinase inhibitor that reversibly inhibits the intracellular tyrosine kinase activity of both HER2 and EGFR, suppressing tyrosine autophosphorylation and thereby downstream pathways. Preclinical studies showed that lapatinib could inhibit the growth of HER2-positive breast cancer cells that were resistant to trastuzumab and that this small molecule could enhance the apoptotic effect of anti-HER2 antibodies (Arteaga et al., 2012; Wolff et al., 2013). In advanced-stage disease, pertuzumab (another monoclonal antibody) is approved in conjunction with trastuzumab. This monoclonal antibody binds to the HER2 extracellular domain in a different site to trastuzumab, and is able to inhibit ligand-induced dimerization of HER2 with its receptor partners (Arteaga et al., 2012; Wolff et al., 2013).

I.1.7.1 HER2 Status and Germline *TP53* Mutations

Some recent studies have shown that HER2 amplification or overexpression in a young woman may be a useful marker for identifying germline *TP53* mutations (Lee et al., 2012; Melhem-Bertrandt et al., 2012; Rath et al., 2013; Wilson et al., 2010). Wilson et al. (2010) and Masciari et al. (2012) showed that 83% and 63%, respectively, of breast cancers in women with germline mutations in *TP53* gene were positive for HER2 (Masciari et al., 2012; Wilson et al., 2010), which is significantly higher than the proportion of sporadic HER2-positive breast carcinomas (about 20%) (Hanna et al., 2014). Melhem-Bertrandt and co-workers (2012) tested 109 female breast cancer patients with invasive carcinoma and

compared the pathological characteristics from patients testing positive for a germline mutation in *TP53* gene with patients testing negative for that gene. They detected that the presence of HER2-positive tumors was significantly different between the group with germline *TP53* mutation (20/30) and the group without mutations (20/79). Estrogen receptor and progesterone receptor status were equally distributed between groups. Furthermore, in the multicovariate logistic regression analysis, they observed that for each increase in age at breast cancer diagnosis there is a decreased likelihood of having a *TP53* mutation by 5%, and in young women diagnosed with HER2-positive disease the odds of having a *TP53* germline mutation increased by nearly 7-fold (Melhem-Bertrandt et al., 2012). However, another study reported germline *TP53* pathogenic mutations in only 5% of HER2-positive breast cancer patients diagnosed before the age of 35 years (Rath et al., 2013).

II. AIMS

The general aim of this work is to study the mechanisms of inherited predisposition of early-onset HER2-positive breast cancer. The specific aims are:

- ✓ To identify the contribution of germline *TP53* mutations for early-onset HER2-positive breast cancer;
- ✓ To identify other genes with germline mutations in patients with early-onset HER2-positive breast cancer;
- ✓ To define the genetic testing criteria to complement established recommendations;
- ✓ To compare the *HER2* amplification pattern in breast carcinomas from patients with pathogenic *TP53* germline mutations with that of patients with other germline mutations.

III. MATERIALS AND METHODS

III.1 Patients and Sample Collection

A consecutive series of DNA samples from 88 women with HER2-positive invasive breast cancer diagnosed before the age of 41 years were retrospectively selected for analysis by Sanger sequencing. Those patients had been referred to the Genetics Department of IPO-Porto between June 2006 and June 2014 for *TP53* and/or *BRCA1* and *BRCA2* germline mutation analysis. Two of these patients had already done the research of germline mutations in the *TP53* gene once they meet the Chompret criteria. However, the test result was negative for mutation in *TP53* gene.

Another consecutive series of DNA samples from 36 women with HER2-positive invasive breast cancer diagnosed before the age of 41 years was collected for gene-panel next generation sequencing (NGS), after being to the Genetics Department of IPO-Porto between November 2014 and February 2016 for *TP53* and/or *BRCA1* and *BRCA2* germline mutation analysis.

III.2 *TP53* Germline Mutation Analysis

Screening for germline mutations of the entire coding region (exons 2-11) and associated splice junctions of the *TP53* gene was performed by Sanger sequencing in 88 peripheral blood samples. For this purpose, approximately 20 ng of DNA were amplified in a solution containing 1x PCR Gold Buffer [Applied Biosystems, Foster City, CA, USA] (150 mM Tris-HCl, 500 mM KCl), 1.5 mM of MgCl2 [Applied Biosystems], 0.75 mM dNTP mix [Applied Biosystems], 1.25 mM of each primer (reverse and forward) [Frilabo, Maia, Portugal], 0.5 U of AmpliTaq Gold [Applied Biosystems] and bidestiled sterile water [B. Braun, Foster City, CA, USA] in a final reaction volume of 20 µL.

Due to the size of the gene in analysis, its amplification was performed using several primer sets (Table III.1).

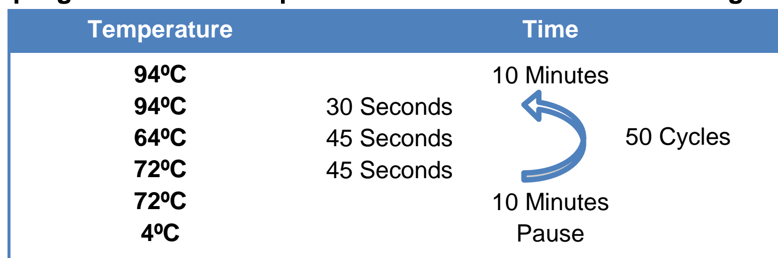
Table III.1. Primers used for PCR

Region amplified	Direction	Primer pairs	Product length
Exons 2-3	F	5'-TGTCTCAGACACTGGCATGG-3'	447 bp
	R	5'-TGAAAAGAGCAGTCAGAGGAC-3'	
Exons 2-3	F	5'-TCTCATGCTGGATCCCCACT-3'	344 bp
	R	5'-AGTCAGAGGACCAGGTCCTC-3'	
Exon 4	F	5'-TGCTCTTTTCACCCATCTAC-3'	353 bp
	R	5'-ATACGGCCAGGCATTGAAGT-3'	
Exons 5-6	F	5'-TGTTCACTTGTGCCCTGACT-3'	467 bp
	R	5'-TTAACCCCTCCTCCCAGAGA-3'	
Exon 7	F	5'-CTTGCCACAGGTCTCCCCAA-3'	237 bp
	R	5'-AGGGGTCAGAGGCAAGCAGA-3'	
Exons 8-9	F	5'-TTGGGAGTAGATGGAGCCT-3'	445 bp
	R	5'-AGTGTTAGACTGGAAACTTT-3'	
Exon 10	F	5'-CAATTGTAACTTGAACCATC-3'	260 bp
	R	5'-GGATGAGAATGGAATCCTAT-3'	
Exon 11	F	5'-AGACCCTCTCACTCATGTGA-3'	245 bp
	R	5'-TGACGCACACCTATTGCAAG-3'	

F: Forward; R: Reverse.

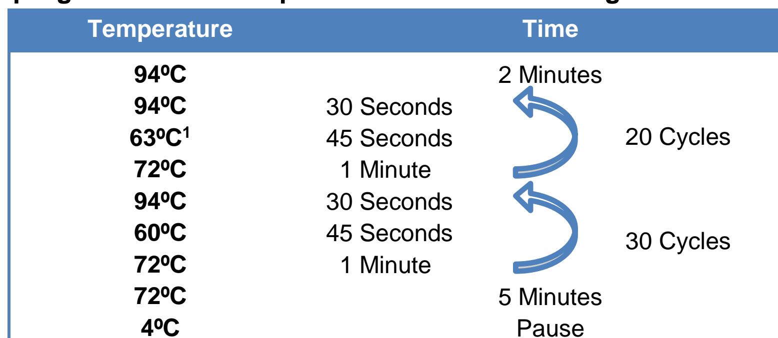
The amplification of exons 2 and 3 was performed with two different primer sets because there is a polymorphism in this region that hinders DNA amplification/sequencing. PCR reactions were performed in a thermocycler [Gene Amp PCR Systern 9700, Perkin-Elmer, Waltham, Massachusetts, USA] according to the conditions of Table III.2 for the second primer set of Table III.1 and according to the conditions of Table III.3 for the remaining primer sets used during this work.

Table III.2. PCR program used for amplification of exons 2-3 of the *TP53* gene



Temperature	Time	
94ºC	10 Minutes	
94ºC	30 Seconds	50 Cycles
64ºC	45 Seconds	
72ºC	45 Seconds	
72ºC	10 Minutes	
4ºC	Pause	

Table III.3. PCR program used for amplification of the remaining exons of *TP53*



Temperature	Time	
94ºC	2 Minutes	
94ºC	30 Seconds	20 Cycles
63ºC[1]	45 Seconds	
72ºC	1 Minute	
94ºC	30 Seconds	30 Cycles
60ºC	45 Seconds	
72ºC	1 Minute	
72ºC	5 Minutes	
4ºC	Pause	

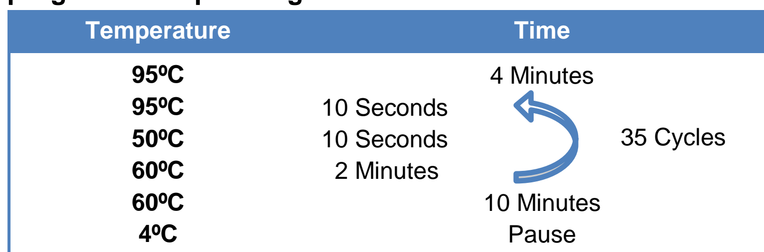
[1] -0.5ºC every 3 cycles

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

Before the sequencing reaction, the PCR amplification products were purified using the ExoSAP-IT method to remove excess of primers, salts, enzymes and dNTPs from the previous reaction. Samples were purified adding 2 µL of ExoSAP solution (Exonuclease I [Thermo Fisher Scientific, Waltham, Massachusetts, USA] (20 U/µL) and Fast Thermosensitive Alkaline Phosphatase [Thermo Fisher Scientific] (1 U/µL), in a proportion of 1:2) to 5 µL of the PCR product, followed by incubation at 37ºC for 50 minutes, and enzyme inactivation at 80ºC for 15 minutes.

The purification was followed by the sequencing reaction in which the BigDye® Terminator v3.1 Cycle Sequencing Kit [Applied Biosystems] was used. 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 µL of the previously purified DNA to reach a final reaction volume of 10 µL. The sequencing reaction was performed according to the conditions of Table III.4.

Table III.4. PCR program of sequencing reaction



Temperature	Time	
95ºC	4 Minutes	
95ºC	10 Seconds	35 Cycles
50ºC	10 Seconds	
60ºC	2 Minutes	
60ºC	10 Minutes	
4ºC	Pause	

In order to remove excess of dNTPs, labeled 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-DiTM 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, State College, PA, USA] by two independent observers.

Approximately 75% of disease-causing mutations can be identified in exons 5 to 8 of the *TP53* gene by sequence analysis. Variants identified in the sequence can be

classified as pathogenic mutations, benign variants, or variations of uncertain clinical significance (Ganguly and Chen, 2016). All *TP53* variants described are according to the LRG_321 (NM_006231.3) and to the Human Genome Variations Society guidelines. The polymorphisms found in *TP53* gene were not reported.

III.3 Gene-panel Analysis by Next-Generation Sequencing

Screening for germline mutations in a panel of 94 genes associated with cancer predisposition (Table III.5) was performed by next-generation sequencing in 36 peripheral blood samples. For this purpose, the Nextera DNA transposome [Illumina, San Diego, CA, USA] was used to convert input genomic DNA (gDNA) into adapter-tagged indexed libraries. Approximately 50 ng of input gDNA was used in the tagmentation process, which involves simultaneous fragmentation and adapter tagging of gDNA followed by adapter ligation.

Table III.5. Trusight Cancer gene list

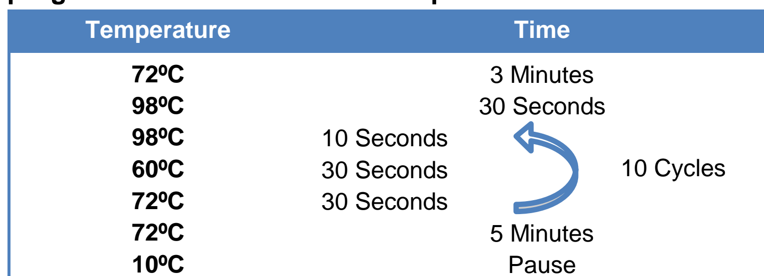
Genes					
AIP	*CEBPA*	*FANCA*	*KIT*	*PRF1*	
ALK	*CEP57*	*FANCB*	*MAX*	*PRKAR1A*	*SLX4*
APC	*CHEK2*	*FANCC*	*MEN1*	*PTCH1*	*SMAD4*
ATM	*CYLD*	*FANCD2*	*MET*	*PTEN*	*SMARCB1*
BAP1	*DDB2*	*FANCE*	*MLH1*	*RAD51C*	*STK11*
BLM	*DICER1*	*FANCF*	*MSH2*	*RAD51D*	*SUFU*
BMPR1A	*DIS3L2*	*FANCG*	*MSH6*	*RB1*	*TMEM127*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

The tagmented DNA was purified by Sample Purification Beads [Illumina], and then it was analyzed by high-resolution capillary electrophoresis in a QIAxcel Advanced system [QUIAGEN]. The electrophoresis results were analyzed using the QIAxcel ScreenGel software [QUIAGEN].

The purification reaction was followed by the first PCR amplification, in which the purified tagmented DNA was amplified and index 1 and index 2 were added. This PCR

reaction is required for cluster generation and sequencing. 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 III.6.

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

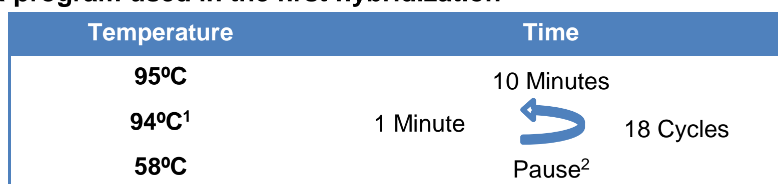


Temperature	Time	
72ºC	3 Minutes	
98ºC	30 Seconds	
98ºC	10 Seconds	10 Cycles
60ºC	30 Seconds	
72ºC	30 Seconds	
72ºC	5 Minutes	
10ºC	Pause	

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

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 III.7.

Table III.7. PCR program used in the first hybridization



Temperature	Time	
95ºC	10 Minutes	
94ºC[1]	1 Minute	18 Cycles
58ºC	Pause[2]	

[1] -2ºC per cycle

[2] for at least 90 minutes and up to a maximum of 24 hours

The first hybridization was followed by capture of 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 by Sample Purification Beads [Illumina], 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 III.8.

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

Temperature	Time	
72ºC	3 Minutes	
98ºC	30 Seconds	
98ºC	10 Seconds	12 Cycles
60ºC	30 Seconds	
72ºC	30 Seconds	
72ºC	5 Minutes	
10ºC	Pause	

The PCR products were purified using the Sample Purification Beads [Illumina]. The tagged and amplified sample libraries were quantified using a Qubit 2.0 Fluorometer [Invitrogen] and the quality of the library was checked using the high-resolution capillary electrophoresis in QIAxcel Advanced system [QUIAGEN]. The pools were diluted to 4 nM in a final volume of 10 µL with HT1 (Hybridization Buffer) [Illumina] and subsequently joined 5 µL of each pool. The pooled library (12 pM) was loaded and sequenced on the MiSeq platform [Illumina], according to the manufacturer's instructions.

The trimmed FASTQ files were generated using MiSeq Reporter [Illumina]. Reads were aligned against the whole-genome build Isaac Enrichment v.2.1 [Illumina]. All variants found were confirmed by Sanger sequencing using the primer sequences of the respective genes (Table III.9).

Table III.9. Primers used for PCR of *BRCA2* exons 23-24 and *FANCA* exon 4

Gene	Region amplified	Direction	Primer pairs	Product length
BRCA2	Exons 23-24	F	5'-TCCACTACTAATGCCCACAAAGAGA-3'	613 bp
		R	5'- CAAATTTGCCAACTGGTAGCTCC-3'	
FANCA	Exon 4	F	5'-AGGTGTTGCCACCAGTTTTATTG-3'	404 bp
		R	5'-CAGCTTAAAAGTAACAACGGGCA -3'	

F: Forwrad; R: Reverse.

The *BRCA2* mutation was described according to the LRG_293 (NM_000059.3) and the *FANCA* mutation was described according to the LRG_495 (NM_000135.2), both taking into account the Human Genome Variation Society guidelines. The polymorphisms found were not reported.

III.4 Fluorescence *In Situ* Hybridization in Tissue Sections

Screening of *HER2* amplification was performed by Fluorescence *In Situ* Hybridization (FISH) in sections from paraffin-embedded tumor from patients with germline variants. Slides were deparaffinized in two passages through xylol, followed by other two passages through 100% ethanol, 5 minutes each. For pre-treatment, slides were incubated in 2x SSC for 3 minutes, followed by the incubation with NaSCN 1M at 80ºC for 10 minutes and then rinsed in 2xSSC for 2 minutes. The enzymatic digestion was made through incubation of a pepsin solution (4mg/mL) with each slide at 37ºC for 6 minutes. In order to finish the digestion, the slides were placed two times in a 2xSSC solution for 2 minutes each, followed by an increasing series of ethanols, 70%, 85% and 100%, for 2 minutes each. After dehidratation, the specific probe sets were applied onto each sample. The *HER2* probe was combined with a centromeric probe for chromosome 17 (ON ERBB2 Her-2/Neu (17q12) / SE 17, Kreatech). Slides were placed in a ThermoBrite denaturation/hybridization system (Leica Biosystems, Nussloch, Germany) and co-denaturated at 80ºC for 8 minutes, followed by hybridization for 18 hours at 37ºC. The slides were then washed in a 2xSSC/0.5% IGEPAL (Sigma Aldrich) solution at 74º for 5 minutes and 2XSSC/0.1% IGEPAL at room temperature (RT) for 3 minutes. Finally, slides were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, California, USA).

The slides were analyzed and fluorescent images corresponding to DAPI, Spectrum Green and Spectrum Orange were automatically captured in a GSL-120 Automated Cytogenetics Platform with a CytoVision Software Version number 7.4 (Leica Biosystems). For amplification scoring the image processing uses a fixed Modal Average (ModeSA) of 0.25 microns squared (μm^2) for the reference size of a test signal. CytoVision image processing evaluates the signal size, breaking up large or clustered signals greater than 0.5 μm^2 by dividing by 0.25 to calculate the signal count.

Results were evaluated according to ASCO/CAP recommendations for *HER2* testing in breast cancer (Wolff et al., 2013). *HER2* gene amplification by FISH assay was performed in the invasive component of a breast cancer specimen. Amplification in a dual-probe FISH assay was defined by examining first the HER2/CEP17 ratio followed by the average *HER2* copy number. If there was a second contiguous population of cells with increased *HER2* signals per cell, and this cell population consists of more than 10% of tumor cells on the slide (defined by image analysis or visual estimation of the slide), a separate counting was also performed.

A ratio of average *HER2* and centromeric probe signals of at least two representative non overlapping cancer cell populations (at least 60 cells) were computed for each sample. Cases were categorized as negative for *HER2* amplification whenever HER2/CEP17 < 2.0 with an average *HER2* copy number < 4.0 signals/cell, and as having *HER2* amplification

when HER2/CEP17 ≥ 2.0 or HER2/CEP17 < 2.0 with an average *HER2* copy number ≥ 6.0 signals/cell. Cases were categorized as equivocal when HER2/CEP17 < 2.0 with an average *HER2* copy number ≥ 4.0 and < 6.0 signals/cell.

IV. RESULTS

IV.1 *TP53* Germline Variants in the Retrospective Cohort

DNA samples from 88 women with HER2-positive invasive breast cancer were screened for germline mutations in all coding exons of the *TP53* gene (exons 2 to 11) by Sanger sequencing. Three heterozygous variants (Table IV.1) were found in three cases, corresponding to a frequency of *TP53* germline variants of 3.41% in this retrospective cohort.

Table IV.1. Germline variants found in the *TP53* gene

Sample number	cDNA description	Protein description	Exon number	Effect	Biological Significance
#32	c.642T>G	p.(His214Gln)	Exon 6	Missense	VUS
#60	c.524G>A	p.(Arg175His)	Exon 5	Missense	Pathogenic
#72	c.559+19_559+35del	p.?	Intron 5	Intronic	VUS

VUS: Variant of uncertain significance

IV.1.1 Evaluation of *TP53* variants

The first variant consists of a nonsynonymous substitution of a Thymine for a Guanine (transversion, c.642T>G) in the third position of codon 214 (CAT → CAG), resulting in a nonconservative substitution of a Histidine for a Glutamine, p.(His214Gln) (figure IV.1a). This variant has already been described (Kakudo et al., 2005; Lacroix et al., 2006), but the disease-association remains unclear and is therefore classified as a variant of uncertain significance (VUS) for the time being.

The second variant consists of a nonsynonymous substitution of a Guanine for an Adenine (transversion, c.524G>A) in the second position of codon 175 (CGC → CAC), resulting in a nonconservative substitution of an Arginine for a Histidine, p.(Arg175His) (figure IV.1b). This variant occurs in a mutation hotspot and is classified as a deleterious mutation (Freed-Pastor and Prives, 2012; Kakudo et al., 2005).

The third variant consists of a deletion of 17 base pairs (c.559+19_559+35del) in intron 5 (figure IV.1c). This variant has not yet been included in any databases, such as COSMIC (Catalogue of Somatic Mutations in Cancer), Ensemble, LOVD (Leiden Open Variation Database) or IARC *TP53* databases and is therefore classified as a VUS.

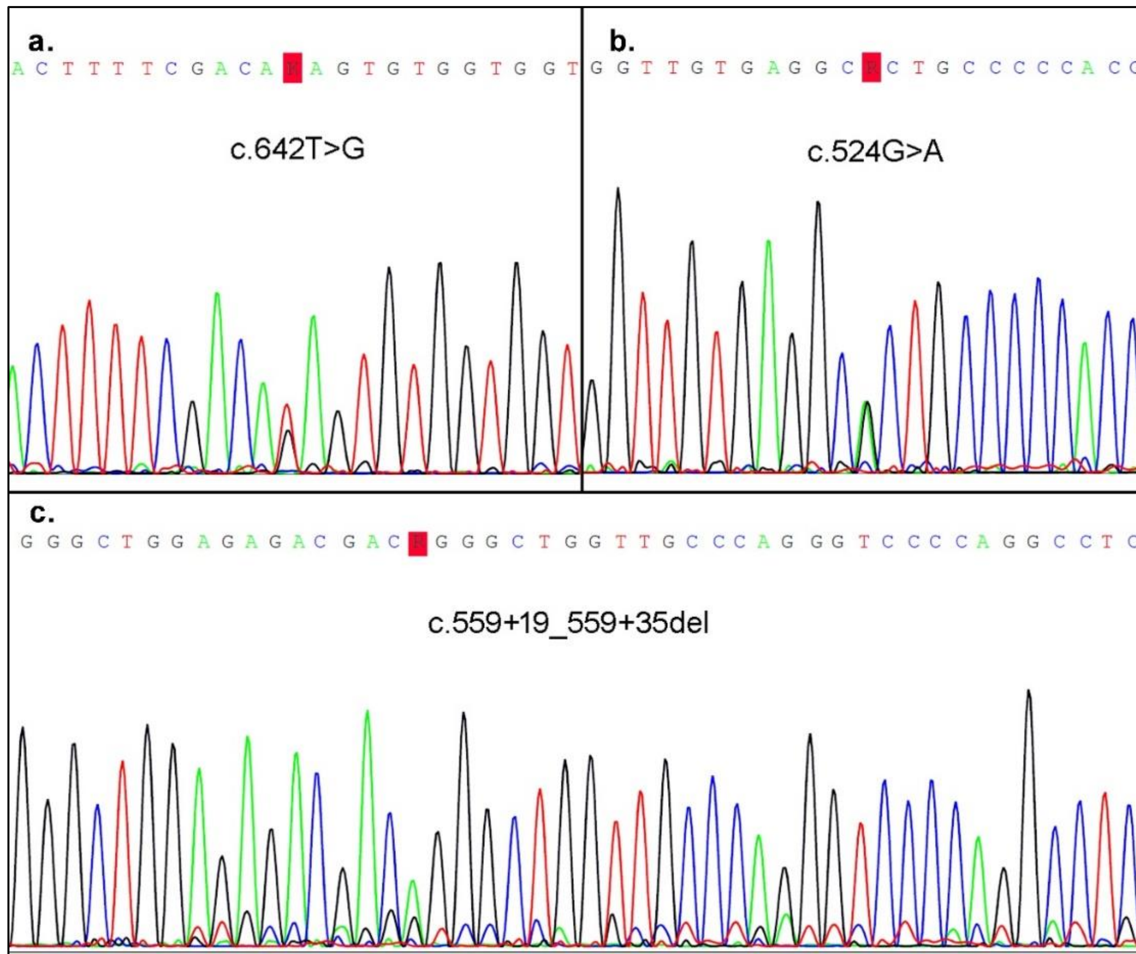


Figure IV.1. DNA sequence electropherograms obtained from peripheral blood samples: a. *TP53* c.524G>A, p.(Arg175His); **b.** *TP53* c.642T>G, p.(His214Gln); **c.** *TP53* c.559+19_559+35del, p.?.

IV.1.2 Clinicopathological Characteristics of *TP53* variant carriers

Patient #32, with the *TP53* variant c.642T>G, p.(His214Gln), is a woman who was diagnosed at age 26 years with an invasive ductal breast carcinoma, grade III. Immunohistochemical analysis showed negative expression of estrogen and progesterone receptors, and HER2 was classified as 3+. According to the TNM classification, the tumor stage was pT2N2M0. The patient had previously been tested for germline mutation in *BRCA1* and *BRCA2* genes and did not present any mutation. This patient has no family history of cancer, as it is shown in the pedigree (Figure IV.2a).

Patient #60, with the *TP53* mutation c.524G>A, p.(Arg175His), is a woman who was diagnosed at age 31 years with a medullary carcinoma, grade III. Immunohistochemical analysis showed negative expression of estrogen and progesterone receptors, and HER2 was classified as 3+. According to the TNM classification, the tumor stage was pT1N0M0. The patient's peripheral blood had previously been tested for mutations in *BRCA1* and *BRCA2* genes and three VUS in the *BRCA1* gene were found. This patient's family comprises many relatives affected with gastric, colon, pancreas and breast tumors. From

the mother's side, there are three relatives in the first-degree and one relative in second-degree affected with gastric cancer at a young age; in the father's side has a cousin affected with breast cancer at a young age and grandmother with colon cancer (Figure IV.2b).

Patient #72, with the *TP53* variant c.559+19_559+35del, is a woman who was diagnosed at age 35 years with an invasive ductal breast carcinoma, grade III. Immunohistochemical analysis showed positive expression of estrogen receptor (75-100%), positive expression of progesterone receptor (50-75%), and HER2 was classified as 3+. According to the TNM classification, the tumor stage was pT2N0(sn). The patient's peripheral blood had previously been tested for *BRCA1* and *BRCA2* Portuguese founder mutations and the results were negative. This patient has no family history of cancer (Figure IV.2c).

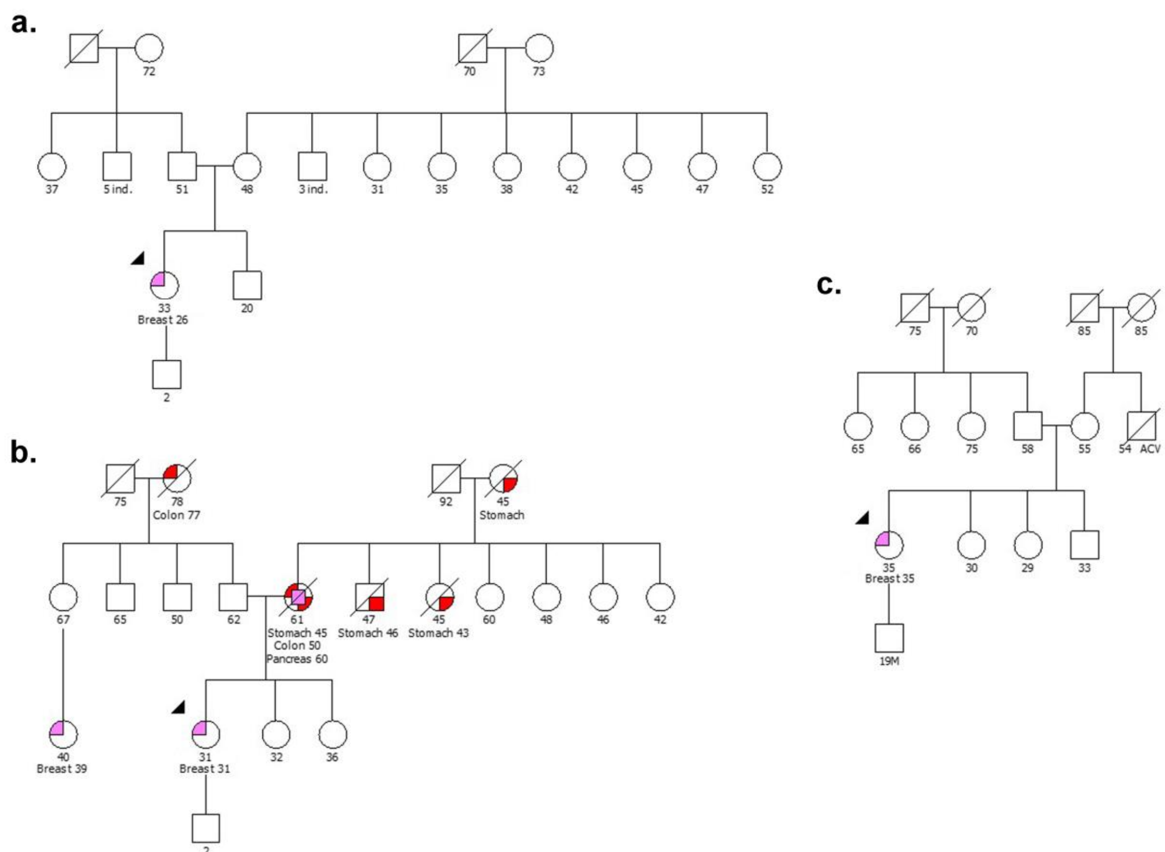


Figure IV.2. Pedigrees of the three patients with germline variants in the *TP53* gene in the retrospective series: a. patient #32; **b.** patient #60; **c.** patient #72

IV.2 Germline Mutation Spectrum in the Prospective Cohort

DNA samples from 36 women with HER2-positive invasive breast cancer were screened by next-generation sequencing for germline mutations in 94 genes known to play

a role in cancer predisposition. Four heterozygous variants (Table IV.2) were found in four cases.

Table IV.2. Germline variants found by next-generation sequencing

Sample number	Gene	cDNA description	Protein description	Exon number	Effect	Biological Significance
#93	*BRCA2*	c.9105T>A	p.(Tyr3035Ter)	Exon 23	Nonsense	Pathogenic
#104	*TP53*	c.383delC	p.(Pro128LeufsTer42)	Exon 5	Frameshift	Pathogenic
#107	*FANCA*	c.295C>T	p.(Gln99Ter)	Exon 4	Nonsense	Pathogenic
#122	*TP53*	c.935C>G	p.(Thr312Ser)	Exon 9	Missense	VUS

VUS: Variant of uncertain significance

IV.2.1 Evaluation of Germline Carriers

Patient #93 presents a mutation in the *BRCA2* gene consisting of a nonsynonymous substitution of a Thymine for an Adenine (transversion, c.9105T>A) in the third position of codon 3035 (TAT → TAA) and resulting in a nonsense mutation: p.(Tyr3035Ter) (Figure IV.3a). This mutation has not yet been included in any database, such as the COSMIC or Ensemble databases, but by its nature and localization it is classified as pathogenic.

Patient #104 has a mutation in the *TP53* gene consisting of a deletion of a Cytosine (c.383delC) in the second position of codon 128 (CCT → C-T) and resulting in a frameshift deletion: p.(Pro128LeufsTer42) (Figure IV.3b). This mutation was reported in the COSMIC database as a somatic event in breast invasive carcinoma, but by its nature it is classified as pathogenic.

In Patient #107 a mutation in the *FANCA* gene was found. This mutation consists of a nonsynonymous substitution of a Cytosine for a Thymine (transversion, c.295C>T) in the first position of codon 99 (CAG → TAG), resulting in a nonsense mutation: p.(Gln99Ter) (Figure IV.3c). This mutation has been described in unrelated Spanish Gypsy patients with Fanconi anemia (Callen et al., 2005; Gille et al., 2012) in which it is considered deleterious.

Patient #122 presents a variant in the *TP53* gene consisting of a nonsynonymous substitution of a Cytosine for a Guanine (transversion, c.935C>G) in the second position of codon 312 (ACC → AGC) and resulting in a nonconservative substitution of a Threonine for a Serine: p.(Thr312Ser) (Figure IV.3c). This variant has been described as having an uncertain biological significance by ClinVar and LOVD databases.

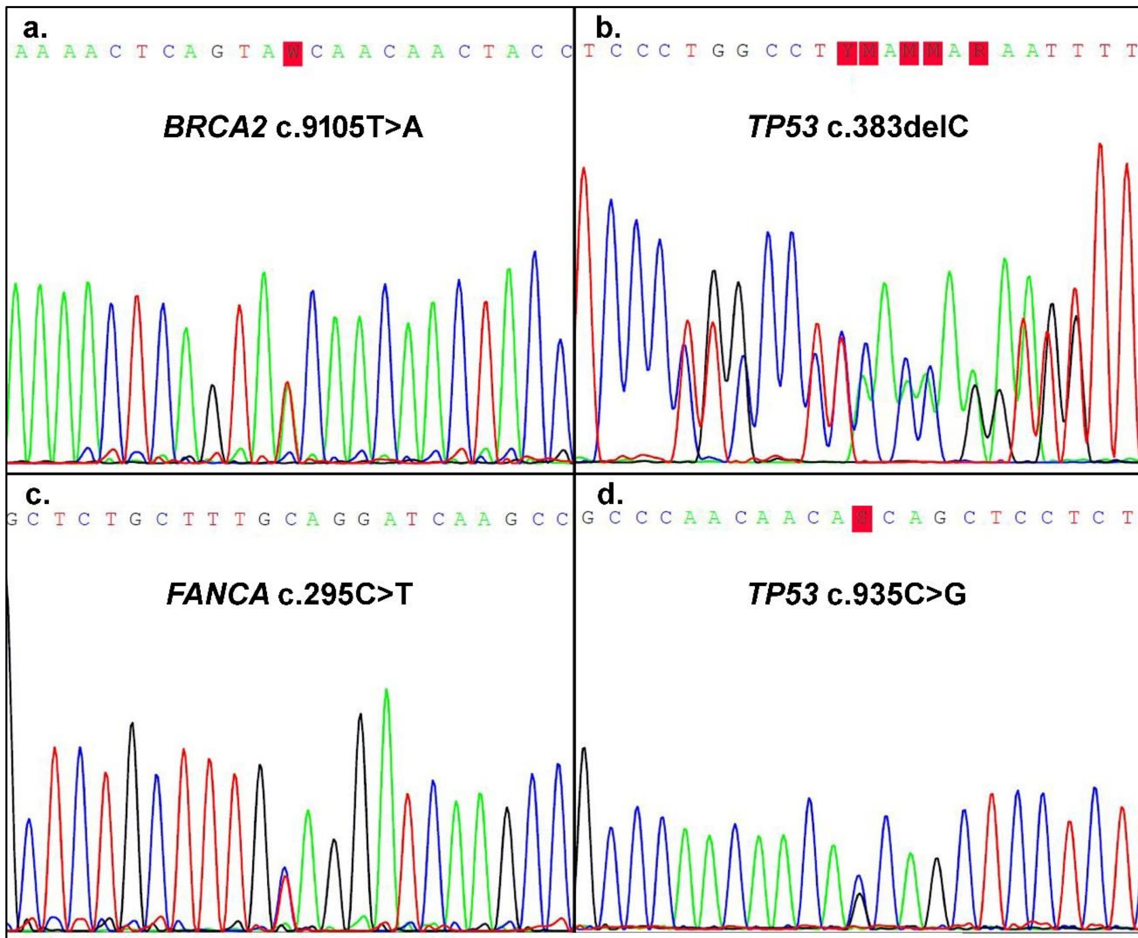


Figure IV.3. DNA sequence electropherograms obtained from peripheral blood samples: a. *BRCA2* c.9105T>A, p.(Tyr3035Ter); **b.** *TP53* c.383delC, p.(Pro128LeufsTer42); **c.** *FANCA* c.295C>T, p.(Gln99Ter); **d.** *TP53* c.935C>G, p.(Thr312Ser).

IV.2.2 Clinicopathological Characteristics of Variant Carriers

Patient #93, with the *BRCA2* mutation c.9105T>A, p.(Tyr3035Ter), is a woman who was diagnosed at age 30 years with an invasive micropapillary carcinoma, grade III. Immunohistochemical analysis showed positive expression of estrogen receptor (75-100%), positive expression of progesterone receptor (75-100%), and HER2 was classified as 2+ (amplification was confirmed by FISH). According to the TNM classification, the tumor stage was pT1cN0(sn)Mx. This patient presented scant family history of cancer, namely a maternal uncle affected with gastric cancer at age 54 years, and a second-degree cousin affected with breast cancer at age 40 years (Figure IV.4a).

Patient #104, with the *TP53* mutation c.383delC, p.(Pro128LeufsTer42), is a woman who was diagnosed at age 33 years with an invasive ductal breast carcinoma, grade III. This woman had previously been diagnosed with contralateral breast cancer at age 29 years, but the HER2 status of this first tumor is unknown. Immunohistochemical analysis showed positive expression of estrogen and progesterone receptors, and HER2 was

classified as 3+. According to the TNM classification, the tumor stage was cT1N0M0. This patient presents family history of early-onset cancer, namely, a sister with three primary tumors (teratoma, breast and colon cancer, respectively at ages 19, 25 and 33) the father with lung cancer at age 40, and a paternal grandmother who died with an unspecified gynecologic cancer (Figure IV.4b).

Patient #107, with the *FANCA* mutation c.295C>T, p.(Gln99Ter), is a woman who was diagnosed at age 29 years with an invasive ductal breast carcinoma, grade III. Immunohistochemical analysis showed positive expression of estrogen receptor (30%), positive expression of progesterone receptor (25%), and HER2 was classified as 3+. This patient presented scant family history of cancer, with only the paternal grandmother with thyroid cancer at age 70 years (Figure IV.4c).

Patient #122, with the *TP53* variant c.935C>G, p.(Thr312Ser), is a woman who was diagnosed at age 38 years with an invasive micropapillary carcinoma, grade II/III. Immunohistochemical analysis showed positive expression of estrogen receptor (75-100%), positive expression of progesterone receptor (1-10%), and HER2 was classified as 2+ (amplification was confirmed by FISH). According to the TNM classification, the tumor stage was cT3N1M0. This patient has family history of cancer, namely two paternal aunts with breast cancer at age 50 years (Figure IV.4d).

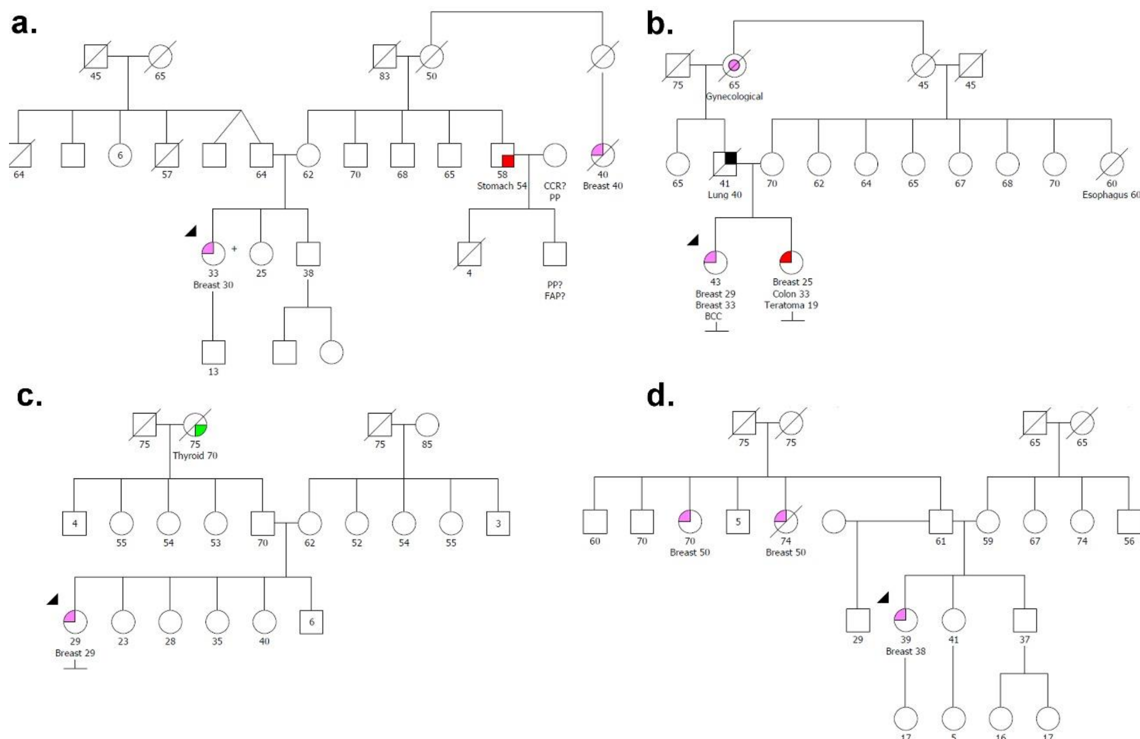


Figure IV.4. Pedigrees of the four patients positive for germline variants in *BRCA2*, *TP53* or *FANCA* genes in the prospective cohort: a. patient #93; **b.** patient #104; **c.** patient #107; **d.** patient #122

IV.3 Pattern of *HER2* Amplification in Mutations Carriers

HER2 amplification was screened by dual-probe FISH (HER2 and CEP17 probes) in seven tumors from patients with germline variants. The results are summarized in Table IV.3 and illustrated by Figure IV.5.

Table IV.3. *HER2* amplification in paraffin-embedded tumor

Sample number	IHC	HER2 signal	CEP17 signal	HER2/CEP17	% amplified cells
VUS in *TP53*					
#32	3+	13.40	5.10	2.63	100
#72	3+	9.72	2.80	3.47	90
#122	2+	6.80	2.93	2.32	80
Pathogenic Mutations in *TP53*					
#60	3+	10.32	2.47	4.18	100
#104	3+	9.62	2.55	3.77	100
Pathogenic Mutations in *BRCA2*					
#93	2+	5.33	2.62	2.04	76.6
Pathogenic Mutations in *FANCA*					
#107	3+	7.70	2.12	3.64	100

VUS: Variant of uncertain significance

According to ASCO/CAP recommendations, all cases were classified as HER2-positive (HER2/CEP17 $\geq$ 2.0). The patients with *HER2* test result as equivocal by IHC (2+) (#93 and #122) had ratios by FISH lower than patients with 3+ by IHC. The patients with pathogenic mutation in the *TP53* gene (#60 and #104) presented higher ratios than the other patients.

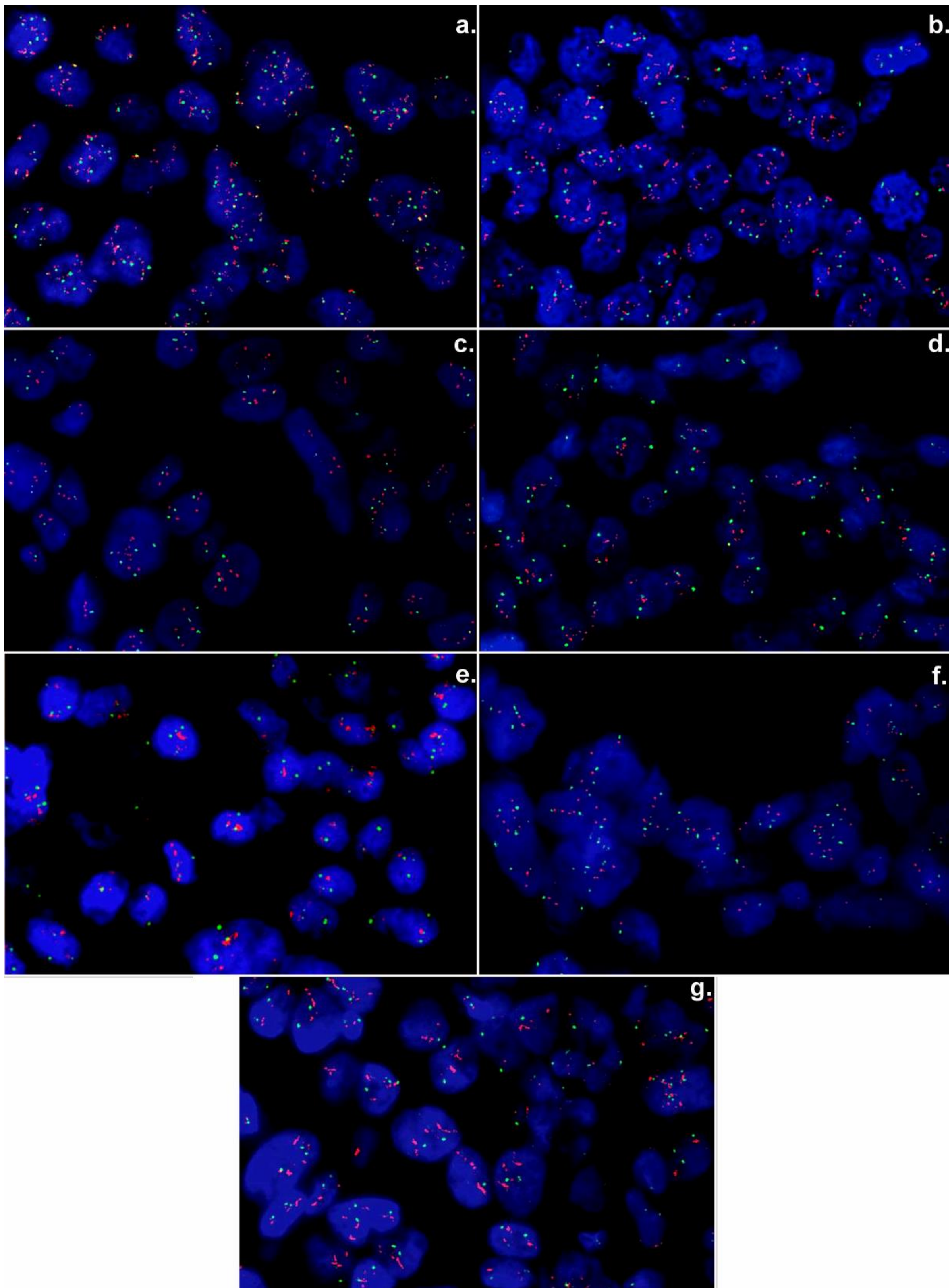


Figure IV.5. Representative FISH images from 7 tumors from patients with germline variants: a. patient #32; **b.** patient #72; **c.** patient #122; **d.** patient #60; **e.** patient #104; **f.** patient #93; **g.** patient #107. Green corresponds to CEP17 signals and red corresponds to HER2 signals.

V. DISCUSSION

Some data in recent years have suggested that the proportion of HER2-positive breast cancer is much higher in Li-Fraumeni syndrome patients than that in sporadic or Hereditary breast/ovarian cancer patients (Lee et al., 2012; Melhem-Bertrandt et al., 2012; Wilson et al., 2010), but the limited data available indicates that germline *TP53* mutations may have a limited overall contribution to early-onset HER2-positive disease (Rath et al., 2013). The identification of germline *TP53* mutations carriers is essential for offering them screening or prophylactic measures appropriate for their high breast cancer risk (Sorrell et al., 2013) and no formal recommendations yet exist in the genetic testing criteria taking into consideration this clinicopathological association. Therefore, this study aimed to identify the contribution of germline *TP53* mutations for early-onset HER2-positive breast cancer, to identify other genes with germline mutations in patients with early-onset HER2-positive breast cancer, to define the genetic testing criteria to complement established recommendations, and to compare the *HER2* amplification pattern in women with deleterious *TP53* germline mutations compared with that in women with other germline mutations. We identified five germline variants in the *TP53* gene (5/124 = 4.03%) and two truncating germline mutations in other genes (2/36 = 5.56%) in patients diagnosed with HER2-positive breast cancer diagnosed until the age of 40 years.

V.1 Pathogenic Germline Mutations in the *TP53* Gene

In the present work, two pathogenic germline *TP53* mutations were found in 124 patients with HER2-positive breast cancer diagnosed until the age of 40 years (1.6%), accounting for two of 79 patients (2.53%) diagnosed before the age of 36 years (one of them with a contralateral breast cancer diagnosed before the age of 30 years). The deleterious *TP53* mutations identified were the c.524G>A, p.(Arg175His), and the c.383delC, p.(Pro128LeufsTer42), one identified by Sanger sequencing in the retrospective series and the other by NGS in the prospective series of patients. The first mutation is quite common and has been described as a mutation hotspot. In breast cancer patients, according to the IARC TP53 Database, p.(Arg175His) has been detected as germline mutation twenty-one times and the UniProtKB/Swiss-Prot reports that this mutation is associated with Li-Fraumeni syndrome. This mutation is able to confer increased proliferation, inhibition of apoptosis, invasive ability, and changes in metabolism, among others, through the transcriptional activation of target genes (*MYC*, *EGFR*, *EGR1*, *NFKB2*, *MMP3*, *MMP13*, *CYP24A1*, *DHCR24,* and others) (Freed-Pastor and Prives, 2012). Lacroix

and colleagues (2006) also report that this mutation has the ability to strongly inhibit transcription of the FAS pro-apoptotic gene (Lacroix et al., 2006). On the other hand, the *TP53* mutation c.383delC, p.(Pro128LeufsTer42), had only been identified once and it was reported as a somatic event (http://cancer.sanger.ac.uk/cosmic). This mutation occurs at the beginning of the DNA-binding domain that is essential to protein-DNA interaction and encodes a truncated protein with only 168 amino acids, which strongly suggests its pathogenicity. The identification of these deleterious mutations establish the genetic diagnosis of Li-Fraumeni syndrome in these two families and predictive testing is available to relatives. Interestingly, none of these two families comply with the classical clinical criteria of Li-Fraumeni syndrome or with the Chompret criteria for germline *TP53* testing due to the lack of other cancers typical of this syndrome, but both index cases have first degree relatives with early-onset and/or multiple primary cancers, which are features that fit well with the genetic diagnosis.

The study of Rath et al. (2013), the only one in the literature having early-onset HER2-positive breast cancer as the primary selection criterion, evaluated the prevalence of germline *TP53* mutation in a cohort of 213 women with HER2-positive breast cancer diagnosed before 50 years, and found three patients with germline mutation in *TP53* (ages at diagnosis of 23, 32, 44 years) (1.4%) (Rath et al., 2013). Among the 40 women diagnosed before 36 years with HER2-positive breast cancer, two had mutation in the *TP53* gene (5%) (Rath et al., 2013). Our study found two germline *TP5*3 pathogenic mutations among 79 women diagnosed with (2.53%). Although our study was more restrictive regarding the age of diagnosis (before 40 years), it included almost double the number of patients HER2-positive breast cancer diagnosed before age 35, so the proportion we report may be closer to reality in this setting.

V.2 Variants of Uncertain Significance in the *TP53* Gene

In addition to the clearly deleterious mutations, we found also three variants of uncertain significance in the *TP53* gene, namely, c.642T>G, p.(His214Gln), c.559+19_559+35del, p.?, and c.935C>G, p.(Thr312Ser) (3/124 = 2.4%). The missense variant c.642T>G, p.(His214Gln), has already been reported as a somatic event in four sporadic carcinomas of the esophagus, cervix uteri, sinuses and colon (http://p53.iarc.fr/), but never as a germline change. Some studies reported that the variant p.His214Gln has higher ability to induce apoptosis than the wild-type p53 and it has been referred to as a super p53 (Kakudo et al., 2005; Lacroix et al., 2006). These studies and the absence of history of cancer in the relatives of our patient with this variant question the pathogenicity of this variant, although we have not yet excluded that it is a *de novo* variant. On the other

hand, the variant c.559+19_559+35del has never been reported before and it occurs in intron 5 away from the highly conserved 5' splice site. The Human Splice Finder program predicts that this deletion does not have an impact on splicing activity (http://www.umd.be/HSF3/), suggesting that this variant might have no impact on the biological activity of the protein. The patient with this variant is the only affected with cancer in her family, a fact that reinforces the nondeleterious nature of this variant or its low penetrance (if it did not occur *de novo*). Finally, the missense variant c.935C>G, p.(Thr312Ser), has already been reported as a somatic event in four sporadic neoplastic samples of the lung, ovary, mouth and hematopoietic system (http://p53.iarc.fr/), but never as a germline change. No study has yet addressed the biological significance of this variant and our patient has only two second-degree relatives affected with breast cancer at an older age. In fact, there is a striking difference regarding cancer history in first-degree relatives between the two patients with clearly deleterious *TP53* mutations and these three patients with VUS. Further segregation data on a research basis or determination of its *de novo* nature may contribute to a better evaluation of these variants, which at this stage have no clinical relevance for these families and cannot be used for predictive testing.

V.3 *BRCA2* Germline Mutation in HER2-positive Breast Cancer

The *BRCA2* mutation c.9105T>A, p.(Tyr3035Ter) has never been reported in the scientific literature or in databases. This mutation is predicted to result in a truncated protein with only 3034 amino acids (the BRCA2 wild-type contains 3418 amino acids), if the mutated mRNA is not affected by nonsense mediated decay. Although this mutation is quite distal, there are other downstream deleterious mutations described in the *BRCA2* gene, such as the c.9924C>G, Y3308X (Kuznetsov et al., 2008), something that strongly supports its pathogenic nature. A mutation in this gene was unexpected since *BRCA1/BRCA2*-associated tumors rarely overexpress or show amplification of *HER2*. In the large study from the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA), only 10% of *BRCA1* mutation carriers and 13% of *BRCA2* mutation carriers had HER2-positive tumors (Mavaddat et al., 2012), percentages that are much lower than that in sporadic breast carcinomas. This is supported by our own data in our population, where only 5% of *BRCA1* and 10% of *BRCA2* index breast cancer patients had HER2-positive carcinomas (Peixoto et al., 2015).

According to the NICE guidelines, genetic testing of the *BRCA1/BRCA2* genes should be offered when the *a priori* likelihood of a germline mutation in these genes is equal or higher than 10% (Evans et al., 2013). In the patient in whom we found a *BRCA2* mutation, the combined mutation probability is well below the 10% threshold, both using the

BRCAPRO Bayesian calculation (Berry, 2002) and the updated Manchester score (which includes a penalization for HER2+ tumors). This relatively low likelihood is further supported by the fact that only one of the 36 patients (2.8%) tested with NGS in this study was shown to carry a deleterious *BRCA1*/*BRCA2* mutation. This means that, in this case, we would normally offer testing only for the Portuguese founder mutations in the *BRCA1*/*BRCA2* genes, which represent nearly 50% of the mutations in our population, a strategy that reduces the likelihood of missing a deleterious mutation to about 5% (Peixoto et al., 2015). Therefore, this *BRCA2* mutation was found only because the proband was included in this study for *TP53* mutation analysis by gene-panel NGS, highlighting the potential of NGS to increase the molecular diagnosis yield in situations in which different syndromes have overlapping clinical features (for example, Hereditary breast/ovarian cancer and Li-Fraumeni syndromes) and in which genetic testing criteria do not have 100% sensitivity (Pinto et al., 2016). Another example of overlapping clinicopathological features between different syndromes is provided the patient with a deleterious *TP53* mutation that presented a medullary breast carcinoma, a relatively rare histological subtype that is strongly associated with germline *BRCA1* mutations (Mavaddat et al., 2012; Peixoto et al., 2015). However, this medullary breast carcinoma was atypical for being HER2-positive.

V.4 *FANCA* Mutation in Early-onset HER2-positive Breast Cancer

The mutation identified in *FANCA*, c.295C>T, p.(Gln99Ter), results in a predicted truncated protein with only 98 amino acids (the FANCA wild-type contains 1455 amino acids). This mutation has been found in homozygosity in unrelated Spanish Gypsy patients with Fanconi anemia (Callen et al., 2005; Gille et al., 2012), therefore demonstrating that it is deleterious. Although an initial study suggested that mutations in Fanconi anemia genes other than *BRCA2* (*FANCD1*) would not be associated with increased risk of breast cancer (Seal et al., 2003), it is today clear that heterozygous truncating mutations in *PALB2* (*FANCN*), another gene that causes Fanconi anemia when biallelic inactivating mutations occur, significantly increases the risk of breast cancer. Antoniou and co-workers (2014) reported that the risk of breast cancer for female *PALB2* mutation carriers, as compared with the general population, was eight to nine times as high among those younger than 40 years of age (Antoniou et al., 2014), resulting in cumulative penetrance that is similar to that of *BRCA* mutations, especially in the presence of family history of breast cancer. Mutations in other FANC genes are so rare that a clear evidence of their association with breast cancer and robust penetrance estimates are lacking (Kleibl and Kristensen, 2016). Interesting in this context is the study of Solyom and co-workers (2011), who assessed the *FANCA* gene for breast cancer susceptibility and identified a novel heterozygous deletion removing the

promoter and 12 exons in a single family (Solyom et al., 2011). Our finding of one truncating mutation in 36 patients (2.8%) tested by NGS, a frequency that is similar to those of *TP53* and *BRCA2* mutations, indicates that a more extensive evaluation of the association of *FANCA* mutations with breast cancer susceptibility is required, namely through the inclusion of this gene in NGS panels used for genetic testing of breast cancer patients.

V.5 Genetic Testing Criteria of Early-onset HER2-positive Breast Cancer

Since the initial description of the Li-Fraumeni syndrome, several criteria have been suggested to identify high-risk families (McCuaig et al., 2012; Sorrell et al., 2013). The classic clinical criteria for Li-Fraumeni syndrome require families with proband diagnosed with sarcoma before age 45, and a first-degree relative with cancer before age 45, and also another first- or second-degree relative with any cancer diagnosed under age 45 or with sarcoma at any age (Li et al., 1988). On the other hand, more sensitive but less specific criteria are used for selecting patients for germline *TP53* testing, the most commonly used being the revised Chompret criteria. These criteria require families with proband diagnosed with a tumor belonging to the Li-Fraumeni syndrome spectrum (soft tissue sarcoma, osteosarcoma, brain tumor, premenopausal breast cancer, adrenocortical carcinoma, leukemia, lung bronchoalveolar cancer) before age 46 and at least one first- or second degree relative with a Li-Fraumeni syndrome cancer (except breast if the proband has breast cancer) under the age of 56 years or with multiple tumors at any age; or proband with multiple primary tumors (except multiple breast), two of which belong to the Li-Fraumeni syndrome tumor spectrum and the first of which occurred before age 46; or proband with adrenocortical carcinoma or choroid plexus tumor at any age, irrespective of family history (Gonzalez et al., 2009; McCuaig et al., 2012). Both patients identified in this work with a pathogenic mutation in the *TP53* gene did not meet these testing criteria, leaving room to recommend the use of less stringent criteria that takes in consideration also the HER2 status in breast cancer patients.

Of note, patient #60 has several relatives affected with early-onset gastric cancer, a cancer that has been observed before in the context of Li-Fraumeni syndrome (Horio et al., 1994; Pinto et al., 2009; Varley, 2003), especially in countries with high gastric cancer incidences (Pinto et al., 2009). Portugal is a high-risk country for gastric carcinoma with an incidence of 13.1 new cases per 100 000 inhabitants per year (IARC, GLOBOCAN 2012), so this tumor type should perhaps also be considered in the genetic testing criteria in our population. Furthermore, both probands with deleterious *TP53* mutations in this study, besides early-onset HER2-positive breast cancer, have first degree relatives with early-onset and/or multiple primary cancers. Based on literature (McCuaig et al., 2012) and our

data, we propose that *TP53* germline testing should be offered also to patients that, even if not complying with the Chompret criteria, present HER2-positive breast cancer diagnosed until the age 35 with at least one first-degree relative with cancer before age 45 (including tumors that do not belong to the Li-Fraumeni syndrome spectrum), as well as to all cases of breast cancer diagnosed before the age of 30 irrespective of family history (to take into account the frequent occurrence of *de novo* mutations in the *TP53* gene and the fact that not all breast carcinomas in Li-Fraumeni patients are HER2-positive).

On the other hand, as discussed above, it is clear that inherited predisposition to early-onset HER2-positive breast cancer is not restricted to *TP53* mutations, since we found a similar proportion of *BRCA2* and *FANCA* deleterious mutations in this study. However, we currently see no compelling reason to modify the genetic testing criteria for the *BRCA1*/*BRCA2* genes, and more data are required to substantiate the role of *FANCA* mutations in breast cancer predisposition.

V.6 Pattern of *HER2* Amplification in Carriers of Deleterious *TP53* Mutations Compared with Those with Other Mutations

Some studies have suggested that germline *TP53* mutations encourage breast cancer development along a fairly specific oncogenic pathway that frequently includes *HER2* amplification and/or overexpression (Masciari et al., 2012; Melhem-Bertrandt et al., 2012; Wilson et al., 2010). Wilson and co-workers (2010) reported that ten out of twelve cases with pathogenic germline *TP53* mutations were positive for HER2 (83%), contrasting with the percentage of *HER2* amplification in sporadic breast cancer (15-20%) (Wilson et al., 2010). In another study with more patients, Melhem-Bertrandt and co-workers (2012) compared the pathological characteristics of breast tumors from patients testing positive for a germline mutation in *TP53* gene (n=30) with those of breast tumors from patients testing negative (n=79), and found *HER2* amplification and/or overexpression in 67% (20/30) of the former and 25% in the latter (Melhem-Bertrandt et al., 2012). Finally, Masciari and co-workers (2012) sought to complement the existing small literature with histopathologic analysis of breast cancers from women with Li-Fraumeni syndrome, and reported that 63% (20/32) of the invasive breast cancers and 73% (8/11) of DCIS were positive for HER2 (Masciari et al., 2012).

According to the latest ASCO/CAP guidelines, positive HER2 status is defined as a score of 3+ by IHC, a HER2/CEP17 ratio of 2.0 or more by dual-probe ISH, as well as an average *HER2* copy number of ≥ 6.0 signals per cell even when the HER2/CEP17 ratio is below 2.0 (Wolff et al., 2013). In routine clinical practice the most common approach for

HER2 testing is to screen for protein overexpression with IHC (Mano et al., 2007), a strategy that was also followed routinely at our institution. In fact, of the cases with germline variants, only the two cases classified as 2+ by IHC had previously been tested by FISH. To the best of our knowledge, the issue of whether the pattern of *HER2* amplification in carriers of deleterious *TP53* mutations is different from those without has not been addressed before. We therefore decided to test all cases with germline variants with the same quantitative software analysis of digital images in order to compare the *HER2* amplification pattern in breast carcinomas from patients with pathogenic *TP53* germline mutations with that of patients with other germline mutations. We found that, while amplification was confirmed in all cases, the highest *HER2* amplification ratios were detected in the breast carcinomas from patients with deleterious *TP53* germline mutations, followed closely by that of the breast carcinoma in the truncating *FANCA* mutation carrier. Conversely, the lowest amplification ratio was seen in the breast carcinoma of the patient with the *BRCA2* germline mutation, whereas the breast tumors from patients *TP53* VUS showed intermediate amplification scores. However, we have so far analyzed only a few breast carcinomas of patients with germline variants (n=7), so clearly more data are necessary to conclusively show if such differences are real.

HER2-positive breast cancers are currently treated with a humanized monoclonal antibody, trastuzumab, alone or coupled to emtansine, a cytotoxic agent (T-DM1). An alternative therapy is based on a small molecule tyrosine kinase inhibitor, lapatinib. Despite the remarkable effectiveness of these therapies, breast cancers frequently become resistant, resulting in disease progression (Vicario et al., 2015; Wolff et al., 2013). HER2 overexpression is routinely used to predict response to anti-HER2 therapy and only patients with a score of 3+ in IHC and gene amplification should receive anti-HER2 therapy. It has been demonstrated that tumors with moderate HER2 (2+) expression without gene amplification do not respond to trastuzumab therapy (Eggemann et al., 2015). We can therefore speculate that patients with higher degree of *HER2* amplification in their tumors might respond better to anti-HER2 therapies when compared with patients with lesser degrees of amplification, although increasing levels of amplification under treatment with anti-HER2 therapy might theoretically be a mechanism of resistance. Be that as it may, Vicario and co-workers (2015) showed that gene amplification in double minutes occurs in approximately 30% of HER2-positive breast tumors and that the loss of HER2 protein expression due to loss of double minutes containing *HER2* isn't a likely mechanism of resistance to anti-HER2 therapies, since the number of double minutes containing *HER2* is maintained in different models of resistance to anti-HER2 therapies (Vicario et al., 2015). Further studies are therefore needed to identify the mechanism of *HER2* amplification pattern in breast carcinomas of Li-Fraumeni patients, as well as to find out if their response

to anti-HER2 targeted therapies differs from that of other patients with HER2-positive breast cancer.

VI. CONCLUSION

After the completion of this study we conclude that:

- ✓ Deleterious germline *TP53* mutations are present in about 1.6% of patients with HER2-positive breast cancers diagnosed until the age of 40 years, and in 2.5% of those diagnosed before the age of 36 years;
- ✓ Other genes besides *TP53* are involved in predisposition to early-onset HER2-positive breast cancer, namely *BRCA2* and *FANCA*;
- ✓ *TP53* germline testing should be extended beyond those complying with the Chompret criteria, namely, to those presenting HER2-positive breast cancer diagnosed until the age of 35 with at least one first-degree relative with any cancer before age 45, as well as to all cases of breast cancer diagnosed before age 30 irrespective of family history;
- ✓ Breast carcinomas from patients with deleterious *TP53* germline mutations might show higher *HER2* amplification ratios than those from patients with other germline variants.

VII. FUTURE PERSPECTIVES

This study may benefit from further analysis to support our conclusions and to allow a more specific evaluation of the genes with germline mutations in patients with early-onset HER2-positive breast cancer. Thus, we plan:

- ✓ To complete segregation analysis in order to obtain more data for better pathogenicity evaluation of the identified variants;
- ✓ To extend the gene panel NGS study to a larger series of patients with early-onset HER2-positive breast carcinomas in order to establish the mutation frequency in other genes besides *TP53*;
- ✓ To extend the *HER2* amplification analysis by FISH to carcinomas from more mutation carriers, including from families already with genetic diagnosis of Li-Fraumeni syndrome;
- ✓ To test the *HER2* amplification pattern in breast carcinomas from patients without germline variants and with similar age at diagnosis, to be used as controls for comparison with that of variant carriers.

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