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THE ROLE OF THE HOXB13 GENE IN BREAST CANCER

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

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"Every pro was once an amateur...

Every expert was once a beginner...

So dream big and start now"

Inkhorn theme

Acknowledgments

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Relevant abbreviations

A - Adenine

ADP - Adenosine diphosphate

AI - Aromatase inhibitor

AKT - v-akt murine thymoma viral oncogene homolog 1 (gene)

ATM - Ataxia telangiectasia mutated (gene)

BAC - Bacterial artificial chromosome

BCL2 - B-cell lymphoma 2 (gene)

BCS - Breast conserving surgery

Bp - Base pairs

BRCA1/2 - Breast cancer 1/2 (gene)

BUB1B - Budding uninhibited by benzimidazoles 1 homolog beta (gene)

C - Cytosine

CBP - CREB binding protein

CCND1 - Ciclin D-1(gene)

CDH1 - Cadherin-1(gene)

cDNA - Complementary DNA

CENPA - Centromere protein A (gene)

CEP17 - Chromosome 17 centromere

CGH - Comparative genomic hybridization

CHDH - Choline dehydrogenase (gene)

CHEK2 - Checkpoint kinase 2 (gene)

CMF - Cytoxan, methotrexate, fluorouracil (regimen)

COX-2 - Ciclo-oxigenase-2 (gene)

CYP - Cytochrome p450

DCIS - Ductal carcinoma *in situ*

ddNTP - Didesoxyribonukleosid-triphosphate

DNA - Deoxyribonucleic acid

DNMT - DNA methyltransferases

dNTP - Deoxyribonucleotide triphosphate

DSB - Double strain break

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

ER - Estrogen receptor

ERBB2 - See HER2

EU - European Union

EXO1 - Exonuclease 1

FANCN - Fanconi anemia (gene)

FGF-3 - Fibroblast growth factor-3 (gene)

FISH - Fluorescent *in situ* hybridization

G - Guanine

Glu - Glutamic acid

Gly - Glycine

GSTM1 - Glutathione S-transferase Mu 1

GSTT1 - Glutathione S-transferase theta 1 (gene)

GUSB - Glucuronidase beta (gene)

HDAC - Histone deacetylase

HER2 - Human epidermal growth factor receptor 2/3

HOX - Homeobox (gene)

HOXB13 - Homeobox cluster B 13 (gene)

HPRT - Hypoxanthine-guanine phosphoribosyltransferase (gene)

HR - Hazard racio

HRT - Hormone replacement therapy

IDC - Invasive ductal carcinoma

IDC-NOS - Invasive ductal carcinoma not otherwise specified

IL17BR - Interleukin 17B receptor

IL6 - Interleukin 6

ILC- Invasive lobular carcinoma

INT-2 - See FGF-3

LCIS - Lobular carcinoma *in situ*

MGI - Molecular grade index

miRNA - microRNA

MRI - Magnetic resonance imaging

mTOR - Mammalian target of rapamycin (gene)

MYC - Myelocytomatosis (gene)

NEK2 - Serine/threonine-protein kinase 2 (gene)

PARP - Poly ADP ribose polymerase

PCR - Polymerase chain reaction

PLC - Pleomorphic lobular carcinoma

PR - Progesterone receptor

PTEN - Phosphatase and tensin homolog (gene)

RACGAP1 - Rac GTPase activating protein 1 (gene)

RNA - Ribonucleic acid

RRM2 - Ribonucleotide reductase M2 (gene)

SERM - Selective estrogen receptor modulators

STK11 - Serine/threonine kinase 11(gene)

T - Thymine

TP53 - Tumor protein 53 (gene)

UTR - Untraslated region

VEGF - Vascular endothelial growth factor

Summary

Breast cancer is the most common and most lethal cancer in women worldwide, affecting 1.38 million women in 2008 and being responsible for 23% of all cancers cases in this gender, with an associated mortality rate of 13.7% (460.000 deaths). There are currently several treatment strategies, but a large number of cases stands without adequate therapy or develop resistance to it. Deregulation of the "homeobox" gene *HOXB13*, crucial in cell division and differentiation, has been associated with several cancer models. This gene is known to be overexpressed in breast cancer cells, associated with an increased tumoral proliferation and a worse clinical outcome, suggesting an oncogenic role for this gene. Furthermore, it has been shown that high *HOXB13* expression is associated with resistance to tamoxifen therapy, through a not well studied mechanisms, related with estrogen receptors suppression and induction of alternative pathways.

The main goal of this master thesis was to try to understand better the role and relevance of *HOXB13* in breast cancer. More specifically, one goal was to find out if germline *HOXB13* mutations occur in familial breast cancer (some of which having also prostate cancer), negative for mutations in *BRCA1* or *BRCA2*. Additionally, the relevance of copy number changes and amplification for *HOXB13* expression in breast carcinomas was evaluated. Finally, the findings were correlated with clinico-pathological parameters.

To address these aims, DNA from 95 peripheral blood samples was extracted and amplified and both *HOXB13* exons were sequenced to verify if there were any germline mutations. Next, to quantify the *HOXB13* expression levels in breast cancer cases with and without 17q21 gain (CGH available data), a qRT-PCR was done in 84 samples of cDNA, adjusting the values for the endogenous control gene, *GUSB*. To directly evaluate copy number changes in *HOXB13*, the cytogenetic technique FISH was performed, using a probe that was able to cover our gene of interest (located in 17q21) and a control probe to the centromeric region of chromosome 17.

No deleterious germline mutations were found in any of the 95 cases of familial breast cancer, wildtype for mutations in the BRCA1/2 genes. Furthermore, a statistical association was found between the 17q21 status, measured by CGH, and the expression levels of *HOXB13*. By FISH analysis, although *HOXB13* amplifications were found in 8 tumor samples, no significant association was found between copy number changes and *HOXB13* expression. However, there was a significant association

between higher levels of *HOXB13* expression in breast carcinomas with *HOXB13* amplifications, when compared with breast carcinomas with and without 17q21 gain, by CGH, but no *HOXB13* amplification. Concerning clinico-pathological associations, we found no prognostic or predictive value of *HOXB13* expression in the limited series of breast cancer patients analyzed but there was indeed an association with tumor grade and HER2 status.

In conclusion, no evidence was found that the *HOXB13* gene is a significant contributor to inherited predisposition to familial breast cancer in Portugal, both regarding the recurrent G84E mutation or any other. Furthermore, mean *HOXB13* expression is higher in breast carcinomas with 17q21 gain and also in tumors with *HOXB13* amplification, especially when compared with tumors without 17q21 gain, but and no *HOXB13* amplification, this difference suggests that genetic copy number gain, can be the main mechanism for *HOXB13* overexpression in breast cancer.

Resumo

O cancro da mama é o mais comum e mais letal na mulher mundialmente, tendo afectado 1.38 milhões de mulheres em 2008 e sendo responsável por 23% de todos os casos de cancro neste género, com uma taxa de mortalidade associada de 13.7% (460.000 mortes). Actualmente existem várias estratégias de tratamento, mas um elevado número de casos permanece sem uma terapêutica adequada, ou desenvolve uma resistência à mesma. A desregulação do gene "homeobox" *HOXB13,* crucial na divisão e diferenciação celular, tem sido associada a vários modelos de cancro. Sabe-se que este gene está sobrexpresso nas células de cancro da mama, associado a um aumento na proliferação tumoral e a uma maior agressividade clínica, sugerindo assim um papel oncogénico para este gene. Foi também já provado, que a elevada expressão de *HOXB13* está associada com a resistência ao tamoxifen, por mecanismos ainda pouco estudados, relacionados com a supressão dos receptores de estrogénio e indução de vias alternativas.

O maior objectivo desta tese de mestrado foi tentar perceber melhor o papel e relevância do *HOXB13* no cancro da mama. Mais especificamente, um dos objectivos foi saber se mutações germinativas ocorriam em cancro da mama familiar (alguns dos quais com casos de cancro da próstata) negativos para mutações no *BRCA1* e *BRCA2*. Adicionalmente, avaliar a relevância de alterações no número de cópias e amplificações do *HOXB13*, para a sua expressão em carcinomas da mama. Por último, os resultados foram correlacionados com os parâmetros clinico-patológicos.

Para realizar estes objectivos, extraiu-se e amplificou-se o DNA de 95 amostras de sangue periférico e sequenciaram-se ambos os exões do *HOXB13*, para verificar se havia mutações germinativas. Seguidamente, para quantificar os níveis de expressão do *HOXB13* em casos com cancro da mama, com e sem ganho do 17q21 (dados disponíveis de CGH), fez-se qRT-PCR em 84 amostras de cDNA, ajustando os dados para o gene de controlo endógeno, *GUSB*. Para avaliar directamente alterações no número de cópias do *HOXB13,* foi feita a técnica citogenética de FISH, usando uma sonda que capaz de cobrir o nosso gene de interesse (localizado no 17q21) e uma sonda controlo para a região centromérica do cromossoma 17.

Não foram encontradas mutações germinativas no *HOXB13* nos 95 casos de cancro de mama familiar, negativos para mutações no BRCA1/2. Foi encontrada uma associação estatisticamente significativa entre o ganho da região 17q21, dados fornecidos por CGH, e o nível de expressão do *HOXB13*. Por análise de FISH, apesar

de terem sido descobertas amplificações do *HOXB13* em 8 amostras de tumor, não foi encontrada nenhuma associação significativa entre as alterações no seu número de cópias e a expressão do mesmo. No entanto, verificou-se novamente uma associação positiva entre os valores de expressão do *HOXB13* mais elevados e carcinomas da mama com amplificações do *HOXB13,* especialmente quando comparados com carcinomas da mama sem ganho do 17q21 por CGH, assim como sem amplificação deste gene. No que diz respeito às associações clinico-patológicas, não encontramos qualquer valor preditivo ou de prognóstico nos níveis de expressão do *HOXB13,* na limitada série estudada, apenas uma associação com o grau do tumor e o receptor HER2.

Concluindo, não foi encontrada nenhuma evidência de que o gene *HOXB13* contribua significativamente para uma predisposição hereditária para cancro da mama familiar em Portugal, quer no que diz respeito à mutação recorrente G84E, ou qualquer outra. Os níveis médios de *HOXB13* revelaram ser mais elevados em carcinomas da mama com ganho da região 17q21 assim como nos casos com amplificação deste gene, especialmente quando comparados com tumores sem ganho do 17q21 e sem amplificação do *HOXB13*. Esta diferença estatisticamente significativa, sugere então o aumento do número de cópias como um mecanismo responsável para a sobrexpressão do *HOXB13* em cancro da mama.

I. Introduction

1. Breast cancer

1.1 Epidemiology

Cancer continues to increase its numbers every year across the world, in part because of the aging of the population and its continuous growth, alongside with an augmented adoption of cancer-causing behaviors. About 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 [1], but there has also been an improvement in survival rates [2].

Breast cancer is the most common and remains the most lethal cancer in women worldwide, being responsible for almost 23% (1.38 million) of the cancers affecting women and with an associated mortality of 13.7%, corresponding to 460,000 deaths (2008) [1]. About 20% of breast cancers occur among women aged younger than 50 years, while 40% occur among women aged 65 years and older [2]. Mortality rates are also highest in young women (<35 years), associated with a more aggressive disease, and in the very old (>75 years) because they are not treated aggressively [3]. It is estimated that a million women will develop breast cancer each year [4]. Breast carcinoma in men represents approximately 1% of all breast cancers and 1% of all malignancies in men, but the incidence of male breast cancer appears to be increasing [5].

About half of the breast cancer cases and 60% of the deaths are estimated to occur in economically developing countries, associated with a higher prevalence of the already known risk factors for the disease [1], which will be discussed ahead. The highest incidence rates of breast cancer are observed in Northern and Western Europe, North America, Australia, New Zealand and in the southern countries of South America; intermediate incidence rates appears in South America, the Caribbean, and Northern Africa; and low incidence rates are seen in sub-Saharan Africa and Asia [2] **[Figure 1.1]**.

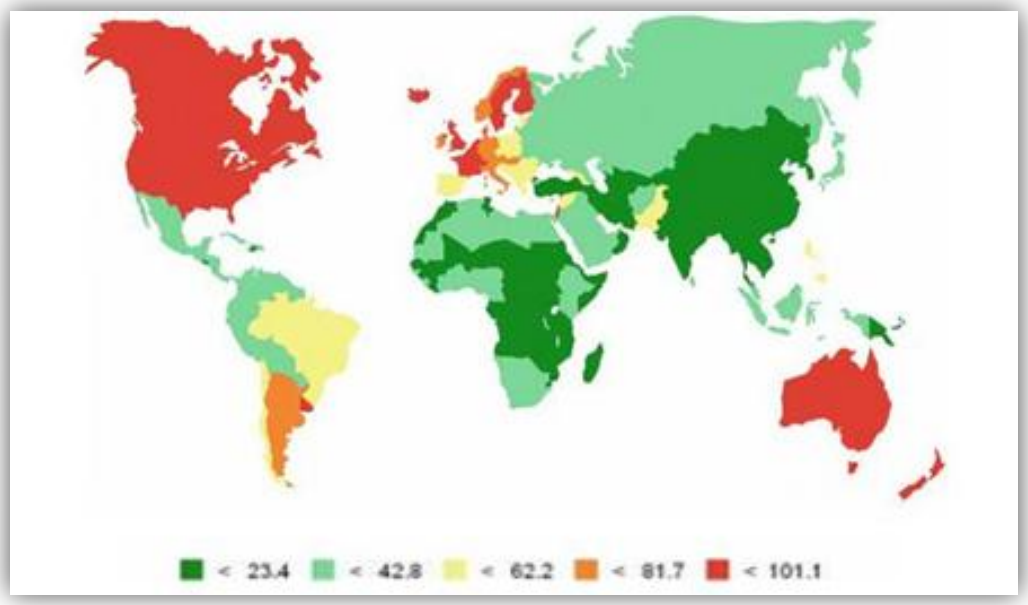


Figure 1.1 - **The global burden of breast cancer in 2002: age-standardized incidence rates per 100.000 [adapted from:** *Li* (2010)**].**

In Europe, approximately 426,000 (28.2%) women were diagnosed with the disease in 2008 and 30.3% of them actually died because of it, representing the second cause of cancer related deaths (17%) [6] **[Figure 1.2]**. Considering the European Union (EU-27), in the same year, breast cancer represented 13.5% of all cancer cases in women, ranking the leading cause of cancer death (89,801; 16.6%), followed by the lung (71,100, 13.5%) and colorectal (69,000, 12.8%) cancers [7,8].

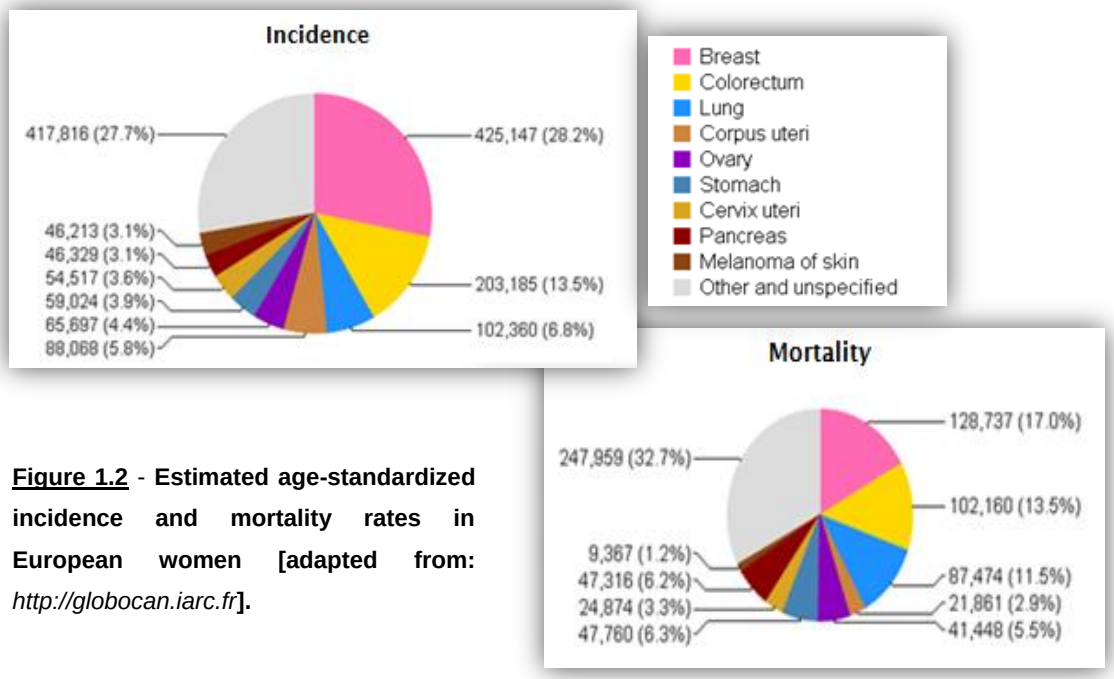


Figure 1.2 - **Estimated age-standardized incidence and mortality rates in European women [adapted from:** *http://globocan.iarc.fr***].**

A fall in breast cancer mortality rates, in most European and other developed countries in the 1990s, was reported by several studies. These declines have been attributed to the combined effect of changes in reproductive factors, earlier detection and improving treatment [9], but it was observed mainly in young women. Because of the aging of the European population, the number of deaths from breast cancer is still disturbingly high (130,000 in 2004, 132,000 in 2006 and 129,000 in 2008). The introduction of organized mammography screening programs throughout Europe, has lead to a reduction in breast cancer mortality, being the short-term consequence, an increased incidence [10].

In Portugal, breast cancer is the most incident type of cancer, both considering only women (27.6%) or both sexes (12.3%). It is the cancer with higher associated mortality if we consider only female gender (16%), being responsible for 1,537 deaths in 2008 in this country [6].

1.2 Etiology and risk factors

Various risk factors for breast cancer have been recognized through the years, some with more impact than others. Among them, it is known that besides genetic and lifestyle variables, reproductive factors are strongly involved in the etiology of breast cancer, mainly for being a hormone dependent malignancy and its risk associated with the endogenous level of estrogens [11] **[Table 1.1]**.

Gender: Is the greatest risk factor. Breast cancer is 100 times more frequent in women than men [12].

Age: The incidence increases with age, doubling about every 10 years until menopause, when the rate of increase dramatically slows. By the ages 75 to 80 the curve actually decreases [13].

Race/ethnicity: Incidence rates are higher in non-Hispanic white woman compared to women with African origins for most age groups. However, African women have a higher incidence rate before 40 years of age and are more likely to die from breast cancer at every age. Incidence and death rates are lower among women of other racial and ethnic groups (Hispanic/Latina; Indian; Asian) [10].

Age at menarche and menopause: Women who start menstruating early in life or who have a late menopause have an increased risk of developing breast cancer. The risk decreases by about 15% for each year of delay in age at menarche and increases by 3% for each year of delay in age at menopause [11].

Age at first pregnancy: Nulliparity and late age at first birth increase the lifetime incidence of breast cancer. The highest risk group are those who have a first child after the age of 35. These women appear to be at even higher risk than nulliparity women [14].

Family history: Only 5% to 10% of breast cancers are considered hereditary. Mutations in the *BRCA1* and *BRCA2* genes account for an estimated 80% of the hereditary cases and confer a 45% to 80% lifetime risk of developing this type of cancer [15]. Familial breast cancer is considered a risk if a first degree relative develops this malignancy before menopause, if it affected both breasts or if it occurred in conjunction with ovarian cancer [16].

Previous benign breast disease: Women with severe atypical epithelial hyperplasia have a four to five times higher risk of developing breast cancer than women who do not have any proliferative changes [12].

Radiation: A doubling of risk of breast cancer was observed among teenage girls exposed to radiation. Ionizing radiation also increases risk later in life, particularly when exposure is during rapid breast formation (prepubertal years of 10 to 14) [17].

Lifestyle: Although there is a close correlation between incidence of breast cancer and dietary fat intake, the true relationship does not appear particularly consistent [18]. Obesity is associated with a two fold increase in the risk in postmenopausal women whereas among premenopausal women is associated with a reduced incidence [19]. The relation with smoking status is inconsistent, but recent evidence has suggested a potential role of active smoking in breast cancer development, particularly with long-term heavy smoking and smoking initiation at an early age [20]. Alcohol has been linked to increased blood levels of estrogen, thus increasing the risk of breast cancer development [21].

Oral contraceptive: When women are taking oral contraceptives and for the 10 years after stop using these agents, there is a small increase in the relative risk of developing breast cancer. Duration of use, age at first use, dose and type of hormone within the contraceptives appear to have no significant effect on breast cancer risk. Women who begin use before the age of 20 appear to have a higher relative risk than women who begin oral contraceptive use at an older age [22].

Hormone replacement therapy (HRT): Among current users of HRT and for those who have previously used this therapy during a long term, the relative risk of developing breast cancer increases by a factor of 1,023 for each year of use. The risk of breast cancer appears higher with combined estrogen and progesterone therapy. Moreover, HRT increases breast density and reduces the sensitivity and specificity of breast screening [23].

Table 1.1 - Factors that increase the risk for breast cancer development in women [adapted from: *DeSantis et al* (2012)**].**

RELATIVE RISK	HIGH RISK GROUP
>4	Age (>65 vs <65 years, although risk increases across all ages until age 80)
	Biopsy-confirmed atypical hyperplasia
	Certain inherited genetic mutations for breast cancer (*BRCA1* and/or *BRCA2*)
	Mammographically dense breasts
	Personal history of breast cancer
2.1-4.0	High endogenous estrogen or testosterone levels
	High bone density (postmenopausal)
	High-dose radiation to chest
	Two first-degree relatives with breast cancer
1.1-2.0	Alcohol consumption
	Ashkenazi Jewish heritage
	High socioeconomic status
	Late age at first full-term pregnancy (>30 years)
	Early menarche (<12 years) and late menopause (>55 years)
	Never breastfed a child
	No full-term pregnancies
	Obesity (postmenopausal)/adult weight gain and height (tall)
	One first-degree relative with breast cancer
	Personal history of endometrium, ovary, or colon cancer
	Recent and long-term use of menopausal hormone therapy containing estrogen and progesterone
	Recent oral contraceptive use

1.3 Anatomy and pathology

Breast starts developing between the ages of 9-14 years for most women, when hormonal changes associated with puberty begin to occur. The breast is the tissue overlying the chest (pectoral) muscles. Women's breasts are made of specialized tissue that produces milk (glandular tissue) as well as fatty tissue. The amount of fat determines the size of the breast [24]. During pregnancy the breasts grow further and this growth is much more uniform than that at adolescence. The amount of milk-producing tissue is essentially the same in all women [25].

Each breast has 15 to 20 sections called lobes. Each lobe has many smaller lobules where milk is produced. These structures are linked by thin tubes called ducts that lead to the nipple in the center of a dark area of skin called the areola. Fat fills the spaces between lobules and ducts, and connective tissue and ligaments provide support to the breast and give it its shape. The breast also contains blood vessels, lymph vessels and lymph nodes **[Figure 1.3]**. The mammary glands exist in the male as well as in the female, but in the former, only in the rudimentary state [24].

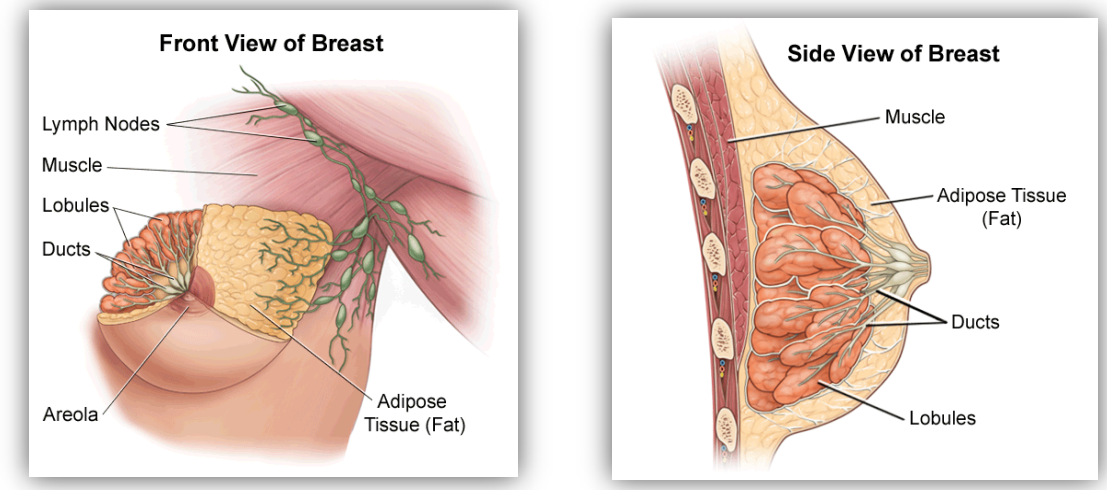


Figure 1.3 - Anatomy of the female breast. Front and side view [adapted from: *http:// www.yalemedicalgroup.org***].**

Among benign lesions related to cancer of the breast, inflammatory lesions, fibrocystic, proliferative and granulomatous diseases can be distinguished [12] **[Table 1.2]**.

Table 1.2 - Benign lesions of the breast [adapted from: *Richie et al* (2003)**].**

Inflammatory lesions	Fibrocystic disease	Proliferative diseases	Granulomatous disease
Acute mastitis	Apocrine metaplasia	Ductal/Lobular hyperplasia	Foreign bodies
Fat necrosis	Epithelial metaplasia	Sclerosing adenosis	Infections
Mammary duct ectasia	Fibrosis and cyst formation	Radial scar	Systemic conditions
Other granulomatous lesions	-----	-----	----

Ninety-five percent of the breast cancers are carcinomas, which arise from breast epithelial elements. Breast cancers are divided into two major types, *in situ* carcinomas and invasive (or infiltrating) carcinomas. The *in situ* carcinomas may arise in either ductal (DCIS) or lobular epithelium (LCIS), but remain confined, with no invasion of the underlying basement membrane. As would be expected with such localized and confined malignancy, there is little potential for metastases [12]. When there is extension of the malignancy beyond the basement membrane, then the malignancy is considered invasive and consists of several histological subtypes: invasive ductal *not otherwise specified* (76%), invasive lobular (8%), mixed ductal/lobular (7%), mucinous (2.4%), tubular (1.5%), medullary (1.2%) and papillary (1%) **[Figure 1.4]**. Other subtypes including cribriform carcinoma, metaplastic breast cancer and invasive micropapillary breast cancer, account for fewer than 5% of the cases. The potential for metastases and ultimately death occurs in invasive disease [26].

There are two major "arms" in the multi-pathway model of breast cancer progression: one comprising well differentiated DCIS that progresses to grade I invasive ductal carcinoma (IDC) and the second comprising poorly differentiated DCIS that progresses to grade III IDC [27]. About 85% of all intraductal cancers, often with less than 1 centimeter, are detected by the appearance of clustered microcalcifications on mammography and their morphology is important to differentiate between benign and malignant calcification [28]. Also interesting is the comedo-type DCIS, which is more malignant than other types of DCIS and is probably between DCIS and invasive cancer [29].

Recently a pleomorphic variant of lobular carcinoma (PLC) has been described, in which cells show the typical discohesiveness of lobular neoplasia, but they are of high grade and show features of apocrine differentiation [27].

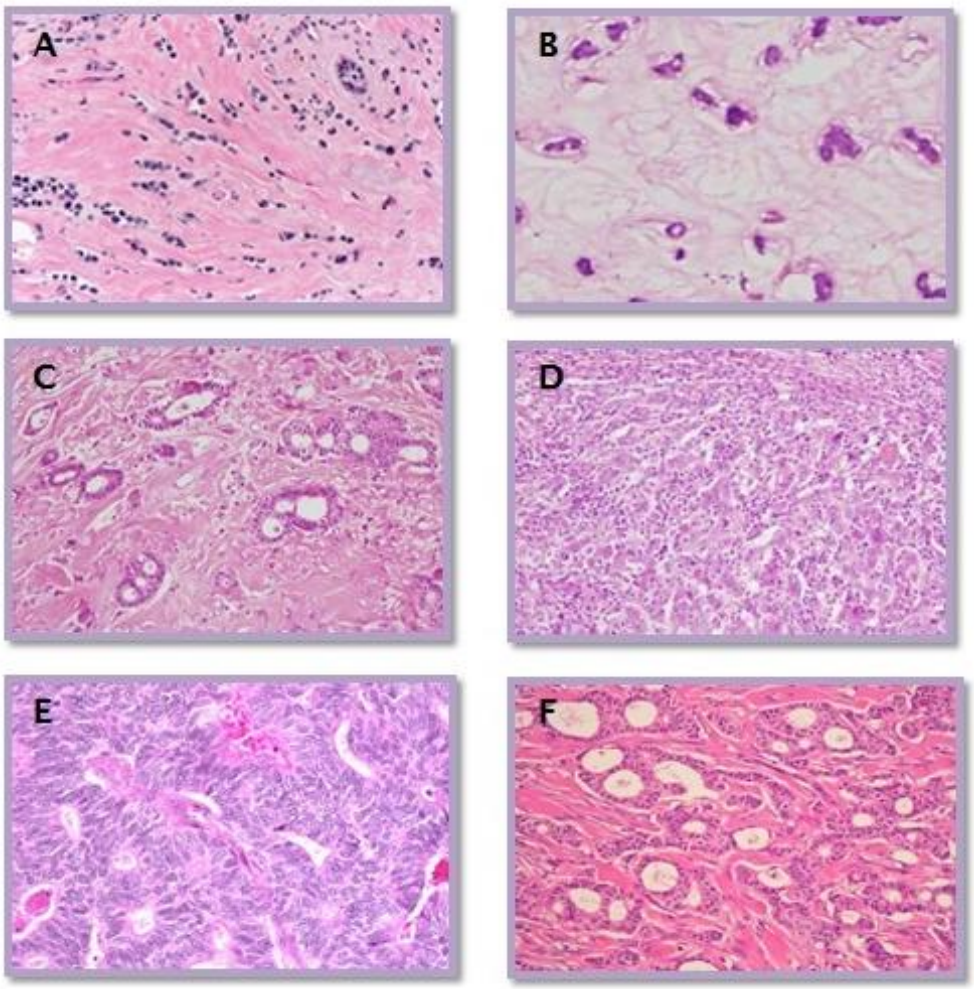


Figure 1.4 - Histological based tumor subtyping classification. A- Invasive lobular carcinoma; **B-** Mucinous carcinoma; **C-** Tubular carcinoma; **D-** Medullary carcinoma; **E-** Papillary carcinoma; **F-** Cribriform carcinoma **[adapted from:** *http://www.breastpathology.info*]**.**

1.4 Screening, detection and staging

The underlying premise for breast cancer screening is that it allows the detection of small tumors that are more likely to be at an early stage of the disease, have a better prognosis, and have a more successful treatment. Women at increased risk of breast cancer might benefit from additional screening strategies, beyond those offered to women of average risk [30] **[Table 1.3]**.

Table 1.3 - Recommended guidelines for breast cancer screening for women with a hereditary risk. [adapted from: *Robson & Offit* (2007)**].**

Organization	Risk Group	BSE	CBE	MMG	MRI
National Comprehensive Cancer Network	≥1.7% at ≥35 y	Encouraged	Every 6–12 m	Annual (35 yr)	NA
	Strong family history	Encouraged	Every 6–12 m	Annual (varies)	NA
	Genetic high risk	Monthly (18 yr)	Every 6 mo (25 yr)	Annual (25 yr)	Annual (25 yr)
Cancer Genetics Studies Consortium	BRCA mutation	Monthly (18 yr)	Every 6 mo (20–25 yr)	Annual (20–25 yr)	NA
National Institute for Health and Clinical Excellence	>8% (30–39 yr) or >20% (40–49 yr) or >12% (40–49 yr) plus dense breast tissue or BRCA mutation or p53	NA	NA	Annual digital (40 yr)	Annual (30 yr; 20 yr for p53)
American Cancer Society	BRCA mutation; untested close relative of carrier; lifetime risk of at least 20–25%; chest radiation at age 10–30; Li–Fraumeni and close relative with breast cancer; Cowden and close relative with breast cancer	Annual	NA	NA	Annual

BSE- breast self-examination, **CBE-** clinician-performed breast examination, **MMG-** mammography, **MRI-** magnetic resonance imaging and **NA-** not available.

The most common symptom of breast cancer is a new mass [31] and several methods can be used to find out if the disease is present: the "triple diagnosis", which includes clinical examination, mammography and/or ultrasonography, and fine-needle aspiration for cytology or core needle biopsy for histopathological examination [32]. Widespread use of screening mammograms has increased the number of breast cancers found before they cause any symptoms, despite the window of opportunity between mammogram detection and possible palpation due to augmented breast

tumor density or size, being very small. In fact, more than 90% of breast cancer diagnoses are made early in the disease [33].

Still, some breast cancers are not found by mammogram, either because the test was not well done or because, even under ideal conditions, mammograms do not find every breast cancer [34]. Some MRI can detect invisible tumors for mammography screening **[Figure 1.5]**. This type of technique is recommended as a screening tool for women who have a 20% or greater increased lifetime risk of breast cancer [35].

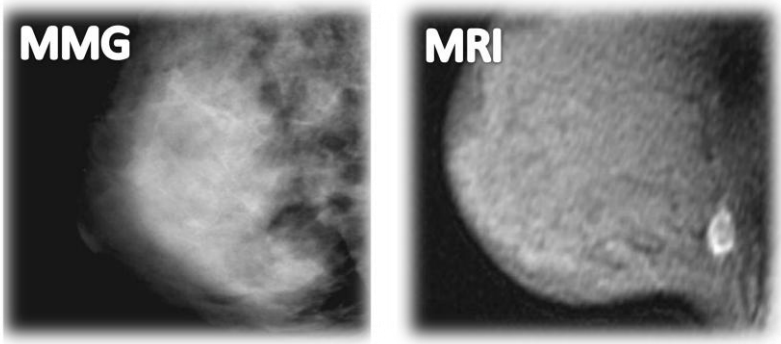


Figure 1.5 - Mammographically (MMG) occult breast cancer detected in the carrier of a *BRCA2* mutation on magnetic resonance imaging (MRI) [adapted from: *Robson* (2007)**].**

After breast cancer has been diagnosed, tests are done to find out if cancer cells have spread within the breast or to other parts of the body [36].

The **TNM classification** for staging breast cancer **[Table 1.4]** consists in three main evaluations:

The tumor size (T) -

TX - Primary tumor cannot be assessed;

T0 - No evidence of primary tumor;

Tis - Carcinoma in situ **[Figure 1.6];**

T1 - Tumor with 2 cm or less in greatest dimension;

T2 - Tumor with more than 2 cm but not more than 5 cm in greatest dimension;

T3 - Tumor with more than 5 cm in greatest dimension;

T4 - Tumor of any size with direct extension: (a) chest wall or (b) skin [31].

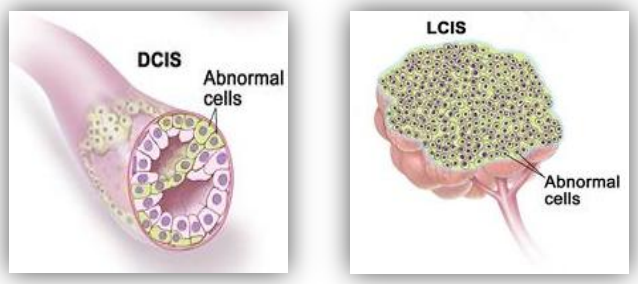


Figure 1.6 - Ductal and lobular carcinoma *in situ* [adapted from: *http://www.cancer.gov***].**

The regional lymph node involvement (N) -

NX - Regional lymph nodes cannot be assessed;

N0 - No regional lymph node metastasis;

N1 - Metastasis to movable ipsilateral axillary lymph node(s);

N2 - Metastasis to ipsilateral lymph node(s) fixed or matted, or in clinically apparent ipsilateral internal mammary nodes in the absence of evident axillary node metastasis;

N3 - Metastasis to ipsilateral infraclavicular lymph node(s) with or without clinically evident axillary lymph nodes, or in clinically apparent ipsilateral internal mammary lymph node(s) and in the presence of clinically evident axillary lymph node metastases, or metastasis in ipsilateral supraclavicular lymph nodes with or without axillary or internal mammary nodal involvement [31].

Distant metastasis (M) -

MX - Distant metastasis cannot be assessed;

M0 - No distant metastasis;

M1 - Distant metastasis [31] **[Figure 1.7].**

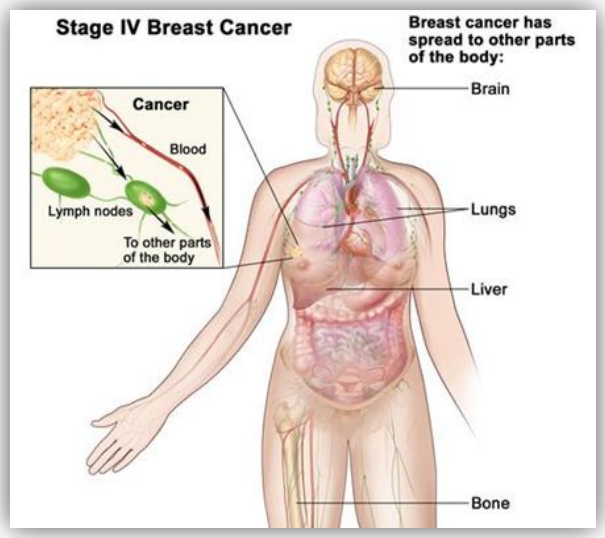


Figure 1.7 - Stage IV of breast cancer (any T; any N;M1) [adapted from: *http://www.cancer.gov*]**.**

Table 1.4 - Breast cancer TNM staging groups [adapted from: *Kalogerakos et al* (2008)]**.**

Stage groupings
Stage 0 - Tis N0 M0
Stage I - T1 N0 M0
Stage IIA - T0 N1 M0; T1 N1 M0; T2 N0 M0
Stage IIB - T2 N1M0; T3 N0 M0
Stage IIIA - T0 N2 M0; T1 N2 M0; T2 N2 M0; T3 N1 M0; T3 N2 M0
Stage IIIB - T4 Any N M0
Stage IIIC - Any T N3 M0
Stage IV - Any T any N M1

At initial diagnosis, over 50% of the breast cancers are stages 0 or I, and 75% are stage 0, I or II. The quantity of lymph node involvement has a profound impact on survival. Stage IIA cancer with only 1 involved lymph node has a 10 year disease free survival of 71% and a 20 year disease free survival of 66%. If 2 to 4 lymph nodes are involved, the 10 year disease free survival decreases to 62% and the 20 year disease free survival to 56% [37]. The 5 year relative survival rate for women diagnosed with localized breast cancer is 98.6%, but it declines to 83.8% for regional stage and to 23.3% for distant stage [2]. The overall 5 year relative survival rate for female breast cancer patients, has improved significantly in the last decades, from 75.1% between 1975 to 1977, to 90.0% for 2001 through 2007 [38].

In addition to stage, factors that influence prognosis and survival include: tumor grade - well differentiated tumors present better prognosis; histological type - specified types (tubular, mucinous, medullar, etc) have better prognosis than not specified types (IDC-NOS); proliferation index - high levels of proliferation (mitotic index) are associated with worse prognosis; ploidy - aneuploid tumors have worse prognosis; hormone receptor status - tumors that are positive for this receptors have better prognosis; human epidermal growth factor receptor (HER2) status - overexpression of this gene is associated with a worse prognosis, but it can be used as a therapeutic target for Herceptin [2]. Actually, the 5 year survival rate for Triple Negative (ER-; PR-; HER2-) breast cancer is about 77% [39].

1.5 Treatment strategies

Improvement in breast cancer treatment has undoubtedly increased the long-term survival of patients as reflected by the increased overall survival, across all breast cancer stages [31]. Nowadays, personalized medicine also seeks to offer the proper treatment to the right person, at the right time.

1.5.1 Surgical therapy

Surgical treatment for breast cancer involves Breast Conserving Surgery (BCS) or mastectomy. When BCS is appropriately used for localized or regional cancers, long-term survival is the same as with mastectomy [40]. However, some patients require mastectomy for clinical conditions, and others elect it because of personal reasons [2]. Mastectomy is also recommended if the risk of recurrence, over the next 5 to 10 years is >10% to 15%, even after surgery and radiation [41,31]. Regardless of the method used, an axillary lymph node dissection is always needed because is the most important prognostic factor for recurrence and survival [42].

The following graphic shows treatment patterns among women diagnosed with early or late stage breast cancer, concerning surgery and adjuvant options [19] **[Figure 1.8]**.

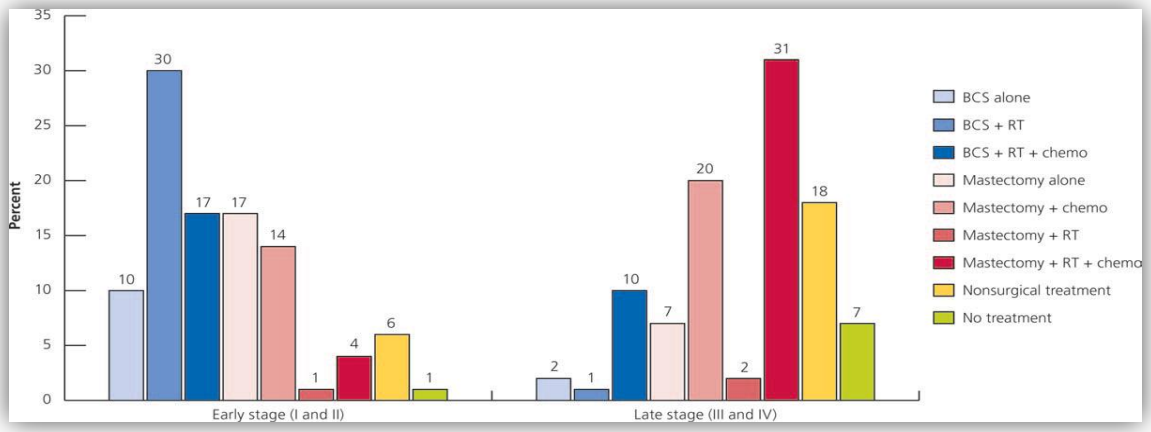


Figure 1.8 - Female breast cancer treatment patterns by stage [adapted from: *Siegel et al* (2008)**].**

1.5.2 Radiation therapy

Usually is done after the surgery and is known to substantially reduce the risk of locoregional recurrence by 19% after 5 years, and improves breast cancer mortality by 5.4% after 15 years [43]. Doses of 45 to 50 Gray (Gy) are typically given to the whole breast in daily fractions over a 5-week period, followed by a tumor boost over 1 to 2 weeks [44]. There are side effects but adjuvant radiation therapy after mastectomy is recommended to patients with tumors >5 centimeters regardless of lymph node status and those who have four or more positive lymph nodes [45]. If systemic chemotherapy is indicated, it will usually be completed before the initiation of radiotherapy [46].

1.5.3 Chemotherapy

Adjuvant chemotherapy shows results in terms of delayed tumor recurrence [47]. In most cases, chemotherapy is more effective when combinations of more than one drug is used. The original regimen was CMF (cytoxan, methotrexate, fluorouracil), but now many are being used, and it is not clear that any single combination is clearly the best. Some of the most used groups of drugs are the anthracyclines and the taxanes, but due to increased toxicity, taxanes are only recommended for patients at moderate/high risk of recurrence (node positive, HER2 positive, young age) [31].

1.5.4 Hormone therapy

A lot of research has been carried out on the mechanism of action of steroid hormone receptors, which has helped to identify potential molecular targets, that could be used to prevent or treat breast cancer. However, there is still ongoing search for drugs with maximal benefits and minimal undesirable effects. Briefly, they can be used to reduce the production of hormones or block them from working [48]. Some of the most important groups are:

Selective Estrogen Receptor Modulators: This group of drugs act as receptor binding competitors of estrogen and block their effects. The most common and successfully used member of this group is tamoxifen, which is a non-steroidal anti-estrogen that antagonizes the effects of estrogens **[Figure 1.9]**. It is used in both prevention and treatment of breast cancers that are responsive to estrogen, although sometimes tumors develop resistance to this type of treatment [49].

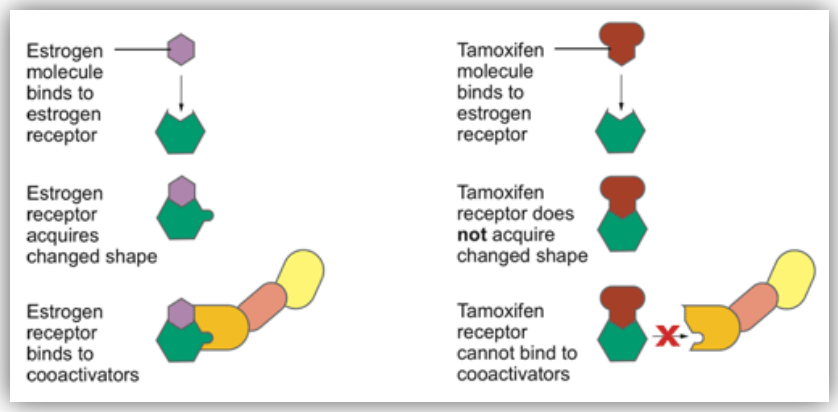


Figure 1.9 - Tamoxifen action on estrogen receptors [adapted from: *http://www.cancer.gov*].

Selective Estrogen Receptor Down-Regulators: These are anti-estrogens with no agonist activity and are more potent than SERMs. One of the most widely used members is fulvestrant, which is a steroidal anti-estrogen that has a 100-fold higher affinity to the ER than tamoxifen, with no agonist activity in the uterus [50].

Aromatase Inhibitors: These agents are superior to tamoxifen in both efficacy and toxicity, and have the potential to reduce receptor-negative tumors by synergy with *COX-2* inhibitors [51]. Aromatase inhibitors block the production of estrogens from androgens, which is the main pathway of estrogen production in post-menopausal and non-pregnant women, as well as from other tissues throughout the body [52].

1.5.5 Targeted therapy

It is a type of treatment that uses drugs or other substances to identify and attack specific cancer cells, without harming normal cells. Monoclonal antibodies and tyrosine kinase inhibitors are two types of targeted therapies used in the treatment of breast cancer [53].

The most known agent in this group is Herceptin (trastuzumab), a monoclonal antibody against the external domain of HER2 receptor, overexpressed in around 20%

of the breast cancers [54]. In some studies, trastuzumab showed a reduction of mortality, recurrence and metastases rates compared to patients never treated with the drug [55]. However, not all patients benefit from this drug, around 15% of women relapse after trastuzumab-based therapy [56]. Other used agents can be: Perjeta (pertuzumab), a humanized monoclonal antibody that binds to the extracellular domain II of HER2. Its mechanism is complementary to trastuzumab, inhibiting ligand-dependent HER2-HER3 dimerization [57]; Tyberb (lapatinib) competes with adenosine triphosphate for its binding site, on the tyrosine kinase domain of HER2 and EGFR inactive form, being a dual inhibitor useful for a wider range of patients [58]; Avastin (bevacizumab) is a humanized monoclonal antibody, designed to block vascular endothelial growth factor A (VEGF-A). It can be used for the treatment of metastatic breast cancer [59]; Afinitor (everolimus) is a mTOR inhibitor that works against hormone-receptor-positive breast cancers that have stopped responding to other drugs, by stopping the cancer cells from getting the energy they need [60]; PARP inhibitors are a type of targeted therapy being studied for the treatment of triple-negative breast cancer, because the absence of poly(ADP-ribose) polymerase leads to spontaneous single strand breaks, which are usually repaired by homologous recombination. Individuals with a defect in *BRCA1/2* are extremely sensitive to these inhibitors [61].

2. Genetic and molecular alterations in breast cancer

2.1 Molecular classification of breast cancer subtypes

Breast cancer is a heterogeneous group of several subtype entities, displaying distinct biological and clinical differences. Besides histopathological assessment and the hormonal receptors status, comprehensive molecular profiling using microarray-based technology has resulted in useful clinical information [62]. Five distinct intrinsic subtypes have been identified, based solely on gene expression, with distinct prognostic signatures: luminal A, luminal B, HER2 overexpressing, basal-like and normal breast tissue-like [63] **[Figure 1.10]**. Recent studies have also identified a new intrinsic subtype known as Claudin-low or mesenchymal-like [7].

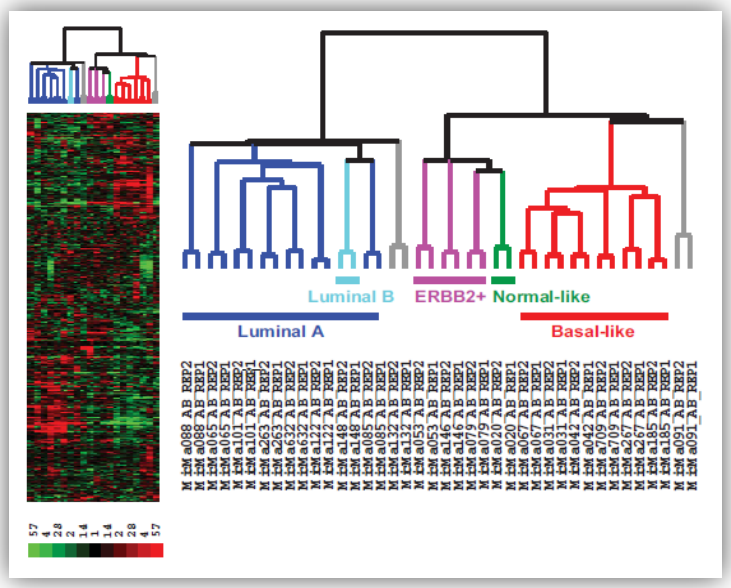


Figure 1.10 - Hierarchical clustering of 20 breast tumor tissues analyzed by arrays using 526 mapped intrinsic genes [adapted from: *Sorlie et al* (2006)**].**

The emergence of next-generation sequencing has now allowed the characterization of the mutational landscape of this disease. Based on significant genetic diversity due to both inherited genetic variation and acquired genomic aberrations, that contributes to cancer initiation and progression [64], Dawson *et al* [62] created a new classification consisting in ten integrative clusters, with distinct clinical features and outcomes.

The 70-gene signature MammaPrint and the 21-gene signature OncoType, are being used in selected patients, to obtain more accurate prognostic information [7].

2.2 Multistep carcinogenesis pathway of breast cancer

Breast cancer emerges by a multistep transformation of normal cells via hyperplasia, premalignant change and *in situ* carcinoma **[Figure 1.11]**. The understanding of the molecular role in cancer development, progression and metastases, is important for new prevention and treatment methods, but in breast cancer it is not easy to establish a single and definitive carcinogenesis model. Breast cancer is a hormone related type of cancer, associated with a carcinogenesis mechanism in which hormones (endogenous and exogenous) drive cell proliferation, along the progression pathway, increasing the number of cell divisions and the chance for random genetic errors [65].

It was already demonstrated that tumor development involves the accumulation of various genetic alterations, including amplification and activating mutation of oncogenes and inactivating mutation or loss of tumor suppressor genes. This loss of function can occur through sequential gene mutation events (somatic alterations), or

through a single mutation event of a remaining normal copy when there is a germline mutation [66].

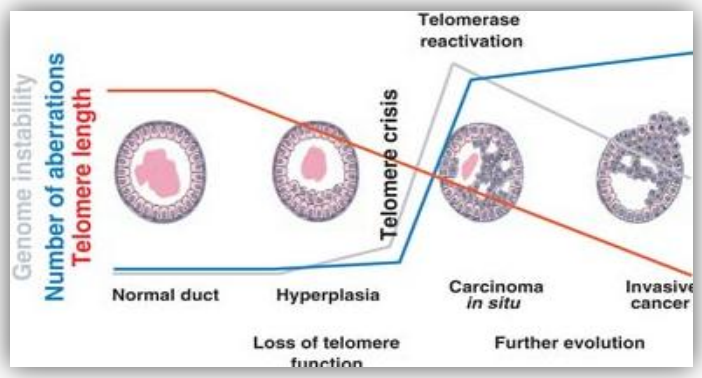


Figure 1.11 - Schematic representation of some genomic events associated with breast cancer progression [adapted from: *Chin K et al* (2004)**].**

The evolution of breast cancer and the interactions of genetic predisposing factors with somatic changes are summarized in **Figure 1.12**. In breast cancer, oncogene amplification is a common mechanism, but only few oncogenes seem to be essential for cancer development. These include three chromosome locations, 8q24 (*c-myc*), 11q13 (*int2, BCL1, EMS1, FGF3/4, CCND1*), and 17q12 (*c-erbB2* or *HER2*). Oncogene amplification, however, is not an early event in the multistep carcinogenesis pathway of breast cancer [4].

A consistently mutated tumor suppressor gene, in sporadic breast cancer, is *TP53*, with point mutations in approximately 22-34% of the cases [67]. Loss of heterozygosity incidences higher than 10%, have been observed in familial and sporadic breast cancer, with frequencies ranging between 20% and 79%. As seen for oncogene amplification and breast cancer development, some loci seem to be pathognomonic for the development or progression of a specific histological subtype [68].

Some studies defend that more than any other clinico-pathological parameter, the tumor grade strongly reflects the extent, complexity and type of genomic aberrations. For instance, grade I and tubular breast carcinomas, show a low number of genomic alterations with highly recurrent losses of 16q, whereas grade III breast carcinomas show complex genotypes frequently harboring loss of 11q, 14q, 8p, 13q; gain of 17q, 8q, 5p; and high level gains (amplifications) on 17q12, 17q22-24, 6q22, 8q22, 11q13 and 20q13. A lower number of genetic changes are found in ILCs relative to IDC [27].

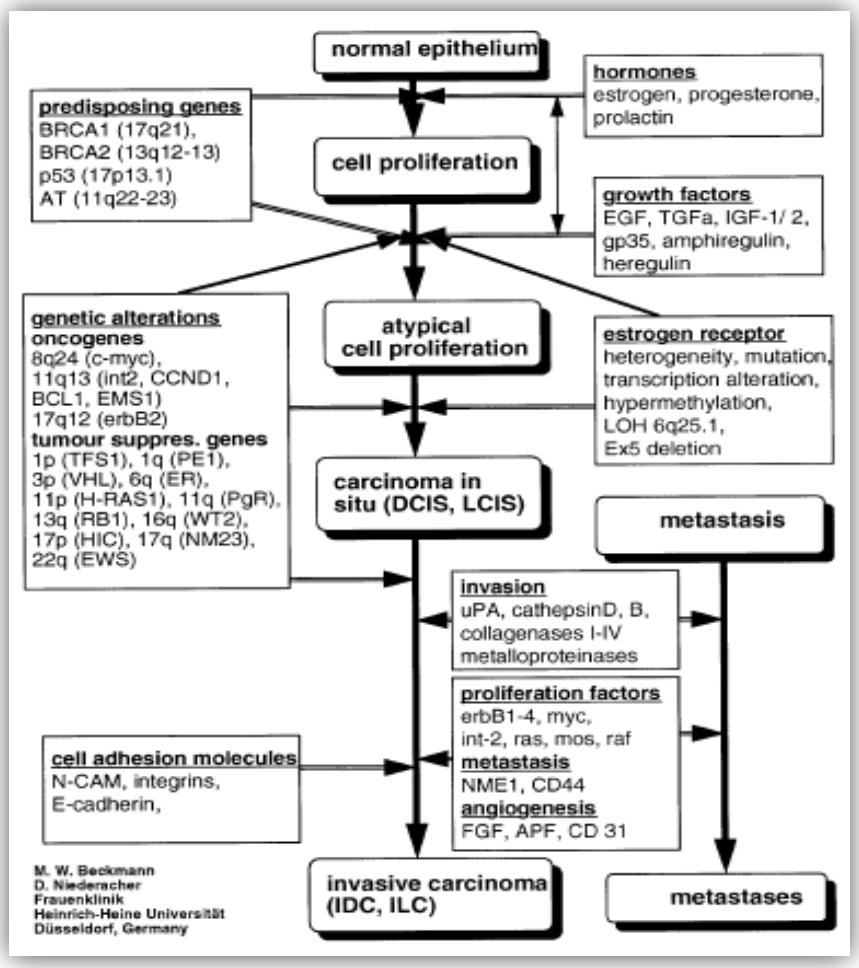


Figure 1.12 - Multistep model of breast cancer carcinogenesis [adapted from: *Beckmann et al* (1997)**].**

2.3 Predisposing genes

It is known that a portion of breast cancers are associated with genetic susceptibility. Some genes have already been identified, conferring an increased risk of developing this malignancy **[Table 1.5]**. As previously said, hereditary breast cancer constitutes only about 5−10% of total breast cancers, but the genes known to be involved in familial breast cancer (*BRCA1*, *BRCA2* and others), account for only about 15-20% of the familial risk **[Figure 1.13]**. On the other hand, most of the genetic variants that contribute to the risk of developing sporadic breast cancer are unknown, and recent studies suggest that much of the remaining variation in genetic risk, is probably caused by combinations of more common, lower penetrance, genetic variants [66] and their interaction with environmental agents [69].

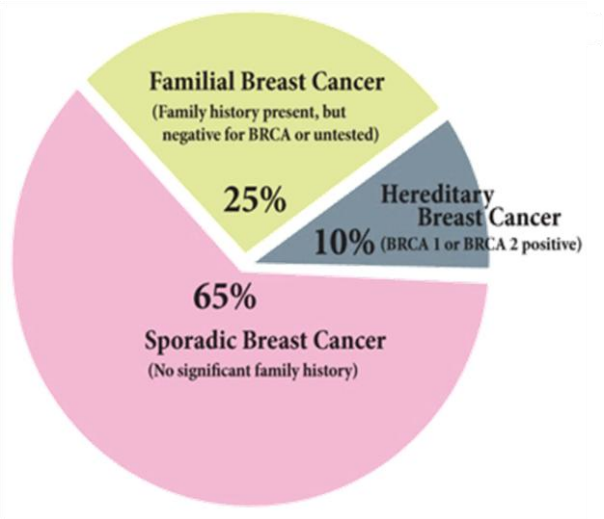


Figure 1.13 - Pie chart representing breast cancer distribution [adapted from: *www.breastcare.com**].***

2.3.1 High penetrance breast cancer susceptibility genes:

BRCA1 and *BRCA2* are two large tumor suppressor genes, with 24 and 27 exons, respectively, found in families with breast/ovarian cancer at early onset and inherited in an autosomal dominant pattern. Since their identification, several high risk mutations have been found that can originate hereditary breast-ovarian cancer syndrome [70].

***BRCA1* -** This gene has been implicated in DNA repair, cell-cycle regulation, transcriptional regulation and chromatin remodeling [4]. Tumors with *BRCA1* mutations are often triple negative and a high proportion show a "basal" aggressive phenotype that is associated with invasive ductal carcinomas (74%) [71] with a higher frequency of lung and brain metastases [72]. As previously said, *BRCA1* mutations confer a lifetime risk for developing breast cancer of 60% to 80% and by the age of 40, the risk for developing ovarian cancer is 17%, increasing to 39% by the age of 70, and to 54% by the age of 80 [73].

***BRCA2* -** Mutations in *BRCA2* are frequently found in families with high frequencies of male breast cancer [71]. It has been implicated in DNA recombination and repair with a role in the regulation of RAD51 activity [4]. The most frequent histological type of tumors with *BRCA2* alterations is also invasive ductal carcinoma (76%) [74], related to the luminal type, with expression of ER and more frequently

metastases to the bone and soft tissues [72]. Mutations in this gene confer a lifetime risk for developing breast cancer of 45% up to 80% [71].

These two genes form complexes that activate the repair of double strand breaks (DSBs) and initiate homologous recombination **[Figure 1.14]**. RAD51 is the key component of this mechanism. Cells defective in *BRCA1*/*BRCA2* (BRCAs) are hypersensitive for agents that produce DSBs. Overall, 3,492 distinct mutations, polymorphisms and variants were found in the BRCAs, most of them being predicted to truncate the protein, and some of them being population specific [74]. Women with BRCAs mutations can manage the risk by surveillance, prophylactic surgery and chemoprevention [75].

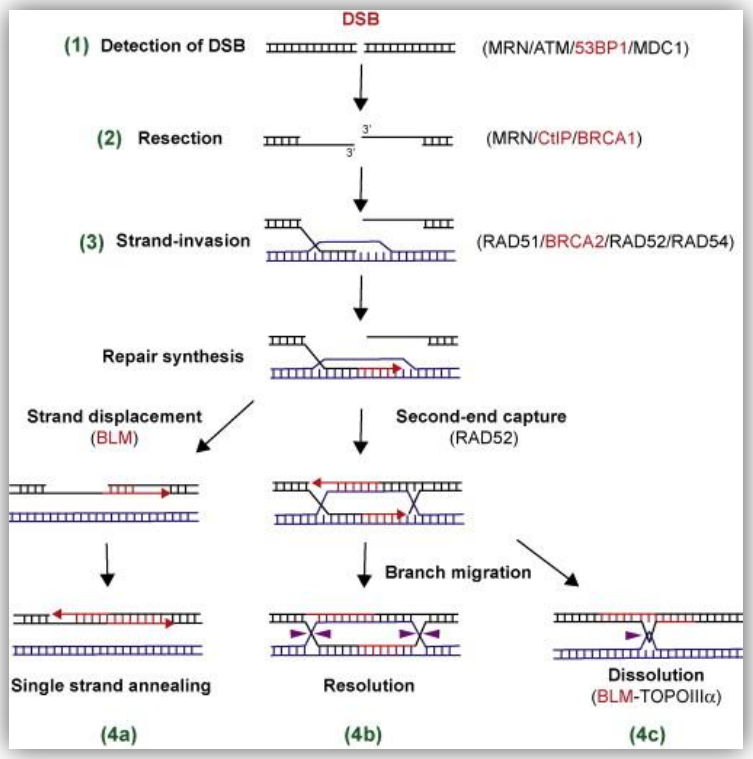


Figure 1.14 - Molecular mechanism of homologous recombination [adapted from: *Yata & Esashi* (2008)**].**

Some referral criteria for *BRCA1/2* testing include: three breast cancer cases in first degree relatives (or second if from paternal side) from the same side of the family, with at least one diagnosed before the age of 50 years; or two breast cancer cases at

any age, if there is a case with ovarian cancer; or one breast cancer diagnosed before the age of 45 years and one ovarian cancer at any age [76].

***TP53* -** Tumor suppressor gene encoding p53. p53 acts as a transcription factor, involved in the control of cell cycle progression, repair of DNA damage, genomic stability and apoptosis [77]. This gene is constitutionally mutated in the Li-Fraumeni syndrome (autosomal dominant predisposition to breast cancer among others) [78]. It is more commonly altered somatically in *BRCA1* and *BRCA2* related breast cancers [79]. Most mutations are point mutations [80].

2.3.2 Moderate to low penetrance breast cancer susceptibility genes:

Mutations in known high penetrance breast cancer susceptibility genes, account for only a portion of the familial aggregation of the disease. Linkage studies suggest that the remaining breast cancer susceptibility can be polygenic and a relative large number of moderate to low penetrance genes can be involved [66] **[Table 1.5]**.

ATM - Ataxia teleangiectasia mutated gene encodes a checkpoint kinase that plays a role in DNA repair. Biallelic mutations in this gene are linked to the rare human autosomal recessive disorder called ataxia teleangiectasia [81]. Carriers have a 2 to 5-fold risk of breast cancer [82].

CDH1 - Gene encoding E-cadherin, a calcium dependent cell adhesion glycoprotein, important for cell-to-cell adhesion [83]. Mutations in this gene lead to familial diffuse gastric cancer (autosomal dominant) and a predisposition to lobular breast cancer, with a risk of 50% [84].

PTEN - It is a tumor suppressor gene that encodes the protein phosphatidylinositol phosphate phosphatase with multiple roles in cellular regulation [85]. Germline mutations can lead to Cowden syndrome (autosomal dominant), characterized by a high risk of breast cancer, among others [86].

STK11 - This gene encodes a serine/theronine kinase and functions through inhibition of the mTOR pathway. It is mutated in the autosomal dominant condition Peutz-Jeghers syndrome, with a 30-50% risk of developing breast cancer [87].

***PALB2/FANCN* -** Encodes a protein that interacts with *BRCA2* in homologous recombination and double-strand break repair. Biallelic *PALB2* mutations are responsible for a subset of Fanconi anemia cases, characterized by a phenotype similar to that caused by biallelic *BRCA2* mutations [88].

Table 1.5 - List of the main susceptibility genes for breast cancer development and syndromes associated [adapted from: *van der Groep et al* (2011)**].**

Gene involved	Cytoband	Breast cancer risk	Syndrome
BRCA1	17q21	High	Hereditary breast cancer and ovarian syndrome
BRCA2	13q12.3	High	Hereditary breast cancer and ovarian syndrome
TP53	17p13.1	High	Li-Fraumeni syndrome
ATM	11q22.3	Intermediate	Ataxia teleangiectasia
CDH1	16q22.1	Intermediate	Familial diffuse gastric cancer syndrome
PTEN	10q23.31	Intermediate	Cowden syndrome
STK1	19p13.3	Intermediate	Peutz-Jeghers syndrome
NBS1	8q21	Intermediate	Njimegen breakage syndrome
BRIP/FANCJ	17q22	Intermediate	Fanconi anemia
PALB2/FANCN	16p12	Intermediate	
FANCA	16q24.3	Low	
FANCE	6p22-p21	Low	
MSH2	2p22-p21	Low	
MSH3	5q11-q12	Low	Lynch syndrome
MSH6	2p16	Low	
MLH1	3p21.3	Low	
PMS1	2q31-q33	Low	
PMS2	7p22	Low	

2.4 Genetic polymorphisms

The search for other genetic markers that confer susceptibility to breast cancer development, has led to an increase in epidemiological studies of relatively common genetic polymorphisms that may have a role in the metabolism of estrogen or in the activation or detoxification of environmental carcinogens [89].

Genes involved in the metabolism of sex hormones are strong candidates for breast cancer susceptibility genes. Those in the sex hormones biosynthesis pathway (CYP17, CYP19 and 17 B-hydroxysteroid dehydrogenase type2) may affect production of, and thus exposure to, the most active estrogen, estradiol. As some of the proteins involved in this pathway are regulated through specific receptor binding, steroid hormone receptor genes, such as *ER*, *PR* and *AR*, are also good candidates for breast cancer susceptibility [90] **[Table 1.6]**.

Several enzymes have evolved for the detoxification of xenobiotic compounds and their gene expression is induced in response to the presence of that compound. The action of phase I (CYP1A1 and CYP2D6) and phase II (GSTM1 and GSTT1) enzymes make susceptible compounds more soluble and more easily excreted, reducing breast cancer risk [90]. Phase I enzymes are induced by, and act on, carcinogens found in tobacco smoke [91], phase II enzymes, detoxify carcinogens and their reactive intermediates [92], and N-acetyl transferases participate in the detoxification of components in tobacco smoke and in cooked meat, among others [93].

Polymorphisms in common alleles of high penetrance genes like *TP53* and *BRCA1* that results in decreased protein activity, are associated with high lifetime risk of breast and other cancers [94].

Table 1.6 - Genetic polymorphisms and their relation with breast cancer risk [adapted from: *Dunning et al* (1999)**].**

Gene	Base change	Functional effect
Steroid hormone metabolism genes		
COMT	Exon 4 G → A	Reduced activity
CYP17	Promoter T → C (T1931C)	Creates a fifth *Sp1* site and might increase transcription
CYP19	Intron 4 $(TTTA)_n$ microsatellite	Unlikely
	Intron 4 TCT insertion/deletion	Unlikely
CYP2D6	2367delA (*A* allele)	Nonfunctioning enzyme
	Intron 3 G → A (G1934A) (*B* allele)	Nonfunctioning enzyme
	Del Lys281 (*C* allele)	Catalytically normal enzyme, but wrong cellular compartment
	17.5-kb deletion (*D* allele)	No enzyme
EDH17B2	Exon 6 A → G	Unlikely
ER	CCC325CCG	None
	Intron 1/exon 2 *Xba*I site	Unlikely
PR	Alu repeat insertion in intron G	Unlikely
Carcinogen metabolism genes		
CYP1A1	Exon 7 A → G (A4889G)	Uncertain, possible increase in enzyme activity
	3′ UTR T → C (T6235C)	None
	Exon 7 C → A (C4887A)	Unknown
	3′ UTR T → C (T5639C)	None
CYP2E1	Intron 6 unspecified	Unlikely
GSTM1	Gene deletion	Null individuals have no enzyme
GSTP1	A313G	Reduced enzyme activity
GSTT1	Gene deletion	Null individuals have no enzyme activity
NAT1	A1088T	Possible increase in enzyme activity
NAT2	G191A	Low activity allele
	C481T	Low activity allele
	G590A	Low activity allele
	G857A	Low activity allele

2.5 Epigenetics

During the past decade, the somatic mutation theory of cancer has been changed because it became evident that epigenetic malfunctions, play an equally important role as genetics in cancer development. Epigenetic mechanisms coordinate important biological processes like X-chromosome inactivation, genomic imprinting, position effect variation, reprogramming of genomes during differentiation and development among others [95].

DNA hypomethylation is evident in breast cancer, with up to 50% of cases showing reduced 5-methylcytosine content, compared with normal tissue. Hypomethylation in breast carcinomas affects mainly repetitive sequences, which are normally heavily methylated (for instance retrotransposons), contributing to genomic instability [96], but it can also affect individual genes. Besides, *de novo* gene

methylation can also occur, which has been shown to be a non-random process, specific of different cancer types and affecting genes like *BRCA* and other tumor suppressor genes, steroid receptor genes and cell cycle-related genes [97]. DNA methyltransferases (DNMTs) are known to be overexpressed in various types of tumors, including breast cancer. Approximately 30% of patients, revealed overexpression of *DNMT3B* in tumor tissue, 5% overexpressed *DNMT1* and 3% *DNMT3A*. Indeed, several polymorphisms were detected in the *DNMT3B* gene promoter and were associated with an increased risk of developing breast cancer [98]. miRNAs are globally downregulated in several cancers including the breast type. A panel of 15 miRNAs was reported to be able to distinguish between breast cancer and normal breast tissue, besides its ability to correlate with clinicopathological features. This deregulation can be due to aberrant DNA methylation or copy number variations [99]. As epigenetic changes are potentially reversible, the use of DNMT inhibitors and histone diacetylase (HDAC) inhibitors is a possible therapeutic strategy that is being explored [95].

3. The *HOXB13* gene and breast cancer

3.1 HOXB13

Homeobox (HOX) genes are crucial regulators of cell growth and differentiation. These genes initiate and control gene expression cascades, that drive development. They encode transcription factors that function to establish basic body pattern, during embryogenesis and maintain the function of specific organs in the adult, providing intercellular information for the determination of identity, lineage and fate of the cells [100]. The absent or aberrant expression of tissue-specific HOX genes has been implicated in cancer development [101].

Vertebrates HOX genes are clustered in four unlinked complexes in the genome (the HOXA, B, C and D clusters) with 39 members [100]. They arose from an ancestral complex amplification of some paralogs, followed by large-scale duplications, and each cluster has maintained a different subset of 13 paralogs. Genes located at the 3' ends of the complexes (paralog groups 1 and 2) are expressed at anterior positions within the hindbrain, while genes at the 5' positions (paralog groups 9-13) are expressed in progressively more posterior regions in the main body axis [102] **[Figure 1.15]**.

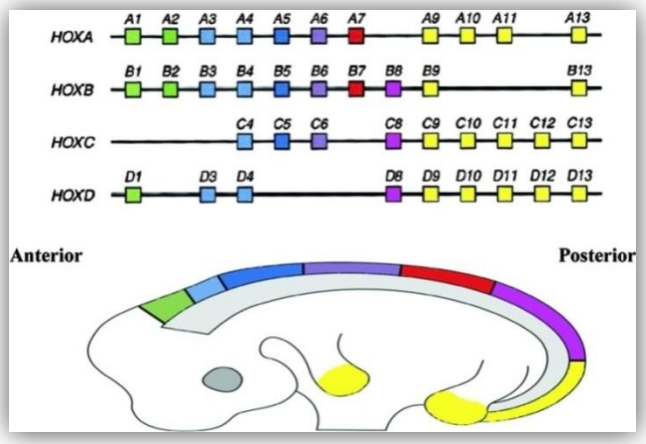


Figure 1.15 - Schematic representation of the genomic organization of the mammalian HOX clusters and their embryonic expression pattern [adapted from: *Daftang and Taylor* (2006)**].**

HOXB13 maps to chromosome 17q21 and it is a paralog of the *HOX13* gene, being the most 5' member of the HOXB cluster and the last HOX gene to be identified [102]. It consists of exon 1, with 601bp, and exon 2 with 254bp, divided by one intron of 949bp. The 5' UTR region contains 585bp and the 3' UTR region 2.027bp [103]. Its expression is highly specific of the tail bud and posterior domains: the spinal cord, digestive tract and urogenital system [102]. More recently, deregulated expression of *HOXB13* has been associated with aggressive cancer [104]. Changes in *HOXB13* gene expression, have been documented in small-cell lung cancer, breast cancer, prostate cancer, colorectal cancer, esophageal cancer and kidney cancer [105].

3.2 HOXB13 and breast cancer

The expression of *HOXB13* is known to be upregulated in breast cancer cells, compared with normal breast epithelium, suggesting a possible oncogenic role [106]. It has also been shown that *HOXB13* is an estrogen regulated gene, negatively correlated to estrogen receptor (ER) status and its expression is positively correlated with HER2 status in ER positive but not in ER negative tumors [107]. However, a subset of normal breast specimens demonstrated expression of *HOXB13*, in the terminal duct lobular unit, raising the possibility that it may play a role in normal mammary physiology. Cells expressing *HOXB13* displayed distinct morphological changes, characterized by a reduction in epithelial-type junctions [108].

Observations suggest that *HOXB13* may regulate a pathway that functions synergistically with *EGF*-dependent signaling, to stimulate cell motility and invasion *in vitro*, supporting the idea that it may directly contribute to tumor invasion and metastasis. Functional cooperation between *HOXB13* and *EGFR* signaling pathways may be relevant in the context of tamoxifen resistance, since activation of growth factor

signaling pathways can cause tamoxifen-resistant tumor growth [109]. *HOXB13* may also have a direct effect on ER signaling, since homeobox proteins have been shown to inhibit the histone acetyltrasferase activity of CBP/p300, a key coactivator of ER-dependent transcriptional regulation [110]. Interestingly, the gene encoding HER2 also maps to this chromosomal location (17q), which raises the question about possible coamplification of this two genes [111].

HOXB13 has been shown to be also a susceptibility gene for other types of cancer, namely prostate cancer, in which the mutation p.Gly84Glu was described to increase the risk of having cancer [112]. Although there are no confirmed mutations in *HOXB13* in breast cancer cases, some studies report that the p.Gly84Glu mutation, found in prostate, also increases the risk in familial breast cancers [113], while others claim that this association is not valid [114].

3.3 HOXB13 and tamoxifen resistance

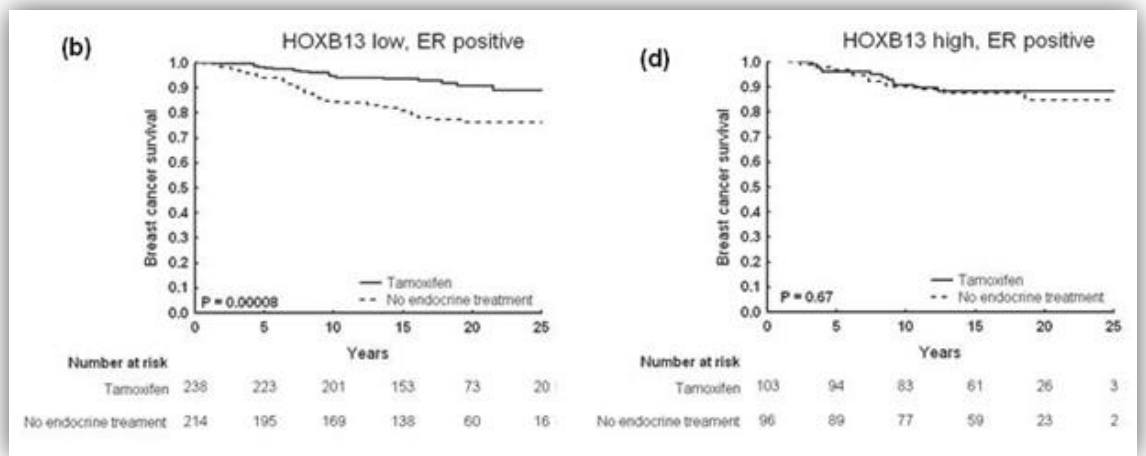
Several recent studies aimed to discover novel biomarkers and gene profiles for predicting risk of recurrence and response to endocrine therapy of breast cancer. With the development of reliable genetic markers for this purpose, it would be possible at an early stage, to predict which patients would benefit from alternative hormonal therapies [111].

Tamoxifen is the antiestrogen agent most prescribed in women, with both early stage and metastatic hormone receptor positive breast cancer [115]. As previously shown, tamoxifen is thought to act as a competitive inhibitor of estrogen binding to its receptor, with no effect on tumor cells lacking ER expression [116]. Prognosis has been based on clinical and pathologic parameters, however a subset of patients fail to respond or develop early resistance to tamoxifen (25% of ER+/PR+ tumors, 66% of ER+/PR- cases and 55% of ER-/PR+ cases), through diverse mechanisms [117].

Patients with tumors expressing high levels of *HOXB13* are more likely to be unresponsive to the therapy **[Figure 1.16]**, suggesting that this gene is somehow involved in tamoxifen resistance [111]. A recent study by Shah *et al* (2013) [118], actually concluded that *HOXB13* promotes tamoxifen resistance, directly downregulating the expression of ERα and driving tumor proliferation by inducing expression of IL6, leading to independent ER growth, by activation of alternative *AKT* and *mTOR* pathways.

The use of alternative hormonal therapies in such patients, such as the aromatase inhibitors, rapamycin, chemotherapeutic agents or inhibitors of other signaling pathways, may improve clinical outcome [103].

Figure 1.16 - Cumulative survival for patients with ER-positive tumors and with low or high levels of *HOXB13* [adapted from: *Jerevall et al* (2010)**].**



3.4 HOXB13/IL17BR index and clinical outcome

To discover novel biomarkers, predicting tamoxifen response, in the adjuvant setting, Ma and colleagues (2004) [108] conducted a microarray-based survey of gene expression patterns, that correlate with clinical outcome. Three genes were identified, *HOXB13*, *IL17BR* (interleukin 17 receptor B; induces the production of pro-inflammatory cytokines) and *CHDH* (choline dehydrogenase, an enzyme that belongs to the family of oxidoreductases), which were significantly associated with clinical outcome. They hypothesized that a two-gene expression index (*HOXB13/IL17BR*) might be a novel biomarker, for predicting treatment outcome in tamoxifen monotherapy **[Figure 1.17]**. They concluded that higher expression of *HOXB13* and lower expression of *IL17BR* or *CHDH* and a higher *HOXB13/IL17BR* index were associated with a higher risk of relapse and correlated significantly with prediction of poor prognosis (*HER2* amplification, S-phase fraction and number of positive lymph nodes). *HOXB13/IL17BR* was reported as a much better predictor in lymph node-negative patients than in lymph node-positive patients and, surprisingly, these genes predicted relapse in untreated cases.

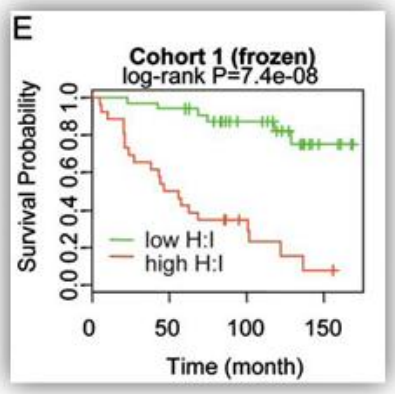


Figure 1.17 - Survival probability with low or high *HOXB13/IL17BR* ratio [adapted from: *Ma et al* (2004)**].**

Because ER functions to suppress *HOXB13* and increase *IL17BR* expression, either directly or indirectly, hormone responsive tumors should have a low index. When estrogen signaling is impaired by either loss of ER, aberrant expression of certain cofactors, or by HER2 signaling, suppression of *HOXB13* and induction of *IL17BR* expression is lost, resulting in a high index [107].

Ma *et al* [119] also concluded, in another study, that *HOXB13* was overexpressed in tamoxifen recurrence cases, whereas *IL17BR* was overexpressed in tamoxifen nonrecurrence cases, and that the two gene ratio had a stronger correlation with treatment outcome than either gene alone. The reduction of the tamoxifen response signature to two genes, raised the possibility that these genes may not only be markers of clinical outcome, but may be involved in breast tumorigenesis.

Among ER-positive, lymph node-negative patients not treated with adjuvant chemotherapy, a molecular grade index (MGI) of five gene (*BUB1B, CENPA, NEK2, RACGAP1, RRM2*) and the *HOXB13:IL17BR* (H:I) index shown to be associated with risk of breast cancer death [120].

In a recent study, Sgroi *et al* [121] concluded, through an analysis of previous trials, that patients with primary tumors with a high H/I index, who are disease-free after 4.5 to 6 years of adjuvant tamoxifen therapy, receive benefit from extended adjuvant letrozole therapy, reducing the relative and absolute risk of recurrence at 5 years by 67% and 16.5%, respectively.

3.5 Epigenetic repression of HOXB13 in breast cancer

Genetic analysis found that increased promoter CpG islands methylation of *HOXB13*, correlate with the decreased expression of its transcript in breast cancer ER-

positive cell lines. This aberrant epigenetic event, is associated with shorter disease free survival in a subset of patients. Transcriptional silencing of this gene can be reversed by a demethylation treatment, showing a prognostic value [122]. Because of this controversial results comparing with the studies about *HOXB13* and *HOXB13/IL17BR* index, the author of this epigenetic study suggests that this findings must be interpreted separately, as only an epigenetic marker and attributes a dual role of this gene in breast tumorigenesis. On one hand, the expression of *HOXB13* can be enhanced in ER-positive tumors exposed to tamoxifen treatment and, on the other hand, *HOXB13* may undergo epigenetic silencing by hypermethylation, triggered by estrogen signaling in other ER-positive tumors and being probably a later event in tumor progression [122].

II. Aims of the study

The main goal of this study was to clarify the role of *HOXB13* in breast cancer development. For that purpose, the specific aims were:

❖ Search for germline alterations in *HOXB13* in familial breast cancer cases (including some also with prostate cancer) that have been tested, negative for the *BRCA*1/2 genes.

❖ Determine the levels of *HOXB13* expression in a series of breast carcinomas, with and without 17q21 gain, previously determined by CGH.

❖ Evaluate if *HOXB13* gene amplification is a major driver of upregulation of this gene, in breast carcinomas.

❖ Test if there are clinico-pathological associations with *HOXB13* overexpression in breast cancer patients.

III. Experimental methodology

1. Germline mutation analysis

1.1 Samples

The selected DNA samples were extracted from blood of 100 patients with breast cancer at the Portuguese Oncology Institute of Porto, between 1990 and 2008. After an initial medical consultation, these patients with family history of breast cancer, some of which also had relatives affected with prostate cancer, were shown to have the referral criteria for *BRCA1/2* mutation analysis, but had a negative genetic test.

1.2 DNA extraction from peripheral blood

1.2.1 Isolation of nucleated cells

The samples were collected in sterile tubes containing EDTA and conserved at 4ºC or -80ºC for storage. A hypotonic solution was added (AKE: 155mM NH_4Cl; 10mM $KHCO_3$; 0.1mM EDTA; pH=7.4) to 3-5ml of blood in a proportion of 9-10 times that volume, followed by an incubation period of 30 minutes at 4ºC. The tubes were then centrifuged at 800g for 10 minutes. The supernatant was discarded and the procedure repeated until there was no significant hemoglobin in the pellet. Finally, those pellets were transferred to 1.5ml tubes and AKE was once again added, followed by a centrifugation at 3.200g for 5 minutes. The supernatant was rejected and the sample was stored at -80ºC until extraction.

1.2.2 DNA extraction

The DNA isolation was done by the salting out/chloroform protocol (Mullenbach, Lagoda et al 1989). Briefly, 400µl of SE buffer (6M NaCl; 0.5M EDTA; pH=8) and 400µl of 10% SDS were added to the previously precipitated nucleated cells. This mixture was well agitated in a vortex and 40µl of proteinase K (20mg/ml) [GIBCO BRL, California,

USA] were added for digestion to occur in an overnight incubation at 56ºC. The next day, 1ml of 6M NaCl was added and the samples were incubated for 10 minutes at 56ºC again. Then, 1ml of chloroform was added to the tubes and those were agitated for 30 minutes at room temperature. Samples were centrifuged at 2.000g at 4ºC for 10 minutes and the supernatant was collected and precipitated with a volume of isopropanol [Merck, USA]. The resulting DNA was washed with ethanol 70% (v/v) [Merck] two times, and finally eluted in bidestilled water. The DNA concentration and possible protein contamination of the samples was measured by NanoDrop ND-1000 spectrophotometer [NanoDrop Technologies, USA].

1.3 Polymerase chain reaction (PCR)

PCR was performed on the samples to amplify the *HOXB13* region in the DNA. Primers were designed using Primer-BLAST from NCBI database [123] **[Table 3.1]** and acquired from Thermo Scientific [Massachusetts, USA].

The PCR reaction was made in a 30µl volume, adding 1X of Taq Reaction Buffer containing 75mM Tris-HCL, 20mM $(NH_4)_2SO_4$ [Thermo Scientific], 2,5mM $MgCl_2$ [Thermo Scientific], 10µM dNTP's [Applied Biosystems, California, USA], 0.5M of primer forward and 0.5M of primer reverse, 1.5U of Taq DNA Polimerase [Thermo Scientific], 100 to 300ng of DNA sample, completing the final volume with ddH_2O. The touchdown PCR reaction was carried out in the Verity thermocycler [Applied Biosystems,California,USA] with the following program: 97ºC during 10 minutes; 6 cycles consisting in 1 minute of denaturation at 97ºC, 1 minute of annealing at 64ºC and 2 minutes of extension at 72ºC; 6 cycles consisting in 1 minute of denaturation at 97ºC, 30 seconds of annealing at 62ºC and 2 minutes of extension at 72ºC; 6 cycles consisting in 1 minute of denaturation at 97ºC, 30 seconds of annealing at 60ºC and 2 minutes of extension at 72ºC; 22 cycles consisting in 1 minute of denaturation at 97ºC, 30 seconds of annealing at 56ºC and 2 minutes of extension at 72ºC; finalizing with 10 minutes at 72ºC. The final products were analyzed using a 2% (w/v) agarose [Lonza, Basel, Switzerland] gel stained with 5µl of ethidium bromide (5µg/µl) [Sigma, Missouri, USA].

Table 3.1 - **Primers used for polymerase chain reaction and sequencing.**

	Exon 1	Exon 2
Primer forward	5'-GACGGCCGTGCTGAGCGAAT-3'	5'-GGACCCACAACCCCAGGCTCA-3'
Primer reverse	5'-AGGCCTGGGTCTGCATGGGT-3'	5'-GCCTGGGCTTGGCAGGTTCC-3'

1.4 DNA sequencing

DNA sequencing began with a first enzymatic purification (ExoSAP) of the PCR product to remove the excess of nucleotides, primers or other components of the previous reaction. The enzymes Exonuclease I 20U/µl [Thermo Scientific] and FastAP Thermosensitive Alkaline Phosphatase 1U/µl [Thermo Scientific] were mixed in the ratio of 1:2, respectively, and 1µl of this mix was added to 10µl of the amplified product. The reaction took place at 37ºC for 30 minutes fallowed by enzymatic inactivation 15 minutes at 85ºC.

Then, a new PCR was done using the BigDye^{TM} Terminator v3.1 Cycle Sequencing Reaction Kit [Applied Biosystems]. The sequencing reaction was performed in a 10µl volume adding 1µl of Terminator Ready Reaction Mix (with dNTP's, ddNTP's-fluorocromes, $MgCl_2$ and Tris-HCl buffer), 1.9µl of 5X sequencing buffer, 3.5M of primer forward, 1µl of ExoSAP purified product and ddH_2O until reaching the required volume. The PCR reaction was carried out in the Verity thermocycler with the following program: 96ºC during 10 minutes and 35 cycles consisting in 10 seconds of denaturation at 96ºC, 5 seconds of annealing at 52ºC and 6 minutes of extension at 60ºC.

The final product was again purified, this time with Illustra Sephadex® G-50 Fine DNA Grade [GE Healthcare Life Sciences, Ohio, USA] columns. Briefly, Sephadex® and ddH_2O filled the 96 wells of a plate provided by the manufacturer, and was centrifuged at 3900g for 4 minutes. The flow-through was discarded and 12µl of each sample were added to the resin and purified through it with other centrifugation at 3900g for 4 minutes. To stabilize the single strand DNA and elute the samples, 12µl of deionized formamide [Applied Biosystems] were added to each samples that was then sequenced by

capillary electrophoresis **[Figure 3.1]** in the 3500 Genetic Analysis automatic sequencer [Applied Biosystems].

The electropherograms were analyzed by the Sequencing Analysis Software v5.2 [Applied Biosystems] and the Mutation Surveyor DNA Variant Analysis Software [Softgenetics, Pennsylvania, USA].

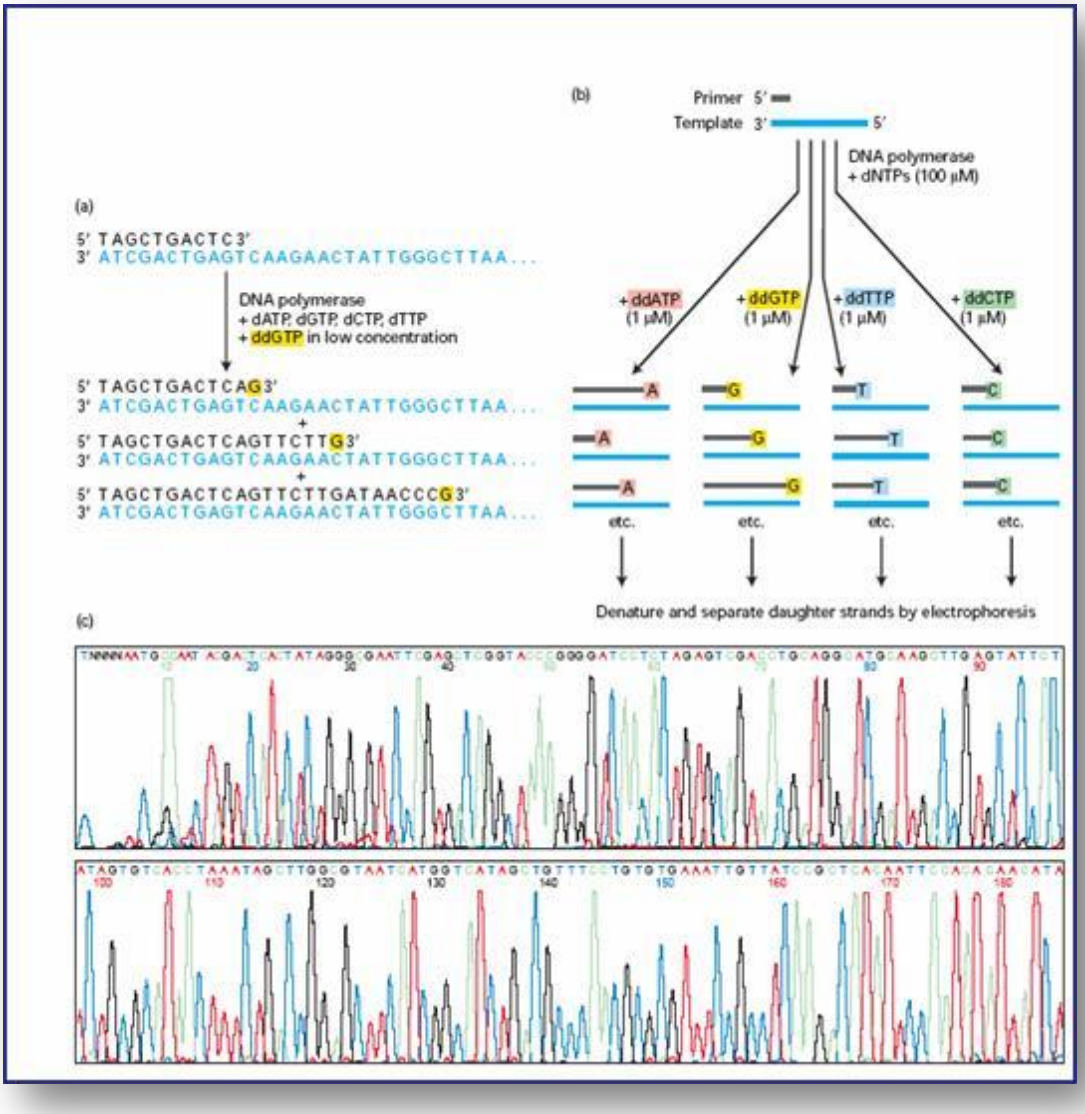


Figure 3.1 - Schematic representation of DNA sequencing molecular principles. (a) The incorporation of ddNTPs truncates the DNA new chain in certain locations, during primer elongation. **(b)** Each ddNTP is labeled with a different fluorescent molecule. **(c)** In a sequencing machine, the fragments suffer electrophoresis and the different fluorescences are registered **[adapted from:** *Ladish et al* (1990)**].**

2. *HOXB13* quantitative expression analysis

2.1 Samples

For this purpose, 84 samples collected from patients with breast cancer at the Portuguese Oncology Institute of Porto, between 1999 and 2001, were available. All tissue fragments had been fresh-frozen and the equivalent paraffin-embedded sections were stained with hematoxylin-eosin and examined by a pathologist to determine the diagnosis and evaluate the histopathological features.

Within the context of a previous study done in this research center, comparative genomic hybridization (CGH) was performed on those samples, to find possibly relevant genetic gains and losses. Since *HOXB13* localizes in the 17q21 chromosome region, for this study we have chosen all the cases with 17q21 gain (28 cases) and 56 cases without 17q21 gain **[Table 3.2]**.

Table 3.2 - Clinical characterization of the samples from patients with breast cancer used in this study, according to the 17q21 gain status.

	With 17q gain (%)	Without 17q gain (%)
Number of patients	28	56
Mean age (at diagnosis)	56	62
Receptor status:		
HER2+	14 (50)	5 (9)
ER+	19 (68)	41 (73)
PR+	13 (46)	38 (68)
Grade:		
I	0 (0)	8 (14)
II	11 (39)	29 (52)
III	16 (57)	19 (34)

2.2 RNA extraction

The piece of tumor was macerated in Petri plates with a scalpel and then transferred to matrix lysing tubes [MP Biomedicals, California, USA] with 1ml of TRIzol. They were agitated in Savant FastPrep FP 120 Cell Disruptor [MP Biomedicals] at 6.5m/s for 45 seconds, followed by a resting period to cool down in ice for a couple of minutes. The samples with TRIzol were transferred to 1.5ml tubes and 200µl of chloroform were mixed, then they were agitated in a vortex and centrifuged for 15 minutes at 12.000g at 4ºC. The resulting RNA phase was separated for different tubes, 500µl of isopropanol [Merck] were added and the tubes were agitated and incubated at room temperature 10 minutes for precipitation to occur. A centrifugation was done at 12.000g for 10 minutes as well, and the supernatant rejected. 1ml of ethanol 75% (v/v) was added to wash the pellet and waited 10 minutes, then centrifuged for 5 minutes at 7500g. The ethanol was rejected and the wash and centrifugation procedures were repeated. The ethanol was rejected again and the tubes were left to dry for 30 minutes at room temperature. Finally, RNAs were eluted in a volume of nuclease free water according to the pellet size (60 to 180µl) and were left to rest on ice for 20 minutes. The evaluation of RNA concentration was assessed using NanoDrop ND-1000 spectrophotometer. RNA was stored at -80ºC.

2.3 cDNA synthesis

The cDNA synthesis was performed following the Transplex Whole Transcriptome Amplification (WTA) kit [Rubicon Genomics, Michigan, USA] protocol. Briefly, to 300ng of template RNA, 2.5µl of WTA Library Synthesis buffer and 2.5µl of WTA Library Stabilization Solution were added and mixed for a final volume of 24µl. The samples were denatured in the thermocycler for 5 minutes at 70ºC and chilled on ice for 2 minutes. Afterwards, 1µl of WTA Library Synthesis enzyme was added, and mixed samples were placed back at the thermocycler for cDNA synthesis, using the following program: 15 minutes at 24ºC, 2 hours at 42ºC and 5 minutes at 95 ºC, and were chilled immediately on ice when finished.

For amplification, each reaction contained 7,5µl of WTA Amplification Master Mix, 1,5µl of dNTP Mix, 5U of TITANIUM Taq DNA polymerase (5U/ µl) and 5µl of Library Synthesis product and nuclease free water to perform a final volume of 75µl. The tubes

were placed in the thermocycler and the following program was performed: 3 minutes at 95ºC, and 17 cycles of 20 seconds at 94ºC and 5 minutes at 65ºC.

The cDNA was purified using the QIAquick PCR Purification Kit [QIAGEN, California, USA] according to the manufacturer´s protocol. Briefly, 350µl of Buffer PB were added to the PCR product, and this solution was transferred to a QIAquick column placed in a 2ml collection tube. The flow through was rejected after a centrifugation at 17.900g for 60 seconds and the column was placed again in the tube. To wash the sample, 750µl of Buffer PE were added to the column, followed by two centrifugations at 17.900g for 60 seconds. The flow through was discarded after each centrifugation and the columns were placed in 1.5ml tubes. The cDNA was eluted with 50µl of nuclease free water and a centrifugation at 17.900g for 60 seconds was performed. This procedure was repeated to reach a final volume of 100µl and the purified cDNA was stored at -80ºC.

2.4 Quantitative reverse-transcriptase PCR

The reactions were performed using TaqMan gene expression assays **[Figure 3.2]** for *HOXB13* (Hs00197189_m1) and the endogenous control assays for *GUSB* (Hs00939627_m1), from Applied Biosystems.

The reaction was done in 96-well plates in a 7500 Real-Time PCR System [Applied Biosystems]. In each well were added 2µl of the previously synthesized cDNA (15µg/µl), 10µl of SensiFast Probe Lo-Rox mix (2x) [Bioline, London, UK], 1µl of Taqman *HOXB13* expression assay and 7µl of bidestilled water for a total volume of 20µl. PCR conditions were the ones predefined by the manufacturer: 50ºC for 2 minutes, 95ºC for 1 minute and 45 cycles at 95ºC for 15 seconds and 60ºC for 1 minute. cDNA samples were run in duplicate. cDNA from LNCaP prostate cell line was used to prepare four consecutive dilutions (dilution factor: 10X) that were used as standards, run in triplicate on each plate, allowing the construction of a standard curve to evaluate amplification efficiency. Water blanks were also added in duplicate for each gene.

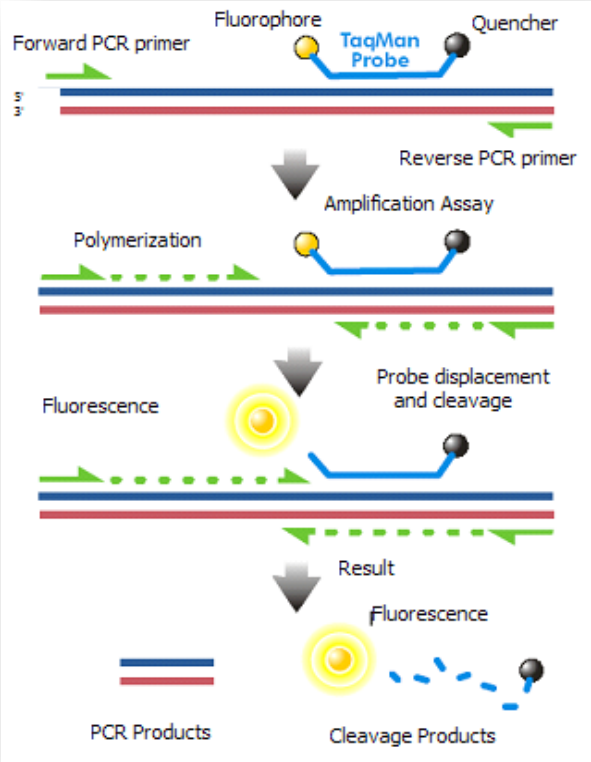


Figure 3.2 - TaqMan biochemistry of the probe reaction. The displacement between the quencher and the fluorophore (reporter), originates fluorescence that is captured as the products are being amplified.

The results were analyzed using the 7500 Software v2.0.6 [Applied Biosystems]. The standard curves slopes for each gene did not vary more than 0.05 and the efficiency was between 90% and 98% per plate. The mean quantity of *HOXB13* transcripts was normalized for the mean quantity of the endogenous control *GUSB*, applying the comparative Ct method [124].

The statistical analysis and respective significance was calculated using the SPSS v.16 program [IBM Software, USA] and also GraphPad Prism 5 [California, USA]. Results were considered significantly different when $p < 0.05$.

3. Fluorescent in situ hybridization (FISH)

3.1 Samples and strategy used

A series of 22 paraffin-embedded tumors was chosen for cytogenetic analysis, based on the levels of *HOXB13* expression, determined in the previous quantitative expression analysis. These included 16 samples that revealed high expression of this gene and 6 with low expression. The 17q21 gain status was also considered **[Table 3.3].**

Table 3.3 - Sample distribution according to *HOXB13* levels of expression and 17q21 gain status.

	HOXB13 high expression	*HOXB13 low expression*
17q gain: positive	10 (62.5%)	3 (50%)
negative	6 (37.5%)	3 (50%)
Total	16	6

To access if there was any *HOXB13* copy number gain in this series, a BAC clone covering the entire gene was selected (ID: 2200B24 [Invitrogen, California, USA]), using the University of California, Santa Cruz (UCSC) Human Genome Browser. A commercial probe for chromosome 17 centromeric region [Abbot Molecular] was used as control for ploidy.

3.2 Isolation and amplification of plasmid DNA from E. coli

Bacteria were transferred for a 50ml Falcon with 10ml of LB culture (1% (w/v) of Bacto-Triptone; 0,5% (w/v) of Bacto-Yeast Extract; 0,25% (w/v) of NaCl; 100ml of bidestilled water) and 20µl of chloranphenicol (12.5µg/ml) to select only the resistant bacteria with the desired transformed plasmid. They were allowed to grow for 16 hours at 37ºC with orbital shaking in an agitator [ROSI 1000 Thermolyne, Iowa, USA] overnight. The

tubes were then centrifuged at 3100g for 30 minutes and after drying for 15 minutes, plasmids were extracted according to the NucleoSpin Plasmid kit protocol [Macherey-Nagel, Düren, Germany]. Briefly, bacteria were resuspended with A1 buffer (with RNAse), then the plasmid DNA was released of the cells with buffer A2 addition (by SDS/alkaline lysis). To neutralize the resulting lysate, buffer A3 was added and the unwanted cell remains were discarded after a 10 minutes 11.000g centrifugation. The supernatant was then transferred to NucleoSpin^{R}Plasmid columns and a centrifugation of 1 minute at 11.000g was performed. Ethanolic buffer A4 was used to wash the column membrane and plasmid DNA was eluted with Alkaline Elution (AE) Buffer (5mM Tris/HCl, pH 8.5) by centrifugation at 11.000g for 1 minutes.

DNA content was quantified using NanoDrop ND-1000 spectrophotometer and diluted with AE buffer [Macherey-Nagel] for a final concentration of 10ng/µl.

For plasmid amplification the Illustra GenomiPhi V2 DNA Amplification Kit [GE Healthcare, UK] was used according to the manufacturer's instructions. Briefly, 9µl of Sample Buffer (with non-specific random primers) was added to 1µl of DNA and the samples were heated to 95ºC using in the thermocycler [Biometra, Gottingen, Germany] for denaturation during 3 minutes and then cooled at 4ºC, then, for each tube, 10µl of a master mix with 9µl of Reaction Buffer and 1µl of Enzyme Mix were added to gather all the components required for DNA amplification. The reaction was carried out in the thermocycler for 15 hours at 30ºC, followed by enzyme inactivation at 65ºC for 10 minutes.

3.3 Nick translation and precipitation

First, DNA content was quantified and eluted in water for a final concentration of 1000ng/µl. For labeling the DNA with SpectrumRed [Abbott Laboratories, UK], the Nick translation DNA Labeling System kit was used [ENZO Life Sciences, USA] and the following reagents were mixed in 0,5ml ambar tubes: 1.6µl of the previous eluted DNA, 5µl of Reaction Buffer, 5µl of dNTP mix, 2.5µl of dTTP, 0.75mM of green fluorophore-dUTP, 5µl of DNA Polymerase I and freshly diluted DNase I (8µl of 10X DNase I Dilution Buffer + 72µl of nuclease free water + 1µl of DNase I). The reaction took place in the thermocycler at 15ºC for 120 minutes **[Figure 3.3]**. At the end, 5µl of Stop Buffer were added and tubes were placed back in the thermocycler at 65ºC for 5 minutes.

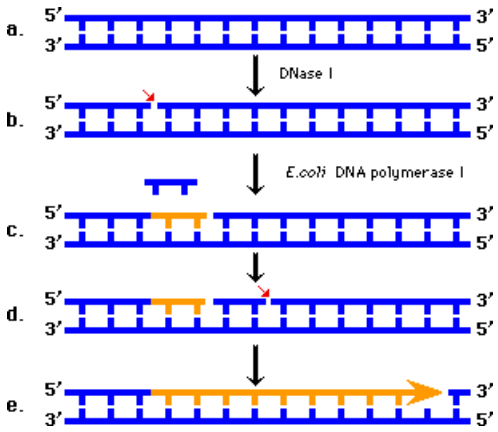


Figure 3.3 - DNase nicks the double strand DNA and E. coli DNA polymerase I removes and polymerizes the new chain, due to its exonuclease and polymerize activity.

For precipitation, 11µl of the labeled probe were transferred to a fresh 1.5ml tube, following an addition of 4µl of Human Cot-1 DNA (1mg/ml) [Life Technologies, Rockville, MD], 1µl of bidestilled water (to a total volume of 16µl), 1.6µl of 3M sodium acetate and 40µl of ice-cold precipitated, dried and dissolved in hybridization buffer [Abbott Molecular] ethanol. The tubes were placed at -22ºC overnight and the next day were centrifuged at 4ºC and 15.300g for 30 minutes to create a DNA pellet. The supernatant was removed carefully and the tubes were left to dry in a heating plate at 45ºC for 42 minutes, in the dark. The probe was, then, re-hydrated with 3µl of water and 7µl of LSI/WCP Hybridization Buffer [Abbot Molecular] and 0,5µl of SpectrumGreen Vysis CEP17 SGn Probe [Abbot Molecular] were added. This mix was placed in a water bath for 30 minutes and stored at room temperature in a dark place before hybridization.

The used probes were tested in metaphases to ensure their specificity for the chromosome region of interest and to evaluate the quality of the signals.

3.4 Treatment and digestion

The paraffin-embedded samples were placed in the heater, at 60ºC for 10 to 30 minutes, to melt the paraffin. Then, the slides were transferred 2 times through xylol (first 10 minutes, second 8 minutes) and 2 times through ethanol 100% (10 minutes each), and washed 3 minutes in 2xSSC. Slides were then incubated in a solution of 0.06g/ml NaSCN [Sigma-Aldrich] at 80ºC for 10 minutes, followed by two washes, 2

minutes each, in 2xSSC. The slides were covered with pepsine 4mg/ml (100µl pepsine 10mg/ml [Sigma-Aldrich], 100µl ddH_20, 50µl 1M HCl) and a lamella and placed in Hybrite Vysis [Abbot Molecular] for 13 to 20 minutes (depending on the tumor sample) at 37ºC for digestion of the nucleated cells.

3.5 Denaturation, hybridization and contrast

After digestion, the lamella was removed and the slides ware washed twice in 2xSSC, 2 minutes each. Next, dehydration was performed through a series of alcohols: 3 minutes in 70% alcohol, then the same time in 85% and finally 3 minutes in 100% alcohol. Before hybridization slides were left to dry at room temperature. Probes were heated for 30 minutes in a water bath at 37ºC, and 5 to 10µl were added to the slides that were then covered with a lamella and sealed with easy removable glue [Marabu, Tamm, Germany]. Slides were placed in Thermobrite StatSpin denaturation/hybridization system [Abbott molecular, Ilenois, USA] with the following program: 80ºC for 5 minutes (co-denaturation) and 37ºC for 18 hours (hybridization) **[Figure 3.4]** .

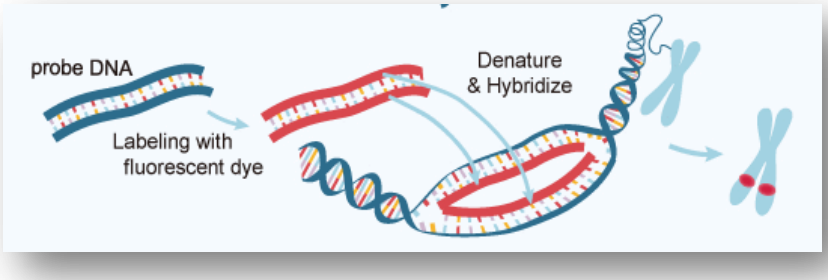


Figure 3.4 - Fluorescent in situ hybridization principle. The labeled probe hybridizes with the high complementary DNA in the desired chromosome region and the signals are then revealed by fluorescence microscopy.

When finished, the glue and the lamella were removed and the slides were washed in 2xSSC/0.5% Igepal solution [Sigma-Aldrich] at 73ºC for 5 minutes and in 2xSSC/0,1% Igepal solution for 3 minutes [Sigma-Aldrich]. Finally, the slides were counterstained with 10µl of 4´, 6-diamidino-2-phenylindole (DAPI) [Vector Laboratories, Burlingame, California, USA] were and stored at 4ºC. The observation and analysis was performed in a Zeiss Axioplan fluorescence microscope [Zeiss, Oberkochen, Germany]

coupled with a Cohu 4900 CCD camera and a CytoVision system version 3.9 [Applied Imaging, Santa Clara, California, USA].

For each slide, 60 cells were counted (30 in two different regions) and for each cell the mean HOXB13/CEN17 ratio was calculated, by adding all the values and dividing by 60.

IV. Results

1. *HOXB13* germline mutation analysis

From the 100 DNA samples, 5 could not be amplified by PCR due to DNA quality and 95 were sequenced. No germline mutations were found in either the two exons of *HOXB13*, only previously described polymorphisms with the expected frequencies **[Table 4.1]**.

Table 4.1 - Main polymorphisms found in exon 1 and exon 2 of *HOXB13* and frequencies (fr).

Ref Allele/ Variant Allele	dbSNP ID	Variant Type	Substitution	N (fr)
A/G	rs9900627	Synonymous	S171S	20 (0,21)
G/A	rs8556	Synonymous	S122S	24 (0,25)

2. *HOXB13* mRNA expression by 17q21 status

Significant differences were observed in the transcript expression levels of *HOXB13* between breast cancer cases with or without gain of 17q21 chromosome band. The Mann-Whitney non-parametric test was performed to assess the significance of the differences (p= 0.006) (N=84) **[Figure 4.1]**.

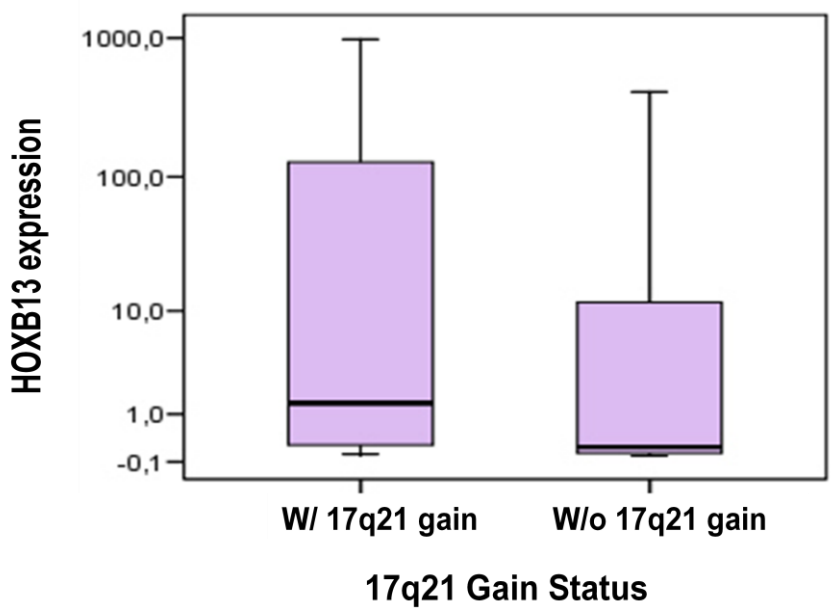


Figure 4.1- Boxplot representing *HOXB13* expression levels (logarithmic scale) in samples with and without 17q21 gain.

3. *HOXB13* copy number changes

Among the 22 selected tumor samples, eight were found to have *HOXB13* amplification with values of HOXB13/CEP17 ratio between 2.0 and 3.0 **[Table 4.2]**. However, no statistically significant difference was found between the group of samples with and without *HOXB13* amplification concerning the levels of expression of this gene (p= 0.145), although there is a visible tendency for the mean expression values to be higher in the group where amplification was found **[Figure 4.3]**.

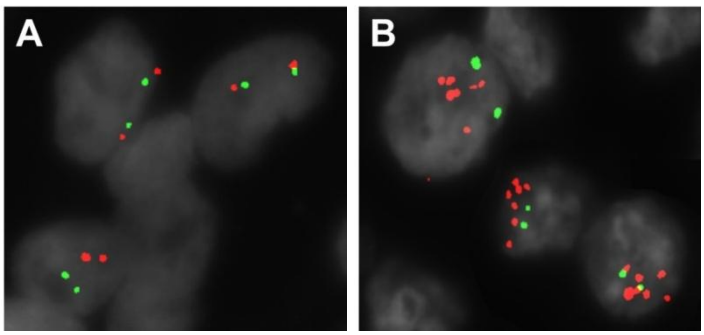


Figure 4.2 - Examples of cell images captured by fluorescence microscopy, after fluorescent in situ hybridization (FISH). Red signals correspond to the gene (*HOXB13*) and green signals to the chromosome 17 centromeric region (CEP17). (**A**) Tumor cells with normal copy number; (**B**) Tumor cells with *HOXB13* amplification.

Table 4.2 - Fluorescent in situ hybridization results. HOXB13/CEP17 relative ratio and 17q21 status, analyzed by CGH, for each sample. Cases with *HOXB13* amplification are highlighted.

Samples	CGH 17q21	Ratio
87_99	no gain	1.1
381_99	no gain	1.0
509_00	gain	2.8
531_99	gain	2.1
876_01	gain	2.0
1024_99	gain	2.5
1048_99	no gain	1.5
1580_01	no gain	1.1
1951_00	gain	2.5
2473_00	no gain	1.1
3664_00	gain	1.6
3868_00	gain	1.2
5941_99	no gain	1.1
5980_99	gain	1.1
6406_99	gain	2.3
7016_00	gain	3.0
7166_00	gain	1.0
7270_99	no gain	1.1
7586_00	gain	1.1
7690_00	gain	1.2
7775_00	no gain	1.3
7835_00	gain	2.4

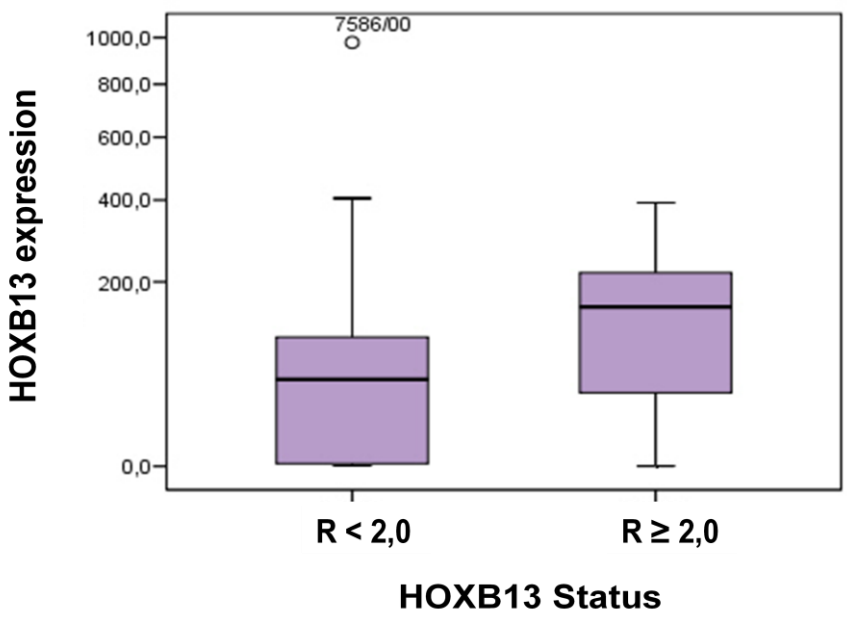


Figure 4.3 - Boxplot representing *HOXB13* expression levels (logarithmic scale) in tumors with amplification (Ratio>2) of this gene and tumors with no amplification (Ratio<2).

4. *HOXB13* expression by 17q21 and *HOXB13* copy number

When comparing the 17q21 status, defined by the CGH data previously obtained, and the *HOXB13* gene copy number status, we can consider three groups. The first group that includes all the samples with *HOXB13* amplification and that also presents 17q21 gain, as expected, and the remaining two groups with the samples that showed no amplification, but that were separated by 17q21 status. Samples without 17q21 gain, that were not analyzed by FISH, were considered to have no *HOXB13* amplification and were also included in the statistical test (N=70). To compare these three groups regarding *HOXB13* levels of expression at the same time, the Kruskal-Wallis test was performed, and a statistical significance was found (p= 0,010) **[Figure 4.4].** However, if we compare the group with 17q21 gain and no amplification of *HOXB13* with the remaining groups, using the Mann-Whitney test, there are no statistical significant differences (p= 0.245*;* p= 0.228), which are only observed between the group of samples with 17q21 gain/amplification and without 17q21 gain/no amplification (p= 0.006).

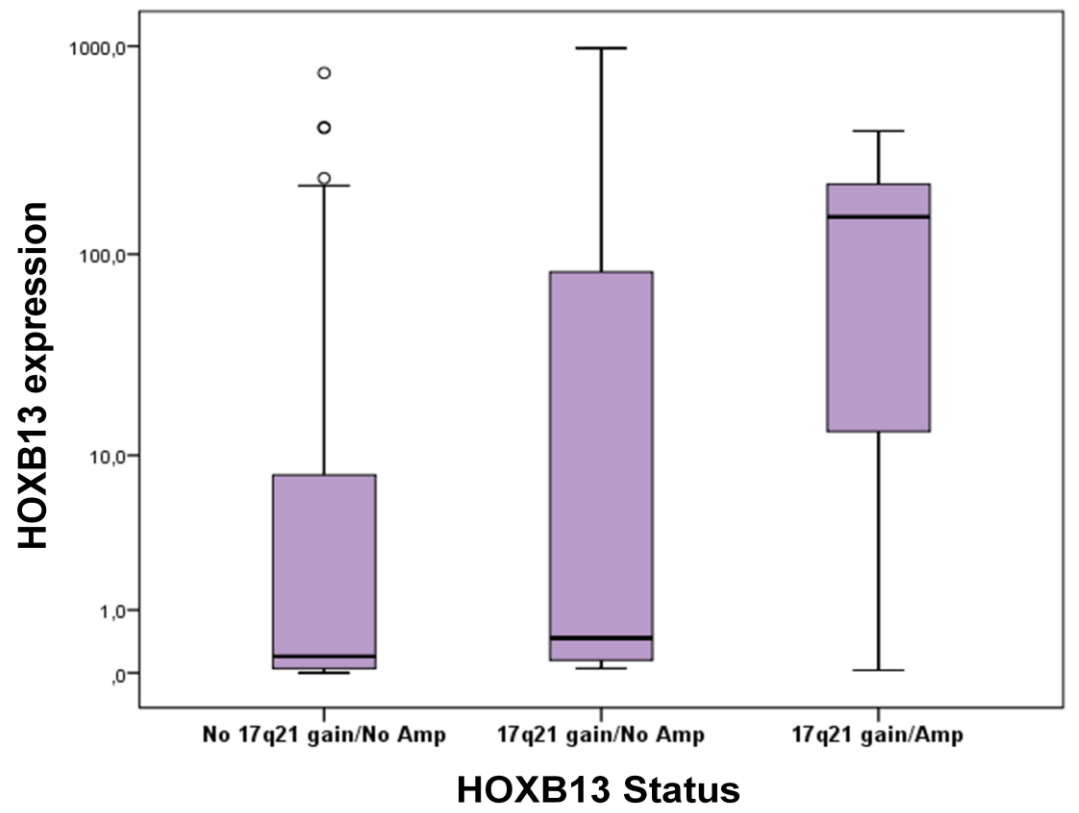


Figure 4.4 - Boxplot representing *HOXB13* expression levels (logarithmic scale) in cases with and without *HOXB13* amplification and with or without 17q21 gain.

5. Clinico-pathological associations

There were no significant associations between the levels of *HOXB13* expression and some clinical features, namely: ER status (p= 0.395), PR status (p= 0.193), molecular subtyping (p= 0.114) and histological type (p= 0.762).

A significant and progressive association was observed between *HOXB13* levels of expression and tumor grade (N=83) (p=0.015) **[Figure 4.5]**, as well as a positive association concerning HER2 status (N=83) (p=0.019) **[Figure 4.6]**.

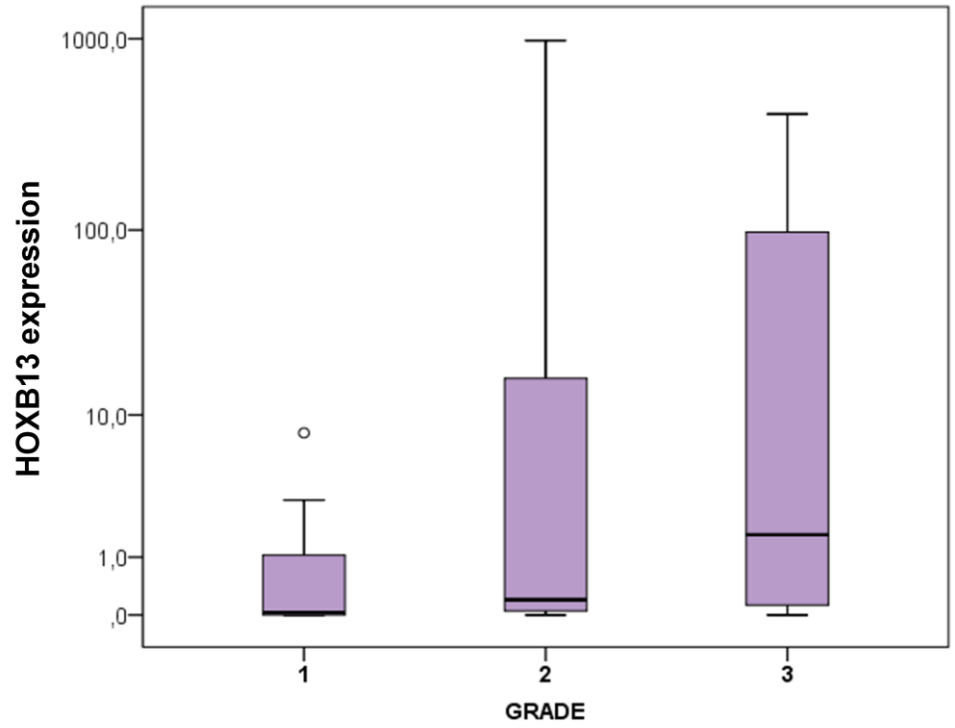


Figure 4.5 - Boxplot representing *HOXB13* expression levels (logarithmic scale) in groups with different tumor grades.

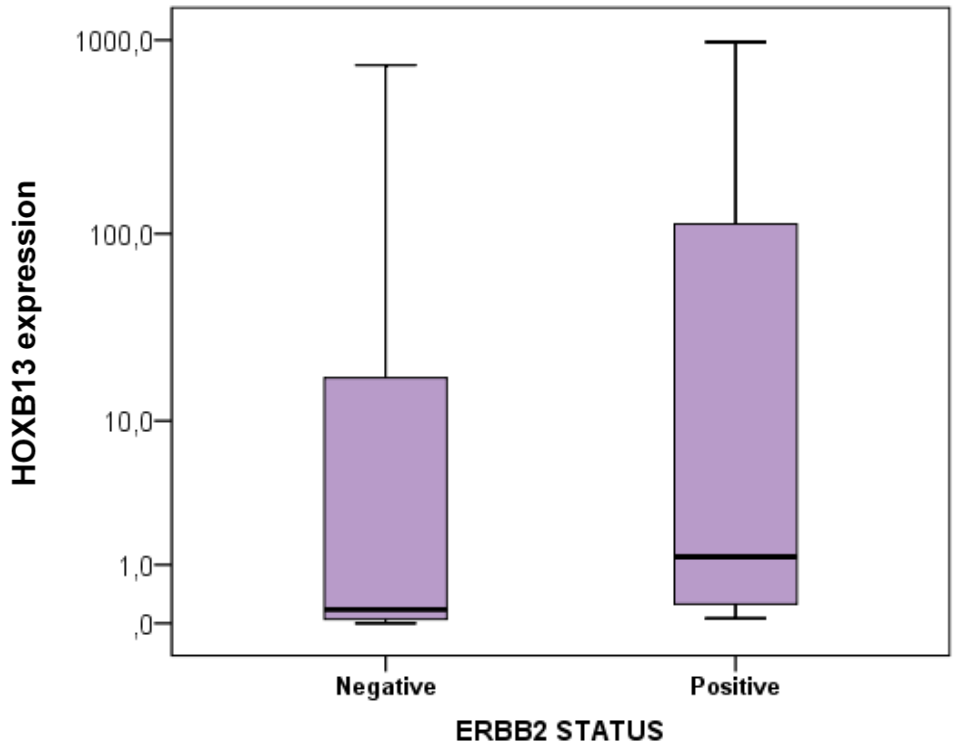


Figure 4.6 - Boxplot representing *HOXB13* expression levels (logarithmic scale) concerning ERBB2 status.

Considering the recurrence data until 2009, a relapse in the disease occurred in only 14 of the 54 patients. Although there was no statistical significant association between this event and the expression levels of *HOXB13* transcript (*p*= 0.132), expression levels tend to be higher in cases with tumor recurrence **[Figure 4.7]**.

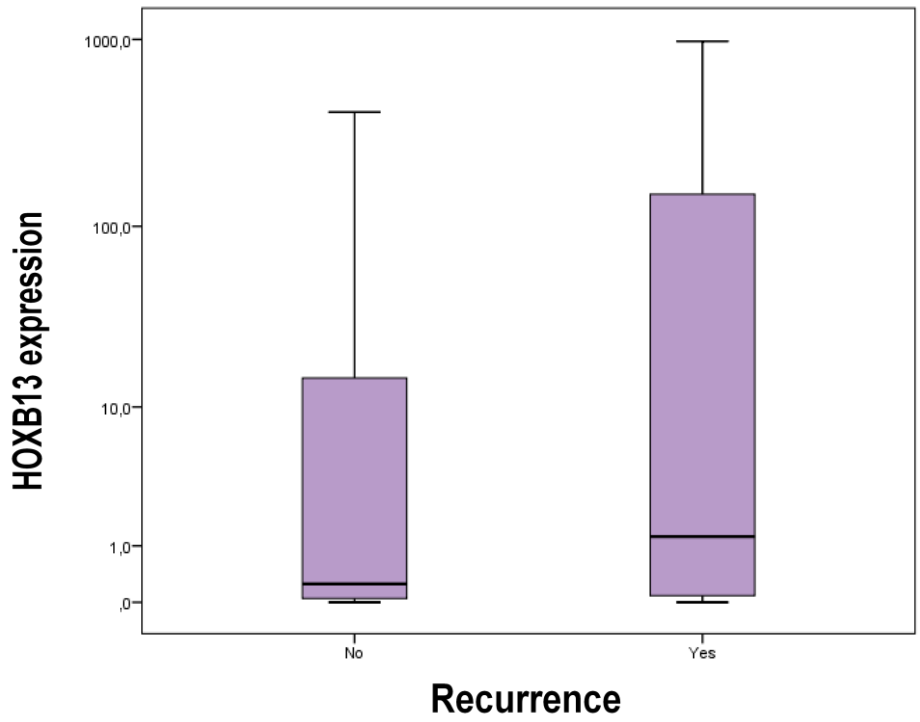


Figure 4.7 - Boxplot representing *HOXB13* expression levels (logarithmic scale) in cases where there was recurrence and no recurrence of the disease.

Regarding disease survival data, Kaplan-Meier survival analysis was performed with a total of 82 patients from the series used for qRT-PCR, with information on the diagnosis data and time of death (with evidence of the disease) or last appointment data. Forty-nine cases were considered to have low values of *HOXB13* expression and 33 to have high values (below or above median value = 1.94, respectively) **[Figure 4.8]**. Mantel-Cox test reveals no statistical significance between the survival probability of this two groups (p = 0.997), in fact the mean survival of patients considered to have expression levels of *HOXB13* ≤ 1.94 was 119,130 months, while patients with expression levels of *HOXB13* > 1.94 was 118,275 months.

Considering only patients that were receiving tamoxifen treatment (N=59), Kaplan-Meier survival analysis was again performed to search for a relation between therapy response and *HOXB13* levels of expression, but there was no difference (Mantel-Cox, *p*= 0.577) **[Figure 4.9]**.

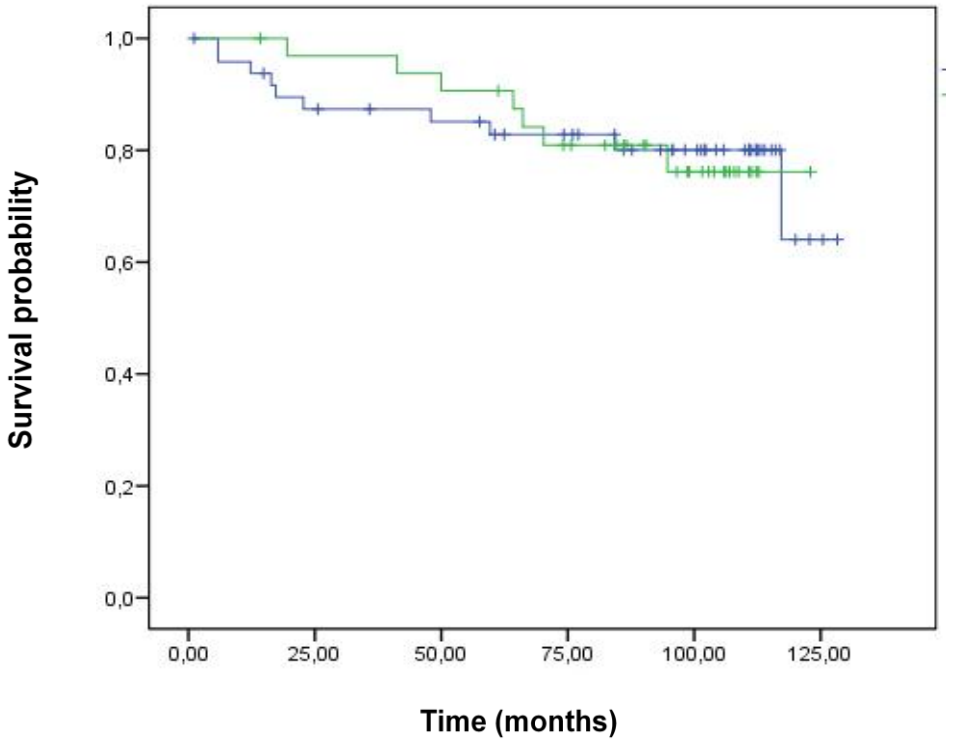


Figure 4.8 - Disease specific survival probability representation between cases with low and high expression levels of *HOXB13*.

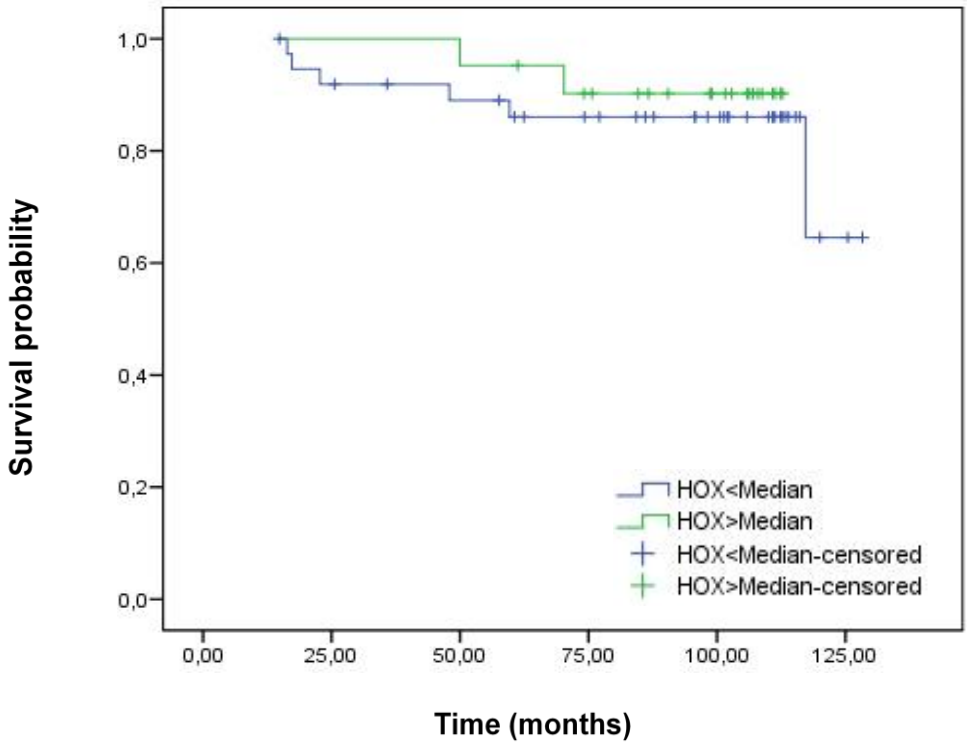


Figure 4.9 - Disease specific survival probability representation, in patients that were under tamoxifen therapy and with high or low levels of *HOXB13* expression levels.

V. Discussion

Breast cancer presents a challenge to prognosis and therapeutic choices due to its heterogeneous nature. Improving the understanding of the molecular pathways involved in breast cancer development is an opportunity for predicting tumor responsiveness to treatment as well as tumor aggressiveness [111]. The search for novel tumor biomarkers for breast cancer development led to some interesting, relatively recent, discoveries based on genome-wide microarray analysis, and *HOXB13* was one of the genes that came out as significantly associated with clinical outcome [104]. Furthermore, since *HOXB13* was recently discovered as a prostate cancer susceptibility gene and men carriers of *BRCA1/2* mutations have increased risk for prostate cancer [112], it became important to look for the role of this gene in hereditary predisposition to breast cancer.

HOXB13, like many other homeobox genes, encodes a highly conserved transcription factor, essential in embryonic development. It presents temporal and spatial colinearity in the body axis and its expression should be at basal levels in breast tissue of developed individuals [100,102].

HOXB13 in breast cancer is described as being overexpressed [106]. Its predictive value in estrogen receptors positive (ER+), node negative, breast cancers was already demonstrated by numerous studies that correlate high levels of expression of this gene with disease aggressiveness, poor clinical outcome and tamoxifen therapy failure, in many studies measured together with other gene (*IL17BR*) [107,108,121]. However, not much is known about how it contributes to disease progression and there are no publications at all about what is the mechanism that triggers *HOXB13* overexpression. In this study, we tried to understand if *HOXB13* could be involved in any case of familial breast cancer (some of which also with prostate cancer cases), wildtype for *BRCA1/2*, and if copy number changes in this gene could be responsible for its upregulation in breast carcinomas.

1. HOXB13 germline mutation analysis

Little is known about germline mutations in *HOXB13* in breast cancer patients. In this study, no deleterious germline mutations were found in the 95 samples that were fully sequenced. In the literature, until now, only the p.G84E mutation (rs138213197), first described in prostate cancer, was sought in two publications, but the results were contradictory. Alanee *et al* (2012) [112] found an association between this *HOXB13*

variant and breast cancer, with seven times higher prevalence of the p.G84E mutation in patients with familial breast cancer wild type for *BRCA1/2*, than in control cases. On the other hand, Akbari and colleagues (2012) [113] claim no association between this mutation and breast cancer risk, since in their study similar frequencies in cases with the disease and controls were found. Both used DNA from peripheral blood of more than 4.000 cases, which, in fact, contrasts with the number of cases analyzed in our study, although we have performed full sequencing instead of single mutation analysis.

2. *HOXB13* mRNA expression by 17q21 status

Since *HOXB13* localizes in chromosome 17q21 [102], we evaluated whether or not copy number changes explained expression levels of this gene in breast carcinomas, by studying distinct groups of cases with and without 17q21 gain. Our study shows that there was statistically significant differences between the two groups of tumors, when we first considered only the available CGH data. One should remember that the CGH technique in chromosome metaphases has its intrinsic limitations, which includes the exclusive detection of gross and unbalanced abnormalities and not copy number alterations of individual genes. It is also important to notice that, although the series of samples used was not very large, this study was the first to evaluate the role of 17q21 and *HOXB13* copy number changes as the genetic mechanism behind *HOXB13* deregulation in breast cancer, with this results increasing the strength of this hypothesis.

3. *HOXB13* expression by 17q21 and *HOXB13* copy number

As mentioned above, the presence of 17q21 copy number gain by CGH does not necessarily mean that a particular gene in that band is gained or amplified. We therefore decided to search for amplifications of the *HOXB13* gene using FISH with specific probes in samples with low and high values of *HOXB13* expression and with or without gain of 17q21. With this strategy we could directly evaluate if the expression level our gene of interest, was indeed associated with gene copy number changes. Surprisingly, the eight cases with *HOXB13* amplification did not show statistically significant higher levels of *HOXB13* expression compared with those with no amplification, although the mean value was higher in the former. In fact, one of the cases considered to have low expression of our gene of interest had the highest

HOXB13/CEP17 amplification ratio and, on the other hand, 9 cases considered to have high expression of *HOXB13* had low HOXB13/CEP17 ratios. Another interesting fact was that, when we compared the two groups with no amplification but with and without 17q21 gain, including the 48 samples without 17q21 gain and no amplification, not analyzed by FISH, the values of *HOXB13* expression gained statistical significance. These data, once again, goes in line with our first results concerning *HOXB13* expression analysis and the CGH data and with our assumptions. However, additional studies would be necessary to identify alternative mechanisms involved in *HOXB13* overexpression in the cases where there was no copy number gain, maybe further exploring mutational and epigenetic events or chromosome translocations, which are also mechanisms of oncogene activation.

4. Clinico-pathological associations

The majority of the available studies involving *HOXB13* and breast cancer were aimed for its prognostic role and capacity of predicting tamoxifen therapy benefit, as already said [111,117,118]. In our study *HOXB13* revealed no prognostic or predictive value, because we found no statistically significant differences in recurrence, disease survival and other clinico-pathological parameters like molecular and histological subtyping and ER/PR status, regarding *HOXB13* expression levels. This is most likely due to insufficient power of our study to detect such differences, and also due to differences in the methodology and techniques used for diagnosis and therapy choice 10 years ago, since most literature data in larger series support *HOXB13* expression as having a predictive value regarding hormonal therapy.

A study by Wang (2007) [107] describes a significant association between high levels of expression of *HOXB13* and estrogen receptors negative (ER-) tumors, which goes in line with another recent study by Shah (2013) [118], clarifying that *HOXB13* directly suppresses ERα and promotes an ER independent pathway. Wang also found an ER dependent association with *HER2* status and progesterone receptors (PR), in which ER+ tumors with this particular type of receptors also had higher levels of *HOXB13* expression. The association with grade was also expected since *HOXB13* is overexpressed mainly in tumors with a more aggressive phenotype and clinical course [118], a fact that was indeed observed in our study.

Concerning disease recurrence, survival and therapy resistance, multiple publications [107,108,11,117,118,121] reported that primary tumors with high expression of *HOXB13* had a significantly higher risk of disease recurrence (HR=3.55) and death

(HR=3.438) in treated and untreated patients with tamoxifen compared to those with low *HOXB13* expression. A publication involving cell lines that express higher levels of *HOXB13* reported that these type of cells, that were the ones with more aggressive features, grow faster and display tamoxifen resistance through estrogen independent proliferation [118]. It has also been described by a mechanistic investigation [118] that *HOXB13* transcriptionally upregulates IL-6 and its mediated pathways (which act as mediators of disease progression) by tumoral proliferation and stromal recruitment, possibly increasing the risk of relapse. This information is actually important, since patients with this activated alternative pathways can benefit from *mTOR* inhibitors instead of tamoxifen, aimed for ER+ tumors. The same study also refers that *HOXB13* was found to be overexpressed in distant metastases from breast cancer of nonsurvivors compared with survivors and with normal breast organoids.

Finally, but not less interesting, Jerevall *et al* (2010) proposed a possible coamplification of the *HOXB13* gene and *HER2*, since he observed a positive association between the expression of *HOXB13* and *HER2* amplification, as we also did. This theory was however not supported by our data, because the majority of cases with *HOXB13* amplification did not revealed HER2 amplification.

VI. Conclusions

Having in mind the results obtained in this study, we can conclude that:

❖ No evidence was found that the *HOXB13* gene is a significant contributor to inherited predisposition to familial breast cancer in Portugal, both regarding the recurrent G84E mutation or any other.

❖ *HOXB13* expression levels seem to be higher in breast carcinomas with *HOXB13* amplification and are significantly higher in tumors with 17q21 gain and *HOXB13* copy number gain, when compared with tumors without this gain and no amplification, suggesting that copy number gain can be a mechanism responsible for *HOXB13* overexpression.

❖ There is a progressive association between *HOXB13* expression and tumor grade, as well as a significant association with HER2 status that does not seem to be caused by co-amplification.

VII. Future Perspectives

❖ Increase the size of the sample series for germline mutation analysis.

❖ Search for somatic mutations in breast cancer samples.

❖ Increase the size of the sample series for FISH analysis and include some samples of normal tissue in the expression analysis to verify the *HOXB13* levels in normal tissue.

❖ Quantify the levels of expression of *IL17BR* and compare the results of the *HOXB13/IL17BR* ratio with that of *HOXB13* alone.

❖ Clarify the epigenetic methylation status of this gene in normal tissue and breast cancer tissue.

VIII. References

1- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011). Global cancer statistics 2011. CA Cancer J Clin 61:69-90.

2- Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, Cooper D, Gansler T, Lerro C, Fedewa S, Lin C, Leach C, Cannady RS, Cho H, Scoppa S, Hachey M, Kirch, R, Jemal A, Ward E (2012). Cancer treatment and survivorship statistics, 2012. CA Cancer J Clin 62:220-241.

3- Smith H, Kammerer-Doak D, Barbo D, Sarto G (1996). Hormone replacement therapy in the menopause: A pro opinion. CA- A cancer journal for clinicians. 46: 343.

4- Da Silva L, Lakhani SR (2010). Pathology of hereditary breast cancer. Modern Pathology 23: 46-51.

5- Meguerditchian A, Falardeau M, Martin G (2002). Male breast carcinoma. J Can Chir 45: 4.

6- http://globocan.iarc.fr/

7- Espinosa Arranz E, Vara JA, Gámez-Pozo A, Zamora P (2012). Gene signature in breast cancer: Current and future uses. Translational Oncology 5(6): 398-403.

8- Li C (2010). Breast cancer epidemiology. Springer.

9- Althuis MD, Dozier JD, Anderson WF, Devesa SS, Brinton LA (2005). Global trends in breast cancer incidence and mortality 1973-1997. Int J Epidemiol 34:405-412.

10- Jemal A, Center MM, DeSantis C (2010). Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol Biomarkers Prev 19: 1893-190.

11- IARC world cancer report 2008

12- Richie RC, Swanson JO (2003). Breast cancer: A review of the literature. J Insur Med 35: 85-101.

13- McPherson K, Steel CM, Dixon JM (2000). ABC of breast diseases. Breast cancer epidemiology, risk factors, and genetics. BMJ 321: 624-8.

14- Willett WC, Rockhill B, Hankinson SE (2000). Epidemiology and nongenetic causes of breast cancer. Disease of the breast 175-220.

15- Struewing JP, Tarone RE, Brody LC, Li FP, Boice JD (1996). BRCA1 mutations in young women with breast cancer. Lancet 347: 1493.

16- Hoskins K, Stopfer J, Calzone K (1995). Assessment and counseling for women with a family history of breast cancer. A guide for clinicians. JAMA 273: 577-585.

17- Jonh E, Kelsey J (1993). Radiation and other environmental exposures and breast cancer. Epidemiol Rev 15: 157.

18- Sieri S, Krogh V, Ferrari P, Berrino F, Pala V, Gullberg B, Hallmans G, Lenner P, Bueno-de-Mesquita HB, van Duijnhoven FJ, Peeters PH, van Gils CH, Key TJ, Crowe FL, Bingham S, Khaw KT, Rinaldi S, Slimani N, Jenab M, Norat T, Riboli E (2008). Dietary fat and breast cancer risk in the European Prospective Investigation into Cancer and Nutrition. Am J Clin Nutr 88(5):1304-12.

19- DeSantis C, Siegel R, Jemal A (2012). Cancer treatment & survivorship facts & figures 2012-2013. American cancer society.

20- Terry PD, Rohan TE (2002). Cigarette smoking and the risk of breast cancer in women: a review of the literature. Cancer Epidemiol Biomarkers Prev 11: 953–971.

21- Singletary K, Gapstur S (2001). Alcohol and breast cancer: review of epidemiologic and experimental evidence and potential mechanisms. JAMA 286: 2143.

22- Kiley J, Hammond C (2007). Combined oral contraceptives: a comprehensive review. Clin Obstet Gynecol 50: 868-77.

23- Evans DG, Howell A (2007). Breast cancer risk-assessment models. Breast Cancer Res 9: 213-220.

24- Gray H (2008). Gray's anatomy. The anatomical basis of clinical practice. Elsevier.

25- http://www.medicinenet.com/breast/article.htm

26- Li CI, Uribe DJ, Daling JR (2005). Clinical characteristics of different histologic types of breast cancer. Br J Cancer 93:1046.

27- Simpson PT, Reis-Filho JS, Gale T, Lakhani SR (2005). Molecular evolution of breast cancer. J Pathol 2005; 205: 248–254.

28- Holland R, Hendriks JH (1994). Microcalcification associated with ductal carcinoma in situ: mammographic-pathologic correlation. Semin Dign Pathol 11: 181.

29- Patchefsky AS, Schwartz GF, Finkelstein SD (1989). Heterogeneity of intraductal carcinoma of the breast. Cancer 63: 731.

30- Smith RA, Saslow D, Sawyer KA, Burke W, Costanza ME, Evans III WP, Foster RS, Hendrick E, Eyre HJ, Sener S (2003). American Cancer Society guidelines for breast cancer screening: update 2003. CA Cancer J Clin 53:141-169

31- Kalogerakos K, Sofoudis C, Baltayiannis N (2008). Early breast cancer: A review. Cancer Therapy 6: 463-476.

32- Soares D, Jonhson P (2007). Breast imaging update. West Indian Med J 56: 351-4.

33- Mirshahidi HR, Abraham J (2004). Managing early breast cancer prognostic features guide choice of therapy. Postgrad Med 116: 23-24.

34- Donegan W (1992). Evaluation of a palpable breast mass. N Engl J Med 327: 937-942.

35- Robson M, Offit K (2007). Management of an Inherited Predisposition to Breast Cancer. N Engl J Med 357;2.

36- http://www.cancer.gov/cancertopics/pdq/treatment/breast/Patient/page2

37- Moore M, Kinne D (1995). Highlights from the national cancer data base. CA-Cancer J Clin 49(3): 145-158.

38- Berry DA, Cronin KA, Plevritis SK, Fryback DG, Clarke L, Zelen M, Mandelblatt JS, Yakovlev AY, Habbema JD, Feuer EJ (2005). Effect of screening and adjuvant therapy on mortality from breast cancer. N Engl J Med 353:1784-1792.

39- Krishnamurthy S, Poornima R, Challa VR, Goud YG (2012). Triple negative breast cancer- our experience and review. Indian J Surg Oncol3(1):12-16.

40- Jatoi I, Proschan MA (2005). Randomized trials of breast-conserving therapy versus mastectomy for primary breast cancer: a pooled analysis of updated results. Am J Clin Oncol 28:289-294.

41- Alderman AK, Hawley ST, Waljee J, Morrow M, Katz SJ (2007). Correlates of referral practices of general surgeons to plastic surgeons for mastectomy reconstruction. Cancer 109:1715-1720.

42- Orr RK (1999). The impact of prophylactic axillary node dissection on breast cancer survival - a Bayesian meta analysis. Ann Surg Oncol 6: 109-116.

43- EBTCG (2005). Effects of radiotherapy and of difference in the extent of surgery of early breast cancer on local recurrence and15-year survival an overview of the randomized trials. Lancet 366: 2087-2106.

44- Owen JR, Ashton A, Bliss JM, Homewood J, Harper C, Hanson J, Haviland J, Bentzen SM, Yarnold JR (2006). Effect of radiotherapy fraction size on tumor control in patients with early-stage breast cancer after local tumor excision long term results of a randomized trial. Lancet Oncol 7: 467-71.

45- Recht A, Edge SB, Solin LJ, Robinson DS, Estabrook A, Fine RE, Fleming GF, Formenti S, Hudis C, Kirshner JJ, Krause DA, Kuske RR, Langer AS, Sledge GW, Whelan TJ, Pfister DG, American Society of Clinical Oncology (2001). Postmastectomy radiotherapy clinical practice guidelines of the American Society of Clinical Oncology. J Clin Oncol 19: 1539-1569.

46- Whelan T, MacKenzie R, Julian J, Levine M, Shelley W, Grimard L, Lada B, Lukka H, Perera F, Fyles A, Laukkanen E, Gulavita S, Benk V, Szechtman B (2002). Randomized trial of breast irradiation schedules after lumpectomy for women with lymph node-negative breast cancer. J Natl Cancer Institute 94: 1143.

47- Fisher B, Carbone P, Economou SG, Frelick R, Glass A, Lerner H, Redmond C, Zelen M, Band P, Katrych DL, Wolmark N, Fisher ER (1975). 1-Phenylalanine mustard (L-PAM) in the management of primary breast cancer; a report of early findings. N Engl J Med 292: 117-122.

48- Goldhirsch A, Colleoni M, Gelber RD (2002). Endocrine therapy of breast cancer. Ann Oncol 13 Suppl 4:61-8.

49- Abdulkareem IH, Zurmi IB (2012). Review of hormonal treatment of breast cancer. Nigerian Journal of Clinical Practice 15:1.

50- Jordan VC, O'Malley BW (2007). Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. J Clin Oncol 25: 5815-5824.

51- Brueggemeier RW, Hackett JC, Diaz-Cruz ES (2005). Aromatase inhibitors in the treatment of breast cancer. Endocrine Reviews 26(3): 331–345.

52- Smith IE, Downsett M (2003). Aromatase inhibitors in breast cancer. N Engl J Med 348: 2431-2442.

53- Higgins MJ, Baselga J (2011). Targeted therapies for breast cancer. *J Clin Invest.* 121(10): 3797–3803.

54- Vlastos G, Verkooijen H (2007). Minimally invasive approaches for diagnosis and treatment of early-stage breast cancer. The oncologist 12: 1-10.

55- Viani GA, Afonso SL, Stefano EJ, De Fendi LI, Soares FV (2007). Adjuvant transtuzumab in the treatment of her2-positive early breast cancer a meta-analysis of published randomized trials. BMC Cancer 7: 153.

56- Nahta R, Esteva FJ (2006). HER2 therapy: molecular mechanisms of trastuzumab resistance. Breast Cancer Res 8: 215.

57- Capelan M, Pugliano L, De Azambuja E, Bozovic I, Saini KS, Sotiriou C, Loi S, Piccart-Gebhart MJ (2013). Pertuzumab: new hope for patients with HER2-positive breast cancer. Annals of Oncology 24: 273–282.

58- Bilancia D, Rosati G, Dinota A, Germano D, Romano R, Manzione L (2007). Lapatinib in breast cancer. Annals of Oncology 18: 26–30.

59- Rossari JR, Metzger-Filho O, Paesmans M, Saini KS, Gennari A, Azambuja E, Piccart-Gebhart M (2012). Bevacizumab and Breast Cancer: A Meta-Analysis of First-Line Phase III Studies and a Critical Reappraisal of Available Evidence. J Oncol 2012: 417673.

60- Baselga J, Campone M, Piccart M, Burris III HA, Rugo HS, Sahmoud T, Noguchi S, Gnant M, Pritchard KI, Lebrun F, Beck JT, Ito Y, Yardley D, Deleu I, Perez A, Bachelot T, Vittori L, Xu Z, Mukhopadhyay P, Lebwohl D, Hortobagyi GN (2012). Everolimus in Postmenopausal Hormone-Receptor–Positive Advanced Breast Cancer. N Engl J Med 366;6.

61- Plummer R (2011). Poly(ADP-ribose) polymerase inhibition: a new direction for *BRCA* and triple-negative breast cancer? Breast Cancer Research 13: 218.

62- Dawson SJ, Rueda OM, Aparicio S, Caldas C (2013). A new genome-driven integrated classification of breast cancer and its implications. The EMBO Journal 32: 617-628.

63- Perou CM, Sorlie T, Eisen MB, Van de RM, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamensschikov A, Williams C, Zhu SX, Lonning PE, Borrensen-Dale AL, Brown PO, Botstein D (2000). Molecular portraits of human breast tumours. Nature 406: 747-752.

64- Curtis C, Shah SP, Chin S, Turashvili G, Rueda OM, Speed M, Lynch AG, Samarajiwa S, Yuan Y, Graf S, Ha G, Haffari G, Bashashati A, Russell R, McKinney S, METABRIC Group, Langerød A, Green A, Provenzano E, Wishart G, Pinder S, Watson P, Markowetz F, Murphy L, Ellis I, Purushotham A, Børresen-Dale A, Brenton JD, Tavare S, Caldas C, Aparicio S (2012). The genomic and transcriptomic architecture of 2000 breast tumours reveals novel subgroups. Nature x:xxx

65- Henderson BE, Feigelson HS (2000). Hormonal carcinogenesis. Carcinogenesis 21: 427-433.

66- Beckmann MW, Niederacher D, Schnurch H, Gusterson BA, Bender HG (1997). Multistep carcinogenesis of breast cancer and tumour heterogeneity. J Mol Med 75: 429-439.

67- Scherneck S (1995). Contribution of molecular genetic changes for improved management of breast cancer. Onkologie 18: 189-201.

68- Devilee P, Cornelisse CJ (1994). Somatic genetic changes in human breast cancer. Biochimica et Biophysica Acta 1198: 113-130.

69- Pasche B (2008). Recent advances in breast cancer genetics. Cancer Treat Res 141: 1-10.

70- Smith TM, Lee MK, Szabo CI, Jerome N, McEuen M, Taylor M, Hood L, King MC (1996). Complete genomic sequence and analysis of 117 kb of human DNA containing the gene BRCA1. Genome Res 6: 1029–1049.

71- Stratton MR (1997). Pathology of familial breast cancer: differences between breast cancers in carriers of BRCA1 or BRCA2 mutations and sporadic cases. Lancet 349, 1505–1510.

72- Kriege M, Seynaeve C, Meijers-Heijboer H, Collee JM, Menke-Pluymers MB, Bartels CC, Tilanus-Linthorst MM, van den Ouweland A, van Geel B, Brekelmans CT, Klijn JG (2008). Distant disease-free interval, site of first relapse and postrelapse survival in BRCA1- and BRCA2-associated compared to sporadic breast cancer patients. Breast Cancer Res Treat 111: 303–311.

73- Antoniou A, Pharoah PD, Narod S, Risch HA, Eyfjord JE, Hopper JL, Loman N, Olsson H, Johannsson O, Borg A, Pasini B, Radice P, Manoukian S, Eccles DM, Tang N, Olah E, Anton-Culver H, Warner E, Lubinski J, Gronwald J, Gorski B, Tulinius H, Thorlacius S, Eerola H, Nevanlinna H, Syrjakoski K, Kallioniemi OP, Thompson D, Evans C, Peto J, Lalloo F, Evans DG, Easton DF (2003). Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet 72:xxx–xxx.

74- van der Groep P, van der Wall E, van Diest PJ (2010). Pathology of hereditary breast cancer. Cell Oncol 34: 71-88.

75- Mincey B (2003). Genetics and the management of women at high risk for breast cancer. The Oncologist 8: 466- 473.

76- Balmanã J, Díez O, Rubio IT, Cardoso F (2011). BRCA in breast cancer: ESMO Clinical Practice Guidelines. Annals of Oncology 22(6):31-34.

77- Vogelstein B, Lane D, Levine AJ (2000). Surfing the p53 network. Nature 408: 307–310.

78- Sellers T (1997). Genetic factors in the pathogenesis of breast cancer: Their role and relative importance. J Nutr 127: 929-932.

79- Chappuis PO, Nethercot V, Foulkes WD (2000). Clinico-pathological characteristics of BRCA1- and BRCA2-related breast cancer. Semin. Surg. Oncol. 18: 287–295.

80- Gasco M, Yulug IG, Crook T (2003). TP53 mutations in familial breast cancer: functional aspects. Hum. Mutat. 21: 301–306.

81- Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazi M, Pecker I, Frydman M, Harnik R, Patanjali SR, Simmons A, Clines GA, Sartiel A, Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NG, Taylor AM, Arlett CF, Miki T, Weissman SM, Lovett M, Collins FS, Shiloh Y (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science 268: 1749–1753.

82- Renwick A, Thompson D, Seal S, Kelly P, Chagtai T, Ahmed M, North B, Jayatilake H, Barfoot R, Spanova K, McGuffog L, Evans DG, Eccles D, Easton DF, Stratton MR, Rahman N (2006)**.** ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. Nat Genet 38: 873–875.

83- Becker KF, Atkinson MJ, Reich U, Becker I, Nekarda H, Siewert JR, Hofler H (1994). E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. Cancer Res 54: 3845–3852.

84- Keller G, Vogelsang H, Becker I, Hutter J, Ott K, Candidus S, Grundei T, Becker KF, Mueller J, Siewert JR, Hofler H (1999). Diffuse type gastric and lobular breast carcinoma in a familial gastric cancer patient with an E-cadherin germline mutation. Am J Pathol 155: 337–342.

85- Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275: 1943–1947.

86- Nelen MR, Padberg GW, Peeters EA, Lin AY, van den Helm B, Frants RR, Coulon V, Goldstein AM, van Reen MM, Easton DF, Eeles RA, Hodgsen S, Mulvihill JJ, Murday VA, Tucker MA, Mariman EC, Starink TM, Ponder BA, Ropers HH, Kremer H, Longy M, Eng C (1996). Localization of the gene for Cowden disease to chromosome 10q22-23. Nat Genet 13: 114–116.

87- Giardiello FM, Brensinger JD, Tersmette AC, Goodman SN, Petersen GM, Booker SV, Cruz-Correa M, Offerhaus JA (2000). Very high risk of cancer in familial Peutz-Jeghers syndrome. Gastroenterology 119: 1447–1453.

88- Rahman N, Seal S, Thompson D, Kelly P, Renwick A, Elliott A, Reid S, Spanova K, Barfoot R, Chagtai T, Jayatilake H, McGuffog L, Hanks S, Evans DG, Eccles D, The Breast Cancer Susceptibility Collaboration (UK), Easton DF, Stratton MR (2007). *PALB2*, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat Genet 39(2): 165–167.

89- Smith G, Stanley LA, Sim E, Strange RC, Wolf CR (1995). Metabolic polymorphisms and cancer susceptibility. Cancer Surv 25: 27-65.

90- Dunning AM, Healey CS, Pharoah P, Teare MD, Ponder B, Easton DF (1999). A systematic review of genetic polymorphisms and breast cancer risk. Cancer Epidemiol Biomarkers Prev 8: 843-854**.**

91- Hunter DJ, Willett WC (1996). Nutrition and breast cancer. Cancer Causes & Control 7: 56-68.

92- Rebbeck TR (1997). Molecular epidemiology of the human glutathione S-transferase genotypes *GSTM1* and *GSTT1* in cancer susceptibility. Cancer Epidemiol Biomarkers Prev 6:733-743.

93- Hein DW, Doll MA, Rustan TD, Gray K, Feng Y, Fergunson RJ, Grant DM (1993). Metabolic activation and deactivation of arylamine carcinogens by recombinant human NAT1 and polymorphic NAT2 acetyltransferase. Carcinogenesis 14: 1633-1638.

94- Borresen AL, Andersen TI, Garber J, Barbier-Piraux N, Thorlacius S, Eyfjord J, Ottestad L, Smith-Sorensen B, Hovig E, Malkin D, Freind SH (1992). Screening for germline *TP53* mutations in breast cancer patients. Cancer Res 52: 3234-3236.

95- Veeck J, Esteller M (2010). Breast cancer epigenetics: from DNA methylation to miRNAs. J Mammary Gland Biol Neoplasia 15: 5-17.

96- Bernardino J, Roux C, Almeida A, Vogt N, Gibaud A, Gerbault-Seureau M (1997). DNA hypomethylation in breast cancer: an independent parameter of tumor progression? Cancer Genet Cytogenet 97: 83-9.

97- Esteller M, Corn PG, Baylin SB, Herman JG (2001). A gene hypermethylation profile of human cancer. Cancer Res 61: 3225-9.

98- Roll JD, Rivenbark AG, Jones WD, Coleman WB (2008). DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. Mol Cancer 7: 15.

99- Zhang L, Huang J, Yang N, Greshock J, Megraw MS, Giannakakis A (2006). microRNAs exhibit high frequency genomic alterations in human cancer. Proc Natl Acad Sci U S A 103: 9136-41.

100- Yamashita T, Tazawa S, Yawei Z, Katayama H, Kato Y, Nishiwaki K, Yokohama Y, Ishikawa M (2006). Suppression of invasive characteristics by antisense introduction of overexpressed HOX genes in ovarian cancer cells. International J of Oncol 28(4): 931-938.

101- Chen H, Sukumar S (2003). HOX genes: emerging stars in cancer. Cancer Biol Ther 2:524-5.

102- Zeltser L, Desplan C, Heintz N (1996). Hoxb-13: a new HOX gene in a distant region of the HOXB cluster maintains colinearity. Development 122:2475-84.

103- https://portal.biobase-international.com/hgmd/pro/cdna.php

104- Cross DS, Burmester JK (2008). Functional characterization of the HOXB13 promoter region. Med Oncol 25: 287-293.

105- Nunes FD, de Almeida FC, Tucci R, de Sousa SC (2003). Homeobox genes: a molecular link between development and cancer. Pesqui Odontol Bras 17: 94-8.

106- Cantile M, Pettinato G, Procino A, Feliciello I, Cindolo L, Cillo C (2003). In vivo expression of the whole HOX gene network in human breast cancer. Eur J Cancer 39: 257-264.

107- Wang Z, Dahiya S, Provencher H, Muir B, Carney E, Coser K, Shioda T, Ma XJ, Sgroi DC (2007). The prognostic biomarkers HOXB13, IL17BR and CHDH are regulated by estrogen in breast cancer. Clin Cancer Res 13: 6327-6334.

108- Ma X, Wang Z, Ryan PD, Amon P, Wang W, Wang W, Enright E, Stecker K, Estepa-Sabal E, Smith B, Younger J, Balis U, Michaelson J, Bhan A, Habin K, Baer TM, Brugge J, Haber DA, Erlander MG, Sgroi DC (2004). A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. Cancer cell. 5:607-616.

109- Nicholson RI, McClelland RA, Gee JM, Manning DL, Cannon P, Robertson JF, Ellis IO, Blamey RW (1994). Epidermal growth factor receptor expression in breast cancer: Association with response to endocrine therapy. Breast Cancer Res Treat 29: 117-125.

110- Shen WF, Krishnan K, Lawrence HJ, Largman C (2001). The HOX homeodomain proteins block CBP histone acetyltransferase activity. Mol Cell Biol 21: 7509-7522.

111- Jerevall PL, Jansson A, Fornander T, Skoog L, Nordenskjold B, Stal O (2010). Predictive relevance of HOXB13 protein expression for tamoxifen benefit in breast cancer. Breast Cancer Research 12: R53.

112- Ewing CM, Ray AM, Lange EM, Zuhlke KA, Robbins CM, Tembe WD, Wiley KE, Isaacs SD, Johng D, Wang Y, Bizon C, Yan G, Gielzak M, Partin AW, Shanmugam V, Izatt T, Sinari S, Craig DW, Zheng SL, Walsh PC, Montie JE, Xu J, Carpten JD, Isaacs WB, Cooney KA (2012). Germline mutations in HOXB13 and prostate cancer risk. N Engl J Med 366: 141-9

113- Alanee S, Couch F, Offit K (2012). Association of a HOXB13 variant with breast cancer. N Eng J Med 367;5.

114- Akbari MR, Kluz´niak W, Rodin R, Li S, Wokołorczyk D, Royer R, Kashyap A, Menkiszak J, Lubinski J, Narod SA, Cybulski C (2012). The HOXB13 p.Gly84Glu mutation is not associated with the risk of breast cancer. Breast Cancer Res Treat 136:907–909.

115- Clarke R, Liu MC, Bouker KB, Gu Z, Lee RY, Zhu Y, Skaar TC, Gomez B, O'Brien K, Wang Y, Hilakivi-Clarke LA (2003). Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. Oncogene 22: 7316-7339.

116- EBCTCG (2001). Tamoxifen for early breast cancer. Cochrane Database Syst Rev 1 CD000486.

117- Nicholson RI, Gee JM, Knowlden J, McClelland R, Madden TA, Barrow D, Hutcheson I (2003). The biology of antihormone failure in breast cancer. Breast Cancer Res Treat 80: S29-S34.

118- Shah N, Jin K, Cruz LA (2013). HOXB13 mediates tamoxifen resistance and invasiveness in human breast cancer by supressing ERα and inducing IL-6 expression. Cancer Res xx:xxx.

119- Ma X-J, Hilsenbeck SG, Wang W, Ding L, Sgroi DC, Bender RA, Osborne CK, Allred DC, Erlander MG (2006). The *HOXB13:IL17BR* expression index is a prognostic factor in early-stage breast cancer. J Clin Oncol 24: 4611-4619.

120- Habel L, Sakoda L, Achacoso N, Ma X-J, Erlander M, Sgroi D, Fehrenbacher L, Greenberg D, Quesenberry C (2013). HOXB13:IL13BR and molecular grade index and risk of breast cancer death among patients with lymph nodenegative invasive disease. Breast Cancer Res 15: R24.

121- Sgroi D, Carney E, Zarrella E, Steffel L, Binns S, Finkelstein D, Szymonifka J, Bhan A, Shepherd L, Zhang Y, Schnabel C, Erlander M, Ingle J, Porter P, Muss H, Pritchard K, Tu D, Rimm D, Goss P (2013). Prediction of late disease recurrence and extended adjuvant letrozole benefit by the HOXB13/IL17BR biomarker. J Natl Cancer Inst.

122- Rodriguez BA, Cheng AS, Yan PS, Potter D, Agosto-Perez FJ, Shapiro CL, Huang TH (2008). Epigenetic repression of the estrogen-regulated Homeobox B13 gene in breast cancer. Carcinogenesis 29(7):1459-65.

123- http://www.ncbi.nlm.nih.gov/tools/primer-blast/

124- Shmittgen T, Livak K (2008). Analyzing real-time PCR data by the comparative Ct method. Nature Protocols 3(6):1101-1108.

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