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HER2 Status in Male Breast Cancer: a comparison between protein expression by immunohistochemistry and gene amplification by fluorescence *in situ* hybridization

Sónia Macedo Barros

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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HER2 STATUS IN MALE BREAST CANCER: A COMPARISON BETWEEN PROTEIN EXPRESSION BY IMMUNOHISTOCHEMISTRY AND GENE AMPLIFICATION BY FLUORESCENCE *IN SITU* HYBRIDIZATION

Dissertação de Candidatura ao grau de **Mestre em Oncologia** – Especialização em Oncologia Laboratorial – Submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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Success is not final; failure is not fatal:

It is the courage to continue that counts.

Winston Churchill

Table of Contents

Figures Index xi
Tables Index xii
Abbreviations List xiii
Abstract xv
Resumo xvii
I. Introduction 1
I.1. Male Breast Cancer 3
I.1.1. Epidemiology 3
I.1.2. Aetiology and Risk Factors 3
I.1.3. Male Breast Anatomy 5
I.1.4. Screening and Counselling 6
I.1.5. Diagnosis 6
I.1.5.1. Histological Type 7
I.1.5.2. Histological Grade 7
I.1.5.3. TNM System 8
I.1.5.4. Biomarkers 8
I.1.6. Molecular Classification 8
I.1.7. Prognosis 9
I.1.8. Treatment 10
I.1.8.1. Anti-HER2 Antibody 12
I.1.8.2. Anti-HER2 Small Molecule 12
I.1.9. Gender Differences 13
I.2. HER2 15
I.2.1. Assessment of the HER2 Status 16
I.2.2. Correlation Between FISH and IHC 17
II. Aims 19
III. Material and Methods 23
III.1. Patients and Samples 25
III.2. Immunohistochemistry 25
III.3. Fluorescence in situ Hybridization 25
III.3.1. Protocol 25
III.3.2. Analysis 26

III.4. Statistical Methods 27
IV. Results 29
IV.1. Characteristics of the Study Cohort 31
IV.2. Assessment of the HER2 Status by IHC and FISH 32
IV.3. Characteristics of the Discrepant and Concordant Cases 37
IV.4. Association Between *HER2* Amplification and Tumour Clinicopathologic Features 40
IV.5. Overall Survival 40
V. Discussion 43
V.1. Clinicopathological Features 45
V.2. HER2 Status 45
V.3. Possible Therapeutic Strategies 48
V.4. Clinicopathological Association with Amplified *HER2* 49
V.5. Overall Survival 49
VI. Conclusions 51
VII. Future Perspectives 55
VIII. References 59

Figures Index

Figure 1. Anatomical differences in female and male breasts. 5

Figure 2. Adjuvant therapy options according to the molecular subgroups. 11

Figure 3. Structure of the HER2 (extracellular domains (I, II, III and IV), transmembrane domain and intracellular domain), shedding cleavage sites and trastuzumab and lapatinib binding sites. 13

Figure 4. EGFR family members, heterodimer formation and downstream signalling pathway..... 15

Figure 5. Groups of *HER2* gene amplification tested by FISH. 27

Figure 6. IHC and FISH images of the discrepant cases. 39

Figure 7. Representative images of #19. 40

Figure 8. Overall survival in the discrepant and concordant HER2 positive cases. 41

Tables Index

Table 1. Breast cancer intrinsic subgroups classification..... 9

Table 2. New classification proposed for MBC..... 14

Table 3. Clinicopathological features of the study cohort. 31

Table 4. Parameters for evaluation of the HER2 status by IHC and FISH. 33

Table 5. FISH groups determined in the cohort. 36

Table 6. HER2 status analysed by IHC and FISH..... 36

Table 7. Sensitivity, specificity, positive predictive value and negative predictive value of IHC. 36

Table 8. Clinicopathological characteristics of the cases with discrepant and concordant HER2-positive results. 37

Table 9. Summary of the vital status of the patients from the two groups in study. 41

Abbreviations List

AR	Androgen Receptors
ASCO	American Society of Clinical Oncology
ATP	Adenosine Triphosphate
CAP	College of American Pathologists
CEP17	Chromosome Enumeration Probe 17
CISH	Chromogenic *in Situ* Hybridization
DAPI	4', 6-diamidino-2-phenylindole
DCIS	Ductal Carcinoma *in situ*
DMs	Double Minutes
DNA	Deoxyribonucleic Acid
ECD	Extracellular Domain
EGFR	Epidermal Growth Factor Receptor
ER	Oestrogen Receptors
ERBB2	Erb-b2 Receptor Tyrosine Kinase 2
FBC	Female Breast Cancer
FISH	Fluorescence *in Situ* Hybridization
HER2	Human Epidermal Growth Factor Receptor 2
HSRs	Homogeneously Staining Regions
ICD	Intracellular Domain
IHC	Immunohistochemistry
ISH	*In Situ* Hybridization
MBC	Male Breast Cancer
mRNA	Messenger Ribonucleic Acid
NST	No Special Type
PR	Progesterone Receptors
RNA	Ribonucleic Acid
SISH	Silver enhanced *in situ* Hybridization
TNM	Tumour, Node, Metastases
WHO	World Health Organization

Abstract

Male breast cancer (MBC) is a very rare disease, corresponding to 1% of all male cancers, and with a mortality higher than the observed in females. For therapeutic decisions, MBC is classified similarly to female breast cancer (FBC), being divided in four different subgroups, by the evaluation of biomarkers' expression, such as HER2. It is very important to determine the HER2 status of the patients with breast cancer with a quick, precise and cost-effective methodology, so the patients could benefit from an anti-HER2 therapy. Nowadays, guidelines recommend that HER2 should be tested by immunohistochemistry (IHC) and only in the equivocal cases fluorescence *in situ* hybridization (FISH) should be made. Studies comparing both techniques showed a good overall concordance. HER2 is positive in a higher proportion of FBC than MBC, but the reason is unclear.

The aim of the present study was to test for *HER2* amplification with FISH in a consecutive series of MBC and to compare with the results previously obtained by IHC, to define the frequency of HER2 positivity in MBC, to determine the concordance between IHC and FISH in the determination of the HER2 status in MBC, to correlate HER2 status with clinicopathological characteristics of MBC, and to compare the frequency of HER2 positivity in MBC with that of FBC.

All patients treated in IPO-Porto from 1991 to July 2020 and with formalin-fixed paraffin-embedded tissue blocks were included in this study. FISH was performed in 98 cases and the findings were compared with those obtained with IHC.

We observed that, in our cohort, *HER2* is amplified in 15.3% of the cases. We also found a moderate correlation between IHC and FISH, with a significant proportion of cases with amplification by FISH not showing overexpression by IHC. We did not find any correlation between the *HER2* status and clinicopathological characteristics of the patients. We show evidence that MBC and FBC are biologically different entities, with *HER2* being amplified in a lower proportion and with a higher percentage of discrepancy with IHC in males. Furthermore, we concluded that it might be important to test the HER2 status by both IHC and FISH in all MBC patients at diagnosis in order to identify the patients with amplification without overexpression and enrol them in clinical trials to optimize their treatment.

This work makes evident the need to perform more studies in MBC to better characterize this disease and to allow appropriate treatment of these patients.

Resumo

O carcinoma da mama masculina (MBC) é uma doença rara, que corresponde a 1% de todas as neoplasias masculinas, com uma mortalidade superior à observada nas mulheres. Para decisões terapêuticas, o MBC é classificado de forma semelhante ao cancro da mama feminina (FBC), sendo dividido em quatro subgrupos diferentes pela avaliação da expressão de biomarcadores, tal como o HER2. É muito importante determinar o estado do HER2 dos pacientes com carcinoma da mama, com uma metodologia rápida, precisa e económica, para que os doentes possam beneficiar de uma terapia anti-HER2. Atualmente as *guidelines* recomendam que o HER2 deve ser testado por imunohistoquímica (IHC) e apenas nos casos equívocos a hibridação *in situ* fluorescente (FISH) deve ser realizada. Estudos que compararam as duas técnicas mostraram uma boa concordância global. O HER2 é positivo numa proporção muito maior de casos de FBC do que de MBC, mas a razão não é conhecida.

O objetivo do presente estudo foi testar a amplificação do *HER2* com FISH numa série consecutiva de carcinomas de mama masculinas e comparar com os resultados obtidos previamente por IHC, para definir a frequência de positividade do HER2 no MBC, determinar a concordância entre a IHC e o FISH na determinação do estado do HER2 em MBC, correlacionar o estado do HER2 com características clinico-patológicas de MBC e comparar a frequência de positividade do HER2 em MBC com a do FBC.

Todos os pacientes tratados no IPO-Porto de 1991 a julho de 2020 e com bloco de tecido fixado em formol e incluído em parafina foram incluídos neste estudo. O FISH foi realizado em 98 casos e os resultados foram comparados com os da IHC.

Observamos que, na nossa coorte, o HER2 está amplificado em 15.3% dos casos. Encontramos uma correlação moderada entre IHC e FISH, com uma proporção significativa de casos com amplificação por FISH não apresentando sobrexpressão por IHC. Não encontramos correlação entre o estado do HER2 e as características clinico-patológicas dos pacientes. Mostramos evidência de que o MBC e FBC são entidades biologicamente diferentes, com o HER2 amplificado numa menor proporção e com maior percentagem de discrepância com IHC no homem. Concluímos que poder ser importante testar o estado do HER2 por IHC e FISH em todos os pacientes com MBC ao diagnóstico, a fim de identificar os pacientes com amplificação e sem sobrexpressão para os incluir em ensaios clínicos para otimizar o seu tratamento.

Este trabalho evidencia a necessidade de realização de mais estudos em MBC para melhor caracterizar esta doença e para se poder oferecer o tratamento mais adequado a estes doentes.

I. Introduction

Tumours result from stepwise genetic and epigenetic alterations of normal cells, which enable a succession of hallmark capabilities, such as: I. sustaining proliferative signalling; II. evading growth suppressors; III. activating invasion and metastasis; IV. allowing replicative immortality; V. inducing angiogenesis; VI. resisting cell death; VII. deregulating cellular energetics; and VIII. avoiding immune destruction. All these *cancer hallmarks* are possible due to genomic instability, mutations and tumour-promoting inflammation, which lead to alterations in the cells behaviour (1, 2).

According to the World Health Organization (WHO), cancer is the first or second leading cause of death in 91 of 172 countries (3), such as Portugal (4). Its incidence and mortality has been increasing worldwide, regardless of the level of human development, probably due to aging, growth of the population, prevalence of risk factors (3) and improvement of diagnostic tools (4).

I.1. Male Breast Cancer

I.1.1. Epidemiology

Male breast cancer (MBC) is a rare disease, corresponding to 1% of all male cancers (5) and less than 1% of all breast malignancies (6). In Portugal, in the year of 2010, the incidence of MBC was 67 per 100.000 and the mortality was 19 per 100.000 cases (7). The mortality is higher than that observed in females (8, 9) and the incidence differs according to the geographical areas and ethnic groups, being higher in North America and Europe, and lower in Asia. African Americans present a higher incidence than Caucasians, Hispanics or Asian/Pacific Islanders (10, 11). Uganda and Zambia have a higher rate (5% and 15%, respectively) due to endemic infectious diseases, which lead to hyperoestrogenism, and Japan presents an incidence of less than five per million. Jewish men, regardless of living in the USA or in Israel, are the only population with a higher incidence than the average (12). It has been observed that the incidence of MBC has increased over the past 25 years by about 26% (13–15).

I.1.2. Aetiology and Risk Factors

The aetiology of MBC is uncertain (16). However, some of the risk factors are:

Family history: It was reported that 15% to 20% of males with breast carcinoma present a first-degree relative with breast or ovarian cancer (5). When a person has a first-

degree relative affected, the risk of developing breast cancer doubles and it is progressively higher with the increasing number of affected relatives (17, 18).

Personal history: The personal history, such as the history of breast trauma and nipple discharge, also increases the risk (19). The age of the patient is also an important risk factor (12), being the incidence higher in those with 55 years or older (17). It was also observed an increased incidence in black men (5).

Genetic Predisposition: Up to 10% of MBC are hereditary forms caused by inherited germline mutations (18). The frequency of these mutations varies according to the ethnicity (10). The presence of both *BRCA1* and *BRCA2* germline mutations increases the risk of MBC, at all ages, when compared to noncarriers. The cumulative risk for *BRCA1* mutation carriers is 1.2% and for *BRCA2* carriers it is 6.8%, at the age of 70 years old (20). The relative risk is higher at the age of 30 and 40, decreasing with older ages, especially in the *BRCA2* mutation carriers (20). *BRCA2* is the most frequently mutated gene in MBC patients, since it occurs in 4% to 14% of the patients (21) (22).

Mutations in other genes associated with breast cancer predisposition have also been observed, such as *ATM, CHEK2 and PALB2*, but they are only present in rare cases and for that reason are usually not tested (22).

Hormonal Imbalances: Hormonal changes, such as high levels of oestrogen and low levels of testosterone, are important risk factors in MBC (10).

In obesity, the blood levels of total and free testosterone are reduced (23), so this condition doubles the risk of developing MBC because it increases the peripheral aromatization of androgens, causing hyperestrogenization (10).

Diabetes is a risk factor because testosterone levels are also reduced (23). In a comparison of testosterone levels in men, between type 1 and type 2 diabetes, the levels are significantly lower in the latter (24).

Hepatic disease may also lead to an hyper estrogenic state and testicular dysfunction, like orchitis, orchiectomy and congenital inguinal hernia, is also an important risk factor (10, 23).

Klinefelter's syndrome, present in 5% of men with breast cancer (21), results from an extra X chromosome (47,XXY) and includes gynecomastia, testicular dysgenesis and sterility. The total and free testosterone levels in plasma are low and the oestradiol levels are high, due to increased peripheral conversion of testosterone into oestradiol (23). Individuals who presents this syndrome have a risk 20 to 50 times higher to develop MBC than the general population (10) and a higher probability of death. However, this syndrome does not accelerate the progression of the disease (23).

Although gynecomastia is not a risk factor, it is coexistent with MBC in more than 40% of the cases (16), probably due to high oestrogen levels present in both conditions (25).

It seems that androgens are protective factors in males (26), but mutations in the deoxyribonucleic acid (DNA) binding domain of androgen receptors (AR) are also a main risk factor in males (27). Nevertheless, the cross-sex hormone treatment, which reduces androgen and increases oestrogen, does not appear to be related with an increased risk of developing MBC (28).

Radiation: The exposure to ionizing radiation, due to professional exposure or therapy with X-rays, is a possible causal cofactor in MBC, possibly due to the exposure to high ambient temperature that can lead to testicular failure (10).

Lifestyle: Apart from alcohol consumption, which increases the risk and has a dose-response gradient (29), dietary factors do not seem to have a significant role in the development of MBC (10).

Bone fractures: A greater risk of developing MBC was observed when a bone fracture occurs in men with 45 years or older (17).

I.1.3. Male Breast Anatomy

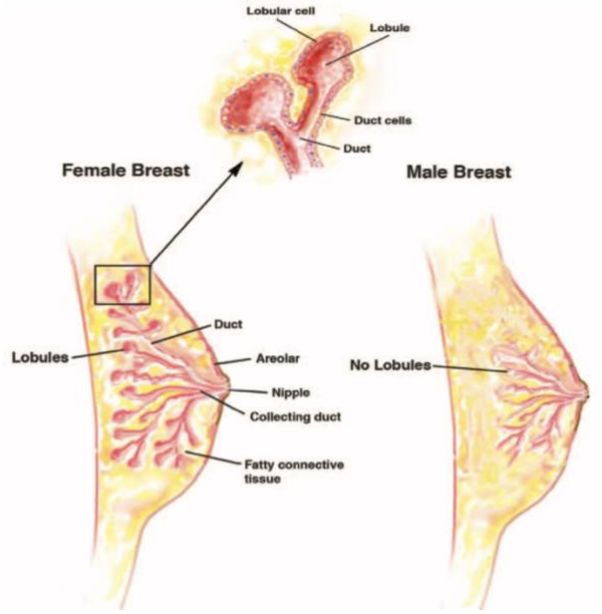

Figure 1. Anatomical differences in female and male breasts. From (30).

The tissue of the male breast is different from that of the female breast (30) **(Figure 1),** being located predominantly in the subareolar region (31). It includes mostly fatty tissue and a few stroma and mammary ducts, without terminal duct lobular elements or mammary

lobules (30, 31). Male mammary epithelium normally expresses oestrogen receptors (ER), progesterone receptors (PR) and AR. The epithelial elements are affected by several hormonal stimuli during life that could lead to the development of lobules (31).

I.1.4. Screening and Counselling

There are no breast cancer screening programs for men, even in cases with personal or family history (32), because there is no evidence for the benefit of performing selective screening in high risk men, in a disease with such a low prevalence (33, 34). There is also a lack of public education about the existence of breast cancer in males (33).

Since MBC is more strongly associated with *BRCA* germline mutations than FBC (35), and because *BRCA2* carriers are associated with an early age at diagnosis, positive axillary nodal status and a non-triple-negative histology (34), the American Society of Clinical Oncology (ASCO) recommends that all men with breast cancer should be referenced for genetic counselling, regardless of the family history (5).

I.1.5. Diagnosis

Men normally do not have any breast awareness, since cancer is rarely detected by screening mammography, and, therefore, the diagnosis is often made belatedly, with a larger tumour size and at a later stage (23, 36). The most common symptoms are a nonmobile painless mass (16), nipple retraction (19), and bloody nipple discharge (37). In men, breast cancer is more common in the left side than in the right side (19, 37, 38).

Usually, diagnosis is made by triple evaluation, as in women, comprising clinical assessment, imaging, and biopsy (9).

Clinical breast examination comprises bimanual palpation of the breasts and regional lymph nodes (39). It is very sensitive for detection of MBC, but presents a lower specificity (37).

Men with classic signs of gynecomastia usually do not need imaging, but when it is not possible to differentiate malignancy from benign disease by the clinical findings, or if the clinical presentation is doubtful, it is recommended the use of imaging tools (40). The principal imaging techniques used in males are mammography and ultrasound, as in females (16), and the main determinants to choose the image technique are the patient's age and the clinical suspicion based on the symptoms and physical examination (40).

When clinical examination and mammography findings are in agreement about the benign nature, biopsy may be unnecessary (41). However, pre-treatment evaluation includes a pathological diagnosis, based on biopsy (39), namely to infer the prognosis (42).

The pathological report should include information about the presence or absence of ductal carcinoma *in situ* (DCIS), the histological type and grade, immunohistochemistry (IHC) assessment of several biomarkers' expression, and others (39).

I.1.5.1. Histological Type

The histological type denotes the growth pattern of the tumours (43). This classification is made according to the WHO criteria (13) and includes invasive breast cancers, precursor lesions, lesions of low malignant potential, benign epithelial proliferations, among others (45).

MBC can appear almost everywhere in the breast tissue (46) and nearly all breast lesions identified in females have also been described in males (31).

The majority of breast carcinomas are classified as invasive ductal carcinoma of no special type (NST), which represents a carcinoma with no sufficient characteristics to belong to one of the special types. According to WHO, there are at least seventeen distinct histological special types (43).

In men, the commonest histological type is invasive ductal carcinoma NST (85% to 95%), followed by DCIS (5% to 10%) (5). The other histopathological types, such as invasive papillary carcinoma, medullary carcinoma and mucinous carcinoma, are very rare in males, occurring in approximately 2%, 2% and 1%, respectively (12). Since male breast does not present lobular elements, unless it is exposed to high levels of oestrogen (endogenous or exogenous), the lobular histological type is quite rare (10, 47), corresponding to 1% (12).

I.1.5.2. Histological Grade

Grade shows the similarity of the tumour cells to normal breast tissue (42), by an evaluation of the degree of differentiation (tubular formation and nuclear pleomorphism) and proliferative activity (mitotic index), showing the aggressiveness of the tumour (43).

The College of American Pathologists (CAP) recommends the use of the Nottingham combined histologic grade (Nottingham modification of the Scarff-Bloom-Richardson (SBR) grading system). For each morphologic feature a score is calculated, adding from one to three points, if it is close to normal breast (favourable) or distant to normal breast (unfavourable), respectively (42, 48).

I.1.5.3. TNM System

Staging for men respects the same guidelines as for women (35) and can be made according to the TNM system (39). It is an anatomic system that categorizes the tumour according to the extent of the disease (T), the status of the surrounding lymph nodes (N), and the presence of distant metastases (M). The T group is based on the size of the invasive component of the tumour and can be measured by clinical and/or pathological findings. The N classification is different if based on clinic (cN) or pathology (pN). Clinical classification comprises nodes found by clinical examination or imaging and pathologic classification comprises the suspicious of malignancy based on biopsy or sentinel node biopsy. The M group is based on clinical history, physical examination and imaging. The staging system is valid for *in situ* and invasive carcinomas, with the presence or absence of microinvasion (48).

I.1.5.4. Biomarkers

Despite the prognostic value of the number of lymph node metastases, tumour size, and tumour grade, it is essential to analyse molecular biomarkers, who can add prognostic information and predict response to therapy (42).

ER is the oldest and most used predictive biomarker in breast cancer. It is a transcription factor that regulates gene expression, binding to regulatory elements in the genome, and when deregulated can lead to tumour growth (42, 49).

PR and AR are commonly expressed in ER-positive tumours and it seems that they affect the ER transcriptional activity. ER can stimulate PR expression (42, 49) and, when in presence of progesterone, PR changes the binding position of ER to chromatin. This alteration causes a switch in the regulated genes, leading to cell cycle arrest, apoptosis, and differentiation, and being associated with a good prognosis in females. PR is generally tested simultaneously with ER (42).

Ki67 is a nuclear antigen only present in dividing cells, used to measure and monitor tumour proliferation (50) and add prognostic information (51).

Human epidermal growth factor receptor 2 (*HER2*) status assessment is also compulsory in the prognostic and predictive evaluation.

I.1.6. Molecular Classification

A study from Perou *et al.*, showed that FBC is an heterogeneous disease with different gene expression patterns, and proposed a molecular classification that divided the tumours

into four intrinsic subgroups (luminal A, luminal B, HER2-enriched and basal) (52). In daily clinical practice, the election of the treatment of choice is made essentially by the use of the previously mentioned four biomarkers (ER, PR, *HER2* and Ki67), from which a surrogate classification into the different intrinsic subgroups can be derived (39, 50).

For therapy decision, MBC is classified similarly to FBC, namely, as luminal A-like (ER- and PR-positive, *HER2*-negative and low Ki67), luminal B-like (ER-positive, *HER2*-negative, PR-negative or low or high Ki67; or ER-positive, *HER2*-positive, any PR or Ki67), HER2-enriched non-luminal (ER- and PR-negative, *HER2*-positive and high Ki67) and basal-like (triple negative) (ER-, PR- and *HER2*-negative and high Ki67) (52, 53). Ki67 and PR help do discriminate luminal A-like from luminal B-like *HER2*-negative (51). In the first one, PR are positive and Ki67 is low while in the latest either PR is low or negative or Ki67 is high (53) **(Table 1)**.

Table 1. Breast cancer intrinsic subgroups classification. Adapted from (53).

Intrinsic Subtype	**Biomarkers**			
	ER	**PR**	***HER2***	**Ki67**
Luminal A-like	+	+ (≥1%)	-	Low
Luminal B-like	+	Any	++	Any
		- or Low*****	-	High*****
***HER2*-enriched non luminal**	-	-	++	High
Basal-like	-	-	-	High

Abbreviations: ER - oestrogen receptor; **PR** - progesterone receptor; ***HER2*** - human epidermal growth factor receptor 2; **++** - amplified/overexpressed; **+** - positive; **-** - negative; ***** - either a high Ki67 value or a negative/ low PR value.

I.1.7. Prognosis

MBC is associated with poor prognosis, with a high risk of disease recurrence, mostly due to the delay in the detection of the cancer, the older age at diagnosis and the lack of studies about this disease in men (42, 54). The five-year survival rates varies between 36% and 66% (5). It seems that there is a scarcity of information about prognostic factors in MBC, but some female prognostic factors seem to be male prognostic factors as well (6), including age, tumour size, stage, nodal status, and lymph vascular invasion (5, 44).

Luminal A-like patients present a better prognosis, contrarily to basal-like patients who are five times more probable to die. A higher tumour grade, more advanced stage and younger age at diagnosis are also present in the basal-like group. Luminal B-like and *HER2*-

enriched non-luminal patients have intermediate prognosis, being worst in the latter, with a risk of death 1.9 times higher when compared with HER2-negative patients (55).

Patients with ER-positive carcinomas exhibit a reduction of 30% in the risk of death compared with ER-negative patients (56), with PR status being a less important prognostic factor (6) associated with a poor prognosis when negative (57). *HER2* prognostic value in males has not been defined (58) or it is controversial (6), with some studies showing that *HER2* overexpression is associated with a poor prognosis (8, 59) and other studies showing that there is no correlation between gene expression and prognostic features (60). This controversy is probably due to the paucity of data (5).

The risk of developing a contralateral breast cancer is higher, especially if the diagnosis of the first breast cancer was made earlier than 50 years old (61).

I.1.8. Treatment

Personalized medicine in oncology was firstly used in breast cancer, since the status of the biomarkers have been used as predictive factors to elect patients for endocrine therapy or anti-*HER2* treatment (39). It is therefore very important to make sure that the molecular subtype is correctly identified in MBC patients (62).

MBC is currently treated based on the existing knowledge from post-menopausal FBC (63). There are several options to treat breast cancer, depending on the general health status, age, preferences of the patient, and on the location (39) and biological features of the tumour (10).

When the disease is found in an early-stage, the treatment combines local modalities, such as surgery and radiotherapy, systemic anticancer therapies, and supportive measures (39). Most of the patients are treated with modified radical mastectomy rather than breast conservative surgery techniques (15, 35), since the last is less appropriate in men due to the shortage of breast tissue, the usual advanced stage at diagnosis (9), and the subareolar location of the tumour (36). The execution of the sentinel lymph node biopsy presents low morbidity and allows an improving in staging of the regional lymph nodes. This procedure is feasible and accurate, but when the result is positive for metastatic disease, complete axillary lymph node dissection is needed (36). When lumpectomy is done, it is recommended to perform radiotherapy, according to female guidelines (64). Men usually wait less time since diagnosis to radiotherapy than women, possibly due to the concern over locoregional recurrence or the preference to perform chemotherapy in females (65). In early-stage patients there is a survival benefit for postoperative radiotherapy (64). However, it is important to avoid unnecessary toxicity and costs, being mandatory to identify when

radiotherapy is not required (63). To prevent the risk of metastasis, usually adjuvant therapy is used, according to each molecular classification **(Figure 2)** (66).

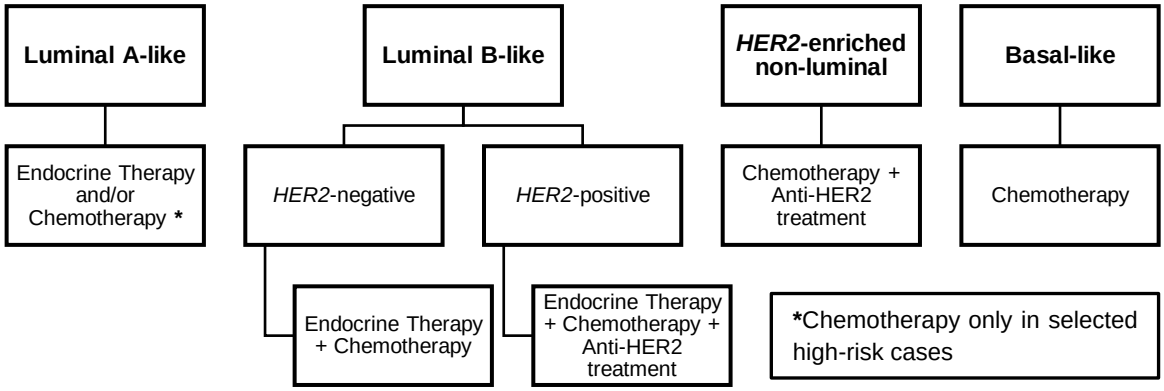

Figure 2. Adjuvant therapy options according to the molecular subgroups. Adapted from (62) and (66).

Luminal A-like: Endocrine therapy, for 5 to 10 years, is recommended and chemotherapy should be considered in high risk patients (62, 66). Yet, men are usually more undertreated and adjuvant chemotherapy is not normally used (63).

Endocrine therapy shows a large efficacy difference between men and women because of intrinsic differences in hormone production (35). α-ER is present mostly in neoplastic breast tissues while β-ER is mostly present in normal breast tissues. During breast carcinogenesis, the expression of β-ER decreases and the expression of α-ER becomes predominant (67). *Tamoxifen* is the most used hormonal treatment and is a selective ER modulator that blocks α-ER in breast tissue (23), and consequently blocks the proliferative action of oestrogen, acting like an antagonistic (67). When ER is positive, a better overall survival is observed if *tamoxifen* is used (57).

Luminal B-like: a combination of chemotherapy and endocrine therapy should be chosen. When in presence of *HER2* positivity, anti-HER2 treatment, such as antibodies or small molecules, should be administered as well (66). However, protein overexpression is necessary for the responsiveness of anti-HER2 therapies (68). If there is any contraindication for the use of chemotherapy, the other two therapies should be used in combination (62).

***HER2*-enriched non-luminal:** the treatment of choice should be a combination of chemotherapy and anti-HER2 treatment (66).

Basal-like: chemotherapy is the only treatment option (66).

I.1.8.1. Anti-HER2 Antibody

Trastuzumab is an humanized recombinant monoclonal antibody (Herceptin®; Genentech, Inc.; South San Francisco, CA) (69) that has the ability to prevent the activation of the intracellular tyrosine kinase of the *HER2* receptor, binding with high affinity to the juxta membrane portion of its extracellular domain (ECD) (70), specifically in the C-terminal region of domain IV **(Figure 3)** (71). The mechanisms of action are not completely understood (69).

Since there is no specific data regarding the use of *trastuzumab* in men, it should be used according to female guidelines and the patients' and tumour's characteristics (10). There is small evidence that *trastuzumab* works in males in the same way as in females (58). The results are better in patients with IHC score of 3+ than 2+ (72). When used alone, this drug may cause an acute hypersensitivity, but alopecia was not reported (70).

Trastuzumab can also be used in all metastatic breast cancers with an HER2-positive status, even as a single agent therapy (70). Usually, it is administered with other drugs, such as chemotherapy or hormonal agents. Nowadays, there are a number of new drugs that have been developed and that are in clinical trials, to treat *trastuzumab*-resistant breast cancer patients (69).

I.1.8.2. Anti-HER2 Small Molecule

Lapatinib (Tykerb®, GlaxoSmithKline, Philadelphia, PA) is a tyrosine kinase inhibitor of *HER1* and *HER2*, who binds reversibly to the intracellular domain (ICD) of the receptor **(Figure 3)**, in the adenosine triphosphate (ATP) binding site. The activation of the receptor is blocked, inhibiting the downstream pathways (73–75). One of the benefits of this drug is the presence of a slower dissociation, having a higher duration effect (75).

Even in the cases were shedding occurs (see below), *lapatinib* is still efficient and, since it is the first approved small molecule inhibitor that can cross the blood-brain barrier, it can target brain metastases (75). Thus, this oral drug (69), in *trastuzumab*-resistant patients, is efficient and can have an synergistic effect when combined with *trastuzumab* (74).

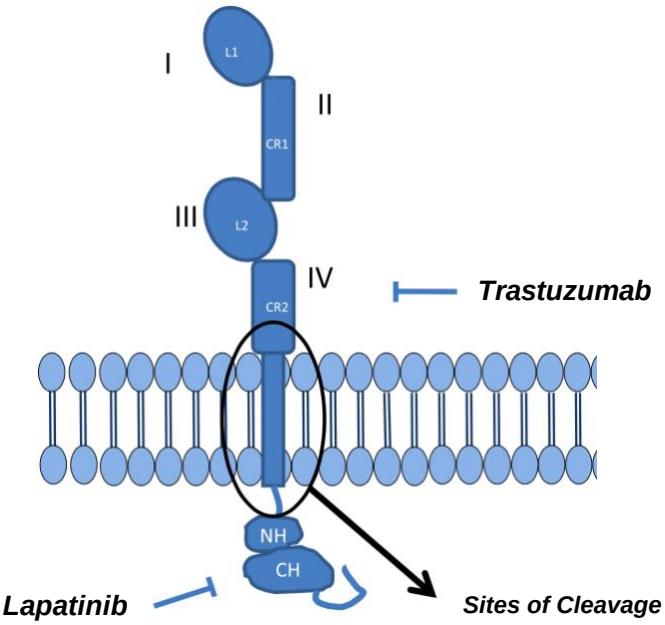

Figure 3. Structure of the *HER2* (extracellular domains (I, II, III and IV), transmembrane domain and intracellular domain), **shedding cleavage sites and *trastuzumab* and *lapatinib* binding sites.** Adapted from (89).

I.1.9. Gender Differences

Clinicopathological features of MBC do not overlap completely with FBC (18). The main differences between genders are the age at diagnosis, with males being diagnosed at an older age (mean ages from 63 to 71 years old in different studies) than females (52 to 63 years old), the histological type proportions, and the hormonal receptors status (49, 55, 77).

Initially the biology of MBC was thought to be like the late onset (post-menopausal) FBC. The early onset (premenopausal) is almost female-specific, since it is rarely observed in men, in whom it is associated with Klinefelter's syndrome and with *BRCA2* mutations (77, 78). However, MBC seems to be a different disease from FBC (44). Some differences in DNA aberrations (6) were observed, such as the presence of genomic gains involving whole chromosomes being more common and genomic losses less common in MBC (79). There are approximately 1000 genes differentially expressed between FBC and MBC (44).

Men usually present a poorer prognosis and a lower overall survival than women (80), probably due to a delay in the diagnosis or the more aggressive behaviour of the tumours (47). They present characteristics of advanced stage tumour, such as an advanced TNM stage at diagnosis (tumor size 2 to 5 cm and positive axillary lymph nodes) (5, 33, 38).

MBC show ER positivity, especially α-ER, at higher rates (superior to 90% in males *versus* 76% in females) (87, 89). PR are positive in more than 81% of the male cases *versus*

67% in females (33), and AR are positive in more male than female cases (81). In females, HER2 is overexpressed in 25% to 30% of the cases (58). However, there is no consensus about the proportion of HER2-positive cases in MBC.

If breast cancer carcinogenesis was the same in females and males, we should expect similar occurrence patterns. However, this is not a reality (77) and there is a significant difference in HER2 positivity between genders, with a lower proportion of HER2-positive cases in males (60).

There are conflicting studies about the proportion of the intrinsic subtypes in males. Some studies reported a predominance of the luminal A-like subgroup (luminal A-like: 75-98%; luminal B-like: 0-21%; HER2-enriched non-luminal: 0%; basal-like: 0-4%) (6), but others reported a predominance of the luminal B-like subgroup (luminal A-like: 42%; luminal B-like (HER2-negative): 49%; luminal B-like (HER2-posiitve): 9%; HER2-enriched non-luminal and basal-like: less than 1%) (15). On the other hand, in females, there is a predominance of the luminal A-like subgroup (luminal A-like: 44-66%; luminal B-like: 6-19%; HER2-enriched non-luminal: 8-10%; basal-like: 10-17%) (6). Since the proportions of the distinct subtypes are so different between genders, the direct transposition of the female subgroups to male is not necessarily identifying correctly the aggressive forms of MBC (82).

Johansson and co-workers proposed a new classification to MBC, dividing the tumours in two subgroups (luminal M1 and luminal M2) with different biological and clinicopathological features, according to gene expression profiles and alterations in copy number. The differences showed that these groups do not overlap with the female subgroups and suggested that they have their own biological and clinical characteristics and may require distinct therapies (83) **(Table 2)**. Therefore, MBC is probably an independent tumour entity of FBC (60).

Table 2. New classification proposed for MBC. Adapted from (83).

MBC Subgroup	Characteristics
Luminal M1*	Upregulated genes in tumour invasion, metastasis and cell proliferation. *HER2* upregulated. Downregulated ER signalling-associated genes.
Luminal M2**	Upregulated ER-signalling associated genes and immune response genes.

Abbreviations: ***HER2*** – Human Epidermal Growth Factor Receptor 2; **ER** – Oestrogen Receptors; * - More aggressive phenotype and worse prognosis; **** - Similarities with female intrinsic subtypes luminal A-like, luminal B-like, HER2-enriched non-luminal and basal-like.

I.2. *HER2*

The epidermal growth factor receptor (EGFR) family is composed of four receptors (*EGFR/HER1/ErbB1*, *HER2/ErbB2*, *HER3/ErbB3* and *HER4/ErbB4*), which present an ECD with ligand-binding function (84), except for *HER2* that does not present a known ligand (70), and an ICD with tyrosine kinase activity, with exception of *HER3* (84) **(Figure 4)**. They normally exist as monomers (inactive) and when bound to the ECD they stabilize the formation of dimers (active), which can either be with the same receptor type (homodimerization) or other receptor of the EGFR family (heterodimerization) (69, 85). When the signal transduction pathway is activated, they present an important role in controlling epithelial cell growth, differentiation (80), invasion (60), survival, adhesion and migration (84).

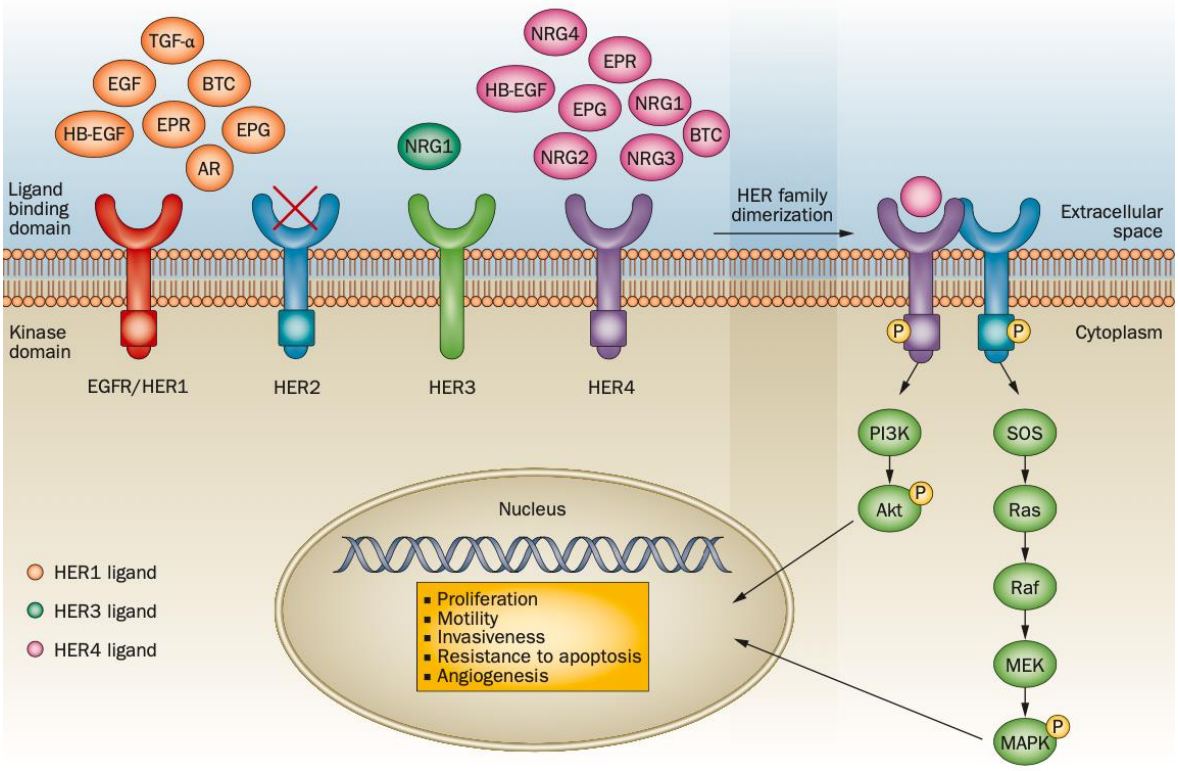

Figure 4. EGFR family members, heterodimer formation and downstream signalling pathway. The downstream pathway of HER2 is activated by homodimerization or heterodimerization. Since HER2 does not present a known ligand, it is always in an open conformation that allows dimerization. From(74).

A proto-oncogene is a gene that when is activated is transformed into an oncogene and can lead to cancer. Its activation can occur by gene amplification (1, 86), point mutations or gene fusions (1).

Erb-b2 receptor tyrosine kinase 2 (*ERBB2*), alias *HER2*, is the approved name according to HUGO Gene Nomenclature Committee (58). It is a proto-oncogene located in the long arm of the chromosome 17 (38) and encodes a 185kD cell transmembrane growth receptor with tyrosine kinase activity (80, 87), named $p185^{HER2}$ (88). The ECD presents four regions (71), where domains II and IV are responsible for receptor dimerization (89) **(Figure 3)**. Since HER2 has no known ligand, activation may only occur by heterodimerization (85).

HER2 is the preferred partner for interaction, so heterodimeric signals including this receptor are especially potent. These complexes present more stability at the cell surface than complexes without HER2, stay in the surface for longer periods of time and are recycled after their internalization (84, 85). The most potent dimer is HER2-HER3 (85).

Gene amplification of this biomarker (multiple copy numbers of a limited region of a chromosome arm) (90), which usually leads to overexpression of the protein (levels 10 to 100 times greater than normal), leads to a constitutive activation of the downstream pathway (60, 87). The DNA amplification of the *HER2* gene occurs after a double-strand break, when the cell does not have the ability to stop the cell cycle. When amplified, it can form in tandem arrays within a chromosome (head-to-tail or head-to-head repeats), and form homogeneously staining regions (HSRs), or can be stored outside the chromosome in double minutes (DMs). In breast cancer, *HER2* amplification can occur in both HSRs or DMs (86).

HER2 activation may also occur by shedding, by the proteolytic cleavage of the ECD that forms a truncated membrane-associated fragment ($p95^{HER2}$) with tyrosine kinase activity (89), which lead to the phosphorylation of the tyrosine in the cytoplasmatic domain of the receptor (72). The ICD has the ability to bind ATP and catalyse receptor autophosphorylation, activating downstream pathways, such as PI3K/AKT and RAS/MAPK (42, 91).

HER2 amplification is rare in other than breast cancer, although it has been described in different neoplasms, such as gastric and ovarian carcinomas (70, 85).

I.2.1. Assessment of the HER2 Status

Gene amplification was firstly detected in frozen tumour specimens by southern hybridization, and later found to be correlated with overexpression at the messenger ribonucleic acid (mRNA) and protein levels (73).

Nowadays, ASCO/CAP guidelines recommend that HER2 protein expression should be tested by IHC (92), which measures the membrane staining intensity (93), and classifies the samples as 0 (negative), 1+ (negative), 2+ (equivocal) and 3+ (positive). It is only in the cases where the IHC results are equivocal that gene amplification must be tested by an *in*

situ hybridization (ISH) technique, such as fluorescence *in situ* hybridization (FISH), chromogenic *in situ* hybridization (CISH) or silver enhanced *in situ* hybridization (SISH) (60, 92). Thus, IHC assay is the first screening step and measures protein expression. Scoring includes the percentage of positive tumour cells, the intensity of staining and the type of membrane staining (94, 95).

Established in the 1980s, FISH is a molecular cytogenetic technique that works by specific recognition of denatured targeted DNA sequences, by fluorescent labelled sequence pairing probe (96, 97). It is considered the *gold standard* for *HER2* testing and uses the mean *HER2*/Chromosome Enumeration Probe 17 (CEP17) ratio in a population of tumour nuclei and also the mean number of *HER2* copies (94). It presents a higher sensitivity and specificity than IHC (98) as it is not dependent the individual interpretation of the staining (72). Since DNA is more stable than proteins, it is not as susceptible to adverse effects of tissue handling and fixation (99). Although FISH is the *gold standard*, allowing the accurate and correct identification of *HER2* amplified tumours, it is more expensive than IHC and for that reason is not the method of choice in the majority of the centres (72, 95). Both techniques have the advantage of preserving the original tissue architecture, allowing the analysis in its histologic context (98).

The correct determination of the HER2 status is mandatory, because false negative results lead to undertreated patients, and false positive results to a waste of resources and disappointed patients, since the treatment is unlikely to be effective (72).

I.2.2. Correlation Between FISH and IHC

A overall good correlation between IHC and FISH techniques was found in FBC samples (62, 94, 95), with sensitivity varying between 84.7% and 97.9% (72, 87, 88, 100). A systematic review conducted by Cuadros and colleagues showed a very high correlation between IHC scores of 0 and 1+ and FISH, reporting a median percentage of discordance of 3.5 and 5.8, respectively. However, in IHC score 3+, FISH was only positive in 83.5% (median), leading these authors to concluded that the concordance between IHC and the FISH (considered the *gold standard*) was not the desirable (102).

Although studies executed in males are scarse, a good correlation between both techniques was also demonstrated (60, 88). On the other hand, Nilsson and colleagues, in a cohort of 197 MBC, reported that the majority of *HER2* amplified cases by SISH were IHC 0/1+ or 2+, and only a small percentage was IHC 3+. The authors hypothesized that those results could be explained by epitope loss during fixation or that protein expression is not as highly correlated with gene amplification in MBC as it is in FBC (6, 72). Interestingly, the

distribution of the different breast cancer molecular subtypes is quite different between males and females, raising the question of what might be the explanation (6).

II. Aims

The general aim of the present study was to test for amplification of *HER2* with FISH in a large series of male breast carcinomas and to compare the results with the previously obtained IHC findings.

The specific aims were:

- To define the frequency of *HER2* positivity in MBC.
- To determine the concordance between IHC and FISH in the determination of the *HER2* status in MBC.
- To correlate *HER2* status with clinicopathological characteristics of MBC patients.
- To compare the frequency of *HER2* positivity in MBC with that of FBC.

III. Material and Methods

III.1. Patients and Samples

All 152 MBC patients diagnosed with invasive carcinoma and treated in the Portuguese Institute of Oncology of Porto (IPO-Porto), from 1991 to July 2020, with age over 18 years old, were selected, regardless the disease stage and treatment received. The availability of good quality formalin-fixed, paraffin-embedded tissue blocks was required for study inclusion. Therefore, 98 samples obtained by biopsy, mastectomy or lumpectomy, were included in this retrospective study. The clinicopathological data was collected through a systematic revision of the patients records. We included follow-up information up to July 2020.

III.2. Immunohistochemistry

All cases were evaluated by the Department of Pathology of IPO-Porto. For IHC assay, two different anti-HER2 antibodies (polyclonal rabbit antibody 0485, Dako, Hamburg, Germany, and monoclonal rabbit antibody 4B5, Ventana Medical Systems, USA) were used.

Cases were classified, according to 2018 ASCO/CAP guidelines, as 0 if there was no membrane staining or incomplete membrane staining hardly observable in 10% of the tumour cells or less, 1+ if there was an incomplete membrane staining hardly observable in more than 10% of the tumour cells, 2+ if there was weak to moderate complete membrane staining observed in more than 10% of the tumour cells, and 3+ if there was a strong circumferential membrane staining in more than 10% of the tumour cells (92).

For 67 specimens IHC was already performed and the findings reported by Abreu *et al.* (82).

III.3. Fluorescence *in situ* Hybridization

The FISH assay was performed in all cases irrespective of the IHC score. Since the IHC-equivocal cases were previously evaluated at the Genetics Department of IPO-Porto during patient's diagnosis, these cases were not retested. The categorization of those cases was updated according to the 2018 ASCO/CAP guidelines.

III.3.1. Protocol

Assessment of *HER2* amplification was performed by FISH in sections from paraffin-embedded tumour. Slides were placed twice in xylol and 100% ethanol for 5 minutes each,

for deparaffinization. Pre-treatment was made by placing the slides in 2xSSC for 3 minutes, then in NaSCN 1M at 80ºC for 10 minutes and the rinsed in 2xSSC solution at room temperature for 2 minutes. The enzymatic digestion was made through incubation of a pepsin solution (4mg/mL) with each slide at 37ºC. In order to finish the digestion, the slides were placed in 2xSSC for 2 minutes and then in increasing series of ethanol (70%, 85% and 100%) for 2 minutes each. After dehydration, the specific probe sets were applied onto each sample. The *HER2* probe was combined with a centromeric probe for chromosome 17 (ON ERBB2 HER-2/Neu (17q12)/SE 17, Kreatech). Slides were placed in a ThermoBrite denaturation/ hybridization system (Leica Biosystems, Nussloch, Germany), and co-denaturaturated for 8 minutes at 80ºC, followed by hybridization at 37ºC for 18 hours. Washing was made in 2xSSC/0.5% IGEPAL (Sigma Aldrich) solution at 74ºC for 5 minutes followed by 2xSSC/0.1% IGEPAL at room temperature for 3 minutes. In the end, were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, California, USA).

Green signals, red signals and DAPI, obtained by FISH assay, were automatically captured in a GSL-120 automated cytogenetics platform using the *CytoVision* software version number 7.4 (Leica Biosystems).

For amplification scoring, the image processing uses a fixed Modal Average (ModeSA) of 0.25μm^2 for the reference size of a test signal. CytoVision image processing evaluates the signal size, breaking up large or clustered signals greater than 0.5μm^2 by dividing by 0.25 to calculate the signal count.

III.3.2. Analysis

FISH assay was performed in the invasive component of MBC specimens. Amplification in a dual-probe FISH assay was made by counting the *HER2*/CEP17 ratio followed by the average *HER2* copy number. At least 60 cells were counted from at least two representative non overlapping cancer cell populations. If there was a second population of contiguous cells with increased *HER2* signals per cell, and this cell population consists of more than 10% of tumour cells on the slide (defined by image analysis or visual estimation of the ISH or IHC slide), a separate counting was also performed in this cell population (92).

Cases were categorized in five groups, according to 2018 ASCO/CAP guidelines **(Figure 5)**:

Group 1: HER2/CEP17 ratio ≥ 2.0 with average HER2 copy number ≥ 4.0 signals/cell – Positive;

Group 2: HER2/CEP17 ratio ≥ 2.0 with average HER2 copy number < 4.0 signals/cell – result is dependent from IHC and classified as Negative if the IHC was not 3+;

Group 3: HER2/CEP17 ratio < 2.0 with average HER2 copy number ≥ 6.0 signals/cell – result is dependent from IHC and classified as Negative if the IHC was 0 or 1+ and as Positive if IHC was 2+ or 3+;

Group 4: HER2/CEP17 ratio < 2.0 with average HER2 copy number ≥ 4.0 and < 6.0 signals/cell – result is dependent from IHC and classified as Negative if the IHC was not 3+;

Group 5: HER2/CEP17 ratio < 2.0 with average HER2 copy number < 4.0 signals/cell – Negative.

When the IHC was 2+ and the FISH result corresponded to group 2, 3 or 4, a second count was made, by another observer blinded to previous results (92). When both counts were not in agreement, an average value was determined.

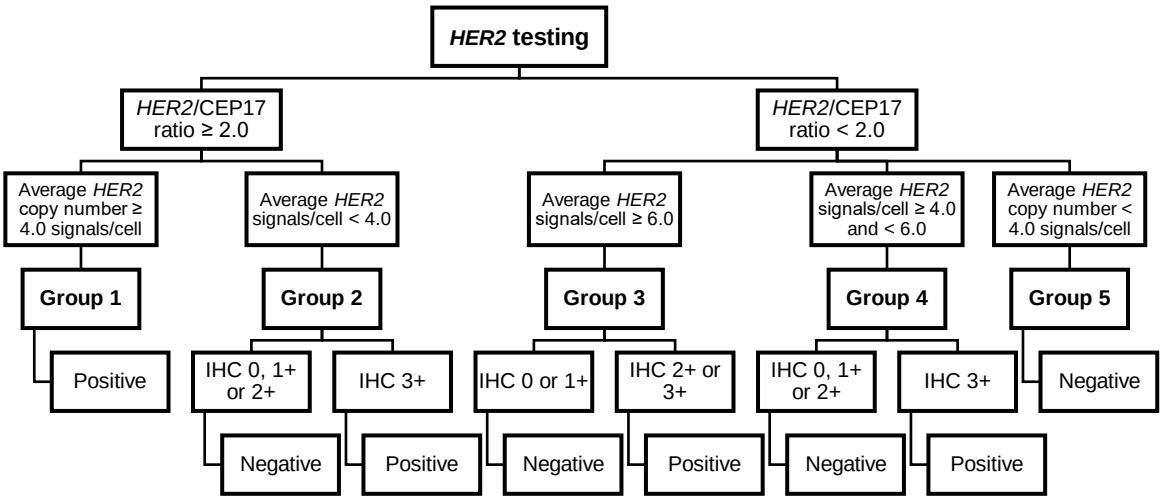

Figure 5. Groups of *HER2* gene amplification tested by FISH. Adapted from (93).

III.4. Statistical Methods

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software (IBM Corp., version 26).

Clinicopathological variables were summarized using frequencies, proportions, means and ranges. To analyse the association between clinicopathological parameters and the HER2 status, the Student's t-test was used for quantitative variables and the Pearson Chi-Square or Fisher's Exact Test were used for categorical variables. A p value < 0.05 was set as the limit for statistical significance.

To evaluate the concordance between techniques, Cohen's Kappa test was calculated. Values between 0-0.20 were considered as indicative of poor concordance, 0.21-0.40 fair concordance, 0.41-0.60 moderate concordance, 0.61-0.80 good concordance and 0.81-1.00 very good concordance (103).

Kaplan-Meier survival curves were calculated for *HER2*-positive concordant and discrepant cases.

IV. Results

IV.1. Characteristics of the Study Cohort

For this study, 98 tissue samples of male breast carcinomas were used, after the institutional ethics committee's approval (CES 159/020).

All 98 patients selected for the study had invasive breast cancer and were diagnosed between 30 and 88 years old, with a mean age of 65 years, 82.6% of them having 55 years or more at presentation. A predominance of left-sided tumours (55.3% versus 44.7%) was found.

The anatomopathological diagnosis revealed that most of the cases were classified as invasive ductal carcinoma NST (80.6%), 14 cases as mixed carcinoma, three cases as papillary carcinoma, one case as micropapillary carcinoma, and another as mucinous carcinoma.

ER were positive in 98% of the cases and PR in 93.9% (92 patients presented ER+/PR+ (93.9%), 2 patients ER-/PR- (2%), and 4 patients ER+/PR- (4.1%)).

The clinicopathological characteristics of the patients are summarized in **Table 3**.

Table 3. Clinicopathological features of the study cohort.

Tumour characteristics		No of patients	%
Age (years) **mean 65.1 (range 30-88)**		86	
	< 55 years old	15	17.4
	≥ 55 years old	71	82.6
Tumour Side	Left	47	55.3
(n=85)	Right	38	44.7
Histological Type	Ductal Invasive NST	79	80.6
(n=98)	Mixed	14	14.3
	Papillary	3	3.1
	Micropapillary	1	1
	Mucinous	1	1
Grade	1	7	7.1
(n=98)	2	49	50
	2/3	3	3.1
	3	39	39.8

Continues on next page

Table 3. (continuation) Clinicopathological features of the study cohort.

Tumour characteristics		No of patients	%
Tumour size (cm), mean 2.6 (range 0.80-8)		97	
	< 2 cm	36	37.1
	2-5 cm	54	55.7
	> 5 cm	7	7.2
T Stage	pT1	30	34.9
(n=86)	pT2	28	32.6
	pT3	3	3.5
	pT4	25	29.1
N Stage	pN0	39	45.3
(n=86)	pN1	28	32.6
	pN2	10	11.6
	pN3	8	9.3
	pNx	1	1.2
M Stage	pM0	63	80.8
(n=78)	pM1	6	7.7
	pMi	1	1.3
	pMx	8	10.3
Oestrogen Receptors	Positive	96	98
(n=98)	Negative	2	2
Progesterone Receptors	Positive	92	93.9
(n=98)	Negative	6	6.1

Abbreviation: NST – No Special Type.

IV.2. Assessment of the HER2 Status by IHC and FISH

All 98 cases were analysed by IHC and classified according to the 2018 ASCO/CAP guidelines (92). In our cohort, HER2 was firstly analysed by IHC and classified as 0 or 1+ (negative) in 79 cases (80.6%), 2+ in 15 (15.3%) and 3+ in 4 (4.1%). *HER2* amplification was assessed by dual-probe FISH (HER2 and CEP17 probes) and results are summarized in **Table 4**.

Table 4. Parameters for evaluation of the HER2 status by IHC and FISH.

Case	IHC	IHC Score	FISH	Group	Cells Analysed	*HER2*/CEP17	*HER2* Copy Number	CEP17 signal
1	Equivocal	2+	Negative	5	60	1.38	3.67	2.67
2	Equivocal	2+	Negative	4	75	1.23	4.20	3.43
3	Negative	1+	Negative	5	100	1.40	2.25	1.61
4	Equivocal	2+	Negative	5	60	1.21	3.82	3.17
5	Negative	0	Negative	5	100	1.21	2.10	1.73
6	Equivocal	2+	Negative	5	60	1.59	3.27	2.05
7	Negative	0	Negative	5	100	1.69	3.11	1.84
8	Equivocal	2+	Positive	1	40	2.13	4.95	2.33
9	Negative	1+	Negative	5	100	1.26	2.31	1.84
10[a]	Negative	0	Positive	1	200	2.14	5.26	2.46
11	Negative	0	Negative	5	150	1.51	3.43	2.28
12	Negative	0	Negative	5	100	1.60	2.96	1.85
13	Negative	1+	Negative	5	100	1.39	3.01	2.17
14	Negative	0	Negative	5	70	1.24	3.26	2.63
15	Equivocal	2+	Negative	4	100	1.62	4.22	2.60
16	Equivocal	2+	Negative	5	80	1.05	1.94	1.85
17	Equivocal	2+	Positive	1	60	3.05	8.65	2.83
18	Negative	NA	Negative	4	115	1.46	4.09	2.81
19*****	Equivocal	2+	Positive	1	50	5.05	8.88	1.76
20	Equivocal	2+	Negative	4	85	1.33	4.26	3.21
21	Negative	0	Negative	5	100	1.14	2.24	1.96
22	Equivocal	2+	Positive	1	100	7.05	10.85	1.54
23[a]	Negative	1+	Positive	1	105	7.79	7.42	0.95
24	Negative	0	Negative	5	60	1.41	2.12	1.50
25	Negative	1+	Negative	5	115	1.22	2.11	1.73
26	Negative	NA	Negative	5	100	1.53	3.85	2.52
27[a]	Negative	1+	Positive	1	100	2.07	5.44	2.63
28	Negative	NA	Negative	5	100	1.65	2.59	1.57
29	Negative	0	Negative	5	100	1.40	2.64	1.89
30	Negative	NA	Negative	5	70	1.24	1.90	1.53
31	Negative	NA	Negative	5	100	1.51	2.74	1.81
32	Negative	1+	Negative	5	100	1.30	2.48	1.91
33[a]	Negative	0	Positive	1	100	2.15	4.32	2.01
34	Negative	0	Negative	5	100	1.03	1.90	1.84
35	Equivocal	2+	Negative	5	60	1.73	3.00	2.50
36	Negative	NA	Negative	5	100	1.08	2.19	2.03

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Table 4. (continuation) Parameters for evaluation of the HER2 status by IHC and FISH.

Case	IHC	IHC Score	FISH	Group	Cells Analysed	*HER2*/CEP17	*HER2* Copy Number	CEP17 Signal
37	Negative	NA	Negative	5	100	1.29	2.75	2.13
38	Equivocal	2+	Positive	1	100	NA	NA	3.00
39	Negative	NA	Negative	5	100	1.19	2.03	1.70
40	Negative	NA	Negative	5	100	1.38	3.76	2.73
41	Negative	NA	Negative	5	100	1.26	3.54	2.82
42	Equivocal	2+	Negative	5	100	1.00	2.00	2.00
43	Negative	NA	Negative	4	100	1.93	4.42	2.29
44	Negative	NA	Negative	5	100	1.24	2.34	1.89
45[b]	Positive	3+	Positive	1	65	3.52	13.22	3.75
46	Negative	1+	Negative	5	100	1.26	2.60	2,07
47	Negative	NA	Negative	5	100	1.30	3.41	2.62
48	Negative	NA	Negative	5	100	1.23	2.50	2.03
49	Negative	NA	Negative	5	100	1.16	2.18	1.88
50	Negative	NA	Negative	4	100	1.34	4.34	3.23
51	Negative	NA	Negative	5	100	1.26	2.93	2.33
52	Negative	NA	Negative	5	100	1.21	3.24	2.67
53	Negative	NA	Negative	5	100	1.18	2.18	1.85
54	Negative	NA	Negative	5	100	1.00	3.50	3.50
55	Negative	NA	Negative	5	100	1.22	2.37	1.94
56	Negative	NA	Negative	3	100	1.32	7.08	5.14
57	Negative	NA	Negative	5	100	1.40	3.89	2.77
58[b]	Positive	3+	Positive	1	65	5.38	11.92	2.22
59	Negative	1+	Negative	5	100	1.19	3.36	2.82
60	Negative	NA	Negative	5	100	1.27	2.57	2.03
61	Negative	NA	Negative	5	100	1.39	3.30	2.38
62	Negative	NA	Negative	5	100	1.15	3.33	2.89
63	Negative	NA	Negative	5	100	1.39	2.16	1.55
64	Negative	NA	Negative	5	100	1.22	3.73	3.05
65	Negative	NA	Negative	5	100	1.29	3.78	2.93
66	Negative	NA	Negative	5	100	1.17	2.74	2.35
67	Negative	NA	Negative	5	100	1.19	2.07	1.74
68[a]	Negative	0	Positive	1	100	3.04	7.90	2.60
69	Negative	NA	Negative	5	100	1.29	2.24	1.73
70	Equivocal	2+	Negative	4	100	1.32	4.09	3.09
71	Negative	NA	Negative	5	100	1.32	3.06	2.31
72	Negative	NA	Negative	5	100	1.10	2.09	1.90

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Table 4. (continuation) Parameters for evaluation of the HER2 status by IHC and FISH.

Case	IHC	IHC Score	FISH	Group	Cells Analysed	*HER2*/CEP17	*HER2* Copy Number	CEP17 Signal
73	Negative	NA	Negative	5	100	1.12	2.34	2.09
74	Negative	NA	Negative	5	100	1.31	3.17	2.42
75	Negative	NA	Negative	5	100	1.18	2.20	1.86
76	Negative	NA	Negative	5	100	1.15	2.91	2.52
77[a]	Negative	1+	Positive	1	100	2.28	4.09	1.79
78	Negative	NA	Negative	5	100	1.23	2.82	2.30
79[b]	Positive	3+	Positive	1	65	2.17	8.06	3.72
80	Negative	NA	Negative	4	100	1.40	4.63	3.30
81	Negative	NA	Negative	5	85	1.17	3.22	2.75
82[b]	Positive	3+	Positive	1	100	2.57	9.45	3.67
83	Negative	NA	Negative	5	100	1.01	2.86	2.84
84	Negative	NA	Negative	4	100	1.17	4.55	3.88
85	Negative	NA	Negative	5	100	1.25	3.41	2.72
86	Negative	NA	Negative	5	100	1.31	3.87	2.96
87	Negative	NA	Negative	5	100	1.56	3.89	2.50
88	Negative	NA	Negative	5	100	1.84	2.97	1.61
89	Negative	NA	Negative	5	100	1.14	2.39	2.09
90	Negative	NA	Negative	5	100	1.18	3.38	2.87
91	Negative	NA	Negative	4	100	1.54	5.02	3.27
92	Negative	NA	Negative	5	100	1.16	2.57	2.21
93	Negative	NA	Negative	4	100	1.50	4.44	2.96
94	Negative	NA	Negative	4	100	0.96	4.04	4.20
95	Negative	NA	Negative	5	100	1.10	2.76	2.52
96	Negative	NA	Negative	5	100	1.20	3.73	3.10
97	Negative	NA	Negative	5	100	1.18	3.77	3.20
98	Negative	NA	Negative	4	100	1.63	4.47	2.75

Abbreviations: IHC – Immunohistochemistry; **FISH** – Fluorescence *in situ* Hybridization; ***HER2*** – Human Epidermal Growth Factor Receptor 2; ***CEP17*** – Chromosome Enumeration Probe 17**; NA** – Data Not Available; [a] – discrepant cases; [b] – concordant cases; ***** – case previously analysed by SISH and classified as negative.

Summing up, by FISH, 15 of the samples belonged to group 1 (15.3%), one to group 3 (1%), 13 to group 4 (13.3%) and 69 to group 5 (70.4%) **(Table 5)**.

Table 5. FISH groups determined in the cohort.

Group	n	Percentage
1	15	15.3
2	0	0
3	1	1
4	13	13.3
5	69	70.4

Comparing the HER2 status by both IHC and FISH techniques, we observed six cases (6.1%) in which HER2 was negative by IHC but positive by FISH. There were no cases with a positive IHC score and a negative FISH result. Among the IHC-equivocal (2+) cases, 66.7% were negative (10/15) and 33.3 (5/15) were positive by FISH **(Table 6)**.

Table 6. HER2 status analysed by IHC and FISH.

IHC	FISH			Concordance
	Positive (%)	Negative (%)	Total (%)	
0/1+	6 (6.1)	73 (74.5)	79 (80.6)	92.4% (73/79)
2+	5 (5.1)	10 (10.2)	15 (15.3)	33.3% (5/15)
3+	4 (4.1)	0	4 (4.1)	100% (4/4)
Total (%)	15 (15.3)	83 (84.7)	98 (100)	

Abbreviations: IHC – Immunohistochemistry; **FISH** – Fluorescence *in situ* Hybridization.

The sensitivity and specificity of IHC were calculated, using FISH as *gold standard*. For this analysis we excluded the IHC-equivocal cases. We found a positive agreement (sensitivity) of 40% and a negative agreement (specificity) of 100% for IHC, as well as a positive predictive value of 100% and a negative predictive value of 92.4% **(Table 7)**.

We obtained a kappa value of 0.54 ($p < 0.001$), with an overall agreement of 92.8%, representing a moderate agreement between techniques.

Table 7. Sensitivity, specificity, positive predictive value and negative predictive value of IHC.

	Percentage
Sensitivity	40
Specificity	100
Positive Predictive Value	100
Negative Predictive Value	92.4

IV.3. Characteristics of the Discrepant and Concordant Cases

Interestingly, of the 10 FISH-positive cases (excluding the IHC 2+), only four (40%) were IHC 3+. The clinicopathological characteristics from the discrepant cases (#10, #23, #27, #33, #68 and #77) and concordant cases (#45, #58, #79 and #82) are summarized in **Table 8**.

For the discordant cases, the mean age at diagnosis was 69 years old (range 46-88). All cases corresponded to invasive ductal carcinomas NST, with exception of one that was a mixed carcinoma. The majority of the carcinomas were grade 3 (66.7%) and were located in the left breast (66.7%). ER and PR were positive in all cases.

For the concordant *HER2*-positive cases, the mean age at diagnosis was 55 years old (range 43-69). Tumours corresponded to invasive ductal carcinoma NST, mixed carcinoma and micropapillary carcinoma, mostly grade 3 (75%) and all were located in the left breast. ER were positive in all cases, and one case presented negative PR.

Table 8. Clinicopathological characteristics of the cases with discrepant and concordant HER2-positive results. The discrepant cases correspond to #10, #23, #27, #33, #68 and #77 and the concordant cases correspond to #45, #58, #79 and #82.

Case	Age at Diagnosis	Breast	Histology Type	Grade	ER	PR
10	46	Right	Invasive Ductal NST	3	+ (90-100%)	+ (90-100%)
23	88	Right	Mixed	3	+ (75-100%)	+ (75-100%)
27	62	Left	Invasive Ductal NST	3	+ (75-100%)	+ (10-25%)
33	75	Left	Invasive Ductal NST	2	+ (75-100%)	+ (75-100%)
68	83	Left	Invasive Ductal NST	2	+ (75-100%)	+ (50-75%)
77	61	Left	Invasive Ductal NST	3	+ (75-100%)	+ (50-75%)
45	44	Left	Mixed	3	+ (75-100%)	+ (50-75%)
58	65	Left	Micropapillary	2	+ (75-100%)	+ (50-75%)
79	69	Left	Invasive Ductal NST	3	+ (75-100%)	+ (25-50%)
82	43	Left	Mixed	3	+ (25-50%)	-

Abbreviations: + - Positive; **-** - Negative**; NST** – No Special Type.

All the six discrepant amplified cases corresponded to group 1, with *HER2*/CEP17 ratios varying from 2.07 to 7.79 (mean: 3.25) and *HER2* copy numbers varying from 4.09 to 7.90 (mean: 5.74).

The concordant group had *HER2*/CEP17 ratios varying from 2.17 to 5.38 (mean: 3.41) and *HER2* copy numbers varying from 8.06 to 13.22 (mean: 10.66).

We observed no statistical differences in the *HER2*/CEP17 ratio between those groups (p= 0.901) but the *HER2* copy number was higher in the group with an IHC score of 3+ (p= 0.004).

Illustrative images of IHC and FISH of the discrepant cases are presented in **Figure 6**. All discrepant cases were revised by an experienced pathologist.

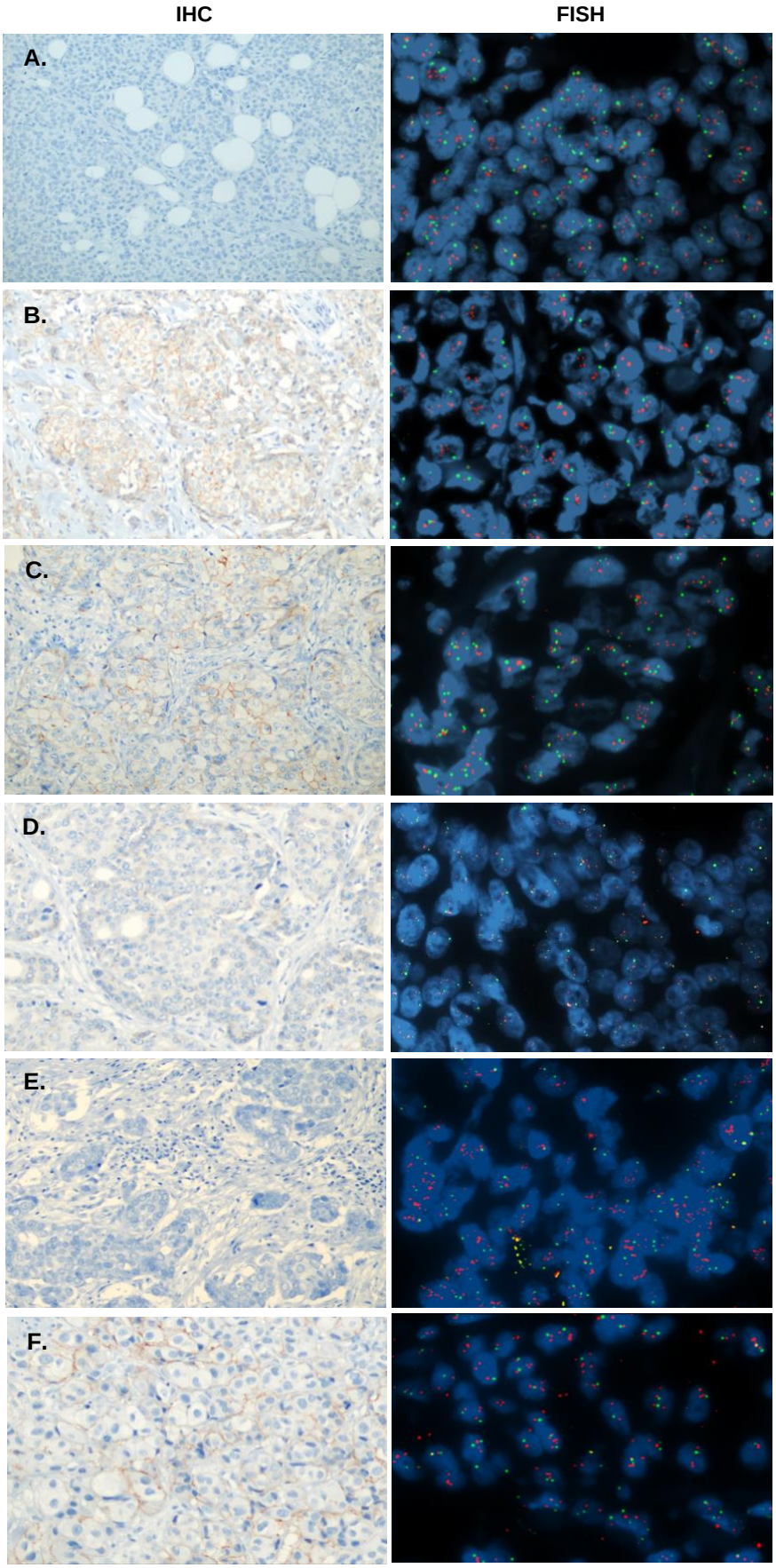

Figure 6. IHC and FISH images of the discrepant cases. A. #10; **B.** # 23; **C.** # 27; **D.** #33; **E.** #68; **F.** #77. Green signals correspond to chromosome 17 centromere and red signals correspond to the *HER2*.

Patient #19 is a particular case of agreement result between IHC and FISH techniques. This patient was excluded from the concordant cases since IHC was considered equivocal, with areas corresponding to 1+ and others corresponding to 2+. FISH result was amplified in the areas corresponding to 2+, and the case was classified as amplified with heterogeneity. Illustrative IHC and FISH images are present in **Figure 7**. This case was referred to our institution with a previous negative SISH result.

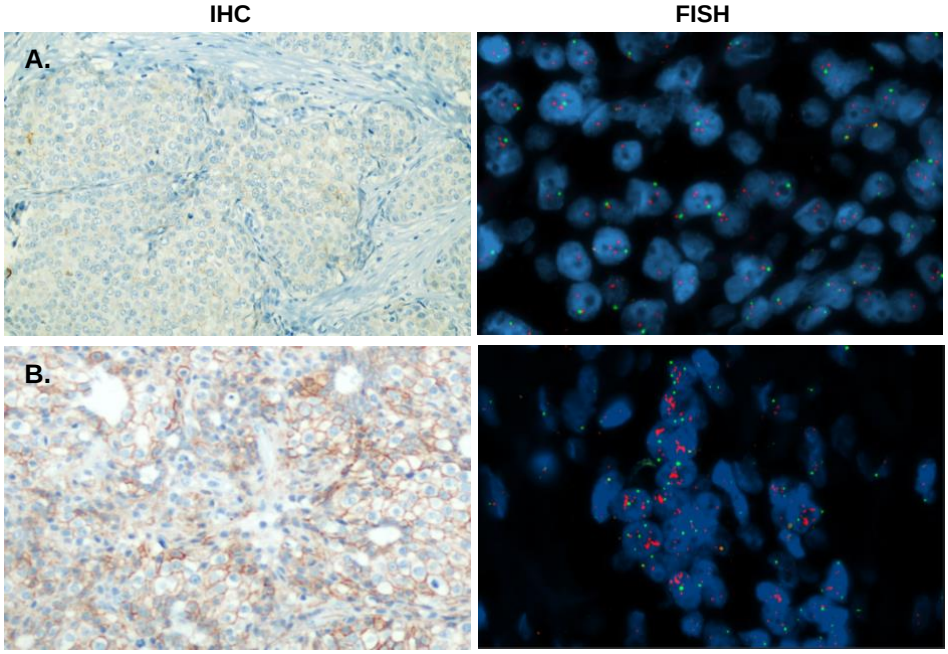

Figure 7. Representative images of #19. Different areas of the tumour showing **A.** IHC 1+ and **B.** IHC 2+, and the corresponded FISH results. Green signals correspond to chromosome 17 centromere and red signals correspond to the HER2.

IV.4. Association Between *HER2* Amplification and Tumour Clinicopathologic Features

There was no association between *HER2* amplification and clinicopathological features, such as age at diagnosis (p= 0.663), tumour size (p= 0.519), histological type (p= 0.265), histological grade (p= 0.462), ER (P= 0.716), PR (p= 0.228), pT (p= 0.947), pN (p= 0.103) and pM (p= 0.684).

IV.5. Overall Survival

Kaplan-Meier survival analysis was performed in the 10 *HER2*-positive cases with concordant or discrepant results **(Figure 8)**. In all cases, data about the diagnosis and time of death (with evidence of the disease) was available. At the time of the last follow-

up, six patients were alive without evidence of disease (60%) and four patients (40%) were deceased, one of them due to breast cancer. The only patient with metastasis is the same who deceased by cancer **(Table 9)**. Due to the low number of cases, the survival analysis does not have statistical power to detect eventual differences.

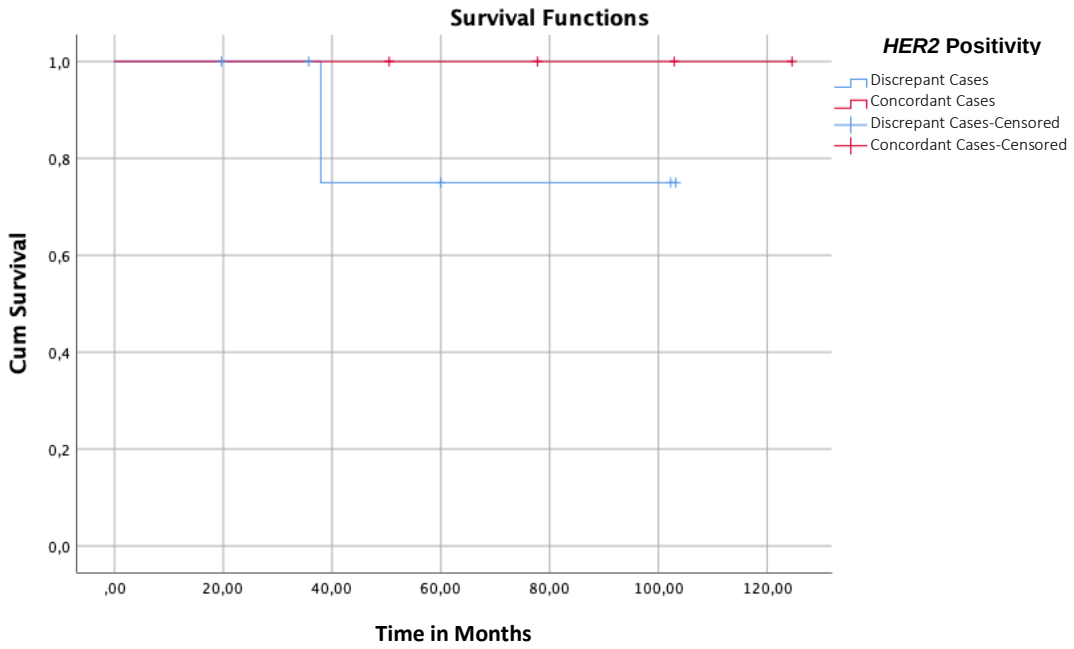

Figure 8. Overall survival in the discrepant and concordant *HER2* positive cases.

Table 9. Summary of the vital status of the patients from the two groups in study.

	Alive Without Evidence of Cancer	Death by Cancer	Death by Other Cause	Metastasis
Discrepant (n= 6)	4	1*	1	1*
Concordant (n= 4)	2	0	2	0

Abbreviation: * – same patient.

V. Discussion

Data from the literature showed that, although MBC and FBC share some similarities, important differences in prognosis and survival were observed. There is an increasing appreciation of differences in the tumour biology of MBC *versus* FBC that might explain the higher mortality and the worse survival rates in males, highlighting the need for studies focused on this unique population (60, 80).

In this retrospective study we aimed to obtain more data on the correlation of HER2 protein expression and *HER2* gene amplification in MBC, by comparing HER2 status by both FISH and IHC in a consecutive cohort of MBC patients treated in our reference centre. A worse correlation between protein expression and gene amplification may place MBC patients at risk of undertreatment.

V.1. Clinicopathological Features

The clinicopathological characteristics of our cohort are in agreement with other large MBC studies, such as the studies from Vermeulen and co-workers (104), Sun and co-workers (38) and Cardoso and colleagues (15), showing that it is representative of the general MBC population.

MBC and FBC differ quite a lot regarding the age at diagnosis, histological type and hormonal receptors status (76). In our cohort, the median age at diagnosis was 65 years old, which is line with the literature (63 to 71) (54, 76) and is in fact higher than the mean age (52 to 63) reported in FBC (47). The incidence of breast cancer increases at 55 years old (17) and the majority of our patients (83.5%) were, as expected, within this group. The advanced age at diagnosis can be explained, at least in part, by the absence of screening in males (10).

The most frequent histological subtype was invasive ductal carcinoma NST, with none of the patients presenting lobular carcinoma, which confirms the rarity of this histological subtype in males (12).

The hormonal receptors status is another major difference observed between genders. In our study, as expected, most of the cases presented ER positivity (98%) as well as PR positivity, but with a slightly lower ratio (93.9%), in agreement with the literature (44).

V.2. HER2 Status

In our retrospective study, the percentage of *HER2* amplification determined by FISH was 15.3%, which is lower than the 25-30% described for FBC (58). This lower rate of *HER2* amplification was also found by others (6, 60).

It is of major importance to determine the *HER2* status in breast cancer patients with a quick, accurate and cost-effective technique, to identify the patients who may benefit from an anti-HER2 therapy. Nowadays, breast cancer samples are analysed firstly by IHC and only the equivocal cases are analysed by FISH, to determine the presence or absence of amplification of the *HER2* (92). There is no unanimity on which test is more predictive of response (102), although FISH is considered the *gold standard* for evaluation of the *HER2* status (97).

This study shows that IHC and FISH present an overall moderate agreement, with 92.8% concordance when the IHC 2+ cases are excluded. Patient #19 from our cohort was a good example of that. The IHC was negative with focal equivocal areas, and the FISH analysis showed concordant positive areas (*HER2*/CEP17 ratio: 5.05; *HER2* copy number: 8.88), such that the case was classified as positive with heterogeneity. Case #19 had previously been evaluated by SISH in an external laboratory and the result was negative for *HER2* amplification. Although previous studies in FBC reported that FISH and SISH present an excellent concordance, with a correlation of 92-96% (105–107), the only case from our cohort analysed by both FISH and SISH demonstrated a discrepant result – presence of *HER2* amplification by FISH and absence of *HER2* amplification by SISH.

Furthermore, we observed that all cases classified as positive by IHC with a score of 3+ were also positive by FISH. Although we have only four cases with IHC 3+ in our series, we observed 100% concordance, which is higher than the 83.5% described by Cuadros *et al.* (102) or the 76.4% described by Bloom *et al*. (88), in FBC. However, in our cohort, not all cases classified as IHC 0/1+ were negative by FISH, with 6 of the 79 IHC 0/1+ cases (7.6%) presenting gene amplification. Those results seem to be in accordance with the literature for FBC, where FISH was found to be positive in 9.3% of the IHC 0/1+ cases (102).

Interestingly, we observed that among the 10 *HER2* amplified cases by FISH (excluding IHC 2+), only four were IHC 3+, representing a positive agreement of only 40%, whereas all 73 cases not amplified by FISH were IHC-negative. Despite IHC showing a 100% positive predictive value for amplification by FISH, we found that it has very low sensitivity (40%), meaning that most *HER2* amplified cases by FISH were IHC-negative. Therefore, although the Kappa Cohen's test demonstrated a moderate agreement between techniques, the overall moderate agreement in fact masks the low sensitivity of IHC to identify the cases with HER2 amplification due to the relatively low frequency of *HER2* amplification in MBC as compared with FBC. Our findings are supported by the data of Nilsson and co-workers, who evaluated HER2 status by IHC and SISH in a MBC cohort and found out that of nine *HER2* amplified tumours by SISH, only three were IHC 3+ (33.3%) and six were IHC 0/1+ (66.7%) (6). These findings in MBC are in stark contrast with has

been reported for FBC, in which the majority of FISH-positive cases were IHC 3+ (84.7%-97.9%) (72, 87, 88, 100).

The reason(s) underlying the HER2 IHC-negative/FISH-positive discrepancies, both in MBC and FBC, are not clear. Dowsett *et al.* proposed that a possible explanation for that discrepancy could be an artifact of the IHC technique, due to the loss of epitope during fixation of the tissue (72). There are rabbit monoclonal antibodies that present high affinity to the HER2 epitope, binding to the ECD of the receptor (108) and polyclonal antibodies that bind to the ICD of the receptor. In our cohort, we used two different anti-HER2 antibodies, depending on the year of diagnosis. Antibody A0485, which binds to the ICD of HER2, was used until 2015 and antibody 4B5, which binds to the ECD of HER2, was used thereafter. In three out of six discrepant cases, scored before 2015, IHC was repeated with the currently used antibody (4B5), and no differences in the score were found. If we considered that there was a loss of epitope during fixation, HER2 overexpression should be detected by the A0485 antibody, since it binds to the ICD. Since this antibody is no longer available in our institution, it was not possible to confirm this hypothesis in the three more recent cases and the low number of cases analysed by both antibodies makes it difficult to make a conclusion. Several mono and polyclonal antibodies, with different sensitivities and specificities, are commercially available for IHC. There is a need of a compromise between sensitivity and positive predictive value to elect the most accurate antibody for detection of HER2 protein expression. The use of one antibody instead of another could in theory lead to differences in the results. Besides the choice of the antibody, there are other possible explanations for discrepancies in IHC scoring, like the different staining techniques, the time it takes for the sample to be submerged in formalin, the duration of fixation or the use of inappropriate fasteners (108).

The discrepancy between FISH and IHC could also be explained by the fact that the FISH probe covers a large genomic segment of chromosome 17q11-12 and not only the *HER2* gene. Therefore, a FISH-positive result may happen when only a part of the HER2 probe covering other gene(s) present in the amplicon is amplified, as proposed by Luoh and colleagues (68). In FBC, the *HER2* amplicon (17q12-21) is very well studied and it is known that other genes beyond *HER2* can be co-amplified and have an impact in the phenotype and clinical characteristics of the tumour (109). Genomic instability is more frequent in *HER2*-positive than in *HER2*-negative tumours (110) and carcinogenesis is not only dependent on the primary oncogene of the amplicon, but also on other genes that are co-amplified (111). However, there is a lack of studies about the *HER2* amplicon in MBC.

This notwithstanding, Nilsson *et al.* proposed that a lack of correlation between protein expression and gene amplification in MBC may be real (6). We suggested that a possible explanation could be that in those tumour cells the *HER2* activation occurs by the proteolytic

cleavage of the ECD (*HER2* shedding), which forms a truncated membrane-associated fragment with tyrosine kinase activity (89) that leads to phosphorylation of the tyrosine in the cytoplasmatic domain of the receptor (72). Having in consideration the lower frequency of *HER2* amplification and the worse sensitivity of IHC for its detection in MBC as compared to FBC, one might speculate that there are in fact biological differences of the disease arising in the two genders and that there is a need for a new MBC-specific classification, such as that proposed by Johansson *et al.* (83).

V.3. Possible Therapeutic Strategies

Trastuzumab is an HER2 downregulator with proved success in the treatment of FBC (9). Information about the use of trastuzumab in MBC is restricted due to the smaller number of HER2-positive cases. Some case reports described the benefit of the use of trastuzumab and chemotherapy in metastatic MBC (112–114). By extrapolating from the success of adjuvant therapy in FBC and also based on the results obtained in metastatic MBC, it seems that HER2 may have a role also in MBC treatment and the use of anti-HER2 therapy could be a wise option in the adjuvant setting (9, 112–114).

As previously discussed, there are some theories that could explain the significant discrepancies observed between IHC and FISH in MBC. If the epitope was lost during fixation but the receptor is intact *in vivo*, the protein would not be detected in IHC but trastuzumab could still be efficient, due to the presence of the binding site. Although the use of trastuzumab in combination with adjuvant chemotherapy did not improve disease-free survival in FBC patients with low levels of HER2 expression (IHC 1+ and 2+) or that were FISH-negative (115), the probability of not responding to trastuzumab was higher when gene amplification was present with normal protein expression than when there was HER2 overexpression without gene amplification. Furthermore, a linear dose-effect was not observed between the level of *HER2* amplification and trastuzumab response (116). Thus, if there is a lack of the ECD *in vivo*, treatment with trastuzumab, which binds to the ECD of the HER2, may in fact not be beneficial due to the absence of the binding site.

If the above is true, it would be important to search for an alternative targeted therapy, such as a small molecule targeting the catalytic domain instead of the antibody targeting the ECD of *HER2*. Lapatinib binds reversibly to the ICD of the receptor, instead of binding to the juxta membrane portion of its ECD as it happens with trastuzumab (70, 74), and showed modest clinical activity in trastuzumab-resistant patients, (117, 118). On the other hand, Sánchez-Muñoz *et al.* found that only one out of seven tumours, in a cohort of MBC classified as HER2-enriched by PAM50, was HER2-positive by IHC and CISH, with the remaining cases being negative by both techniques (81). Data from the *Cancer Genome*

Atlas proposed that the activation of the HER2/EGFR signalling pathway is higher when both HER2-positive by IHC and HER2-enriched gene expression by PAM50 are present, and may benefit the most from anti-HER2 therapy (119). Prat *et al.*, in a retrospective analysis from a phase 3 clinical trial, observed a benefit from lapatinib therapies in a small group of metastatic FBC HER2-negative by IHC but HER2-enriched by PAM50 (81, 120). Information provided by gene expression profiling may lead to less misclassification of the tumours than the use of immunohistochemical markers, although the genetic landscape of MBC is not yet completely characterized (81).

V.4. Clinicopathological Association with Amplified *HER2*

In our cohort there was no association between *HER2* amplification and the clinicopathological variables considered to be associated with poor prognosis in males, such as the nodal involvement or the tumour size, as observed in females. Rudlowski and co-workers observed the same lack of association in males and hypothesised that these distinctions could be due to differences in pathogenesis and biological behaviour in males (60). However, due to the low number of *HER2*-positive patients in our cohort, and in MBC in general, data should be interpreted with caution.

V.5. Overall Survival

Males present more often characteristics related with better prognosis, such as positive hormonal receptors and lower *HER2* amplification. However, they present higher morbidity and mortality when compared with females (9, 46).

In our cohort we did not find significant differences in the overall survival between the patients with a concordant HER2 status and the patients with a discordant HER2 status. Survival was expected to be different between groups, since only the concordant cases patients were eligible for anti-HER2 targeted therapy by routine IHC testing first. Although the absence of survival differences between the two groups could be due to a different role of the *HER2* in MBC, Sánchez-Muñoz *et al.* observed that HER2-enriched tumours by PAM50 presented a worse outcome (81). On the other hand, the small number of patients studied, combined with the differences in the treatment that may have occurred in the thirty-year period, including the fact that trastuzumab was only approved as adjuvant therapy in 2006, might explain by themselves the lack of correlation in the overall survival.

VI. Conclusions

The main conclusions of this study are:

- In our cohort, *HER2* was amplified in 15.3% of MBC patients.
- We observed a moderate overall correlation between IHC and FISH in the determination of the HER2 status, with a significant proportion of cases with amplification by FISH not showing overexpression by IHC.
- We did not find a correlation between the HER2 status and clinicopathological characteristics of the patients.
- MBC is a biologically different entity from FBC, with *HER2* amplification being present in a lower proportion and with higher discrepancy between FISH and IHC.
- It might be important to test the HER2 status by both IHC and FISH in all MBC at diagnosis in order to identify the patients with amplification without overexpression and enrol them in clinical trials to optimize their treatment.

VII. Future Perspectives

Many questions remain about the impact of *HER2* in the prognosis and treatment of MBC. In order to better understand the biology of MBC, we plan to:

- To increase the size of the MBC cohort for *HER2* amplification analysis.
- To determine the gene expression patterns with PAM50 in all the FISH *HER2*-positive MBC (concordant and discordant with IHC) and to compare them with a similar series of FBC.
- To compare the status of the signalling pathway dependent on HER2 activation in all the FISH *HER2*-positive MBC (concordant and discordant with IHC) and to compare them with a similar series of FBC.

VIII. References

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