Biological Characterisation of *HER2* amplified breast cancer

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ABSTRACT

Breast carcinoma is the most frequent type of cancer affecting women. Among the recently described molecular and phenotypic classes of breast cancer, human epidermal growth factor receptor 2 (HER2)-positive tumours are associated with a poor prognosis. HER2 status is currently assessed in routine breast cancer reporting using immunohistochemistry (IHC) in addition to *in situ* hybridisation (ISH) in borderline cases. The ability of *HER*2 gene status to predict response to targeted therapy (Trastuzumab) is well documented. However, prognostic information provided by IHC expression categories and prognostic value added by using ISH in borderline cases remains unclear. HER2 plays an important role in cancer progression being targeted to provide predictive and prognostic information. Moreover, HER2 is related to cancer resistance against a variety of therapies; however, trastuzumab has proved successful in treatment of this subgroup. Nevertheless, patients may acquire resistance to this drug after a period of treatment, which indicates that other molecular mechanisms might influence success of this therapy. Dimerisation between members of the HER family may contribute to resistance against treatments due to different combinations that trigger different downstream pathways. This is promoted by ligands, which are expressed as transmembrane precursor protein molecules and have a conserved epidermal growth factor-like domain. Through resistance to trastuzumab, other drugs are being developed to interact in different domains of HER2 protein. The study of interaction between receptors/ligands will characterise specifically their signalling pathway and understand which strategy to acquire.

The main aim of this thesis was to assesses the status of HER2 protein (IHC), HER2 gene (chromogenic ISH) and HER heterodimers (in situ proximity ligation assay (PLA)), including HER2/EGFR, HER2/HER3 and HER2/HER4, in two BC series prepared in tissue microarray format; a series of consecutive primary operable BC cases (n = 1858) including HER2+ trastuzumab naïve cases (TrN, n = 221), the second series of HER2+ trastuzumab adjuvant treated cases (TrT, n = 143). Therefore determining the biological characterisation of these biomarkers by associating against clinicopathological parameters, survival outcome and understand the trastuzumab therapy value.

There was excellent overall concordance between HercepTest negative (scores 0/1+) and positive (3+) with CISH positive/negative (defined as *HER2*/Chr17 copy number ratio of ≥ 2; $p < 0.001$). Kaplan-Meier analysis for breast cancer specific survival (BCSS) and disease free interval (DFI) revealed statistically significant differences between HER2 positive and negative cases detected by HercepTest and CISH ($p < 0.001$). Interestingly, it was identified that HercepTest 2+ non-amplified cases were not significantly different with those amplified 2+ or 3+ cases with respect to their behaviour (BCSS and DFI).

The results revealed an inverse association between the HER heterodimerisation status and hormone receptor status ($p < 0.001$), and a significantly worse outcome amongst cases revealing high levels of all heterodimers ($p < 0.001$). Among ER+ cases, the heterodimers high levels were significantly associated with worse prognosis ($p < 0.001$) overall.

However amongst the two HER2+ populations dimerisation status did not show an association with patient outcome.

The overall concordance between HercepTest and CISH analysis for HER2 status was excellent. All HercepTest 2+ cases identified were observed to have poor outcomes similar to those HercepTest 3+ cases regardless of gene amplification status. In the current clinical environment cases exhibiting IHC 2+ and non-amplified gene HER2 status will not be offered targeted HER2 therapy but do exhibit aggressive clinical behavioural characteristics. Even though those patients with high levels of the HER2 truncated form, p95HER2, have shown poor outcome, this biomarker does not reveal any extra findings comparing with the HER2 expression results. Beside HER2, EGFR is the only monomer that reveals prognostic value amongst the breast cancer patients. Tumours exhibiting high levels of HER heterodimerisation have an adverse prognosis, however in the context of HER2+ breast cancer no association with clinical outcome was observed regardless of use of trastuzumab treatment. HER2/HER3 heterodimerisation status assessed in multivariate analyses has shown that this protein-protein interaction is associated with a poor prognostic outcome, which needs further investigation and assessment of clinical utility to use in the future in breast cancer treatment decision. Further quantification analysis of dimer/ligand complex using PLA of other HER family members may be useful to identify subset of patients associated with distinct outcome, response to treatment and relationships with HER signalling related biomarkers.

PUBLICATIONS

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ABBREVIATIONS

ADCC	Antibody Dependent Cell Cytotoxicity
Ang-1	Angiopoietin-1
AP-1	Activator Protein 1
AREG	Amphiregulin
ASCO	American Society of Clinical Oncology
ASK1	Apoptosis Signal Regulating Kinase 1
ATP	Adenosine Triphosphate
Bcl-2	B Cell Lymphoma 2
BCSS	Breast Cancer Specific Survival
BP	Basal Phenotype
BRCA1	Breast Cancer 1
BTC	Betacellulin
CAF	Cyclophosphamide, Adriamycin and Fluorouracil
CAP	College of American Pathologists
CDK	Cyclin Dependent Kinase
CGH	Comparative Genomic Hybridisation
CISH	Chromogenic *in situ* Hybridisation
CK	Cytokeratin
CMF	Cyclophosphamide containing regime
CTFs	Carboxy Terminal Fragments
DC	Dendritic Cell
DFI	Disease Free Interval
DNA	Deoxyribonucleic Acid
ECD	Ectodomain

EDTA	Ethylene Diamine Tetra-acitic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EPI	Epigen
EPR	Epiregulin
ER	Oestrogen Receptor
FDA	Food and Drug Administration
FISH	Fluorescent *in situ* Hybridisation
FFPE	Formalin Fixed Paraffin Embedded
GFP	Green Fluorescent Protein
HB-EGF	Heparin Binding EGF-like Growth Factor
HER	Human Epidermal Growth Factor Receptor
IL	Interleukin
µm	Micrometre
MAPK	Mitogen Activated Protein Kinase
mRNA	messenger Ribonucleic Acid
mTORC2	mammalian Target of Rapamycin Complex 2
MUC	Mucin
NF-**κ**B	Nuclear Factor **κ**B
NK cell	Natural Killer cell
NPI	Nottingham prognostic index
NRG	Neuregulin
p17	Polysomy 17
PAI-1	Plasminogen Activator Inhibitor 1
PI3K	Phosphatidylinositol 3-kinases

PIP2	Phosphatidylinositol 4,5 – biphosphate
PIP3	Phosphatidylinositol 3,4,5 – triphosphate
PLA	*in situ* Proximity Ligation Assay
PgR	Progesterone Receptor
PLC-γ1	Phospholipase C-γ1
PTEN	Phosphatase and Tensin Homolog
ROI	Region of Interest
RTK	Receptor Tyrosine Kinase
shRNA	Short Hairpin Ribonucleic Acid
STATs	Signal Transducer and Activation of Transcription
TBS	Tris-Buffered Saline
TGF-α	Transforming Growth Factor – alpha
TKI	Tyrosine Kinase
TKIs	Tyrosine Kinase Inhibitors
TMA	Tissue Microarray
TN	Triple Negative
TNF	Tumour Necrosis Factor
TSP-1	Thrombospondin 1
VEGF	Vascular Endothelial Growth Factor

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1. CHAPTER 1: Introduction

1.1 Cancer

Throughout history cancer has been present in human nature. Egyptians first described the disease, even though the word cancer was not used, in approximately 3000BC. Several manuscripts were found revealing some cases of tumours, including breast, and was described as a non-treatable disease. Carcinos, which refers to the Latin word 'crab', or in ancient Greek 'cancer', was the term firstly described by Hippocrates (460-370 BC) as an ulcer-forming tumour.

Nowadays, in developing countries cancer is the second most common cause of death and the first in developed countries. Different factors as smoking, pollution, sedentary lifestyle and western food style together with population aging have been described as the mains factors for the rise of this disease[1]. In 2008 it was estimated there were nearly 12.7 million cancer cases worldwide, and 7.6 million deaths caused by cancer[2]. Males have been affected more by lung cancer with 17% of the total new cancer cases and 23% of the total cancer deaths. In developing countries lung cancer affects 11% of women. Though this disease mostly affects the world's developed countries for both genders, the cancer mortality percentage is comparable with developing countries. This fact might be related to an earlier diagnostic stage and access to the latest and innovative treatments in the developed countries. Additionally, preventive measures like tobacco control or vaccination to avoid cervical and liver

carcinomas and promotion of physical exercise together with healthier diet behaviours are being applied in these countries[3].

Of the 26 different types of cancer, breast carcinoma is the most common in women with approximately 1.38 million new cases worldwide, which corresponds to 23% of the total cases and 14% of cancer death[3]. During the last decade of the twentieth century cervical cancer was the most common type of cancer in developing countries, however in 2008 breast cancer became more common amongst women with 50% of the breast cancer cases and 60% of the deaths worldwide[3].

Breast carcinomas are heterogeneous with a variable prognosis depending on different histological characteristics and molecular characteristics, which influence patient prognosis and tumour behaviour[4]. The central interest was always to evaluate and differentiate breast cancer patients to understand the different prognosis. In order to score the breast carcinomas, tumour size, tumour stage and histological grade are the three main variables used to calculate the Nottingham Prognostic Index (NPI)[5]. NPI offered a more completed scoring method to predict the survival time amongst the patients carrying this disease[6]. This system has been amongst others currently used to decide which adjuvant treatments are appropriate[7,8]. However this histologic scientific method, based on Scarff-Bloom Richardson system, was questioned due to supposed inconsistency[9]. For breast cancer prognosis, the method developed by Bloom and Richardson involves microscopic analysis of tubule formation, mitosis frequency and nuclear pleomorphism[10]. Even though NPI

was seriously questioned, different studies were performed and validated this approach[11,12] and has been adopted by all Europe[13] and USA[14]. In the last two decades of the last Century, breast cancer research focussed on biomarkers documentation and understanding. Techniques such as immunohistochemistry made possible this mode of research by using highly specific antibodies to react against certain antigens, such as hormone receptors (HR), epidermal growth factor receptors (EGFR), tumour suppressor genes, angiogenic and adhesion factors, matrix metalloproteinases amongst others, some revealing prognostic applicability[15].

The vast quantity of genes that are involved in cancer cell differentiation, development, and death made it possible to understand the complexity and heterogeneity of the cellular and molecular structures. Scientific studies took place to understand these genetic discrepancies. The use of cDNA microarrays related the tumour genes and their phenotypic distinctions providing a great potential to a more descriptive taxonomy of carcinomas[16,17]. Molecular biology establishment made possible a new approach for cancer research, without which it would not be possible to describe the mechanisms involved in cancer cells biology. Understanding the mode of cancer cells development is constantly being improved by different discoveries developed on molecular biology and genetics field. Nowadays the approach for cancer, either for diagnostic or research purposes, using molecular tumour morphology analysis is made possible by the characterisation of at least four breast cancer groups allowing treatment discrimination. The cancer cases were separated into two main groups and well defined. One group revealing low or absent expression of

Oestrogen receptor (ER) and the second group involving ER gene high expression. Two distinct subgroups are described amongst the ER positive cases, Luminal A with the highest expression for ER as well as GATA-3 and Progesterone receptor (PgR) and Luminal B that reveal low to moderate expression of ER, PgR and might reveal high expression of Ki67. Patients expressing ER revealed better outcome, especially Luminal A. The two subgroups that do not express ER are the basal-like tumours or also named as triple-negative, which do not express ER, PgR or human epidermal growth factor receptor 2 (HER2) but high expression of cytokeratins (CK) revealing the worse prognosis, and the HER2 subgroup revealing no expression of ER or PgR but high expression of several genes localised in the amplicon HER2, which includes HER2 and GRB7. The outcome for this subgroup is amongst the worst in breast cancer[17].

New genomic analysis technologies applied on the study of human genome and more particular tumour tissues, like comparative genomic hybridisation (CGH), made it possible to divulge mutated regions of the chromosomes specifically present in breast carcinomas[18] including oncogenes as *HER2* amongst others[19]. Lately by genome analysis of 2000 breast cancer tumours, revealed a cluster of 10 discriminative groups with distinct genetic features. Furthermore each group was specifically correlated with a different prognosis even though further studies are required to start implementing this taxonomy in clinical treatments discrimination[20].

1.2 General introduction to HER family: Type 1 Tyrosine Kinase Growth Receptors

1.2.1 Epidermal Growth Factor Receptor Family

Human epidermal growth factor receptor (EGFR or HER) family is composed of four different types: HER1 (EGFR or c-erbB-1), HER2 (neu, p185 or c-erbB-2), HER3 (c-erbB-3) and HER4 (c-erbB-4) (Figure 1.1)[21]. These four molecules compile type I group of 20 families of Receptor Tyrosine Kinases (RTKs) and regulate several cellular metabolic reactions[22,23]. This RTK family has an important role in cell proliferation, differentiation, adhesion, survival and migration. The organisation of these molecules permits a contact from side to side of the plasma membrane. Furthermore, this communication encourages gene expression, provoking a reaction by the cell to external stimulus performing intracellular docking sites for signalling complexes. These complexes are then released into the cytoplasm for activation of several signal transduction cascades[24]. RTKs can function as controllers of cellular progress, however they may influence the development and improvement of different types of carcinomas[25] being present in 30% of the epithelial cancers[26]. The entire group of 20 RTK families share some structural relationships consisting of extracellular and intracellular segments connected by a transmembrane fragment[27], showing high functional and structural identities[28]. Amongst the HER family, the similarity of these molecules ranges from 53%, involving EGFR and HER3, up to 64% between EGFR and HER2. More specifically the most conserved sequence is located on the tyrosine kinase domain with a range of 59-81% of identity and the C-terminal sequence showing a poor similarity of between 12-30%[29].

HER proteins are composed of a highly conserved ectodomain (ECD) or extracellular binding region where the ligand fragment interacts and directs molecular structure alteration, promoting receptor dimerisation, enhancing kinase activity[30]. Four domains named L1, S1 (CR1), L2 and S2 (CR2), also known by domain I, II, III and IV, respectively, compose the ECD[31]. Domains I and III interact with the respective ligand[32]. Additionally the domains II and IV, which the whole HER group shares, are composed by two high reactive cysteine-rich domains[25] and interact to each other and with the cell membrane[27]. The transmembrane hydrophobic segment is composed of 20-30 amino acids molecules[33], with special attention for five residues included in the α-helices that is critical on dimerisation reaction[34]. It has critical role in dimerisation where a mutation on valine amino acid is enough to result in a dramatic transformation[35], i.e., originating a dimer with constant activation[36]. Forces of van der Waals[37] and hydrogen bonds[38] are also significantly involved in the dimerisation reaction comprising the α-helices. An intracellular tyrosine kinase domain compose the remain structure of the entire complex[27]. This last domain exhibits an extended C-terminal tail with high levels of conservation comprising the ATP-linking position for receptor autophosphorylation and phosphorylation of respective substrates[25]. This fragment of the receptor structure is the most conserved possessing a motif GlyX GlyX XGlyX (15-20) lys, which is the linkage location for ATP[39]. Finally the carboxy terminal tail (including in the intracellular domain), possesses tyrosine residues that are the phosphorylation spots, which react under ligand-monomer/monomer interactions[40]. However, within the HER family it is possible to register some exceptions, such as HER3 not possessing intramembranous kinase activity and

HER2 missing a ligand binding site[24]. However these receptors may cooperate with each other to form active heterodimers and trigger downstream pathways that can lead to cell development[41].

Within the RTK group, the HER family is the only able to achieve dimerisation triggering their own stimulation. The general RTK families drive dimerisation by a kinase transphosphorylation through a covalent stimulation that will trigger certain downstream pathways until exhaustion of the phosphatases. Nonetheless HER monomers are activated in a allosteric manner, which the kinase domain from the first monomer interacts with the kinase domain of the second monomer triggering downstream pathways with no need for additional phosphorylation reactions[42]. This approach builds a whole reaction system that increases the activation specificity of the downstream signalling. Consequently the great variety of arrangements that the HER family possess to form dimers, either homodimers or heterodimers, will deliver a diversity of distinctive purpose[43].

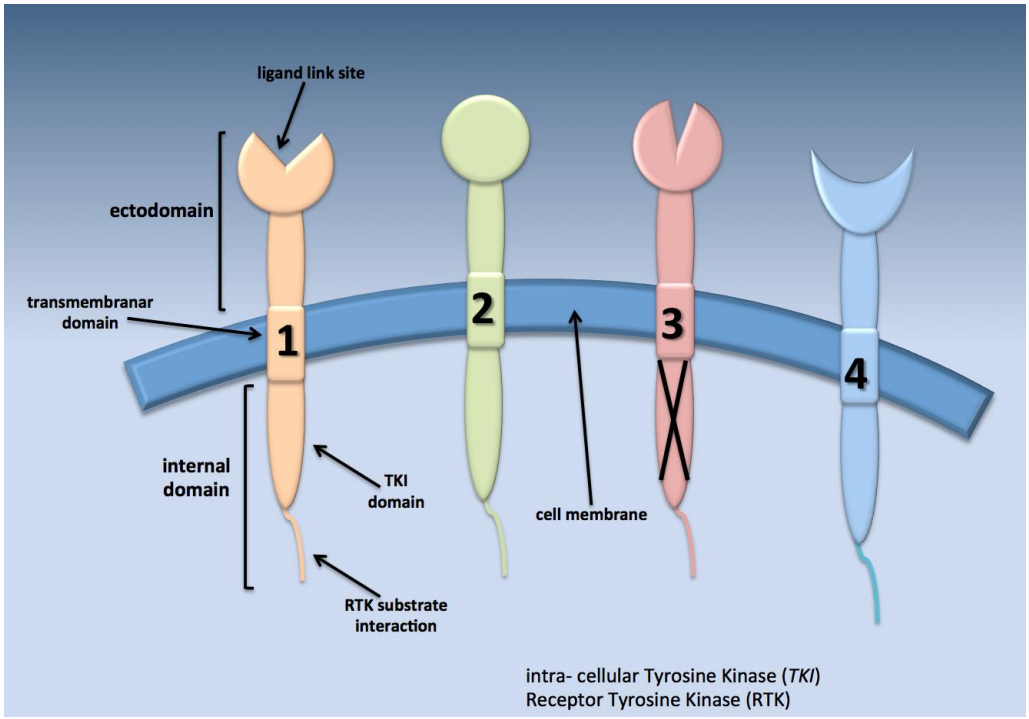

Figure 1.1– The four members of HER family is represented showing some differences and similarities amongst them. HER2 (2) is the only member of this family that does not possess any ligand-binding site on its ectodomain. On the other hand, HER3 (3) show some lack of tyrosine kinase (TKI) region located onto the internal domain. EGFR (1) and HER4 (4), contain both, ligand link site and TKI. The receptor tyrosine kinase domain is a conserved region present in all family members, except HER3, which is dependent of the other members to form a dimer and trigger downstream pathways.

1.2.1.1 HER2 Receptor

HER2 is the member amongst its RTK family more intensively studied in breast cancer and other solid cancers due to it being a crucial systematic tool in a clinical perspective for prognostic analysis and treatment target.

Firstly identified by Schechter et al 1984[44], HER2 is a transmembrane 185kDa protein[44] and its gene is located on chromosome 17q21[45]. The HER2 protein frequency in breast epithelial cells can be between 20,000-50,000 per cell however carcinoma cells might express in excess of 2,000,000 receptors per cell[46]. Overexpression of HER2 is also associated with poor prognosis in ovarian[47], stomach[48] and uterus carcinoma with worse prognosis amongst the uterine serous papillary carcinoma[49]. Also a mutation at codon 655 of the *HER2* gene modifies the structure of the receptor transmembrane domain resulting in an active form, promoting development of HER2 homodimers[50]. Cells that either possess this mutation on the *HER2* gene or overexpress HER2 protein have a high possibility to show HER2 homodimerisation resulting in continuous uncontrolled growth and division, and apoptosis avoidance[21]. This is further supported by the fact that this genetic mutation is associated with a higher risk of breast carcinoma[51].

Approximately 10%-34% of breast tumours possess cells that show amplification of *HER2* and overexpression of HER2, which is associated with reduced overall survival[21,52-54]. It is also involved in driving malignant growth of breast cancer cells, i.e., HER2 protein is greatly linked with metastatic occurrences[55] and with an aggressive phenotype[56]. Additionally approximately

40% of the inflammatory breast carcinomas might reveal HER2 overexpression[57]. A residual number of cases (1-2%) show HER2 protein overexpression without gene amplification[58]. This occurrence may take place due to modification in system of control of gene expression[59]. Amplification of *HER2* results in an overexpression of the protein that might affect the activity of this receptor characterised either by ligand-independent dimerisation, where HER2 reacts by itself, or it dimerises with any other receptor of the same family[60]. HER2 does not include an ectodomain where a ligand can connect, but is able to act as a co-receptor with high affinity for receptors of the same family, thus forming heterodimers[25]. Within RTKs Type I group, HER2 is the most favourable molecule to interact with the others due to extend ligand/heterodimer linkage and therefore prolonging activation of the mitogen-activated protein kinase (MAPK) pathway[21,61,62] relating these cases with high biological activity. Additionally HER2 does not interact with any ligand, as its conformation arm is constantly predisposed to exist in a competent form and ready to connect with any of the other monomers from the same family[63].

HER2 overexpression can make available some mitogenic and anti-apoptotic signals[64]**,** more specifically it can promote different signalling reactions downstream, such as proliferation when RAS-MAPK pathway is activated, inhibition of apoptosis by phosphatidylinositol 3-kinase (PI3K)/Akt pathway[24] and promotion of angiogenic reaction by production of vascular endothelial growth factor (VEGF)[65]. More specifically it encourages PI3K/Akt signalling cascade promoting cell cycle and survival[66] due to high presence of cyclin D1 (Cdk1) and inhibition of p27. Cdk1 is a critical cyclin-dependent kinase in

G1/S phase shift, being a regulator of cell cycle. This cyclin was distinguished as it is upregulated in HER2 overexpressing cells and moreover showing reduced activity when HER2 expression is blocked, decreasing tumour growth due to cells being detained in G1 stage[67]. The molecule p27, which inhibits *Cdk2* gene that is related to cell cycle progression, is arrested by high levels c-Myc and D-cyclins when cells show a HER2 overexpression[68,69]. Therefore HER2 overexpression can affect cell cycle by arresting Cdk2 activity and promoting p21 phosphorylation[70]. PI3K/Akt pathway, stimulated by HER2 overexpression, can inhibit cell apoptosis by interaction of p21 phosphorylated with apoptosis-signal-regulating kinase 1 (ASK1), blocking its activity[25,71]. Additionally HER2 overexpressing breast cancer cells showed an association with high levels of Ki67 expression. This might explain the high proliferative phenotype of these types of cancer cells and a link between HER2 gene and MKI67 gene upregulation[72]. Others have reported this relationship using immunohistochemistry assays revealing an association of Ki67 and HER2 protein intensities[73,74].

1.2.1.2 **HER2 *Gene***

HER2 positive (HER2+) breast carcinomas do not follow a linear approach due to a range of abnormalities present in the genome. Moreover, not only *HER2* is amplified others, including *MYC*, *FGFR1*, *ZNF217* or *CCND1* might be present at high levels of amplification[75]. Therefore it is possible to think that HER2+ breast cancers are biologically heterogeneous. This is exemplified by the population of HER2+ tumours can be separated by positive or negative ER expression[76].

Through the DNA microarray technique, Bertucci *et al* attempted to distinguish gene amplifications between HER2+ and HER2- cohorts to demonstrate any discrepancies. Considering the HER2+ cases, an additional 29 genes were identified to be overexpressed. The origin of these overexpressions might not be the same for all genes, being either a co-amplification due to close proximity to *HER2*, or regulation of these genes by identical factors that stimulate HER2 expression, or even genes regulated by *HER2* itself. On a distance lower than one megabase from *HER2* gene, on chromosome 17,q12, six genes were identified including *GRB7*[72,77], which is also involved in gastric[78], oesophageal[79], testicular[80] and breast carcinoma[81]. The aberrant expression of these genes might explain the different outcomes to trastuzumab treatments. *GRB7* is involved in cell migration dependent on integrins[77] and PPARBP that is involved in downregulation of p53 and consequently a decrease in apoptosis[82]. However genes localised in different regions of the same chromosome might be affected by *HER2* amplification indirectly, i.e., these genes activated only by HER2 overexpression and not by *HER2* amplification itself such as CD41, CD61, CD31 and MAP2K6[72]. Furthermore genes, such as CDH15 (M-Cadherin) and GATA4, not present on chromosome 17 might be amplified in HER2 positive cancer cells[72]. On the other hand, genes located in close proximity to *HER2* and therefore co-amplified, might not overexpress their respective proteins due to possess different members on their transcription mechanism such as its promoters or repressors[72]. This indicates that even though a gene is amplified, is not the only factor to incite overexpression.

1.3 Limitations of the current classification system

In the last years significant important innovations were achieved not only to prevent breast but in diagnosis and therapy progress. Also research made significant progresses on detection of potential biomarkers and genetic abnormalities to characterise new types of breast cancers[20]. Nowadays the major difficulty is to understand whether a patient will benefit from a specific treatment strategy or not. In breast cancer treatment only a small number of targets are accessible, like trastuzumab and endocrine therapies that are used depending on which receptor is identified as the target[83]. Though, different groups have questioned HER2 classification methods. HER2 gene amplification analysis is not accurate and the selection of patients might not be correct.

Chromosome 17 polysomy (p17) is characterised by a minimum of one or more copies of the chromosome than observed in normal diploid cells. Polysomy is associated with only moderate overexpression of HER2 which suggests a diminutive control of this polysomy on *HER2* gene expression in breast cancer development[84]. This polysomy control is null in HER2-negative cases[85]. It is also possible to frequently observe a high copy number of chromosome 17 centromere (CEP17) in the lack of *HER2* gene amplification[86]. Those cases holding p17 and treated only with chemotherapy revealed a higher benefit when compared with breast cancer patients with no p17. However both groups possess similar disease free interval (DFI) (3-5 years) when submitted to trastuzumab, which might reveal that p17 does not predict if patients either benefit or not from this antibody therapy[87]. Patients that exhibit p17 are not

candidates for adjuvant trastuzumab therapy except those showing strong HER2 overexpression [88,89]. By the use of microarray-based CGH technique Marchio et al was able to suggest that p17 is an extremely uncommon incident in breast carcinoma cases, showing that only one case proved to be polysomic by CGH among 18 fluorescent *in situ* hybridisation (FISH) polysomic cases. Moreover CGH results showed two among 12 samples with FISH polysomy revealing *HER2* amplification ratio lower than 2. Therefore although these patients might benefit from trastuzumab therapy they are denied to such treatment[90]. On the other hand, patients that possess *HER2* amplification are denied to trastuzumab treatment because of high number of CEP17 due to gain or amplification of this centromere and consequently a ratio HER2/CEP17 < 2[91]. It might be more precise placing into consideration only the mean number of *HER2* signals ignoring the number of CEP17[90]. Being suggested that above 6 *HER2* signals as the mean cut-off to be considered to characterise a patient as *HER2* gene amplified and be able to benefit from trastuzumab or alternative HER2 mark therapy[90].

Studies developed to this point are more directed to gene amplification or protein levels of each HER receptor. However some protein analysis relate HER3 expression with poor survival outcome[92,93], others relate its expression with good prognosis[94,95]. Still not very clear if analysis of DNA, mRNA or protein levels of these receptors is the best way to assess in order to define the patients outcome[96,97]. Some studies even suggest the evaluation of co-expression of different members of HER family[98,99]. However the study of not only coexpression but the interaction of these receptors, like dimerisation,

stimulated by different ligands might be a way to assess this complex problem and provide some answers. Interaction amongst this family is so complex and diverse that cancer cells expansion might be more dependent on this variability of processes than with each receptor individually.

1.4 HER2 and hormone receptors

1.4.1 Crosstalk - ER, PgR and HER2

Nearly 65% of primary breast carcinomas possess positive levels of ER and tamoxifen was the first permitted drug to be applied in treatments where ER is the target. This drug competes with oestrogen to bind with ER and therefore represses the high transcriptional action of ER. Subsequently ER positive breast cancer cells lose their proliferative phenotype, which is successful in approximately 60% of the ER positive tumours[100]. Aromatase inhibitors and other anti-steroidal drugs are also effective against ER positive carcinomas[101]. Some ER positive cases however demonstrate resistance to endocrine therapy and others develop resistance after a certain period of time on therapy[102]. The mechanisms of resistance are not entirely understood. Nonetheless crosstalk between the ER/HER family are involved in mechanisms of resistance in patients with breast carcinoma when submitted to endocrine treatments[103-105].

Dimerisation between HER family members triggers several downstream pathways as a consequence of phosphorylation of kinases, resulting in anti-apoptotic encouragement and enhancement of cell proliferation or even cell migration[106-108]. PI3K/Akt or MAPK might be involved in the activation of

these responses; however they can be also implicated in the activation of ER or even its co-regulators. This way ER is able to develop its cellular outcomes, which means higher stimulation on tumour development and endocrine treatment resistance in those cells showing high expression of HER2 and ER[105,109-111].

ER expression is primarily located in the nucleus, where it acts as a transcriptional regulator for several genes. Its structure is composed of a ligand linkage site, transcription activator sites and a DNA binding site in order to cooperate with promoters of the target genes[112,113]. The target genes transcription include the recruitment of AIB1 (SRC3), an ER coactivator[114]. On the other hand the structure of ER is highly reactive with tamoxifen, which stimulates the enrolment of corepressors and therefore the transcription of the target genes becomes compromised[105,115]. Depending on the tissue involved, tamoxifen can possess both antagonist and agonist roles, which might indicate that in different tissues different corepressors and coactivators are involved. In case of those cells holding high levels of coactivators, as AIB1 or SRC1, tamoxifen acts as an agonist[115].

ER can act as a coactivator of proteins such as cyclin D1, involved on cell cycle, insulin-like growth factor receptor (IGFR), PgR, transforming growth factor alpha (TGF-α) and VEGF. This mode of "tactic" from ER on cell biology is named as ER genomic mediated mechanism[116]. Additionally ER might be located in the cytoplasm of breast tumour cells and in the membrane,

where it is able to react with Src, EGFR and HER2, known as ER non-genomic mediated mechanism (Figure 1.2)[105,117,118].

When tamoxifen is applied in cancer cells expressing high levels of ER, AIB1 and HER2, it works as an agonist resulting in tumour development. However if this tumour suffers estrogen deficiency this development is compromised. Therefore aromatase inhibitors, which deprive the cell of oestrogen, indicate to posses a more efficient effect on ER/HER2 positive cancer cells[119,120]. Moreover, crosstalk between ER and HER has been demonstrated via the EGFR/HER2 downstream pathways, MAPK, is compromised if the concentration of ER drops significantly, but is enhanced in case of tamoxifen or oestrogen levels increase[105].

Supporting Tyrosine Kinase Inhibitors (TKIs), such as gefinitib, on cancer cells expressing ER, HER2 and AIB1, prevents the phosphorylation of the HERs changing the effects mediated by oestrogen and tamoxifen. Hence is possible to understand that the HERs are able to influence the outcome of oestrogen and tamoxifen due to a crosstalk occurrence[105]. Also MAPK when activated is able to phosphorylate AIB1, which enhances the agonist effect of tamoxifen. Therefore the overexpression of HER2 and dimerisation with EGFR stimulates downstream pathway MAPK with tumour development as a consequence of the presence of tamoxifen[109]. A different study using MCF7/HER2-18 cells (expressing ER, AIB1 and HER2) revealed high levels of AIB1 activity when submitted to heregulin, oestrogen or tamoxifen. However if gefinitib is applied, this AIB1 phosphorylation is prevented,

confirming crosstalk amongst ER and HER2 occurs[121]. When submitted to both gefinitib and tamoxifen, AIB1 activation is repressed, the antagonist effect of tamoxifen is observed and moreover the tumour cells development inhibited[105].

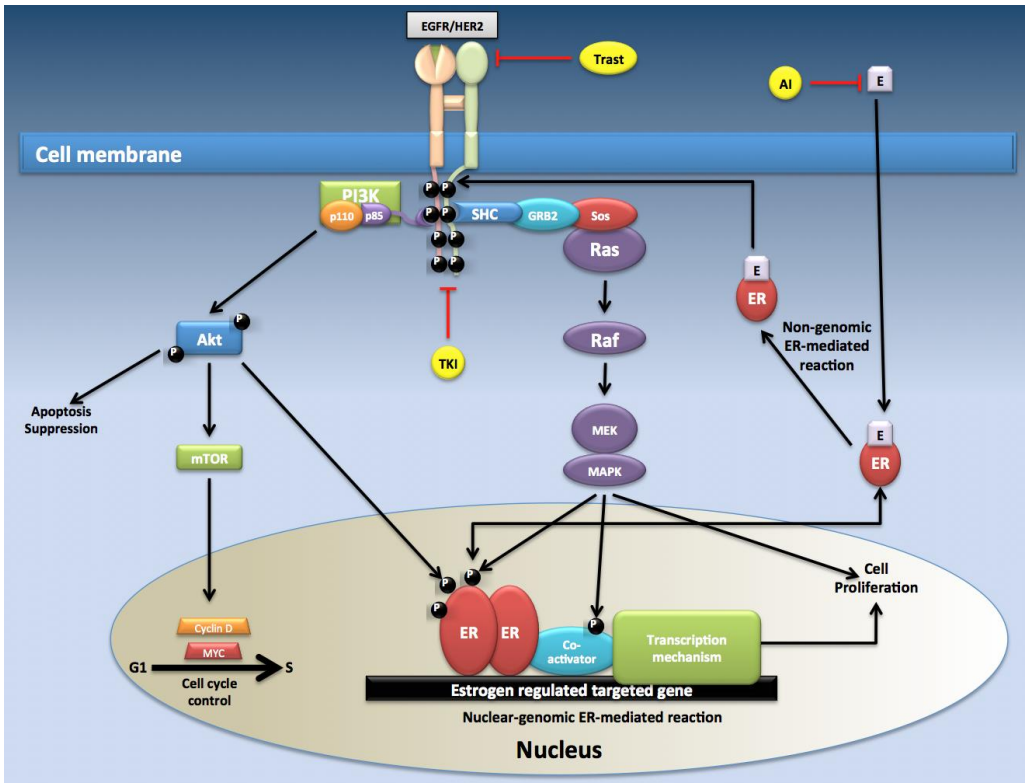

Figure 1.2 – The crosstalk HER2/ER and its effects on breast cancer cells biology, understanding at the same time the significance of respective transduction pathways. Also different certain drug elements used in cancer therapy are represented in this diagram, including trastuzumab (Trast), aromatase inhibitors (AI) and tyrosine kinase inhibitors (TKI). Two ER main pathways are represented, the non-genomic ER mediated reaction – intervened by the hormone oestrogen (E) – and the nuclear-genomic ER mediated reaction.

1.5 HER2 breast cancer therapy

1.5.1 Trastuzumab (Herceptin[tm]; Genentech; South San Francisco, Ca)

Though the expression of HER2 is inversely associated with ER/PgR status[122], those tumours revealing both ER and HER2 positivity tend to be resistant to tamoxifen therapy[120] and moreover when tamoxifen is applied alone results in a negative effect on patients prognosis[123]. Adjuvant C chemotherapy cocktail that combines Cyclophosphamide, Adriamycin and Fluorouracil (CAF), showed that this adverse effect is not observed comparing with only tamoxifen[124]. Additionally, aromatase inhibitors, like letrozole, possess a greater reaction than tamoxifen in both ER+/EGFR+ and ER+/HER2+[119]. Regimes including anthracycline revealed a correlation of improved therapy frequency and HER2 positivity[125]. When Cyclophosphamide containing regimens (CMF)[126] and taxanes[127] are used as adjuvant chemotherapy on HER2 positive carcinomas it revealed lack of effectiveness. However later studies contradict this statement when concerning the taxane as adjuvant therapy[128]. Finally those HER2+ cases submitted to paclitaxel following adjuvant chemotherapy exhibit promising results independently of ER status[129]. Regarding all these studies, it is possible to understand that HER2 has prognostic value even though for some chemotherapy administrations it remains ineffective. Therefore HER2 targeted therapy is the most effective strategy or in combination with the most effective chemotherapy and/or in combination with endocrine therapy.

HER2 positive breast tumours commonly exhibit numerous genomic abnormalities, however this type of carcinoma is strictly conditioned on HER2

expression and its downstream pathways to develop and survive. Therefore targeting this receptor is an appropriate strategy in order to treat patients with HER2+ cancer. Numerous strategies are currently in development not only to react with HER2 itself in several of its domains, either extra or intracellular, but also some of its downstream pathways elements.

Trastuzumab is already approved by US Food and Drug Administration (FDA), and is applied in those HER2 overexpressing breast cancer patients; it acts on the HER2 domain IV avoiding the formation of a heterodimer or homodimer[56]. This drug was initially developed from a mouse monoclonal antibody, which reacts with HER2. This receptor is not itself a tumour specific antigen, but when expressed 10 to 100 times above normal level it becomes a target of trastuzumab. This attribute is useful when the purpose is to target only cancer cells and therefore this discrimination may contribute for success of trastuzumab in cancer treatments. The mouse constant region does not react as an antigen to provoke a reaction by human immune system. Additionally the use of this mouse antibody in humans would cause an anaphylactic shock therefore the constant region from the anti-mouse antibody (HAMA) was "humanised", resulting in a chimerical molecule - anti-HER2 MoAb (Figure 1.3)[56]. From clinical trials, it is conclusive that trastuzumab has a significant effect on patients that diagnosed with tumours showing high intensity staining of HER2 protein (HercepTest 3+) or a high gene amplification (≥2) using FISH method[130] or chromogenic *in situ* hybridisation (CISH)[131] (scoring system explained in methods chapter). However, patients showing moderate protein expression (HercepTest 2+) do not show a response to treatment if no gene

amplification was additionally present[132]. These cases revealing weak overexpression are not necessarily correlated with *HER2* gene amplification but with p17[84]. When compared with chemotherapy alone or use of trastuzumab plus chemotherapy, the disease-free survival and the overall survival of HER2+ patients can increase 20%[133].

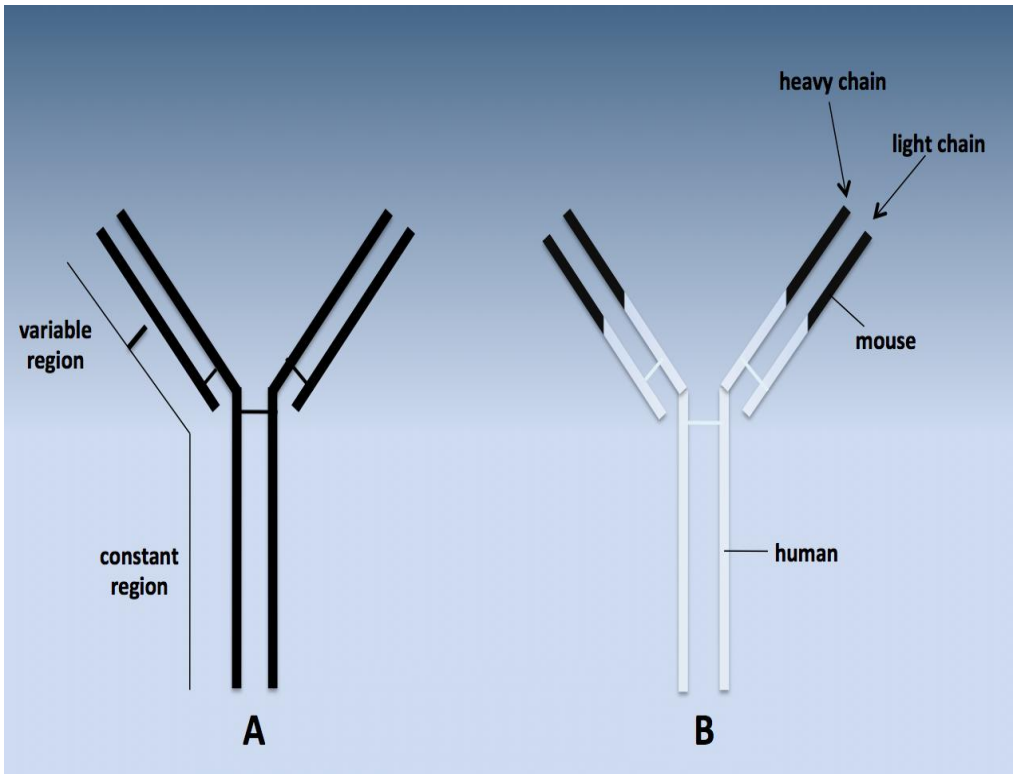

Figure 1.3 – Humanised monoclonal antibody. Anticancer monoclonal antibodies (MoAbs) produced in mice invoke an immune response in human patients due to antigenic constant regions of the heavy and light chains (black) (**A**). Therefore, the mouse MoAb was humanised by designing non-immunogenic heavy and light chains resulting in a chimeric antibody (**B**).

1.5.2 Trastuzumab role in breast cancer treatment

The role of this therapeutic antibody could be separated into two main mechanisms as stimulation of the immunologic structure mediating an anti-tumour reaction or inhibition of HER family downstream pathways. However the mechanism of action of trastuzumab is still not completely understood, but it has been suggested that internalisation of the complex trastuzumab/HER2 and degradation of the respective receptor occurs[134,135]. Trastuzumab/HER2 interaction is characterised by internalisation and denaturation of the receptor[135,136]. Other groups suggest that the PI3K/Akt pathway is triggered by HER2/HER3 heterodimer, which is affected, and downregulated by trastuzumab in cells with HER2 overexpression[137,138].

1.5.2.1 Immune system

When trastuzumab is given to patients, the immune system is activated by recruiting the effector cells responsible for detection of the antibodies and as a consequence results in a cytotoxic response[139]. Trastuzumab promotes apoptosis of cancer cells by antibody-dependent cellular cytotoxicity (ADCC)[140] and the immune system responds to this stimulus provoked by trastuzumab. Hence the natural killer (NK) cells may develop the key role for this cytotoxic reaction. These white blood cells possess an Fc gamma receptor which interacts with the Fc domain on trastuzumab, resulting in NK-mediated cell lysis[141]. However in cancer patients in an advanced stage with an immune system immunosuppressed, the levels of cytotoxicity reactions are very low[142,143].

A modification of trastuzumab is showing promising results *in vitro* on enhancing ADCC when compared against the original and patented therapeutic antibody. The transformation of the ordinary trastuzumab molecule consists on elimination of the fucose (defucosylation) from the oligosaccharides linked to the heavy chain of Asn297[144]. These results were confirmed by in vivo where an improved median progression free survival is observed in preclinical models of HER2+ breast carcinomas[145]. The use of vaccines targeting the HER2 ectodomain is a promising strategy as these will decrease significantly any functional autoimmune reaction and destruction of the normal tissue will be limited[146].

1.5.2.2 Inhibition of angiogenesis

Several pro-angiogenic factors are expressed in breast carcinomas to promote development of new blood vessels to provide more nutrients and oxygen that consequently encourage tumour growth. However, Izumi Y et al demonstrated that trastuzumab can act as an anti-angiogenic drug[147]. The vessel development becomes compromised and therefore rapid expansion of the tumour does not take place. In addition the evolution to metastasis is reduced due to a lower migration by endothelial cells. When combined with chemotherapy, trastuzumab is even more effective in preventing angiogenesis[148]. Moreover, it has a double effect reducing the expression of pro-angiogenic factors VEGF[149], TGFα[150], Angiopoietin-1 (Ang-1)[151] and Plasminogen-Activator Inhibitor-1 (PAI-1)[152], and enhancing anti-angiogenic factors as Thrombospondin-1 (TSP-1)[153].

1.5.2.3 Induction of cell cycle arrest and apoptosis

The tumour volume of HER2+ breast tumours can decrease enormously when submitted to trastuzumab therapy due to enhancement of apoptosis cells[154]. It induces apoptosis and cell cycle arrest due to dramatically reducing the mitogenic signalling downstream pathways including PI3k and MAPK[155,156]. The cycle arrest of the cells treated with trastuzumab occurs through G1 stage of the cell cycle[156] and the levels of $p27^{KIP1}$ also increase inhibiting cell cycle development[157]. The expression of gene $p27^{KIP1}$ produces a cell cycle inhibitor functional protein, which interacts with different classes of cyclin dependent kinase (Cdk) molecules inhibiting the termination of the cell cycle (Figure 1.4)[158].

in vitro studies performed with only trastuzumab suggest that the levels of apoptosis are barely affected[159,160]. However when this antibody is combined with some chemotherapy agents these levels of apoptosis increase and the cell growth inhibition can vary among 20%-90%[161].

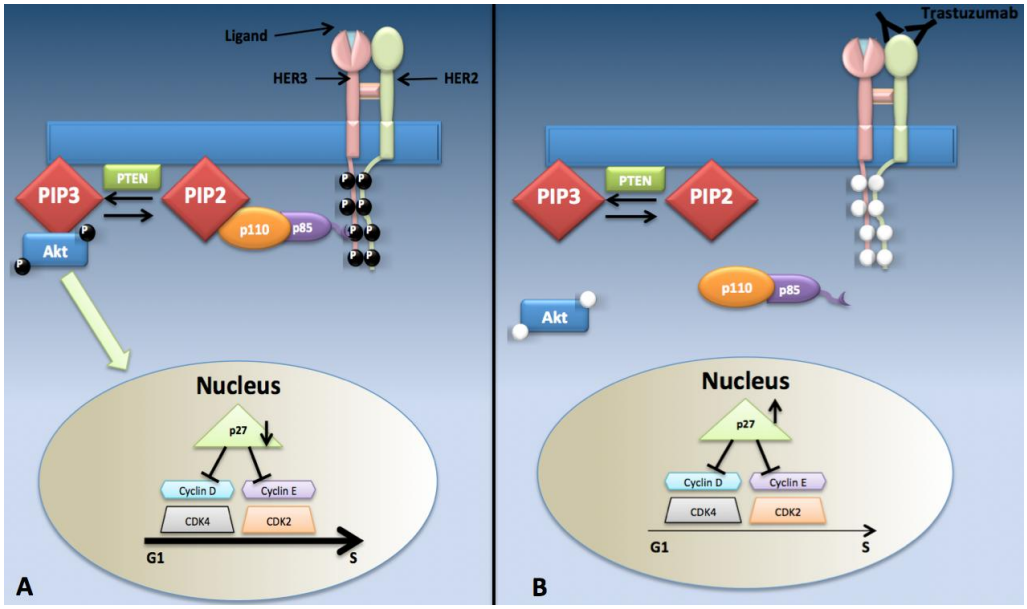

Figure 1.4 – The mechanism suggested of trastuzumab against HER2 membranal protein. By linkage of the monoclonal antibody on HER2 monomer the whole complex HER2/HER3 dimer/PI3K is interrupted and consequently the levels of p27 increase due to its decrease of degradation. By increase of the P27 levels, cell cycle arrest takes place.

1.5.2.4 Other mechanisms of action

Although it remains unclear, trastuzumab appears to downregulate HER2 by promoting its internalisation and denaturation[155,156]. However it trastuzumab does not have any influence on HER2 endocytosis but when recycled the complex trastuzumab/HER2 receptor, an inactive complex, returns to cell surface[162,163]. Also trastuzumab in combination with chemotherapy can block DNA repair as demonstrated through a study using HER2 overexpressing breast cancer cell lines BT-474 and SK-BR-3, which suffer high incidence of DNA breaks in a different approach of other cell lines[164]. Finally, a study performed with the same breast cancer cell lines by Molina et al[165] suggested that trastuzumab may obstruct the HER2 (p185) ectodomain cleavage avoiding development of the HER2 active form - $p95^{HER2}$, a truncated membrane bound fragment related with occurrence of lymph node metastasis[166].

One active effect by trastuzumab was reported to be the inhibition of HER3/HER2 and HER3/p85 dimerisation that consequently downgraded the levels of PI3K and Akt pressure for cell survival[159]. Ritter CA et al confirmed this when the appliance of gefinitib reduced the levels of p85 and HER3 association inhibiting the activation of Akt[167]. Moreover there is dependency of PI3K pathway on HER3 phosphorylation.

1.6 Mechanism of resistance to trastuzumab

1.6.1 Overview

Although trastuzumab is used for therapies against HER2 metastatic breast cancer cases, employment of this drug on its own demonstrated to be successful in only up to 35% of these cases[168]. However when combined with first-line chemotherapy, the success of therapy can range 50%-84%[169,170]. The efficacy of trastuzumab when applied either as a single agent or in combination with chemotherapy can be temporary due this development of resistance. This suggests that other mechanisms are involved in cancer development, which are independent of this therapy[171]. Those cases submitted to trastuzumab alone or chemotherapy alone revealed a disease progression to progression of 4.6 – 8.3 months against 7.4 – 14.2 months for those receiving chemotherapy plus trastuzumab and a median survival time of 20.3 – 22.2 months against 25.1 – 32.2 months, respectively[169].

The unsuccessful results may be caused by failure of trastuzumab to block the dimerisation when high levels of ligands are present[172]. It is not clear what contributes for trastuzumab resistance, however some additional mechanisms may be involved and influence the final therapy results.

1.6.1.1 Truncated HER2 (p95^{HER2})

Since trastuzumab links to the ectodomain of HER2 receptor, it was proposed the possible mechanism of resistance involving p95^{HER2}. This molecule does not possess the external domain and therefore trastuzumab does not attach to

$p95^{HER2}$ and promote its degradation. Moreover a proteolytic cleavage of the ectodomain results in a generation of a phosphorylated tyrosine kinase HER2 $p95^{HER2}$ [165,166]. This molecule constantly activated might trigger downstream pathways improving the influence of the cancer cells and moreover higher presence of this molecule in node positive cases comparing with the negative cases[173]. Within HER2 overexpression breast cancer cell lines the presence of heregulin, $p95^{HER2}$ promotes HER3 transphosphorylation which is not inhibited by trastuzumab, however with lapatinib the transphosphorylation reaction is repressed[174]. Confirming this, breast cancer cells transfected with either HER2 or $p95^{HER2}$, showed sensitivity to trastuzumab and lapatinib in those transfected with HER2, however the cells transfected with $p95^{HER2}$ were resistant to trastuzumab but not to lapatinib[175]. Some mechanisms of resistance are associated with $p95^{HER2}$ as the expansion of downstream pathways activated from other members of HER family and consequently PI3K activation, or also IGFR might also over react[176]. The incidence of PI3K over activation can reach 70% of these breast carcinomas[177], reducing the efficacy of trastuzumab and lapatinib[176,178]. The heterodimer HER2/HER3 is a PI3K activator, however the dimer resulting from $p95^{HER2}$ is more operative when compared with the full length HER2[179]. It was additionally reported that TGFα levels increase too as a consequence of $p95^{HER2}$ presence[179].

1.6.1.2 PI3K/Akt pathway & PTEN expression

In order to achieve a phosphorylated Akt the membrane associated phosphatidylinositol 4,5 – biphosphate ($PtdIns(4,5)P_2$ or PIP2) is converted, by result of HER2/HER3 heterodimerisation, into Phosphatidylinositol 3,4,5 –

triphosphate (PIP3)[180]. The PI3k/Akt pathway, highly present in HER2 overexpressing cancer cells, stimulates inhibition of cell cycle arrest and/or an anti-apoptotic incident[159]. In contrast Phosphatase and tensin homolog (PTEN) is an important anti-tumour gene that is highly correlated with trastuzumab. Its protein is the phosphatase with the important role of converting the reactive PIP3 to PIP2, being a natural antagonist of PI3K activation[180]. Furthermore, cancer cells expressing small amounts of PTEN exhibit high activity of PI3K and therefore develop resistance to trastuzumab. The use of PI3K inhibitors is a possible strategy in order to achieve higher sensitivity to trastuzumab[181]. It was shown that those cell lines showing HER2 positivity and no PTEN expression develop some resistance to trastuzumab, however the combination of trastuzumab with a PI3K inhibitor (LY294002) the cells did not survive[178]. The molecular mechanisms of trastuzumab identified comprise the inhibition of PI3k and Akt pathways due to HER2 ectodomain being blocked and the impossibility of cleavage[159,165]. PTEN is a tumour suppressor gene on chromosome 10 that, either through mutations or haploinsufficiency, is not expressed in standard levels within half of breast cancer cases[182,183]. As a phosphatase it reacts with PI3, dephosphorylating it on position D3 with the purpose of recruiting Akt[184]. PTEN influences PI3k to promote the production of PI3 and Akt activity[185], inducing apoptosis and cell cycle arrest.

On the other hand, the tyrosine kinase Src, possesses an important role as a pro-oncogenic factor and when associated with HER2 it becomes reactive and inactivates the main role of PTEN [181,186]. Therefore when trastuzumab interacts with HER2 in the same domain as Src, PTEN can perform its tumour

suppressive functions, which contributes for trastuzumab anti-cancer progression [181]. When the interaction of this oncogene with HER2 is blocked by trastuzumab, PTEN can contribute against the uncontrolled and high-proliferated cancer cells. The lack of PTEN results in resistance to trastuzumab in HER2 overexpressing patients resulting in poor prognosis. Absence or low expression of PTEN is a great indicator of trastuzumab resistance [181].

1.6.1.3 EGFR involved in trastuzumab resistance

EGFR might confer a trastuzumab resistance phenotype when it is expressed in high levels in HER2+ cell lines[187]. Furthermore this resistance is conducted by either the presence of EGFR/EGFR homodimers or EGFR/HER2 heterodimers, which both trigger several downstream pathways including MAPK and AKT[187]. The levels of HER2/EGFR reduced the trastuzumab therapeutic effects and it is in part responsible for the trastuzumab resistance acquired by tumour cells[167]. Moreover expression of HER ligands like EGF, TGFα, betacellulin, and heregulin has also been associated with trastuzumab resistance[172,188,189]. Cells directed to overexpress TGFα developed high levels of resistance to trastuzumab. Therefore TGFα might be significantly involved in trastuzumab resistance due to its levels being increased dramatically comparing pre- and post-therapy periods in breast cancer patients[190].

Long periods of exposure to trastuzumab encourage EGFR expression creating resistance to this monoclonal antibody however further anti-EGFR agents, such as gefinitib or cetuximab further increase sensitivity[191]. However lapatinib showed to be more effective due to obstruction of cell proliferation despite

EGFR expression, demonstrating independency of lapatinib on EGFR range[192]. Moreover lapatinib demonstrated to be more effective in antagonising Akt activation. MAPK phosphorylation is not significantly affected by lapatinib, which suggests this pathway is a compensation for the lack of Akt phosphorylation[187,193]. Additionally two studies have implied that the presence of lapatinib enhances HER2 levels while trastuzumab has an opposite effect[194]. Therefore trastuzumab resistance might be partly explained by EGFR/EGFR homodimerisation, which is present in high levels when cell lines were submitted to trastuzumab, which indicates a direct association between EGFR phosphorylation and trastuzumab resistance[187].

HER2/EGFR heterodimer levels are affected in different ways by the presence of lapatinib, increasing with low levels of lapatinib, however showing an opposite effect for high concentrations of this drug[187,193].

1.6.1.4 IGF-1R involved in Trastuzumab Resistance

The main PI3K upstream triggering involves another RTK, which is not part of the HER family – Insulin-like growth factor (IGF-1R), however might be associated with some of the HER family reactions. Cells that overexpress both HER2 and IGF-1R reveal high levels of resistance against trastuzumab. Even though addition of insulin-like factor binding protein-3 (a-IR3) supplementing the trastuzumab treatment, it significantly reduces the resistance of these specific cell lines (MCF-7/HER2-18). Also IGF-1R is able to trigger downstream pathways increasing the concentrations of SKP2, which interacts

with p27, reducing its levels and consequently leading to cell cycle progression[195,196].

1.6.1.6 HER3 involved in treatment resistance

HER3 being completely dependent on any other member of HER family, the cancer therapies were not significantly directed on HER3 role but on the same family members. There is some evidence that some HER2+ tumours associate HER3 as the responsible for trastuzumab resistance[197]. However, trastuzumab does not disturb HER2/HER3 downstream cascades in HER2 amplified breast cancers[175,198]. Moreover the use of tyrosine kinase inhibitors as an alternative approach shows that is not effective as desired due to short time ability on restriction of the catalytic activity of the receptors[199-201]. Notwithstanding that the therapeutic strategies are driven towards HER2 overexpression, these inhibitors block HER2/HER3 dimerisation action for just a few hours and thus is unsuccessful on suppression of HER3 activation and with a final consequence on maintain PI3K/Akt downstream pathway operational, promoting proliferation and survival[202].

HER2/HER3 heterodimerisation activity may be inhibited in order to inactivate HER2 metabolic role. However this requires high concentrations of TKIs that would not be supportable *in vivo*[203]. HER3 plays a critical role in protection of HER2/HER3 dimerisation function against TKIs action due to a high range of different mechanisms that are able to regulate HER3 expression and signalling. In fact HER3 levels are increased as a compensatory response in HER2 amplified breast cancer cells with the presence of TKIs[202]. More precisely the

increase in HER3 levels is stimulated by the dramatic decrease of Akt signalling[203]. HER3 signalling is stimulated by different mechanisms to increase of its expression, translation expansion, which involves proliferation of the raptor complex of mTor[204]. Additionally the half-life can be extended by HER3 avoiding dephosphorylation through alteration of HER family anti-regulators such as NRDP1 or LRIG1[205]. Finally, HER3 expression can be affected by microRNA, e.g. miR205, which reduces HER3 activity and reinstates the initial sensitivity to TKIs in HER2 positive cancer cells [206] .

1.7 Trastuzumab toxicity and alternative therapies

1.7.1 Overview

Exceptionally, rare cases of heart disease have been reported where trastuzumab is used in cancer treatments. This type of reaction, although rare, has a higher frequency when trastuzumab is used in combination with other therapeutic compounds such as paclitaxel or docetaxel[132,207,208]. These cardiac effects are not understood, but it is thought to cause dilated cardiomyopathy[209-212]. HER-2 is highly expressed in cardiac cells to avoid apoptosis and consequently increase cell survival to avoid cardiac dysfunction[213]. It promotes synthesis of several proteins such as nuclear factor κB (NF-κB), which is involved in control of proteins during cells stress and activator-protein-1 (AP-1) that controls proteins responsible for the progress of cardiac hypertrophy[214]. Other secondary effects can emerge when trastuzumab is used for cancer treatment such as myelosuppression, nausea, emesis and hypersensitivity, however these reactions can be prevented using antihistamines, anti-inflammatory drugs and corticosteroids[215].

1.7.1.1 Trastuzumab-DM1 (TDM1)

New strategies to improve trastuzumab effectiveness are being addressed by linking a cytotoxic agent in order to intensify and discriminate the target cells increasing their cytotoxicity. Thus, this new molecular complex composed by trastuzumab, the cytotoxic element and a connector forms trastuzumab-DM1 (T-DM1)[216]. T-DM1 is an antagonist of tubulin polymerisation originated from maytansine and MCC linker, which links trastuzumab to DM1, composes the connector. Some studies already showed some effectiveness of this molecule in HER2+ cell lines resistant to trastuzumab alone[217]. This complex is chemically very stable and shows positive results with the addition of low toxicity levels amongst the patients during phase I study[218]. The action resides on its cytotoxicity role only when it is internalised by the cell once the linkage against HER2 occurs. In case this reaction does not occur, T-DM1 will stay inactive and therefore not affect the whole system[216]. The higher concentration level for this drug tolerated by patients is limited to 3.6mg/kg every 3 weeks. However tiredness, sickness, anaemia and high levels of liver enzymes occurred in patients submitted to T-DM1[219].

1.7.1.2 Pertuzumab

A new generation of monoclonal antibodies developed to inhibit HER dimerisation are currently being developed. Pertuzumab, like trastuzumab, blocks the interaction between HERs family members, preventing heterodimerisation of HER2 with HER3 or EGFR [220], even though HER2/HER3 heterodimer is the main target[221]. However the difference

between pertuzumab and trastuzumab is regarding the locale of interaction in the HER2 ectodomain. While trastuzumab binds on epitope in domain IV, pertuzumab reacts with domains I, II and III[222,223]. It is possible to conclude that this drug could be valuable in trastuzumab resistant cases and use of pertuzumab in combination with trastuzumab could be a good strategy due to their different target epitopes on HER2 ectodomain. However it has been reported that pertuzumab does not have an additional benefit[224]. Even though in the presence of high levels of heregulin, the heterodimer HER2/HER3 is not disturbed by trastuzumab, however pertuzumab is capable to disrupt this heterodimer in similar conditions[225].

Trastuzumab and pertuzumab act on the extra-membrane domain of the receptor preventing ligand interaction or cleavage of this domain creating the oncoprotein $p95^{HER2}$. Therefore an alternative strategy may reside on use of small molecules that compete for ATP preventing phosphorylation and consequently activation of TK domains in the cytoplasm. Thus, the main objective of the treatments is the use of antibodies that interact directly with the external domain of the receptors, although small interfering molecules could be used to disrupt the PI3k pathway[226]. Among these small molecules gefitinib, erlotinib and lapatinib are used in this type of therapy[171]. Several studies demonstrated that lapatinib presented successful results where trastuzumab did not have any effect, being suggested to use it with trastuzumab treatment[227].

1.7.1.3 Small Molecules or tyrosine kinase inhibitors (TKIs)

As referred above, the consequence of ligand/receptor linkage is the formation of either HER homodimer or heterodimers developing an allosteric activation of certain tyrosine residues[24], which triggers several downstream pathways responsible for survival as PI3K or proliferation via MAPK[228,229].

Other strategies targeting HER2+ breast cancer are being developed particularly surrounding the use of tyrosine kinase inhibitors (TKIs). These molecules compete against ATP to bind on HER family catalytic site, reducing downstream pathways activation[230]. Amongst the group of TKIs there are irreversible inhibitors, e.g. canertinib (Pfizer, Groton, USA)[231] and reversible inhibitors, e.g. lapatinib (GlaxoSmithKline, King of Prussia, USA)[230]. Although these inhibitors are HER family specific, the majority possess an unselective approach. Amongst a high number of these agents that have been tested to inhibit tyrosine kinases, lapatinib is the most specific small molecule against EGFR and HER2 and extra two kinases STK10 and SLK. The small molecule EKB-569 reacts against 56 kinases amongst 113 examined, which included non-HER family targets[232]. Carnetinib reacted positively against 36 kinases even though reported to be a specific inhibitor of EGFR, HER2 and HER4[232]. Although these small molecule inhibitors reveal promise, the lack of specificity may cause high levels of toxicity. Additionally in some HER2 positive breast carcinomas is not appropriate to target all the HER family. This argument is supported by those reporting HER4 correlates with better survival outcome and therefore should not be inhibited[233]. Unlike monoclonal antibodies such as trastuzumab, TKIs only reduces levels of phosphorylation

but do not limit the number of HER2 receptors in tumour cells[174,230]. Lapatinib disturbs both HER2 and EGFR phosphorylation a month after the first application[234]. Additionally the levels of pErk1/2, pAkt and cyclin D decreased, promoting cancer cell apoptosis[234]. Another factor that plays an important role on patient's prognosis is survivin, which is an inhibitor of apoptosis and downregulated when HER2 amplified breast cancer patients are submitted to lapatinib[235].

Trastuzumab therapies might result in some toxicity including in the cardio system, however lapatinib demonstrated to involve lower risks on this level[236]. Other small molecules, provoke a stronger EGFR signalling inhibition, the frequency of toxicity increased considerably being characterised by diarrhoea and skin rash[237,238].

Lapatinib has been demonstrated to be the most promising small molecule to be applied in HER2+ breast cancer therapy where a partial reaction is observed when submitted to lapatinib[238].

Currently, several strategies are being addressed to increase the levels of therapy success, which include the combination of lapatinib with trastuzumab, aromatases inhibitors, anti-oestrogens and taxanes. These combinations might reveal some advantages due to complementation of each other. HER2 positive breast cancer patients when submitted to trastuzumab possess better survival outcome, however the frequency of metastases localised in central nervous system is high[239]. The reason is due to the high molecular weight of

trastuzumab, which is unable to pass the blood brain barrier and therefore is not able to act on metastatic cells. Small molecules are able to permeate this barrier and act on brain metastasis[239]. Additionally lapatinib may complement trastuzumab therapy against the mechanisms of resistance originated during trastuzumab monotherapy. IGF-1R is linked to trastuzumab resistance amongst the HER2+ breast cancers, however it does not affect lapatinib influence improving the patients outcome[240]. The lack of PTEN also reveals as a mechanism of resistance for trastuzumab[241], as lapatinib is not dependent on PTEN, and is therefore not affected by this factor[242]. Since $p95^{HER2}$ does not possess an ectodomain, trastuzumab is not able to interact in order to disrupt $p95^{HER2}$ uncontrollable action. Therefore lapatinib offers an alternative way to increase the chances of control $p95^{HER2}$ reacting via intracellular domain[174].

In a clinical trial, some patients were submitted to either trastuzumab/lapatinib or lapatinib alone. The combination of both drugs revealed a significant improvement on the risk of disease progression and better survival prognosis[243].

Finally the combination of TKIs with other current therapies might be able to increase the levels of success amongst the HER2 amplified breast cancers. The combination of trastuzumab and lapatinib showed to be more effective against cancer cell lines which survivin activity was inhibited, when compared with the use of any of the drugs singly[244].

1.8 Transmembrane precursor proteins molecules

1.8.1 Overview

All members of HER family, apart from HER2, are activated by a group of ligands, expressed as transmembrane precursor proteins, which possess a conserved epidermal growth factor (EGF)-like domain or neuregulins. This domain is composed by six cysteine residues localised on a region of 40 amino acids[245]. The cysteine residues form disulphide bonds in order to confine the secondary structure into a three loop conformation, which will be vital for their activity and respective specificities [246]. Expressed by 11 different genes[229] the HER ligands group includes EGF, betacellulin (BTC), TGFα, heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPR), amphiregulin (AREG), epigen (EPI) and a subfamily of four neuregulins (NRG), which after splicing reaction originates six different proteins (NRG 1α, 1β, 2α, 2β, 3 and 4)[229,247-249]. All HER family members with exception for HER2 possess on their ectodomain four subdomains that assume an auto-inhibition form through deficiency of ligands[63,250]. Each HER member has specific ligands that they interact with which in turns encourages dimerisation[130]. Some of these ligands only interact with EGFR such as EGF, TGFα and AREG[229]. Other ligands specifically interact either with EGFR or HER4 including HB-EGF, BTC and EPR[251]. NRG 1 and 2 cooperate with both HER3 and HER4, but NRG3 and NRG4 only bind to HER4 (Figure 1.5)[229]. Even though HER4 interacts directly with its respective ligands, recent studies suggested that HER4 when existing as a heterodimer might be able to interact with ligands such as EGF, TGFα, HB-EGF and EPR[248,252].

An interesting study by Révillion et al[247] revealed high levels of ligand expression in 85% of breast carcinoma cases independently of HER2 eminence. Moreover the same group demonstrated that a co-expression occurs between receptors and ligands indicating autocrine or paracrine pathways triggered by ligands on breast cancer cells.

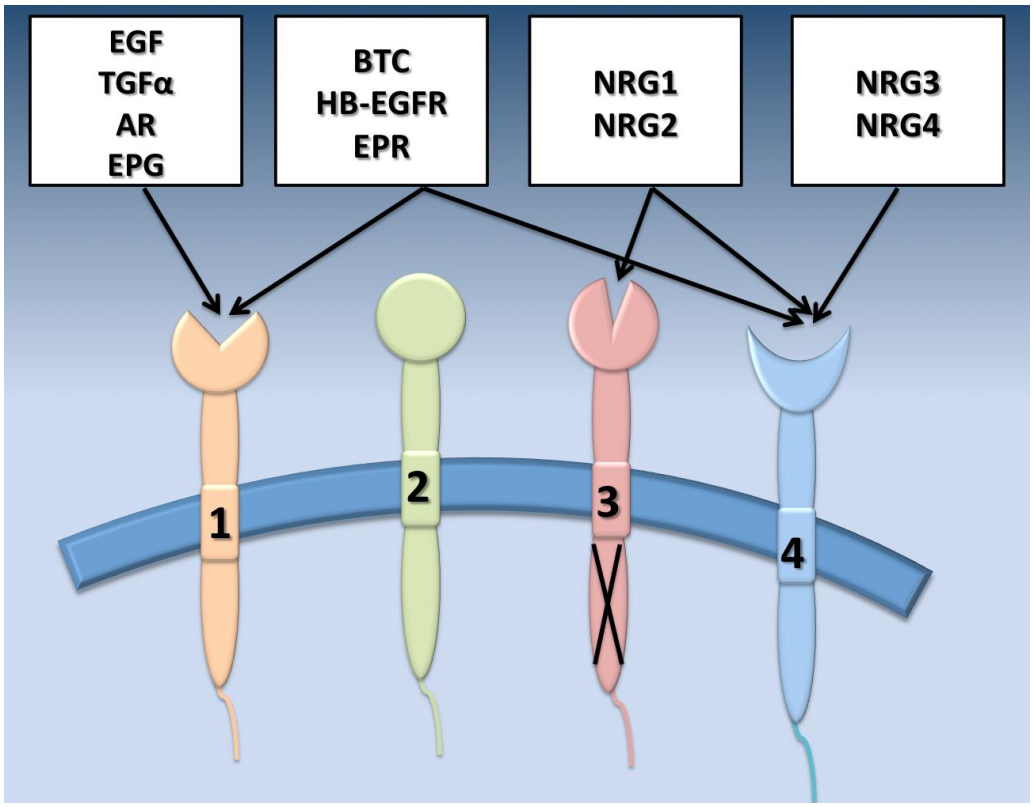

Figure 1.5 – Interaction specificity with Ligands. EGFR interacts with specific ligands as EGF (Epidermal Growth Factor), TGFα (Transforming Growth Factor-Alpha), Epigen (EPG) and AREG (Amphiregulin), which do not bind to any other receptor of HER family. Nevertheless this receptor shares some ligands interaction specificity with HER4, HB-EGF (Heparin-Binding EGF-like Growth Factor) and EPR (Epiregulin). On the other hand HER4 and HER3 interact with both NRG1 (Neuregulin) and NRG2, but NGR3 and NGR4 only create a linkage with HER4. BTC (Betacellulin) is the only ligand able to interact with EGFR, HER3 and HER4 (misrepresented in the diagram for HER3).

1.8.1.1 Epidermal Growth Factor (EGF)

EGF and TGFα were firstly identified due to their effects on inhibition of acid gastric inhibition[253] and a "sarcoma growth factor"[254] respectively. EGF when introduced in neonatal mice, affects eyelid opening tooth eruption[255] and neurobehavioral progress[256]. The protein is expressed by a gene of 110kb located on chromosome 4q25[257] and consists of a 1217 amino acid precursor, which is glycosylated and secreted by adjacent cells[258]. EGF is expressed in a wide range of normal tissues and is highly correlated with promotion of cell proliferation. EGF overexpression occurs in several types of cancer such as breast, liver or pancreas[29]. *In vitro,* proliferation and differentiation of mammary epithelial cells is highly controlled by EGF performing an important role as a mitogenic factor in both normal and malignant cells[259]. EGF, like other growth factors, encourages phosphorylation of tyrosine kinase domain and autokinase action of $p185^{C\text{-}erB\text{-}2}$ that is linked to poor prognostic breast cancers[260]. EGF expression is expressed in approximately 15% - 30% of invasive carcinomas[261,262], revealing a negative association with ER expression but not with tumour size, differentiation or even metastasis development even though it is linked with poor outcome[261]. Additionally overexpression of HER2 increases linkage affinity between EGF and heregulins to their respective receptors due to a reduction of their rates of ligand dissociation[263]. Moreover the reduction or suppression of HER2 diminishes the ligand/receptor connection and for that reason the HER family downstream pathways are not triggered[61].

1.8.1.2 Transforming Growth Factor-alpha (TGFα)

TGFα is composed of a 50 amino acid peptide achieving a total weight of 5.6kDa with an identical secondary structure when compared to EGF including three sulphide bonds[264]. However it shows only 30-40% of homology to EGF in the tertiary conformation but has a similar affinity for EGFR[265] interacting with both the subdomains I and III of the receptor's ectodomain[266,267]. Furthermore EGF and TGFα expression are highly associated with EGFR expression, however when co-expression of EGFR and HER3 occurs, TGFα is the ligand with higher affinity[247]. TGFα is highly expressed throughout gestation and lactation in breast epithelial cells. Likewise in tumour cells, high expression is associated with high tumour grade, recurrence and aggressive outcome[268] and is detected in 40% - 70% of cancer cases[269,270].

1.8.1.3 Heparin-Binding EGF-like Growth Factor (HB-EGF)

Amongst the EGF-like family, HB-EGF was the first to be identified in secretions of macrophages-like cells. Its transmembrane precursor is composed by 208 amino acids including a weight of 14 to 20 kDa[271]. In order to accomplish soluble HB-EGF activation of metalloproteinases has to take place[272]. In fibroblast and smooth muscle cells, HB-EGF holds a higher mitogenic and chemotactic influence when compared with EGF and TGFα[273]. This ligand is able to interact with EGFR[271] promoting cell proliferation and motility and with HER4 the interaction results only in cell motility[274]. Its role is significantly important in gastric[275], bladder[276] and prostate carcinomas[277]. Although HB-EGF expression is highly expressed in breast cancer cells, one study has demonstrated that the levels of expression is reduced dramatically in

those patients with a worse prognosis, large tumour size, elevated cell proliferation and high tumour grade[278]. It can also promote apoptosis avoidance, when anti-cancer treatments are employed or even the immune system attempt to execute destruction of the cell[279]. Moreover this ligand can promote regulation of p21, which inhibits cell cycle[280].

1.8.1.4 Betacellulin (BTC)

BTC is distinguished from other ligands by its unique capacity to trigger heterodimerisation of all the HERs[281]. Its human precursor is composed of 178 amino acids, which is reduced to 80 amino acids with a weight of 32kDa as the active molecule. Similarly to all EGF group, BTC possesses a standard EGF-like domain composed of six cysteine residues[282]. BTC interacts with EGFR however the affinity is ten times lower comparing with the connection of the receptor with EGF, and an additional similarity involves the activation of the heterodimer HER2/HER3 in certain cell lines[283]. mRNA analysis revealed a high expression of BTC in pancreatic tissue, heart and lung of mice[284]. In breast, BTC can stimulate cell growth[285]. Moreover it promotes differentiation of breast epithelial cells to express neutral lipid including droplets[284].

1.8.1.5 Amphiregulin (AREG)

AREG 10.2 kb gene is located on chromosome 4q13, which originates a precursor with molecular weight of 34 – 36 kDa[286]. It only interacts with EGFR[287] and shows a different behaviour when compared with the other ligands due to performing two different functions; either improving some normal or cancer cell lines growth or inhibiting this process in other cancer

cells[288]. Similarly to BTC the interaction with EGFR, which is exclusive for AREG, is characterised by a lower affinity when compared with EGF[288] or even TGFα[289]. However this ligand is highly connected to growth of normal and breast cancer cell lines and some breast primary breast cancer cases, showing superior expression in invasive breast carcinoma[287,290]. Moreover, when AREG expression is suppressed the development of breast tumour is affected and reduction of angiogenesis is also observed[291,292]. Certain correlations of AREG with other biomarkers and clinicopathological parameters remain ambiguous due to discrepancy of observations between different studies. Some studies did not observe any correlation between AREG and ER status, tumour stage, grade and size[293], others found an association with ER levels[290].

1.8.1.6 Epiregulin (EPR)

EPR is a protein mainly expressed by macrophages, placenta and tumour epithelial cells[294]. This protein, with low affinity against the HERs, retains 46 amino acids residues and a molecular weight of 5.4kDa with high levels of similarity with other EGF ligands reaching up to 50%[294]. The gene responsible for EPR expression is located on chromosome 4q13[295]. Amongst breast cancer cell lines this ligand links directly with EGFR and HER4 only[296]. Interestingly EPR promotes inhibition of some tumour cells growth but promotes cell growth amongst fibroblasts[294].

1.8.1.7 Epigen (EPG)

Epigen was the last EGF-like ligand to be documented[297], with a molecular weight of 14.7kDa is expressed by its gene localised on chromosome 4q13.3[298,299]. The EGF-like ligand family is characterised into two different groups, the low affinity group including EPG, EPR and AREG and the high affinity group composed by EGF, TGFα and HB-EGF[297]. However the low affinity group does not possess high influence and is less like to be affected by antagonist mechanisms. If EPG possesses low affinity against its main receptor, the same group reveals an association with high mitogenic features by evading the physiological machinery due to a faint phosphorylation. This way interaction levels with c-Cbl, an ubiquitin enzyme, drops dramatically avoiding downregulation of the growth complex. Finally this ligand was described as possessing specificity to EGFR but also a fragile ability to activate HER3 and HER4[297].

1.8.1.8 Neuregulins (NRGS)

Neuregulins are a diverse family of ligands, expressed by four genes (NRG 1 - 4), which has been highly described due to be present in a high number of cancer tumours. The first of the group to be detected was NRG1 and using the same primers it was possible to detect the second member NRG2[300]. NRG3[300] and NRG4[301] were detected by the use of bioinformatics techniques when the NCBI database was scrutinised. NRG1 gene is located on chromosome 8p12, which expression can undergo splicing resulting in 15 different isoforms. However, NRGα and β are the main isoforms involved specifically in receptor recognition by their c-terminal[248]. NRG2 gene also expresses two isoforms

NRG2α and NRG2β, which possess a distinctive property of recognising and activate HER3 and HER4[302]. NRG3 gene is located in chromosome 10q22 expressing a protein with EGF-like domain 39% similar to NRG1 and the cytoplasmic domain only 12% similar to NRG1 suggesting that NRG3 is not a slice isoform of NRG1[300]. NRG4 is encoded by a gene located on chromosome 15q24[298] and the closest similarity is with NRG3 with 42% of their EGF-like domain, which explains their common and only growth factor target HER4[301].

In breast, they have an important function in development of breast glands[303,304], however they are also expressed in several tissues where they have different functions being involved in improvement of vascular endothelial cells[305], pancreas, muscles[306] and nervous system where different NRG act in different areas of this structure[307]. NRG2 is expressed in neurone system by Schwann cells presenting in high concentrations in synaptic spots[308]. During normal development of breast, all four NRGs are expressed with NRG3 having the most important role[309] and shows the highest expression levels when EGFR and HER4 are co-expressed[247]. Furthermore levels of NRGs are increased in invasive breast cancer[304]. Although the cause remains unknown, the genes responsible for synthesis of NGR1 and NGR3 proteins experience rearrangement in breast cancer cells[310]. These molecules have an important role in breast cell cycle and survival, controlling differentiation of cells, growth and proliferation. NRG1 is also involved on proliferation of several tissues[311] and consequently may have an important function in interaction of ligands and receptors. NRG1 also interacts with HER3 and HER4 stimulating materialisation of the heterodimers alongside HER2. However the mechanisms

involved in this complex reaction(s) remain unclear[312]. The different members of NRG family are present at high concentrations in different incidence, where high levels of NRG2β are found in large breast tumours (> 25 mm) and, along with NRG4, in high-grade tumours. On the contrary, NGR3 is associated with low grade-tumours[311]. Due to NRG family possess a strong mitotic outcome have been receiving high interest as potential therapeutic targets, more specifically targeting the inhibition of their signalling[313]. An autocrine activation of HER3 by NRG revealed development of resistance to gefitinib therapy[226]. Additionally high levels of NRG1 promote resistance to trastuzumab in MCF-7 cell lines[314]. Patients submitted to trastuzumab and revealing low levels of HER2 but high expression of NRG1 possess a better outcome, suggesting that this group of patients might benefit from this therapy even though the low expression of HER2[314].

1.8.1.9 Other Ligands

Neuroglycan C[315] has more recently been identified as a member of the NRG family. Tomoregulin 1 and 2[316] have also been suggested as possible ligands that interact with HER family, however further studies need to conducted in order to confirm this.

The EGF domain is amongst one of the most common recognised in the human genome existing in more than 3000 genes (www.sanger.ac.uk). Therefore it is reasonable to surmise that more ligands and possibly new HER family splicing forms may be identified in the future, revealing an even higher variation on

possible combinations and more and diverse biological effects might be understood currently (Figure 1.6).

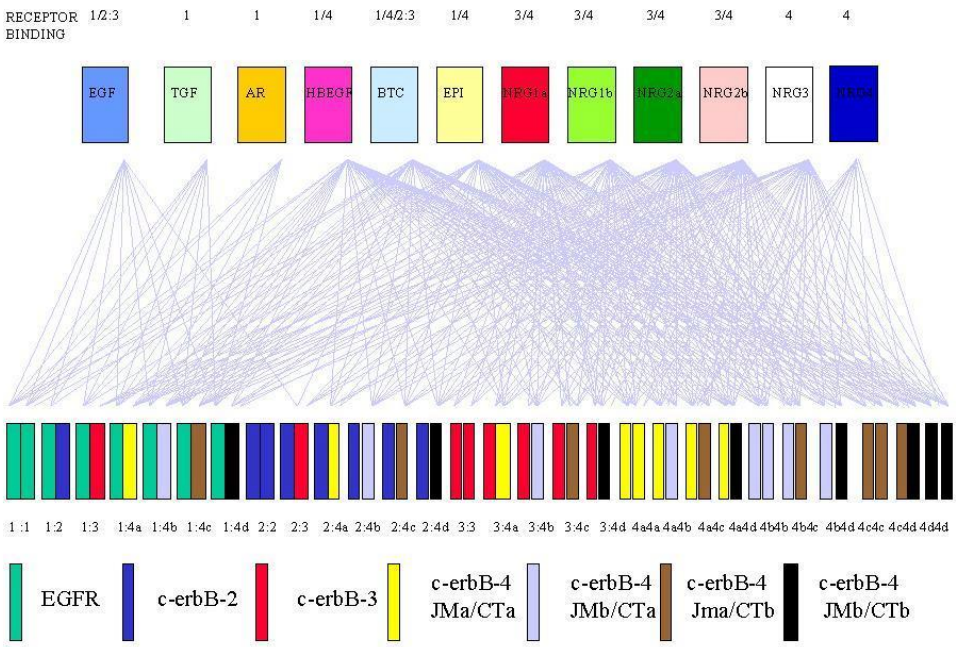

Figure 1.6 – Possible dimer combinations amongst the HER family.
(From: http://www.kent.ac.uk/bio/gullick/?tab=research)

1.9 HER family dimerisation

1.9.1 Overview

A ligand-ectodomain interaction encourages dimerisation, which is characterised by contact either of two HER proteins from the same family – heterodimerisation – or of two identical molecules – homodimerisation. The nature of EGFR, HER3 and HER4, molecular conformation is unable to interact with any other RTK. The dimerisation arm remains in an auto-repressed conformation due to an intramolecular linkage inside a protein situated in domain IV of the ectodomain (ECD). However when the ligand connects to one of these glycoproteins, the dimerisation arm located in domain II of the ECD, converts into a competent conformation (Figure 1.7)[32]. Even though an interaction with a ligand is not enough to provoke the reaction[41]. This is the primary approach to incite interaction between two monomers, however it is not unique. The kinase region localised on HER molecule tail (except for HER3) is activated by a transphosphorylation reaction. Two lobes, C and N, which cooperate to generate the active conformation, compose this domain. The activation of kinase occurs when the C-lobe of the first kinase contacts the N-lobe of the second one, which becomes allosterically activated (Figure 1.8)[42]. Multiple pathways are triggered as a consequence of dimerisation of the HER family, as phospholipase Cγ (PLCγ), signal transducer and activation of transcription (STATs), Ras/MAP kinase (MAPK) and PI3K[317], with the last two being well documented to be associated in cell survival and proliferation, by activation of several transcription factors and Cdk1 and decreasing the levels of active p27 with consequent cell cycle arrest is evaded[70,106].

In cancer cells, mutations of the kinase domain on HER2 triggers EGFR resistance to some inhibitors[318]. The structure of the dimers can manipulate downstream pathways where some arrangements are present in aggressive carcinomas, like HER2/EGFR, EGFR/HER3 and HER2/HER3[283,319]. However the heterodimerisation of HER2/EGFR is reported to be present in the most aggressive carcinomas and worst prognosis confirmed by cancer shortest disease free survival and cancer specific survival[320]. Even though the differences amongst these heterodimers, they are more stable[321] and produce downstream pathways which are more effective[263] when compared with other heterodimers not including HER2.

After the biological reaction, the interacting ligand receptor is broken by an endocytic reaction, inactivating receptors. The unphosphorylated HER molecules are then either degraded or recycled to return onto the cell membrane[322]. Ubiquitin ligases can react with kinase domain playing the role of negative regulators. Furthermore the molecule RALT also known as Mitogen-inducible gene 6 (Mig6), is a protein that has the ability of binding to HER family molecules in their kinase domain slowing down the kinase activity[323]. Expression knockout of this protein may influence the cell cycle and promotes cancer development[324,325].

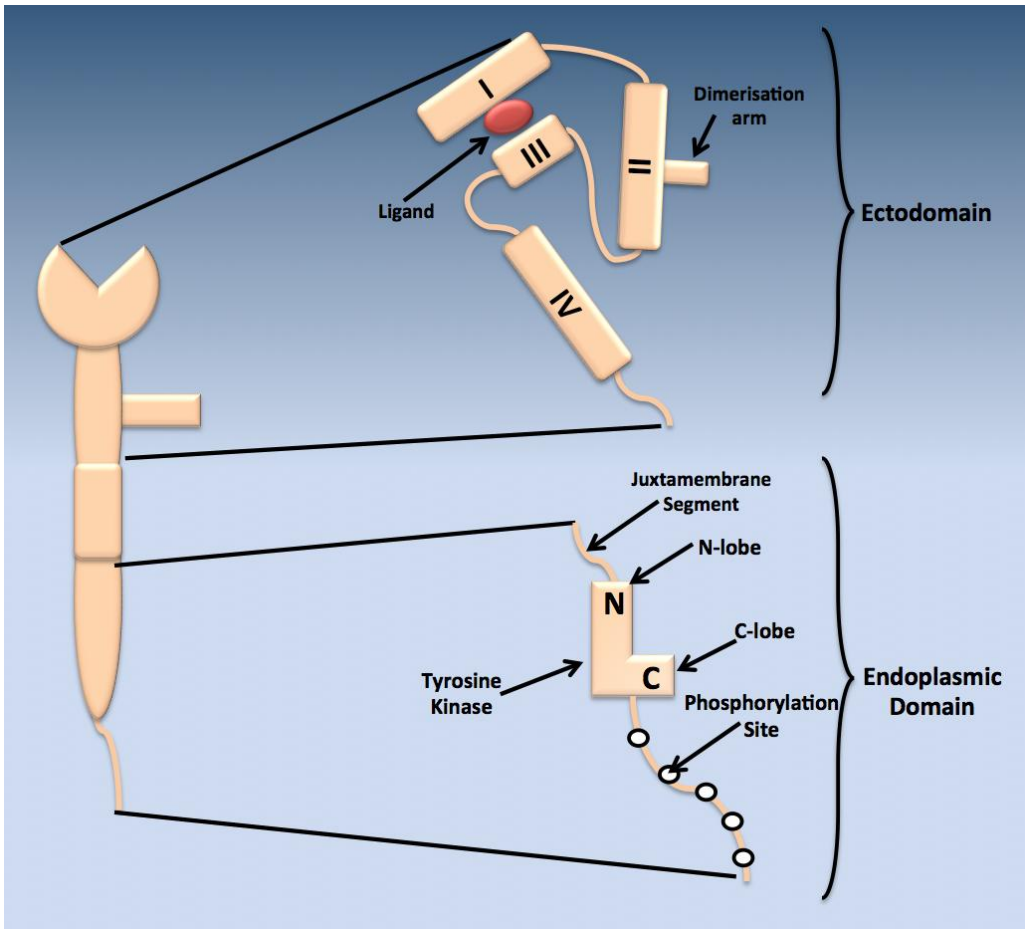

Figure 1.7 – The ectodomain and endoplasmic domain of a member of HER family. The ectodomain has four substructures that together are the competent form due to a ligand molecule interaction with substructures I and III. Also the substructure II is where the interaction with another receptor takes place due to the localisation of the motif arm and where pertuzumab interacts with the receptor during treatments. The last substructure, IV, is the target from trastuzumab avoiding competent conformation of the ectodomain. The endoplasmic domain includes the N-lobe and C-lobe of the tyrosine kinase involved in the dimerisation process, at cytoplasmic level, which will trigger several downstream pathways by phosphorylation reaction.

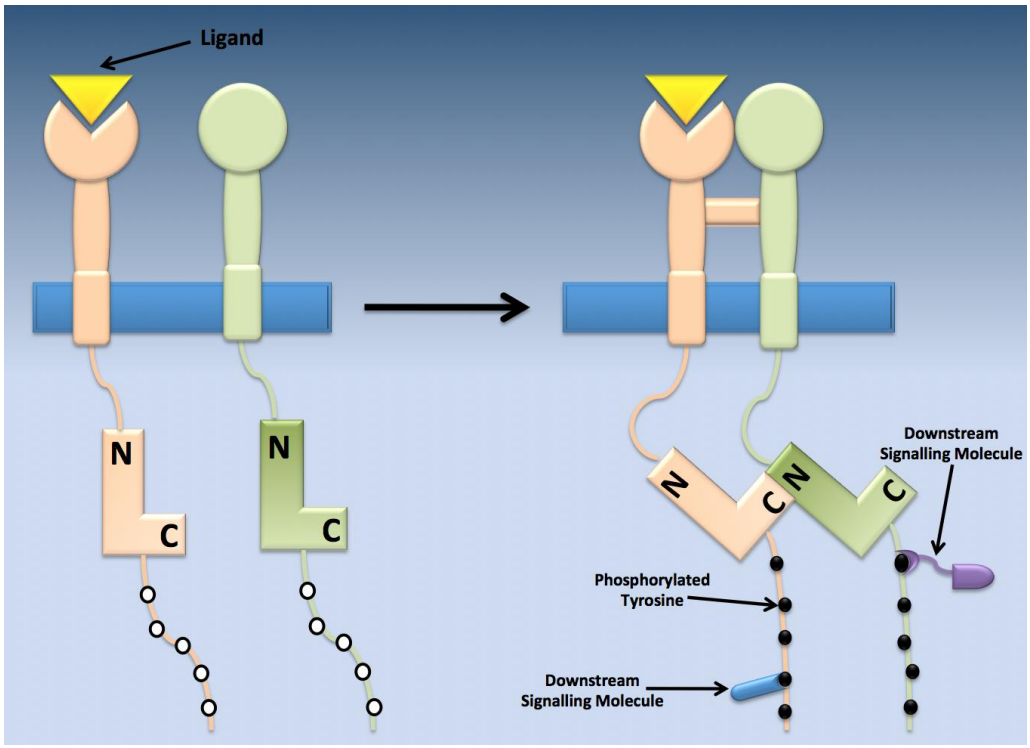

Figure 1.8 – Induced HER Dimerisation. Ligand/Receptor connection that triggers dimerisation reaction that takes place in the internal domain showing the N-lobe of one monomer reacting with the C-lobe of the second monomer. Phosphorylation of the tyrosine sites takes place to activate downstream pathways.

1.10 Hypothesis

Accurate quantification of protein expression and other biological characteristics, such as HER heterodimerisation, could be important to elucidate and predict patient outcome, particularly with HER targeted therapies. The action of these molecules are controlled by a complex system that includes structures modifications or connections with other molecules such as ligands; the subsequent heterodimer arrangements will trigger different pathways and consequently different outcomes[30,31]. It is therefore hypothesised that the different HER heterodimers might possess distinct associations with relevant biomarkers including hormone receptors and moreover how it influences survival outcome amongst the patients. If any of these heterodimers is associated with trastuzumab resistance development and in which period of time after the first treatment this resistance is developed. Three techniques are going to be utilised for a more complete understanding of the heterodimers biochemistry, which includes Immunohistochemistry, chromogenic *in situ* hybridisation and a novel technology named *in situ* Proximity Ligation Assay. Classes of biomarkers never identified before discriminated amongst breast cancers can be characterised. An innovative and more robust classification system of breast carcinoma is possible to be developed and discrimination of patients can be more reliable, with reduce number of relapses or even death occurrence originated by wrong diagnosis and therapy decision.

1.11 Aims

Therefore the aims of this study are four fold:

1) To accurately establish HER2 status of a large historical series of breast cancers, by comparing correlation between protein overexpression and gene amplification (determined by CISH) in order to determine and understand their specific contributions to patient outcome.

2) Perform quantification of HER2 as a monomer, and heterodimers (HER2/HER3, HER2/EGFR and HER2/HER4) in both Unselected HER2+ breast cancer series.

3) Determine the biological characterisation of these heterodimers in both Unselected and HER2+ breast cancer series by correlating to clinicopathological parameters, patient's survival outcome and trastuzumab therapy effectiveness.

4) Understand how and if $p95^{HER2}$ demonstrates any different outcome from the wild-type HER2 molecule and the correlation between them.

2. CHAPTER 2: Patients and Methods

2.1 Patient samples

2.1.1 Unselected Series

The first patient series comprised 1858 unselected primary operable invasive breast carcinoma cases between 1986-1998, from the well-characterised Nottingham-Tenovus Primary Breast Carcinoma Series (Unselected Series). The entire series was evaluated for clinicopathological variables as age, menopausalcc status, tumour type[326], histological grade[327], tumour size, tumour recurrence, presence of distance metastasis, lymph node status, vascular invasion and Nottingham Prognostic Index (NPI)[328]. The NPI was obtained using the formula: 0.2 x tumour size (cm) + grade (1-3) + lymph node stage (1-3). Patients were assessed in three prognostic groups: ≤ 3.4 – good prognosis, 3.41-5.4 – moderate prognosis and > 5.4 – poor prognosis. The treatment strategy was established on tumour characteristics including NPI and hormone receptor status. The patients exhibiting an NPI score ≤ 3.4 did not receive adjuvant therapy, those patients with NPI score > 3.4 received, Tamoxifen if ER positive (n = 1272, 73.2%) (± Zoladex if premenopausal), or classical cyclophosphamide, methotrexate and 5-fluorouracil if ER negative (n = 465, 26.8%) and fit enough to tolerate chemotherapy[329].

Table 2.1 summarises the clinicopathological parameters. A total of 1256 (67.6%) were 50 years old or over and 601 (32.4%) were under 50 years old. The age range was 18-72 years, mean age situated on 54 years and the median

55 years. Histopathological description of the disease varied, which is illustrated with 354 (19.1%) tumours grade I, 616 (33.3%) grade II and 880 (47.6%) grade III. Tumour size ranged between 0.1 cm and a maximum size of 15.0 cm. A total of 1367 (73.9%) of patients tumours were 1.5cm or larger and 484 (26.1%) under 1.5cm. Additionally 1187 (64.1%) revealed tumour stage N0, 505 (27.3%) stage N1 and 159 (8.6%) stage N2.

Follow-up data was collected for the first time at 3 months after surgery, followed by an interval of 6 months and then each 12 months (median 160 months; range 1-224 months). Throughout this time, relapse occurred in 718 (39.6%) cases, distant metastasis in 559 (30.3%) cases and 483 (31.7%) patients died from breast cancer. Local recurrence, defined as an additional malignancy occurring in the same mammary tissue, and regional recurrence, defined as an extra malignant incidence in lateral axillary lymph nodes, occurred in 216 (11.9%) and 178 (9.8%) patients, respectively. Characterisation, including immunoreactivity, marking and categorising of ER, progesterone receptor (PgR), p53, breast cancer 1 (BRCA1), basal phenotype (BP) and Triple Negative Phenotype (TN) were previously determined in this series[330-333].

2.1.2 HER2+ Series

2.1.2.1 HER2+ adjuvant trastuzumab series

The HER2+ adjuvant trastuzumab series consisted of 143 primary operable breast tumours from patients presenting between 2003 and 2010 who received adjuvant trastuzumab. A total of 79 (56.4%) patients were aged over or equal

to 50 years, and 61 (43.6%) were less than 50 years old. The age of patients at presentation ranged between 31 and 79 years old (median and mean of 52 years). At primary diagnosis 3 (2.1%) tumours were grade 1, 37 (25.9%) grade 2, and 103 (72.0%) cases were grade 3. A total of 120 (83.9%) patients had tumours 1.5cm or larger, (ranging from 0.1 cm and 8 cm). Relapse occurred in 23 (16.1%) cases, distant metastasis in 17 (12.1%) cases, and 10 (7.0%) patients died from breast cancer. Local recurrence occurred in 7 (4.9%) of cases and regional recurrence in 1 (0.8%) case.

2.1.2.2 HER2+ trastuzumab naïve series

The HER2+ trastuzumab naïve series (n = 224) is composed by all HER2 positive cases part of Unselected Series. A total of 142 (63.4%) of patients were 50 years old or over and 82 (36.6%) less than 50 years old, with an age range between 27 and 71 years old and median of 53. This series primarily consisted of grade III tumours (186, 83.0%) of cases the remaining being either grade II (33, 14.8%) or grade I (5, 2.2%). The tumour size ranged between 0.5 cm and 8.0 cm, including 186 (83.0%) of patients having tumours larger than 1.5 cm. The tumour lymph node (LN) stage N3 was present in 34 (15.2%) cases, N2 in 67 (30.0%) patients and the majority 122 (54.8%) acquired LN stage 1. Finally relapse occurred in 113 (51.1%) of patients, distant metastases occurred in 102 (45.5%) cases and 97 (49.7%) patients died from breast cancer. Local and regional recurrence occurred in 31 (14.0%) and 28 (12.7%) of the patients, respectively.

2.1.2.3 HER2+ Series

Combining the HER2+ adjuvant trastuzumab and HER2+ trastuzumab naïve series resulted in the HER2+ Series, which included 367 patients. Clinicopathological parameters for this series are summarised in Table 2.1 patients.

Table 2.1 – Clinicopathological characteristics of the Unselected series (n = 1858), HER2+ trastuzumab naïve series (n = 224), HER2+ adjuvant trastuzumab series (n = 143) and HER2+ Series (n = 367).

Characteristics	**Unselected Series**	**HER2+** (Trastuzumab Naïve)	**HER2+** (Adjuvant Trastuzumab)	**HER2+** (Naïve + Adjuvant)
	N Patients (%)	N Patients (%)	N Patients (%)	N Patients (%)
Age at Diagnosis (Years)				
<50	601 (32.4)	82 (36.6)	61 (43.6)	143 (39.3)
≥50	1256 (67.6)	142 (63.4)	79 (56.4)	221 (60.7)
Menopausal				
Pre	700 (37.7)	92 (41.1)	26 (18.6)	118 (32.4)
Post	1154 (62.3)	132 (58.9)	114 (81.4)	246 (67.6)
Death				
No	1043 (68.3)	98 (50.3)	133 (93.0)	231 (68.3)
Yes	483 (31.7)	97 (49.7)	10 (7.0)	107 (31.7)
Tumour Size (cm)				
<1.5	484 (26.1)	38 (17.0)	23 (16.1)	61 (16.6)
≥1.5	1367 (73.9)	186 (83.0)	120 (83.9)	306 (83.4)
Tumour Grade				
1	354 (19.1)	5 (2.2)	3 (2.1)	8 (2.2)
2	616 (33.3)	33 (14.8)	37 (25.9)	70 (19.1)
3	880 (47.6)	186 (83.0)	103 (72.0)	289 (78.7)
Lymph Node Stage				
1	1187 (64.1)	122 (54.8)	53 (37.3)	178 (47.9)
2	505 (27.3)	67 (30.0)	56 (39.4)	123 (33.7)
3	159 (8.6)	34 (15.2)	33 (23.2)	67 (18.4)
Distant Metastasis				
No	1288 (69.7)	122 (54.5)	124 (87.9)	246 (67.4)
Yes	559 (30.3)	102 (45.5)	17 (12.1)	119 (32.6)
Mitotic Frequency				
1	684 (38.3)	15 (6.8)	36 (25.9)	51 (14.2)
2	322 (18.0)	43 (19.4)	43 (30.9)	86 (23.9)
3	781 (43.7)	163 (73.8)	60 (43.2)	223 (61.9)
Pleomorphism				
1	51 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)
2	718 (40.3)	26 (11.9)	12 (8.6)	38 (10.6)
3	1011 (56.8)	193 (88.1)	127 (91.4)	320 (89.4)
Tubule Formation				
1	119 (6.7)	2 (0.9)	0 (0.0)	2 (0.6)
2	583 (32.6)	46 (20.8)	23 (16.5)	69 (19.2)
3	1085 (60.7)	173 (78.3)	116 (83.5)	289 (80.2)
Vascular Invasion				
Negative	1061 (58.2)	118 (53.4)	79 (56.0)	197 (54.4)
Positive	763 (41.8)	103 (46.6)	62 (44.0)	165 (45.6)
Relapse				
No	1093 (60.4)	108 (48.9)	119 (83.9)	227 (62.5)
Yes	718 (39.6)	113 (51.1)	23 (16.1)	136 (38.5)
Local Recurrence				
Negative	1595 (88.1)	190 (86.0)	136 (95.1)	326 (89.5)
Positive	216 (11.9)	31 (14.0)	7 (4.9)	38 (10.5)
Regional Recurrence				
Negative	1633 (90.2)	193 (87.3)	124 (99.2)	317 (91.6)
Positive	178 (9.8)	28 (12.7)	1 (0.8)	29 (8.4)

2.2 Tissue microarrays (TMAs)

2.2.1 Technique

Using TMA technology representative tissue cores acquired from formalin-fixed paraffin embedded (FFPE) tissue block (donor block) can be transferred to a new paraffin block (recipient block). Firstly portrayed by Wan et al[334] after the technology was advanced allowing the inclusion of a high number of cases in one single paraffin block allowing high-throughput downstream analysis such as immunohistochemistry[335].

2.2.2 TMA construction

Two separate TMA collections were constructed:

1) Unselected Series TMA – consisting of peripherally sampled tumour cores, with one core representing each patient. Construction of this TMA has been previously described[330,331].

2) HER2+ trastuzumab adjuvant TMA – consisting of three cores sampled from each tumour: two cores from peripheral regions and one from the central region of the tumour.

From each donor FFPE block, the quality of each block was considered, selecting those holding a satisfactory tumour thickness (≥ 3mm). Full-face H&E sections were evaluated and the most representative area for each case were selected and marked. Each representative area marked on the H&E slide

was subsequently marked on the surface of the corresponding donor block using a permanent marker.

To construct the TMAs, a semi-automatic puncher/arrayer instrument was used (Alphelys Minicore Tissue Arrayer, Alphelys SAS, Ferme de Ebisoires, France, Figure 2.1). The punching device employs the technology of coaxial punches where the donor punch, recipient punch and stylet are concentric (Figure 2.2). The TMA cores were 0.6 mm diameter and 3 mm in height. After each core was obtained from the donor, the respective block was removed and the cycle repeated with the next donor block (Figure 2.3). Each recipient block holds 150 TMA cores being spaced 1mm from each other. Additionally three cores from control kidney tissue were included in each recipient block at the start of each construction in order to assist on orientation.

To perform the different techniques of analyses in histopathology sectioning had to be implemented by the use of a standard manual rotary microtome for routine paraffin sectioning (Shandon, Finesse 325 Microtome, Thermo Scientific, UK) on X-tra^{TM} adhesive micro slide (Surgipath, Leica).

Figure 2.1 – Alphelys Minicore Tissue Arrayer used to construct the TMAs showing the main mechanisms **A)** Recipient block holder, **B)** donor block holder, **C)** Selector, **D)** Button to rotate **E)** Punching device.

Figure 2.2 – MiniCore® punching device that employs the expertise of coaxial punches. **A)** donor punch, **B)** recipient punch and **C)** style. The reason for this method is to simplify and reduce the possibility of misalignment concerning both punches.

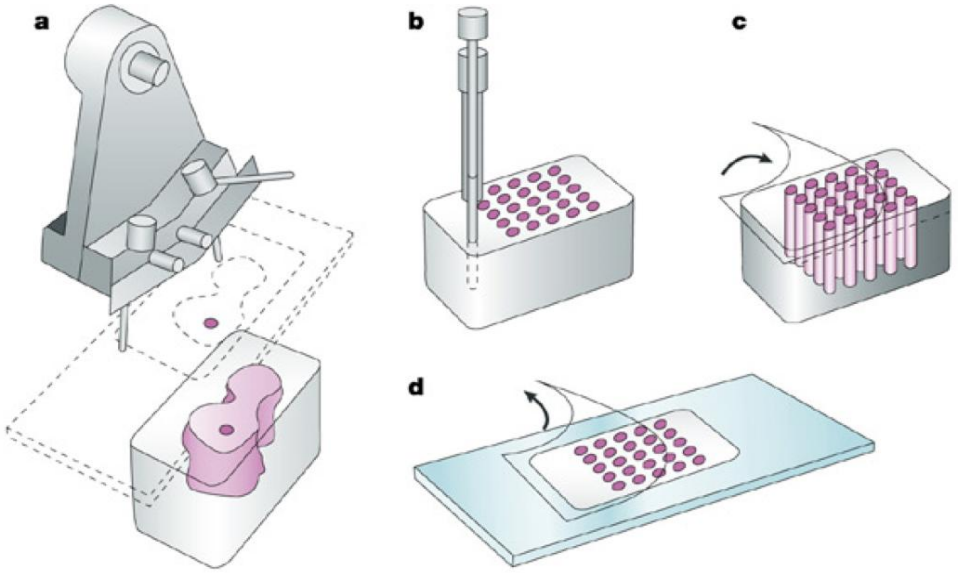

Figure 2.3 – Tissue Microarrays were constructed using a technique of transferring a sample **a)** from a patient's whole tumour section **b)** to a new recipient block possessing multiple samples from different patients. **c** & **d)** These patients' samples can then be observed in the same slide after sectioning and respective staining process (diagram from Sauter et al 2003)[336]

2.3 Immunohistochemistry

2.3.1 Technique

2.3.1.1 Dewaxing and Rehydration

Tissue or TMA sections, 4µm thickness, were placed onto a 60˚C hotplate for 10 minutes. Dewax and rehydration was performed automatically device (Leica Auto Stainer XL, Leica, Germany) consisting of immersion in 2 sequential xylene baths for 5 minutes each, proceeded by rehydration by immersing in a series of three 100% alcohol baths for 2 min each. Finally the slides were left in running tap water for at least for 5 min.

2.3.1.2 Antigen Retrieval

Sections were submitted to an antigen-retrieval process to unmask the antigenic sites, either using Citrate (pH = 6.0) or EDTA (pH = 9.0) buffers. This was accomplished placing the respective pre-treatment buffer for 20 min using a microwave keeping the temperature always below boiling point (Whirlpool 6^{th} sense JT356/JT359, Whirlpool, USA). Then the slides were cooled in tap water, for at least 5 minutes, to stop the retrieval process. Immunohistochemistry was performed using either Envision (Dako, Denmark) or Novolink (Leica, Newcastle, UK) at room temperature.

2.3.1.3 Envision Kit

Endogenous peroxidase activity was blocked using peroxidase-blocking agent (Dako, Denmark) for 5 minutes. Slides were washed well in Tris-Buffered

Saline (TBS) and Ultra V Block (Thermo Scientific, USA) was applied and incubated for 5 minutes. Each primary antibody, at a specific optimised concentration, was added to each slide and incubated for the specific time (Table 2.2). Envision A solution (HRP labelled polymer/secondary antibody – Envision Kit) was applied for 30 minutes and then washed in TBS. Envision B (Buffer Substrate – Envision Kit) and Envision C (3,3'-diaminobenzidine (DAB) Chromogen – Envision Kit), mixed to a ratio of 1:50, was added for 5 minutes. This process was repeated. Finally the slides were washed twice in TBS and left for 5 minutes. The slides were counterstained with freshly filtered Mayer's haematoxylin (Lillie's Modification, Dako, Denmark) for 6 minutes and washed well in tap water for 5 minutes.

2.3.1.4 Novolink Polymer Detection Systems (Novolink kit)

Initially Peroxidase Block (Novolink Kit) was applied onto slide for 5 minutes and then washed twice with TBS for 5 minutes. Protein Block (Novolink Kit) was added for 5 minutes and again washed twice in TBS for 5 minutes. The respective primary antibody was applied at specific concentrations/period of time (Table 2.2). Slides were washed in TBS twice for 5 minutes. Subsequently Post Primary (Novolink kit) was added for 5 minutes, washed in TBS twice. Novolink Polymer (Novolink kit) was added for 30 minutes and the slides washed 5 minutes twice in TBS. Finally a DAB working solution, consisting of 1:20 DAB chromogen in DAB substrate buffer (Novolink kit), was applied for 5 minutes and the slides washed for 5 minutes twice in TBS. Haematoxylin (Novolink kit) was added in a volume of 100 µl for 6 minutes and the slides

washed in running tap water for at least 5 minutes in a rack ready to be submitted to dehydration in the autostainer machine.

2.3.1.5 Counterstaining, Dehydration and Mounting

Slides were placed in the Autostainer for dehydration by incubating three times for 2 minutes in 100% alcohol and cleared in Xylene for 5 minutes twice. Subsequently of a complete dehydration process, the slides were mounted in DPX, a viscous solution composed by distyrene, a plasticiser (tricresyl phosphate) and xylene.

Table 2.2 – Dilution, Source and pretreatment of antibodies used on both immunohistochemistry and *in situ* proximity ligation assay and the cut-off used to classify each biomarker.

Antibody [Clone]	Dilution	Incubation time (min)	Source	Cut-off point
Hormone receptor proteins				
ER [SP1]	1:50	30	Dako	0
PgR [PgR 636]	1:125	30	Dako	0
Human epidermal growth factor receptor proteins				
EGFR [31G7]	1:30	30	Invitrogen	10
EGFR† [EGFR-R2]	1:10	120	Santa Cruz	N/A
HER2	1:400	30	Dako	10
HER2†	1:200	120	Dako	N/A
HER3 [RTJ1]	1:30	30	Leica	10
HER3† [2F12]	1:40	120	Neomarkers	N/A
HER4 [H4.77.16]	1:100	30	Neomarkers	10
HER4† [HFR1]	1:50	120	Abcam	N/A
p95^{HER2}	1:50	30		10
Luminal associated cytokeratins				
CK7/8 [CAM 5.2]	1:2	30	BD	50*
CK18 [DC10]	1:50	30	Dako	50*
Basal cytokeratins				
CK5/6 [D5/16 B4]	1:20	30	Dako	10
CK14 [LL002]	1:100	30	Novocastra	10
Cell Cycle associated proliferation and apoptosis related proteins				
Ki67 [MB-1]	1:100	30	Dako	10-50*
PI3K	1:50	60	Sigma Aldrich	30-100*
Akt^{pS473} [14-5]	1:10	30	Dako	60*
P21 [SX118]	1:100	30	Dako	0
Bcl-2 [124]	1:100	30	Dako	30
Tumour suppressor genes				
PTEN [6H2.1]	1:100	60	Dako	10-100*
P53 [DO7]	1:50	30	Novocastra	5
BRCA1 [MS110]	1:100	40	Calbiochem	100
Apomucins				
MUC-1 [MA695]	1:350	30	Novocastra	20-200*

* H-Score
† Used for in situ PLA

2.3.1.6 Microscopy observation and Immunohistochemical scoring

All the slides were examined using a brightfield microscope (Eclipse 80i, Nikon, Japan) with 20x and 40x power magnification. The positive and negative controls were observed carefully to approve and accept the immunoreactivity. Specific immunoreactivity semi-quantitation was considered using intensity, percentage or H-Score using different cut-off (Table 2.2)[337,338]. Only cancer cells localised within tissue cores were considered, and only those cores revealing at least 15% of the area composed by invasive breast cancer cells. TMAs were scored using high-resolution digital images (NanoZoomer; Hamamatsu Photonics, Welwyn Garden City, UK), at x20 and x40 magnification via a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland).

The intensity of staining was assessed on a range of 0 – 3: 0 for negative staining, 1 for weak staining, 2 for moderate staining and 3 for strong staining. The first considerations were accepted as negative and the two higher classifications as positive. H-Score was determined by adding the intensity score (above) with the percentage of cells stained. Using the following formula: 3 x (percentage of cells showing strong staining) + 2 x (percentage of cells showing moderate staining) + 1 x (percentage of cells showing faint staining) = range 0 – 300[338]. The H-Score system as well as other semi-quantitative systems was validated for TMAs in previous studies[74,339,340]. For HER2 IHC assessment, The American Society of Clinical Oncologists (ASCO) established guidelines were used[341]. Immunohistochemistry results were established as 0 (negative) where a faint membranous immunoreactivity was

observed in <10% of the invasive breast cancer cells, 1+ (negative) where >10% of the cells present in the tumour showed a barely perceptible membranous reactivity, 2+ (equivocal) where >10% of the cells showed moderate but complete membranous reactivity, and 3+ (positive) where >30% of the cells showed strong complete membranous reactivity.

2.4 *HER2* Gene amplification

2.4.1 Fluorescent *in situ* hybridization (FISH)

2.4.1.1 Technique

This method was carried out using the *HER2* FISH pharmDx™ kit (Dako, Denmark). The assay was performed according to the manufacturer's recommendations. FFPE sections were deparaffinised by xylene for 5 minutes twice, ethanol 96% and ethanol 70% for 2 minutes twice and finally rehydrated in wash (Tris/HCl) buffer for 2 minutes. The pre-treatment was performed in pre-treatment buffer and microwaving for 10 minutes. The slides were cooled at RT and rinsed in wash buffer for 3 minutes twice. After pre-treatment the slides were submitted to enzymatic digestion, using pepsin incubating at 37°C for an optimal 6 minutes followed by being immersed in wash buffer for at least 3 minutes twice. Dehydration was performed by immerging the slides in 70% and 96% ethanol for 2 minutes each and left to air dry. The phase of denaturation and hybridisation was performed using 10 µl of probe mix applied onto the slide and a coverslip placed added which was sealed around the periphery using sealant in order to avoid any drying out during the

hybridisation incubation period. The slides were incubated) for denaturation at 85℃ for 5 minutes and hybridisation at 45℃ for 20 hours (Dako Hybridiser) (Dako, Denmark). Slides were washed using Stringent wash buffer (saline-sodium citrate) twice, the first was at 65℃ for 10 minutes and the second at RT for 3 minutes. The slides were dehydrated in 70% and 96% ethanol for 2 minutes and mounted with fluorescence mounting medium. Slides were observed on a fluorescent microscope using DAPI and Texas Red filters Axioskop2 (Carl Zeiss, Germany) with Leitz Weltzar 100x/1.25 oil immersion lens (Germany).

2.4.2 Chromogenic *in situ* hybridization (CISH)

2.4.2.1 Technique

The Dual-colour *HER2* CISH pharmDx™ Kit (Dako, Denmark) protocol used is an extension of the *HER2* FISH pharmDx™ protocol; however the final dehydration, air-drying and mounting steps were not performed. Instead, the slides were immersed in a CISH wash buffer (TBS solution with 0.05% Tween 20) (DAKO, Denmark) for 3 minutes. The sections were covered with 200 µl of peroxidase block solution (3% H_2O_2, 15 mmol/L NaN3) for 5 minutes and washed twice in CISH wash buffer for 3 minutes. CISH antibody mix (HRP-conjugated antibody to FITC and an AP-conjugated antibody to Texas Red in 50 mmol/L Tris buffer, pH 7.5) (*HER2* CISH pharmDx™ Kit) was applied onto the section, incubated in a humidified chamber for 30 minutes at RT and the slides were rinsed twice in the wash buffer. To visualise the AP-conjugated antibodies, the sections were covered with 200 µl of red chromogen solution

(Fast Red KL Salt) (*HER2* CISH pharmDx™ Kit) in a humid chamber for 10 minutes and washed twice for 3 minutes. Afterwards, to visualise the HRP-conjugated antibodies a volume of 200 µl blue chromogen solution (5-amino-2-[3-[5-amino-1,3-dihydro-3,3-dimethyl-1-(4-sulfobutyl)-2H-indol-2-ylidene]-1-propenyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-Indolium, ter-trifluoroacetate) (*HER2* CISH pharmDx™ Kit) was added to each slide in a humidified chamber for 10 minutes and washed twice for 3 minutes. The filtered Mayer's haematoxylin (Lillie's Modification, Dako, Denmark) diluted 1:5 with distilled or deionised water was applied for 5 minutes onto the sections, rinsed with wash buffer and immersed in fresh wash buffer for at least 5 minutes. Slides were rinsed thoroughly with distilled water and dried at 37°C for 30 minutes, mounted with permanent xylene-free mounting media (Tissue-Mount™, Sakura Finetek Europe B.V.) and evaluated using as a brightfield microscope at Axioskop2 (Carl Zeiss, Germany) with Leitz Weltzar 100x/1.25 oil immersion lens (Germany).

2.4.2.2 Scoring HER2 gene amplification

The evaluation of the dual-colour FISH or CISH slides were performed according to the guidelines complementing the *HER2* FISH pharmDx™ Kit (Dako, Denmark). For all tumour specimens the *HER2* signals (red) and CEN-17 signals (green for FISH or blue for CISH) from 20 nuclei in invasive breast cells were counted and the *HER2*/CEN-17 ratios were calculated. All signals of similar size detached from each other by a gap equivalent or less than the diameter of one signal were counted as only one signal. Furthermore those cells revealing only one of the colours were ignored and not included on this

study. The cases where the ratio result was below 2 were classified as *HER2* non-amplified. On the other hand the carcinomas showing a ratio of 2 or higher were accepted as *HER2* amplified. However when a ratio value between 1.8 and 2.0 was observed, the samples were submitted to a recalculation by scoring a further 20 nuclei to obtain an additional rate, resulting in a final mean value between both ratios.

2.5 HER2 heterodimerisation quantification analysis

2.5.1 Duolink® *in situ* Proximity Ligation Assay (PLA)

2.5.1.1 Overview

HER2 protein overexpression and gene amplification are the current methods to discriminate those breast cancer cases to trastuzumab therapy. However semi-quantitative methods, such as IHC, might not be adequate and/or sufficient to achieve high levels of therapy success for HER2 overexpressing breast cancers. Thus, accurate quantification of protein expression and other characteristics such as type 1 growth factor receptor heterodimerisation could be important to elucidate their roles for detailed biological outcome. The action of these molecules is controlled by a complex system that includes structures modifications or connections with other molecules such as ligands; the consequence heterodimer arrangements will trigger different pathways and consequently different outcomes[30,31]. Protein interaction measurements can be used to explore sub-cellular processes, and can serve to reveal molecular pathways to be targeted for pharmaceutical intervention. Currently, protein interactions are being investigated to understand processes at cellular

dimension helping to disclose both upstream and downstream pathways, which might be used to achieve better diagnosis and consequently successful therapies.

To detect, visualise and quantify protein expression or protein interactions, it is used Duolink® *in situ* proximity ligation assay (PLA) for brightfield microscope (Olink, Sweden). With this technique it is feasible to identify distinct signals that symbolise distinct proteins, protein interactions or even protein alterations such as phosphorylation. In order to achieve an effective detection, the approach is to generate certain DNA representations with the assistance of specific antibodies. This method allows detection of specific proteins after a polymerase chain reaction is created to amplify a DNA molecule. This technique needs two specific primary antibodies that identify the protein target or protein interaction target. These detections are possible once a primary antibody of different species suitable for detection of each protein is available[82]. The detection is performed using primary antibodies from different species against each protein followed by incubation with species-specific secondary antibodies, which are linked to an oligonucleotide chain (PLA probes). Each of these probes is linked with a short DNA strand. One of the secondary antibodies is linked to a minus probe and the second to a plus probe. The minus probe is merely a complementary DNA sequence of the plus probe, which will act as a template for DNA amplification. After incubation with the antibodies, a hybridisation reaction will occur, however only successfully achieved if both probes are close enough (<40 nm) revealing protein interaction. Within this distance both DNA strands are able to interact within a following addition of a circle-forming DNA oligonucleotide. To

visualise the discrete signals, a ligation, amplification (via rolling circle amplification - RCA) and detection reactions must occur. The circular molecule is used as an infinite template for RCA. The amplification reaction is primed in one of the PLA probes and by the end of the first circle (the end of the second PLA probe) the polymerase dislocates the new DNA strand, resulting in the production of a single stranded DNA. The detection reaction is characterised by hybridisation of the horseradish peroxidase (HRP) labelled complementary oligonucleotides with the rolling-circle DNA product. Finally the signal is possible to visualise as a individual brown blob throughout an enzymatic conversion of NovaRED substrate, being possible to detect with a standard microscope (Figure 2.4)[83].

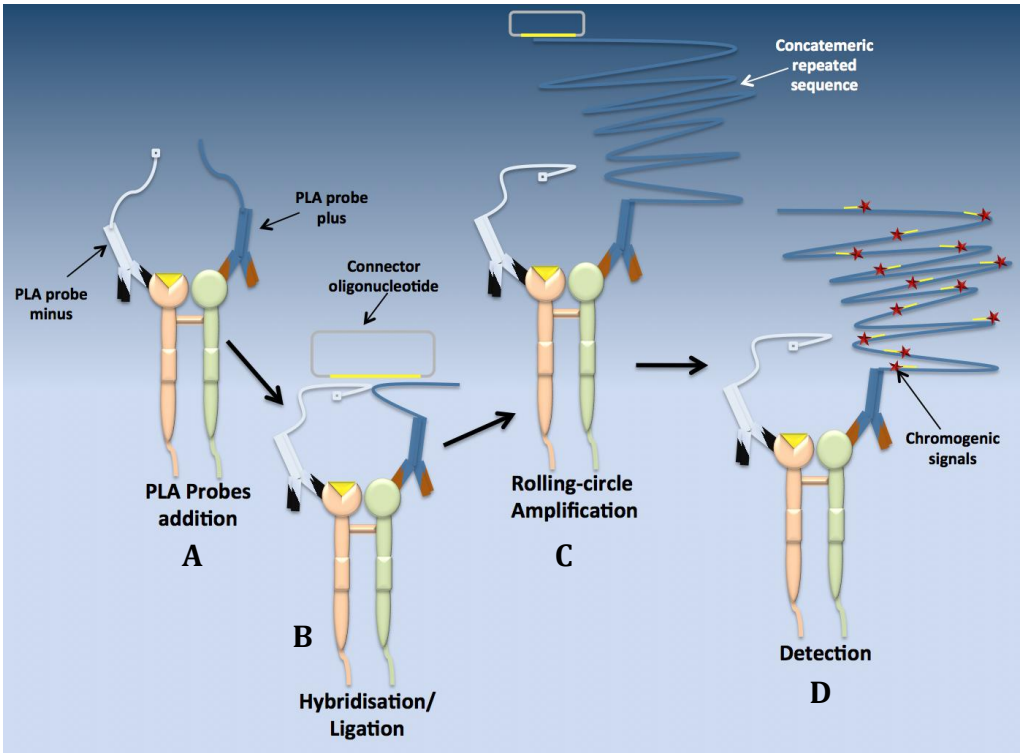

Figure 2.4 – Duolink® *in situ* proximity ligation assay (PLA). **A)** Double binding of PLA probes (secondary antibodies linked to oligonucleotide strands) to primary antibodies (not represented) already connected onto respective protein target. **B)** Hybridisation is characterised by addition of a connector oligonucleotide that will react with the PLA probes only in case they are in close proximity. **C)** After ligation, rolling-circle amplification (RCA) takes place resulting in a concatemeric repeated sequence. **D)** Hybridising this sequence with horseradish peroxidase (HRP) labelled oligonucleotides and perform an enzymatic reaction with NovaRed substrate the signals become visible under brightfield microscope.

2.5.1.2 Technique

Quantification of HER2 heterodimers in primary invasive breast cancer cases was performed using *in situ* PLA for brightfield microscope (Olink, Sweden). FFPE sections were deparaffinised and rehydrated as above followed by heat induced antigen retrieval for 20 minutes using the respective buffers (Table 2.2) followed by peroxidase quenching using 0.3% hydrogen peroxide for 5 minutes at RT and

C

in a humidity chamber. To detect any heterodimer, two antibodies from two different species were applied. For all HER2 heterodimers, the anti-HER2 antibody (Dako, diluted at 1:200) was used. In case of HER2/HER3 heterodimer, anti-HER3 (clone 2F12, Neomarkers, diluted at 1:40) was used, which was incubated for 30 min at RT. For HER2/EGFR the anti-EGFR antibody (clone EGFR-R2, Santa Cruz, diluted at 1:10) was used whereas for the HER2/HER4 heterodimer, anti-HER4 antibody (clone HFR1, abCam diluted at 1:50) was used. Both were incubated for 60 minutes at RT. Slides were rinsed with Wash Buffer A for 2 x 5 min under gentle agitat -

- C.

Again the slides were washed with Wash Buffer A for 2 x 5 min under gentle agitation. Applied oligonucleotides hybridise to both PLA probes during Hybridisation and Ligation incubation, which took place for 30 minutes at 37˚C. Slides were washed with Wash Buffer A for 2 x 2 min under gentle agitation and amplification, performed by appliance of nucleotides and polymerase, took place for 120 minutes at 37˚C. To detect the signals, all slides were washed with Wash Buffer A for 2 x 2 min under gentle agitation and

submitted to a detection stage using Horse Radish Peroxidase (HRP) labelled probes incubated for 30 minutes at RT followed by substrate solution for 10 minutes at RT. Subsequently the slides were washed with Wash Buffer A for 2 x 2 min under gentle agitation and the nuclear staining was performed using Duolink nuclear staining for 2 min at RT and washing the slides under running tap water for 10 min. To finish slides were mounted using Duolink® *in situ* Brightfield Mounting Medium (Duolink, Sweden) after dehydration process already mentioned above.

2.5.1.3 Image analysis

To quantify the HER heterodimers in breast cancer cells the software Duolink ImageTool® (Olink, Sweden) adapted to brightfield version (Figure 2.5) was employed. High-resolution digital images at 40x were obtained using high definition digital camera (Digital Sight-DS-SM, Nikon, Japan), three images were obtained from each case and exported to be analysed using Duolink ImageTool®. Different variables, such as total number of signals in nuclei, total number of signals in cytoplasm, total number of signals, total number of cells, region of interest area (ROI), were achieved through the bioinformatics software (Table 2.3). An average three analysis for each case was obtained for the number of signals per cell and used for all statistic analysis.

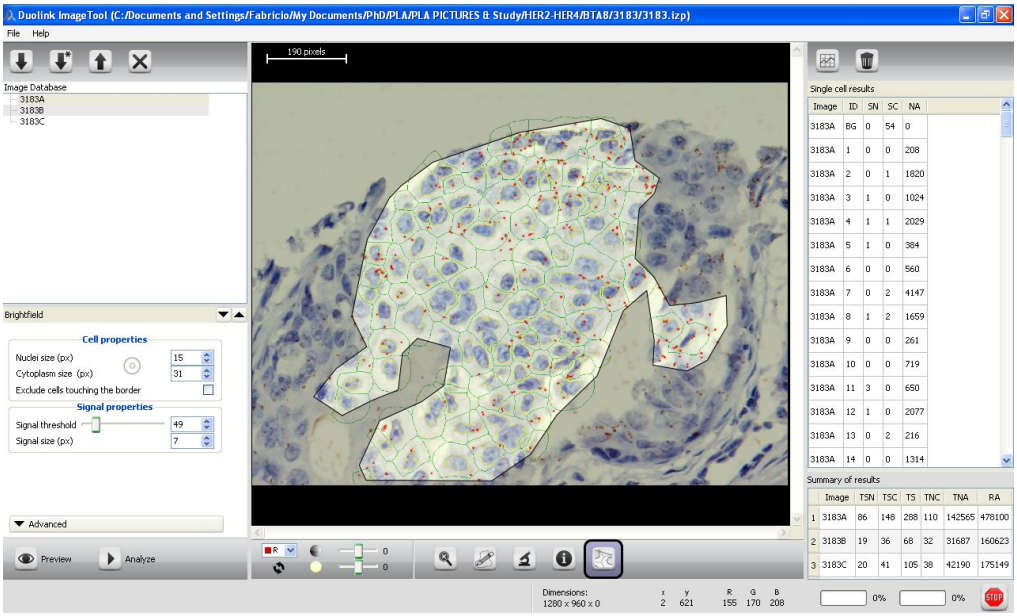

Figure 2.5 – Duolink Image Tool Analysis for brightfield microscopy. One of the three images for the case 3183 is represented showing the area of interest selected for the analysis.

Table 2.3 – Example of summary of the results exported (case number 2895) from Duolink Image tool including calculation performed by the researched (highlighted), obtaining final average number of signals per cell used on statistical studies.

Image file	Total of Signals in Nuclei	Total of Signals in Cytoplasm	Total of Signals	Total of Cells	Total Nuclei Area	ROI Area	Signals /ROI	Normalised *1000	Average	Signal/Cell	Average
2895A	492	2095	2750	181	180034	594267	0.00462755	4.62754957		15.19337	
2895B	73	733	890	57	39652	204440	0.00435336	4.35335551	4.031841151	15.614035	13.80723
2895C	260	1131	1486	140	127375	477105	0.00311462	3.11461838		10.614286	
Min Nucleus Diameter:	15										
Cytoplasm Diameter:	31										
Signal Threshold:	41										
Signal Size:	7										
Signal Channel:	R										
Nuclei Channel:	B										
Ignore cells at border:	0										
Nuclei separation:	0										
Nuclei intensity threshold:	16										

2.5.1.4 Quantification of HER family interactions using **in situ** *PLA*

After following the *in situ* PLA methods explained above we acquired visualisation under brightfield microscope of HER2 protein expression by using two PLA probes against the same primary antibody (PLA detection of single protein). However HER2+ cases are characterised by overexpression of this monomer, which turns impossible to distinguish any individual blob representing each protein structure. Both human eyes and the software available were not skilled enough to distinguish and quantify each blob (Figure 2.6A). Therefore it was possible to understand that this innovative technique possesses some limitations, when concerns single protein expression quantification.

In Figure 2.6B is possible to observe the detection of HER3 monomer, revealing as an example that displays distinguishable blobs possible to quantify due to lowers levels comparing with HER2+ cases. Therefore detection of dimers such HER2/HER3, will be quantifiable due to the low number of HER3 monomer that limits the quantity of blobs possible to observe and quantify. Furthermore heterodimerisation is not a cellular physical structure but a reaction dependent on several molecules. Therefore any signal detected only represents reactions that were occurring at that moment, which reduces the number of signals and the possibility of quantification (Figure 2.6C).

This technique had to be adapted and optimised to our study. Antibodies concentrations were submitted to several trials until individual and cancer specific discreet signals were observed. The background was also an issue and

was reduced by using double volume of wash buffer and lower antibody concentrations.

During every PLA procedure negative controls were characterised by the absence of only one primary antibody. These samples were observed cautiously in order to find whether any false reaction occurred and accept the PLA reaction results.

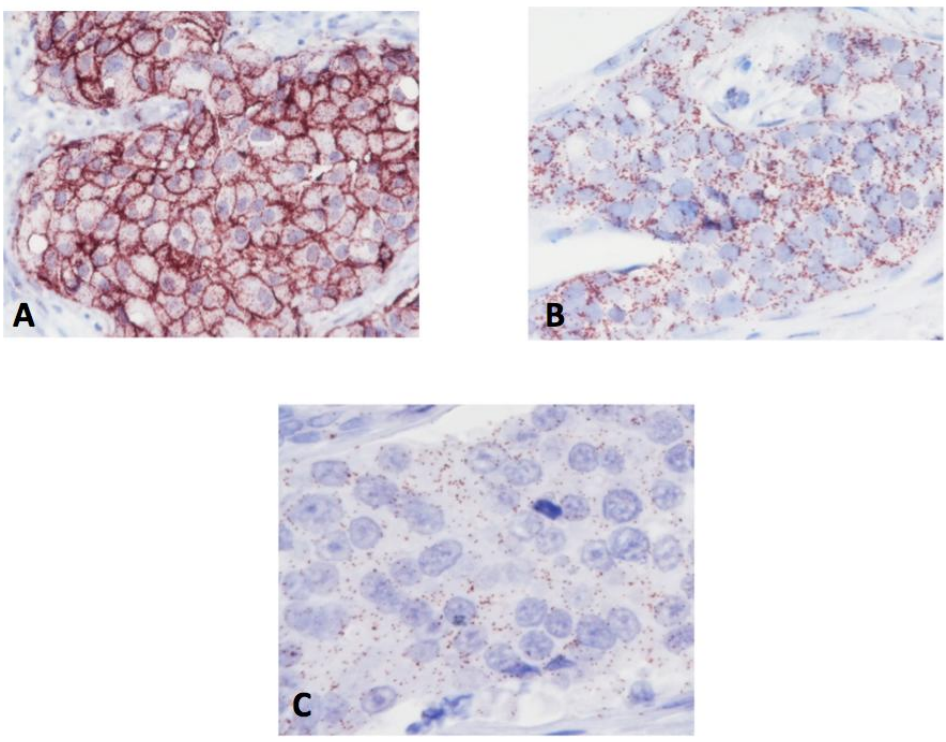

Figure 2.6 – *in situ* Proximity Ligation Assay (PLA) analysis. Examples of breast cancer cells revealing detection of single proteins and proteins interactions by hybridisation of chromogenic labelled oligonucleotides; the brown/red signals represent the specific event for each case. **A)** *in situ* PLA using primary antibody against HER2 only and impossible to be quantified due to signal distinguish impossibility. **B)** *in situ* PLA using primary antibody against HER3 only. **C)** *in situ* PLA using primary antibodies against both HER2 and HER3, being revealed only the dimerisation events.

2.6 Statistical analyses

2.6.1 Correlation Analyses

Relationship between different variables was performed using Spearman rank order correlation in order to understand the strength and direction of the linear correlation. Pearson's chi-square association (χ^2) test was also employed to verify the independency used to relate frequencies amongst the various parameters across the frequencies of another variable. Kappa (κ) measurement was employed to understand the concordance between scores of the same variable in order to validate the method used[342].

2.6.2 Survival Analyses

The Kaplan-Meier method was used to determine the Breast Cancer Specific Survival (BCSS) and Disease Free Interval (DFI) analyses. The respective significances were determined by the log-rank test[343]. This type of analysis stipulates an evaluation of the fraction surviving to a separately period of time. By relating the survival curves of either two or more patient clusters, the statistical disparity amongst the groups replicated the probability that the detected variances could happen by chance. Multivariate analyses operating Cox proportional hazard regression model[344] was applied to determine the influence of the HER2 monomer and HER heterodimers, adjusting to different variables, on BCSS and DFI. It was assessed the proportional hazard regression models of each covariate individually (tumour size, tumour grade and tumour stage).

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS 19.0 Inc. for Windows, Chicago, Illinois). Frequency histograms were examined to group the expression groups, which were used throughout the analyses. All p-values of <0.05 (two-sided) was considered statistically significant.

2.6.3 Cut-off points

To select the ideal cut-off it was used X-tile bio-informatics software (version 3.4.7. Robert L Camp, 2005). The ideal cut-off point for each biomarker or heterodimers reaction was achieved with the p-value and relative risk against BCSS[345]. To evade the issue of achieving several cut-points, X-Tile executes standard Monte Carlo cross-validation in order to achieve accurate p-values[345]. The cut-off values presented in Table 2.2 for EGFR, HER3, HER4, ER, PgR, CK7/8, CK18, CK5/6, CK14, p21, Bcl-2, p53, BRCA1 and MUC-1 have been previously described[346]. The values of Ki67 cut-off were also previously determined by a different project[347].

2.7 Ethics

Nottingham Research Ethics Committee 2 approved this research project under the title of "Development of a molecular genetics classification of breast cancer".

3. CHAPTER 3: The clinical relevance of HER2 and p95^{HER2} in breast cancer

3.1 Introduction

3.1.1 HER2 in breast cancer

HER2 overexpression and gene amplification is detected on 10%-23% of total breast cancer cases being described as HER2 positive breast cancers[21,348,349]. These cases reveal very poor prognosis, however can benefit from a specific treatment by using a monoclonal antibody, trastuzumab, which interacts specifically with cell overexpressing HER2 protein[130].

3.1.2 Clinical determination of HER2 in breast cancer

Analysis of *HER2* amplification and overexpression in patients with breast cancer is essential for an appropriate clinical assessment; moreover it is decisive in categorising patients that are suitable to receive trastuzumab treatment. Though some discordance is noted between immunohistochemistry (IHC) that evaluates protein overexpression and Fluorescent *in situ* Hybridisation (FISH) that classifies levels of gene amplification[350,351]. Guidelines have been established by the College of American Pathologists (CAP), American Society of Clinical Oncologists (ASCO) and by the UK NHS Breast Screening Programme for diagnostic laboratories to determine an accurate and comparable HER2 status in breast tumours[86,352].

Immunohistochemistry

HER2 assessment using IHC in routine FFPE tissues has several advantages in clinical diagnostics. With this relatively economical procedure is possible to preserve the tissue morphology and to identify areas with higher expression *in situ*, which is important for classification in heterogeneous samples. Even though this technique requires standardisation in terms of methodology as type of tissue fixation[353], type of antibody and its concentration[354], and incubation time amongst other variables to avoid inconsistency, it produces reliable results.

Fluorescent in situ Hybridisation

FISH technology evaluation is well established for identification of denatured specific DNA sequences using fluorescently labelled complementary probes. Using this technique it is possible to visualise gene amplification, translocations or even deletions. Determination of such gene aberrations that occur in cancer cells, make possible the understanding of the prognosis and direct the patient to a specific therapy[355]. FISH is utilised in routine laboratories to detect gene amplification in those cases with moderate HER2 protein overexpression[356]. This is particularly important as only those breast carcinomas designated as IHC 2+ (equivocal) that also show gene amplification will possibly benefit from trastuzumab[168]. However FISH results are greatly affected by fixative techniques and the morphology of the cells is not perceptible scoring process[357,358].

Chromogenic in situ Hybridisation

Chromogenic *in situ* Hybridisation (CISH) has emerged over the last few years as an alternative to FISH and is based on a simple bonding reaction of a specific targeted probe to an enzymatic indicator for chromogenic reaction – horseradish peroxidase (HRP). Peroxidase reaction only requires brightfield microscopy, a common instrument in all pathological laboratories unlike the more expensive fluorescent equipment, which also requires specific expertise to evaluate. CISH, in similarity to FISH, can incorporate dual-colours, which allows comparison to a centromeric probe (Figure 3.1). Moreover this dual-colour method is more reliable when compared with one-colour version due to being able to accurately distinguish real amplification from aneuploidy. Additionally a chromogenic reaction is more permanent whereas fluorescent signals are lost within days/weeks [359] and the cells morphology is perceptible not being necessary an expertise to perform a plausible scoring [358].

3.1.3 p95^{HER2} or HER2 carboxy terminal fragment

p95^{HER2} or the HER2 carboxy terminal fragments (CTFs) is a HER2 active fragment that might become a unique biomarker identifying a subtype of HER2 (p185^{HER2}) positive breast cancer with exclusive biological and prognosis characteristics[173,360]. p95^{HER2} is related with resistance to trastuzumab treatment due to lack of ectodomain, which is crucial for trastuzumab to attach[175].

There are two mechanisms that instigate p95^{HER2} creation: a proteolytic shedding of the ectodomain of HER2 protein or by translation of mRNA encoding of internal codons[166,361]. The exclusion of the ectodomain is carried

out by the metalloprotease ADAM10 (α-secretase), results in a membrane p95^{HER2} molecule (648-CTF) of 95 to 100 kDa[362,363] (Figure 3.1). The translation process results in two different types of p95^{HER2} fragment, the 611-CTF and the 687-CTF, which are respectively 100 to 115 kDa and 90 to 95 kDa. Their names are linked with the codons where the translation process begins. The extra 76 amino acids in 687-CTF results in the possession of a transmembrane domain and a small cysteine-rich ectodomain[361]. Additionally 687-CTF and 611-CTF are different in their potency of reaction, 611-CTF has activity highly similar to the full length HER2 but is considerably more reactive to trigger downstream pathways[179]. Moreover the efficiency of driving cell migration is much higher by this hyperactive fragment when compared with the HER2 (p185) molecule[364]. Even though these HER2 structures are small and abnormal, it does not avoid holding into the cellular membrane, 687-CTF may also be detected in the cytoplasm[361]. The main difference amongst these two small fragments resides in the presence of 5 cysteine located on the diminutive ectodomain of 611-CTF. These cysteines form disulphide bonds and therefore the activated homodimer is conserved by covalent bonds[179]. The fragment 687-CTF, is not activated in the same way due to it not possessing any cysteines in the ectodomain, which does not encourage dimerisation[42]. Moreover this small member, 687-CTF, also may react with HER2[174].

611-CTF expression has been suggested to be able to discriminate a subclass of *HER2* amplified breast cancers, which possess a clear and particular biological and clinical outcome[365]. The frequency of ER positivity amongst 611-CTF positive cancer cells, compared with HER2 positive, is considerably lower

reaching only 30%[365,366]. The same p95^{HER2} fragment is highly associated with metastasis localised in lungs, moreover the relapse time and survival time were considerably shorter when compared with full length HER2 positive carcinomas[366]. Comparing both highly reactive forms of p95^{HER2}, 611-CTF and 648-CTF, the first triggers not only proliferative signalling pathways like Akt[179] but also cell migration[364] as it tends to form homodimers by disulphide bonds. Conversely, 648-CTF does not form homodimers and therefore its reactions are vey similar to those develop by HER2[173].

p95^{HER2} is a very reactive molecule[166] and easily reacts with identical molecules forming homodimers[179]. All this high reactivity might be associated with the lack of ectodomain, which might perform as a deplete factor on development of heterodimers[367]. Therefore and as expected cases expressing p95^{HER2} molecules are associated with worse outcome and metastasis development when compared with those patients overexpressing HER2[173].

The current system of HER2 breast cancer classification has been revealing lack of accuracy on diagnosis and therefore selection of patients to be submitted to trastuzumab need an even more specific classification. This way in this chapter by characterising HER2 amongst both series it is hypothesised that some variances on HER2 classification might reveal different prognosis or even development of resistance under trastuzumab therapy. In this chapter it is going to be analysed the correlation between IHC and CISH, and understand outcome among different HER2 status, paying more attention to amplified or not amplified cases. Moreover we will give a special attention on survival

outcome in HercepTest™ 2+ cases both amplified and not amplified. Additionally examining p95^{HER2} status amongst these series might reveal to be an additional marker that significantly impacts on HER2 patients' outcome.

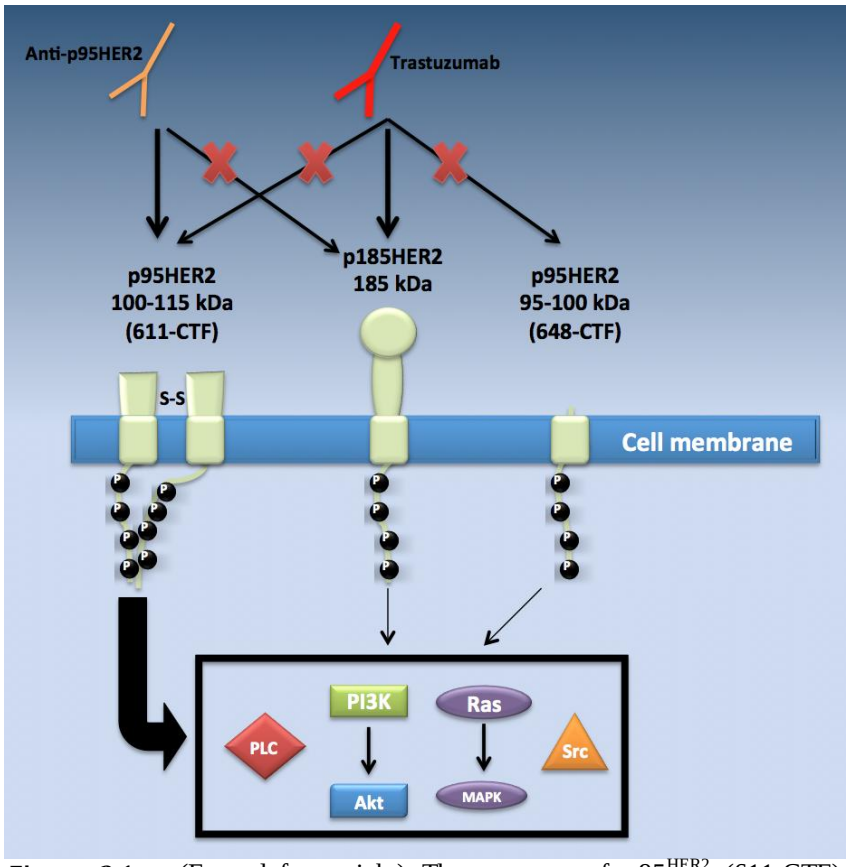

Figure 3.1 – (From left to right) The structure of p95^{HER2} (611-CTF) originated by expression of a codon localised in position 611, the structure of full length HER2 or p185^{HER2} and the structure of p95 ^{HER2} created by cleavage process of a p185^{hER2} molecule and does not possess any ectodomain substructure. Trastuzumab is not able to interact with any structure without the entire ectodomain. The anti-p95^{HER2} antibody is able to interact with the epitopes localised in 100-115kDa, which are masked in the full length HER2 molecule.

3.2 Methods

Two breast cancer series were analysed for HER2: Unselected series (n = 1858) and the HER2+ trastuzumab adjuvant series (n = 143). The methodology including immunohistochemical detection, *in situ* hybridisation and pre-treatments are described in chapter 2 and it was performed in TMAs for both series, with exception for Ki67, which had to be performed in full sections to detect the hotspot of the tumour, i.e., the high proliferative regions. Additionally the classification method for HER2 protein expression and gene amplification is also described in chapter 2. The $p95^{HER2}$ IHC scoring was performed in the same way as HER2 (p185) due to similar membrane staining. Therefore four groups were obtained on $p95^{HER2}$ scoring, classified as weak staining (negative) or as strong staining (positive).

3.3 Results

3.3.1 HER2 expression in breast cancer

In the Unselected series of breast cancers, a total of 1604 (86.3%) cases showed negative (0) or very weak (1+) membrane protein expression, 180 (9.7%) cases show overexpression of HER2 (3+) protein and were classified as HER2 positive. The remaining 74 (4.0%) cases were classified as borderline or equivocal (2+) due to revealing a weak to moderate protein expression. In the HER2+ Trastuzumab series, 30/143 (21%) cases were scored as equivocal and 113/143 (79%) cases were classified as positive. All the equivocal cases revealed HER2 gene amplification as well and therefore classified as HER2+ (Figure 3.2).

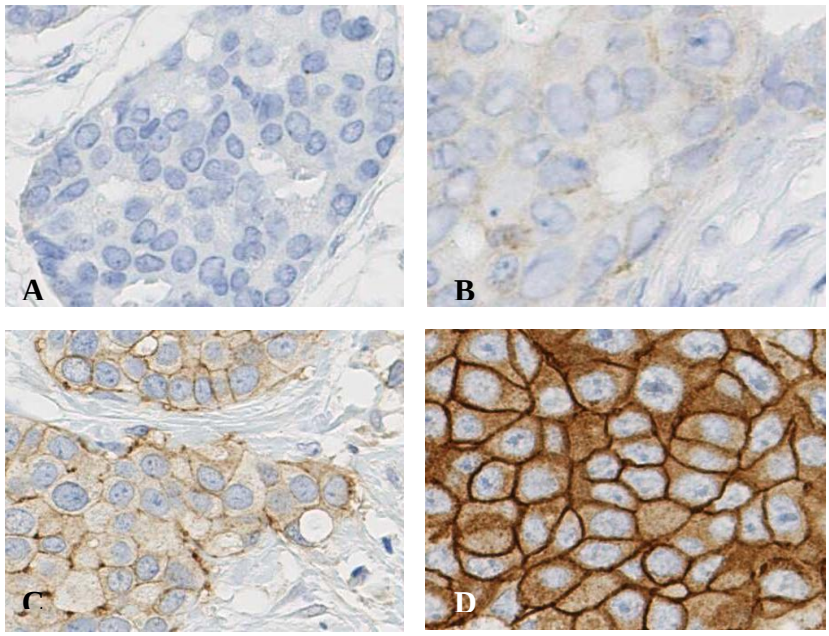

Figure 3.2 – Immunohistochemistry analysis. Detection of HER2 protein expression in breast cancer cores by immunohistochemistry (HercepTest™) and scored according to American Society of Clinical Oncology guidelines. The cells were labelled with a specific antibody against HER2 to detect the intensity of expression of this protein present in the cell membrane. The cells are scored in four different classes of protein expression: (A) 0 when a faint membranous immunoreactivity was observed in less than 10% of the cancer cells, (B) 1+ when more than 10% of the invasive carcinoma cells show a barely perceptible membranous reactivity, (C) 2+ when more than 10% of the tumour cells show moderate but incomplete membranous reactivity, and (D) 3+ when more than 30% of the cells show strong and complete membranous reactivity.

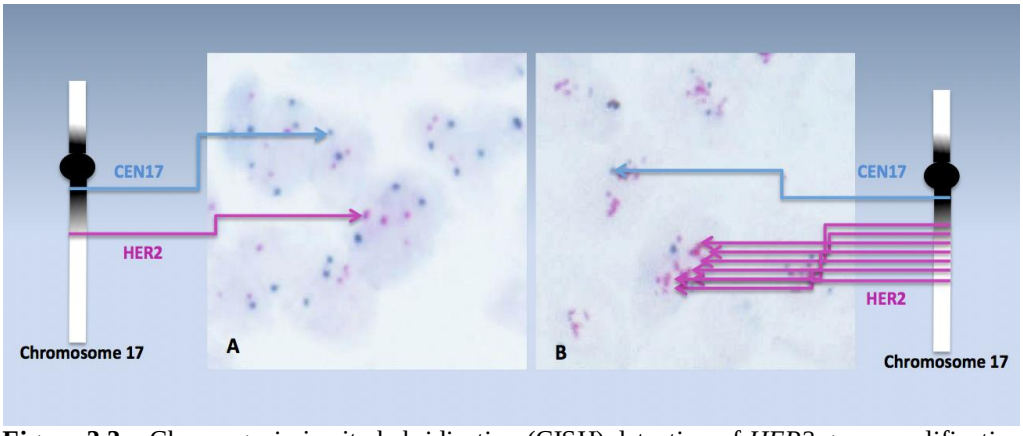

Figure 3.3 – Chromogenic *in situ* hybridization (CISH) detection of *HER2* gene amplification in breast cancer cells. A – Breast cancer cells revealing no *HER2* amplification (*HER2*/CEN17 ratio < 2). B – Breast cancer cells revealing *HER2* gene amplification (*HER2*/CEN17 ratio ≥ 2).

3.3.2 HER2 amplification in breast cancer

A total of 1115 Unselected cases were available for *HER2* amplification analysis determined by CISH (**Figure 3.3**). Amongst them 163 (14.6%) cases showed HER2/CEN17 ≥ 2 and were deemed cases that had *HER2* amplification and the remaining 952 (85.4%) cases were not amplified for HER2 (Table 3.1).

3.3.3 HER2 status in breast cancer

HER2 protein expression data was analysed against *HER2* ratio data to define the HER2 status as positive or negative according to ASCO guidelines, even though in this project CISH was used instead of FISH. Within the borderline cases from the Unselected series, a total of 30/74 (40.5%) do not reveal gene amplification and were classified as negative. Therefore a total of 1634 cases (87.9%) in the Unselected Series were deemed HER2 negative whereas 224 (12.1%) were HER2 positive (Table 3.1). These cases formed the HER2 trastuzumab naïve series. Combining the HER2 trastuzumab naïve series and the HER2 trastuzumab series, it resulted in the HER2+ series consisting of 367 tumours.

3.3.4 Correlation between HER2 expression with *HER2* amplification

There was a significant correlation between HER2 protein expression and *HER2* gene amplification determined by CISH ($p < 0.001$). Among the 952 (85.4%) *HER2* non-amplified carcinomas, only one case (0.1%) was associated with overexpression of the protein. A total of 921 (96.7%) *HER2* non-amplified samples were associated with negative HER2 protein expression (0

or 1+) and 30 (3.2%) cases that showed an equivocal protein expression were non-amplified. In contrast, only three cases (1.8%) amongst a total of 163 *HER2* amplified cases demonstrated negative HER2 protein expression. Nevertheless a total of 160 (98.2%) cases exhibiting *HER2* amplification were associated with moderate to strong HER2 protein expression (Table 3.1).

Table 3.1 – Relation between HER2 protein expression and *HER2* gene amplification in breast cancer.

	HER2 IHC					
	0+ (%)	**1+** (%)	**2+** (%)	**3+** (%)	**Total** (%)	**P value** (Chi-Square)
HER2 CISH (Ratio)						
Non-Amplification	756 (100)	165 (98.2)	30 (40.5)	1 (0.9)	952 (85.4)	
Amplification	0 (0.0)	3 (1.80)	44 (59.5)	116 (99.1)	163 (14.6)	<0.001 (940.539)
Total	756 (100)	168 (100)	74 (100)	117 (100)	1115 (100)	
HER2 Status						
Negative	1329 (100)	275 (100)	30 (40.5)	0 (0.0)	1634 (87.9)	
Positive	0 (0.0)	0 (0.0)	44 (59.5)	180 (100)	224 (12.1)	<0.001 (1689.758)
Total	1329 (100)	275 (100)	74 (100)	180 (100)	1858 (12.1)	

3.3.5 Correlation of HER2 protein, *HER2* amplification and HER2 status with clinicopathological parameters

Overexpression of HER2 protein in the Unselected series was associated with a higher tumour stage ($p = 0.002$), larger tumour size ($p = 0.006$) higher tumour grade, more nuclear pleomorphism, higher mitotic frequency, less tubule formation and presence of distant metastases (all $p < 0.001$). HER2 protein overexpression was also significantly associated with poor NPI prognostic groups ($p < 0.001$) and death due to breast cancer ($p < 0.001$). The age of the patients did not reveal significant association with HER2 overexpression ($p = 0.149$) (Table 3.2).

There was a highly significant association between HER2 protein overexpression and histological tumour type where the majority of cases were invasive ductal carcinoma ($p < 0.001$) (Table 3.4 and Table 3.5)

As expected, the presence of *HER2* amplification and HER2 positive status showed similar significant associations with the same parameters as HER2 protein overexpression (Table 3.3).

Table 3.2 – The relation between HER2 protein expression with clinicopathological parameters.

Parameters	HER2 IHC				
	0+ (%)	**1+** (%)	**2+** (%)	**3+** (%)	**p value** (Chi-Square)
Tumour Grade					
1	280 (21.2)	65 (23.8)	4 (5.4)	5 (2.8)	<0.001 (139.041)
2	457 (34.5)	115 (42.1)	21 (28.4)	23 (12.8)	
3	586 (44.3)	93 (34.1)	49 (66.2)	152 (84.4)	
Tubule Formation					
1	101 (7.9)	14 (5.5)	2 (2.8)	2 (1.1)	<0.001 (42.934)
2	421 (32.8)	107 (42.1)	19 (26.4)	36 (20.3)	
3	762 (59.3)	133 (52.4)	51 (70.8)	139 (78.5)	
Pleomorphism					
1	43 (3.4)	8 (3.2)	0 (0.0)	0 (0.0)	<0.001 (103.870)
2	556 (43.4)	126 (49.8)	17 (23.6)	19 (10.9)	
3	681 (53.2)	119 (47.0)	55 (76.4)	156 (89.1)	
Mitotic Frequency					
1	550 (42.8)	113 (44.5)	8 (11.1)	13 (7.3)	<0.001 (125.939)
2	213 (16.6)	57 (22.4)	18 (25.0)	34 (19.2)	
3	521 (40.6)	84 (33.1)	46 (63.9)	130 (73.4)	
Tumour Stage					
1	861 (65.0)	188 (68.9)	41 (55.4)	97 (54.2)	0.002 (20.938)
2	363 (27.4)	62 (22.7)	26 (35.1)	54 (30.2)	
3	101 (7.6)	23 (8.4)	7 (9.5)	28 (15.6)	
Distant Metastasis					
Negative	956 (72.4)	195 (70.9)	40 (55.6)	97 (53.9)	<0.001 (32.977)
Positive	364 (27.6)	80 (29.1)	32 (44.4)	83 (46.1)	
Local Recurrence					
Negative	812 (62.9)	161 (59.2)	50 (47.2)	86 (48.6)	<0.001 (21.474)
Positive	479 (37.1)	111 (40.8)	56 (52.8)	91 (51.4)	
Regional Recurrence					
Negative	1182 (91.6)	236 (86.8)	91 (85.8)	154 (87.0)	0.015 (10.499)
Positive	109 (8.4)	36 (13.2)	15 (14.2)	23 (13.0)	
Tumour Size					
<1.5cm	371 (28.0)	68 (24.9)	15 (20.3)	30 (16.7)	0.006 (12.326)
≥1.5cm	953 (72.0)	205 (75.1)	59 (79.7)	150 (83.3)	
Vascular Invasion					
Negative	779 (59.9)	149 (54.6)	37 (51.4)	96 (53.9)	0.128 (5.679)
Positive	522 (40.1)	124 (45.4)	35 (48.6)	82 (46.1)	
NPI					
Good	481 (36.7)	112 (41.5)	13 (18.3)	14 (7.9)	<0.001 (84.845)
Moderate	644 (49.2)	137 (50.7)	44 (62.0)	116 (65.5)	
Poor	187 (14.3)	21 (7.8)	14 (19.7)	47 (26.6)	
Age					
<50	413 (31.1)	93 (33.5)	44 (39.3)	67 (37.0)	0.149 (5.337)
≥50	915 (68.9)	185 (66.5)	68 (60.7)	114 (63.0)	
Death					
Negative	776 (72.1)	156 (67.5)	32 (50.8)	79 (50.6)	<0.001 (38.727)
Positive	300 (27.9)	75 (32.5)	31 (49.2)	77 (49.4)	

Table 3.3 – The relation between *HER2* gene amplification and HER2 status in breast cancer with clinicopathological parameters.

Parameters	HER2 CISH			HER2 Status		
	<2 (%)	≥2 (%)	p value (Chi-Square)	Neg (%)	Pos (%)	p value (Chi-Square)
Tumour Grade						
1	171 (18.0)	3 (1.9)	<0.001 (82.018)	349 (21.5)	5 (2.2)	<0.001 (131.835)
2	346 (36.5)	23 (14.4)		583 (35.9)	33 (14.7)	
3	432 (45.5)	134 (83.8)		694 (42.7)	186 (83.0)	
Tubule Formation						
1	51 (5.6)	1 (0.6)	<0.001 (19.952)	117 (7.5)	2 (0.9)	<0.001 (36.135)
2	318 (34.8)	35 (22.2)		537 (34.2)	46 (20.8)	
3	546 (59.7)	122 (77.2)		912 (58.2)	173 (78.3)	
Pleomorphism						
1	21 (2.3)	0 (0.0)	<0.001 (63.542)	51 (3.3)	0 (0.0)	<0.001 (100.475)
2	393 (43.0)	18 (11.5)		692 (44.3)	26 (11.9)	
3	499 (54.7)	138 (88.5)		818 (52.4)	193 (88.1)	
Mitotic Frequency						
1	359 (39.2)	9 (5.7)	<0.001 (73.209)	669 (42.7)	15 (6.8)	<0.001 (117.730)
2	156 (17.0)	29 (18.4)		279 (17.8)	43 (19.5)	
3	400 (43.7)	120 (75.9)		618 (39.5)	163 (73.8)	
Tumour Stage						
1	587 (61.9)	90 (56.6)	0.037 (6.594)	1065 (65.4)	122 (54.7)	<0.001 (17.296)
2	279 (29.4)	45 (28.3)		438 (26.9)	67 (30.0)	
3	82 (8.6)	24 (15.1)		125 (7.7)	34 (15.2)	
Distant Metastasis						
Negative	655 (69.3)	85 (53.1)	<0.001 (16.208)	1166 (71.8)	122 (54.5)	<0.001 (28.165)
Positive	290 (30.7)	75 (46.9)		457 (28.2)	102 (45.5)	
Local Recurrence						
Negative	985 (61.9)	108 (48.9)	<0.001 (13.875)	548 (59.3)	75 (47.5)	0.005 (7.743)
Positive	605 (38.1	113 (51.1)		376 (40.7)	83 (52.5)	
Regional Recurrence						
Negative	1440 (90.6)	193 (87.3)	0.130 (2.292)	827 (89.5)	137 (86.7)	0.298 (1.084)
Positive	150 (9.4)	28 (12.7)		97 (10.5)	21 (13.3)	
Tumour Size						
<1.5cm	231 (24.1)	27 (16.9)	0.039 (4.276)	446 (27.4)	38 (17.0)	0.001 (11.130)
≥1.5cm	718 (75.7)	133 (83.1)		1181 (72.6)	186 (83.0)	
Vascular Invasion						
Negative	523 (55.8)	84 (53.2)	0.535 (0.385)	943 (58.8)	118 (53.4)	0.125 (2.357)
Positive	414 (44.2)	74 (46.8)		660 (41.2)	103 (46.6)	
NPI						
Good	316 (33.7)	12 (7.7)	<0.001 (31.4)	602 (31.4)	18 (31.4)	<0.001 (31.4)
Moderate	481 (51.2)	104 (66.7)		798 (31.4)	143 (31.4)	
Poor	142 (15.1)	40 (25.6)		211 (31.4)	58 (31.4)	
Age						
<50	315 (32.6)	58 (35.6)	0.460 (0.545)	519 (31.8)	82 (36.6)	0.148 (2.095)
≥50	650 (67.4)	105 (64.4)		1114 (68.2)	142 (63.4)	
Death						
Negative	536 (68.6)	69 (49.3)	<0.001 (19.711)	945 (71.0)	98 (50.3)	<0.001 (33.828)
Positive	245 (31.4)	71 (50.7)		386 (29.0)	97 (49.7)	

Table 3.4 – HER2 protein expression with histological tumour type.

Tumour Histology	HER2 IHC				
	0+ (%)	**1+** (%)	**2+** (%)	**3+** (%)	**p Value** (Chi-Square)
Ductal	687 (52.8)	135 (50.4)	85 (75.9)	155 (87.6)	< 0.001 (119.400)
Lobular	170 (13.1)	31 (11.6)	5 (4.5)	1 (0.6)	
Medullar	33 (2.5)	4 (1.5)	2 (1.8)	7 (4.0)	
Mixed	83 (6.4)	15 (5.6)	8 (7.1)	4 (2.3)	
Other	38 (2.9)	5 (1.9)	1 (0.9)	0 (0.0)	
Tubular	289 (22.2)	78 (29.1)	11 (9.8)	10 (5.6)	
Total	1300 (100)	268 (100)	112 (100)	177 (100)	

Table 3.5 – *HER2* gene amplification and HER2 status association with histological tumour type

Tumour Histology	HER2 CISH Ratio			HER2 Status		
	< 2 (%)	**≥2** (%)	**p Value** (Chi-Square)	**Neg** (%)	**Pos** (%)	**p Value** (Chi-Square)
Ductal	510 (54.1)	140 (87.0)	< 0.001 (68.048)	838 (52.5)	193 (87.7)	< 0.001 (106.380)
Lobular	115 (12.2)	1 (0.6)		203 (12.7)	1 (0.5)	
Medullar	26 (2.8)	6 (3.7)		38 (2.4)	7 (3.2)	
Mixed	56 (5.9)	4 (2.5)		101 (6.3)	6 (2.7)	
Other	12 (1.3)	0 (0.0)		43 (2.7)	0 (0.0)	
Tubular	223 (23.7)	10 (6.2)		372 (23.3)	13 (5.9)	
Total	942 (100)	161 (100)		1595 (100)	220 (100)	

3.3.6 Correlation of HER2 expression, *HER2* amplification and HER2 status with hormone and HER family receptors

Overexpression of HER2 protein showed a significant negative association with both ER and PgR positivity ($p < 0.001$). A positive association was observed between HER2 protein expression and those tumours expressing HER4 ($p < 0.001$), but not with HER3 ($p = 0.505$) and EGFR ($p = 0.066$). Additionally, no association was observed between HER2 protein overexpression and basal phenotype ($p = 0.918$) (Table 3.6).

The results of *HER2* amplification (≥ 2 ratio) and HER2 status revealed similar significant associations with the respective biomarkers as protein expression (Table 3.7).

Table 3.6 – Relationship between HER2 expression with hormone receptors and HER family receptors.

Biomarker	HER2 IHC				
	0+ (%)	1+ (%)	2+ (%)	3+ (%)	p value (Chi-Square)
Basal					
Negative	974 (79.3)	204 (77.9)	52 (76.5)	136 (79.1)	0.918
Positive	255 (20.7)	58 (22.1)	16 (23.5)	36 (20.9)	(0.505)
EGFR					
Negative	932 (83.1)	176 (81.9)	40 (75.5)	112 (75.2)	0.066
Positive	189 (16.9)	39 (18.1)	13 (24.5)	37 (24.8)	(7.203)
HER3					
Negative	135 (12.7)	30 (14.9)	8 (15.4)	14 (9.7)	0.505
Positive	924 (87.3)	171 (85.1)	44 (84.6)	130 (90.3)	(2.340)
HER4					
Negative	255 (23.9)	49 (23.6)	9 (17.3)	13 (9.0)	<0.001
Positive	813 (76.1)	159 (76.4)	43 (82.7)	131 (91.0)	(17.173)
ER					
Negative	306 (24.7)	37 (14.2)	16 (25.4)	106 (60.6)	<0.001
Positive	935 (75.3)	224 (85.8)	47 (74.6)	69 (39.4)	(125.208)
PgR					
Negative	467 (37.9)	84 (33.3)	33 (53.2)	126 (72.8)	<0.001
Positive	764 (62.1)	170 (66.7)	29 (46.8)	47 (27.2)	(86.914)

Table 3.7 – Relationship between *HER2* gene amplification and HER2 status with hormone receptors and HER family receptors.

Biomarker	HER2 CISH (Ratio)			HER2 Status		
	<2 (%)	≥2 (%)	p value (Chi-Square)	Neg (%)	Pos (%)	p value (Chi-Square)
Basal						
Negative	717 (79.7)	121 (79.1)	0.869	1197 (79.0)	169 (78.6)	0.905
Positive	183 (20.3)	32 (20.9)	(0.027)	319 (21.0)	46 (21.4)	(0.014)
EGFR						
Negative	666 (83.0)	92 (74.2)	0.017	1125 (82.9)	135 (74.6)	0.006
Positive	136 (17.0)	32 (25.8)	(3.723)	232 (17.1)	46 (25.4)	(7.461)
HER3						
Negative	97 (12.6)	13 (10.7)	0.551	168 (13.1)	19 (10.8)	0.386
Positive	675 (87.4)	109 (89.3)	(0.356)	1112 (86.9)	157 (89.2)	(0.750)
HER4						
Negative	183 (23.7)	12 (10.0)	0.001	308 (23.8)	18 (10.2)	<0.001
Positive	590 (76.3)	108 (90.0)	(11.379)	988 (76.2)	158 (89.8)	(16.472)
ER						
Negative	206 (23.0)	81 (52.6)	<0.001	345 (22.7)	120 (56.1)	<0.001
Positive	690 (77.0)	70 (46.4)	(61.015)	1178 (77.3)	94 (43.9)	(106.916)
PgR						
Negative	321 (36.3)	107 (72.3)	<0.001	559 (37.1)	151 (71.6)	<0.001
Positive	564 (63.7)	41 (27.7)	(67.817)	948 (62.9)	60 (28.4)	(90.697)

3.3.7 Correlation of HER2 expression, *HER2* amplification and HER2 status with biomarkers

HER2 expression revealed high association with CK18, pAkt, PI3K, p53, PTEN and Ki67 (all cases $p < 0.001$). The negative association was observed against Bcl-2 ($p < 0.001$), BRCA1 ($p < 0.001$) and p21 ($p = 0.008$) (Table 3.8).

The relationship of both *HER2* amplification and HER2 status against the biomarkers is summarised in Table 3.9. In all cases the association between these two variables and the biomarkers are very similar with HER2 protein expression.

Table 3.8 – Relationship between HER2 expression with hormone receptors and biomarkers.

Biomarkers	HER2 IHC				
	0+ (%)	**1+ (%)**	**2+ (%)**	**3+ (%)**	**p value** (Chi-Square)
CK5/6					
Negative	1065 (84.5)	221 (84.0)	59 (85.5)	148 (85.1)	0.987
Positive	195 (15.5)	42 (16.0)	10 (14.5)	26 (14.9)	(0.138)
CK 7/8					
Negative	29 (2.3)	1 (0.4)	1 (1.5)	1 (0.6)	0.099
Positive	1232 (97.7)	263 (99.6)	67 (98.5)	173 (99.4)	(6.279)
CK14					
Negative	1109 (89.2)	228 (86.4)	62 (88.6)	149 (86.1)	0.430
Positive	134 (10.8)	36 (13.6)	8 (11.4)	24 (13.9)	(2.762)
CK 18					
Negative	221 (18.5)	19 (8.3)	6 (9.7)	11 (6.9)	<0.001
Positive	971 (81.5)	211 (91.7)	56 (90.3)	149 (93.1)	(28.089)
Bcl-2					
Negative	65 (35.1)	17 (28.8)	2 (22.2)	19 (76.0)	<0.001
Positive	120 (64.9)	42 (71.2)	7 (77.8)	6 (24.0)	(19.117)
pAkt					
Negative	222 (27.3)	28 (16.3)	6 (11.5)	28 (22.4)	0.002
Positive	591 (72.7)	144 (83.7)	46 (88.5)	97 (77.6)	(14.788)
PI3K					
Negative	260 (28.0)	60 (28.2)	12 (17.4)	16 (11.9)	<0.001
Moderate	256 (27.6)	57 (26.8)	21 (30.4)	22 (16.3)	(39.497)
Strong	413 (44.5)	96 (45.1)	36 (52.2)	97 (71.9)	
P21					
Negative	114 (85.1)	28 (63.6)	10 (83.3)	15 (93.8)	0.008
Positive	20 (14.9)	16 (36.4)	2 (16.7)	1 (6.3)	(0.008)
MUC-1					
Negative	120 (11.1)	23 (10.8)	5 (10.0)	9 (6.3)	0.406
Moderate	432 (40.0)	84 (39.6)	26 (52.0)	62 (43.1)	(6.157)
Strong	528 (48.9)	105 (49.5)	19 (38.0)	73 (50.7)	
BRCA1					
Negative	540 (51.5)	97 (48.5)	27 (51.9)	97 (69.8)	<0.001
Positive	509 (48.5)	103 (51.5)	25 (48.1)	42 (30.2)	(18.326)
P53					
Negative	936 (75.2)	193 (74.5)	45 (68.2)	90 (52.3)	<0.001
Positive	309 (24.8)	66 (25.5)	21 (31.8)	82 (47.7)	(40.798)
PTEN					
Weak	104 (23.8)	16 (14.8)	4 (10.8)	5 (6.5)	<0.001
Moderate	131 (30.0)	49 (45.4)	18 (48.6)	31 (40.3)	(24.537)
Strong	202 (46.2)	43 (39.8)	15 (40.5)	41 (53.2)	
Ki67					
Weak	454 (43.0)	105 (50.5)	10 (16.4)	29 (20.7)	
Moderate	315 (29.8)	75 (36.1)	28 (45.9)	61 (43.6)	<0.001
Strong	288 (27.2)	28 (13.5)	23 (37.7)	50 (35.7)	(61.399)

Table 3.9 – Relationship between HER2 gene amplification and HER2 status with biomarkers.

Biomarkers	HER2 CISH (Ratio)			HER2 Status		
	<2 (%)	≥2 (%)	p value (Chi-Square)	Neg (%)	Pos (%)	p value (Chi-Square)
CK5/6						
Negative	782 (85.4)	130 (83.9)	0.627	1310 (84.6)	183 (84.3)	0.927
Positive	134 (14.6)	25 (16.1)	(0.236)	239 (15.4)	34 (15.7)	(0.008)
CK 7/8						
Negative	19 (2.1)	2 (1.3)	0.512	30 (1.9)	2 (0.9)	0.294
Positive	894 (97.9)	153 (98.7)	(0.430)	1520 (98.1)	215 (99.1)	(1.100)
CK14						
Negative	817 (90.1)	137 (89.0)	0.671	1361 (88.7)	187 (86.6)	0.355
Positive	90 (9.9)	17 (11.0)	(0.181)	173 (11.3)	29 (13.4)	(0.856)
CK 18						
Negative	142 (16.7)	13 (9.5)	0.032	242 (16.7)	15 (7.6)	0.001
Positive	710 (83.3)	124 (90.5)	(4.601)	1205 (83.3)	182 (92.4)	(10.911)
Bcl-2						
Negative	50 (31.3)	14 (70.0)	0.001	83 (33.3)	20 (69.0)	<0.001
Positive	110 (68.8)	6 (30.0)	(11.650)	166 (66.7)	9 (31.0)	(14.140)
pAkt						
Negative	170 (24.8)	22 (18.0)	<0.107	252 (25.0)	32 (20.6)	<0.238
Positive	516 (75.3)	100 (82.0)	(2.604)	755 (75.0)	123 (79.4)	(1.395)
PI3K						
Negative	212 (24.9)	17 (11.6)	<0.001	326 (27.9)	22 (12.5)	<0.001
Moderate	246 (28.8)	26 (17.8)	(29.756)	325 (27.8)	31 (17.6)	(40.844)
Strong	395 (46.3)	103 (70.5)		519 (44.4)	123 (69.9)	
p21						
Negative	122 (78.2)	16 (88.9)	0.289	148 (80.0)	19 (90.5)	0.246
Positive	34 (21.8)	2 (11.1)	(1.123)	37 (20.0)	2 (9.5)	(1.349)
MUC-1						
Negative	87 (11.4)	10 (8.3)	0.282	143 (10.9)	14 (8.0)	0.357
Moderate	308 (39.1)	56 (46.3)	(2.529)	526 (40.1)	78 (78.0)	(2.063)
Strong	393 (49.5)	55 (45.4)		642 (49.0)	83 (83.0)	
BRCA1						
Negative	385 (50.1)	79 (64.8)	0.003	646 (50.9)	115 (66.9)	<0.001
Positive	384 (49.9)	43 (35.2)	(9.103)	622 (49.1)	57 (33.1)	(15.393)
p53						
Negative	690 (76.7)	84 (56.8)	<0.001	1150 (75.1)	114 (54.0)	<0.001
Positive	210 (23.3)	64 (43.2)	(26.092)	381 (24.9)	97 (46.0)	(41.411)
PTEN						
Weak	92 (22.2)	4 (5.1)	0.002	123 (22.0)	6 (6.1)	0.001
Moderate	144 (34.7)	36 (45.6)	(12.729)	186 (33.2)	43 (43.4)	(13.994)
Strong	179 (43.1)	39 (49.3)		251 (44.8)	50 (50.5)	
Ki67						
Weak	306 (39.9)	24 (17.6)	<0.001	566 (43.9)	32 (18.1)	<0.001
Moderate	253 (33.0)	59 (43.4)	(24.998)	402 (31.2)	77 (43.5)	(43.395)
Strong	207 (27.0)	53 (39.0)		321 (24.9)	68 (38.4)	

3.3.8 p95^{HER2} expression in breast cancer

Amongst the Unselected series, p95^{HER2} negative cases represented 492/543 (90.6%) cases and the positive cases 51/543 (9.4%) (Figure 3.4). Amongst the HER2+ series the frequency of the p95^{HER2} increased to 85/177 (48.1%) and the negative cases were identified in 92/177 (51.9%).

The association of p95^{HER2} protein expression with HER2 expression was highly significant ($p < 0.001$) with most of the p95^{HER2} negative cases 325/423 (95.0%) being associated with both HER2 negative classifications (0+ and 1+). Also, almost 80% of the cases showing p95^{HER2} overexpression were associated with overexpression of HER2 (3+), with only 3/51 (5.9%) associated with HER2 negativity. The p95^{HER2} analysis against both HER2 gene amplification and HER2 classification showed very similar results with a strong positive association ($p < 0.001$). In both cases approximately 90% of the p95^{HER2} negative cases were associated with either no HER2 amplification or negative HER2 status. Amongst the p95^{HER2} positive cases nearly 92% revealed an association with HER2 gene amplification and HER2 positive status (Table 3.10).

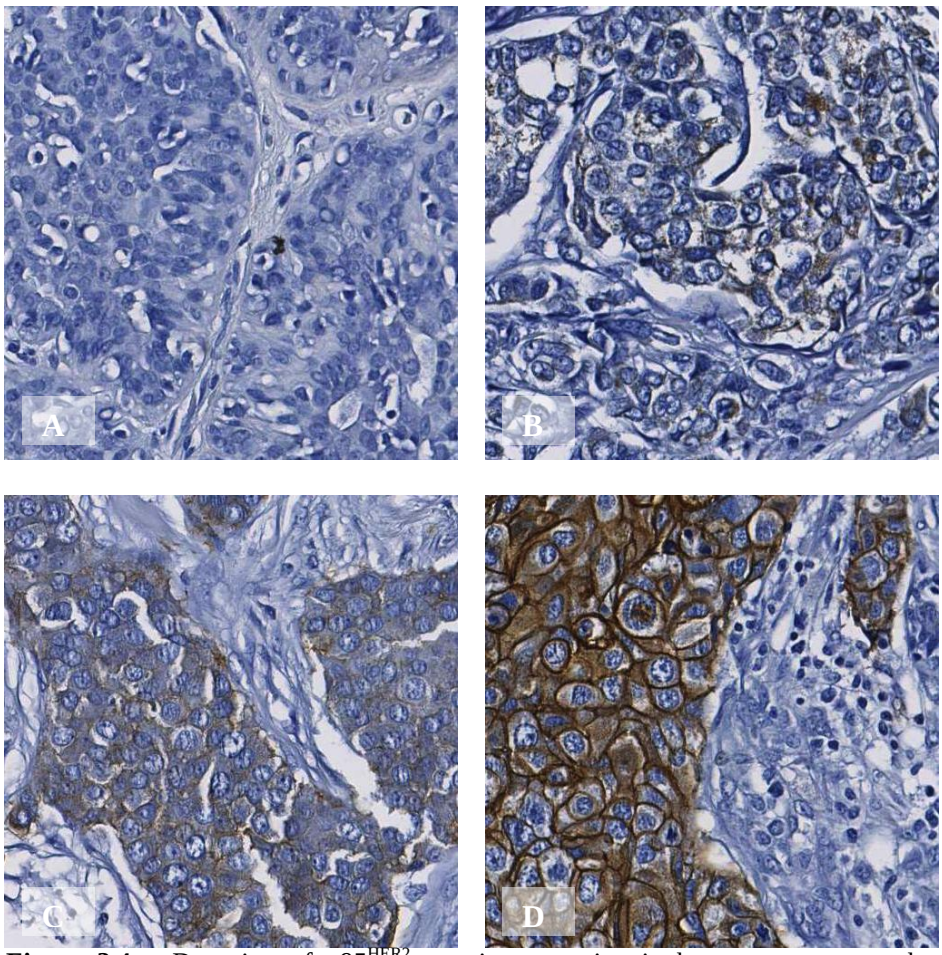

Figure 3.4 – Detection of $p95^{HER2}$ protein expression in breast cancer cores by immunohistochemistry and scored according to intensity. The cells were labelled with a specific antibody against $p95^{HER2}$ to detect the intensity of expression of this protein present in the cell membrane. The cells are scored in four different classes of protein expression: Negative expression – (A), (B) and (C). Positive expression (D) show strong and complete membranous reactivity.

Table 3.10 – Relation between p95^{HER2} protein expression, and HER2 protein expression, *HER2* gene amplification and HER2 status in breast cancer including respective frequencies.

	p95^{HER2} IHC status			
	Negative (%)	**Positive** (%)	**Total** (%)	**p value** (Chi-Square)
HER2 (IHC)				
0+	325 (66.1)	3 (5.9)	328 (60.4)	<0.001 (180.029)
1+	93 (18.9)	0 (0.0)	93 (17.1)	
2+	28 (5.7)	9 (17.6)	37 (6.8)	
3+	46 (39.3)	39 (76.5)	85 (15.7)	
Total	492 (100)	51 (100)	543 (100)	
HER2 CISH				
No amplification	255 (87.0)	3 (8.6)	258 (78.7)	<0.001 (114.652)
Amplification	38 (13.0)	32 (91.4)	70 (21.3)	
Total	293 (100)	35 (100)	328 (100)	
HER2 Status				
Negative	427 (88.2)	4 (8.5)	431 (81.2)	<0.001 (178.079)
Positive	57 (11.8)	43 (91.5)	100 (18.8)	
Total	484 (100)	47 (100)	531 (100)	

3.3.9 Correlation of p95^{HER2} expression with clinicopathological parameters

The p95^{HER2} protein positive expression revealed to be highly associated with higher tumour grade ($p < 0.001$), more nuclear pleomorphism ($p < 0.001$), higher mitotic frequency ($p = 0.008$), occurrence of metastasis ($p = 0.003$), death due to breast cancer ($p = 0.017$) and poor NPI ($p = 0.001$) (Table 3.11).

Table 3.11 – The relation between p95^{HER2} status in breast cancer with clinicopathological parameters.

Parameters	p95^{HER2} IHC Status		
	Negative (%)	**Positive** (%)	**p Value** (Chi-Square)
Tumour Grade			
1	61 (12.4)	2 (3.9)	<0.001 (15.568)
2	156 (31.8)	6 (11.8)	
3	273 (55.7)	43 (84.3)	
Tubule Formation			
1	14 (3.0)	1 (2.0)	0.643 (0.822)
2	145 (30.9)	13 (25.5)	
3	311 (66.2)	37 (72.5)	
Pleomorphism			
1	6 (1.3)	0 (0.0)	<0.001 (16.850)
2	176 (37.5)	5 (9.8)	
3	287 (61.2)	46 (90.2)	
Mitotic Frequency			
1	123 (26.2)	4 (7.8)	0.008 (9.775)
2	100 (21.3)	10 (19.6)	
3	247 (52.6)	37 (72.5)	
Tumour Stage			
1	298 (60.8)	24 (47.1)	0.149 (3.802)
2	143 (29.2)	21 (41.2)	
3	49 (10.0)	6 (11.8)	
Distant Metastasis			
Negative	323 (66.3)	23 (45.1)	0.003 (9.063)
Positive	164 (33.7)	28 (54.9)	
Local Recurrence			
Negative	267 (55.7)	19 (38.0)	0.017 (5.738)
Positive	212 (44.3)	31 (62.0)	
Regional Recurrence			
Negative	411 (85.8)	45 (90.0)	0.413 (0.670)
Positive	68 (14.2)	5 (10.0)	
Tumour Size			
<1.5cm	96 (19.6)	10 (19.6)	0.998 (0.00)
≥1.5cm	394 (80.4)	41 (80.4)	
Vascular Invasion			
Negative	255 (52.6)	21 (42.0)	0.154 (2.031)
Positive	230 (47.4)	29 (58.0)	
NPI			
Good	120 (24.7)	4 (8.0)	0.001 (8.965)
Moderate	285 (58.6)	32 (64.0)	
Poor	81 (16.7)	14 (28.0)	
Age			
<50	177 (36.0)	19 (37.3)	0.856 (0.033)
≥50	315 (64.0)	32 (62.7)	
Death			
Negative	257 (61.8)	19 (43.2)	0.017 (5.734)
Positive	159 (38.2)	25 (56.8)	

3.3.10 Correlation of p95^{HER2} expression with hormone and HER family receptors

Amongst the HER family receptors, positive expression of p95^{HER2} was only highly correlated with HER4 expression ($p = 0.002$). Against basal-type tumours and expression of both ER and PgR the correlation was highly negative with all p values under 0.001 (Table 3.12).

Table 3.12 – Relationship between HER2 expression with hormone receptors and HER family receptors.

Biomarker	p95^{HER2} IHC Status		
	Negative (%)	**Positive (%)**	**p value (Chi-Square)**
Basal			
Negative	26 (19.4)	8 (88.9)	<0.001
Positive	108 (80.6)	1 (11.1)	(22.468)
EGFR			
Negative	331 (80.9)	33 (76.7)	0.510
Positive	78 (19.1)	10 (23.3)	(0.435)
HER3			
Negative	42 (10.7)	2 (4.9)	0.241
Positive	351 (89.3)	39 (95.1)	(1.375)
HER4			
Negative	74 (18.9)	0 (0.0)	0.002
Positive	318 (81.1)	41 (100)	(9.335)
ER			
Negative	146 (31.2)	37 (72.5)	<0.001
Positive	322 (68.8)	14 (27.5)	(34.450)
PgR			
Negative	207 (44.2)	36 (73.5)	<0.001
Positive	261 (55.8)	13 (26.5)	(15.223)

3.3.11 Correlation of p95^{HER2} expression with biomarkers

The positive expression of p95^{HER2} protein revealed to be highly correlated with CK18 ($p = 0.006$), p53 ($p = 0.003$), PTEN ($p = 0.042$) and Ki67 ($p = 0.007$). Additionally p95^{HER2} expression was negatively associated with CK5/6 ($p = 0.029$), Bcl-2 ($p = 0.003$) and BRCA1 ($p = 0.016$) (Table 3.13).

The same analysis was performed amongst the HER2+ series, however p95^{HER2} did not reveal any significant associations with either clinicopathological parameters or biomarkers (table not included).

Table 3.13 – Relationship between p95^{HER2} expression with biomarkers.

Biomarkers	p95^{HER2} IHC Status		
	Negative (%)	**Positive** (%)	**p Value** (Chi-Square)
CK5/6			
Negative	388 (81.5)	46 (93.9)	0.029
Positive	88 (18.5)	3 (6.1)	(4.740)
CK 7/8			
Negative	10 (2.1)	0 (0.0)	0.301
Positive	467 (97.9)	50 (100)	(1.068)
CK14			
Negative	405 (76.5)	44 (90.0)	0.480
Positive	65 (23.5)	5 (10.0)	(0.500)
CK 18			
Negative	81 (18.4)	1 (2.2)	0.006
Positive	359 (81.6)	44 (97.8)	(7.614)
Bcl-2			
Negative	40 (41.7)	7 (100)	0.003
Positive	56 (58.3)	0 (0.0)	(8.949)
pAkt			
Negative	76 (22.8)	6 (14.3)	0.207
Positive	257 (77.2)	36 (85.7)	(1.591)
PI3K			
Negative	99 (24.3)	8 (19.5)	0.553
Moderate	86 (23.6)	9 (19.5)	(1.184)
Strong	212 (52.1)	25 (61.0)	
p21			
Negative	61 (77.2)	2 (66.7)	0671
Positive	18 (22.8)	1 (33.3)	(0.181)
MUC-1			
Negative	44 (11.1)	1 (2.4)	0.219
Moderate	172 (43.3)	19 (46.3)	(3.035)
Strong	181 (45.6)	21 (51.2)	
BRCA1			
Negative	219 (56.9)	32 (76.2)	0.016
Positive	166 (43.1)	10 (23.8)	(5.826)
p53			
Negative	323 (69.0)	24 (48.0)	0.003
Positive	145 (31.0)	26 (52.0)	(0.023)
PTEN			
Weak	60 (18.1)	2 (6.1)	0.042
Moderate	131 (39.6)	10 (30.3)	(6.321)
Strong	140 (42.3)	22 (63.6)	
Ki67			
Weak	128 (32.7)	6 (15.4)	0.007
Moderate	156 (39.9)	18 (46.2)	(5.308)
Strong	107 (27.4)	15 (38.5)	

3.3.12 HER2 and Patient Outcome

Kaplan–Meier survival analysis demonstrated that tumours overexpressing HER2 protein (IHC score of 2+ and 3+), HER2 amplification (ratio > 2) or HER2 positive status showed similar significantly shorter BCSS (Figure 3.5A, Figures 3.6A and C) and DFI (Figure 3.5B, Figures 3.6B and D) compared with those showing unequivocal negative HER2 protein expression, no amplification or HER2 negativity (in all cases $p < 0.001$). Interestingly, for HER2 protein expression, those cases showing moderate status (2+) had slightly better outcome compared with those tumours with overexpression of the protein (3+), though those differences were not significant (BCSS $p = 0.334$, DFI $p = 0.657$) (Figures 3.5C and D).

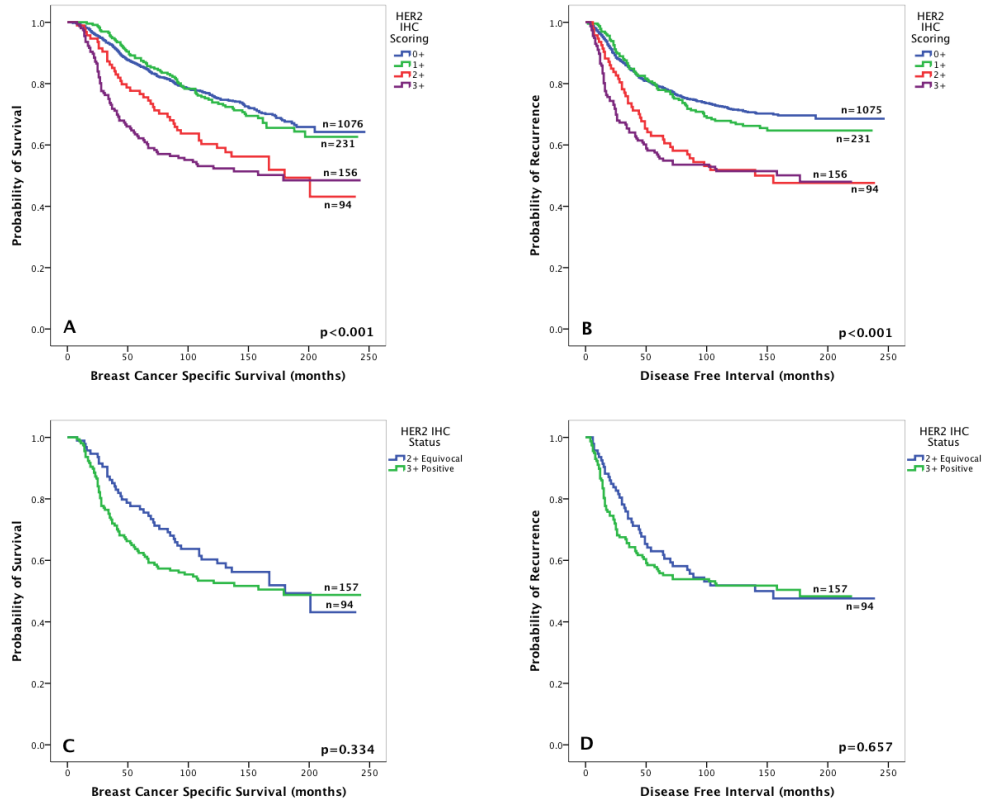

Figure 3.5 – A) BCSS and **B)** DFI analysis for HER2 protein expression status amongst the Unselected series. **C)** BCSS and **D)** DFI analysis for HER2 equivocal (2+) protein expression against positive (3+) cases amongst the Unselected series.

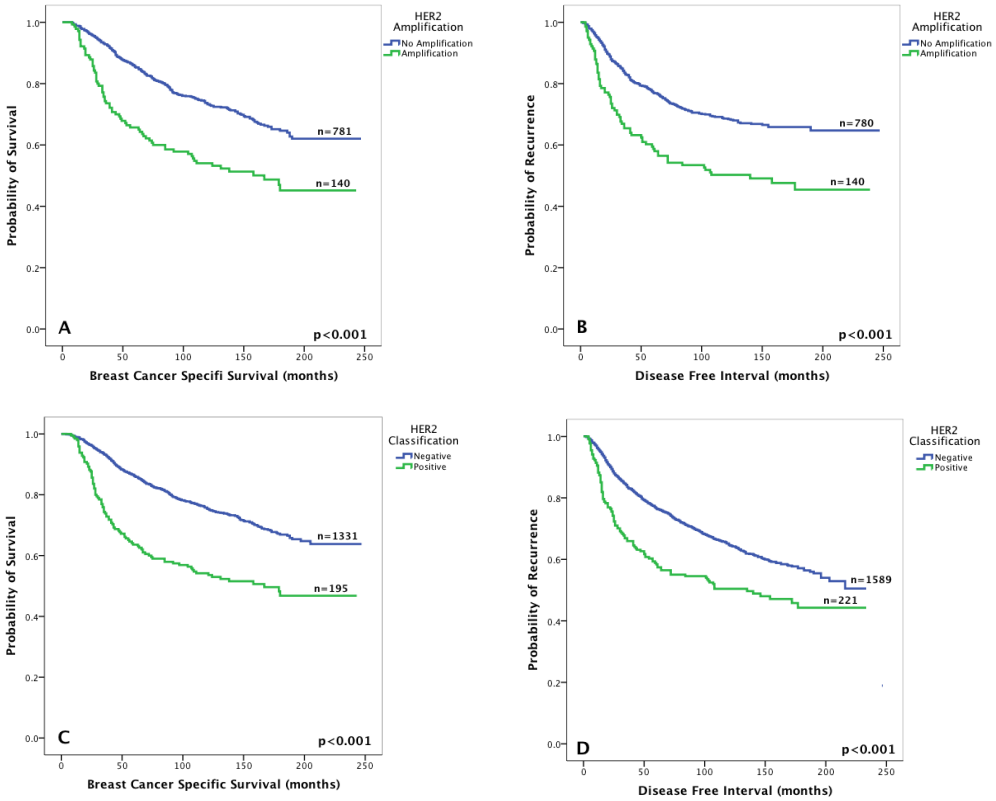

Figure 3.6 – **A)** BCSS and **B)** DFI analysis for *HER2* amplification status amongst the Unselected series. **C)** BCSS and **D)** DFI analysis for HER2 classification amongst the Unselected series

3.3.13 Survival analysis for HER2 expression equivocal cases harbouring either HER2 amplification or not

Combining the HER2 expression and *HER2* gene amplification data, it was observed a better survival outcome within the first 60 months for those patients classified as HER2 equivocal tumours with no gene amplification (IHC 2+ non-amplified), comparing against the equivocal cases harbouring gene amplification and the positive cases (Figure 3.7A). However after this period BCSS was similar between both equivocal with or without gene amplification and IHC 3+ tumours. Analysing only IHC 2+ cases, either with or without gene amplification, it was not possible to observe a significant survival difference between both groups ($p = 0.487$; Figure 3.7B). Regarding the DFI both 0+ and 1+ negative group revealed a better outcome comparing with the positive IHC 3+ and both IHC 2+ groups ($p = 0.001$; Figure 3.7C).

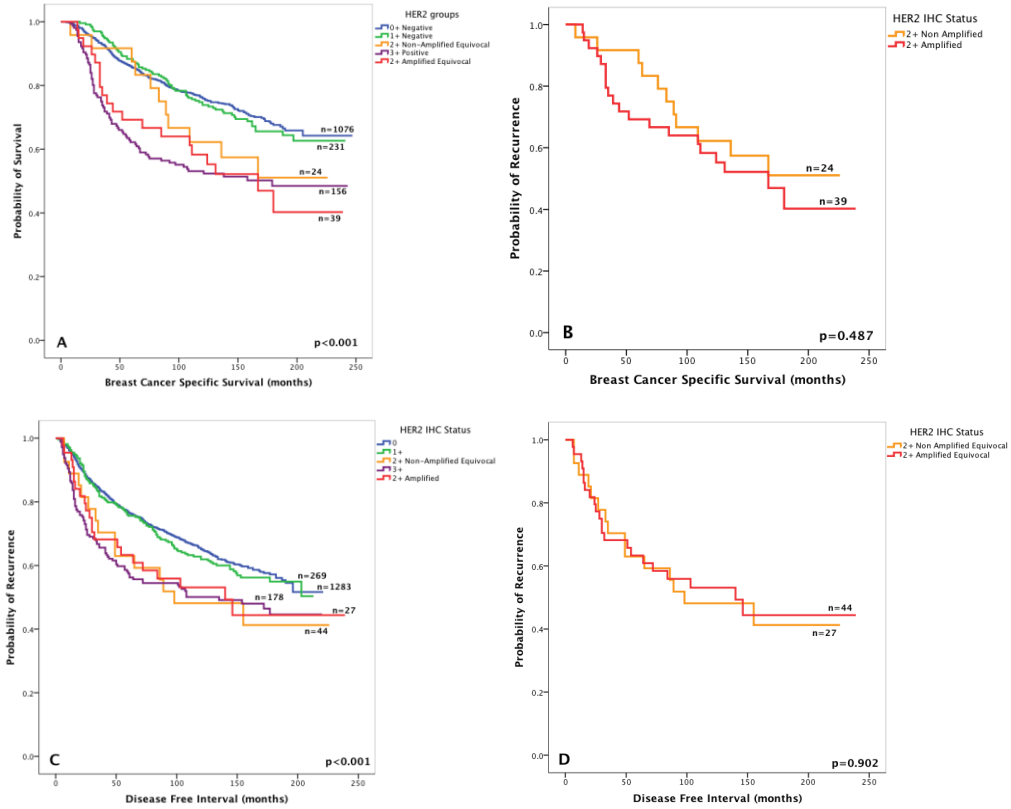

Figure 3.7 – A) BCSS and **C)** DFI analysis for HER2 protein expression discriminating the equivocal cases (IHC 2+) either with or without gene amplification. **B)** BCSS and **D)** DFI analysis for HER2 protein expression discriminating only the equivocal cases (2+) either with or without gene amplification

3.3.14 Survival analysis for ER amongst the HER2+ trastuzumab naïve series

The survival analyses performed the HER2+ trastuzumab naïve either expressing ER or not showed a very similar outcome for both BCSS (p = 0.488) and DFI (p = 0.499) (Figure 3.8A and B, respectively). Interestingly after a period of 110 months the differences became evident with those overexpressing ER possessing a significant worse outcome for both BCSS (p = 0.012) and DFI (p = 0.002) (Figure 3.8C and D, respectively).

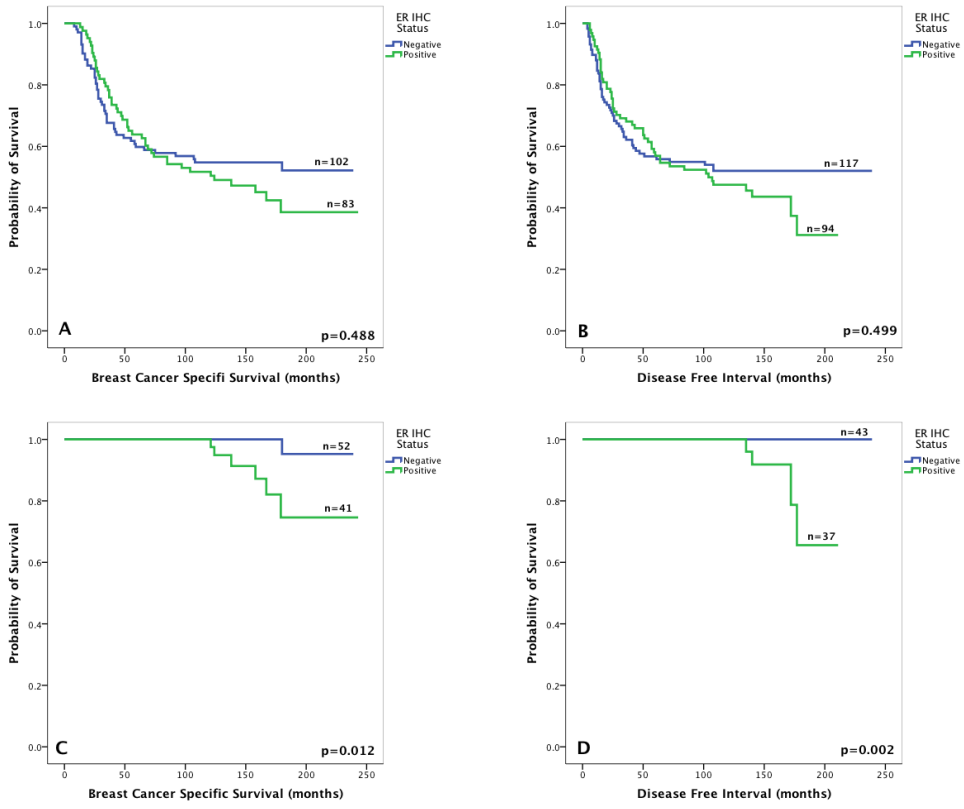

Figure 3.8 – **A)** BCSS and **B)** DFI analyses for ER expression amongst HER2+ trastuzumab naïve cases. **C)** BCSS and **D)** DFI analysis for ER expression amongst HER2+ trastuzumab naïve cases after 110 months from first diagnosis.

3.3.15 Survival analysis for p95^{HER2} expression

Because of such a high association of p95^{HER2} with HER2, survival analysis revealed a significant worst outcome of the p95^{HER2} positive cases against the negative cases for both BCSS (p = 0.003) and DFI (p = 0.011) amongst the Unselected series (Figures 3.9A and B, respectively).

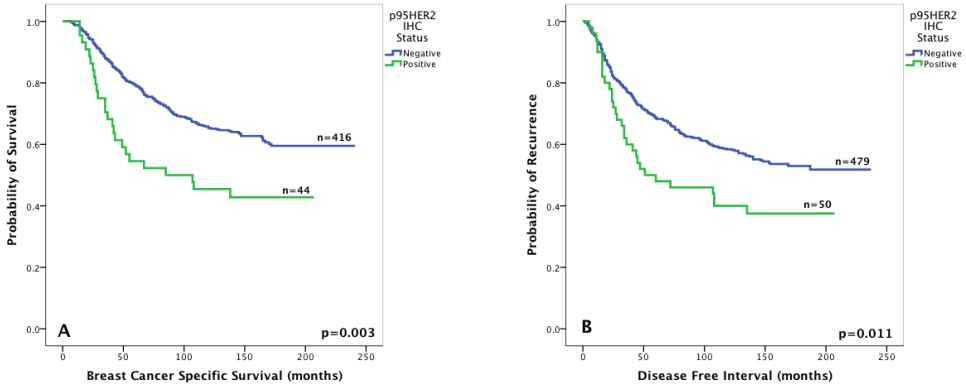

Figure 3.9 – A) BCSS and **B)** DFI analysis for HER2 p95 protein expression in breast cancer.

3.3.16 Survival analysis for HER2 IHC status amongst the HER2+ trastuzumab adjuvant series

For the HER2+ trastuzumab adjuvant series only HER2 IHC status survival analysis was performed. Both *HER2* amplification and HER2 classification status survival analyses were not conceivable due to all patients from this series being *HER2* amplified or HER2 positive. Therefore it was achievable to compare those patients revealing HER2 equivocal expression (2+) versus HER2 overexpression cases (3+). The Kaplan-Meier analysis did not revealed any significant differences for BCSS (p = 0.240) and DFI (p = 0.157) between equivocal and overexpressing patients (Figures 3.10A and B, respectively).

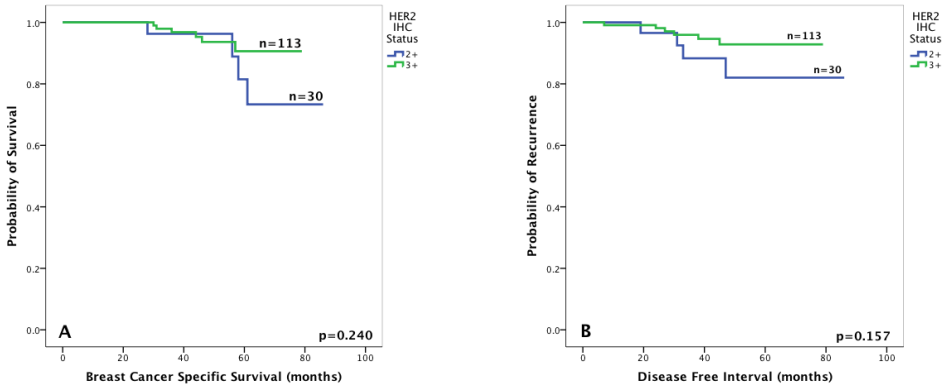

Figure 3.10 – A) BCSS and **B)** DFI analysis for HER2 protein expression for trastuzumab adjuvant series.

3.3.17 HER2 and patient outcome in HER2+ tumours trastuzumab naïve versus adjuvant

Kaplan-Meier survival analyses were performed concerning the BCSS and DFI outcome of those cases submitted to adjuvant trastuzumab treatment against trastuzumab naïve patients. Amongst the HER2 equivocal cases no significant difference was observed between for both BCSS (p = 0.198; Figure 3.11A) and DFI (p = 0.071; Figure 3.11B).

Amongst the HER2 3+ patients BCSS and DFI analyses revealed significant differences on patients' outcome (p < 0.001; Figures 3.11C and D).

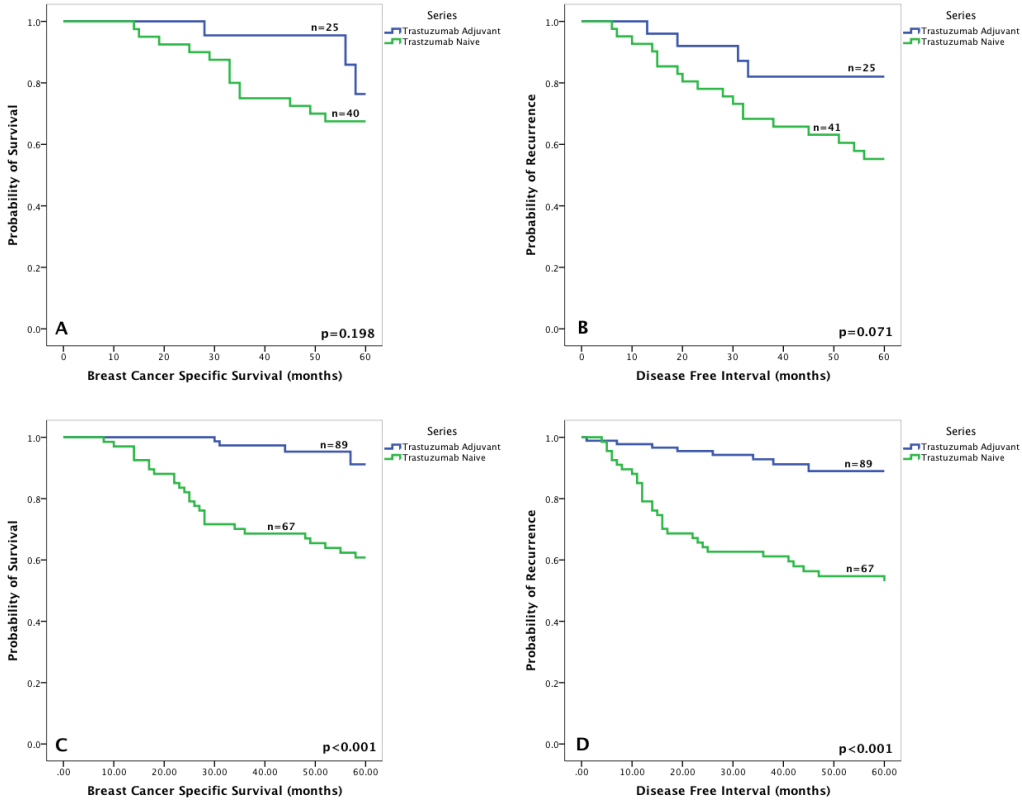

Figure 3.11 – BCSS and DFI analysis for HER2 status amongst the HER2+ trastuzumab adjuvant series against HER2+ trastuzumab naïve series regarding **A)** and **B)** HER2 equivocal status (2+ IHC) but amplified or **C)** and **D)** HER2 positive status (IHC 3+).

3.3.18 Survival analysis for p95^{HER2} expression amongst both the HER2+ trastuzumab naïve and trastuzumab adjuvant series

Kaplan-Meier study was performed for p95^{HER2} amongst both HER2+ trastuzumab naïve series and trastuzumab adjuvant series. This mode it was possible to understand if patients revealing p95HER2 positive expression shown worse prognosis comparing with the other HER2+ patients. Amongst the HER2+ trastuzumab naïve those cases with p95HER2 positive status did not reveal a worse outcome for both BCSS (p = 0.681) and DFI (p = 0.409) (Figures 3.12A and B, respectively). Amongst the trastuzumab adjuvant series the p95HER2 expression did not reveal a significant worse prognosis for both BCSS (p = 0.120) and DFI (p = 0.392) (Figures 3.12C and D, respectively).

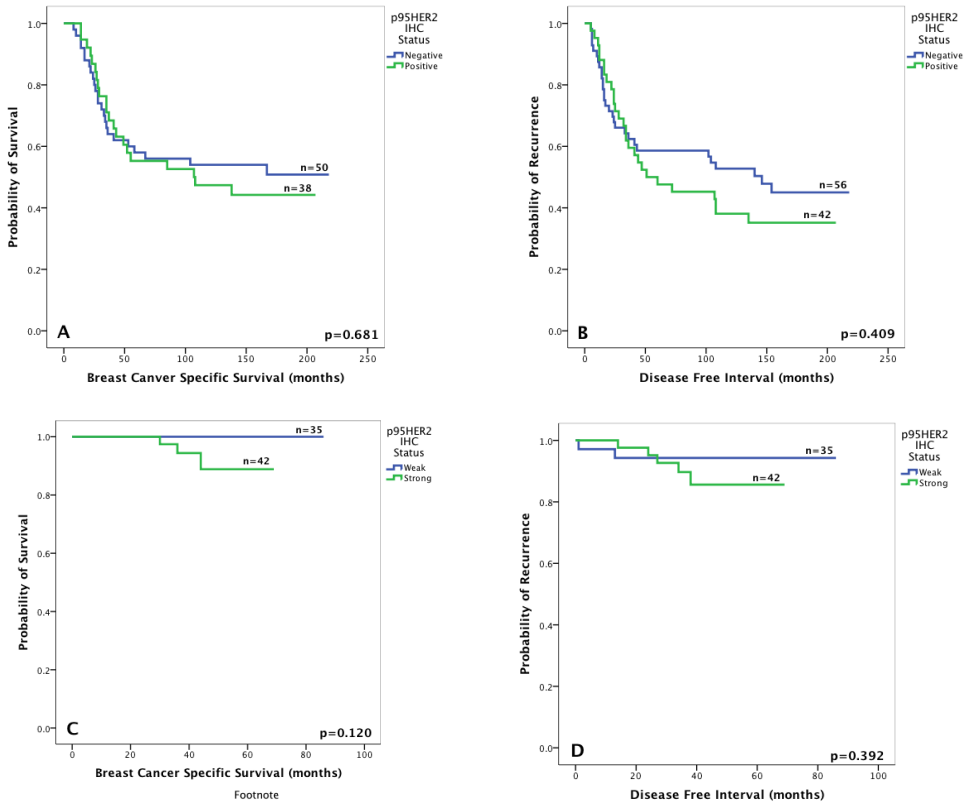

Figure 3.12 – **A)** BCSS and **B)** DFI analysis for p95^{HER2} protein expression for trastuzumab naïve series and **C)** BCSS and **D)** DFI for trastuzumab adjuvant series.

3.4 Discussion

The diversity amongst the different breast carcinomas is astonishingly high when morphology, molecular outline and effects of therapies applied. Genetics studies achieved the identification and distinction of classes of breast carcinomas[17]. *HER2* is of great interest in breast cancer due to its overexpression being associated with dismal innate prognosis and as therapeutic target for trastuzumab[51,55,368-371] and other emerging therapies[219,372]. In this chapter, HER2 protein expression and gene amplification were determined in a large historical cohort of primary breast cancer, prior to the introduction of trastuzumab treatment and also a series of HER2+ tumours from patients treated with adjuvant trastuzumab. Previous studies show concordance of 73% to 98% between IHC and FISH techniques in detection of overexpression of HER2[373-375]. This discrepancy might be due to different IHC techniques used by different groups, which can be influenced by different commercial antibodies that demonstrated diverse specificities and sensitivities to dissimilar epitopes present in HER2[371,376-378].

CISH reveals high concordance with FISH but possesses several advantages over FISH. CISH is relatively low cost, only requires routine equipment present in a regular histopathology laboratory and possible to store for long periods of time and is therefore a useful and more suitable alternative for high-throughput screening of breast cancers prepared as TMAs than FISH[355,359,371,379,380]. Also CISH is suitable to be performed in TMAs because is not affected by the intratumoral heterogeneity of *HER2* amplification. The

concordance between both TMAs and whole tissue sections results reveal to be extremely high[381].

In the case of this study, for detection of each biomarker, instead of performing a couple of thousand immunohistochemistry analyses for each biomarker detection, this was possible to be achieved in only 13 TMAs sections for unselected series and 1 TMA section for HER2+ adjuvant trastuzumab cohort. This way it was possible to avoid time consuming and high quantities of detections solutions, which would reveal to be extremely expensive and unfeasible to obtain a more complete characterisation of both cohorts. Additionally using TMAs, all specimens included into the same block were submitted to exactly the same physical and chemical conditions (temperature, solutions concentrations and the period of time for incubations), providing more consistent results[382,383]. However for each case analysed on a TMA, only 0.3% of a 1 cm cancer tissue is represented. Therefore this method might reveal some limitations due to this small amount of tissue tested. Instead this statistical variability between whole tissue and TMA core results is eliminated by the large amount of cases analysed, which is able to counterbalance any false negative result[340,384]. Different studies suggested that the use of TMAs is enough to represent the whole tissue section from each patient[382,384,385]. However heterogeneity amongst the full tissue section might reveal to be an issue for such a method that only uses an insignificant percentage of each sample. Only one core marked for HER2 able to be scored is representative of more than 90% of the whole tissue section, revealing that in some cases this statistical analysis can achieve 95% concordance[386]. A unique core was

expressive enough in 95% for ER, up to 81% for PgR and up to 74% for p53 against the original full sections[387].

HER2 gene amplification and protein overexpression was observed in 14.6% and 9.7% of a large unselected series of breast cancer cases, respectively. Utilising a combination of both techniques a final frequency of 12.1% of HER2 positive cases being in concordance with a different study[349]. However is clearly lower when compared with another study that revealed HER2 positivity frequency levels of 22.8%[21]. Only four out of a total of 1115 cases studied with IHC against CISH did not show concordance, i.e., 99.6% of our samples revealed protein overexpression harbouring gene amplification, which shows an excellent concordance between these two techniques. Only one case showed high protein expression but no gene amplification, which might be a false positive where IHC was excessively sensitive to antigen retrieval process[58,388], or this fact could be explained by polysomy on chromosome 17 that provokes HER2 overexpression[58,389]. It was observed three cases where there was gene amplification but no protein overexpression, which could be explained by an alteration of the structure in charge of gene expression[59]. Therefore CISH revealed to be reliable technique on *HER2* gene amplification detection with supplementary advantages when compared against FISH.

HER2 positivity is associated with very poor outcome amongst the breast cancer patients[348,390]. In this study HER2 was significantly correlated with poor prognostic clinicopathological parameters including higher stage, larger size, higher grade, high nuclear pleomorphism, higher mitotic frequency, less tubule

formation, higher occurrence of development of distant metastases and poor NPI. Occurrence of death due to breast cancer is highly associated with HER2, was not associated with patient's age. HER2 status was observed more often amongst the ductal carcinomas in contrast to other pathological types that are more correlated with favourable outcome[391].

The molecule $p95^{HER2}$ was previously highly related with poor prognosis clinicopathological parameters as development of metastases and higher tumour stage but not significantly associated with higher tumour size[166]. In this study $p95^{HER2}$ analyses against clinicopathological parameters revealed not only high association with development of distant metastasis but also high correlation with tumour grade, high nuclear pleomorphism, high mitotic frequency and poor NPI but not with tumour stage. Additionally and in agreement with the same study, $p95^{HER2}$ was not correlated with tumour size, which differentiates from HER2+ cases that were highly correlated with this parameter. Tubule formation was not correlated with $p95^{HER2}$ but highly correlated with HER2+ cases as well.

While as anticipated EGFR and HER2 expression are highly correlated[392], in this study HER2 expression was not associated with EGFR expression, even though approximately close to p values under 0.05. However the gene amplification and HER2 status revealed to be highly associated. Again disagreeing with different studies[70,283], HER3 expression is not correlated with any HER2 analyses, even though the overexpression is present in 90% of the HER2+ cases. The last member of HER family revealed to be highly associated

with HER2 positivity in concordance to a different study that also reports increase of sensitivity of the breast carcinomas to trastuzumab if HER4 is overexpressed[393]. The antibody used to detect the HER2 cleaved form was specifically designed to detect 100- to 115-kDa p95^{HER2} in FFPE sections and we could confirm that p95^{HER2} carcinomas comprise a subclass of HER2+ cases with similar biological and clinical descriptions but possible to differentiate. Previously it was possible to show that approximately 80% of the HER2- cases were ER+ and merely 50% of the HER2+ cases were ER+[365,394]. These facts concur with our results revealing the negative association between HER2 and the hormone receptors. The data of ER expression against p95^{HER2} cases revealed an extraordinary linkage between them. Even though the frequency of ER+ versus p95^{HER2} negativity is similar as against HER2- cases, the number of p95^{HER2}+ that are also ER+ is not greater than 30% against the 50% for HER+ cases, which is reflected in previous works[395]. Also becomes even more evident the lack of association of p95^{HER2} expression with EGFR and basal-like breast tumours, which is not in agreement with Pedersen and colleagues that stated a high correlation of p95^{HER2} with EGFR overexpression[179].

HER2 revealed strong correlation with poor prognostic biomarkers and negative association with biomarkers correlated with better outcome, even though some exceptions were observed. HER2 was not correlated with any of the basal cytokeratins (CK5/6 and CK14) and luminal associated cytokeratin CK7/8. As already documented the association of HER2 protein expression with PI3K and pAkt revealed to be very strong[396], which is in concordance

with this study. Moreover incidences as high cell proliferation and transformation had been related with overexpression of PI3K and pAkt arbitrated by overexpression of HER2[396]. However HER2 gene amplification did not reveal any correlation with pAkt. Additionally HER2 was correlated with Ki67, a biomarker highly associated with cancer proliferation and also with breast carcinoma relapse and death[397]. Previous studies have related Bcl-2 and p53 describing their apoptotic role on breast cancer and consequently delivering different prognosis[398,399]. An apoptosis reaction triggered by p53 is inhibited by the anti-apoptotic role developed by Bcl-2. Moreover both biomarkers expression is inversely correlated amongst most of human breast cancer cell lines, suggesting downregulation of Bcl-2 by p53[400]. Breast cancer patients revealing high expression of Bcl-2 and elimination of p53 are related to more aggressive carcinomas[401,402]. Our results revealed a high association of HER2 with p53 and a negative association with Bcl-2. This fact is supported by an anticipated study revealing that the biomarker Bcl-2 is inversely correlated with HER2 and Ki67 and positively associated with ER and PgR [398]. On the other hand and in agreement with this study p53 is inversely correlated with ER and PgR and highly associated with HER2[398]. HER2 also revealed negative association against BRCA1, which contrast with previous studies revealing no correlation between them even though lack of BRCA1 association with poor prognosis[392]. BRCA1 is involved on control of cell cycle and proliferation progression, DNA repair and recombination however absence or mutation drive breast cancer cell malignancy[403].

Interestingly the results achieved for p95^{HER2} demonstrated to be similar to HER2, however not in all cases. The cleaved molecule was correlated with one basal biomarker – CK5/6 – which is related with the most aggressive class of breast cancers[404]. However and similarly to HER2 positivity, p95^{HER2} demonstrated to be highly associated with CK18, which is linked to suppression of tumour development[405]. Another remarkable difference is achieved against pAkt, where p95^{HER2} was not correlated and HER2 protein expression is highly associated.

Expression of HER2 was highly associated with poor BCSS and DFI. Negative or low HER2 protein expression (IHC score of 0+ and 1+) was associated with a better outcome comparing it to 2+ and 3+ during the entire period analysed. However, discrimination of those HER2 tumours with IHC score of 2+ between those showing *HER2* amplification or no-amplification revealed a differential outcome during the first 60 months. However, those cases with HER2 overexpression but not gene amplification had a similar overall outcome (post 60 months) to those with gene amplification. Hence IHC 2+ and 3+ cases revealed similar long-term outcome irrespective of *HER2* gene amplification status. This suggests that despite the gene amplification, the outcome can be affected by the quantity of receptors available to dimerise and trigger downstream pathways and worse outcome is acquired in both 2+ and 3+ regardless gene amplification. In present clinical settings, cases exhibiting IHC 2+ and no *HER2* amplification will not be offered targeted HER2 therapy although they do demonstrate long-term aggressive clinical behavioural characteristics. Even though in trastuzumab adjuvant series all patients possess

HER2 gene amplification, those scored as equivocal HER2 protein expression (2+) for protein expression revealed a worse outcome after a period of 60 months, suggesting development of resistance to trastuzumab therapy. However statistical relevant results were not possible to be achieved due to limitation of samples and short period of treatment of these patients. Concluding, HER2 equivocal patients (2+) possess bad outcome as positive patients (3+) amongst the trastuzumab naïve series but the equivocal patients that harbour gene amplification amongst the trastuzumab adjuvant series might possess worst outcome comparing against the positive patients. This might indicate the possibility that HER2 equivocal patients might not possess enough expression levels to react with trastuzumab. As referred before trastuzumab only reacts with cells expressing HER2 10-100 times above normal levels[56]. Finally amongst the trastuzumab naïve series it was possible to understand that ER overexpression might confer a worse outcome, however that difference is only significant after a period of 120 months, overall the significance is inexistent. The crosstalk HER2/ER is a significant factor for development of resistance to breast cancer treatment[105]. Therefore understand the interaction between these two receptors and its effect on cancer treatment is crucial to develop new therapies that target HER2 overexpressing carcinoams. Lapatinib was approved by FDA to be applied against HER2+ breast carcinomas in combination with chemotherapy[227].

Amongst the trastuzumab naïve series both negative and positive $p95^{HER2}$ status showed similar outcome, though the therapy (chemotherapy adjuvant) was not specifically directed against this cleaved target. This statement is not in

concordance with previous study revealing worse outcome for those patients expressing high levels of p95^{HER2} [406]. Even though amongst the trastuzumab adjuvant series the outcome still not significantly different for both p95^{HER2} statuses, it is possible to understand that those HER2+ patients expressing high levels of p95^{HER2} are starting to uncover a worse prognosis. No action of trastuzumab against p95^{HER2} form due to the lack of the ectodomain takes place[366] and the idea of p95^{HER2} used as a biomarker to define an aggressive subtype of HER2+ breast carcinomas becomes real[173,406]. Therefore and in agreement with Scaltriti and colleagues p95^{HER2} positive patients develop resistance to trastuzumab treatment[175]. However the application of lapatinib is effective duo to its properties of reacting with the tyrosine kinases located on endoplasmic domain and blocking the receptor signalling pathways[179]. Even though p95^{HER2} analysis revealed a high correlation of the positive cases with worse outcome, our study did not reveal any significant difference to turn p95^{HER2} as an alternative biomarker to be selected on breast cancer patient's differentiation. However this molecule does not possess an ectodomain to react with trastuzumab, which might contribute to resistance development against this drug [360].

4. CHAPTER 4: The role of EGFR in HER2+ breast cancer

4.1 Introduction

4.1.1 EGFR in breast cancer

EGFR is a 53 amino acid polypeptide expressed by a gene located on chromosome 7 location 7p12.3-p12.1[407] and its molecular weight is 170KDa[408]. Studies using EGFR knockout mice demonstrated that EGFR is implicated in epithelial cell cycle and essential in mid-gestational stage where animals that survived several weeks after birth had skin, lungs, brain and liver malformation and mammary gland with reduced size and small ducts[29,409-411]. The overexpression of EGFR, similarly to the other HERs, has been highly associated with the development of several types of cancer with poor prognosis, especially amongst aggressive brain tumours, detected in 90% of high grade astrocytic glioma cases and up to 50% of glioblastoma multiforme[412,413]. Also EGFR has been detected and associated with solid tumours progression, becoming a specific target in treatments of bladder, colorectal pancreatic, cervical, prostate, melanoma, lung and breast cancer[414]. The non-small-cell-lung cancer is highly affected by EGFR overexpression reaching up to 80% of the patients[415]. In a normal breast cell, EFGR is crucial for expansion and differentiation of different tissues throughout the last stage of gestation[416]. The frequency of this protein in a normal cell can be between 40,000-100,000[417] against a dramatic increase of this number in cancer cells that possibly reaches 2,000,000[418]. Such a dramatic increase on its expression

triggers a high rate of proliferation and angiogenic and metastasis development[419] resulting in constant growth of the carcinomas[420]. Even though some studies contradict this arguments revealing an association of EGFR expression with reduction of the tumour progress[421], supported by other studies revealing no association between EGFR expression and tumour size, grade or lymph node stage[422,423]. On the cytoplasmic domain if EGFR there are six autophosphorylation spots categorised as major, which includes Y1068, Y1148, Y1173[424,425], and minor including Y992, Y1045, Y1086[31,426,427]. The consequences of these sites being phosphorylated comprise proliferation, angiogenesis and anti-apoptotic improvement[428,429], suggesting that EGFR overexpression as a biomarker revealing poor outcome in breast cancer. However a complete characterisation of EGFR affecting breast cancer development still ambiguous. A later EGFR research study by Rampaul showed that 20% of breast cancer was EGFR positive and possessed a significant association with tumour type, and grade[430].

EGFR overexpression has also been linked with triple negative (TN) invasive ductal breast carcinomas[333,431] revealing a frequency between 10-54% of the cases[432-434] suggesting this receptor as a good target for anticancer therapies[435]. Although expression of EGFR is highly associated with poor prognosis in breast cancer[21] and expressed in about 33% of human carcinomas and 50% of glioblastomas, the presence of this receptor is highly expressed in only 2.7% of breast tumours from patients with poor prognosis[21]. It is being an arguable discussion among those that suggested an association of EGFR with poor prognosis and recurrence high incidence[436,437] and those that revealed no

association[438,439]. It is possible to understand that the discordance between studies still significant and might be explained by small cohort sizes used, diminutive follow up, the use of different techniques to detect this protein are even the different adjuvant therapies applied. However, most studies have applied different cut-off points, which might influence significantly the analysis.

Treatments are being targeted to obstruct EGFR activity, which will target within the extracellular domain by applying monoclonal antibodies like panitumumab or cetuximab. An alternative method is to target the intracellular domain or tyrosine kinase domain using gefitinib or erlotinib known as small molecules or tyrosine kinase inhibitors (TKI)[440,441]. Until now, it is not possible to confirm whether the patients benefit or not with this therapy due to being in early clinical research stage[442]. Reactive oxygen species (ROS), which regulates cellular signalling pathways by association with EGFR-associated tyrosine phosphatase, plays an important role in developing resistance to TKI treatments. Therefore the use of cell-penetrating catalase (specific anti-oxidant enzymes) is crucial to sensitise these carcinoma cell to gefinitib[443].

4.1.2 HER2/EGFR in breast cancer

Approximately 25%-35% of the breast carcinomas revealed co-expression of both EGFR and HER2[442,444], indicating a possible existence of the heterodimer HER2/EGFR, which might be associated with creation of the EGFR active form pEGRF-y992 located at the membrane and the cytoplasmic form Y1068[442]. Patients revealing the co-expression of these two growth factors

showed the worse prognosis[444,445]. This poor outcome might be related with high activity of PI3K/Akt downstream pathways implicated in cell apoptosis evasion, expansion, motility and angiogenesis. Akt is intrinsically associated with PI3K phosphorylation due to react with PIP3, a result of PI3K activity, which phosphorylates Akt at threonine 308. However to be completely activated this molecule requires an extra phosphorylation on serine 473 initiated by mammalian target of rapamycin complex 2 (mTORC2)[446]. Furthermore phosphorylated PI3K is similarly involved on phospholipase C-γ1 (PLC-γ1) instigation. PLC-γ1 reacts also with PIP3 which is recruited to the plasma membrane and activated by reacting with the phosphorylated tyrosine kinases from the growth receptor when in close proximity[447]. It is proposed that EGFR/HER2/PLC-γ1triggers cell motility[107] and HER2/HER3/PI3K may undertake a communication that endorses not only cell proliferation[108] but also motility. This data suggests there is cooperation between the heterodimers EGFR/HER2 and HER2/HER3 (Figure 4.1).

PLA methodology permits the recognition of the heterodimers cell allocation at a molecular level[448-450]. For the first time it was possible to study the heterodimerisation amongst the HER family and their effect over breast carcinoma development. Thus, for further understanding it was decided to analyse the relationships between the expression of each monomer and their interaction with HER2, to further comprehend and predict effectiveness of trastuzumab. It is also hypothesise that the different heterodimers are correlated to other biomarkers in a different manner. With this new classification system

it is possible to discriminate patients and understand in a more consistent way if trastuzumab is the best alternative therapeutic strategy.

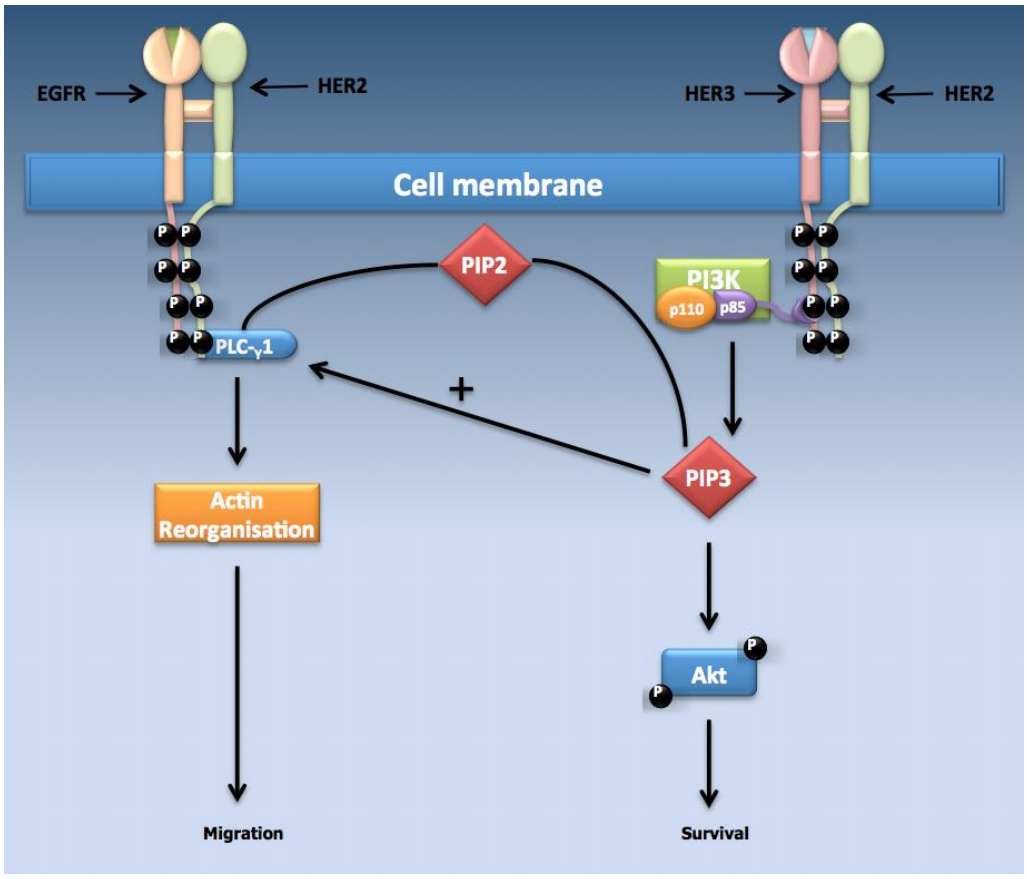

Figure 4.1 – The intercommunication between the heterodimers HER2/EGFR and HER2/HER3 promoting both cell survival and migration.

4.2 Methods

EGFR is a protein receptor localised predominantly in the cell membrane as observed in positive control tonsil tissue. This receptor was detected using the respective antibody anti-EGFR (Clone 31G7, Invitrogen), pre-treatment and the Envision technology as described in Chapter 2.

The detection of HER2/EGFR heterodimers was performed using *in situ* PLA as described in Chapter 2. These heterodimers were detected in TMAs of both series using two primary antibodies from different species, which were stable under the same pre-treatment conditions (rabbit anti-HER2 (Dako) antibody and mouse anti-EGFR (clone EGFR-R2, Santa Cruz) antibody). To quantify the heterodimers levels Duolink ImageTool software was applied. Red signals representing HER2/EGFR heterodimers were localised predominantly in the cell membrane. The cut-off between high and low levels of heterodimerisation was determined by applying X-tile software.

4.3 Results

4.3.1 EGFR

4.3.1.1 Expression in breast cancer

Regarding the EGFR expression, in the Unselected series a total of 1276/1594 (80.1%) cases revealed negative expression and 318/1594 (19.9%) cases expressed positive levels. Amongst the HER2+ series a total of 188/305 (61.6%) patients showed negative expression and 117/305 (38.4%) cases possessed positive levels for EGFR (Figure 4.2).

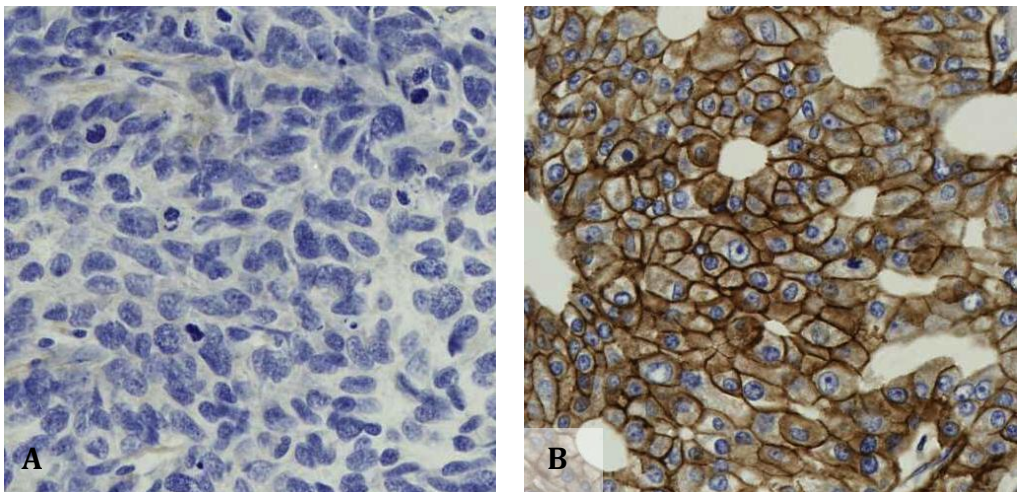

Figure 4.2 – Detection of EGFR protein expression in breast cancer cores by immunohistochemistry. The cells were labelled with a specific antibody against EGFR to detect the intensity of expression of this protein present in the cell membrane. The cells are scored in four different classes of protein expression: (A) Negative when a faint membranous immunoreactivity was observed in less than 10% of the cancer cells and (B) Positive when more than 10% of the invasive carcinoma cells show a weak/moderate perceptible membranous reactivity.

4.3.1.2 Correlation with clinicopathological parameters

Positive expression of EGFR protein in the Unselected series was highly correlated with higher tumour grade, more nuclear pleomorphism, higher mitotic frequency, poor NPI score and death (all $p < 0.001$). Also EGFR expression was correlated with less tubule formation ($p = 0.006$), presence of distant metastasis ($p = 0.001$) and higher tumour size ($p = 0.037$) (Table 4.1)

Amongst the HER2+ series, HER3 positive expression was highly associated with more nuclear pleomorphism ($p = 0.004$), local recurrence ($p = 0.031$), vascular invasion ($p = 0.023$), poor NPI score ($P = 0.027$) and death instigated by breast cancer ($p = 0.038$) (Table 4.1)

Table 4.1 – The relation between EGFR status with clinicopathological parameters in breast cancer.

Parameters	EGFR Status					
	Unselected Series			HER2+ Series		
	Neg (%)	Pos (%)	p value (Chi-Square)	Neg (%)	Pos (%)	p value (Chi-Square)
Tumour Grade						
1	252 (19.7)	26 (8.9)	<0.001 (79.908)	6 (3.2)	0 (0.0)	0.093 (4.746)
2	463 (36.1)	52 (17.9)		36 (19.1)	18 (15.4)	
3	566 (44.2)	213 (73.2)		146 (77.7)	99 (84.6)	
Tubule Formation						
1	87 (5.6)	9 (0.6)	0.006 (10.359)	1 (0.5)	0 (0.0)	0.325 (2.250)
2	420 (34.8)	83 (22.2)		38 (20.5)	17 (14.8)	
3	736 (59.7)	196 (77.2)		146 (78.9)	98 (85.2)	
Pleomorphism						
1	33 (2.7)	2 (0.7)	<0.001 (69.751)	0 (0.0)	0 (0.0)	0.004 (8.257)
2	542 (43.7)	54 (18.8)		25 (13.6)	4 (3.5)	
3	664 (53.6)	231 (80.5)		159 (86.4)	111 (96.5)	
Mitotic Frequency						
1	500 (40.2)	53 (18.4)	<0.001 (81.246)	27 (14.6)	13 (11.3)	0.714 (0.673)
2	250 (20.1)	37 (12.8)		41 (22.2)	27 (23.5)	
3	493 (39.7)	198 (68.8)		117 (63.2)	75 (65.2)	
Tumour Stage						
1	809 (63.2)	179 (61.7)	0.715 (0.671)	88 (46.8)	51 (43.6)	0.632 (0.919)
2	366 (28.6)	83 (28.6)		68 (36.2)	41 (35.0)	
3	105 (8.2)	28 (9.7)		32 (17.0)	25 (21.4)	
Distant Metastases						
Negative	909 (71.3)	176 (61.1)	0.001 (11.475)	122 (71.8)	83 (54.5)	0.254 (1.303)
Positive	366 (28.7)	112 (38.9)		65 (28.2)	33 (45.5)	
Local Recurrence						
Negative	772 (61.8)	159 (56.8)	0.123 (2.276)	119 (64.0)	88 (75.9)	0.031 (4.679)
Positive	478 (38.2)	121 (43.2)		67 (36.0)	28 (24.1)	
Regional Recurrence						
Negative	1143 (91.4)	248 (88.6)	0.131 (2.279)	165 (91.2)	100 (95.2)	0.203 (1.624)
Positive	107 (8.6)	32 (11.4)		16 (8.8)	5 (4.8)	
Tumour Size						
<1.5cm	330 (19.9)	58 (19.9)	0.037 (4.335)	33 (17.6)	14 (12.0)	0.189 (1.727)
≥1.5cm	951 (80.1)	233 (80.1)		155 (82.4)	103 (88.0)	
Vascular Invasion						
Negative	730 (55.8)	146 (53.2)	0.062 (3.488)	91 (48.7)	72 (62.1)	0.023 (5.176)
Positive	544 (44.2)	139 (46.8)		96 (51.3)	44 (37.9)	
NPI						
Good	447 (35.3)	50 (17.5)	<0.001 (33.735)	4 (2.7)	2 (2.1)	0.027 (7.247)
Moderate	636 (50.2)	180 (63.2)		40 (27.4)	42 (44.2)	
Poor	184 (14.5)	55 (19.3)		102 (69.9)	51 (53.7)	
Age						
<50	428 (33.4)	95 (32.6)	0.802 (0.063)	519 (31.8)	82 (36.6)	0.148 (2.095)
≥50	853 (66.6)	196 (67.4)		1114 (68.2)	142 (63.4)	
Death						
Negative	734 (70.2)	135 (57.9)	<0.001 (13.091)	107 (62.9)	83 (74.8)	0.038 (4.295)
Positive	312 (29.8)	98 (42.1)		63 (37.1)	28 (25.2)	

4.3.1.3 Association with patient outcome

EGFR positive expression showed significant shorter BCSS when compared with the negative expression cases ($p < 0.001$; Figure 4.3A). Also high EGFR expression was associated with worst outcome for DFI ($p = 0.001$; Figure 4.3B), revealing higher difference in the first 120 months, remaining constant afterwards. Amongst the HER2+ trastuzumab naïve series, EGFR expression did not reveal any correlation with poor outcome for both BCSS ($p = 0.0515$; Figure 4.2C) and DFI ($p = 0.430$; Figure 4.3D). Similar result was obtained for HER2+ trastuzumab adjuvant. For both BCSS ($p = 0.441$; Figure 4.3E) and DFI ($p = 0.370$; Figure 4.3F).

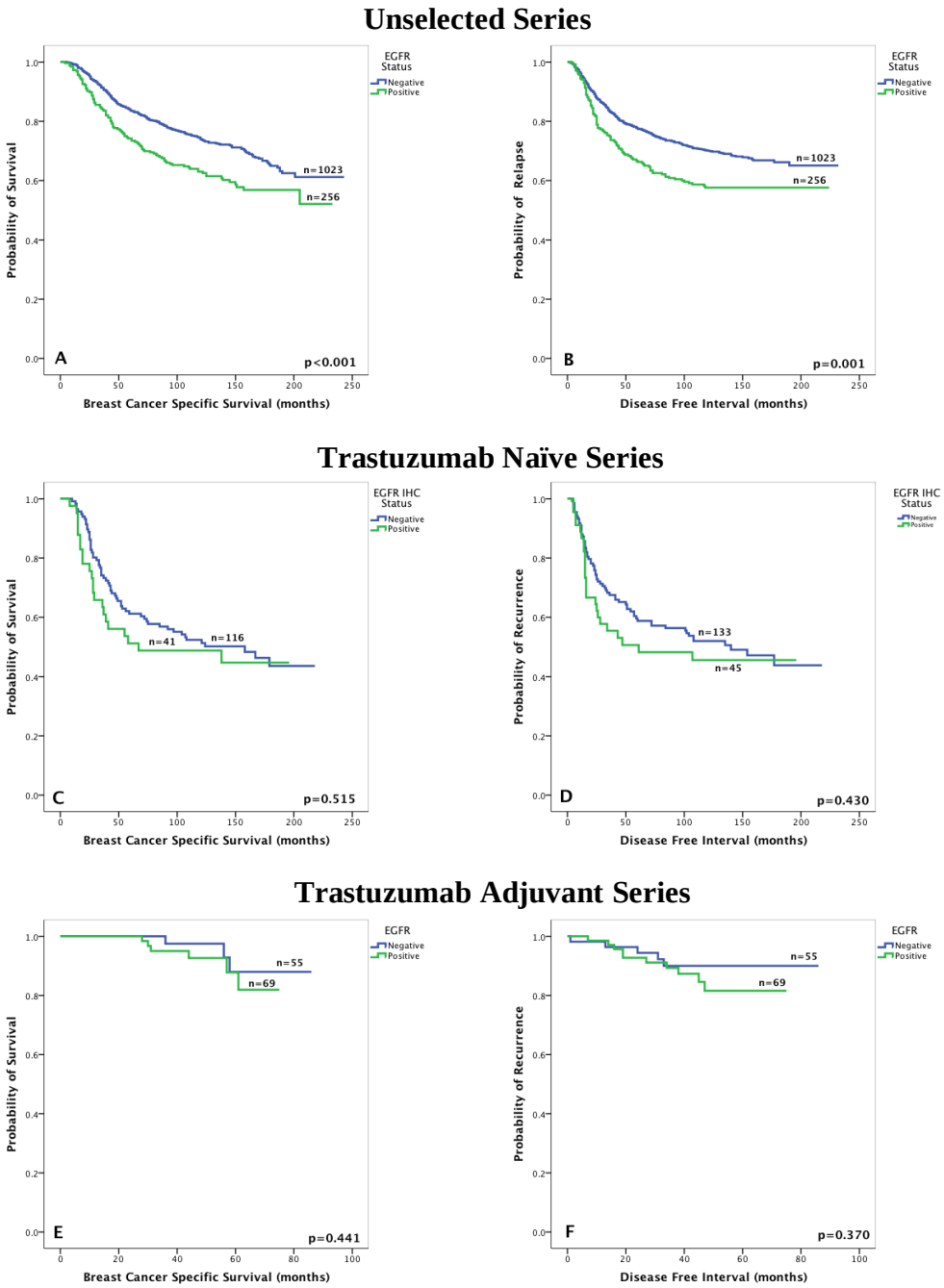

Figure 4.3 – BCSS and DFI survival analyses of EGFR IHC status amongst the Unselected series **A)** and **B)** or HER2+ trastuzumab naïve series **C)** and **D)** or HER2+ trastuzumab adjuvant **E)** and **F)**.

4.3.2 HER2/EGFR

4.3.2.1 HER2/EGFR in breast cancer

The breast cancer cases in general revealed a range of signals/cell for HER2/EGFR heterodimer, which were predominantly detected on the membrane of tumour cells between 0 – 28.7 signals/cell.

Because the frequency of HER2/EGFR levels did not reveal a normal distribution, the cut-off point to define the heterodimer levels status was determined using X-tile[345], which dichotomised data into low and high levels of heterodimerisation. It was therefore considered that low levels of HER2/EGFR heterodimers were in those cases showing less than 1.0 HER2/EGFR signals per cell (Figure 4.4A). Any incidence above these limits were accepted as high levels of heterodimerisation (Figure 4.4B).

The incidence of HER2/EGFR interaction was investigated in 812 breast carcinomas in Unselected series and 229 breast carcinomas amongst the HER2+ series revealing high levels of HER2/EGFR in 104 (12.8%) and 168 (73.4%) cases respectively.

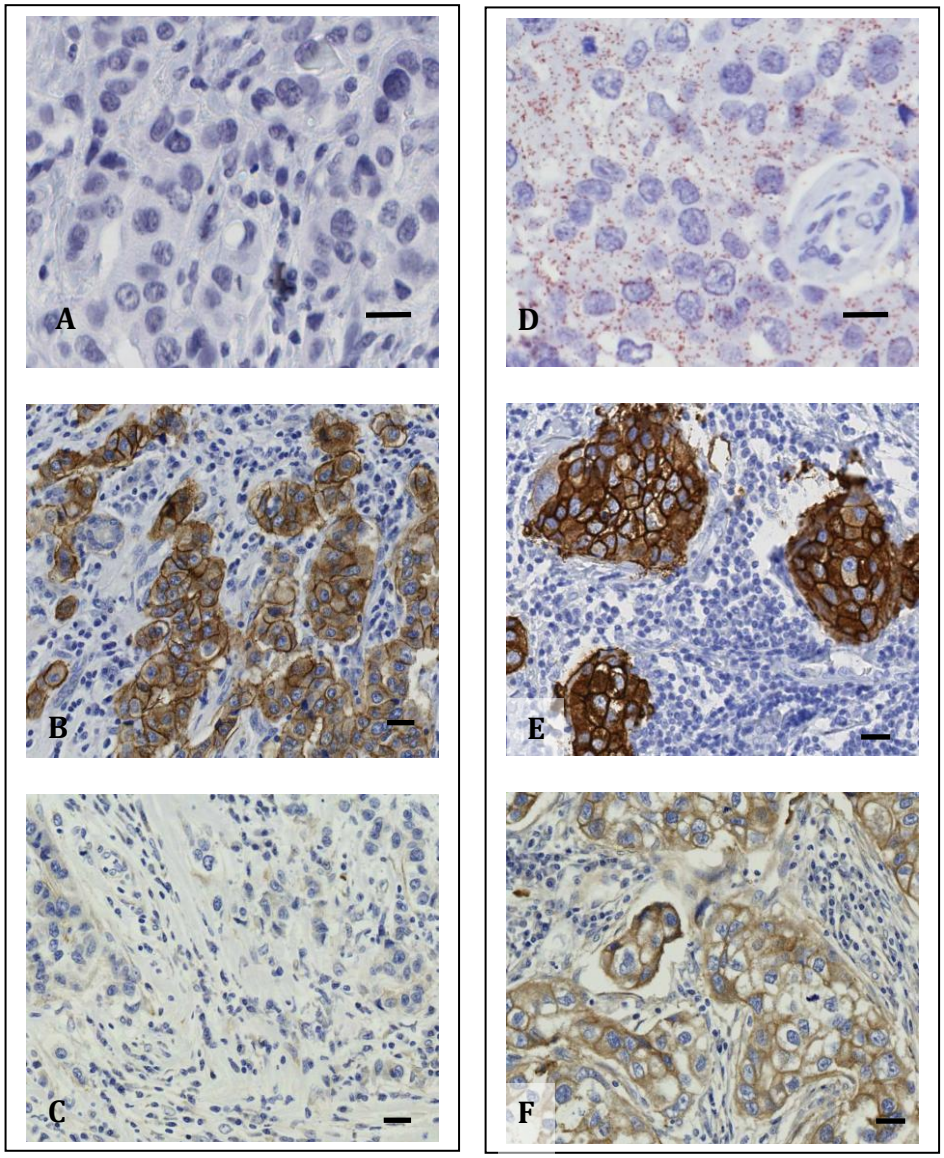

Figure 4.4 – Photomicrographs of *in situ* PLA detection of HER2/EGFR heterodimers at x40 magnification and IHC detection at x20 magnification with a scale bar representing 100 µm. Case 1: Primary breast cancer cells displaying **A)** low levels of HER2/EGFR, **B)** HER2 protein expression and **C)** EGFR protein expression. Case 2: Primary breast cancer cells displaying **D)** high levels of HER2/EGFR, **E)** HER2 protein expression and **F)** EGFR protein expression.

4.3.2.2 Correlation with HER family monomers expression

As expected, there was a strong positive correlation between HER2/EGFR heterodimer and HER2 status ($p < 0.001$) amongst the Unselected series (Table 4.2). A total of 81/96 (84.4%) breast cancer cases exposing HER2/EGFR heterodimers were HER2+. Of the remaining 15 cases that showed HER2/EGFR heterodimers but not HER2 overexpression, the majority (11/15, 73.3%) had very low levels of heterodimers (<2.4 signals/cell). Two cases had HER2 protein expression, regarded as 2+ determined by IHC but did not show gene amplification by CISH. The remaining two cases had high levels of HER2/EGFR heterodimers but no overexpression of HER2. There were a proportion of HER2+ cases (27/108, 25.0%) that did not reveal high levels of HER2/EGFR heterodimers. Interestingly, 59/89 (66.3%) cases with HER2/EGFR heterodimers did not express EGFR protein ($p = 0.002$).

Similar to that observed in the Unselected series, the levels of the HER2/EGFR heterodimer in the HER2+ series was independent of EGFR expression ($p = 0.455$) where approximately half cases showing the heterodimer were negative for EGFR protein. There was 168/229 (73.8%) HER2 overexpressing cases that revealing HER2/EGFR high levels against 61 (26.2%) possessing low levels. Amongst these 61 cases, 31/61 (50.8%) cases were EGFR negative cases and the remaining 30/61 (49.2%) cases revealed low levels of the heterodimers despite HER2 positivity. From these 30 cases 21 (70%) possessed EGFR expression and the remaining 9 no data was available concerning EGFR expression (Table 4.2).

Table 4.2 – Association between HER2/EGFR heterodimer levels and expression of HER family and hormone receptors in breast cancer.

Biomarker	HER2/EGFR heterodimers expression					
	Unselected Series			HER2+ series		
	Low (%)	High (%)	p value (Chi-Square)	Low (%)	High (%)	p value (Chi-Square)
EGFR						
Negative	476 (80.7)	59 (66.3)	0.002	31 (59.6)	81 (53.6)	0.455
Positive	114 (19.3)	30 (33.7)	(9.578)	21 (40.4)	70 (46.4)	(0.558)
HER2						
Negative	655 (96.0)	15 (15.6)	<0.001	0 (0.0)	0 (0.0)	N/A
Positive	27 (4.0)	81 (84.4)	(455.221)	61 (100)	168 (100)	
HER3						
Negative	48 (8.5)	3 (3.6)	<0.119	4 (8.3)	5 (3.6)	0.190
Positive	519 (91.5)	81 (96.4)	(2.427)	44 (91.7)	133 (96.4)	(1.716)
HER4						
Negative	97 (17.1)	6 (7.0)	0.017	0 (0.0)	5 (3.6)	0.184
Positive	471 (82.9)	80 (93.0)	(5.743)	47 (100)	132 (96.4)	(1.763)

4.3.2.3 Correlation with HER2 and EGFR mutual expression

Where there were high levels of HER2 monomer, determined by IHC, the frequency of heterodimers increased dramatically up to 80% (Figure 4.2F). When the HER2 expression status was negative the levels of HER2/EGFR changed dramatically to low status on a frequency of nearly 99% (Table 4.3).

Table 4.3 – Frequency of HER2/EGFR levels against HER2 and EGFR mutual expression status.

Expression Status	**HER2/EGFR**	
	Low (%)	High (%)
HER2+/EGFR+	13 (20.3)	51 (79.7)
HER2+/EGFR-	8 (28.6)	20 (71.4)
HER2-/EGFR+	93 (93.9)	6 (6.1)
HER2-/EGFR-	461 (98.5)	7 (1.5)

4.3.2.4 Correlation with HER2 amplification

Figure 4.5 represents the correlation between HER2 gene amplification with the number of detected signals/cell of HER2/EGFR heterodimers where there was a positive correlation observed ($r^2 = 0.381$, $p < 0.001$). Most of the cases representing HER2/EGFR low levels remained under the HER2/CEN17 ratio 2.

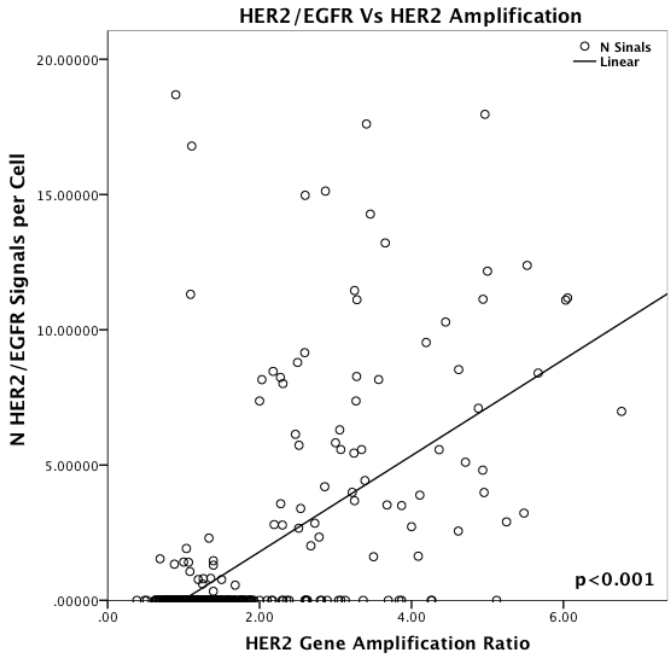

Figure 4.5 – Linear regression analysis between HER2/EGFR and *HER2* gene amplification in breast cancer (Unselected series).

4.3.2.5 Correlation with histological tumour type

There was a very strong correlation between histological types and HER2/EGFR levels ($\chi^2 = 16.297$, $p = 0.003$, Figure 4.6). A significantly higher percentage (20.8%) of medullary carcinomas showed of HER2/EGFR heterodimers. The highest number of HER2/EGFR heterodimers occurred in invasive ductal with 90 cases. No expression of HER2/EGFR heterodimers was observed in invasive lobular or tubular carcinomas.

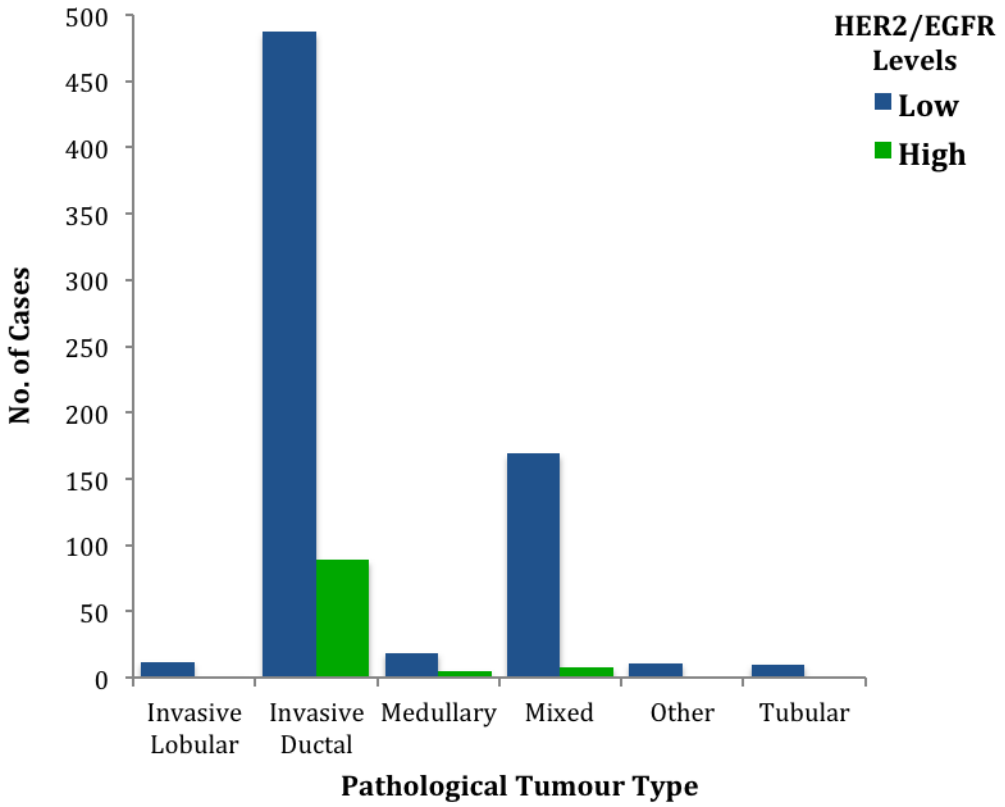

Figure 4.6 – Correlation of histological tumour type and HER2/EGFR dimerisation levels for Unselected series ($p = 0.003$).

4.3.2.6 Correlation with the clinicopathological parameters

Correlations between HER2/EGFR heterodimers and clinicopathological parameters are summarised in Table 4.4. Within the Unselected series, HER2/EGFR heterodimers were significantly associated with higher tumour grade, marked pleomorphism, higher mitotic frequency and poor NPI, (all $p < 0.001$) and lack of tubule formation ($p = 0.010$). High HER2/EGFR heterodimer levels were also associated with presence of distant metastasis ($p = 0.005$). Both local and regional recurrence ($p = 0.033$ and $p = 0.042$, respectively) and death due to breast cancer ($p = 0.042$) were also highly correlated with HER2/EGFR high levels. Though, tumour stage, tumour size, vascular invasion and age of the patient were not correlated with HER2/EGFR levels.

Within the HER2+ series, HER2/EGFR heterodimers were associated with high tumour grade ($p = 0.006$), less of tubule formation ($p = 0.033$) and poor NPI score ($p = 0.048$).

Table 4.4 – Association of HER2/EGFR dimerisation levels with clinicopathological parameters in breast cancer

Parameters	HER2/EGFR heterodimers status					
	Unselected Series			HER2+ Series		
	Low (%)	High (%)	p value (Chi- Square)	Low (%)	High (%)	p value (Chi- Square)
Tumour Grade						
1	94 (13.3)	4 (3.8)	<0.001 (37.203)	1 (1.6)	6 (3.6)	0.006 (9.3267)
2	231 (32.7)	11 (10.6)		20 (32.8)	25 (14.9)	
3	381 (54.0)	89 (85.6)		40 (65.6)	137 (81.5)	
Tubule Formation						
1	21 (3.1)	2 (1.9)	0.010 (9.115)	0 (0.0)	2 (1.2)	0.033 (6.795)
2	226 (33.4)	20 (19.4)		17 (28.3)	23 (13.9)	
3	429 (63.5)	81 (78.6)		43 (71.7)	140 (84.8)	
Pleomorphism						
1	8 (1.2)	0 (0.0)	<0.001 (21.096)	0 (0.0)	0 (0.0)	0.113 (2.506)
2	253 (37.5)	16 (15.5)		10 (16.7)	15 (9.1)	
3	414 (61.3)	87 (84.5)		50 (83.3)	149 (90.1)	
Mitotic Frequency						
1	193 (28.6)	10 (9.7)	<0.001 (23.796)	11 (28.3)	23 (14.5)	0.490 (1.426)
2	132 (19.5)	14 (13.6)		18 (23.3)	42 (26.1)	
3	351 (51.9)	79 (76.7)		31 (48.3)	99 (59.4)	
Tumour Stage						
1	404 (57.3)	53 (51.0)	0.104 (4.522)	23 (39.0)	70 (41.7)	0.926 (0.154)
2	233 (33.0)	34 (32.7)		23 (39.0)	64 (38.1)	
3	68 (9.6)	17 (16.3)		13 (22.0)	34 (20.2)	
Distant Metastases						
No	471 (67.1)	55 (52.9)	0.005 (8.067)	47 (78.3)	118 (70.7)	0.252 (1.310)
Yes	231 (32.9)	49 (47.1)		13 (21.7)	49 (29.3)	
Local Recurrence						
No	406 (58.8)	48 (47.5)	0.033 (4.543)	49 (80.3)	117 (70.1)	0.123 (2.379)
Yes	285 (41.2)	53 (52.3)		12 (19.7)	50 (29.9)	
Regional Recurrence						
No	616 (89.1)	83 (82.2)	0.042 (4.128)	55 (93.2)	140 (90.9)	0.587 (0.295)
Yes	75 (10.9)	18 (17.8)		4 (6.8)	14 (9.1)	
Tumour Size						
<1.5 cm	147 (20.8)	18 (17.3)	0.406 (0.690)	10 (16.4)	26 (15.5)	0.866 (0.028)
≥1.5 cm	559 (79.2)	86 (82.7)		51 (83.6)	142 (84.5)	
Vascular Invasion						
No	367 (52.3)	46 (45.1)	0.175 (1.838)	34 (56.7)	90 (54.2)	0.744 (0.1076)
Yes	335 (47.7)	56 (54.9)		26 (43.3)	76 (45.8)	
NPI						
Good	160 (24.3)	7 (6.8)	<0.001 (31.055)	15 (24.6)	21 (12.5)	0.048 (11.152)
Moderate	402 (57.5)	63 (61.2)		37 (60.7)	95 (56.2)	
Poor	127 (18.2)	33 (32.1)		9 (14.7)	52 (30.9)	
Age						
<50	230 (32.5)	39 (37.5)	0.310 (1.029)	23 (37.7)	77 (46.7)	0.229 (1.450)
≥50	478 (67.5)	65 (62.5)		38 (62.3)	88 (53.3)	
Death						
No	374 (63.4)	47 (52.2)	0.042 (4.130)	47 (79.7)	114 (72.2)	0.261 (1.265)
Yes	216 (36.6)	43 (47.8)		12 (20.3)	44 (27.8)	

4.3.2.7 Correlation with expression of other biomarkers

Concerning other biomarkers that are not included in the HER family, their association with HER2/EGFR heterodimers in the Unselected series are summarised in Table 4.5. High expression of HER2/EGFR heterodimers revealed a very significant association with CK18 ($p < 0.001$), PI3K ($p = 0.002$), p53 ($p < 0.001$), Ki67 ($p = 0.004$) and PTEN ($p = 0.007$). On the other hand, a significantly negative association was observed against Bcl-2 ($p < 0.001$), ER ($p < 0.001$) and PgR ($p < 0.001$).

Within the HER2+ series, HER2/EGFR was positively correlated with only CK18 ($p = 0.012$). There was a significant association between high HER2/EGFR heterodimer status with negative ER and PgR status ($p = 0.002$ and $p = 0.016$, respectively), Bcl-2 ($p = 0.014$) and CK14 ($p = 0.014$) expression. Though HER2/EGFR heterodimers were not associated with PI3K the heterodimers possessed a strong trend with PI3K ($p = 0.051$).

Table 4.5 – Association between HER2/EGFR heterodimer levels and expression of biomarkers in breast cancer.

Biomarkers	HER2/EGFR Status					
	Unselected Series			HER2+ Series		
	Low (%)	High (%)	p value (Chi- Square)	Low (%)	High (%)	p value (Chi- Square)
CK5/6						
Negative	557 (81.4)	83 (83.9)	0.563	33 (57.9)	94 (61.0)	0.679
Positive	127 (18.6)	16 (16.2)	(0.335)	24 (42.1)	60 (39.0)	(0.172)
CK7/8						
Negative	20 (2.9)	0 (0.0)	0.084	1 (3.7)	0 (0.0)	0.086
Positive	662 (97.1)	99 (100)	(2.980)	26 (96.3)	79 (100)	(2.954)
CK14						
Negative	584 (86.5)	89 (89.0)	0.493	35 (81.4)	123 (93.9)	0.014
Positive	91 (13.5)	11 (11.0)	(0.469)	8 (18.6)	8 (6.1)	(6.056)
CK18						
Negative	124 (19.7)	5 (5.3)	0.001	6 (15.0)	5 (3.8)	0.012
Positive	506 (80.3)	90 (94.7)	(11.734)	34 (85.0)	126	(6.367)
Bcl-2						
Negative	210 (40.1)	60 (76.9)	<0.001	26 (47.3)	93 (66.4)	0.014
Positive	314 (59.9)	18 (23.1)	(37.267)	29 (52.7)	47 (33.6)	(6.092)
pAkt						
Negative	127 (25.2)	16 (20.0)	0.311	8 (33.3)	13 (20.3)	0.202
Positive	376 (74.8)	64 (80.0)	(1.027)	16 (66.7)	51 (79.7)	(1.629)
PI3K						
Negative	126 (21.7)	8 (10.0)	0.002	3 (5.8)	3 (2.2)	0.051
Moderate	153 (26.3)	14 (17.5)	(12.346)	10 (19.2)	12 (8.8)	(0.051)
Strong	302 (52.0)	58 (72.5)		39 (75.0)	122	
p21						
Negative	87 (78.4)	9 (90.0)	0.385	10 (41.7)	15 (25.4)	0.144
Positive	24 (21.6)	1 (10.0)	(0.756)	14 (58.3)	44 (74.6)	(2.138)
MUC-1						
Negative	72 (12.5)	6 (7.3)	0.385	4 (7.3)	5 (3.4)	0.466
Moderate	278 (48.4)	43 (52.4)	(1.911)	28 (50.9)	73 (50.0)	(1.527)
Strong	224 (39.0)	33 (40.2)		23 (41.8)	68 (46.6)	
BRCA1						
Negative	326 (58.3)	57 (67.9)	0.097	35 (66.0)	101 (69.2)	0.674
Positive	233 (41.7)	27 (32.1)	(2.759)	18 (34.0)	45 (30.8)	(0.177)
p53						
Negative	473 (70.9)	50 (50.5)	<0.001	20 (34.5)	54 (33.3)	0.874
Positive	194 (29.1)	49 (49.5)	(16.579)	38 (65.5)	108 (66.7)	(0.025)
PTEN						
Weak	98 (19.0)	4 (5.1)	0.007	2 (4.7)	6 (5.6)	0.946
Moderate	191 (37.0)	30 (38.5)	(9.913)	19 (44.2)	49 (45.8)	(0.111)
Strong	227 (44.0)	44 (56.4)		22 (51.2)	52 (48.6)	
Ki67						
Weak	173 (30.6)	12 (14.1)	0.004	2 (11.1)	7 (10.6)	0.216
Moderate	209 (37.0)	34 (40.0)	(11.186)	11 (61.1)	26 (39.4)	(3.062)
Strong	183 (32.4)	39 (45.9)		5 (27.8)	33 (50.0)	
ER						
Negative	197 (29.4)	64 (66.0)	<0.001	23 (39.7)	102	0.002
Positive	474 (70.6)	33 (34.0)	(50.658)	35 (60.3)	59 (36.6)	(9.775)
PgR						
Negative	296 (44.6)	72 (75.8)	<0.001	32 (54.2)	111 (71.6)	0.016
Positive	367 (55.4)	23 (24.2)	(32.266)	27 (45.8)	44 (28.4)	(5.819)

4.3.2.8 Correlation with the other HER heterodimers

There was a strong association between HER2/EGFR heterodimerisation levels with both HER2/HER3 and HER2/HER4 for both the Unselected and HER2+ series (all $p < 0.001$; Figure 4.6).

Table 4.6 – Association of HER2/EGFR heterodimer and HER2/HER3 or HER2/HER4 heterodimer levels in breast cancer.

	HER2/EGFR Status					
Heterodimers	**Unselected Series**			**HER2+ Series**		
	Low (%)	High (%)	p Value (Chi- Square)	Low (%)	High (%)	p Value (Chi- Square)
HER2/HER3						
Low	387 (97.5)	11 (16.7)	<0.001 (306.265)	38 (84.4)	18 (13.7)	<0.001 (77.182)
High	10 (2.5)	55 (83.3)		7 (15.6)	113 (86.3)	
HER2/HER4						
Low	432 (98.0)	24 (31.6)	<0.001 (274.489)	41 (95.3)	36 (26.3)	<0.001 (63.787)
High	9 (2.0)	52 (68.4)		2 (4.7)	101 (73.7)	

4.3.2.9 Association with patient outcome

High levels of HER2/EGFR heterodimer were significantly associated with poor outcome in relation to both BCSS (p = 0.006) and DFI (p = 0.014) in the Unselected series (Figure 4.7A and B). However HER2/EGFR heterodimers amongst trastuzumab naïve and trastuzumab adjuvant cohorts did not reveal any significant association with either BCSS (Figure 4.7C and E, respectively) or DFI (Figure 4.7D and F, respectively).

Amongst the Unselected series the multivariate Cox regression including tumour size, stage and grade, high levels of HER2/EGFR heterodimers was an independent predictor for worse BCSS (HR = 0.64, 95% CI= 0.45-0.89, p = 0.009, Table 4.7) and DFI (HR = 0.64, 95% CI = 0.47-0.88, p = 0.005, Table 4.7).

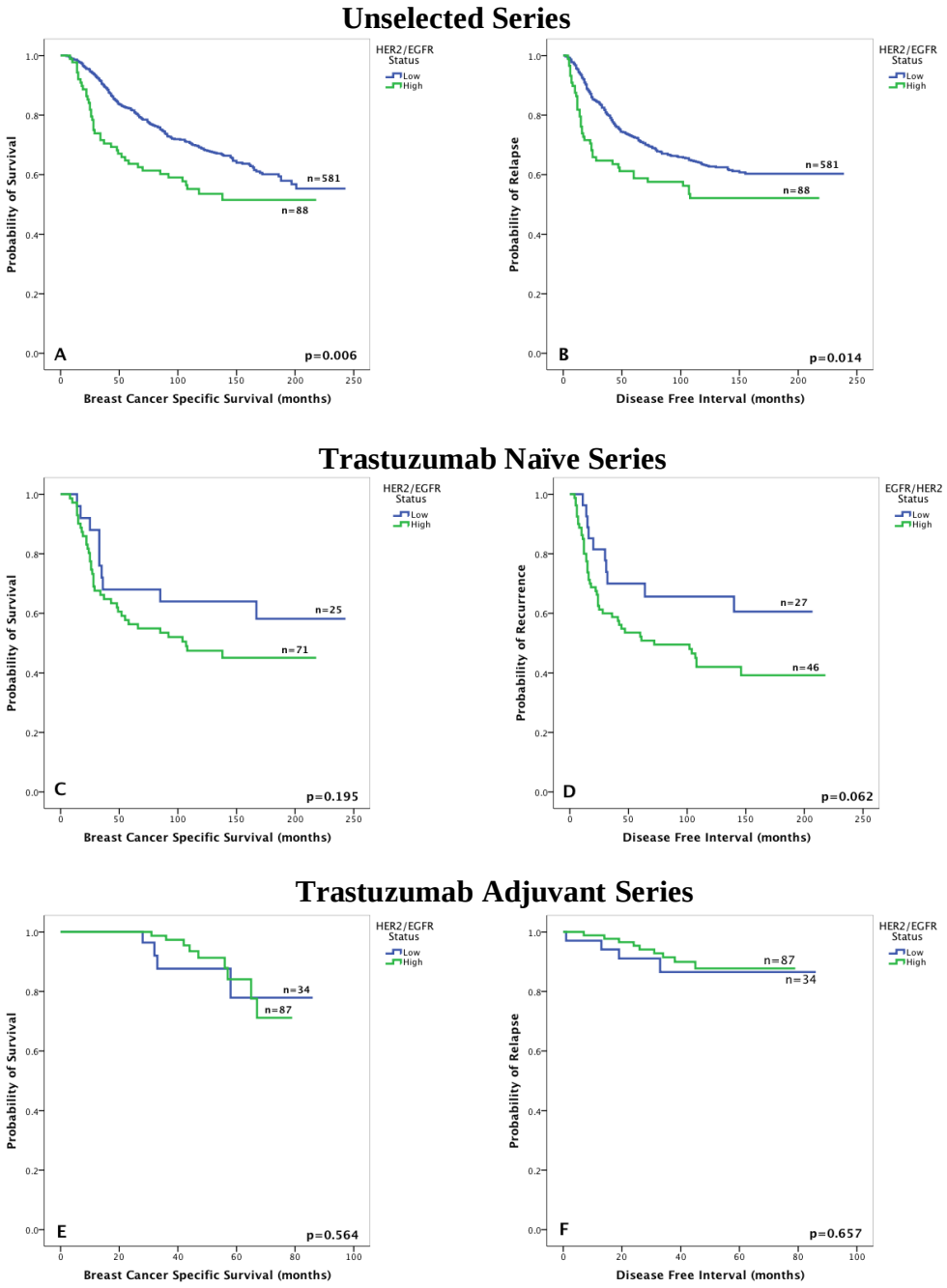

Figure 4.7 – BCSS and DFI analysis for HER2/EGFR status amongst the Unselected series (**A** and **B**), Trastuzumab Naïve Series (**C** and **D**) and Trastuzumab Adjuvant Series (**E** and **F**).

Table 4.7 – Multivariate Cox regression analysis of factors associated with BCSS and DFI for Unselected series.

Variable	BCSS		DFI	
	Hazard Ratio (95% CI)	p Value	Hazard Ratio (95% CI)	p Value
Tumour Size (cm)	0.52 (0.34-0.79)	0.002	0.66 (0.48-0.91)	0.010
Tumour Stage	1.92 (1.62-2.29)	<0.001	1.78 (1.53-2.09)	<0.001
Tumour Grade	1.49 (1.49-1.20)	<0.001	1.23 (0.95-1.34)	0.163
HER2/EGFR	0.64 (0.45-0.89)	0.009	0.64 (0.47-0.88)	0.005

4.3.2.10 Association with ER status and patient outcome

Survival analysis was conducted for HER2/EGFR heterodimers amongst both ER negative and ER positive populations in relation to BCSS and DFI. In the ER negative population, there was no association with either BCSS or DFI (p = 0.138 and p = 0.165, respectively, Figure 4.8A and B). However for those patients with ER positive tumours and high HER2/EGFR heterodimer levels were significantly associated with worse BCSS (p = 0.009; Figure 4.9A) and DFI (p = 0.032; Figure 4.9B).

For the first 60 months the prognosis was significantly different for the different HER2/EGFR levels. Amongst the ER negative population that outcome difference still not significant for both BCSS (p = 0.156; Figure 4.8C) and DFI (p = 0.183; Figure 4.8D). Though amongst the ER positive patients for the first 60 months the difference of survival is highly significant for both BCSS (p = 0.004; Figure 4.9C) and DFI (p = 0.029; Figure 4.9D).

The multivariate Cox regression model including tumour size, tumour stage and tumour grade, revealed that high levels of HER2/EGFR amongst the ER positive patients was not independently associated with worse BCSS (HR = 0.62, 95% CI = 0.36-1.04, p = 0.070, Table 4.8) and DFI (HR = 0.68, 95% CI = 0.42-1.12, p = 0.134, Table 4.8)

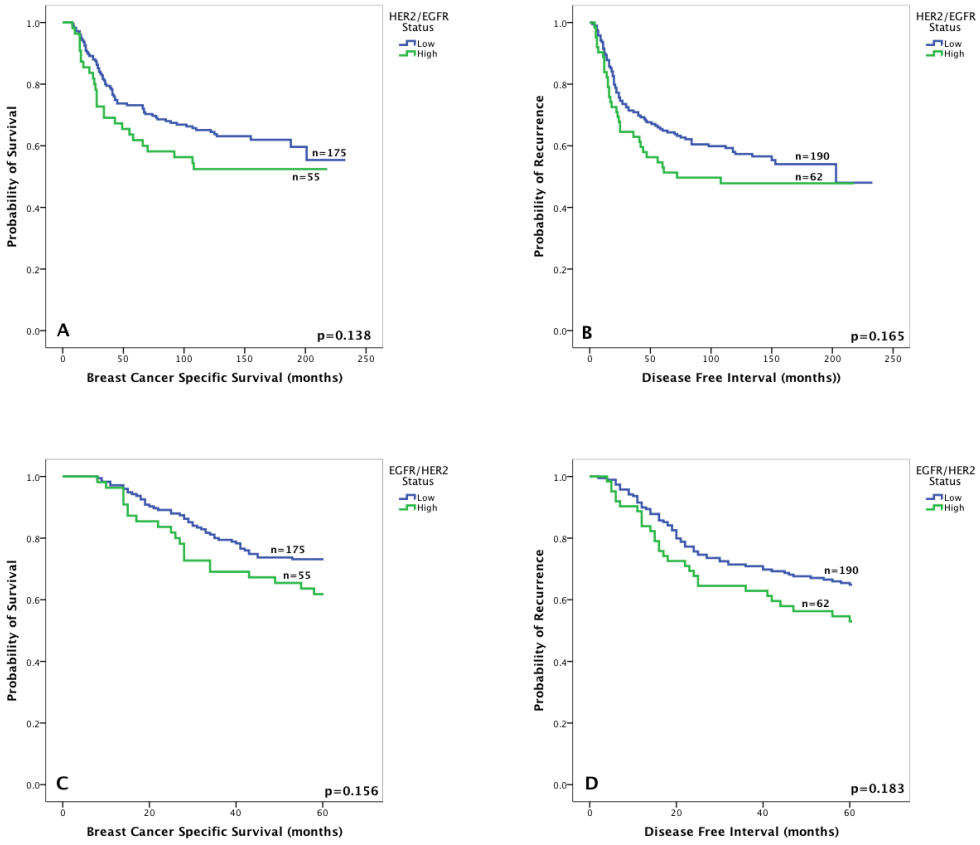

Figure 4.8 – BCSS and DFI analysis for HER2/EGFR status amongst the ER negative cases in Unselected series for a period of 250 months **A)** and **B)** or for a period of 60 months **C)** and **D)**.

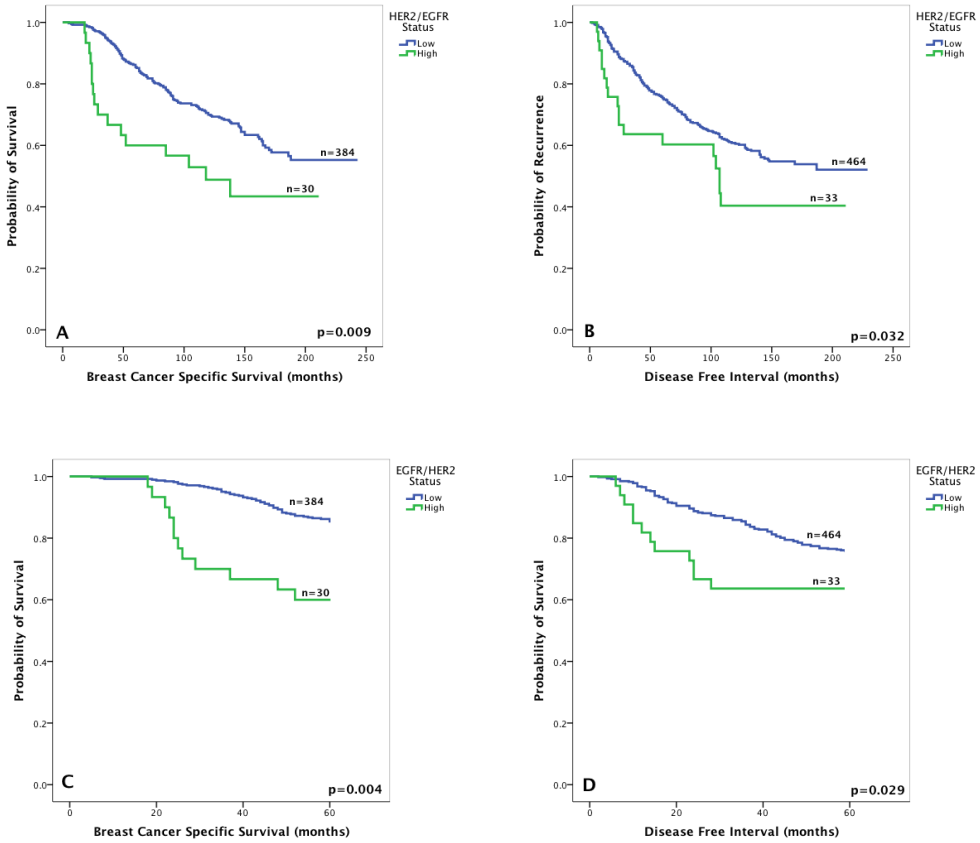

Figure 4.9 – BCSS and DFI analysis for HER2/EGFR status amongst the ER positive cases in Unselected series for a period of 250 months **A)** and **B)** or for a period of 60 months **C)** and **D)**.

Table 4.8 – Multivariate Cox regression analysis of factors associated with BCSS and DFI for ER positive cases in Unselected series.

Variable	BCSS		DFI	
	Hazard Ratio (95% CI)	p Value	Hazard Ratio (95% CI)	p Value
Tumour Size (cm)	0.51 (0.32-0.84)	0.007	0.64 (0.44-1.12)	0.022
Tumour Stage	1.89 (1.50-2.38)	<0.001	1.70 (1.39-2.08)	<0.001
Tumour Grade	1.52 (1.19-1.94)	0.001	1.20 (0.98-1.46)	0.084
HER2/EGFR	0.62 (0.36-1.04)	0.070	0.69 (0.42-1.12)	0.134

4.3.2.11 Association with patient outcome in HER2+ trastuzumab adjuvant versus naïve

Survival analysis of the adjuvant trastuzumab cohort against the trastuzumab naïve was performed and is represented in Figure 4.10A-D. Similar stage and grade patients from both cohorts were selected and matched in order to obtain comparable patients where the significant difference would be the in the treatment plan, which included or not trastuzumab. There was no significant different on patients' outcome when HER2/EGFR levels are low for both BCSS ($p = 0.099$; Figure 4.10A) and DFI ($p = 0.056$; Figure 4.10B). Using HER2/EGFR high levels to understand patients' survival or relapse occurrences, the Kaplan-Meier analysis revealed that those receiving trastuzumab had a better outcome for BCSS ($p < 0.001$; Figure 4.10C) and DFI ($p < 0.001$; Figure 4.10D). At the end of 60 months the probability of survival for those receiving trastuzumab was above 80% comparing with not even 60% for the naïve group.

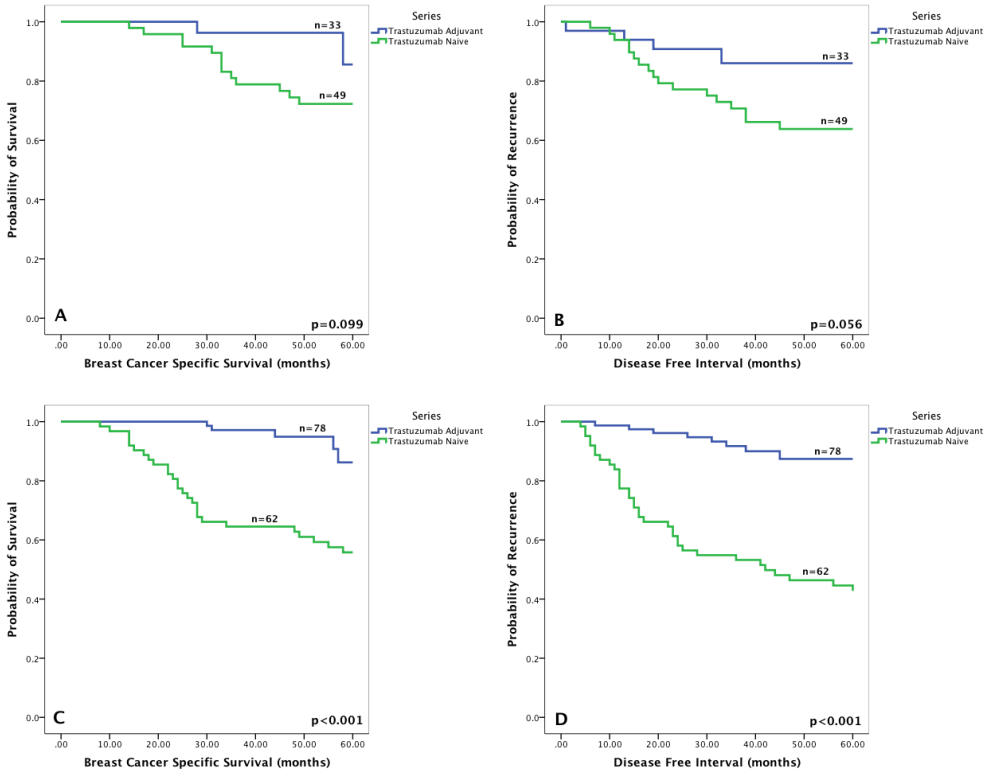

Figure 4.10 – BCSS and DFI analysis for HER2/EGFR status amongst the HER2+ trastuzumab adjuvant series against HER2+ trastuzumab naïve series regarding **A)** and **B)** HER2/EGFR low levels or **C)** and **D)** HER2/EGFR high levels.

4.4 Discussion

EGFR is not only a very critical biomarker in molecular classification but also valuable as a prognostic factor in breast cancer[451]. Mutual expression of EGFR and HER2 has been commonly detected in some types of carcinomas, particularly in ovarian[452] and breast[453]. Moreover constitutive HER2/EGFR heterodimer is determinant on migration signalling of breast cancer cells[454]. The aims of this chapter were to investigate the expression of EGFR in breast cancer and whether its dimerisation with HER2 was associated with patient outcome and response to trastuzumab treatment.

EGFR positive expression detected via IHC revealed to reach 20%, concurrent with previous study developed by Rampaul and colleagues[438]. Furthermore, it was revealed a co-expression of EGFR and HER2 monomers in 25%-35% of tumours[442,444], which is comparable to the results presented, showing 38.4% of the patients with the same condition. Different prognostic suggestions were anticipated regarding EGFR expression associating with high levels of proliferation and development of metastases[419], but also been associated with decline of tumour progression[421]. In this study EGFR was highly associated with presence of metastases, higher tumour grade and high tumour size, which is supported by other studies[423,430]. But contradicting the same studies EGFR expression was not associated with tumour stage. Even though only 2.7% of the EGFR positive cases are associated with poor prognosis[455]. It was demonstrated that 19.3% were associated with poor NPI score and the correlation with death occurrence due to breast cancer was highly significant. Amongst the HER2+ series, EGFR expression did not reveal to be relevant

against clinicopathological parameters as amongst the Unselected series. Understandably the outcome of those patients possessing high expression of EGFR was worse amongst the Unselected series in concordance with previous studies[414] but no significance was observed when co-expressed with HER2, also sustained by Rehim and colleagues[331]. Different studies have been revealing different results regarding EGFR expression with some suggesting a correlation with worse prognosis and recurrence great prevalence[436,437] and those that discovered no association[438,439].

Even though, multiple studies were performed concerning EGFR and HER2 co-expression but none have reported the prognostic values of the heterodimer. HER2/EGFR dimerisation frequency revealed to be present in 12.8% of the Unselected series against the 35% previously reported for HER2 and EGFR co-expression[444]. This difference of frequency indicates that further mechanisms are involved in heterodimerisation such as ligand/receptor interaction[249]. Also supports the idea that PLA detects primarily protein interactions and not co-localisation of monomers as suggested by different studies[456,457].

The frequency of HER2/EGFR heterodimer high levels drops dramatically (from 71.4% to 6.1%) when HER2 monomer scoring turns to negative. The reduction was almost inexistent (from 79.7% to 71.4%) when EGFR monomer expression becomes deficient. This dramatic difference might explain the idea that dimerisation occurrence may not be dependent on EGFR overexpression but can also materialise when its levels are not considered positive using IHC. Furthermore dimerisation is dependent on ligand/EGFR[32] activation reaction

upstream and not on EGFR overexpression individually. The expression of the ligands EGF and TGFα is extremely correlated with EGFR expression[249] promoting cancer development and aggressiveness, though EGFR is dependent on these ligands to become in an activated conformation[268]. In contrast, HER2, which is unable to interact with any ligand, must be only highly expressed in a constant competent form to function as co-receptor[63]. However still some inconsistencies, when is observed high levels of HER2/EGFR while none of the monomers were positively scored, representing 8.3% of the total cases. One explanation might reside on the antibodies used in both techniques for the respective labelling. For both technologies was used the same anti-HER2 antibody, however due to methodology restrictions each technique required a different anti-EGFR antibody. Therefore different antibodies react against different epitopes delivering different levels of sensitivity and specificity. Additionally pre-treatments during the procedure such as antigen-retrieval unmask the epitopes to make them available for interaction with the respective antibody[458]. In this study for both methods the antigen retrieval treatment consists in different methodologies delivering some variances in the results. Though, the correlation between the HER2/EGFR heterodimers levels and the monomers results still highly significant.

The analyses of HER2/EGFR levels against *HER2* amplification revealed to be very interesting by the observation of the significant differences on HER2/EGFR levels between both margins of the cut-off established for *HER2/CEN17* ratio to define the amplification status. Most of the high levels of HER2/EGFR heterodimers were associated with *HER2* gene amplification.

It was observed some cases that whilst there was *HER2* amplification and EGFR expression, heterodimerisation did not occur, supporting the idea that other mechanisms are critical on dimerisation occurrence[167,249,459].

The very high association of HER2/EGFR with distant metastases occurrence and high mitotic frequency is promoted by cell proliferation and apoptosis evasion, which is correlated with PI3K[107,460,461]. Throughout the tumour development, a shift is required to modify the cells behaviour, resulting in motility competence from the main carcinoma cluster invading adjacent tissues. EGFR homodimers and HER2/EGFR heterodimers are assembled by EGF ligand stimulation, but HER2 is the critical to activate PLC-γ1 supporting the idea that the heterodimers are favoured over the homodimers[462]. HER2/EGFR trigger downstream pathways that control PLC-γ1 signal transduction and triggers cell motility[107]. Furthermore PLC-γ1 signal transduction is not only triggered directly by HER2/EGFR heterodimer but also indirectly by PI3K[447]. This indirectly triggering of PI3K is regulated by HER2/HER3 heterodimerisation signalling transduction[108], suggesting a possible crosstalk between HER2/EGFR and HER2/HER3[461]. In this study it was revealed a strong association between HER2/EGFR with PI3K expression and also between both HER heterodimers supporting the idea of a possible crosstalk between them. As anticipated Bcl-2 has an important role in breast carcinomas being highly associated with ER positivity however its lost is associated with other poor prognostic markers such as p53, EGFR and HER2 expressions[463]. It was even suggested that EGFR ligands had an important role on downregulation of Bcl-2[463]. In this study HER2/EGFR high levels

demonstrated a negative association with both Bcl-2 and ER for both series, but highly associated with strong expression of p53 in only the Unselected series. This supports the idea that Bcl-2 anti-apoptotic role might be regulated by the hormone receptors previously defended[463]. However does not necessary supports the idea that cell harbouring high levels of HER2/EGFR are directed to apoptosis. Two studies revealed that the levels of CK18 degrades with apoptosis occurrence[464,465], which confirm the idea that HER2/EGFR is involved in apoptosis evasion processes by being associated with CK18 expression in both cohorts. Also the HER2/EGFR high levels were highly correlated with Ki67, which is involved in high levels of cell proliferation[397].

Although the monomers EGFR and HER2 overexpression amongst breast cancer have been previously correlated with worse outcome[466,467], HER2/EGFR heterodimerisation has not been well documented in breast cancer. In this chapter, it is shown for the first time that HER2/EGFR heterodimers were highly prevalent in HER2+ breast cancer and were correlated with poor outcome. This is in contrast to a previous study which showed that EGFR and HER2 co-expression had no significant association with survival[331]. Moreover the HER2/EGFR high levels were identified as an independent prognostic factor in multivariate analysis. Amongst the HER2+ trastuzumab naïve cohort the prognostic analysis did not reveal to be significant, though nearly significant for DFI outcome. For the first 80 months of trastuzumab adjuvant treatment, HER2/EGFR levels did not demonstrate any worse outcome or development of resistance to this target therapy.

Because of a negative association of HER2/EGFR heterodimer against ER levels it was relevant to understand the implication on patients' survival. Indeed if ER status is negative, the patients with high levels of HER2/EGFR heterodimerisation did not possess a worse outcome. When ER positivity is present, those cases possessing high levels of HER2/EGFR demonstrated a worse outcome, especially significant for BCSS. However HER2/EGFR was not identified as an independent prognostic factor in multivariate analysis. The reason for this fact is probably because of the negative correlation with ER, which is recognised as a prognostic factor in breast cancer[468]. This poor prognosis is developed throughout the first 60 months after diagnosis, suggesting that a even though negatively associated, if both classes of receptors are expressed in breast carcinomas, a signalling crosstalk between them takes place contributing for development of aggressiveness. The association of HER2/EGFR with PI3K might explain this poor outcome, due to PI3K also be connected with activation of ER and its co-regulators. This cycle of HER2/ER crosstalk is involved in carcinomas development and resistant to therapies[105,109].

Finally it was possible to demonstrate the significant role of HER2/EGFR amongst breast carcinomas and moreover the effectiveness of trastuzumab by revealing the similar outcome between both HER2+ series in case the dimer is not present in high levels. Though the prognostic data for those submitted to trastuzumab revealed to be considerable improved comparing with trastuzumab naïve patients.

In conclusion, PLA demonstrated to be a reliable, reproducible and able to quantify HER2/EGFR heterodimers in FFPE clinical cores. The high levels of HER2/EGFR seem to associate with poor outcome confirming the hypothesis proposed. However the results demonstrated no development of resistant to trastuzumab by patients possessing high levels of HER2/EGFR. Possibly the series did not involve enough number of cases to turn the results expressively different. Nonetheless the significance of multiple protein interactions and identification of both upstream and downstream protein interactions, such as ligand/receptor, might still relevant on selection of patients to be submitted to new developed therapies as lapatinib or other monoclonal therapeutic antibodies. Therefore the present results sustain the idea of further investigations to characterise in more detail the biochemistry of HER2/EGFR.

5. CHAPTER 5: The role of HER3 in HER2+ breast cancer

5.1 Introduction

5.1.1 HER3 in breast cancer

The third member of HER proto-oncogene family, HER3, is located on chromosome 12 location 12q13 with a protein molecular weight of 145 kDa[469]. By itself HER3 does not comprise an activated tyrosine kinase due to a storing of extra inactive residues (histidine and asparagine) in its structure that substitute the active residues (glutamate and asparate)[469]. Therefore HER3 requires contact with another HER member to trigger a signalling cascade[64], which makes this monomer as an obligate partner for heterodimerisation[43]**.** A study using mouse mutants deficient in HER3 expression showed that only 20% survived to embryonic stage; however remaining pups died shortly after birth, showing several imperfections on development and differentiation of Schwann cells. They also did not respond to tactile stimulus and showed abnormal development of the lung. Nevertheless, mammary epithelial cells were not affected by lack of HER3 expression, as expression of HER4 compensated for this loss as it possesses similar roles[470].

In breast tumour tissues, HER3 possesses a high affinity to bind with PI3K promoting anti-apoptotic reaction[471]. The SH2 domains acts as a PI3K linkage to the receptor with reasonable to elevated attraction[472]. Levels of HER3 in breast tumour cells can raise considerably compared with their normal

counterparts as a result of either higher protein expression caused by either protein translation or increased half-life of the receptor[470]. When HER2 is inactivated by antagonist antibodies, a decrease of HER3 signalling activity occurs, due to overexpression of HER3 and HER2 being greatly associated[70,283]. Indeed HER3 might have a role with HER2 on promotion of cell transformation and breast cancer progression[470] and is the preferred heterodimer arrangement with proliferative downstream pathways[43]. Lack of HER3 in breast cancer cells are strongly antiproliferative, although still expressing EGFR and HER4 suggesting that EGFR or HER4 are not competent to substitute for the absence of HER3[60]. The heterodimer HER2/HER3 is imperative in cell proliferation by keeping constitutive action of PI3K/PKB downstream signalling[473].

Overexpression of HER3 is associated with poor prognosis in different types of cancer such as breast, lung and ovarian[474]. It was reported that 17.5% of breast cancer patients show high expression of HER3 associated with poor prognosis[21], including association with high grade[475] and recurrence[476]. However its prognostic value still not coherent when other studies reveal an absence of correlation between HER3 overexpression and poor survival in breast carcinomas[476].

The HER3 endoplasmic domain is unique since EGFR and HER2 do not comprise the same kinase domain molecular structure and HER4 only possesses a single PI3K link spot included in one of its isoforms[477]. However another important characteristic of HER3 is in its ectodomain, which is able to

link with numerous ligands contributing to homodimerisation creation avoidance[478]. This increases the limitation on HER3 activation and downstream pathways being triggered. However this feature might be a mode to control PI3K premature activation[197]. Being a very important regulator of cellular processes as cell proliferation, PI3K precipitate triggering has to be avoided. Therefore HER3 activation is limited by the lack of a kinase domain on its structure which is compulsory on the other members of the HER family and their respective ligands[203]. Moreover if ligand activation does not occur, the c-terminal tail is in trans conformation making allosteric activation impossible[479]. HER3 C-terminal possesses 14 tyrosines, which can harbour several SH2 or PTB binding molecules responsible for the trigger of downstream pathways[480,481]. After PI3K is activated by phosphorylated HER3, it is able to activate phosphoinositides present on the cell membrane, which through a subsequent chain of activations will trigger Akt. Akt is able to regulate a cascade of molecules responsible for survival, proliferation and differentiation of the cell[482], including the mTor pathway, which coordinates cell growth and important metabolic reactions depending on the nutrients and energy accessible[483]. Therefore HER3 expression can be extremely significant in making accessible the other members of the same family for the regulation of Akt[482].

HER3 is not itself considered as an oncogene; however it is classified as a compulsory assistant of HER family oncogenesis. HER3 when is overexpressed is not associated with cancer development by itself[484]. Moreover

it has not been reported that any activating mutations or gene transformations of HER3 present oncogenic action[93,485].

In *HER2* amplified breast cancer cell lines, a decline of transformed growth occurs when HER3 is knocked down, whereas no alterations are seen with loss of EGFR expression. Thus, it was possible to suggest that HER3 is critical on the growth of *HER2* amplified breast carcinomas[486]. Coherently, HER3 is consistently coexpressed in the majority of *HER2* amplified breast cancers, however EGFR does not follow the same tendency[475].

HER3 is expressed in different types of cancer, e.g. melanoma, where it is not associated with HER2 expression suggesting HER3 might serve as an allosteric activator of EGFR or HER4[487]. Even though it is not implicated in prostate cancer pathogenesis, HER3 overexpression is critical in most of the transformed phenotypes of prostate carcinomas[488]. In the same way HER3 is detected in a high number of colorectal carcinomas[489] where HER3 is associated with HER4[490]. Within lung carcinomas, HER3 overexpression frequency ranges between 18% and 100% and is linked with poor outcome[491,492]. Similarly, HER3 overexpression in ovarian cancers may vary from 3% to 90%[493-495], although it is not associated with poor survival outcome[474].

5.1.2 HER2/HER3 in breast cancer

The HER2/HER3 heterodimer effectively triggers downstream signalling responsible for proliferation and survival[496], reacting as an oncogenic element

driving breast carcinomas to high levels of propagation[108]. Additionally chemotaxis is indirectly stimulated by this heterodimer creating distant metastasis[497].

HER3 presence in breast carcinomas is highly associated with anti-apoptosis development by promotion of PI3K pathway[471]. Cell transformation and breast carcinoma progression might be triggered by HER2 and HER3 interaction[498] being narrated as the most common heterodimer associated with proliferative downstream pathways[43]. Therefore the heterodimer HER2/HER3 could be essential to sustain a constitutive role of the PI3K downstream pathway[473].

Even though HER2 and HER3 are not able to form homodimers and promote any downstream pathways, the heterodimerisation between these two monomers creates a highly functional triggering intracellular domain[43]. The HER2/HER3 dimer is the combination of HERs with superior effectiveness when compared with any homodimer or heterodimer amongst this receptor tyrosine kinase family. This oncogenic combination promotes phosphorylation of the downstream pathways Erk1/2 (MAPK) and PI3k/Akt promoting cells proliferation (Figure 5.1)[499,500].

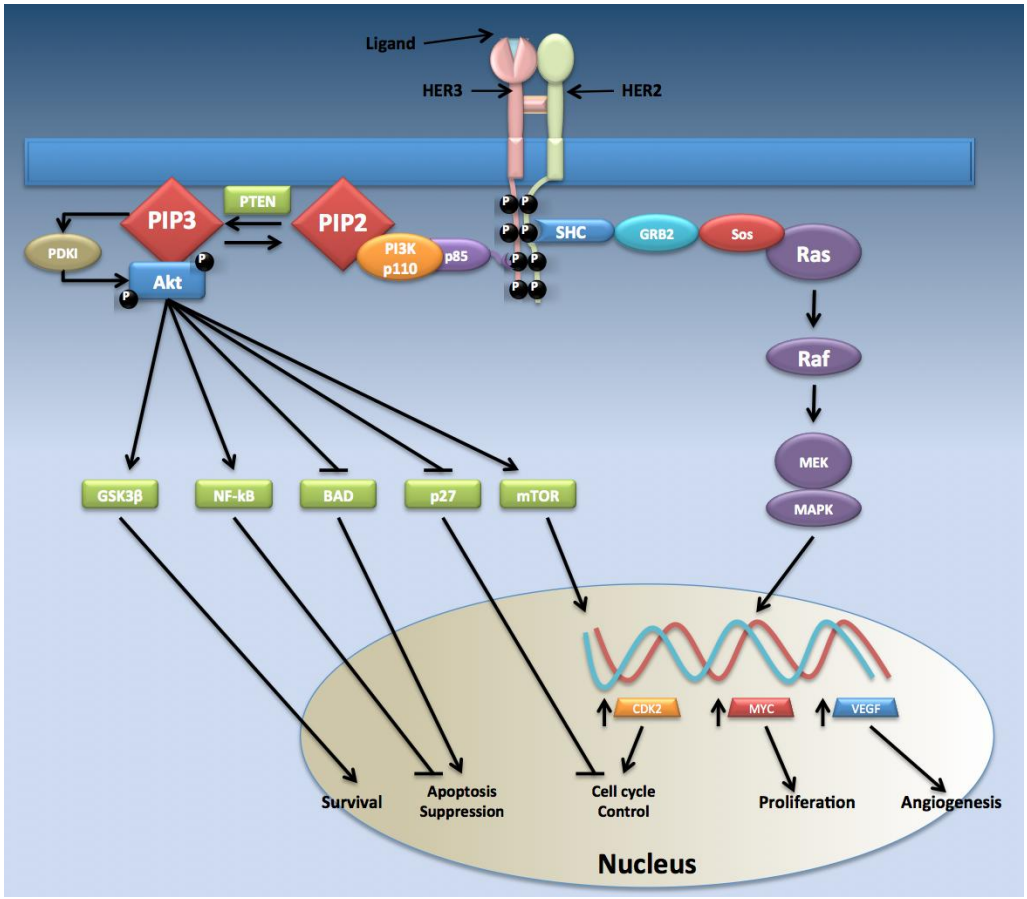

Figure 5.1 – The main downstream pathways triggered by HER family dimers activated by the ligands, with the example of HER2/HER3 dimerisation.

5.2 Methods

Immunohistochemical detection of HER3 was achieved by using the anti-HER3 (clone RTJ1, Leica) antibody and appliance of Novolink Polymer Detection Systems method following the antigen retrieval pre-treatment. In positive control tonsil tissue, HER3 was distributed mostly in the cells membrane. Detection of the heterodimer HER2/HER3 was performed by applying the PLA technology as described in chapter 2 using two antibodies from different species reacting under the same pre-treatment conditions (rabbit anti-HER2 (Dako) and mouse anti-HER3 (clone 2F12, Neomarkers)). The red signals present mostly in the cancer cells membrane were quantified and the cut-off achieved using the same methodology applied for HER2/EGFR heterodimerisation.

5.3 Results

5.3.1 HER3

5.3.1.1 Expression in breast cancer

Amongst the Unselected series HER3 negative expression was detected in 170/1509 (11.3%) cases and the positive expression in 1339/1509 (88.7%) cases. The HER2+ series revealed only 21/278 (7.6%) cases with negative expression for HER3 and 257/278 (92.4%) patients possessed positive expression (Figure 5.2).

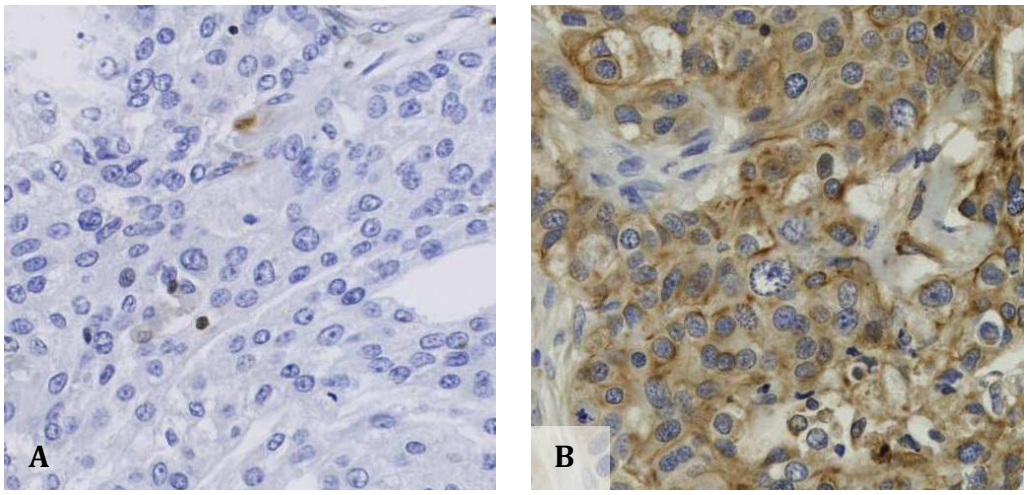

Figure 5.2 – Detection of HER3 protein expression in breast cancer cores by immunohistochemistry. The cells were labelled with a specific antibody against HER3 to detect the intensity of expression of this protein present mostly in the cell membrane. The cells are scored in four different classes of protein expression: **(A)** Negative when a faint membranous immunoreactivity was observed in less than 10% of the cancer cells and **(B)** Positive when more than 10% of the invasive carcinoma cells show a weak/moderate perceptible membranous reactivity.

5.3.1.2 Correlation with clinicopathological parameters

Overexpression of HER3 protein amongst the Unselected series was highly associated with higher tumour grade, more nuclear pleomorphism, higher mitotic frequency and poor NPI (all $p < 0.001$). Additionally HER3 expression was correlated with less tubule formation ($p = 0.003$), higher tumour stage ($p = 0.010$) and higher vascular invasion ($p = 0.007$) (Table 5.1).

For the HER2+ series, the HER3 expression was not significantly associated with any of the clinicopathological parameters (Table 5.1).

Table 5.1 – The relation between HER3 status with clinicopathological parameters in breast cancer.

Parameters	HER3 Status					
	Unselected Series			HER2+ Series		
	Neg (%)	Pos (%)	p value (Chi-Square)	Neg (%)	Pos (%)	p value (Chi-Square)
Tumour Grade						
1	60 (31.4)	208 (15.8)	<0.001 (45.141)	1 (4.8)	5 (1.9)	0.211 (3.113)
2	70 (39.8)	417 (31.7)		6 (28.6)	41 (16.0)	
3	55 (28.8)	691 (52.5)		14 (66.7)	211 (82.1)	
Tubule Formation						
1	18 (9.7)	71 (5.5)	0.003 (11.509)	0 (0.0)	1 (0.4)	0.386 (1.905)
2	76 (40.9)	422 (32.9)		6 (30.0)	45 (17.7)	
3	92 (49.5)	788 (61.5)		14 (70.0)	208 (81.9)	
Pleomorphism						
1	10 (5.4)	19 (1.5)	<0.001 (54.580)	0 (0.0)	0 (0.0)	0.116 (2.475)
2	110 (59.1)	460 (36.1)		4 (20.0)	23 (9.1)	
3	66 (35.5)	797 (62.5)		16 (80.0)	230 (90.9)	
Mitotic Frequency						
1	95 (51.1)	439 (34.3)	<0.001 (21.713)	3 (15.0)	30 (11.8)	0.354 (2.075)
2	33 (17.7)	240 (18.7)		2 (10.0)	61 (24.0)	
3	58 (31.2)	602 (47.0)		15 (75.0)	163 (64.2)	
Tumour Stage						
1	138 (72.3)	804 (61.2)	0.010 (9.224)	13 (61.9)	113 (44.0)	0.184 (3.391)
2	39 (20.4)	400 (30.4)		4 (19.0)	99 (38.5)	
3	14 (7.3)	110 (8.4)		4 (19.0)	45 (17.5)	
Distant Metastases						
Negative	130 (68.4)	913 (69.7)	0.722 (0.127)	14 (66.7)	171 (67.1)	0.971 (0.001)
Positive	60 (31.6)	397 (30.3)		7 (33.3)	84 (32.9)	
Local Recurrence						
Negative	105 (55.9)	800 (62.3)	0.089 (2.885)	13 (61.9)	171 (67.3)	0.612 (0.257)
Positive	83 (44.1)	484 (37.7)		8 (38.1)	83 (32.7)	
Regional Recurrence						
Negative	175 (93.1)	1165 (90.7)	0.292 (1.112)	19 (90.5)	228 (93.1)	0.659 (0.195)
Positive	13 (6.9)	119 (9.3)		2 (9.5)	17 (6.9)	
Tumour Size						
<1.5cm	48 (25.1)	326 (24.8)	0.915 (0.012)	3 (14.3)	37 (14.4)	0.989 (0.001)
≥1.5cm	143 (74.9)	990 (75.2)		18 (85.7)	220 (85.6)	
Vascular Invasion						
Negative	125 (65.4)	718 (55.1)	0.007 (7.304)	13 (61.9)	133 (52.2)	0.390 (0.740)
Positive	66 (34.6)	586 (44.9)		8 (38.1)	122 (47.8)	
NPI						
Good	91 (47.9)	384 (29.5)	<0.001 (28.449)	1 (6.3)	4 (2.0)	0.460 (1.553)
Moderate	84 (44.2)	702 (54.0)		4 (25.0)	68 (33.3)	
Poor	15 (7.9)	214 (16.5)		11 (68.8)	132 (64.7)	
Age						
<50	66 (34.6)	435 (33.0)	0.676 (0.175)	11 (55.0)	112 (43.9)	0.337 (0.921)
≥50	125 (65.4)	882 (67.0)		9 (45.0)	143 (56.1)	
Death						
Negative	91 (63.6)	756 (69.0)	0.191 (1.710)	8 (50.0)	164 (68.0)	0.137 (2.208)
Positive	52 (36.4)	339 (31.0)		8 (50.0)	77 (32.0)	

5.3.1.3 Association with patient outcome

The survival analyses for HER3 monomer amongst the Unselecteed series revealed no significant worse prognosis between those patients that express high levels of HER receptor and those not revealing any expression, for both BCSS (p = 0.762; Figure 5.3A) and DFI (p = 0.286; Figure 5.3B).

Kaplan-Meier test was not performed for both trastuzumab adjuvant and naïve series because over 90% of the cases were HER3 positive, which would not reveal any significant results.

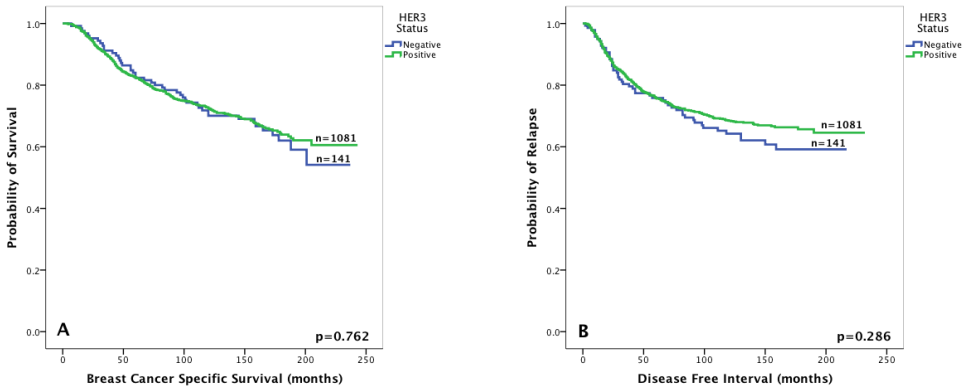

Figure 5.3 – **A)** BCSS and **B)** DFI analysis for HER3 status amongst the Unselected series.

5.3.2 HER2/HER3

5.3.2.1 HER2/HER3 in breast cancer

In breast carcinoma cells the HER2/HER3 heterodimers signals were mostly detected in the cell membrane on a range of 0 to 34.6 signals per cell. The cut-off defined as low levels those cases revealing less than 4.5 HER2/HER3 signals per cell (Figure 5.4A) and high levels were considered for any occurrence above that value (Figure 5.4D).

The frequency of HER2/HER3 heterodimers high levels was detected in 97/743 (13.1%) for the Unselected series and 149/226 (65.9%) amongst the HER2+ series.

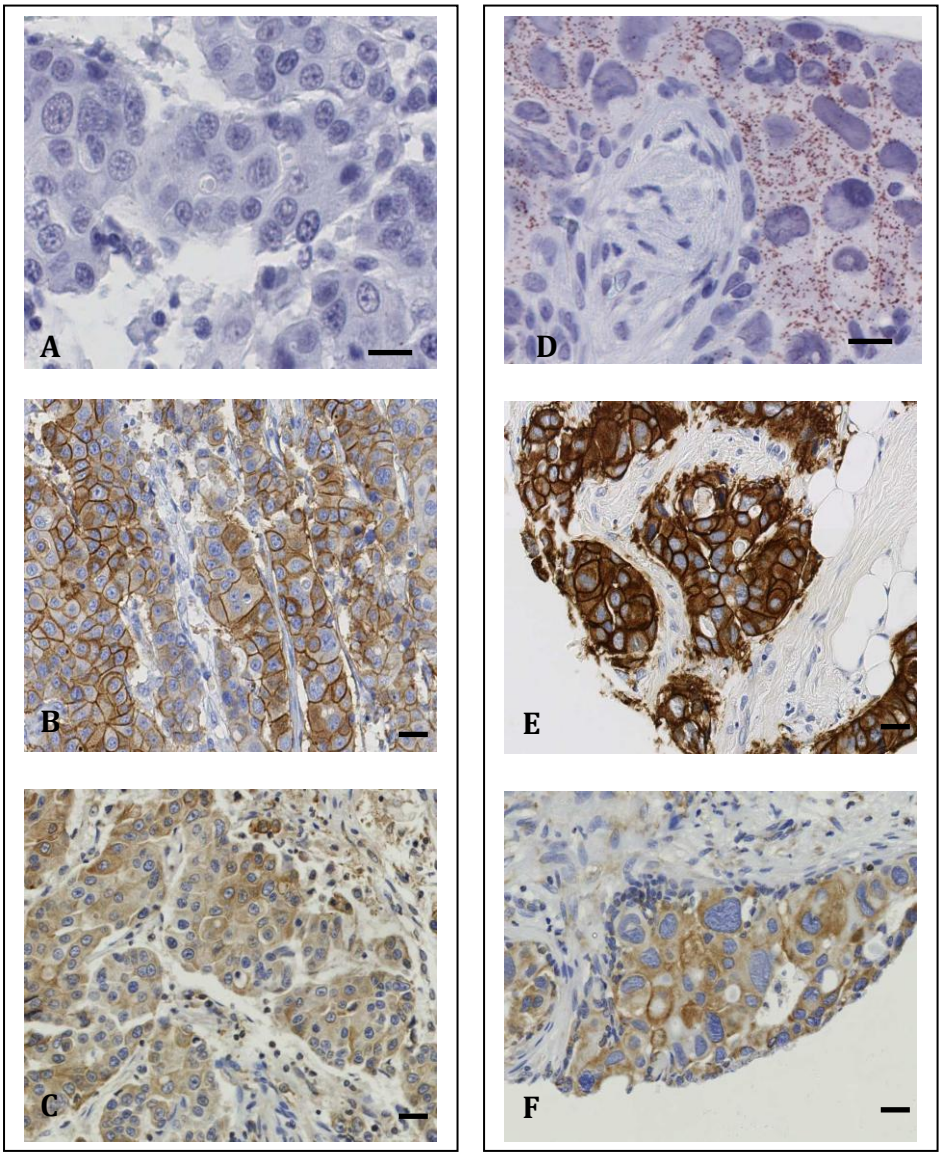

Figure 5.4 – Photomicrographs of *in situ* PLA detection at HER2/HER3 heterodimers at x40 magnification and IHC detection at x20 magnification with a scale bar representing 100 µm. Case 1: Primary breast cancer cells displaying **A)** low levels of HER2/HER3, **B)** HER2 protein expression and **C)** HER3 protein expression. Case 2: Primary breast cancer cells displaying **D)** high levels of HER2/HER3, **E)** HER2 protein expression and **F)** HER3 protein expression.

5.3.2.2 Correlation with HER family monomers expression

The correlation between HER2/HER3 and the HER family monomers is summarised in Table 5.2 A significant proportion ($p < 0.001$) of cases showing HER2/HER3 heterodimers were HER2+ (79/89, 88.8%). Of those remaining, 7/10 (70.0%) cases had relatively low levels of heterodimers as detected by PLA (<6.9 signals/cell) and 3/10 (30.0%) cases had increased HER2 protein expression (2+) but showed no gene amplification by CISH analysis. There were a number of HER2+ cases, 33/112 (29.5%) that did not show dimerisation of HER2/HER3. There was no significant association ($p = 0.999$) between HER2/HER3 heterodimer and HER3+ tumours although the majority of cases with high HER3 displayed high levels of HER2/HER3 heterodimers 71/78 (91.0%). However, 477/548 (87.1%) of HER3+ cases were correlated with low levels of HER2/HER3 heterodimers, which means that amongst a total of 548 HER3+ cases only 71 (12.9%) were associated with high levels of HER2/HER3 heterodimers.

Within the HER2+ series, the majority of the patients (149/226, 65.9%) were associated with HER2/HER3 dimerisation. There was no correlation between HER2/HER3 heterodimers and HER3 expression ($p = 0.450$), with 117/123 (95.1%) of cases with high heterodimer levels and 61/66 (92.4%) of low-level cases being HER3 positive. A total of 6/123 (4.9%) revealing high levels of the HER2/HER3 heterodimers showed negative expression of HER3 despite showing strong HER2 protein expression (IHC score of 3+).

Table 5.2 – Association between HER2/HER3 levels and expression of HER family monomers in breast cancer.

Biomarker	HER2/HER3 Status					
	Unselected Series			HER2+ Series		
	Low (%)	High (%)	p value (Chi-Square)	Low (%)	High (%)	p value (Chi-Square)
EGFR						
Negative	430 (79.2)	60 (74.1)	0.296	45 (66.2)	73 (55.3)	0.139
Positive	113 (20.8)	21 (25.9)	(1.094)	23 (33.8)	59 (44.7)	(2.194)
HER2						
Negative	599 (94.8)	10 (11.2)	<0.001	0 (0.0)	0 (0.0)	N/A
Positive	33 (5.2)	79 (88.8)	(414.975)	77 (100)	149 (100)	
HER3						
Negative	47 (9.0)	7 (9.0)	0.999	5 (7.6)	6 (4.9)	0.450
Positive	477 (91.0)	71 (91.0)	(0.0)	61 (92.4)	117 (95.1)	(0.570)
HER4						
Negative	82 (15.4)	5 (6.8)	0.048	1 (1.8)	3 (2.1)	0.618
Positive	452 (84.6)	69 (93.2)	(3.919)	68 (98.2)	118 (97.5)	(0.249)

5.3.2.3 Correlation with HER2 and HER3 mutual expression

While the expression of both monomers was positive the levels of HER2/HER3 heterodimers was significantly high (70.7%). However if only HER2 expression becomes negative or even both monomers the levels of dimerisation drops considerably to 1.3% (Table 5.3).

Table 5.3 – Frequency of HER2/HER3 levels against HER2 and EGFR mutual expression status.

Expression Status	**HER2/HER3**	
	Low (%)	**High (%)**
HER2+/HER3+	24 (29.3)	58 (70.7)
HER2+/HER3-	2 (25.0)	6 (75.0)
HER2-/HER3+	439 (98.7)	6 (1.3)
HER2-/HER3-	51 (98.1)	1 (1.9)

5.3.2.4 Correlation with HER2 amplification

The relation between HER2/HER3 heterodimer levels with HER2 gene amplification is shown in Figure 5.5 where HER2/HER3 dimerisation levels were significantly associated with gene *HER2* amplification ($r^2 = 0.429$, $p < 0.001$). The majority of the HER2 non-amplified cases were showed low levels of HER2/HER3 heterodimers.

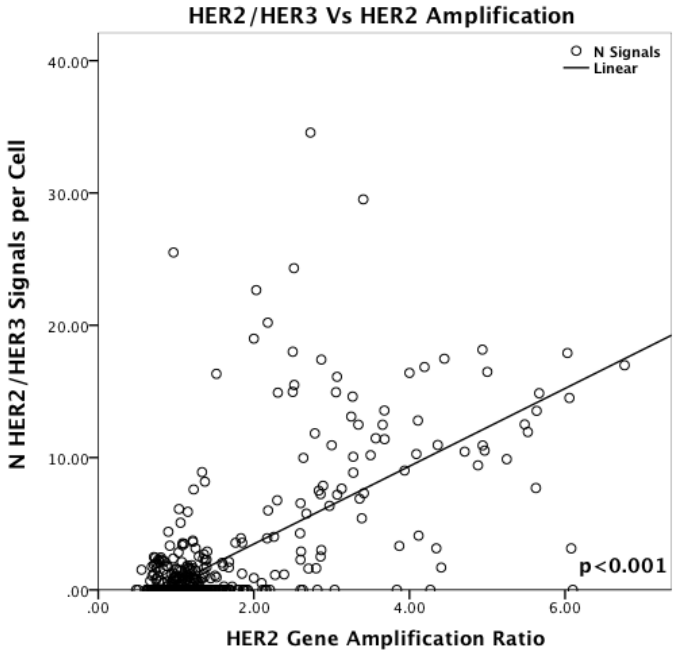

Figure 5.5 – Linear regression analysis between HER2/HER3 and *HER2* gene amplification in the breast cancer (Unselected series).

5.3.2.5 Correlation with clinicopathological parameters

Table 5.4 summarises the correlations between HER2/HER3 heterodimers levels and clinicopathological variables. Within the Unselected series, HER2/HER3 heterodimers were significantly correlated with higher tumour grade ($p = 0.001$), tumour stage ($p < 0.001$), marked nuclear pleomorphism ($p < 0.001$), higher mitotic frequency ($p = 0.001$), lack of tubule formation ($p = 0.017$), development of metastases ($p = 0.004$), poor NPI ($p < 0.001$) and death ($p = 0.004$).

Within the HER2+ series, the association with the clinicopathological parameters was restricted only to tumour grade ($p = 0.017$) but not any of its specific components. Additionally there was no correlation between HER2/HER3 heterodimerisation levels and any histological tumour type ($p = 0.379$).

Table 5.4 – Association of HER2/HER3 levels with clinicopathological parameters in breast cancer.

Parameter	HER2/HER3 Status					
	Unselected Series			HER2+ Series		
	Low (%)	High (%)	p value (Chi-Square)	Low (%)	High (%)	p value (Chi-Square)
Tumour Grade						
1	84 (13.0)	5 (5.2)	<0.001 (21.591)	1 (1.3)	6 (4.0)	0.017 (8.159)
2	196 (30.3)	13 (13.4)		21 (27.3)	19 (12.8)	
3	366 (56.7)	79 (81.4)		55 (71.4)	124 (83.2)	
Tubule Formation						
1	23 (3.7)	1 (1.1)	0.017 (8.143)	0 (0.0)	1 (0.7)	0.455 (1.576)
2	213 (34.1)	21 (22.1)		16 (21.3)	23 (15.6)	
3	388 (62.2)	73 (76.8)		59 (78.7)	123 (83.7)	
Pleomorphism						
1	6 (1.0)	0 (0.0)	<0.001 (18.532)	0 (0.0)	0 (0.0)	0.137 (2.209)
2	219 (35.2)	13 (13.8)		11 (14.7)	12 (8.2)	
3	397 (63.8)	81 (86.2)		64 (85.3)	134 (91.8)	
Mitotic Frequency						
1	173 (27.7)	11 (11.6)	0.001 (13.126)	13 (17.3)	21 (14.3)	0.355 (2.069)
2	115 (18.4)	16 (16.8)		22 (29.3)	33 (22.4)	
3	336 (53.8)	68 (71.6)		40 (53.3)	93 (63.3)	
Tumour Stage						
1	386 (59.8)	49 (50.5)	0.001 (13.086)	35 (46.7)	66 (44.3)	0.334 (2.191)
2	207 (32.1)	29 (29.9)		29 (38.7)	49 (32.9)	
3	52 (8.1)	19 (19.6)		11 (14.7)	34 (22.8)	
Distant Metastases						
No	432 (67.5)	51 (52.6)	0.004 (8.305)	60 (77.9)	100 (68.0)	0.119 (2.424)
Yes	208 (32.5)	46 (47.4)		17 (20.1)	47 (32.0)	
Local Recurrence						
No	363 (57.6)	46 (47.9)	0.074 (3.188)	59 (76.6)	100 (67.6)	0.157 (2.004)
Yes	267 (42.4)	50 (52.1)		18 (23.4)	48 (32.4)	
Regional Recurrence						
No	557 (88.4)	84 (87.5)	0.796 (0.067)	71 (95.9)	127 (91.4)	0.214 (1.547)
Yes	73 (11.6)	12 (12.5)		3 (4.1)	12 (8.6)	
Tumour Size						
<1.5 cm	142 (22.0)	14 (14.4)	0.089 (2.897)	10 (13.0)	24 (16.1)	0.534 (0.387)
≥1.5 cm	504 (78.0)	83 (85.6)		67 (87.0)	125 (83.9)	
Vascular Invasion						
No	357 (55.7)	45 (46.9)	0.106 (2.619)	36 (47.4)	81 (54.7)	0.296 (1.091)
Yes	284 (44.3)	51 (53.1)		40 (52.6)	67 (45.3)	
NPI						
Good	166 (27.8)	7 (7.4)	<0.001 (30.329)	12 (15.6)	17 (11.5)	0.200 (7.295)
Moderate	363 (56.5)	59 (62.1)		51 (66.3)	84 (56.4)	
Poor	113 (17.6)	29 (30.5)		14 (18.2)	48 (32.2)	
Age						
<50	235 (36.4)	33 (34.0)	0.652 (0.203)	33 (43.4)	67 (45.6)	0.759 (0.094)
≥50	411 (63.6)	64 (66.0)		43 (56.6)	80 (54.4)	
Death						
No	350 (65.1)	41 (48.8)	0.004 (8.214)	58 (79.5)	97 (69.8)	0.131 (2.276)
Yes	188 (34.9)	43 (51.2)		15 (20.5)	42 (30.2)	

5.3.2.6 Correlation with expression of other biomarkers

Table 5.5 summarised the correlation between HER2/HER3 levels and additional biomarkers of importance in breast cancer. A significant correlation was observed between high levels of HER2/HER3 heterodimer expression with CK18 ($p < 0.001$), pAkt ($p = 0.044$), PI3K ($P < 0.001$), BRCA1 ($p = 0.005$), p53 ($p < 0.001$) and PTEN ($p = 0.006$). There was a negative association between HER2/HER3 heterodimer expression and Bcl-2, ER, PgR (all $p < 0.001$) and MUC-1 ($P = 0.020$).

Within the HER2+ series there was a significant association between high expression of HER2/HER3 heterodimers with negative ER ($P < 0.001$), PgR ($P = 0.001$) and Bcl-2 ($p = 0.010$) status. A positive correlation was observed between HER2/HER3 heterodimers with CK18 ($P = 0.001$), p21 ($p = 0.027$) and pAkt ($p = 0.013$).

Table 5.5 – Association of HER2/HER3 dimerisation levels with biomarkers expression in breast cancer.

Biomarker	HER2/HER3 Status					
	Unselected Series			HER2+ Series		
	Low (%)	High (%)	p Value (Chi- Square)	Low (%)	High (%)	p Value (Chi- Square)
CK 5/6						
Negative	517 (82.7)	83 (88.3)	0.175	47 (61.0)	92 (63.9)	0.676
Positive	108 (17.3)	11 (11.7)	(1.841)	30 (39.0)	52 (36.1)	(0.175)
CK7/8						
Negative	22 (3.5)	0 (0.0)	0.064	1 (3.0)	0 (0.0)	0.122
Positive	602 (96.5)	94 (100)	(3.419)	32 (97.0)	78 (100)	(2.385)
CK14						
Negative	531 (87.7)	86 (91.5)	0.223	48 (88.9)	114 (94.2)	0.215
Positive	79 (13.0)	8 (8.5)	(1.483)	6 (11.1)	7 (5.8)	(1.540)
CK18						
Negative	124 (21.4)	4 (4.4)	<0.001	9 (17.0)	3 (2.5)	0.001
Positive	456 (78.6)	87 (95.6)	(14.698)	44 (83.0)	117 (97.5)	(11.943)
Bcl-2						
Negative	207 (41.0)	53 (75.7)	<0.001	32 (47.1)	80 (66.1)	0.010
Positive	298 (59.0)	17 (24.3)	(29.925)	36 (52.9)	41 (33.9)	(6.549)
pAkt						
Negative	121 (25.4)	11 (15.5)	0.044	11 (29.7)	11 (19.6)	0.263
Positive	356 (74.6)	60 (84.5)	(3.295)	26 (70.3)	45 (80.4)	(1.255)
PI3K						
Negative	122 (122)	7 (8.6)		4 (5.8)	5 (3.7)	
Moderate	143 (143)	13 (16.0)	<0.001	12 (17.4)	11 (8.2)	0.105
Strong	288 (288)	61 (75.3)	(15.942)	53 (76.8)	118 (88.1)	(4.512)
p21						
Negative	99 (79.2)	10 (90.9)	0.351	17 (45.9)	12 (23.5)	0.027
Positive	26 (20.8)	1 (9.1)	(0.871)	20 (54.1)	39 (76.5)	(4.877)
MUC-1						
Negative	69 (12.9)	2 (2.6)		4 (5.6)	4 (3.1)	
Moderate	243 (45.3)	43 (56.6)	0.020	39 (54.9)	66 (52.0)	0.581
Strong	224 (41.8)	31 (40.8)	(7.834)	28 (39.4)	57 (44.9)	(0.581)
BRCA1						
Negative	317 (61.0)	59 (77.6)	0.005	48 (72.7)	85 (69.1)	0.603
Positive	203 (39.0)	17 (22.4)	(7.913)	18 (27.3)	38 (30.9)	(0.270)
p53						
Negative	435 (71.7)	46 (48.9)	<0.001	27 (36.0)	45 (31.5)	0.499
Positive	172 (28.3)	48 (51.1)	(19.524)	48 (64.0)	98 (68.5)	(0.457)
PTEN						
Weak	79 (22.8)	3 (5.5)		0 (0.0)	5 (5.7)	
Moderate	130 (37.5)	21 (38.2)	0.006	21 (53.8)	40 (45.5)	0.265
Strong	138 (39.8)	31 (56.4)	(10.092)	18 (46.2)	43 (48.9)	(2.653)
Ki67						
Weak	172 (33.8)	21 (25.3)		4 (19.0)	14 (21.2)	
Moderate	176 (34.6)	25 (30.1)	0.062	10 (47.6)	21 (31.8)	0.400
Strong	161 (31.6)	37 (44.6)	(5.573)	7 (33.4)	31 (47.0)	(1.831)
ER						
Negative	169 (28.3)	56 (60.2)	<0.001	23 (31.1)	91 (62.8)	<0.001
Positive	429 (71.7)	37 (39.8)	(37.424)	51 (68.9)	54 (37.2)	(19.699)
PgR						
Negative	264 (44.2)	65 (70.7)	<0.001	36 (48.6)	100 (71.4)	0.001
Positive	333 (55.8)	27 (29.3)	(22.321)	38 (51.4)	40 (28.6)	(10.845)

5.3.2.7 Correlation with HER2/HER4

As stated in Chapter 4, HER2/HER3 dimerisation was significantly correlated with HER2/EGFR in both tumour series. There was likewise a positive correlation between HER2/HER3 and HER2/HER4 heterodimer expression ($p < 0.001$; Table 5.6).

Table 5.6 – Association of HER2/HER3 and HER2/HER4 heterodimer levels in breast cancer.

Heterodimer	HER2/HER3 Status					
	Unselected Series			HER2+ Series		
	Low (%)	High (%)	p Value (Chi-Square)	Low (%)	High (%)	p Value (Chi-Square)
HER2/HER4						
Low	364 (96.6)	26 (37.1)	<0.001 (187.288)	46 (74.2)	38 (32.2)	<0.001 (28.793)
High	13 (3.4)	44 (62.9)		16 (25.8)	80 (67.8)	

5.3.2.8 Association with patient outcome

HER2/HER3 high levels showed a significantly worst outcome for both BCSS ($p < 0.001$; Figure 5.6A) and DFI ($p = 0.001$; Figure 5.6B) in the Unselected series. This association with patient outcome was not significant between HER2/HER3 dimer levels in patients with HER2+ breast cancer trastuzumab naïve: BCSS ($p = 0.235$; Figure 5.6C) and DFI ($p = 0.241$; Figure 5.6D). Similarly, high HER2/HER3 heterodimer levels were not associated with patient outcome in the patients with HER2+ breast cancer receiving adjuvant trastuzumab (Figure 5.6E and F).

The multivariate Cox regression including tumour size, stage and grade, high levels of HER2/HER3 heterodimers was an independent predictor for worse BCSS (HR = 0.62, 95% CI= 0.45-0.62, p = 0.006, Table 5.7) and DFI (HR = 0.72, 95% CI = 0.53-0.98, p = 0.037, Table 5.7).

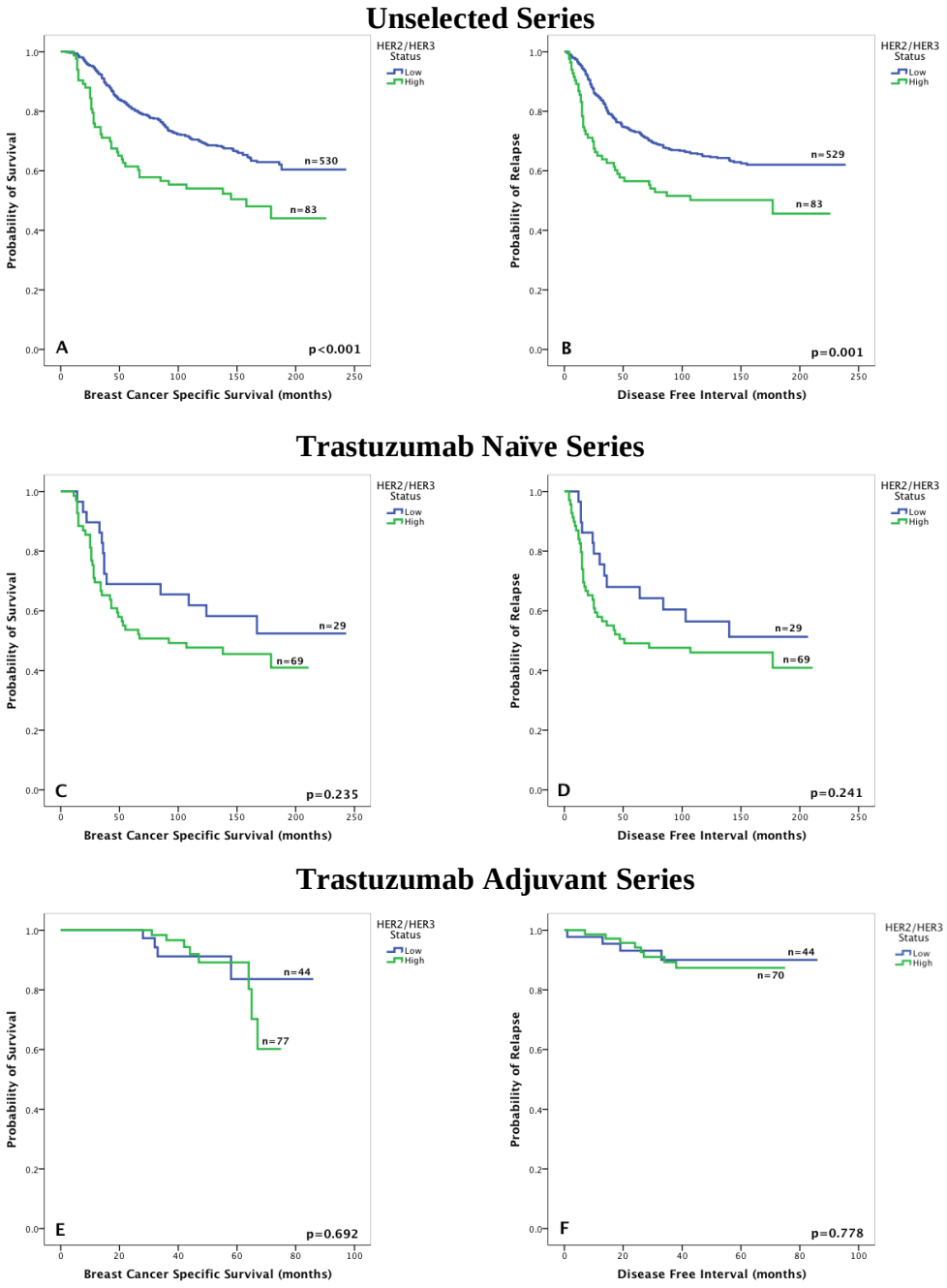

Figure 5.6 – BCSS and DFI analysis for HER2/HER3 status amongst the Unselected series (**A** and **B)**, Trastuzumab Naïve Series (**C** and **D**) and Trastuzumab Adjuvant Series (**E** and **F**).

Table 5.7 – Multivariate Cox regression analysis of factors associated with BCSS and DFI for Unselected series.

Variable	BCSS		DFI	
	Hazard Ratio (95% CI)	p Value	Hazard Ratio (95% CI)	p Value
Tumour Size (cm)	0.55 (0.35-0.85)	0.007	0.60 (0.43-0.83)	0.002
Tumour Stage	1.87 (1.56-2.24)	<0.001	1.74 (1.49-2.04)	<0.001
Tumour Grade	1.64 (1.31-2.05)	0.001	1.12 (0.95-1.33)	0.179
HER2/HER3	0.62 (0.45-0.62)	0.006	0.72 (0.53-0.98)	0.037

5.3.2.9 Association with ER status and patient outcome

There was no correlation between HER2/HER3 heterodimer and patient outcome in the ER negative population for both BCSS ($p = 0.169$; Figure 5.7A) and DFI ($p = 0.141$; Figure 5.7B). The same analysis was performed for the first 60 months to understand if patients survival showed any significant difference between HER2/HER3 levels, however the outcome was not significant for both BCSS ($p = 0.174$; Figure 5.7C) and DFI ($p = 0.079$; Figure 5.7D).

Amongst the ER positive population, high HER2/HER3 dimerisation was significantly associated with shorter BCSS ($p < 0.001$; Figure 5.8A) and DFI ($p = 0.028$; Figure 5.8B). The same type of analysis performed for the first 60 months revealed that the outcome was already significant worse for both BCSS ($p < 0.001$; Figure 5.8C) and DFI ($p = 0.025$; Figure 5.8D) amongst those cases possessing high levels of HER2/HER3.

The multivariate Cox regression model including tumour size, tumour stage and tumour grade, revealed that high levels of HER2/HER3 amongst the ER positive patients was independently associated with worse BCSS (HR = 0.46, 95% CI = 0.29-0.74, $p = 0.001$, Table 5.8) but not with DFI (HR = 0.67, 95% CI = 0.43-1.06, $p = 0.087$, Table 5.8).

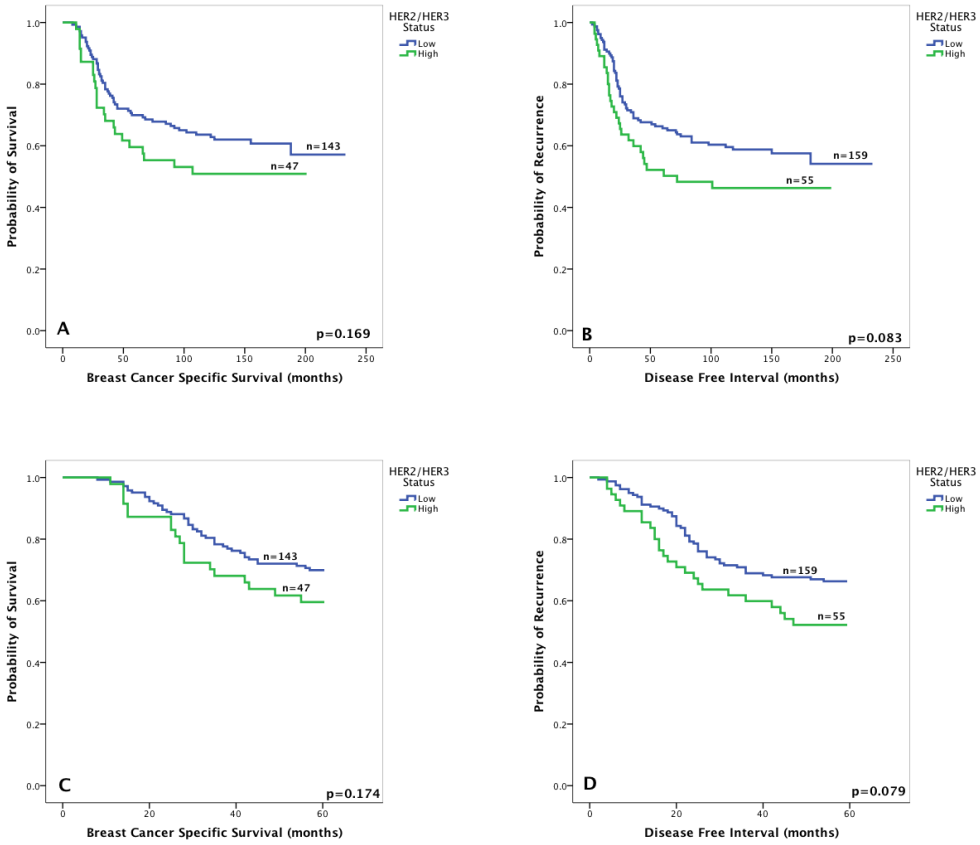

Figure 5.7 – BCSS and DFI analysis for HER2/HER3 status amongst the ER negative cases in Unselected series for a period of 250 months **A)** and **B)** or for a period of 60 months **C)** and **D)**.

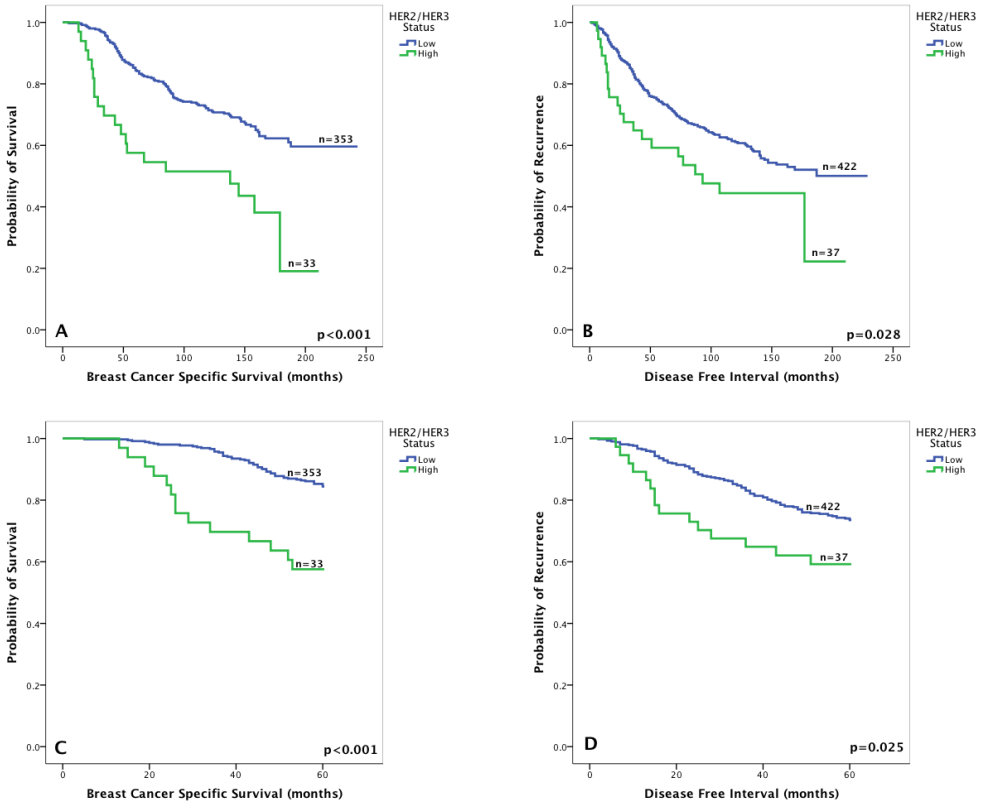

Figure 5.8 – BCSS and DFI analysis for HER2/HER3 status amongst the ER positive cases in Unselected series for a period of 250 months **A)** and **B)** or for a period of 60 months **C)** and **D)**.

Table 5.8 – Multivariate Cox regression analysis of factors associated with BCSS and DFI for ER positive cases in Unselected series.

Variable	BCSS		DFI	
	Hazard Ratio (95% CI)	p Value	Hazard Ratio (95% CI)	p Value
Tumour Size (cm)	0.48 (0.28-0.85)	0.011	0.57 (0.38-0.85)	0.007
Tumour Stage	1.69 (1.33-2.16)	<0.001	1.61 (1.30-1.99)	<0.001
Tumour Grade	1.77 (1.37-2.30)	<0.001	1.22 (0.99-1.49)	0.059
HER2/HER3	0.46 (0.29-0.74)	0.001	0.67 (0.43-1.06)	0.087

5.3.2.10 Association with patient outcome in HER2+ tumours trastuzumab naïve versus adjuvant

The survival analyses regarding the patients' outcome receiving or not trastuzumab adjuvant were performed for both low and high HER2/HER3 heterodimers levels. The trastuzumab naïve patients possessing low levels of heterodimers reveal a significant worse outcome for both BCSS ($p = 0.009$; Figure 5.9A) and DFI ($p = 0.007$; Figure 5.9B).

Amongst the HER2/HER3 heterodimer high levels BCSS and DFI analyses showed significant differences on patients' outcome ($p < 0.001$; Figure 5.9C and D).

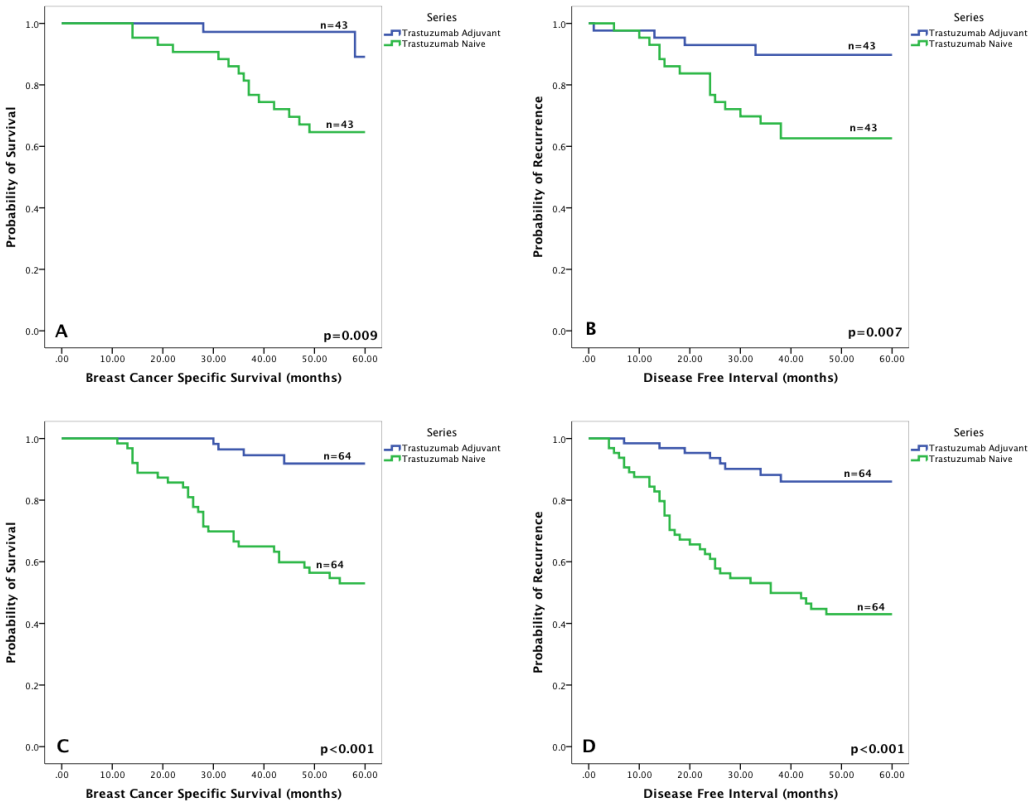

Figure 5.9 – BCSS and DFI analysis for HER2/HER3 status for HER2+ trastuzumab adjuvant series against HER2+ trastuzumab naïve series regarding low levels **A)** and **B)** or high levels **C)** and **D)** of HER2/HER3.

5.4 Discussion

HER3 is distinguished from its family members by the lack of an activated tyrosine kinase domain, which turns impossible the formation of homodimers[43]. Though its ectodomain is extremely reactive with a high range of ligands[478], which classifies this member of HER family as an essential supporter of HER family oncogenesis[484]. The co-expression with HER2 is strictly connected[70] and if HER3 levels decline also cell transformation is restricted[486]. HER2/HER3 heterodimers has been described to be the major interaction amongst the HER2 amplified breast carcinomas[460]. In case HER3 interacts with HER2, downstream pathways responsible for proliferation and survival are triggered[496], also stimulating creation of metastases[497]. Therefore in this chapter it was assessed the expression of HER3 in breast cancer and whether its HER2/HER3 heterodimers were correlated with poor prognosis as already documented[501] but with special attention for the development or not of resistance to trastuzumab therapy. HER3 has been revealed to possess an important and essential role in the development of HER family mediated carcinomas and is overexpressed in a high number of HER2+ breast cancer cases[475]. This is in concordance with the results described within this chapter where an incidence of up to 92% of all HER2+ cases was observed. Additionally HER3 is related with 17.5% of the total breast carcinomas with poor prognosis[455], which in this study it was associated with 16.5%.

Overexpression of HER3 has been inversely correlated with tumour grade[502], which is contradicted by this study showing a strongly positive correlation not only with higher tumour grade but to all its criteria such as reduced tubule

formation, more nuclear pleomorphism and high mitotic frequency. Additionally HER3 monomer high expression was highly correlated with higher tumour stage as anticipated[460,503], however no association with development of metastases in disagreement with Tsai and colleagues[504]. In this study HER3 as been associated with other of the poor prognostic parameters such a poor NPI and vascular invasion, even though not associate with deat occurrence due to breast cancer or local and regional recurrence. Interestingly HER3 monomer was not associated with any of the clinicopathological parameters amongst the HER2 amplified breast carcinomas, revealing no distinctive feature within this group of patients as a monomer.

Regarding survival analysis it was demonstrated no significant association of HER3 as a monomer with worse outcome amongst the unselected series. A prognostic value regarding HER3 overexpression was not proven yet, with some studies associating with worse prognosis[92,455,505], but also correlated with good patients' outcome[95,506]. Some theories were hypothesising to try to explain the differences of prognostic results amongst the different groups. The high affinity of a soluble isoforms of HER3 to the ligands NRGs blocks the interaction with the membranous HER3 receptors, suggesting that subcellular distribution of the receptors might affect cells development[507]. Therefore considering not only the levels of expression but also the localisation and the ligands presence might enhance the clinical outcome.

Although HER2 and HER3 monomers overexpression has previously been reported has the most frequent in breast cancer[455,501], it was found that all three

HER heterodimers were expressed at a similar frequency. HER2/HER3 heterodimers frequency amongst the Unselected series revealed to be significantly lower when compared to a different study documented presence of high levels three times more frequent[501]. However definition of high levels might not correspond to the same criteria and moreover technique procedure still not standardised.

As illustrated in this study dimerisation is highly but not entirely dependent on HER family monomers expression only. Amongst the HER2+ series, 34% of the cases did not reveal high levels of HER2/HER3 heterodimerisation, confirming the influence of supplementary factors, which are involved in dimerisation of these receptors. Interaction with specific ligands incites dimerisation occurrence[130], moreover in case HER3 does not interact with one of its specific ligands, the competent conformation is not accomplished being impossible to dimerise with any receptor[32]. Therefore a characterisation of the ligands class must be performed to understand in more detail all the factors involved in dimerisation multifaceted foundation. An example was presented in subchapter 5.3.2.1 where the photomicrographs suggest that dimerisation is highly but not entirely dependent on HER family monomers expression only, when is observed moderate/high expression of both monomers but residual dimerisation was detected for the same case. Arguing again that PLA detected protein-protein interaction and not monomers co-localisation contradicting a different study[456]. Supporting this idea the analysis of HER2/HER3 dimerisation against the monomers mutual expression status showed that when both monomers were highly expressed, only 70.7% of those cases possessed

high levels of HER2/HER3. Additionally the mutual expression analysis demonstrated that in case HER2 expression is absent the frequency of HER2/HER3 heterodimerisation drops dramatically. HER2 phosphorylation and activation is extremely correlated with high levels of dimerization[508]. Dimers including HER2 are more stable and with elongated active downstream pathways comparing with other dimers[106] making HER2 the preferred growth factor for dimerisation[509]. High levels of HER2/HER3 heterodimers has been associated with poor prognostic parameter such as higher tumour grade and stage, *HER2* gene amplification and ER negativity[501]; this is consistent with the results presented in this study. Additionally HER2/HER3 heterodimerisation revealed to be associated with poor NPI, death occurrence due to breast cancer and development of distant metastases in agreement with previous studies[455].

This heterodimer develops an important role in promotion of cell proliferation and apoptosis avoidance[28]. Even though HER2/HER3 heterodimers are not associated with Ki67, a biomarker involved in cell proliferation[397] and negatively associated with Bcl-2, which is highly correlated with apoptosis avoidance[463], this HER heterodimerisation is related with poor prognostic features. As a monomer, HER2 is not competent of interacting with PI3K subsequently activating the PI3K/Akt pathway. However the HER2/HER3 heterodimer is favourably efficient performing it, which was confirmed by the strong association with both Akt and PI3K expression. This role is stimulated by HER3 that contains six tyrosine-binding points with the highest affinity for PI3K amongst the HER family[471,472,510]. Therefore instigation of HER3 results in a durable and strong trigger of the downstream PI3K/Akt cascade[511].

Furthermore the high association with HER2/EGFR dimer and their crosstalk also contributes for development of metastases[107] as observed in this study. Additionally the high association of HER2/HER3 heterodimers with CK18 supports the idea that apoptosis does not take place amongst these cells due to CK18 high levels of degradation being associated with apoptosis[464,465].

Interestingly it was observed a high association of HER2/HER3 heterodimers with p21 but only amongst HER2+ breast carcinomas, which might help to explain the development of resistance from patients harbouring high levels of HER2/HER3, because the loss of p21 expression is associated with sensitivity to chemotherapy[512]. Therefore those cases expressing high levels of p21 would reveal resistance to chemotherapy and trastuzumab alone reveal low levels of success[513]. p53, which induces p21[514], is a biomarker that its immunopositivity is correlated with worse survival outcome amongst the breast carcinoma patients[512,515]. This supports the idea that the association of HER2/HER3 protein-protein interaction with p21 and p53 contributes for worse outcome. Additionally lack of BRCA1 is associated with poor prognosis[392] and in this study it was confirmed the negative association of HER2/HER3 with this biomarker.

As anticipated Bcl-2 has an important role in breast carcinomas being highly associated with ER positivity however its lost is associated with other poor prognostic markers such as p53, EGFR and HER2 expressions[463]. It was even suggested that EGFR ligands had an important role on downregulation of Bcl-2[463]. In this study HER2/EGFR high levels demonstrated a negative association

with both Bcl-2 and ER for both series, but highly associated with strong expression of p53 in only the Unselected series. This supports the idea that Bcl-2 anti-apoptotic role might be regulated by the hormone receptors previously defended[463]. However does not necessary supports the idea that cell harbouring high levels of HER2/EGFR are directed to apoptosis. Two studies revealed that the levels of CK18 degrades with apoptosis occurrence[464,465], which confirm the idea that HER2/EGFR is involved in apoptosis evasion processes by being associated with CK18 expression in both cohorts. Also the HER/EGFR high levels were highly correlated with Ki67, which is involved in high levels of cell proliferation[397]. HER2/HER3 was the only heterodimer to be associated with strong levels of MUC-1, which is associated with poor prognosis amongst the breast cancer patients being described to be connected with the metastases progression process[516,517].

The Unselected series revealed to possess a robust number of cases, the prognostic value revealed by HER2/HER3 heterodimerisation amongst the trastuzumab adjuvant series should be discussed with caution due to its reduced number of patients. Unsurprisingly the results for Unselected series, which included 1858 cases with primary invasive breast cancer, revealed that high levels of HER2/HER3 protein interaction predicted worse outcome for both BCSS and DFI autonomously of pathological and treatments considerations. This idea is supported by the fact that HER2/HER3 dimerisation occurrence is directly dependent on HER2 overexpression, which is associated with poor prognosis[17,501]. HER2/HER3 high levels was an independent prognostic factor in multivariate analysis, in concordance with previous findings[501]. The high

association between ER and BCL-2 already documented[463] and observed in this study (data not presented) might explain the poor outcome observed amongst those patients revealing high levels of HER2/HER3 and ER+ status. Moreover the multivariate analysis revealed HER2/HER3 high levels as an independent prognostic factor for both BCSS and DFI. This finding is in concordance to a previous study[501], supporting the idea that this protein-protein interaction may deliver additional prognostic information to the obtained by *HER2* gene amplification status. Moreover this worse prognostic eminence was observed within the first 60 months. For those patients with negative ER status no significant difference was observed regarding HER2/HER3.

The results generated specifically within HER2+ tumours revealed that HER2/HER3 dimerisation levels did not discriminate between different outcomes. Even though the survival analysis for HER2+ trastuzumab adjuvant did not reveal a significant prognostic difference regarding the HER2/HER3 heterodimers levels, it was possible to observe that after 60 months the probability of survival drops dramatically. This observation was not observed for any of the other HER heterodimers in study. Cautiously speculating it might indicate the development of resistance to trastuzumab by those showing high levels of HER2/HER3 heterodimerisation. HER2/HER3 is highly correlated with amplified proliferation and development of resistance to treatments[518-520] that can be achieved in up to 32.2 months[169]. Even though, it was observed that trastuzumab still effective comparing with chemotherapy alone. On a period of 60 months the probability to survive amongst the patients treated with trastuzumab was 90 % comparing with only 55% of those not

receiving the monoclonal antibody. Very similar results were obtained for relapsing analysis.

In conclusion it was possible to confirm that PLA delivers a reliable and reproducible assessment of HER2/HER3 heterodimers quantification *in situ* in clinical samples. High levels of HER2/HER3 heterodimers emerge to be associated with worse outcome and the hypothesis that this protein-protein is a poor prognostic factor that requires supplementary research. HER2/HER3 high levels contribute to trastuzumab resistance even though further analysis in a larger series must be performed to validate these results. Additionally characterisation of the ligands amongst the HER amplified breast carcinoma is urgently required to create a more robust classification method in order to avoid high levels of unsuccessfully therapy. PLA technology will permit researchers to investigate the clinical value of various interactions between molecules highly involved in breast cancer development.

6. CHAPTER 6: The role of HER4 in HER2+ breast cancer

6.1 Introduction

6.1.1 HER4 in breast cancer

The last member from HER family to be characterised was HER4 in 1993 using a breast cancer cell line (MDA-MB-453). *HER4* is located on chromosome 2q33[521] resulting in a protein of 180kDa[522]. This transmembrane protein is composed of 625 amino acid ECD, 26 amino acid transmembrane domain and a cytoplasmic domain composed of 633 amino acids, which reveals a normal RTK structure[521]. The cytoplasmic domain in EGFR and HER2 is highly conserved in HER4 (79% and 77% respectively)[521], indicating capacity of autophosphorylation[251]. Also four domains – juxtamembrane region, tyrosine kinase domain, helical domain and a domain including 18 tyrosine residues responsible for autophosphorylation – compose this internal substructure[521]. Therefore HER4 does not require dimerisation with any other HER family member to trigger downstream pathways. HER4 is critical not only in heart development but also in the nervous system and mammary glands[523]. HER4 expression is found in carcinomas of colon, prostate, breast, lung, ovarian, cervix, stomach, thyroid[251]. In breast, HER4 is ubiquitously expressed[96], in both normal and malignant cells but is not as highly expressed in breast tumours as either EGFR or HER2[524]. During pregnancy, HER4 is overexpressed, though its phosphorylation and consequent signalling activation was only highly detected in later stages where breast epithelial cells were guided through the processes of differentiation and lactation, instead of over

proliferation[525,526,527]. In breast tumour cells, contrary to the other HERs which are not associated with ER overexpression, HER4 expression is related[21]. Moreover HER4 expression is related with reduced cell proliferation[369].

The interaction of HER4 with its respective ligands triggers several downstream pathways by interaction with p85 subdomain of PI3K, Shc molecules[528] and PLC-γ1[529]. However this monomer has been correlated with good prognostic results in breast cancer and is present in 70% of tumours[21]. This is partly due to its action in reducing HER2 activity and consequently enhancing apoptosis[530]. In a further study performed by Suo et al[320] HER4 correlates with longer breast cancer specific survival (BCSS) and DFI. On the other hand, *HER4* gene overexpression is correlated with poor prognosis in breast cancer[92]. Furthermore, using differential-display PCR, linked HER4 and HER3 gene expression results in tumour progression[97]. In breast cancer, it is infrequent to observe overexpression of HER4 with any of others of the same family (1.4%). However more and more studies reveal that HER4 develops a critical role in antiproliferation and promotion of apoptosis[233,531].

6.1.2 HER2/HER4 in breast cancer

The biological characterisation knowledge of HER4 and its interaction with other members of the same family remain unclear. HER3/HER4 heterodimers were detected in some breast cancer cases, revealing a better outcome comparing with other dimers, even though a justification for this fact was not possible to be understood. Cells expressing these two monomers showed a high mitotic frequency but low levels of cell transformation[331]. On the other hand in

case of co-expression of HER3 and HER4 with either EGFR or HER2 increases the levels of cell transformation dramatically[484].

HER4 homodimers are highly correlated with better outcome[233]. This idea is supported by *in vitro* observations, using HER2 positive cell lines, demonstrating a reduction in cell proliferation and increase of apoptosis when transfected with *HER4* gene[233,527].

A particularity of HER4 dimerisation is that its downstream pathways usually are complemented by a cleavage and discharge of HER4 endoplasmic domain (4ICD), which is then transferred to the nucleus or mitochondria[526]. The accumulation of this complex in the subcellular compartments results in cellular proliferation[532], differentiation[533] and lactation[534]. Also it can activate gene expression[534], promote cell cycle arrest[535] but also apoptosis[531].

6.2 Methods

HER4 monomer in breast cancer TMAs was detected using ani-HER4 antibody (clone H4.77.16, Neomarkers) and the antigen retrieval preceding the immunohistochemistry technique based on the Envision kit methodology as described in chapter 2. In tonsil positive control HER4 was mainly detected in cells membrane. For detection of HER2/HER4 heterodimers the mouse anti-HER4 antibody (clone HFR1, Abcam) was utilised. This antibody detects the antigen in both membrane and nucleus; however detection of HER2/HER4 was predominantly in the cytoplasmic membrane. To obtain any signal representing HER2/HER4 heterodimers, HER2 and HER4 receptors need to be in close proximity. Therefore the antibody anti-HER2 (Dako), which reacts only against the cell membrane antigens, worked as a control for detection using *in situ* PLA.

6.3 Results

6.3.1 HER4

6.3.1.1 Expression in breast cancer

In the Unselected series HER4 negative expression was observed in 327/1525 (21.4%) patients and overexpression in 1198/1525 (78.6%) cases. Amongst HER2+ series it was revealed only 21/276 (6.5%) cases with negative expression for HER4 and 258/276 (93.5%) patients possessed positive expression (Figure 6.1).

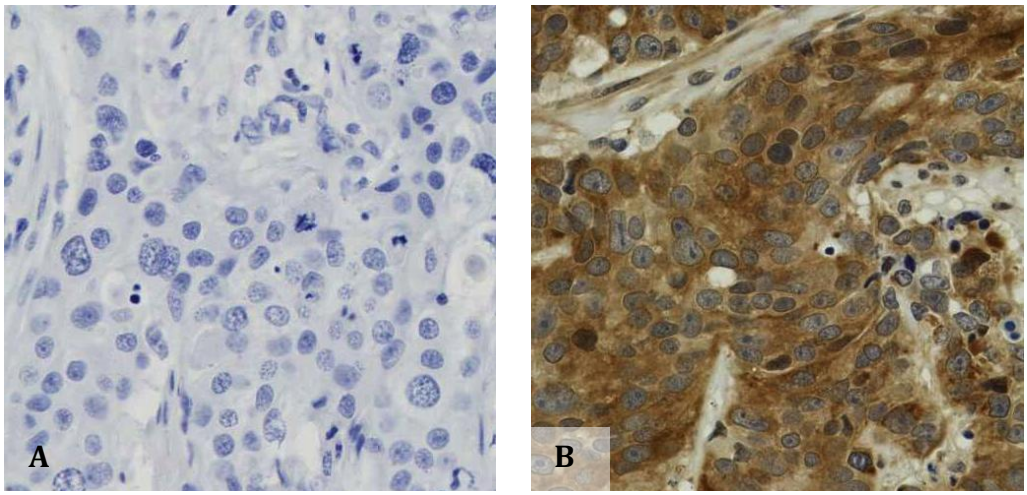

Figure 6.1 – Detection of HER4 protein expression in breast cancer cores by immunohistochemistry. The cells were labelled with a specific antibody against HER4 to detect the intensity of expression of this protein present in both the cell membrane and cytoplasm. The cells are scored in four different classes of protein expression: **(A)** Negative when a faint immunoreactivity was observed in less than 10% of the cancer cells and **(B)** Positive when more than 10% of the invasive carcinoma cells show a weak/moderate perceptible reactivity.

6.3.1.2 Correlation with clinicopathological parameters

HER4 protein overexpression amongst the Unselected series was highly correlated against higher tumour grade, more nuclear pleomorphism, higher mitotic frequency and poor NPI (all $p < 0.001$). Additionally HER4 expression was correlated with less tubule formation ($p = 0.016$) and higher vascular invasion ($p = 0.030$) (Table 6.1).

HER4 positive expression was not significantly associated with any of the clinicopathological parameters amongst the HER2+ series (Table 6.1)

Table 6.1 – The relation between HER4 status with clinicopathological parameters in breast cancer.

	HER4 IHC Status					
	Unselected Series			HER2+ Series		
Parameters	**Neg** (%)	**Pos** (%)	**p value** (Chi-Square)	**Neg** (%)	**Pos** (%)	**p value** (Chi-Square)
Tumour Grade						
1	85 (25.8)	177 (15.1)	<0.001 (61.320)	0 (0.0)	6 (2.3)	0.649 (0.865)
2	143 (43.5)	354 (30.1)		2 (11.1)	43 (16.7)	
3	101 (30.7)	645 (54.8)		16 (88.9)	209 (81.0)	
Tubule Formation						
1	30 (9.4)	59 (5.2)	0.016 (8.275)	0 (0.0)	1 (0.4)	0.967 (0.067)
2	106 (33.2)	377 (32.9)		3 (17.6)	45 (17.6)	
3	183 (57.4)	709 (61.9)		14 (82.4)	209 (82.0)	
Pleomorphism						
1	16 (5.0)	15 (1.3)	<0.001 (54.580)	0 (0.0)	0 (0.0)	0.562 (0.337)
2	181 (56.9)	387 (33.9)		1 (20.0)	26 (9.1)	
3	121 (38.1)	739 (64.9)		16 (80.0)	228 (90.9)	
Mitotic Frequency						
1	166 (52.0)	364 (31.8)	<0.001 (52.974)	0 (0.0)	31 (12.2)	0.099 (4.628)
2	62 (19.4)	213 (18.6)		2 (11.8)	62 (24.3)	
3	91 (28.5)	568 (49.6)		15 (88.2)	162 (63.5)	
Tumour Stage						
1	224 (68.1)	719 (61.2)	0.076 (5.146)	13 (72.2)	112 (43.4)	0.053 (5.888)
2	82 (24.9)	356 (30.3)		4 (22.2)	95 (36.8)	
3	23 (7.0)	99 (8.4)		1 (5.6)	51 (19.8)	
Distant Metastases						
Negative	225 (68.8)	814 (69.5)	0.807 (0.060)	10 (55.6)	171 (66.8)	0.948 (0.948)
Positive	102 (31.2)	357 (30.5)		8 (44.4)	85 (33.2)	
Local Recurrence						
Negative	188 (58.6)	709 (61.8)	0.292 (1.112)	10 (55.6)	169 (66.3)	0.355 (0.856)
Positive	133 (41.4)	438 (38.2)		8 (44.4)	86 (33.7)	
Regional Recurrence						
Negative	296 (92.2)	1036 (90.3)	0.302 (1.065)	17 (94.4)	224 (91.8)	0.690 (0.159)
Positive	25 (7.8)	111 (9.7)		1 (5.6)	20 (8.2)	
Tumour Size						
<1.5cm	81 (24.6)	289 (24.6)	0.987 (0.001)	2 (11.1)	36 (14.0)	0.735 (0.114)
≥1.5cm	248 (75.4)	887 (75.4)		36 (88.9)	222 (86.0)	
Vascular Invasion						
Negative	198 (61.3)	638 (54.5)	0.030 (4.709)	6 (33.3)	138 (53.9)	0.091 (2.855)
Positive	125 (38.7)	532 (45.5)		12 (66.7)	118 (46.1)	
NPI						
Good	141 (47.9)	328 (29.5)	<0.001 (27.829)	0 (0.0)	5 (2.5)	0.082 (4.991)
Moderate	153 (44.2)	638 (54.0)		1 (7.1)	69 (34.0)	
Poor	34 (7.9)	195 (16.5)		13 (92.9)	129 (63.5)	
Age						
<50	109 (33.2)	385 (32.7)	0.851 (0.035)	8 (44.4)	113 (44.3)	0.991 (0.001)
≥50	219 (66.8)	793 (67.3)		10 (55.6)	142 (55.7)	
Death						
Negative	172 (67.7)	663 (68.0)	<0.931 (0.007)	8 (53.3)	159 (66.3)	0.307 (1.042)
Positive	82 (32.3)	312 (32.0)		7 (46.7)	81 (33.8)	

6.3.1.3 Association with patient outcome

HER4 overexpression did not reveal significant worse BCSS (p = 0.580; Figure 6.2A) and DFI (p = 0.834; Figure 6.2B) amongst the Unselected series.

For both trastuzumab naïve and trastuzumab adjuvant cohorts the survival analysis was not possible to be performed due to nearly the total number of cases be characterised as HER4 positive.

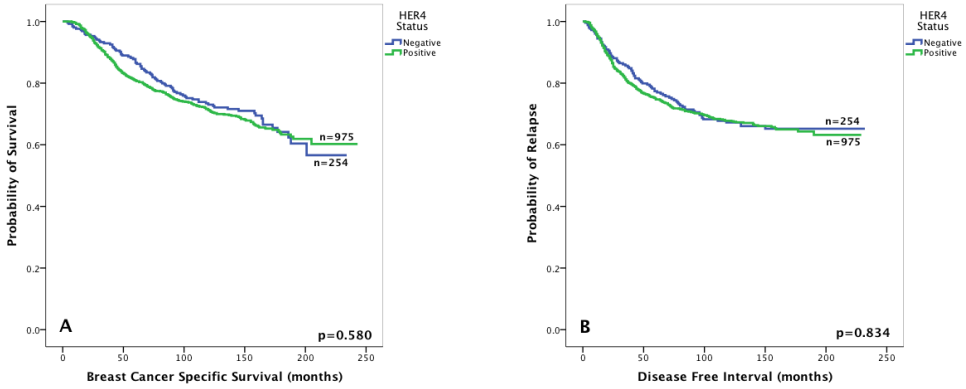

Figure 6.2 – **A)** BCSS and **B)** DFI analysis for HER4 status amongst the Unselected series.

6.3.2 HER2/HER4

6.3.2.1 HER2/HER4 in breast cancer

As expected, the HER2/HER4 heterodimerisation signals were detected predominantly on the cell membrane of breast tumour cells on a frequency number of signals per cell that varied from 0 to 17.2. The cut-off determined using X-tile was obtained defining as low levels of HER2/HER4 heterodimers when less than 3.4 signals per cell was observed (Figure 6.3A). Any value equal or above 3.4 signals per cell was accepted as high level of heterodimerisation (Figure 6.3D).

Amongst the Unselected series the frequency of cases revealing high levels of HER2/HER4 heterodimers was 96/891 (10.8%) and amongst the HER2+ series that incidence was observed in approximately half of the cases with 131/243 (53.9%).

Amongst a total of 149 HER2+ cases that were scored for the three heterodimers, 73 (49.0%) expressed high levels of all heterodimers, whereas 32 (21.5%) cases did not show expression of any of the heterodimers investigated. On behalf of two heterodimers high levels presence, the detection was observed in only 24 (16.1%) cases for HER2/EGFR and HER2/HER3, 11 (7.38%) cases with both HER2/EGFR and HER2/HER4, and only 1 (0.67%) case with both HER2/HER3 and HER2/HER4.

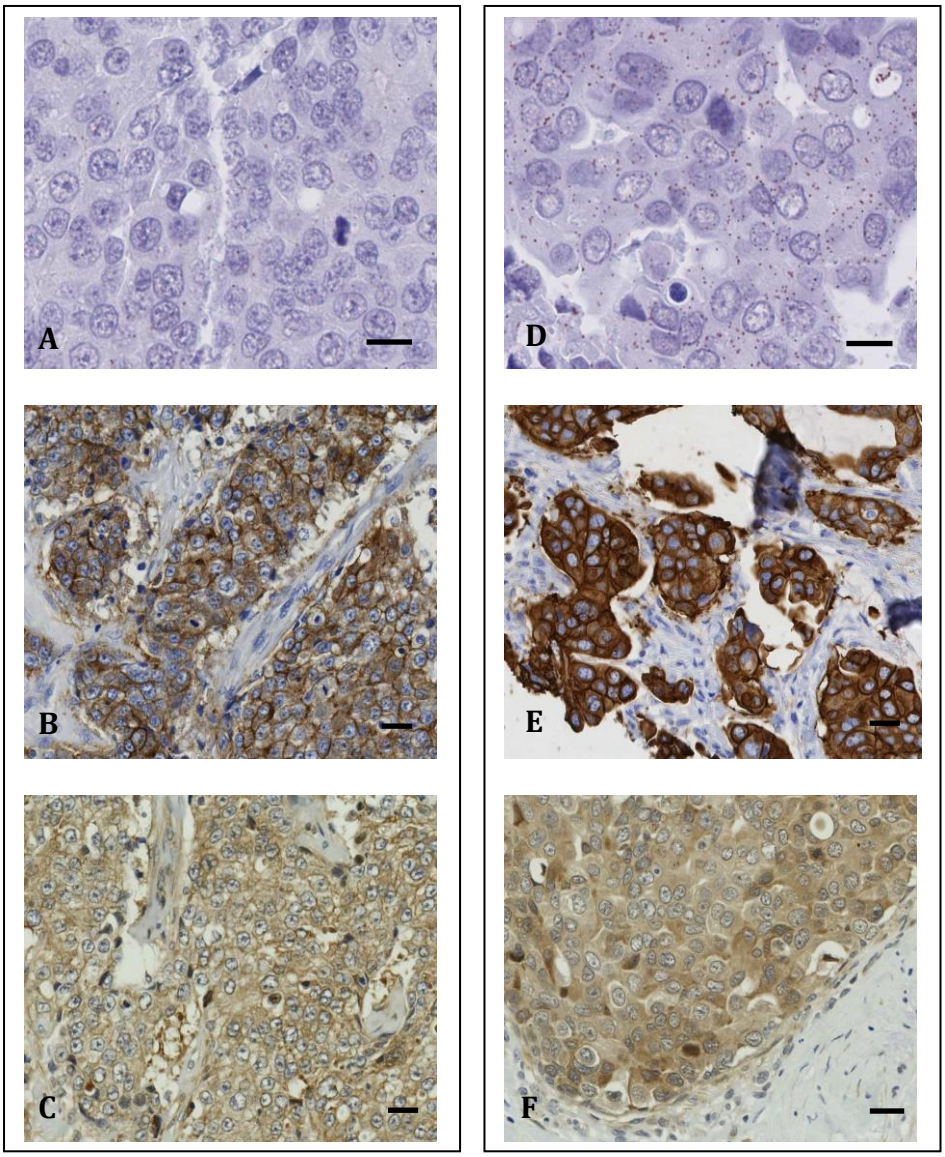

Figure 6.3 – Photomicrographs of *in situ* PLA detection of HER2/HER4 heterodimers at x40 magnification and IHC detection at x20 magnification with a scale bar representing 100 µm. Case 1: Primary breast cancer cells displaying **A)** low levels of HER2/HER4, **B)** HER2 protein expression and **C)** HER4 protein expression. Case 2: Primary breast cancer cells displaying **D)** high levels of HER2/HER4, **E)** HER2 protein expression and **F)** HER4 protein expression.

6.3.2.2 Correlation with HER family monomers expression

Table 6.2 summarised the correlation between HER2/HER4 and the HER family monomers. There was a strong positive correlation between HER2/HER4 and HER2 status ($p < 0.001$). However some HER2/HER4 high-level cases were associated with HER2 negativity (10/86, 11.6%). The majority of these cases had nearly low levels of HER2/HER4 heterodimers (6/10 cases; <4.7 signals/cell) or showed overexpression of HER2 protein but not amplification of the gene (4/10 cases). A total of 62/138 (44.9%) HER2+ cases did not show high levels of HER2/HER4 heterodimers. Likewise, all but 5/74 (6.3%) cases that possessed high levels of HER2/HER4 were positive for the HER4 protein ($p = 0.004$). Amongst the HER2+ cohort, 131/243 (53.9%) cases revealed high levels of the HER2/HER4 heterodimer. There was no association between HER2/HER4 heterodimer expression HER4 protein ($p = 0.936$).

Table 6.2 – Association between HER2/HER4 heterodimer levels and expression of hormone and HER family and hormone receptors in breast cancer.

Biomarker	HER2/HER4 Status					
	Unselected Series			HER2+ Series		
	Low (%)	High (%)	p value (Chi-Square)	Low (%)	High (%)	p value (Chi-Square)
EGFR						
Negative	527 (78.5)	57 (69.5)	0.064 (3.421)	64 (66.0)	64 (54.7)	0.094 (2.807)
Positive	144 (21.5)	25 (30.5)		33 (34.0)	53 (45.3)	
HER2						
Negative	709 (92.0)	10 (11.6)	<0.001 (369.562)	0 (0.0)	0 (0.0)	N/A
Positive	62 (8.0)	76 (88.4)		112 (100)	131 (100)	
HER3						
Negative	74 (11.7)	1 (1.3)	0.006 (7.704)	6 (6.8)	2 (1.9)	0.080 (3.055)
Positive	560 (88.3)	75 (98.7)		82 (93.2)	106 (98.1)	
HER4						
Negative	125 (19.5)	5 (6.3)	0.004 (8.219)	3 (3.5)	4 (3.7)	0.936 (0.006)
Positive	517 (80.5)	74 (93.7)		83 (96.5)	104 (96.3)	

6.3.2.3 Correlation with HER2 and HER4 mutual expression

The high levels of HER2/HER4 revealed to be nearly to 60% when was observed overexpression of both monomers. It was noticed that when one of the HER family receptors is not expressed the frequency of HER2/HER4 low levels reach a minimum of 98.5% (Table 6.3).

Table 6.3 – Frequency of HER2/HER4 levels against HER2 and HER4 mutual expression status.

Expression Status	**HER2/HER4**	
	Low (%)	**High (%)**
HER2+/HER4+	43 (42.2)	59 (57.8)
HER2+/HER4-	3 (42.9)	4 (57.1)
HER2-/HER4+	461 (98.5)	7 (1.5)
HER2-/HER4-	121 (100)	0 (0.0)

6.3.2.4 Correlation with HER2 amplification

The presence of *HER2* gene amplification and HER2/HER4 heterodimers were significantly associated with each other ($r^2 = 0.377$, $p < 0.001$), revealing that the majority of those cases with low levels of HER2/HER4 ($n < 3.4$ signals/cell) did not have HER2 gene amplification (Figure 6.4).

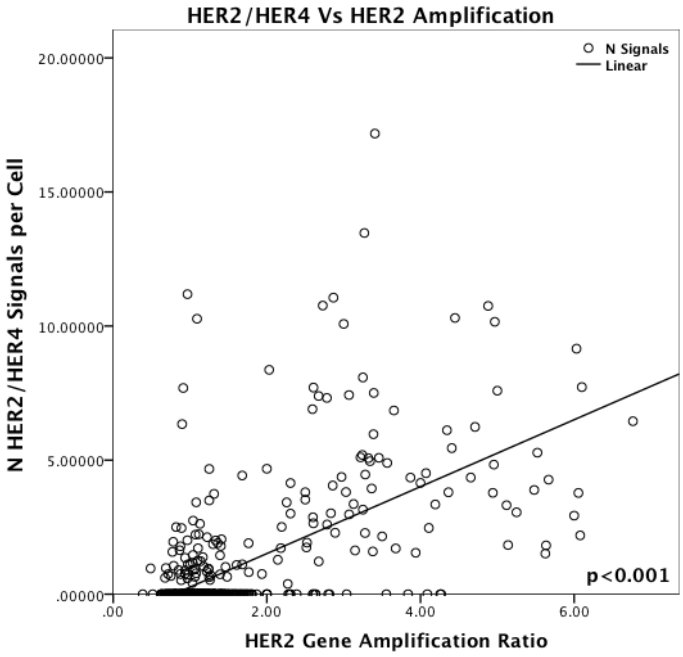

Figure 6.4 – Linear regression analysis between HER2/HER4 and *HER2* gene amplification in the breast cancer (Unselected series).

6.3.2.5 Correlation with clinicopathological parameters

Within the Unselected series, high HER2/HER4 dimerisation levels were significantly associated with higher tumour grade ($p < 0.001$), less tubule formation ($p = 0.007$), marked pleomorphism ($p < 0.001$), higher mitotic frequency ($p<0.001$), higher tumour stage ($p = 0.037$), presence of distant metastasis ($p = 0.002$), local recurrence ($p = 0.036$) poor NPI score ($p < 0.001$), poor NPI score ($p < 0.001$), and death ($p = 0.005$). There was no association between HER2/HER4 heterodimers with regional recurrence ($p = 0.681$), tumour size ($p = 0.419$), vascular invasion ($p = 0.526$) and age at diagnosis ($p = 0.297$).

High HER2/HER4 heterodimers was not associated with any of the clinicopathological parameters investigated within the HER2+ cohort. Similarly to HER2/HER3 this protein interaction did not reveal any significant correlation with this pathological parameter ($p = 0.483$) (Table 6.4).

Table 6.4 – Association of HER2/HER4 dimerisation levels with clinicopathological parameters in breast cancer.

Parameters	HER2/HER4 status					
	Unselected Series			HER2+ Series		
	Low (%)	High (%)	p value (Chi- Square)	Low (%)	High (%)	p value (Chi- Square)
Tumour Grade						
1	101 (12.7)	4 (4.2)	<0.001 (33.835)	0 (0.0)	4 (3.1)	0.075 (5.187)
2	243 (30.6)	8 (8.3)		22 (19.6)	17 (13.0)	
3	451 (56.7)	84 (87.5)		90 (80.4)	110	
Tubule Formation						
1	29 (3.8)	2 (2.1)	0.007 (9.855)	0 (0.0)	2 (1.6)	0.207 (3.147)
2	238 (31.2)	16 (16.8)		21 (19.1)	17 (13.2)	
3	495 (65.0)	77 (81.1)		89 (80.9)	111	
Pleomorphism						
1	12 (1.6)	1 (1.1)	<0.001 (26.714)	0 (0.0)	0 (0.0)	0.265 (1.244)
2	259 (34.2)	8 (8.4)		12 (12.0)	9 (7.0)	
3	487 (64.2)	86 (90.5)		96 (88.9)	120	
Mitotic Frequency						
1	208 (27.3)	10 (10.5)	<0.001 (21.085)	10 (9.1)	18 (14.0)	0.281 (2.538)
2	147 (19.3)	11 (11.6)		31 (28.2)	27 (20.9)	
3	407 (53.4)	74 (77.9)		69 (62.7)	84 (65.1)	
Tumour Stage						
1	485 (61.1)	48 (50.0)	0.037 (6.601)	52 (47.3)	60 (45.8)	0.866 (0.289)
2	240 (30.2)	33 (34.4)		36 (32.7)	47 (35.9)	
3	69 (8.7)	15 (15.6)		22 (20.0)	24 (18.3)	
Distant Metastases						
No	530 (67.0)	49 (51.0)	0.002 (9.623)	81 (72.3)	86 (66.7)	0.343 (0.901)
Yes	261 (33.0)	47 (49.0)		31 (27.7)	43 (33.3)	
Local Recurrence						
No	455 (58.6)	45 (47.4)	0.036 (4.393)	83 (74.8)	85 (65.4)	0.114 (2.500)
Yes	321 (41.4)	50 (52.6)		28 (25.2)	45 (34.6)	
Regional Recurrence						
No	689 (88.8)	83 (87.4)	0.681 (0.169)	103 (94.5)	111	0.411 (0.675)
Yes	87 (11.2)	12 (12.6)		4 (5.5)	10 (8.3)	
Tumour Size						
<1.5 cm	169 (21.3)	17 (17.7)	0.419 (0.653)	17 (15.2)	21 (16.0)	0.855 (0.033)
≥1.5 cm	626 (78.7)	79 (82.3)		95 (84.8)	110	
Vascular Invasion						
No	429 (54.5)	48 (51.1)	0.526 (0.402)	58 (54.5)	73 (56.3)	0.501 (0.453)
Yes	358 (45.5)	46 (48.9)		53 (45.5)	56 (43.4)	
NPI						
Good	200 (25.5)	8 (8.4)	<0.001 (28.617)	14 (12.5)	21 (16.1)	0.259 (6.516)
Moderate	448 (57.2)	58 (60.4)		73 (65.2)	67 (51.1)	
Strong	136 (17.4)	30 (31.2)		25 (22.3)	43 (32.9)	
Age						
<50	280 (35.2)	39 (40.6)	0.297 (1.089)	21 (33.9)	29 (38.2)	0.602 (0.272)
≥50	515 (64.8)	57 (59.4)		41 (66.1)	47 (61.8)	
Death						
No	421 (64.1)	40 (48.2)	0.005 (7.918)	78 (73.6)	81 (67.5)	0.318 (0.999)
Yes	236 (35.9)	43 (51.8)		28 (26.4)	39 (32.5)	

6.3.2.6 Correlation with expression of other biomarkers

Table 6.5 summarises the association of HER2/HER4 heterodimerisation levels with other biomarkers. Within the Unselected series, there was a significant association between HER2/HER4 and CK18 positivity ($p < 0.001$), PI3K ($p < 0.001$), p53 ($p < 0.001$), PTEN ($p = 0.020$) and Ki67 ($p = 0.004$). There was a negative association between HER2/HER4 heterodimers with Bcl-2 ($p < 0.001$), BRCA1 ($p = 0.014$) and both hormone receptors, ER and PR (both $p < 0.001$).

Within the HER2+ cohort, there was a significant association of HER2/HER4 heterodimers with negative Bcl-2 ($p < 0.001$), ER ($p < 0.001$) and PgR status ($p = 0.001$).

In both cohorts the association of HER2/HER4 heterodimers with both HER2/EGFR and HER2/HER3 was highly significant (Table 4.6 all $p < 0.001$).

Table 6.5 – Association between HER2/HER4 heterodimer levels and expression of biomarkers in breast cancer.

Biomarkers	HER2/HER4 Status					
	Unselected Series			HER2+ Series		
	Low (%)	High (%)	p value (Chi- Square)	Low (%)	High (%)	p value (Chi- Square)
CK5/6						
Negative	623 (80.7)	76 (85.4)	0.283	69 (64.5)	78 (65.5)	0.867
Positive	149 (19.3)	13 (14.6)	(1.151)	38 (35.5)	41 (34.5)	(0.028)
CK7/8						
Negative	21 (2.7)	0 (0.0)	0.118	1 (1.6)	0 (0)	0.286
Positive	753 (97.3)	88 (100)	(2.447)	61 (98.4)	70 (100)	(1.138)
CK14						
Negative	652 (85.3)	81 (92.0)	0.086	82 (85.4)	97 (93.3)	0.070
Positive	112 (14.7)	7 (8.0)	(2.952)	14 (14.6)	7 (6.7)	(3.276)
CK18						
Negative	145 (20.2)	3 (3.7)	<0.001	8 (9.1)	4 (4.0)	0.149
Positive	573 (79.8)	79 (96.3)	(13.348)	80 (90.9)	97 (96.0)	(2.082)
Bcl-2						
Negative	254 (41.7)	57 (86.4)	<0.001	51 (51.0)	78 (75.7)	<0.001
Positive	355 (58.3)	9 (13.6)	(47.793)	49 (49.0)	25 (24.3)	(13.394)
pAkt						
Negative	151 (25.2)	14 (20.0)	0.348	11 (28.9)	8 (20.0)	0.357
Positive	409 (74.8)	51 (80.0)	(0.882)	27 (71.1)	32 (80.0)	(0.847)
PI3K						
Negative	137 (22.5)	9 (11.4)	<0.001	8 (8.7)	6 (5.4)	0.063
Moderate	165 (27.0)	9 (11.4)	(20.158)	15 (16.3)	8 (7.1)	(5.545)
Strong	308 (50.5)	61 (77.2)		69 (75.0)	98 (87.5)	
p21						
Negative	94 (81.0)	5 (100)	0.282	13 (34.2)	11 (28.2)	0.569
Positive	22 (19.0)	0 (0.0)	(1.159)	25 (65.8)	28 (71.8)	(0.324)
MUC-1						
Negative	89 (13.6)	7 (9.2)	0.559	5 (5.1)	9 (8.1)	0.375
Moderate	290 (44.3)	35 (46.1)	(1.162)	41 (41.8)	53 (47.7)	(1.963)
Strong	275 (42.0)	34 (44.7)		52 (53.1)	49 (44.1)	
BRCA1						
Negative	371 (58.7)	55 (73.3)	0.014	61 (67.8)	80 (73.4)	0.386
Positive	261 (41.3)	20 (26.7)	(5.993)	29 (32.2)	29 (26.6)	(0.753)
p53						
Negative	544 (72.3)	46 (52.3)	<0.001	41 (38.3)	44 (36.1)	0.725
Positive	208 (27.7)	42 (47.7)	(15.177)	66 (61.7)	78 (63.9)	(0.124)
PTEN						
Weak	79 (20.4)	1 (2.4)	0.020	4 (6.9)	3 (4.4)	0.687
Moderate	150 (38.7)	19 (46.3)	(7.869)	26 (44.8)	35 (51.5)	(0.751)
Strong	159 (41.0)	21 (51.2)		28 (48.3)	30 (44.1)	
Ki67						
Weak	197 (31.7)	12 (15.4)	0.004	7 (15.9)	9 (14.3)	0.404
Moderate	211 (34.0)	27 (34.6)	(11.186)	21 (47.7)	23 (36.5)	(1.811)
Strong	213 (34.2)	39 (50.0)		16 (36.4)	31 (49.2)	
ER						
Negative	234 (30.9)	57 (64.8)	<0.001	47 (42.7)	81 (65.9)	<0.001
Positive	523 (69.1)	31 (35.2)	(40.035)	63 (57.3)	42 (34.1)	(12.545)
PgR						
Negative	335 (44.5)	66 (75.9)	<0.001	62 (56.9)	92 (76.7)	0.001
Positive	418 (55.5)	21 (24.1)	(30.769)	47 (43.1)	28 (23.3)	(10.153)

6.3.2.7 Association with patient outcome

Prognostic analysis were realised with HER2/HER4 levels relating to BCSS and DFI. Within the Unselected series, HER2/HER4 heterodimer levels were significantly correlated with worse outcome for both BCSS and DFI (both $p < 0.001$; Figures 6.5A and B, respectively). Within the trastuzumab naïve series, those cases revealing high levels of HER2/HER4 dimerisation were not associated with either BCSS ($p = 0.249$; Figure 6.5C) or DFI ($p = 0.151$; Figure 6.5D). Likewise, in the HER2+ adjuvant trastuzumab there was no significant association between HER2/HER4 levels and BCSS ($p = 0.890$; Figure 6.5E) or DFI ($p = 0.647$; Figure 6.5F).

The multivariate Cox regression including tumour size, stage and grade, high levels of HER2/HER4 heterodimers was an independent predictor for worse BCSS (HR = 0.66, 95% CI= 0.48-0.92, $p = 0.014$) and DFI (HR = 0.69, 95% CI = 0.51-0.94, $p = 0.017$, Table 6.6).

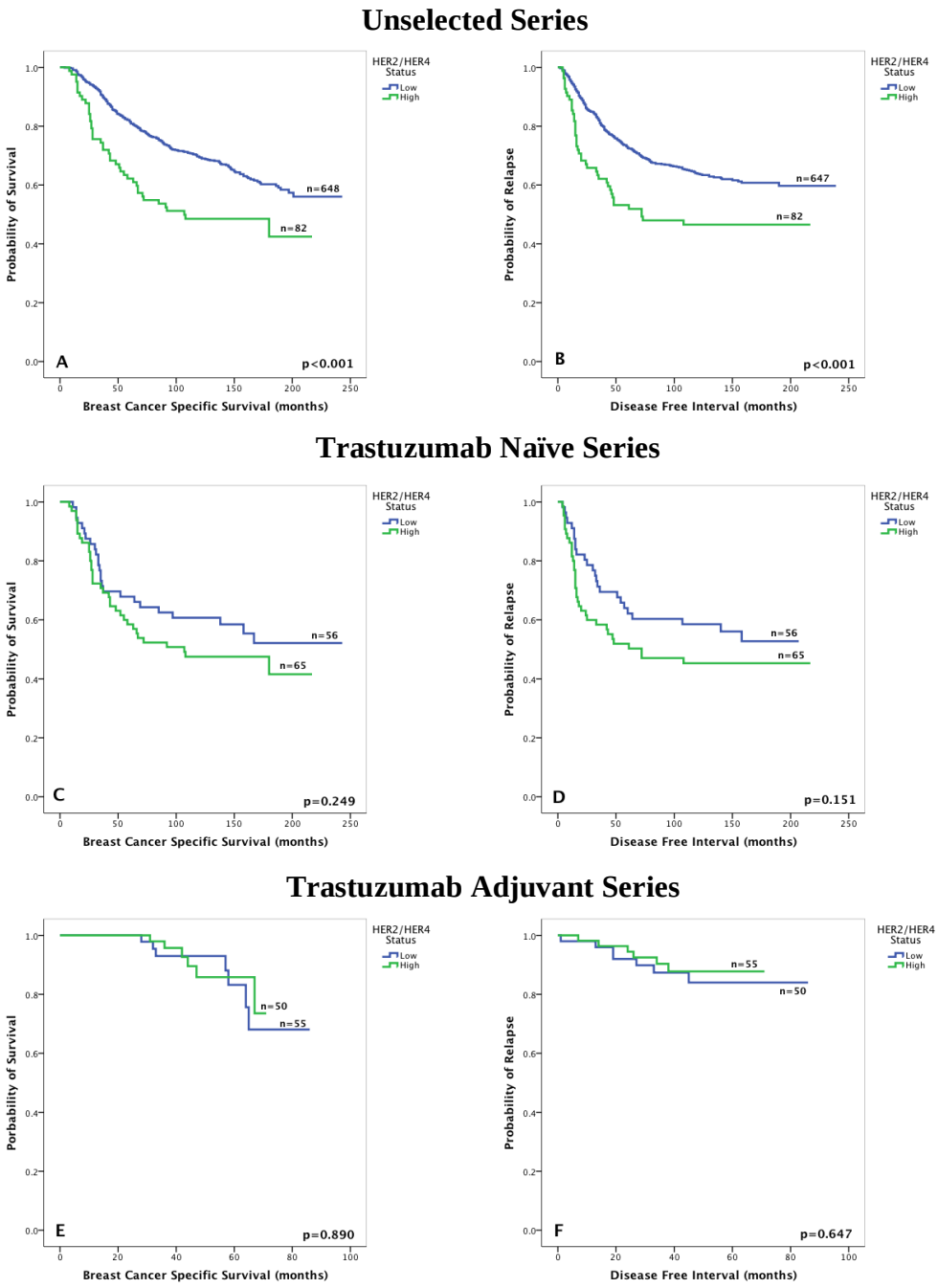

Figure 6.5 – BCSS and DFI analysis for HER2/HER4 status amongst the Unselected series (**A** and **B**), Trastuzumab Naïve Series (**C** and **D**) and Trastuzumab Adjuvant Series (**E** and **F**).

Table 6.6 – Multivariate Cox regression analysis of factors associated with BCSS and DFI for Unselected series.

	BCSS		**DFI**	
Variable	Hazard Ratio (95% CI)	p Value	Hazard Ratio (95% CI)	p Value
Tumour Size (cm)	0.59 (0.40-0.85)	0.005	0.73 (0.55-0.97)	0.032
Tumour Stage	2.16 (1.83-2.54)	<0.001	1.93 (1.66-2.25)	<0.001
Tumour Grade	1.74 (1.41-2.15)	<0.001	1.18 (1.01-1.39)	0.032
HER2/EGFR	0.66 (0.48-0.92)	0.014	0.69 (0.51-0.94)	0.017

6.3.2.8 Association with ER status and patient outcome

For the Unselected series, Kaplan-Meier survival analyses practically did not revealed a significant worse outcome amongst those patients possessing high levels of HER2/HER4 and negative ER status for both BCSS ($p = 0.049$; Figure 6.6A) and DFI ($p = 0.046$, Figure 6.6B). The same analysis, however paying attention to only the first 60 months, revealed no association with both BCSS ($p = 0.061$; Figure 6.6C) and DFI ($p = 0.059$; Figure 6.6D).

For those patients expressing the hormone receptor the significance was incredibly high for both BCSS ($p < 0.001$; Figure 6.7A) and DFI ($p < 0.001$; Figure 6.7B). Moreover during the first 60 months those patients revealing high levels of dimerisation already possessed a significant worse outcome for both BCSS ($p < 0.001$; Figure 6.7C) and DFI ($p = 0.010$; Figure 6.7D).

The multivariate Cox regression model including tumour size, tumour stage and tumour grade, revealed that high levels of HER2/HER4 amongst the ER positive patients was independently associated with worse BCSS (HR = 0.51, 95% CI = 0.30-0.86, $p = 0.012$, Table 6.7) and with DFI (HR = 0.62, 95% CI = 0.37-1.02, $p = 0.017$, Table 6.7).

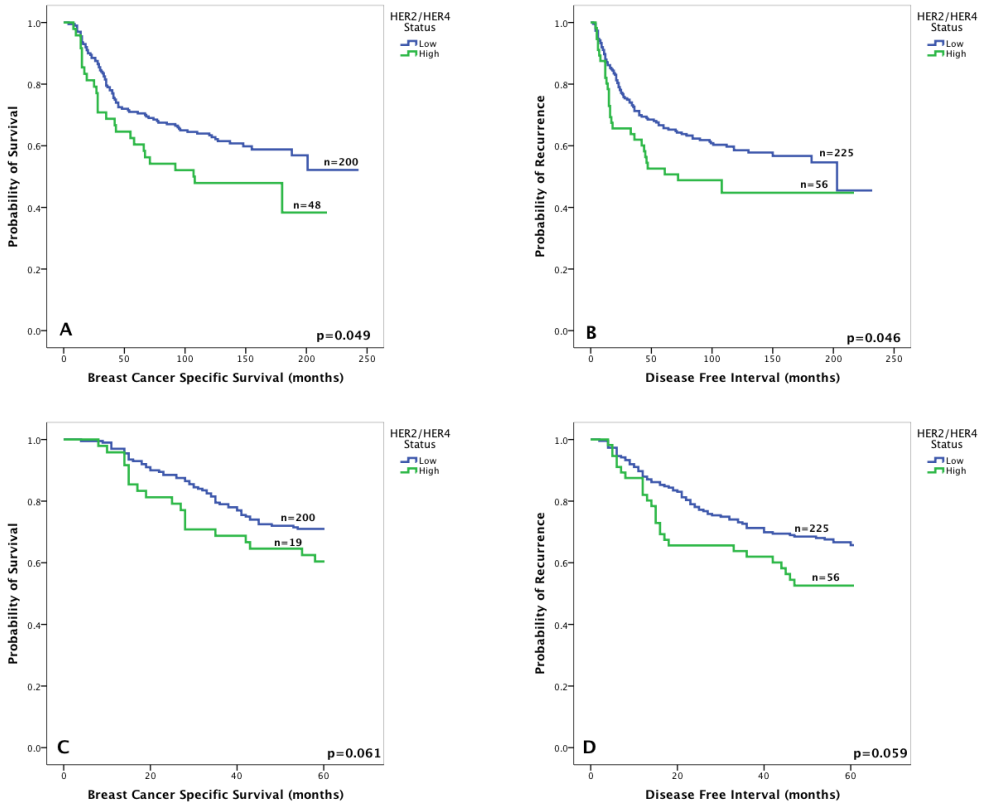

Figure 6.6 – BCSS and DFI analysis for HER2/HER4 status amongst the ER negative cases in Unselected series for a period of 250 months **A)** and **B)** or for a period of 60 months **C)** and **D)**.

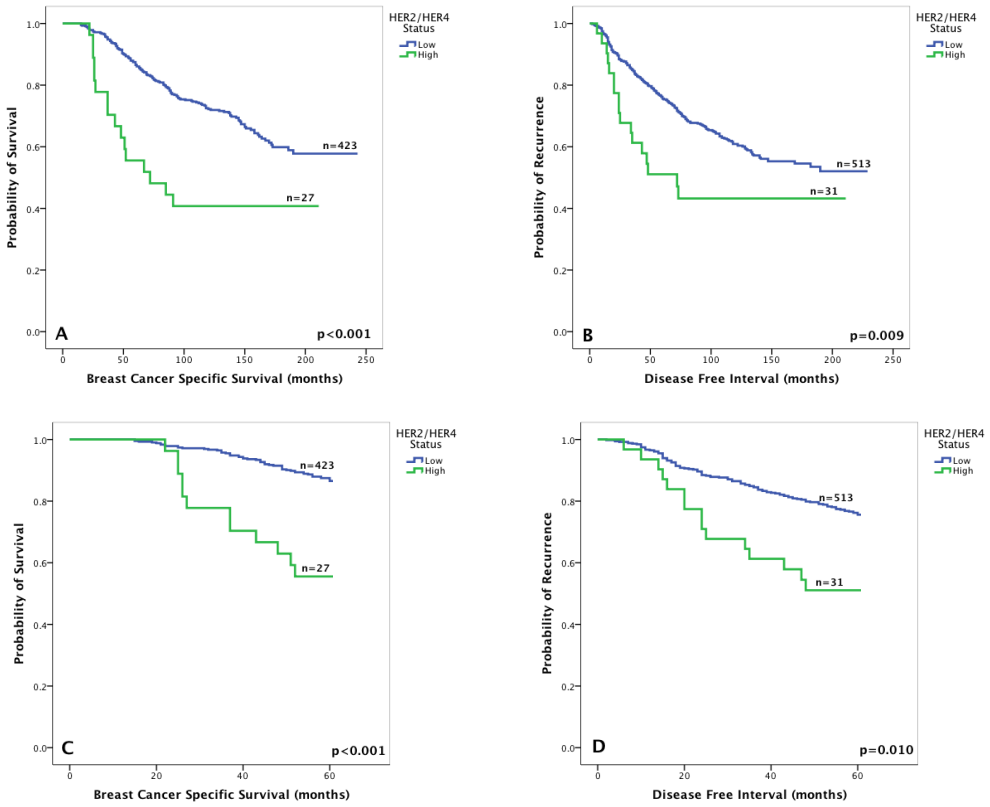

Figure 6.7 – BCSS and DFI analysis for HER2/HER4 status amongst the ER positive cases in Unselected series for a period of 250 months **A)** and **B)** or for a period of 60 months **C)** and **D)**.

Table 6.7 – Multivariate Cox regression analysis of factors associated with BCSS and DFI for ER positive cases in Unselected series.

Variable	BCSS		DFI	
	Hazard Ratio (95% CI)	p Value	Hazard Ratio (95% CI)	p Value
Tumour Size (cm)	0.62 (0.39-0.99)	0.046	0.73 (0.52-1.03)	0.072
Tumour Stage	2.02 (1.62-2.53)	<0.001	1.73 (1.41-2.10)	<0.001
Tumour Grade	1.84 (1.43-2.37)	<0.001	1.18 (0.98-1.43)	0.085
HER2/EGFR	0.51 (0.30-0.86)	0.012	0.62 (0.37-1.02)	0.017

6.3.2.9 Association with patient outcome in HER2+ trastuzumab adjuvant versus naïve

Survival analysis for only those patients possessing low levels of HER2/HER4 revealed that there was a significant worse outcome amongst the cases that were not submitted to trastuzumab adjuvant therapy for both BCSS ($p = 0.015$; Figure 6.8A) and DFI ($p= 0.041$; Figure 6.8B).

High HER2/HER4 levels revealed a highly significant difference in patient outcome between both adjuvant trastuzumab and trastuzumab naïve series, where those receiving trastuzumab did significantly better ($p < 0.001$, Figures 6.8C and D).

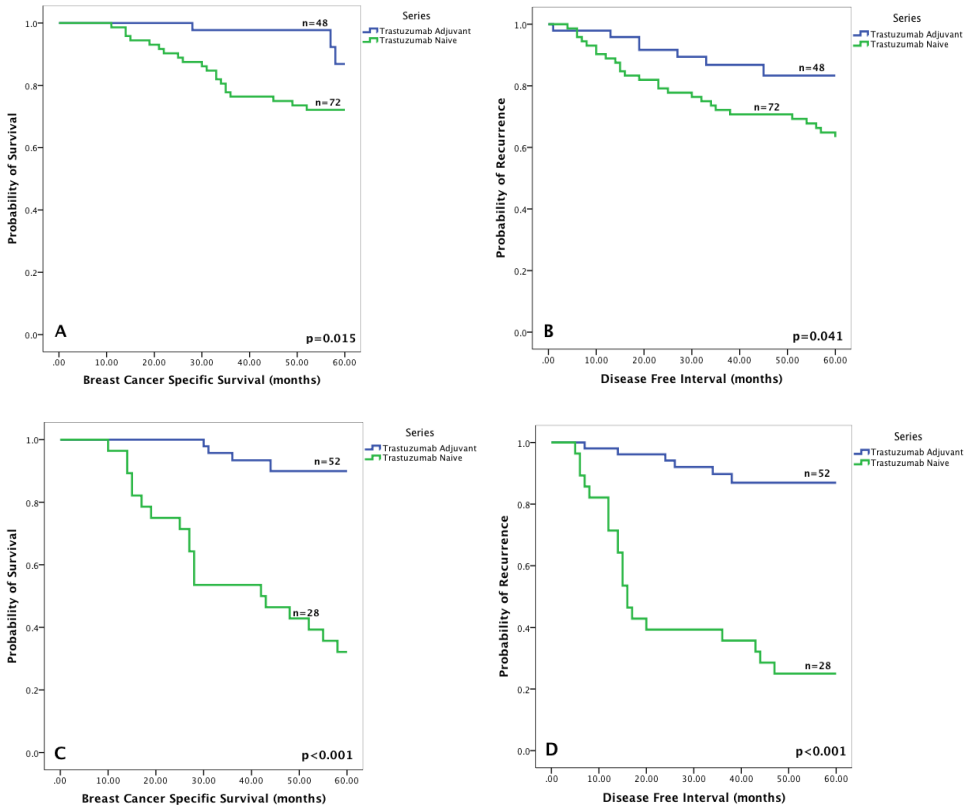

Figure 6.8 – BCSS and DFI analysis for HER2/HER4 status amongst the HER2+ trastuzumab adjuvant series against HER2+ trastuzumab naïve series regarding **A)** and **B)** HER2/HER4 low levels or **C)** and **D)** HER2/HER4 high levels.

6.3.2.10 HER heterodimer frequency in HER2+ breast cancer

A total of 73/142 (51.4%) HER2+ tumours expressed all three heterodimers (Table 6.8) whereas only 32/142 (22.5%) cases did not show expression of any of the heterodimers investigated. Expressions of only one heterodimer or two heterodimers were less common. Though HER2/HER3 plus HER2/EGFR are present in 24/142 (16.9%), being the dual combination more frequent. Also HER2/HER4 heterodimer alone was not observed in any case.

Table 6.8 – Frequency of HER heterodimers in HER2+ breast cancer

HER2/EGFR	HER2/HER3	HER2/HER4	Frequency
+	-	-	4
-	+	-	4
-	-	+	0
+	+	-	24
+	-	+	11
-	+	+	1
+	+	+	73
-	-	-	32

6.4 Discussion

While becomes more evident that overexpression of EGFR, HER2 and HER3 are associated with poor outcome and lead to possible resistance to treatments[187,455,501,536], HER4 is the only monomer from HER family connected with a good prognosis in breast cancer. However, HER4 biology in breast cancer remains unclear. This potentially could be related the pro-apoptotic role of the HER4 intracellular domain[531].

In this chapter, the expression and role of the HER2/HER4 heterodimer was determined to understand whether HER2/HER4 is related with poor prognosis status and if those patients carrying high levels of this heterodimer develop resistance to trastuzumab therapy.

HER4 monomer expression was present in the majority of breast tumours (75%), irrespective of HER2 status, in agreement with previous studies[455]. The prognostic significance of HER4 is controversial. Even though overexpression of HER4 in breast cancer has been reported by different studies, revealing a range 11% to 45% of cases[331,518,537-539]. HER4 is highly correlated with lower tumour grade, good NPI and longer DFI[455,518,537,538]. However, these results are contradicted by others suggesting that HER4 positive expression is correlated with poor prognostic variables[540,541] such as higher tumour grade[542], high tumour stage, poor NPI and vascular invasion but inversely correlated with local recurrence[331]. The results presented in this chapter, confirm that HER4 expression is associated with poor prognostic factors. This discrepancy between studies might be explained by the presence of different HER4

isoforms[543]. Data presented here are in agreement that HER4 positive expression did not distinctly associate with patients' survival rate[518,540]. This suggests that agreement on HER4 scoring still not achieved and standardisation is required.

HER2/HER4 protein-protein interactions have not been previously studied in breast cancer. Though the reliance of the dimerisation between HER2 and HER4 is dependent on the expression of the monomer it was not unique. The frequency HER2/HER4 was significantly lower than other heterodimers. This suggests that amongst breast carcinomas, the affinity of dimerisation between HER2 and HER4 might be weaker than comparing with EGFR or HER3. This idea was already anticipated where it was showed that distinct receptors amongst the HER family noticeably diverge in their preferred interaction partners[481].

As with the previous heterodimers, HER2/HER4 heterodimers were primarily dependent on high levels of *HER2* amplification/protein overexpression and not HER4. However, it is possible that the dimerisation of HER2 and HER4 only occurs when interacting with specific ligands rather than HER4 expression levels. In order to dimerise, HER4 has to interact with one of the ligands including HB-EGF, BTC, EPR[251], NRG3 or NRG4[229]. Several studies have revealed that HER4 is associated with good prognosis and even reduced HER2 activity[537,544]. However, HER2/HER4 heterodimers were tightly associated poor prognostic factors in the Unselected series of breast tumours, with exception for only high tumour size and vascular invasion.

HER2/HER4 was not associated with any basal biomarkers, including as CK5/6 and CK14, which contradicts some studies[537,544], but was strongly associated with luminal CK18 positivity. This association reveals the lack of apoptosis occurrence associated with high expression of CK18[464] in breast carcinomas expressing high levels of HER2/HER4. Also there was high correlation between HER2/HER4 and PI3K revealing apoptosis avoidance and high proliferation. Similar to other members of the HER family, a ligand/receptor interaction promotes dimerisation and subsequently a kinase activation, which in turn will trigger cell-proliferation signalling cascades such as PI3K[477,545]. Even though PI3K interacts with six tyrosines of HER3 against only one in HER4[546]. BRCA1 is expressed in normal breast cells helping to repair the DNA or promoting apoptosis on those cells where damaged DNA is not repairable[547], being related with poor prognosis when not expressed[392]. In this study HER2/HER4 high levels were highly associated with the lack of BRCA1. Finally and supporting the idea that HER2/HER4 high levels is not an indication of good prognosis, this heterodimer was highly associated with p53, which it is related with poor prognosis when highly expressed[512,548,549]. Several studies revealed that p53 IHC is not only prognostic factor for breast carcinoma but also defines the sensitivity to certain treatments[550-552] but also as a predictor for chemotherapy resistance development[553,554]. Patients with worse prognosis were associated with anomalous expression of p53, which is highly associated with the absence of Bcl-2[555]. When Bcl-2 expression is retained, cell survival is extended but does not promote cell proliferation[556]. The main observation was HER2/HER4 was associated with Bcl-2 negative status. Low

Bcl-2 expression has been correlated with development of resistance to certain types of chemotherapy such as paclitaxel[557]. Reason to regard HER2/HER4 heterodimers as a poor prognostic parameter despite some studies associating HER4 (monomer) with good prognosis[455,538].

Survival analysis demonstrated HER2/HER4 predicts worse outcome in the Unselected series although not the HER2+ positive series, suggesting that HER2 prognostic value is not manipulated by HER4. In HER2+ breast cancer, factors such as different heterodimerisations contribute a worse prognosis, showing that HER2/HER4 heterodimers do not develop an independent value for prediction of poor outcome. High levels of HER2/HER4 heterodimer were associated with worse outcome independent of ER status, being the only heterodimer showing this. Though, the poor prognostic value is much stronger amongst ER positive cases. One reason might due to the high expression of HER4 in these cases and therefore on localisation of HER4 intracellular domain (4ICD) in the nucleus, which trigger proliferation reactions by acting as a co-activator of ER[558]. Four different isoforms of HER4, JMa, JMb, Cyt1 or Cyt2, as a result of alternate RNA splicing[477], although this does not influence ligand linkage site or structure of tyrosine kinase. However it affects sensitivity of the HER4 ECD to be released from the cell membrane[251]. Additionally, other HER4 isoforms with sequence mutation on tyrosine kinase domain can influence affinity to PI3K[251]. The JMa isoform possesses an ectodomain that can be cleaved by metalloproteinase[559]. A second intracellular cleavage can also occur resulting in the discharge in the cytoplasm of a soluble form of 4ICD[560], which can be localised in the cytoplasm or nucleus. Depending on the

localisation of 4ICD, the roles of this small molecule include differentiation of the mammary cell, pro-apoptotic reactions, cell cycle arrestor cell proliferation[526]. If 4ICD is localised in the mitochondria, apoptosis reactions are promoted in tumours cell, mediated by the cell-killing BH3 domain[531].

Survival analysis between those HER2+ patients not receiving trastuzumab against those that received trastuzumab revealed that the trastuzumab naïve patients possessed a worse prognosis independent of HER2/HER4 levels. Though, the significance was much higher amongst those patients possessing high levels of HER2/HER4. This supports the idea that HER2/HER4 might be the least influential heterodimer currently studied.

In conclusion, HER2/HER4 heterodimers were associated with poor clinicopathological parameters and poor survival outcome. The data presented within this chapter suggests that the stated hypothesis of HER2/HER4 high levels contribution to trastuzumab therapy resistance is not supported. However expansion of the number of samples and studying the influence of HER4 specific ligands and isoforms might increase a further understanding on these structures and the signalling cascades triggered.

7. CHAPTER 7: General discussion

7.1 Importance of HER2 in breast cancer

The HER family has been showing a considerable importance on breast carcinoma development. HER2 acts as a dimerisation partner with the other members of the same family[561]. All except HER2 become activated upon contact with ligands following a dimerisation occurrence and signalling cascade activation[135], promoting cell proliferation, survival and development of metastases. Because HER2 does not need interaction with any ligand, this fact allows HER2 to join in a number of signal transduction cascades even if a specific ligand is lacking[135]. Therefore when HER2 is overexpressed this may drive cells to uncontrolled proliferation and avoid apoptosis. HER2 overexpression and gene amplification in breast cancer has been studied over 20 years[562]. HER2+ breast carcinomas are present at a frequency of 12.1% and its expression is highly associated with the *HER2* gene amplification. HER2 status offers both prognostic and predictive value. Recent findings have led to development of novel drugs that exclusively target this receptor, changing dramatically the panorama of breast cancer therapy[349].

Immunohistochemistry technology permits the detection of different proteins in tissues and is the first approach for HER2 status assessment in patients identified with invasive breast tumours[563]. However IHC has some disadvantages such as issues with tissue fixation and methodologies. Variation in antigen retrieval and use of different antibodies can influence IHC results[54]. Additionally IHC interpretation is subjective and can vary depending on the

scorer. Those cases in borderline classification for HER2, gene amplification will be assessed by using FISH to determine status[564]. However as already documented a percentage of HER2+ patients develop resistance to trastuzumab and almost all patients experience disease progression while on this treatment[565]. HER2 status assessed using IHC and FISH cannot identify the resistance to trastuzumab. Better characterisation of the variations HER family interactions, downstream pathways, regulators of the cell cycle and apoptosis offers opportunities to understand the mechanisms of HER2 therapy sensitivity and resistance.

Of the other HER family monomers, high expression of EGFR is the only alteration shown to be associated with poor outcome[249,467,566,567]. EGFR expression has been associated with triple-negative breast tumours, which is characterised by a very aggressive phenotype with distinctive patterns of metastasis[435,568,569]. Therefore EGFR might be an important target in breast cancer treatment in this molecular phenotype group. Both HER3 and HER4 when detected as monomers do not reveal any significant association with poor outcome. HER3's prognostic value is still not well documented due to non-conclusive data[95,476,570]. HER3 has been correlated with poor survival outcome by some groups[455,571] but other group have identified a significant association with good prognosis[95]. Several groups have shown HER4 expression to be a good prognostic factor revealing better survival outcome[95,455,544]. Though HER4 has been correlated as well with poor outcome in a different study[92]. Additionally, the truncated form of HER2 $p95^{HER2}$, when assessed in this study did not reveal any additional prognostic outcome when compared with HER2

status analyses alone. Suggesting that more complex reactions involving all HER family members and the respective ligands or key downstream pathways may be important.

7.2 Dimerisation assays

Previously IHC techniques have been used in a semi-quantitative approach to detect the presence of individual proteins. A multifaceted coordination of a complex system of crosstalk is thought to exist with the HER family, including heterodimerisation, resulting in activation of various downstream pathways potentially contributing to the sensitivity or resistance to treatments[247,572]. Conventional IHC is limited in its ability to detect her family dimers.

In this thesis, the technique *in situ* PLA (brightfield version) was used as a novel approach for the high-throughput detection of HER heterodimerisation levels in FFPE primary breast carcinomas prepared as tissue microarrays. Early studies established the successful use of fluorescence-linked PLA in order to detect protein-protein interactions[501,573] and this was further developed for chromogenic detection. PLA has been extensively applied to studying protein interactions amongst a high range of different cell-lines. In this project this technique was successfully applied in fixed primary invasive breast cancer samples. As performed in the past[501] it was confirmed that this technique works efficaciously on FFPE tissues, and moreover using TMAs it can provide evidence of prognostic and predictive significance of the various heterodimers. A study on protein-protein interactions had been performed using VeraTag™ technology[574], however this technique possesses a major limitation of requiring

detection in liquid phase. Thus, it cannot be performed on fixed clinical samples or TMAs of large patient cohorts.

The use of specific primary antibodies in this technique allowed detection of different HER heterodimerisations partners. This study has shown high sensitivity and specificity of this method to detect of the HER heterodimers in a large series of cases. This technique have revealed in clinical arena, although further validation studies are vital. Use of brightfield *in situ* PLA identification of different morphological structures present in each sample, which is not possible amongst fluorescent techniques as Förster (Fluorescence) resonance energy transfer (FRET) that has been used in a different study to identify HER family dimerisation[457]. Because PLA allows identification of heterodimers at subcellular level and permits investigation and identification of any cellular subsection involved in the HER family downstream pathways, which are significantly associated with development of resistant to treatments[575]. Also the possibility to detect the signal allocation at both intracellular and intercellular levels makes possible detection of molecules with critical role at different downstream pathways such as proliferation, survival and motility[575]. However this study was more focussed on quantity of signals and the implications on prognosis and resistance to treatments.

For all three HER heterodimers investigated, it was evident that whilst HER2 overexpression is the dependent factor in dimerisation, the presence of other receptors did not necessarily result in heterodimerisation. HER2 has previously been suggested as the dominant monomer for heterodimerisation comparing

with any others from the same family[500]. It does not include an ectodomain where a ligand can attach, however is competent to undertake as a co-receptor with an extraordinary affinity to interact with the same family members forming heterodimers[25,63]. The lack occurrence of heterodimers in some HER2+ cases, despite both receptors being expressed, could be due to alternative splicing, modification of the transcription mechanism or the lack of ligands[576].

Heterodimerisation is not a cellular physical structure but characterised as a dynamic reaction dependent on several molecules interactions. The detection of HER heterodimers in FFPE breast tumours reflects status at a snapshot of time. Therefore any signal detected only represents reactions that were occurring at a specific moment during formalin fixation. This might reflect that the number of signals detected is low, maximum of 35 heterodimer signals per cell; it is known that the number of HER2 molecules per cell is able to reach 2,000,000[46]. Therefore several questions emerge. Are the dimers stable and preserved during the Formalin fixation process? The heterodimers half-life might be significantly shorter compared with the period of paraffin fixation. Therefore further research studies should be performed in frozen fixed samples and cell-lines to examine this observation. Additionally different primary antibodies could be examined to understand if the technique is primary antibody dependent.

7.3 HER family heterodimerisation and other biomarkers

It was observed that the incidence of all three heterodimers was present in 50% of the cases, whereas a single heterodimer was a rare occurrence. HER2/HER4 heterodimerisation alone was not observed in these series. Additionally HER2/EGFR and HER2/HER3 heterodimerisations were 70% off all cases regardless HER2/HER4 occurrence. These two heterodimers were significantly correlated with PI3K expression and development of metastases. It has been shown that HER2/EGFR triggers cell motility[107] and that HER2/HER3 interacts to enhances cell proliferation and motility indicating that a "teamwork" of two heterodimers could enhance some behaviour characteristics[460]. It can therefore be suggested that HER heterodimerisation may not be a single event but a complex interconnected system. A previous study has suggested that synergistic heterodimer signalling occurs leading to cell migration[461]. This observation could be influential to understand the response in combination of HER family members on therapy using Lapatinib in combination with trastuzumab and chemotherapy. Lapatinib is the second agent to be approved by FDA (2007) for the use in HER2+ breast cancer patients. This reversible inhibitor interacts with both HER2 and EGFR tyrosine kinases and specifically used in the treatment of metastatic breast tumours. Because it acts in a different manner from trastuzumab, it might become useful in those patients with development of resistance to trastuzumab[199,577]. Essentially lapatinib revealed clinical benefit in patients that experienced disease progression after being treated with trastuzumab[199].

Beside ER and PgR, Bcl-2 was the only biomarker with significant negative association with high expression of all three heterodimers in both series of patients. The lack of expression of this apoptosis regulator has previously been associated with poor prognosis[401]. Results obtained *in vitro* by previous studies revealed Bcl-2 as an anti-apoptotic biomarker, contributing to malignant cell accumulation. Thus overexpression of this biomarker would be expected to be correlated with aggressive tumour cell behaviour[578]. In breast cancer specimens, the expression of this biomarker is correlated with factors related to better prognosis such as lower grade, ER positivity and lower proliferation[579]. Also, Bcl-2 low expression is associated with poor prognosis even when patients are treated with modern regimes such as paclitaxel[557]. Its lack of expression is associated with different poor prognostic biomarkers as p53, EGFR and HER2[463]. The same ligands that trigger EGFR to a competent conformation, also downregulate Bcl-2[463]. In this study heterodimers were negatively associated with Bcl-2 and the hormone receptors, which is in concordance with previous observations and in agreement with a previous study reflecting the regulation of Bcl-2 by the hormone receptors[463]. Even though still not understood why the lack of expression of Bcl-2 is associated with poor prognosis, when its main role is promotion of cell survival. The explanation might reside in the fact that Bcl-2 expression status by itself is not adequate to determine the aptitude of this biomarker in apoptosis pathway. Therefore it might be interesting to study the levels of other members of this biomarkers family. Another reason to believe that the HER heterodimers are associated with poor prognostic factors is their association with p53 expression. As documented by Bottini and colleagues "p53 but not Bcl-2

Immunostaining is predictive of poor clinical complete response to primary chemotherapy in breast cancer patients"[398]. Additionally the frequency of mutated p53 gene is present in a high percentage of breast carcinomas and was proposed not only as a prognostic biomarker but also to predict cytotoxicity to anthracyclines[398].

CK18 is the last biomarker identified in this project that was associated with expression of all heterodimers in both series with exception for HER2/HER4. As stated before this biomarker is associated with apoptosis occurrence through apoptosis related degradation via caspase cleavage[464,465,580]. Those cases expressing high levels of HER heterodimers suggest an influence on cell survival. However some differences between the heterodimers was observed. Interestingly HER2/EGFR was the only heterodimer associated with the basal biomarker CK14. Different studies have already associated basal-like tumours with EGFR expression, even though these tumours are characterised by non-amplification of the *HER2* gene[567,581]. CK14 has also reported to be associated with a subpopulation of luminal cells[582,583]. Recently it was reported that the Luminal B subgroup is characterised by the presence of HER2 expression at a frequency of 30%[584]. Additionally HER2/EGFR heterodimerisation not associated with BRAC1 protein loss, which is associated with poor prognosis[392]. BRCA1 is expressed in normal breast cells and is included in DNA homologous recombination repair or promotion of apoptosis in those cells where DNA is not repairable[547,392,585]. Loss of BRCA1 protein is a fundamental factor in disruption of the error free DNA repair pathway[586,587]. The expression of BRCA1 has been critical in prevention of not only breast but

also ovarian cancer[547], but has less impact in other types of cancer[588,589]. Functional lost of BRCA1 results in a lower reliability of DNA repair, growing significantly the risks of cancer development mediated by increase frequency of DNA repair errors[547].

HER2/HER3 heterodimerisation has been shown to be associated with more adverse tumour characteristics when compared with HER2/EGFR and HER2/HER4 dimers. HER2/HER3 was the only heterodimer to be associated with high levels of MUC-1, which is associated with poor prognosis in breast cancer patients as it is connected with the metastases progression process[516,517]. MUC-1 is a protein aberrantly expressed in breast carcinomas and that works as an oncoprotein. This biomarker has been described as a possible drug target, inducing cell death by blocking it, which was already achieved *in vitro*[590]. It interacts with HER2 contributing for PI3K/Akt pathways activation[590]. Additionally HER2/HER3 was the only heterodimerisation to be highly correlated with Akt positive status. This biomarker is not only involved in anti-apoptotic pathways but also on induction of protein synthesis essential on general tissue growth[591]. Beside cell survival Akt has been highly correlated with proliferation and cell motility. Its uncontrolled phosphorylation is one of the most common modifications in human carcinomas. This fact contributes to the high dependency of the cancer cells on this biomarker, becoming a candidate to be targeted on cancer therapy[592,593]. Akt has a protein domain, the PH domain, which binds with high affinity to PIP3[446,594], an activated product resulting in PIP2 phosphorylation by PI3K triggered by HER2/HER3[108] and other receptors such IGF[594]. Intensification of the PI3K/Akt signalling cascade

is associate with development of resistance to trastuzumab, though not to lapatinib[595]. Therefore a drug directed to this downstream pathway might benefit a significant number of patients. Finally HER2/HER3 was revealed to be the only heterodimerisation highly correlated with p21 expression amongst the HER2+ series. High expression of p21 is highly associated with resistance to chemotherapy[512]. Additionally the HER2/HER3 was the only heterodimerisation not highly correlated with Ki67 expression, nevertheless near to high significance. Ki67 is a nuclear protein severely correlated with ribosomal RNA transcription and is expressed mostly in proliferative cells[397,596]. This suggests that HER2/HER3 promotes high rates of cell proliferation, though not related with Ki67 pathway, but clearly triggering PI3K/Akt signalling cascade including the encouragement generated by interaction with MUC-1[590]. These suggestions are in concordance with previous studies recommended PI3K/Akt as the most important tumorigenic signalling pathway promoted by HER3 amongst HER2 amplified breast carcinomas[496], with HER3 being accepted has the main member of HER family with the ability to trigger this pathway[202].

HER2/HER4 high levels had no significant correlation with any biomarker in this study. It was the only heterodimerisation occurrence that was not correlated with CK18 protein expression amongst the HER2+ series. In comparison to other HER heterodimerisation occurrence HER2/HER4 is the protein-protein interaction that promotes the lowest anti-apoptotic signalling activity. HER4 has been correlated with better prognosis and the only member that antagonises the HER2 highly reactive anti-apoptotic nature[530,544].

PTEN is a tumour suppressor gene that encodes its protein with the function of antagonising PI3K and therefore inhibits Akt phosphorylation and consequently tumour progression. Lack of PTEN has been shown by some to drive HER2+ breast carcinomas to develop trastuzumab resistance[178,597]. Although others did not observe development of resistance to trastuzumab to be associated with the lack of PTEN[439]. In this study PTEN was significantly associated with the three HER heterodimers in the Unselected series but not in the HER2+ series. Therefore the results obtained with PTEN expression against the heterodimers did not appear to concur with any literature. Therefore it is recommended that IHC analysis should be preformed once again using a different antibody in order to validate the present results.

7.4 Heterodimerisation and the clinicopathological parameters

In this study it was identified that within HER2+ series HER2/HER4 was the only dimer that was not associated with any of the clinicopathological parameters correlated with worse prognosis. Other studies have already revealed HER4 to be associated with better outcome[544], which supports this observation.

There were limited isolated associations between HER heterodimers and clinicopathological parameters being more difficult to characterise subgroups. HER2/HER3 and HER2/EGFR were correlated with higher tumour grade but not HER2/HER4, supporting the ideas of a crosstalk between these two heterodimers already suggested[460].

HER2/EGFR heterodimerisation was the only occurrence associated with lack of tubule formation and poor NPI. This last association might be linked to EGFR being the only monomer associated with poor prognosis in this study and contributing for a worse NPI score when involved in heterodimerisation.

This lack of distinguishable associations amongst the heterodimers within the HER2+ series suggests a high correlation between them. As already proposed crosstalk between the different heterodimers occurs[108,461] and therefore the correlation between them is too strong, as revealed in this study. Further studies to target the key clinicopathological parameters or/and biomarkers is very important to understand in more detail the nature of these heterodimers and utilise them as a therapeutic target more efficiently.

7.5 HER heterodimerisation crosstalk with hormone receptors

High levels of HER heterodimerisations are associated with lack of ER and PgR, though not amongst all cases. This fact is in concordance with those studies showing an inverse correlation between HER2 and hormone receptors expressions[598,599]. In cases with high HER2/EGFR and HER2/HER3 heterodimers, when ER was expressed, a significant worse outcome for both BCSS and DFI was observed. However when ER was not expressed the survival curves were not significantly different. Furthermore the worse outcome was achieved for all heterodimerisations in the first 60 months. These data imply that crosstalk occurring in ER+/HER2+ breast cancers could

enhancing each other through a genomic and non-genomic manner resulting in endocrine therapy resistance[121]. However, the interaction between these two receptors is still not well understood. This thesis demonstrates for the first time that potential crosstalk between ER/HER2 and HER2 dimers status can influence outcome and may be relevant to respond to treatment. Data presented in this thesis suggests that alternative mechanisms might be present depending on which heterodimer is present; supporting that HER2 monotherapy is perhaps not the optimal solution for treating patients with HER2+ breast cancer. It is already well documented that there is crosstalk between HER2 and ER[600,601]. HER2 positive tumours develop resistance to hormone therapies[105,602-605]. On the other hand ER becomes up regulated after trastuzumab therapy[606,607]. Treatments with lapatinib also demonstrated that it is more effective with HER2+, ER and PgR negative tumours[608]. Cell line experiments revealed an up regulation of ER with exposure to lapatinib[600,536]. These data support the view that anti-HER2 therapy could result in incomplete blockage of the HER family signalling cascades, if conditional HER homo or heterodimers are present and which could be responsible for treatment failure[167,421,536]. The combination of both lapatinib and trastuzumab has been shown to be more effective[603,609] but relapse still occurs[536]. Trastuzumab is known to disrupt HER2/HER3 heterodimers[136]. Trastuzumab exposure in cell line exhibiting EGFR plus HER2 expression, maintain the level of these proteins, although phosphorylated-HER3 levels decrease significantly[536]. However some investigation have suggested that both EGFR and HER3 contribute to trastuzumab resistance[167,486]. Others have identified that HER2 is influential in trastuzumab resistant[536].

7.6 HER family heterodimerisation and prognostic value

In this thesis has been used PLA technology to identify HER2/EGFR, HER2/HER3 and HER2/HER4 heterodimers. HER2+ carcinomas can show increased expression of the three dimers, which is in concordance with a previous study[501].

Unsurprisingly the three heterodimerisations revealed to be associated with poor outcome for both BCSS and DFI amongst the Unselected series, which had been demonstrated before for HER2/HER3[455,460,501,610] and HER2/EGFR[457,461] dimers. However this association was not observed amongst HER2+ cohort. Although, the results for the trastuzumab adjuvant series should be analysed carefully due to the limited number of cases in study, high levels of the HER heterodimers revealed no significant association with worse outcome when patients received trastuzumab. However HER2/HER3 high levels demonstrated a propensity for poor survival after 60 months of treatment. The possibility that these patients might be developing resistance is supported by observations that PI3K/Akt as one of the major downstream pathways for this heterodimers[198,229,461,595], also revealed in this study. Plus, aggressive phenotypes are associated with PI3K/Akt downstream pathway[611-613] that contributes significantly to resistance to chemotherapy, trastuzumab and tamoxifen in breast cell lines[614]. These ideas indicate that PI3K/Akt phosphorylation could be a candidate prognostic indicator and target that could contribute to enhance the success of breast cancer therapy. Some drugs are already in early stage of clinical development such as NVP-BEZ_{235}, which has shown promising results through their antagoniser of PI3K signalling, inducing

cell cycle arrest[615]. Promising results has been observed in phase I/II clinical trial where patients were treated with the drug in combination with trastuzumab[616].

7.7 Research methodology assessment

PLA technology was developed to reveal interaction between proteins where the targets are recognised by antibodies probes attached to DNA strands. This strategy has the potential to allow the detection of multiple protein and/or protein-protein interactions represented by a single DNA motif.

PLA can support exploration of different proteins interactions and their downstream pathway processes. Therefore this advanced technology has the potential to improvement on discrimination of different cases to be submitted to more specific treatments that target not only HER2 but further elements of the same family or key upstream or downstream proteins involved in trastuzumab blockage avoidance and contributing to tumour progression.

However different groups will use different procedures to perform PLA, which might be a critical factor of the PLA technique, such as the use of different antibodies, different cut-off points and/or the designation of the monomers overexpression status. The results of brightfield versus fluorescent could be a reason for evident discrepancies, however these two techniques produce similar results in both cell culture or tissue samples. Brightfield version reveals to be as specific and sensitive on quantification analysis as the fluorescent version[617]. Therefore standardisation of this technology is necessary.

7.8 Future Perspectives

All studies in this thesis are based on FFPE TMAs, which due to fixation process might affect some protein structures and protein-protein interaction could be disrupted. Therefore it is recommended that the use of fresh frozen breast tumours must be used to further validate the method. With this methodology the tissue is frozen immediately after extraction and is not exposed to organic solvents or fixations keeping almost all native properties. Therefore it would be possible to detect and quantify the native levels of heterodimerisation in order to validate the results presented using this novel method.

More detailed determination of the relevance of ER control, interactions of different dimers, the functional effects of the observed dimer patterns and the potential relevance of such patterns to various forms of targeted anti HER therapy could be studied using *in vitro* cell-line methods. For example a series of 8 breast cell lines could be used - HCC1954 and SKBR3 cell lines (HER2 positive, ER and PgR negative), BT-474 and MDA-MB-361 cell lines (HER2 positive, ER and PgR positive), MCF7 and T47D cell lines (HER2 negative, ER and PgR positive), MDA-MB-231 cell line (HER2, ER, and PgR negative) and normal mammary epithelial cell line MCF-10A (HER2, ER, and PgR negative). HER family expression characteristics of each cell line could be examined using western blotting and q-pcr. Behavioural characteristics could be investigated using functional studies. For example cell proliferation and viability could be assessed using standard methods, Wound Healing Assay used for a more complete characterisation of cellular migration behaviour.

Additional characterisation of the cell lines must be performed by a fixation method, using 10% neutral buffered formalin and paraffin embedding.

It is expected that cell line manipulation might be required to mimic the identified clinical situations. In order to control cell lines expression and/or dimerisation occurrence should be use shRNA transfection for gene knockdown and target specific receptors making sure only one dimer is affecting the results or being affected by ER expression or its absence. The robustness and basic functionality of single and double knockout could be assessed using the above methods. Cell lines not expressing any heterodimer or respective ligands could be transfected with plasmid constructs for each respective situation.

7.9 Conclusion

With this research project it was possible to observe that HER2 heterodimers were significantly correlated with poor outcome features and also to identify that HER2 overexpression encourages heterodimerisation, which may stimulate growth, malignant development[618] and migration[619].

The use of PLA through its ability to provide protein-protein interaction quantification might become more important in the future in respect not only to medical research but also clinical analysis and drug response prediction. Using this technique it is possible to recognise, quantify and localise molecular and cellular events.

HER2 has previously been proposed as the dominant monomer for heterodimerisation comparing with other family members[500]. HER2 does not include an ectodomain where a ligand can attach, however is competent to behave as a co-receptor with an extraordinary affinity to interact with the same family members forming heterodimers[25]. Additionally HER2 has not the ability to interact with any ligand and therefore its conformation arm is constantly predisposed to exist in a competent form and ready to connect with any of the other monomers from the same family[63]. It was found that all three HER heterodimers are expressed at a similar frequency[501].

Additionally the hypothesis that different heterodimers had different associations with breast cancer related biomarkers and consequently different clinicopathological parameters was confirmed. High levels of the three HER heterodimers was revealed to be associated with worse DFI and BCSS, and the hypothesis that they are a poor prognostic factors was also confirmed, although in patients receiving adjuvant trastuzumab the heterodimers patterns were not associated with a significantly worse outcome. High levels of HER2/HER3 were observed to be associated with a significantly worse prognosis after 60 months of treatment and this observation requires further investigation.

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