

## 5. HER2 EXPRESSION IS NOT REGULATED BY E2 NOR TAM IN BREAST CANCER CELL LINES

### 5.1 INTRODUCTION

The *HER2* gene is amplified and/or highly overexpressed in 15-20% of all human breast cancers (60-65) and its alteration is associated with recurrence (66), chemoresistance (adriamycin, cytoxan, methotrexate, 5-fluorouracil and TAM), short survival, high histological grade tumors and positive axillary nodes (67), all of which are considered indicators for poor prognosis (58). Therefore, *HER2* gene amplification is considered a bad prognostic marker and a predictive factor in response to chemotherapeutics, anti-estrogenics and therapy using specific antibodies to block the receptor function (68).

Furthermore, it has been reported that *HER2* gene expression is subjected to hormonal regulation ( $17\beta$ -estradiol-E2), since apparently E2 regulates not only the growth factors expression of the HER family (13, 41, 69) but also causes an indirect activation of the HER2 protein, thus confirming the existence of a strong association between HER2 overexpression and the activation of signaling pathways mediated by estrogens (13, 41, 69, 70). In HER2-overexpressing tumors, it has been observed that an estrogen-independent growth and an increased cross-talk between ERs and signaling pathways mediated by EGFR/HER2 contributes to the development of resistance to endocrine therapy (6, 48). In contrast, estrogens mobilize a variety of systems to increase cell proliferation (2-4), invasiveness and proto-oncogene activation (47-49, 51).

The amplification and overexpression of the *HER2* gene, as well as the time of exposure to mitogenic hormone E2, are two molecular factors that have been widely associated with mammary tumorigenesis, which have been additionally used in therapy selection between anti-estrogen agents and specific antibodies that interrupt the receptors function (TAM). Likewise, a strong association between HER2 overexpression and E2 has been observed, which appears to lead not only to the activation of ER in the absence of ligand, but the indirect activation of HER2-mediated by E2 (9, 13). On the other hand, some studies suggest that the expression of the *HER2* gene is subject to hormonal regulation in both normal and breast cancer cells. These studies reported that estrogens inhibit HER2 expression at both the mRNA and protein levels and regulate expression of a number of growth factors in estrogen receptor positive (ER+) cell lines (13, 41, 235). Anti-estrogens (TAM) inhibit the proliferative actions of estrogens (74, 75) and sometimes have been shown to reduce the invasiveness of breast cancer cells stimulated by estradiol (77).

Taking into account that estrogens are known to increase the proliferation rate and invasiveness of breast cells and that some studies have hypothesized that ER can negatively regulate the expression of the HER2 mRNA and protein at high doses in ER+ but not in ER- breast cancer cell lines (13, 235), we have studied the effect of low doses of sex steroid hormones (E2) and anti-hormones (TAM) on HER2 mRNA and protein levels in ER+ and ER- human breast cancer cell lines.

In this study, we report that neither E2 nor TAM significantly regulates the HER2 mRNA or protein levels in breast cancer cell lines.

## **5.2 METHODS**

### **5.2.1 Cell Lines**

The human breast cancer cell lines MCF7, T47D, BT474 and SKBR3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) in March 2010. MCF7, T47D, SKBR3 were cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA), while BT474 was cultured in DMEM medium (Sigma). All culture media were supplemented with 10% fetal bovine serum (FBS) (Sigma), antibiotic-antimycotic solution (1X) (Sigma) and L-glutamine (2 mM) (Invitrogen GmbH, Karlsruhe, Germany). Cells growing in 75-cm<sup>2</sup> flasks were maintained in an incubator at 37°C and 5% CO<sub>2</sub>. The absence of contamination of mycoplasma was demonstrated by PCR assay.

### **5.2.2 Treatment of the cell lines with E2 or TAM**

In order to remove the serum's steroids and eliminate the weak estrogen agonistic activity of phenol red (162), cells were switched to phenol red-free RPMI 1640 (Sigma) containing 10% charcoal-stripped fetal bovine serum (FBS) (Sigma) 48 hours prior to the addition of E2 (E2758, Sigma) or TAM (T5648, Sigma). E2 and TAM were dissolved in absolute ethanol and diluted in the media at 0.01 $\mu$ M and 1 $\mu$ M respectively, then added to the culture medium for 24h, 48h and 96h. Cells without treatment were used as controls.

### **5.2.3 Reverse transcription and quantitative real-time PCR (qRT-PCR)**

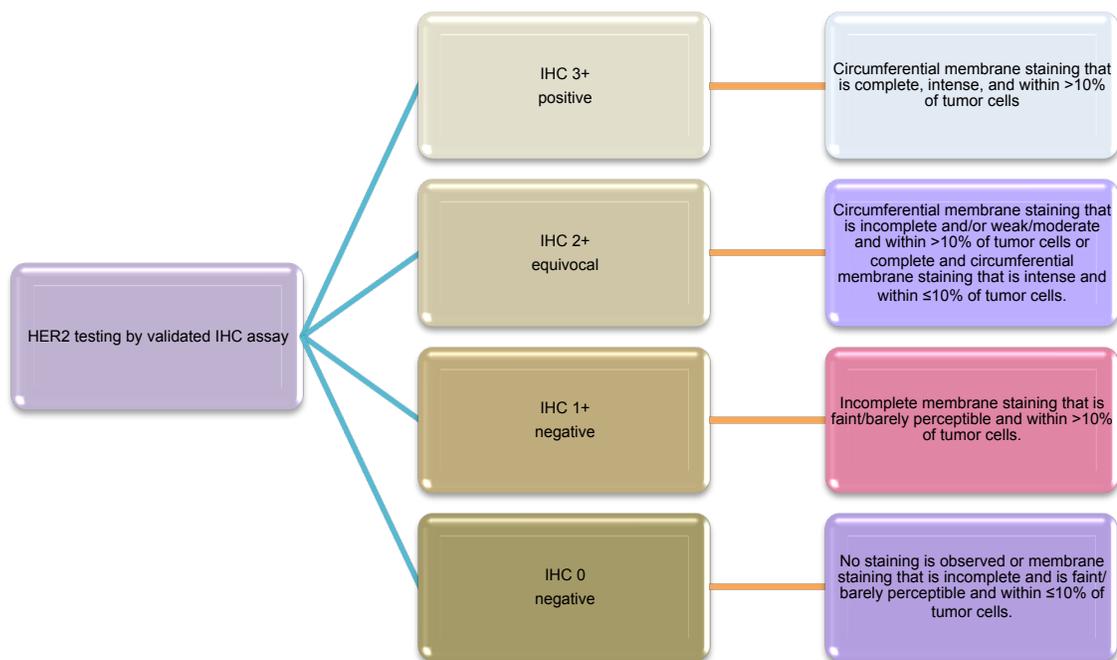
Transcript expressions of *HER2* were analyzed as a part of the Human Breast Cancer RT<sup>2</sup> Profiler PCR Array plate (SABiosciences<sup>TM</sup>-Qiagen). After synthesizing cDNA from 1.0  $\mu$ g total purified RNA using RT<sup>2</sup> First Strand Kit (SABiosciences<sup>TM</sup>-Qiagen), quantitative PCR was performed using RT<sup>2</sup> SYBR Green qPCR Master Mix (SABiosciences<sup>TM</sup>-Qiagen), and equal aliquots of this mixture (25  $\mu$ l) were added to each well of the Human Breast Cancer RT<sup>2</sup> Profiler

PCR Array plate (SABiosciences<sup>TM</sup>-Qiagen). This PCR array plate contained predisposed gene-specific primers for the *HER2* gene; 5 House keeping Genes: *GAPDH* (glyceraldehyde 3 phosphate dehydrogenase), *B2M* (beta 2 microglobulin), *ACTB* (Beta-Actin), *HPRT1* (Hypoxanthine phosphoribosyl transferase 1) and *RPL13A* (ribosomal protein L13A); 1 Genomic DNA Control (Human Genomic DNA Contamination-HGDC); 3 Reverse Transcription Controls (RTC); and 3 Positive PCR controls (PPC). Each sample was analyzed in triplicate using RNA samples from three independent experiments.

The threshold cycle (Ct) values for the *HER2* gene was used to calculate the fold-change in gene expression, using the PCR array data analysis software (SABiosciences<sup>TM</sup>-Qiagen), which is based in the  $2^{-\Delta\Delta C_t}$  method. The *HER2* gene was identified as significantly regulated by E2 or TAM treatments when fold-change was at least  $\pm 2.0$ ; ensuring with this cut-off, we only analyzed highly modified genes.

#### **5.2.4 Immunohistochemistry assays (IHC)**

Immunohistochemistry detection for HER2 were carried out on MCF7, T47D, BT474 and SKBR3 control cells and treated with 10 nM of E2 and 1 $\mu$ M of TAM. Following treatment with E2 and TAM at the indicated time points, cells were fixed in formalin and IHC analyses were performed using the rabbit polyclonal antibody c-erbB2 (A0485, DAKO) and anti-rabbit polyclonal antibody (Dako K403). The reading of the immunohistochemistry tests was performed automatically using the equipment APERIO and subsequently confirmed by expert pathologists applying the algorithm to HER2 (IHC membrane algorithm) (153) (Figure 40)



**Figure 40.** Algorithm for evaluation of human epidermal growth factor receptor 2 (HER2) protein expression by immunohistochemistry (IHC) assay of the invasive component of a breast cancer specimen.

### 5.2.5 Western Blot

Western blot analysis for HER2 protein was performed on MCF7, T47D, BT474 and SKBR3 control cells and treated with 10 nM of E2 or 1 $\mu$ M of TAM. Following treatment with E2 and TAM at the indicated time points, cells were disrupted in a lysis buffer (all components from Sigma, St. Louis, MO), and Western Blot analyses were performed. Briefly, cell lysates were clarified by centrifugation for 15 min at 15,000  $\times$  g. Then protein concentration was measured. From each cell line, 25 - 100  $\mu$ g of protein were loaded on 8% acrylamide/sodium dodecyl sulfate gels. Electrophoresis was performed in TGS buffer; after separation, proteins were transferred to a nitrocellulose membrane. After saturation with TBS and 5% milk, the membrane was immunoblotted either with anti-HER2 (A0485, rabbit polyclonal antibody Dako, Glostrup, Denmark) at 1:1000 or with anti-actin (ACTN05 (C4), mouse monoclonal antibody Abcam, Cambridge, USA) at 1:800 as a loading control. Both antibodies were diluted in TBS with 5% BSA and incubated overnight at 4°C. The membrane was washed and incubated with an anti-rabbit or anti-mouse peroxidase-labelled secondary antibodies (Amersham Pharmacia, Piscataway, NJ). Finally, the membrane was developed with ECL luminol (Bio-Rad, Hercules, CA, USA). Autoradiographs of the blots were quantitated by

densitometry using ImageJ software, which is freely available (<http://rsb.info.nih.gov/ij/>). Mean band intensity was measured and fold change from actin control was calculated. Western blot assays were performed in triplicate using samples from three independent experiments.

### **5.2.6 Statistical analysis**

All data are expressed as mean standard error of three separate experiments. The statistical significance of differences between two groups was analyzed by Student's *t* tests. Differences with *P* values of <0.05 were considered statistically significant.

## **5.3 RESULTS**

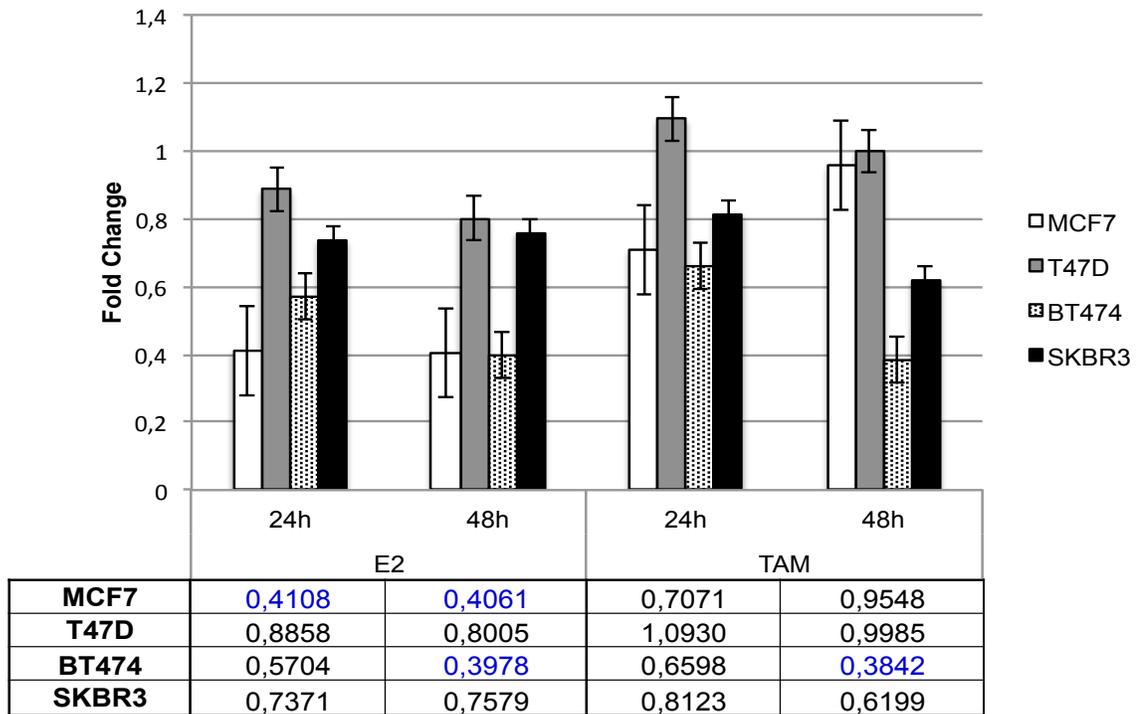
### **5.3.1 Effect of E2 or TAM on HER2 mRNA levels in four-breast cancer cell lines**

In order to test whether E2 or TAM stimulation resulted in modulation of *HER2* mRNA levels, three well characterized ER+ and one ER- human breast cancer cell lines were selected. Each cell line was treated with E2 for 24h and 48h and *HER2* mRNA levels in these cells were then measured using the Human Breast Cancer RT<sup>2</sup> Profiler qPCR Array (SABiosciences<sup>TM</sup>–Qiagen).

The addition of 10 nM of E2 decreased *HER2* mRNA levels in the hormone-dependent, ER+ breast cancer cell line MCF7 (Figure 41); however, this reduction was not statistically significant. This effect was inhibited when the cells were treatment with TAM. In contrast, *HER2* mRNA levels were not altered in T47D (ER+/HER2-) and SKBR3 (ER-/HER2+) cells after the addition of E2. For BT474 cells (ER+/HER2+), the addition of E2 and TAM decreased HER2 mRNA levels at 48h of treatment, which was not significant (Figure 41).

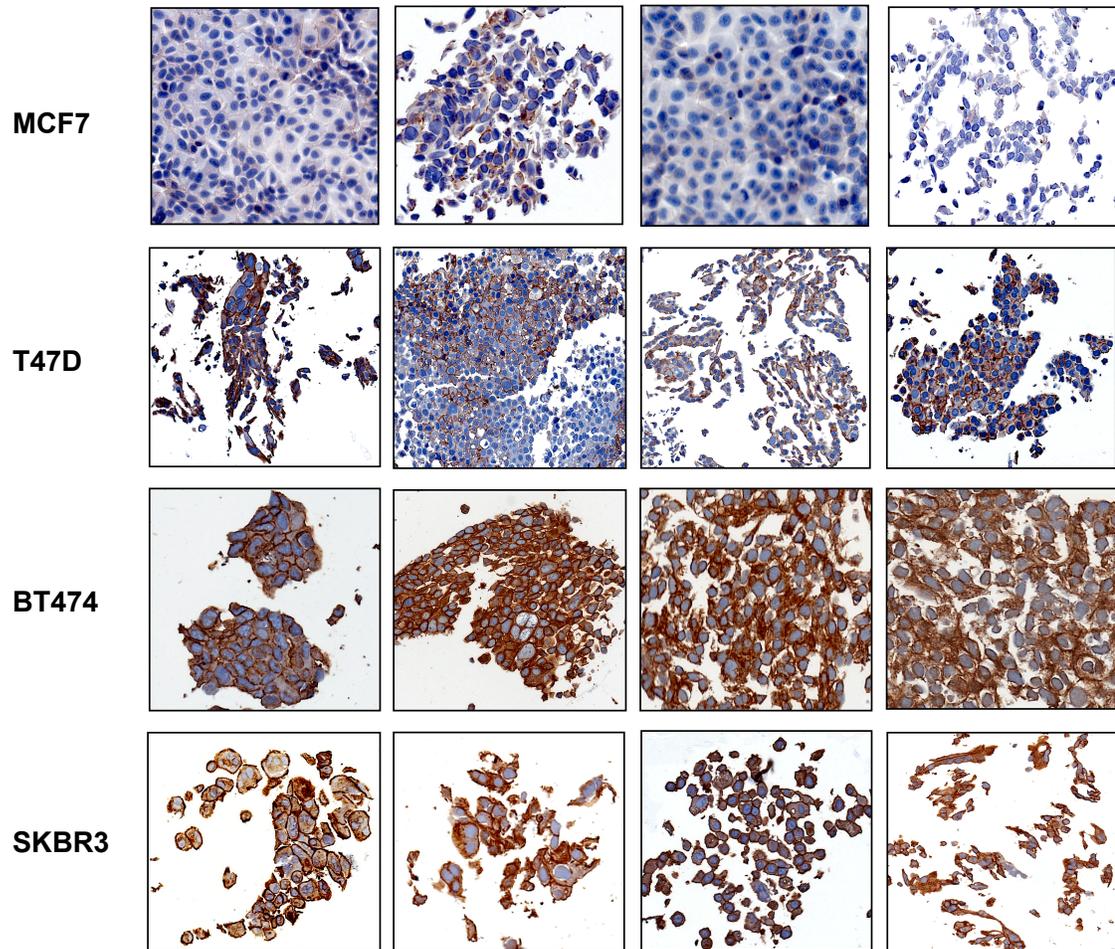
### **5.3.2 Addition of E2 or TAM do not cause alteration in HER2 protein levels in breast cancer cell lines**

The HER2 protein levels as assessed by IHC (Figures 42 and 43) and western blot tests (Figure 44) did not exhibit changes after the addition of E2 or TAM in the four cell lines analyzed. The expression of HER2 was comparable between all cell lines treated and those no treated.



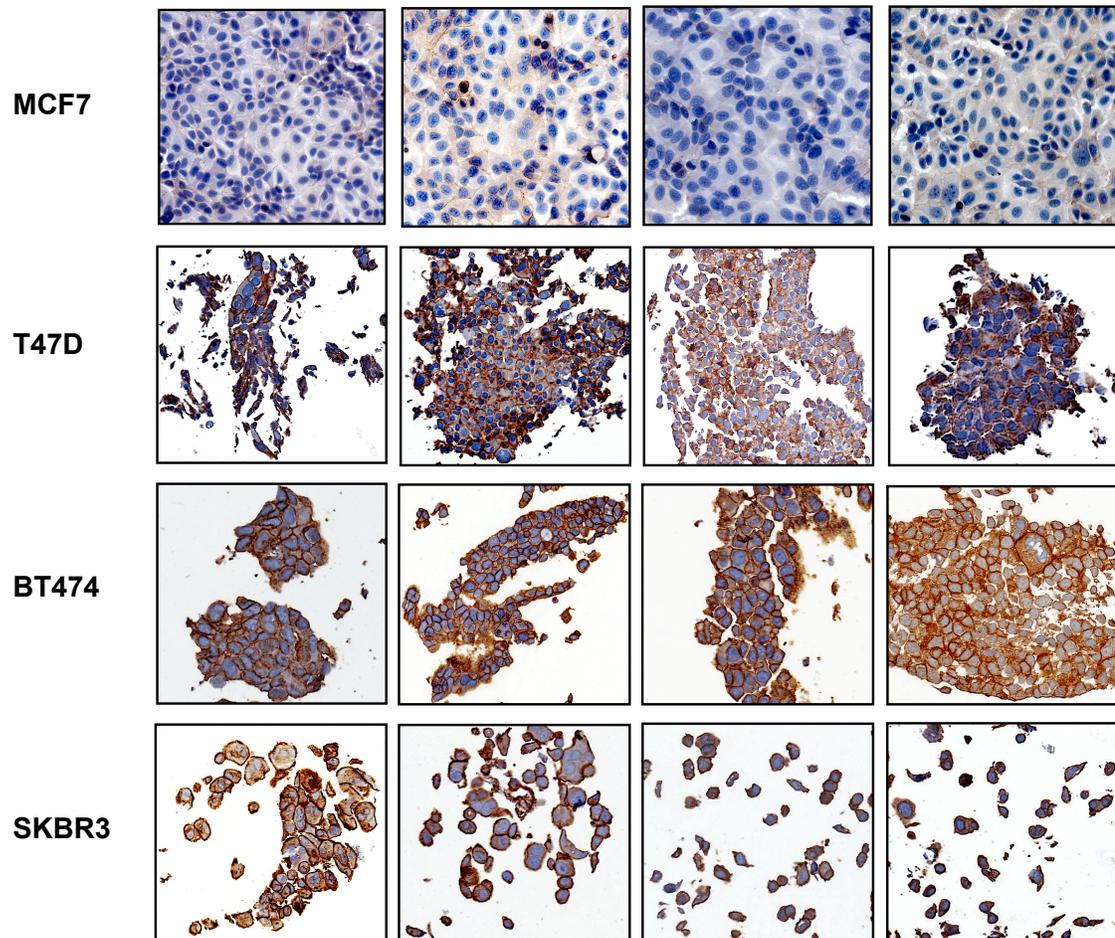
**Figure 41. Expression levels of *HER2* in MCF7, T47D, BT474 and SKBR3 after 24h and 48h of E2 or TAM treatment evaluated by qPCR.** Error bars represent mean standard error of 3 separate experiments. Data was normalized to control cells and 5 house-keeping genes.

CELL LINE	IHC Results: HER2 Score			
	CONTROL	E2. 24h	E2. 48h	E2. 96h
MCF7	1	1	1	1
T47D	2	2	2	2
BT474	3	3	3	3
SKBR3	3	3	3	3

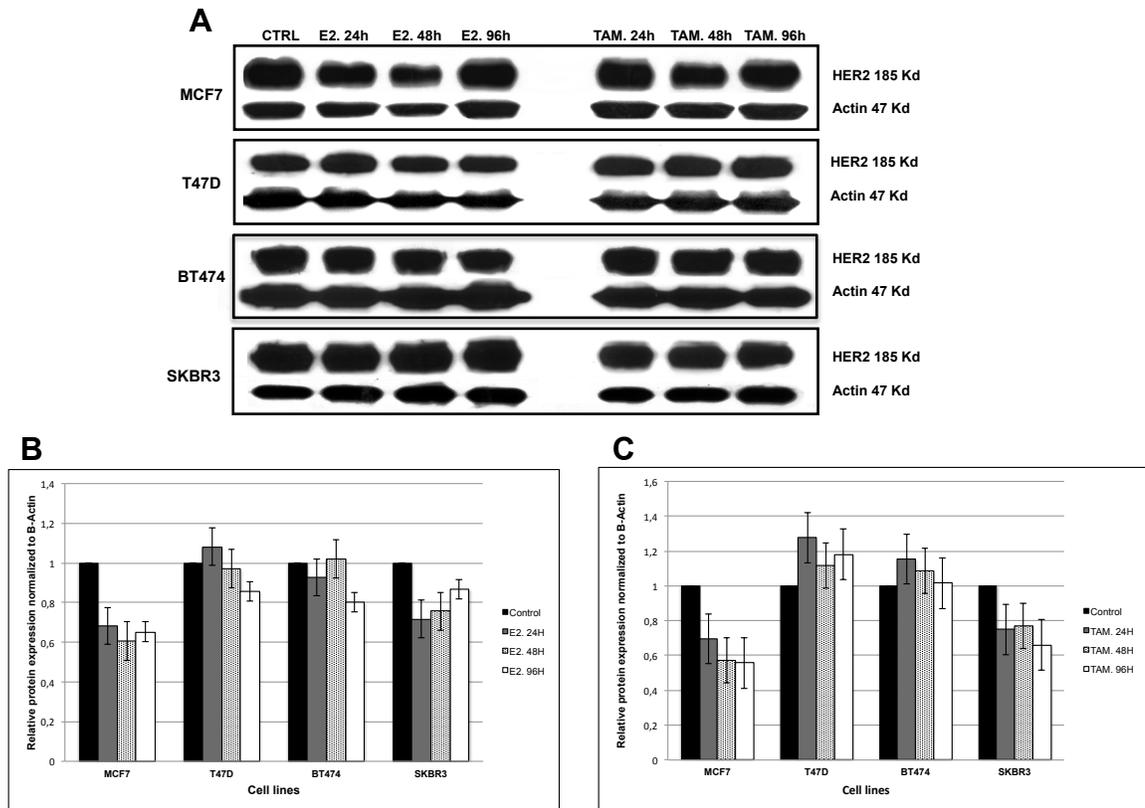


**Figure 42. Effect of E2 on the HER2 protein levels of MCF7, T47D, BT474 and SKBR3 cells.** Cells were exposed to 10 nM of E2 for 24h, 48h and 96h. IHC assays were done at each time point of E2 treatment. The HER2 score was performed automatically using the equipment APERIO and confirmed by expert pathologists applying the algorithm to HER2 (IHC membrane algorithm). All pictures were taken at 40x magnification

CELL LINE	IHC Results: HER2 Score			
	CONTROL	TAM. 24h	TAM. 48h	TAM. 96h
MCF7	1	1	1	1
T47D	2	2	2	2
BT474	3	3	3	3
SKBR3	3	3	3	3



**Figure 43. Effect of TAM on the HER2 protein levels of MCF7, T47D, BT474 and SKBR3 cells.** Cells were exposed to 1 $\mu$ M of TAM for 24h, 48h and 96h. IHC assays were done at each time point of TAM treatment. The HER2 score was performed automatically using the equipment APERIO and confirmed by expert pathologists applying the algorithm to HER2 (IHC membrane algorithm). All pictures were taken at 40x magnification.



**Figure 44. Effects of E2 or TAM on HER2 protein expression.** Immunoblotting (A) from four cell lines, both from the control group and the group treated with 10nM of E2 and 1 $\mu$ M of TAM, was performed for HER2 (*top*) and B-Actin (*bottom*) expression using a rabbit c-erbB2 polyclonal antibody as previously described. HER2 and B-Actin quantification for E2 (B) and TAM (C) was performed by densitometry using ImageJ software. Error bars represent mean standard error of 3 separate experiments. E2 and TAM did not cause alteration in HER2 protein expression at the concentrations used.

## 5.4 DISCUSSION

We have evaluated mRNA and protein HER2 levels on MCF7, T47D, BT474 and SKBR3 breast cancer cell lines after treatments with E2 or TAM and no treated cells. Neither E2 nor TAM had a significant effect on HER2 mRNA or protein levels in these cells over 24h, 48h and 96h of treatment. The changes observed at the level of messenger HER2 were very low and not significant, which did not lead to alteration in the amount of protein expressed after treatments.

We confirmed the results of previous studies by showing that treatment with E2 was able to diminish the HER2 mRNA levels in MCF7 (ER+) but not in T47D (ER+) and SKBR3 (ER-) human breast cancer cell lines. Although our results agreed with those reported by others (235), the observed negative regulation in MCF7 cells was not significant and had no effect on the HER2 protein levels. This is probably due to that *HER2* may be a early response gene and the changes in their expression levels, after treatment with E2 or TAM, can be noticed at earlier times that we used. Indeed previous studies have reported that the reduction of HER2 mRNA level is appreciable within 4-6 hours after E2 treatment (13). On the other hand, the fact that we did not observe any significant effect of E2 on HER2 protein levels in MCF7 cells, despite the observed decrease in mRNA levels, could simply be the result of this decrease having been too small to affect protein levels.

It is interesting to note that the human breast cancer cell line BT474, which is ER+/HER2+, showed similar effects after 48h of E2 or TAM addition: a small, non significant decrease in the HER2 mRNA level was noted. This result suggests that TAM could have estrogen agonist activity, which was enhanced by the presence of ER and HER2 receptors, as it has been postulated for ER+/HER2+ cells (47, 48, 51, 236). The fact that E2 caused a decrease (not significant) in the HER2 mRNA levels in MCF7 (ER+) and BT474 (ER+) cells but not in T47D cells (ER+) could indicate that the effect of E2 on ER+ cells depends of the amount of ER present in these cells. This ER-dependent process of HER2 down-regulation may additionally explain why there was no effect elicited by E2 in T47D cells, which contain very low ER, as has been reported by Linnea D. Read, et al. 1990 (235).

## 5.5 CONCLUSIONS

In summary, these results could indicate that neither E2 nor TAM modulate the mRNA and protein *HER2* levels in these breast cancer cell lines, and may suggest that aggressiveness associated with high levels of HER2 mRNA and protein may be caused by increased cross talk between ERs and signaling pathways mediate by EGFR/HER2, which contributes to increased cell proliferation.