4. CHROMOSOMAL ABNORMALITIES INDUCED BY LOW DOSES OF 17β-ESTRADIOL (E2) AND TAMOXIFEN (TAM) IN BREAST CANCER CELL LINES

4.1 INTRODUCTION

17β-estradiol (E2) is the main estrogenic hormone that acts on mammary epithelial cells; it plays an important regulatory role in a wide variety of biological processes including differentiation, cell proliferation and breast development at puberty and during sexual maturity. More evidence supports the role of E2 as a procarcinogenic agent, which induces genetic damage at a high dose (2, 3, 7-9, 56) thus producing genomic instability prior to inducing alterations such as chromosomal numerical alterations (aneuploidies), gene amplification (*c-MYC*) and chromosomal structural aberrations in animal models (Syriam hamster, mouse genital tract) (2, 7-9, 56).

Tamoxifen (TAM) is a non-steroidal anti-oestrogen with partial agonistic activity, extensively used in the treatment of estrogen receptor (ERα) positive breast cancer. ERα-positivity is a well-established predictor of a good response to TAM treatment, whereas ERα-negative tumors are considered no responders, although 5-10% of these are known to benefit from adjuvant TAM (158-161). Response to TAM is frequently limited in duration because patients can development resistance (36, 76).

Paradoxically, it has been reported that TAM possesses a high mutagenic potential, because it can cause chromosomic ruptures, which later leads to translocations and deletions of chromosomal fragments in animal models (16, 82, 83). However, type and frequency of chromosomal abnormalities induced by TAM are poorly studied (16, 82, 83). Additionally, cytogenetic studies about the effects of TAM at low doses, as it is suggested for treatment of pre-invasive low-grade breast lesions (e.g. low grade in situ ductal carcinomas or lobular intraepithelial neoplasia), are limited (83).

The chromosomal damage induced by E2 or TAM has been explored in model cell lines exposed to high concentrations of these agents, and some numerical aberrations and chromosomal breaks have been found (10, 16, 83, 97). Therefore our goal was to determine if low doses of E2 and TAM would induce chromosomal aberrations and modify the cell proliferation in the human breast cancer cell lines MCF7 and T47D (ER+/HER2-), BT474 (ER+/HER2+) and SKBR3 (ER-/HER2+). Our results demonstrated that all cell lines treated with E2 and TAM at low dose possess a more complex karyotype than control cells, displaying numerical changes (endoreduplications, aneuploidy, polyploidy) as well as additional and

more complex chromosomal rearrangements suggesting karyotypic evolution of certain chromosomal aberrations.

4.2 METHODS

4.2.1 Cell Lines

The human breast cancer cell lines MCF7, T47D, BT474 and SKBR3 were obtained from the American Type Culture Collection (ATCC, Manassas, USA) in March 2010. The MCF7, T47D, SKBR3 cells were cultured in a RPMI 1640 medium (Sigma, St. Louis, MO, USA), while BT474 was cultured in a 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, Karslruhe, Germany). Cells growing in 75-cm^2 flasks were maintained in an incubator at 37ºC and 5% CO2. The absence of mycoplasma contamination was tested by PCR assay.

4.2.2 Treatment of the cell lines with E2 and TAM

In order to remove endogenous serum steroids and eliminate the weak estrogen agonistic activity of phenol red (162), 48 hours prior to the addition to E2 (E2758, Sigma) or TAM (T5648, Sigma) cells were washed with 5 ml phosphate buffered saline (PBS) and then switched to phenol red-free RPMI 1640 (Sigma) containing 10% charcoal-stripped fetal bovine serum (FBS) (Sigma). E2 and TAM were dissolved in absolute ethanol and diluted in the media at 0.01μM (10nM) and 1μM respectively, and then added to the culture medium for 24h, 48h and 96h. Cells without treatment were used as controls.

4.2.3 Proliferation assay- colorimetric assay, BrdU

Cells were seeded at a density of 2.5-5x 10^3 cells per 100 μl of medium, in a 96 multi-well plate. After 24h, cells were treated with E2 (0.01 μM) or TAM (1 μM) for 24h, 48h and 96h; cells without treatment were the controls. At the end of each treatment, cell viability was assessed using the cell proliferation ELISA kit, BrdU (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Measurement of absorbance was performed by using a MultiSkan Bichromatic reader (Labsystems) against a blank background control. Experiments were performed in 24 replicates and expressed as a standard deviation.

4.2.4 Metaphase spread and G-Banding

To determine whether the E2 and TAM treatments resulted in the induction of chromosomal abnormalities, we performed conventional and molecular cytogenetic analyses in parallel with the evaluation of cell proliferation. Metaphases were obtained by standardized harvesting methods in order to perform conventional and molecular cytogenetic analyses (M-FISH and FISH). Briefly, colcemid solution (0.03 μg/ml) (Sigma) was added to cultures 2.5 hours before cell harvesting; cells were then treated with hypotonic solution, fixed three times with Carnoy's fixative (3:1 methanol to acetic acid) and spread on glass slides. For analysis of chromosomal alterations, the slides were G-banded. Glass slides were baked at 70°C for 24h, incubated in HCl and placed in 2xSSC buffer before treatment with Wright's stain. Metaphase image acquisitions and subsequent karyotyping were performed using a Nikon microscope with the cytogenetic software CytoVision System (Applied Imaging, Santa Clara, CA). Between 11 and 26 metaphases with good dispersion and morphology were analyzed for each treatment.
With the aim of determining the level of ploidy, 100 metaphases were analyzed for both control and treatment cells. Chromosomal aberrations were described according to the International System of Human Cytogenetic Nomenclature (ISCN 2013) (122)

4.2.5 Molecular Cytogenetic Analysis - Multi color FISH (M-FISH)

M-FISH was performed to identify complex chromosomal rearrangements unidentified by conventional cytogenetic in both control and treated cell lines. The probe cocktail containing 24 differently labeled chromosome-specific painting probes (24xCyte kit MetaSystems, Altlussheim, Germany) was denatured and later hybridized to denatured tumor metaphase chromosomes according to the protocol recommended by the Human Multicolor FISH kit (MetaSystems). The slides were incubated at 70ºC in saline solution (2xSSC), denatured in NaOH, dehydrated in ethanol series, air-dried, covered with 10 μl of probe cocktail (denatured) and hybridized for two days at 37ºC. Then, the slides were washed with post-hybridization buffers, dehydrated in ethanol solution series and counter-stained with 10μl of DAPI/antifade. Signal detection and subsequent metaphase analysis were done using the Metafer system and Metasytems' ISIS software (software for spectral karyotypes).

4.2.6 Molecular Cytogenetic Analysis - Fluorescence *in situ* hybridization (FISH)

FISH experiments were conducted to define *HER2* gene status on the control, E2 and TAM treated cell lines. Two commercial mix probes - *HER2* (17q11.1-q12)/*CEP17* and Smith-Magenis locus (*SMS*) (17p11.2)/ retinoic acid receptor alpha (*RARA*) (17q21.2) (Abbott Molecular. Illinois, USA) - were used separately

on each cell line (control and treated) in order to investigate *HER2* gene status on metaphases and nuclei. The use of these probes has been proposed as alternative methods to define accurately *HER2* gene status (102, 103, 163-165)

FISH was performed on recently spread slides from methanol acetic acid fixed cells according to the manufacturers' instructions. The slides were washed at 37ºC in 2x saline-sodium citrate buffer (SSC), dehydrated in ethanol series, air-dried, covered with 10 µl of probe, co-denatured in HYBrite System at 70ºC for 5 min and hybridized overnight at 37ºC. Afterwards, the slides were washed with post-hybridization buffer (2xSSC/0.3% Nonidet P-40), dehydrated in ethanol series and counter-stained with 10 ul DAPI/antifade. Metaphases and nuclei were selected with a AxioImager Z1 epifluorescence microscope (Carl Zeiss, Germany) and automated acquisition was performed with the motorized Metafer Scanning System (MetaSystems). Analysis of the signal pattern on nuclei and metaphases was performed on screen with ISIS software.

4.2.7 Data Analysis

The profile of numerical and structural chromosomal changes was determined relative to the control. The Student's t-test was performed to compare cell proliferation between treated and untreated cell lines. Fisher's exact test was applied to compare conventional and molecular cytogenetic results from treated cell lines with results from control cell lines. Confidence limits of 95% (*$p < 0.05$. **$p < 0.01$) were applied. All statistical analyses were performed using the statistical program SPSS v.15

4.3 RESULTS

4.3.1 Effects of E2 and TAM on cell proliferation

In MCF7 and T47D cells (both ER+/HER2-), E2 significantly increased the number of cells ($p<0,0001$, Fisher's exact test) whereas TAM significantly reduced them ($p<0,01$, Fisher's exact test) at all incubation times when compared to the control cells. Moreover, T47D with E2 treatment showed, at 96h, a little higher growth than MCF7 cells (Tables 13A and 13B, Figures 26 and 27). On the contrary, the growth inhibition of T47D by TAM was much lower than that observed in MCF7.

Otherwise, both E2 and TAM increased the BT474 cell proliferation (ER+/HER2+) ($p<0,0001$, Fisher's exact test) at 24h (Table 13C). At 48h they reduced the cell proliferation, and at 96h a rapid increase was observed as compared to control cells (Table 13C, Figure 28). Finally, in SKBR3 (ER-/HER2+) cells, E2 and TAM treatment significantly increased cell growth only at 96h ($p<0,006$ and $p<0,024$,

Fisher's exact test) (Table 13D, Figure 29).

Table 13. Student's t-test results of the proliferation assays for MCF7 (A), T47D (B), BT474 (C) and SKBR3 (D) cell lines control and treated with E2 or TAM at 24h, 48h and 96h.

A) MCF7

Time	Control	E2. 0,01μM	p	TAM 1 μM	p
0h	2500	2500	1	2500	1
24h	13130	17675	0,0001**	10060	0,0001**
48h	15150	18975	0,0001**	11495	0,0001**
96h	17460	21605	0,0001**	12820	0,0001**

Student's t-tests were performed for determination of the significant difference, *p < 0.05. **p < 0.01

B) T47D

Time	Control	E2. 0,01μM	p	TAM 1 μM	p
0h	2500	2500	1	2500	1
24h	14715	17005	0,0001**	14225	0,005**
48h	15205	18645	0,0001**	14570	0,017**
96h	16075	21650	0,0001**	15955	0,508

Student's t-tests were performed for determination of the significant difference, *p < 0.05. **p < 0.01

C) BT474

Time	Control	E2. 0,01μM	p	TAM 1 μM	p
0h	5000	5000	1	5000	1
24h	34050	52100	0,0001**	43950	0,0001**
48h	36100	49850	0,0001**	36692	0,636
96h	40466	56500	0,0001**	43850	0,012*

Student's t-tests were performed for determination of the significant difference, *p < 0.05. **p < 0.01

D) SKBR3

Time	Control	E2. 0,01μM	p	TAM 1 μM	p
0h	2500	2500	1	2500	1
24h	4502	4492	0,946	4784	0,165
48h	8052	8427	0,193	7663	0,116
96h	10733	11427	0,006**	11347	0,024*

Student's t-tests were performed for determination of the significant difference, *p < 0.05. **p < 0.01

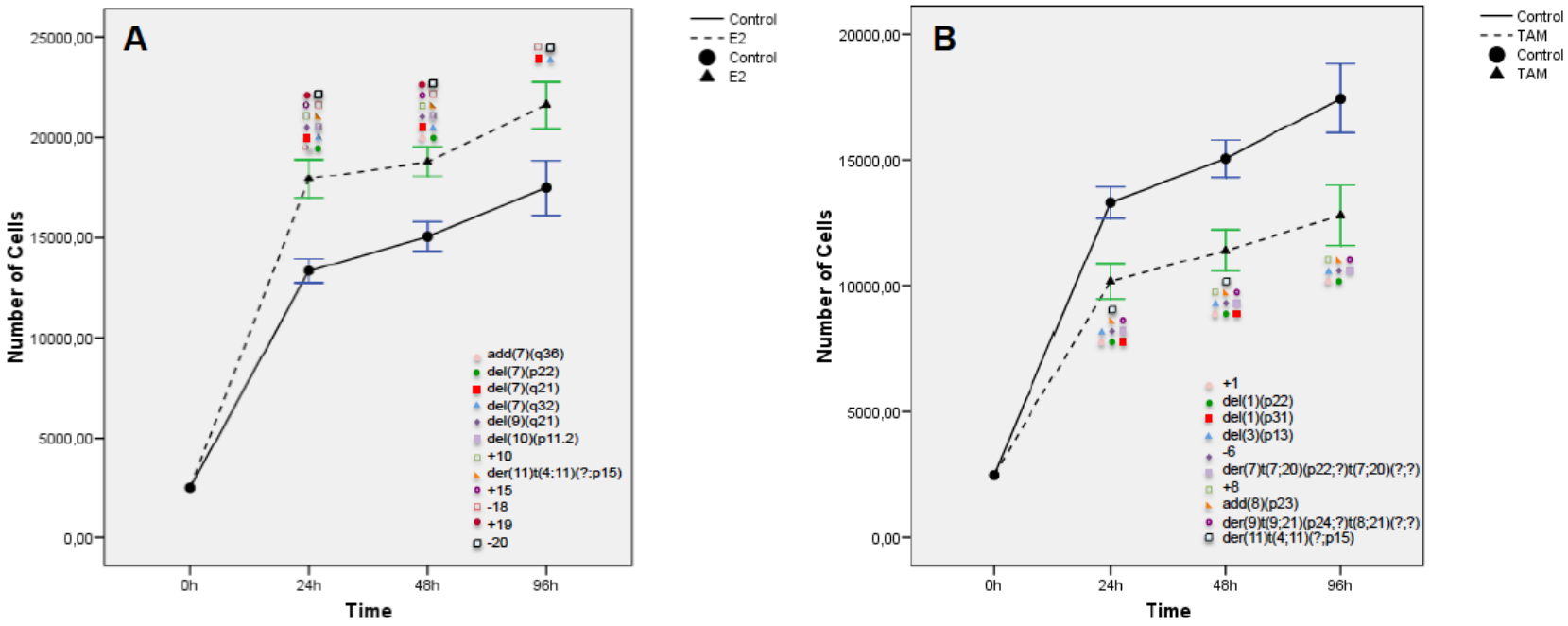


Figure 26. **Effects of E2 (A) and TAM (B) treatment for 24h, 48h and 96h on cell proliferation and chromosomal alterations observed in MCF7 cells**. Number of cells and chromosomal abnormalities are indicated at each time point of E2 and TAM treatment. Error bars represent mean standard error of 24 separate experiments.

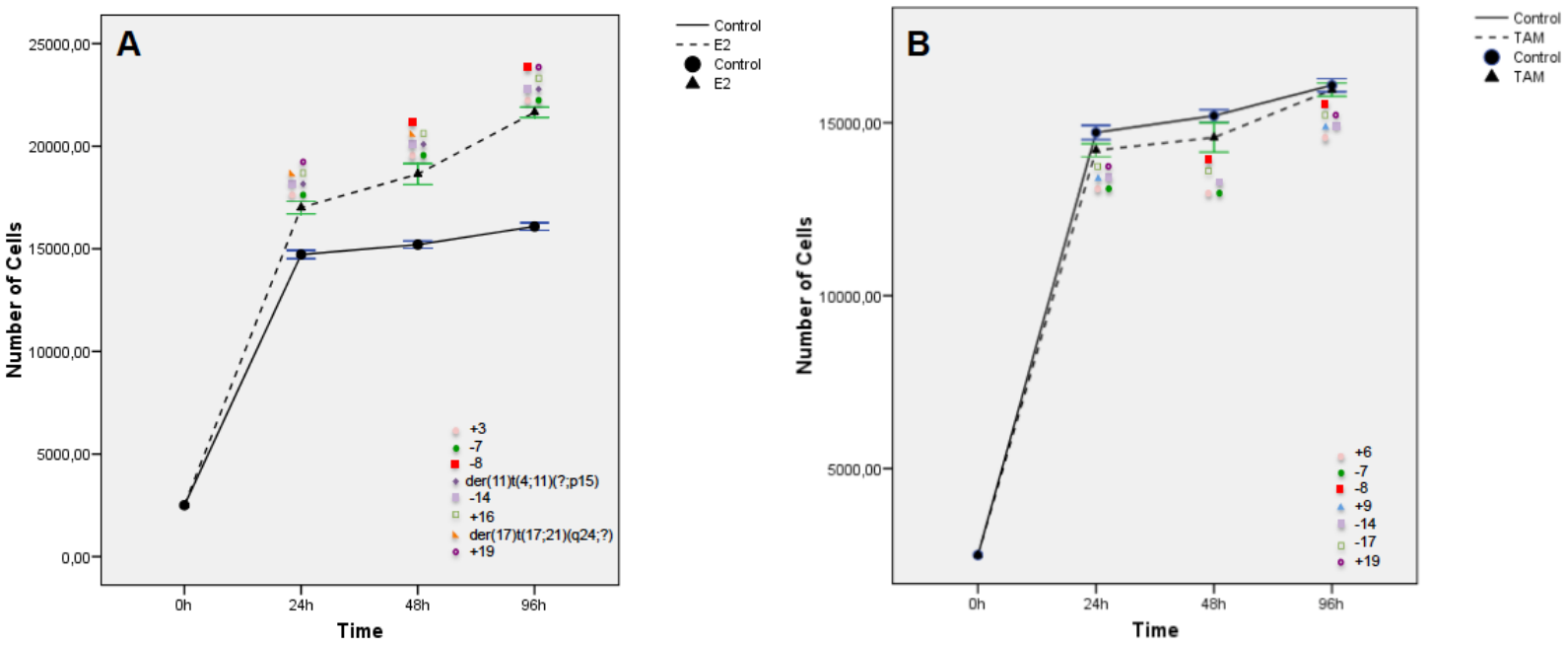


Figure 27. Effects of E2 (A) and TAM (B) treatment at 24h, 48h and 96h on cell proliferation and chromosomal alterations observed in T47D cells. Number of cells and chromosomal abnormalities are indicated at each time point of E2 and TAM treatment. Error bars represent mean standard error of 24 separate experiments.

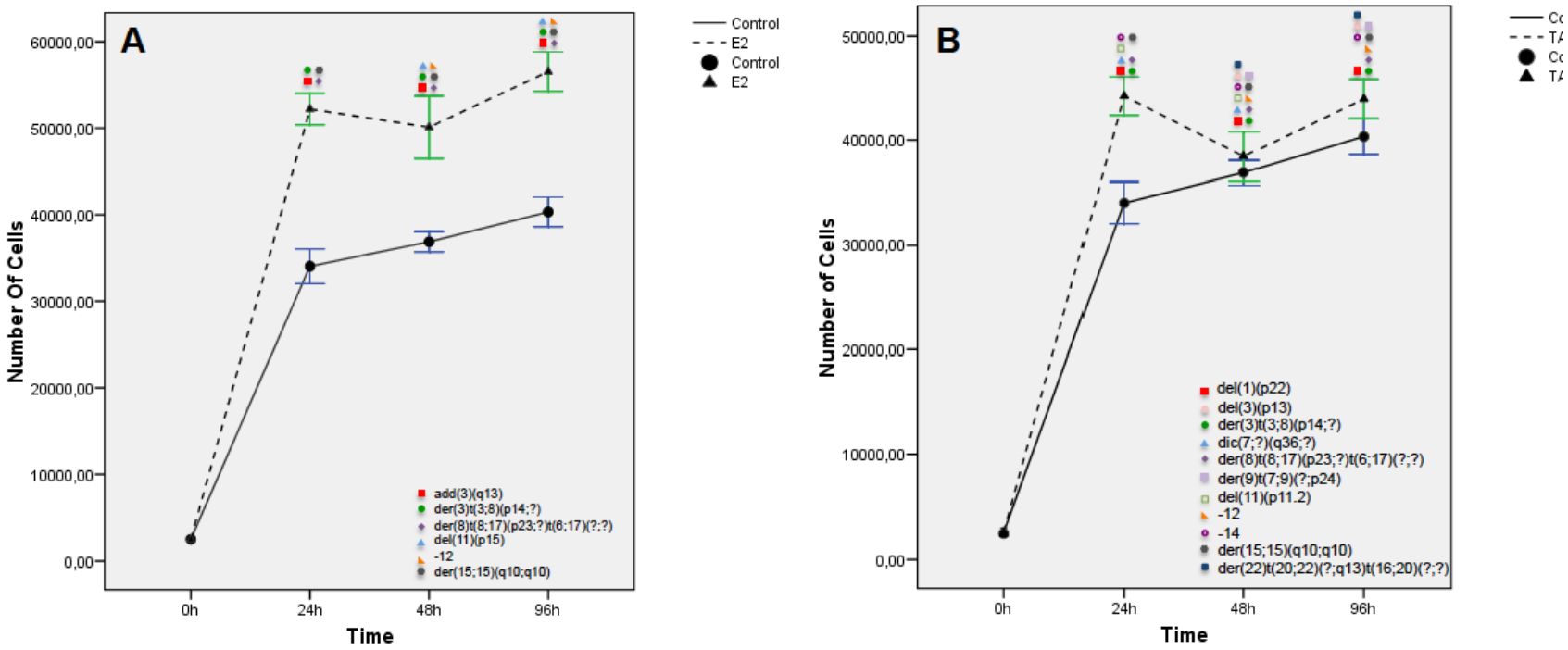


Figure 28. Effects of E2 (A) and TAM (B) treatment for 24h, 48h and 96h on cell proliferation and chromosomal alterations observed in BT474 cells. Number of cells and chromosomal abnormalities are indicated at each time point of E2 and TAM treatment. Error bars represent mean standard error of 24 separate experiments.

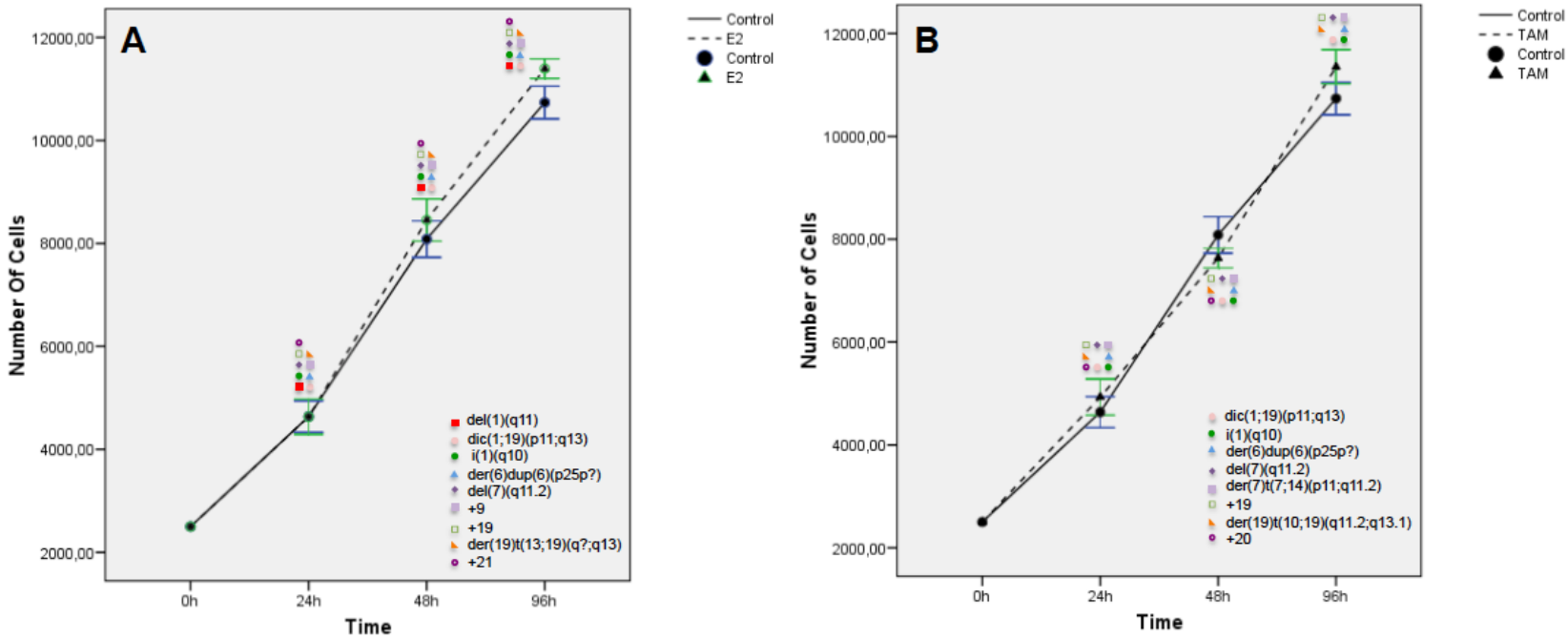


Figure 29. Effects of E2 (A) and TAM (B) treatment for 24h, 48h and 96h on cell proliferation and chromosomal alterations observed in SKBR3 cells. Number of cells and chromosomal abnormalities are indicated at each time point of E2 and TAM treatment. Error bars represent mean standard error of 24 separate experiments.

4.3.2 Effects of E2 and TAM on ploidy

After E2 and TAM treatments, numerical changes were investigated to obtain direct evidence of inducing changes of ploidy in all cell lines. For instance, after E2 treatment at each time point, the number of cells with a sets of chromosomes greater than 4n in all cells lines increased. However, it was greater in SKBR3 cell line (Table 14A). Similarly, results were found for TAM treatment in HER2+ cells (BT474, SKBR3), but in SKBR3 (ER-) this effect was higher (Table 14B).

Table 14. Percentage of cells with polyploidy in MCF7, T47D, BT474 and SKBR3 cell lines. A) Control and E2 treated. B) Control and TAM treated. 100 metaphases were analyzed for both the control and for each one of the treatments with E2 and TAM.

A)

Treatments	MCF7		T47D		BT474		SKBR3	
	4n	>4n	3n	>3n	4n	>4n	4n	>4n
CONTROL	98	2	96	4	100	0	81	19
E2. 24h	85	15	87	13	88	12	63	37
E2. 48h	80	20	78	22	77	23	52	48
E2. 96h	61	39	67	33	70	30	50	50

B)

Treatments	MCF7		T47D		BT474		SKBR3	
	4n	>4n	3n	>3n	4n	>4n	4n	>4n
CONTROL	98	2	96	4	100	0	81	19
TAM. 24h	97	3	85	15	94	6	24	76
TAM. 48h	99	1	98	2	98	2	24	76
TAM. 96h	99	1	100	0	84	16	30	70

4.3.3 E2 and TAM induced numerical and structural chromosomal abnormalities in the four cell lines studied

G-Banding and M-FISH results showed that all cell lines (positives and negatives for ER) treated with E2 or TAM at low dose (0,01μM and 1μM respectively) possessed a more rearranged karyotype than corresponding control cells, which displayed numerical changes (endoreduplications, aneuploidy, polyploidy) as well as new complex chromosomal rearrangements. Our analysis provides strong evidence about karyotypic evolution being driven by certain chromosomal aberrations. G-banding and M-FISH karyotypes are described in detail in Table 15, and the number of metaphases analyzed is also indicated. The stability of the karyotypes of all cell lines analyzed was evaluated by comparing karyotypes between control cell lines at 24h, 48h and 96h which showed identical karyotypes. No additional chromosomal aberrations were detected in control cell lines.

Table 15. **G-Banding and M-FISH composite karyotype from MCF7, T47D, BT474 and SKBR3, control and treated with E2 and TAM**. The number of metaphases analyzed is reported in brackets at the end of each karyotype. Also, the frequency of each additional rearrangement identified is described in brackets.

Treatments	Karyotype	Additional rearrangements
MCF7. Control	76~88<4n>,-X[11],-Xx2[8],-Xx3[4],der(X)t(X;15)(p11.2;q21)[16], der(X)t(X;15)(p11.2;q21)x2[3],der(X)dup(X)(q21qter)[5],-1[22]-1x2[2],der(1)t(1;21)t(9;21)[22],-2[13],-2x2[2],der(2)t(2;3)(q34;?)[19],-3[2],+3[17],del(3)(p14)[22],der(3)t(3;11)(p14;q13)[3],-4[12],-4x2[4], +5[2],-5[13],+6[9],+6x2[8],+6x3[4],add(6)(q27)[2],del(6)(q25)[4],del(6)(q25)x2[8], der(6)t(6;17;16)(q25;q21;?)[26],+7[26],der(7)t(1;7)(?;p15)[23],der(7)t(1;7)(?;p15)x2[2], del(7)(q11.2)[4],dup(7)(p13p15)[7],dup(7)(p13p15)x2[5],dup(7)(p13p15)x3[11],dup(7)(p14p15)[5],dup(7)(p14p15)x2[2],der(7)t(7;7)(p15;?)[19], der(7)t(7;7)(p15;?)[2],-8[8],-8x2[12], der(8)t(8;15)(p11;?)[26], +9[3]-9[7],-9x2[2],der(9)t(8;9)(q13;p22)[22],-10[6],-10x2[10],-10x3[3], der(10)t(7;10)(?;p14)[9] ,der(10)t(7;10)(?;p14)x2[12],-11[14],-11x2[12],del(11)(q23)[2],-12[15], -12x2[4],+12[2],del(12)(p11.2)(5),del(12)(q24)[11],der(12)t(8,12)(q11;p11)[15],-13[12],-13x2[10], -13x3[2],-14[3],+14[14],-15[12],-15x2[10],-15x3[3],-16[3],+16[16], der(16)t(8;16)(q?;q11.2)[8],der(16)t(8;16)(q?;q11.2)x2[17]der(16)t(16;19)(q21;?)[2] ,+17[11],+17x2[10],+17x3[5],der(17)t(8;17)t(1;8)[21],der(17)t(8;17)t(1;8)x2[5],der(17)t(17;19)(p11.1;p12)x2[17],-18[4],-18x2[14],-18x3[5],-18x4[3],-19[7],-19x2[15],-19x3[4], der(19)t(12;19)(q13;p13.3)[21],der(19)t(12;19)(q13;p13.3)x2[2],-20[2],-20x2[5],-20x3[11],-20x4[8], der(20)t(7;20)t(1;7)t(1;7)[21],+21[5],+21x2[2],-21[14],-21x2[2],+22[12],+22x2[3],-22[3],-22x2[2], add(22)(q13)[4],+1~3 mar[17][cp26]	
MCF7. E2. 24h.		+1,del(1)(p13),+2,del(3)(p21),+4, del(6)(q13), del(6)(q22),add(7)(q36), del(7)(p22),del(7)(q32),del(7)(q21),+8,del(9)(q21),+10, del(10)(p11.2),+11,der(11)t(4;11)(?;p15),add(12)(p13),del(12)(p13), +13,+15,+19
MCF7. E2. 48h.		der(7)del(7)(p21)dup(7)(q31q36),del(7)(p22), del(9)(p21),+10,add(10)(p14),del(10)(p11.2), der(11)t(4;11)(?;p15),+15,+19
MCF-7.E2. 96h.		add(7)(q36),del(7)(q32),del(7)(q21), add(10)(p14)
MCF-7. TAM. 24h.		+1,del(1)(p31),del(1)(p21),+2,del(3)(p13),-6,del(6)(q22),-7, der(7)t(7;20)(p22;?)t(7;20)(?;?),add(8)(p23), der(9)t(9;21)(p24;?)t(8;21)(?;?),add(10)(p14),der(11)t(4;11)(?;p15), -17,+19

MCF-7. TAM. 48h.		+1,add(1)(p13),del(1)(p21),del(1)(p32), del(3)(p13),-6x2, der(7)t(7;20)(p22;?)t(7;20)(?;?) ,dic(7;?)(q31;?),+8,der(9)t(9;21)(p24;?)t(8;21)(?;?), +11,der(11)t(4;11)(?;p15) ,del(11)(p11.2),der(14;14)(q10;q10),+15,+19
MCF7. TAM. 96h.		del(X)(q13),+1,del(1)(p21),del(3)(p13),del(3)(q13), -6,del(6)(q22), der(7)t(7;20)(p22;?)t(7;20)(?;?),+8,add(8)(p23), der(9)t(9;21)(p24;?)t(8;21)(?;?),+10,+20
T47D. Control	57~66<3n>,X,-X[24],der(X)t(X;6)(q12;p11)[24],-1[19],-2[22],-3[5],del(3)(p11)[2], del(3)(p14)[2],del(3)(p21)[2],del(3)(q13)[6],del(3)(q22)[3],der(3)ins(3;5)(p14;q13q31)[2],der(3)del(3)(p13)del(3)(q13q25)ins(3;5)(q13;q13q31)[2],-4[19],-5[2],+5[3],-6[17],+7[3],del(7)(p21)[3], del(7)(p13p14)[5],del(7)(p13p14)x2[10],del(7)(p13p15)[8],der(7)t(7;15)(q21;q13)[3],dup(7)(p13p14)[2],+8[12],der(8;14)(q10;q10)x2[24],-9[11],-9x2[9],-10[11],-10x2[10],del(10)(p10)[3], der(10)t(3;10)(q?;q24)del(10)(p11.2)[14],der(10)t(3;10)(q?;q24)del(10)(p11.2)x2[10],+11[9],+11x2[7] ,+11x3[2],der(11)t(11;17)(q23;q?)t(9;17)(q?12;?)[2],-12[2],+12[6],+12x2[4], del(12)(p12)[6],del(12)(q24.1)[5],del(12)(q24.1)x2[3],der(12)del(12)(p12)del(12)(q24)[4], der(12)t(12;13)(p12;q22)[10],der(12)t(12;16)(p11.2;?)[11],-13[16],-13x2[4],+14[3],+14x2[13], +14x3[3],-15[6],-15x2[18],-16[2],der(16)t(1;16)(q12;q12)dup(1)(q21q43)[24], dic(9;17)t(9;17)(p12;p13)[13],dic(9;17)t(9;17)(p12;p13)x2[11],-18[17],-18x2[4],-19[18], +20[9],+20x2[3],der(20)t(10;20)(q21;q13.3)[15],der(20)t(10;20)(q21;q13.3)x2[9],der(20)del(20)(p11)t (10;20)(q21;q13.3)[10],+21[10],+21x2[6],-21[2],-22[14],+1~2 mar[7][cp24]	
T47D. E2. 24h.		+3,-7,der(11)t(4;11)(?;p15),add(11)(q23),-14,+16, der(16)t(1;16)(q12;q12),+17, der(17)t(17;21)(q24;?),+19,+22
T47D. E2. 48h.		+3,dic(5;7)(q35;p22),-7,-8,+9,der(11)t(4;11)(?;p15),- 14,+16,+17,-17, der(17)t(17;21)(q24;?),dic(20;?)(q13.3;?),+22
T47D. E2. 96h.		del(1)(p21],+3,-7,-8,add(11)(q23),-14,+16, +17,+19,+22
T47D. TAM. 24h.		del(X)(q21),+6,del(6)(q25),-7,+9,-14,+16, -17,+17,+19
T47D. TAM. 48h.		+6,-7,-8,-14,-17,+17
T47D. TAM. 96h.		+3,+6,-8,+9,-11,-14,-17,+17,+19

BT474. CONTROL	65~106<4n>,X,-X[9],-Xx2[5],-Xx3[4],der(X)t(X;17)(q13;q11q12)del(X)(p21)[9],der(X)t(X;18;X;12)[2],del(X)(q22)[14],-1[6],-1x2[2],+1[3],del(1)(p36.1)[6],-2[7],+2[7],der(2)t(1;2;7;20)(?;q31;?;?)[18],+3[12],-3[3],del(3)(p11.2)[7],del(3)(p14)[2],del(3)(q11.2)[6],del(3)(q11.2)x2[8],del(3)(q21)[4],del(3)(q13)[2],-4[8],-4x2[9],+4[2],-5[9],-5x2[9],+6[11],+6x3[3],-6[3],del(6)(q13)[3],del(6)(q21)[3],der(6)t(6;7)(q25;q31)[7],der(6)t(6;7)(q25;q31)x2[16],+7[4],+7x2[6],+7x3[9],+7x4[3],der(7)t(7;20)(p13;?)[5],der(7)t(1;7)(?;q11.2)[9],del(7)(q11.2)[7],del(7)(q11.2)x2[3],del(7)(q11.2)x3[3],der(7)t(7;14)(p13;p11.2)[4],-8[10],-9[7],-9x2[4],-9x3[2],der(9)t(3;9)(q33;?)[3],+10[6],-10[5], der(10)t(10;16;19)(q25;?;?)[11],i(10)(q10)[4],+11[9],+11x2[2],-11[3],der(11)t(8;11)(q21.1;p15)[2],der(11)t(8;17)(q21.1;q11q12)t(11;17)(p15;q11q12)hsr(17)(q11q12)[8],der(11)t(8;17)(q21.1;q11q12)t(11;17)(p15;q11q12)hsr(17)(q11q12)x2[12],der(11)t(8;17)(q21.1;q11q12)t(11;17)(p15;q11q12)hsr(17)(q11q12)x3[3],der(11)t(11;17)(q?14;?)t(8;17)(?;q?11.2)hsr(17)(q11q12)[13], der(11)t(11;17)(q?14;q?11.2)hsr(17)(q11q12)[9],+12[8],+12x2[5],del(12)(p11.1)[2],der(12)t(5;12)(q23;q23)[17],der(12)t(5;12)(q23;q23)x2[2],der(12)del(12)(p12)del(12)(q24)[3],-13[7],+13[6],+13x2[3],+13x4[2],der(13)t(13;17)(q10;q11q12)t(13;17)(q10;q11q12)hsr(17)(q11q12)[8],der(13)t(13;17)(q10;q11q12)t(13;17)(q10;q11q12)hsr(17)(q11q12)x2[12],+14[11],+14x2[3],+14x3[2],der(14)t(14;1;14)(q31;?;?)[6],der(14)t(14;1;14)(q31;?;?)x2[5], der(14)t(14;1;14)(q31;?;?)x3[9],der(14)t(14;1;14)(q31;?;?)x4[3],add(14)(p11.2)[2],der(14;14)(q10;q10)[3],der(14;14)(q10;q10)x2[16],-15[6],-15x2[9],-15x3[6],+16[7],+16x2[6],+16x3[3],-16[2],der(16)t(X;16)(q22;q24)[10],+17[16],der(17)t(6;17)(?;p13)t(15;17)(q11.2;q25)hsr(17)(q11q12)[22],-18[10],-18x2[4],-18x3[2],-19[6],-19x2[5],+19[5],-20[6],20x2[6],+20[3],+20x3[2],der(20)t(19;20)(?;q10)[4],der(20)t(19;20)(?;q10)x2[5],+21[2],-21x2[11],-21x3[3],-22[2],-22x2[5],-22x3[2],-22x4[12],der(22)t(16;22)(q12;p11.2)[5],1-4 marker[13][cp23]	
BT474. E2. 24h.		del(X)(q13),add(3)(p21),add(3)(q13),der(3)t(3;8)(p14;?),del(6)(q25),del(7)(q21),+8,der(8)t(8;17)(p23;?)t(6;17)(?;?),+9,der(15;15)(q10;q10)
BT474. E2. 48h.		+X,add(X)(p22.3),add(3)(q13)[2],add(3)(p21),der(3)t(3;8)(p14;?),del(6)(q11),der(8)t(8;17)(p23;?)t(6;17)(?;?),del(11)(p15),-12x2, der(15;15)(q10;q10),+22
BT474. E2. 96h.		del(3)(p14),der(3)t(3;8)(p14;?),der(8)t(8;17)(p23;?)t(6;17)(?;?),del(11)(p15), -12,+15,der(15;15)(q10;q10),add(17)(q25)
BT474. TAM. 24h.		+X,del(1)(p21),add(3)(q21),del(3)(p13),der(3)t(3;8)(p14;?),add(7)(p21),add(7)(p22),dic(7;?)(q36;?),+8,der(8)t(8;17)(p23;?)t(6;17)(?;?),del(11)(p11.2), -14x2,der(15;15)(q10;q10),add(16)(q24)
BT474. TAM. 48h.		der(3)t(3;8)(p14;?),add(3)(p13)x2,del(3)(p13),add(3)(q21),del(6)(q14),del(6)(q25),dic(7;?)(q36;?),der(8)t(8;17)(p23;?)t(6;17)(?;?), der(9)t(7;9)(?;p24),del(11)(p11.2),-12,-14,der(15;15)(q10;q10), +18,+22,der(22)t(20;22)(?;q13)t(16;20)(?;?)
BT474. TAM. 96h.		add(3)(p13),del(3)(p13),der(8)t(8;17)(p23;?)t(6;17)(?;?),der(9)t(7;9)(?;p24),-12,del(12)(p12),,-14,der(15;15)(q10;q10), der(22)t(20;22)(?;q13)t(16;20)(?;?)

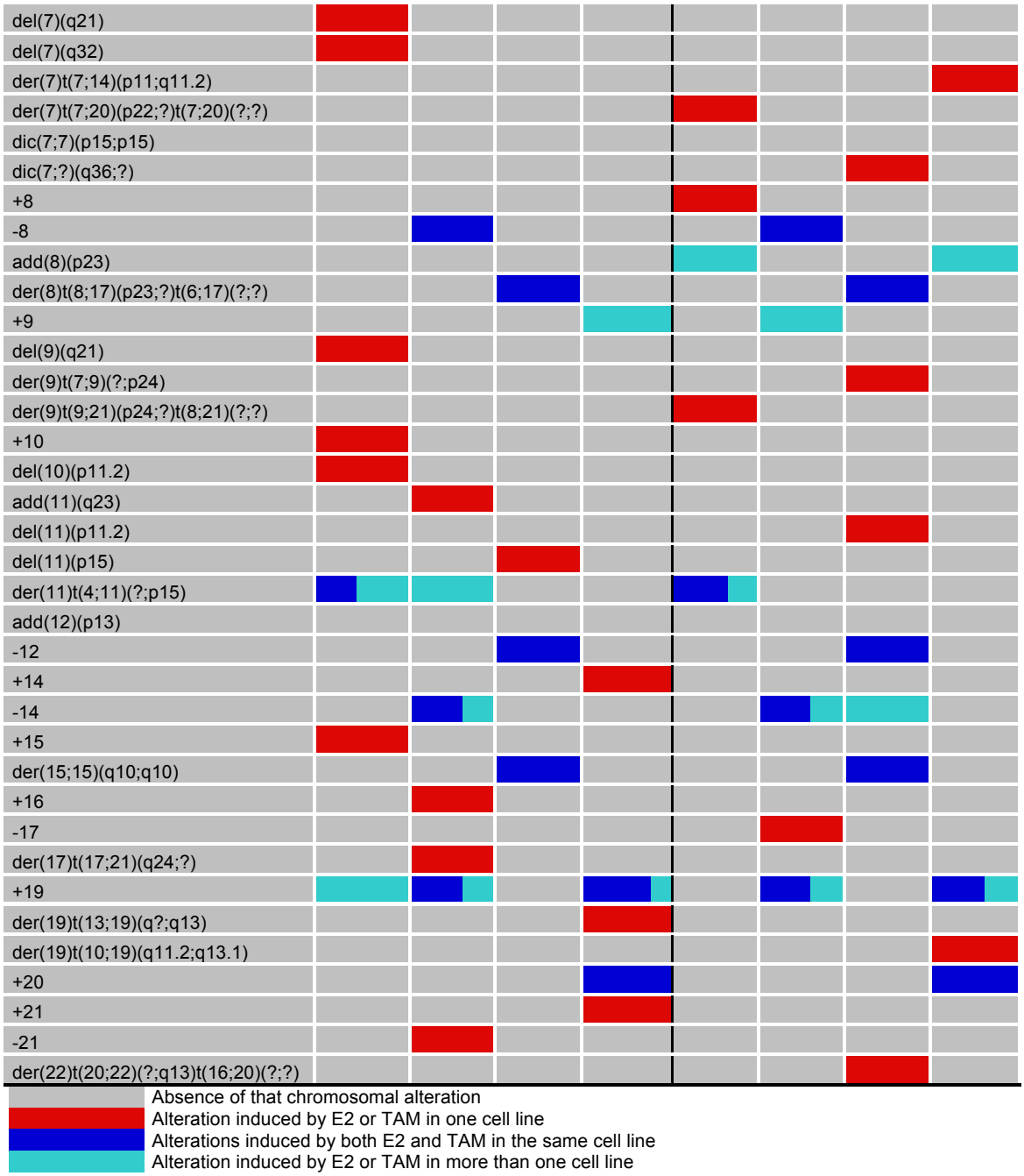
SKBR3. Control	76~83<4n>,XXX,-X[19],der(X)t(X;17)(q21;q?21)hsr(17)(q11q12)[15], der(X)t(X;8;17)(q13;q?21;?)[6],+1[8],+1x3[5],add(1)(p36.3)[4],del(1)(p13)[11], del(1)(p13)x2[6],del(1)(p34)[4],del(1)(p22)[9],del(1)(p36.1)[2], der(1)t(1;4)(q12;q12)[6],-2[6],-2x2[8], -2x3[3],der(2)t(2;6)(p13;?)[5],-3[10],-3x2[6],-4[8],-4x2[8],-4x3[3],der(4;14)t(4;14)(p11;p11.1)[3], -5[8],-5x2[8],-5x3[2],der(5)ins(5;15)(p13;q12q22)[6],-6[4],-6x2[12],-6x3[2], der(6)t(6;14;17)(q21;?;q11q12)del(6)(p23)[8],+7x2[8],+7x3[10],del(7)(q22)[12], del(7)(q32)[3],dup(7)(p14p15)[2],-8[6],+8[8], der(8)t(8;21)(?;?)t(8;21)(p23;?)t(8;21)(q24;?)[11],der(8)t(8;21)(?;?)t(8;21)(p23;?)t(8;21)(q24;?)x2[8], der(8)dup(8)(?)t(8;8)(?;p23)t(8;17)(q24;?)t(11;17)(?;?)[4],der(8;14)t(8;14)(p11.1;p11.1)[15],-9[9], -9x2[7],-10[4],-10x2[13],-10x3[2],+11[2],-11[7],add(11)(p15)[4],add(11)(q25)[2],-12[6],-12x2[5], +12[3],der(12)t(11;12)(p?;p12)[4],der(12)t(5;12)(q23;q23)[10],der(12)t(5;12)(q23;q23)x2[4],-13[6], -13x2[8],-13x3[3],der(13;13)(q11.2;q11.2)[16],-14[6],-14x2[4], der(14;14)(q11.2;q11.2)[18],-15[10], -15x2[7],dic(15;21)(p11.1;p11.1)[3],+16[4],-16[7],-17[3],+17[9], der(17;17)t(17;17)(q25;?)dup(17)(q22q25)t(17;20)(?;?)[5], der(17;17)t(17;17)(q25;?)dup(17)(q22q25)t(17;20)(?;?)x2[7], der(17;17)t(17;17)(q25;?)dup(17)(q22q25)t(17;20)(?;?)x3[7],del(17)(p11.2)[7], der(17)t(8;17)(q12;?)dup(17)(?)hsr(17)(q11q12)[19],der(17)t(8;17)(?;q25)dup(17)(q22q25)[5], der(17)t(8;17)(?;q25)dup(17)(q22q25)x2[2],der(17)t(8;13;14;17;21)(?;q?;q?;q11q12;?)[8],der(17)t(3; 8;13;17;20)(?;?;q12;?p;?)[12],der(17)t(3;8;13;17;20)(?;?;q12;?p;?)x2[2],-18[3],-18x2[11],-18x3[5], der(18)t(18;22)(p11.2;?)[12],-19[4],-19x2[7],-20[8],-20x2[4],-20x3[7],-21[6],-21x2[3], -22[9],-22x2[4],+22[2],der(22)t(19,22)(q?;q13)[5],1~3 mar[9][cp19]	
SKBR3. E2. 24h.		+X[3],i(1)(q10)x2,chrb(1)(p13),chte(1)(q21)[2],del(1)(p34), del(1)(q11),dic(1;19)(p11;q13),+3,+5,+6,der(6)dup(6)(p25 p?), del(7)(q11.2),dic(7;7)(p15;p15),+9,?i(9)(p10)x2, der(12)add(12)(q23)del(12)(p13)[3],+13,+14+15,add(16)(q24),+19x2,der(19)t(13;19)(q?;q13)x2,+20,+21
SKBR3. E2. 48h.		del(1)(q11),der(1)t(1;3)(p34;p21),dic(1;19)(p11;q13), i(1)(q10),der(6)dup(6)(p25p?),del(7)(q11.2),+9,del(9)(q34) ,add(16)(p13.3),+19, der(19)t(13;19)(q?;q13),+21
SKBR3. E2. 96h.		del(1)(q11),dic(1;19)(p11;q13),i(1)(q10),+2,+3,+5, der(6)dup(6)(p25p?),del(7)(q11.2),dic(7;7)(p21;p21),+9, +14x2,+19,der(19)t(13;19)(q?;q13),+20,+21,+22
SKBR3. TAM.		add(1)(p32), del(1)(q11),i(1)(q10),dic(1;19)(p11;q13), +3,del(3)(q13),der(5)t(5;14)(p11;q11.2),+6, der(6)dup(6)(p25p?), del(6)(q14),del(7)(q11.2),der(7)t(7;14)(p11;q11.2),+9,add(14)(p11.2),+19,der(19)t(10;19)(q11.2;q13.1),?dic(19;19)(q 11.1;q11.1),+20,+21
SKBR3. TAM.		i(1)(q10),-3,der(6)dup(6)(p25p?),der(7)t(7;14)(p11;q11.2), del(7)(q11.2),+19,der(19)t(10;19)(q11.2;q13.1),+20x3
SKBR3. TAM. 96h		dic(1;19)(p11;q13),i(1)(q10),der(6)dup(6)(p25p?) ,add(7)(p21),del(7)(q11.2),der(7)t(7;14)(p11;q11.2), ,+19,der(19)t(10;19)(q11.2;q13.1)x2

4.3.3.1 MCF7

G-banding and M-FISH karyotypes of MCF7 cell line after E2 treatment were analyzed and showed some additional numerical and structural chromosomal alterations, which were statistically significant ($p \leq 0.05$. Fisher's exact test) in comparison with those observed in control cells. The new alterations included: add(7)(q36), del(7)(p22), del(7)(q21), del(7)(q32), del(9)(q21), +10, del(10)(p11.2), der(11)t(4;11)(?;p15), +15 and +19 (Tables 15 and 16, and Annexes 3 and 4). However, some of the structural alterations were observed only at 24h and 48h, while only del(7)(q32) and del(7)(q21) were constant at all time points (Figure 26A).

Table 16. Clonal chromosomal abnormalities induced by E2 and TAM in four breast cancer cell lines. Are indicated the chromosomal alterations induced by E2 or TAM observed in at least two of the three times tested and in more than a metaphase (clonal alterations). Gray bars indicate absence of the chromosomal alteration; red bars indicate the alterations present in one cell line; dark blue bars indicate the alterations induced by both E2 and TAM in the same cell line and light blue bars indicate the alterations induced by E2 or TAM in more than one cell line.

NEW CHROMOSOMAL ABNORMALITIES	Treatment with E2. 0,01μM				Treatment with TAM. 1μM			
	MCF7	T47D	BT474	SKBR3	MCF7	T47D	BT474	SKBR3
+X								
+1								
chrb(1)(p13)								
chte(1)(q21)								
del(1)(p22)								
del(1)(p31)								
del(1)(q11)								
dic(1;19)(p11;q13)								
i(1)(q10)								
+3								
der(3)t(3;8)(p14;?)								
add(3)(p21)								
add(3)(q13)								
del(3)(p13)								
+5								
+6								
-6								
der(6)dup(6)(p25p?)								
-7								
add(7)(q36)								
del(7)(p22)								
del(7)(q11.2)								



In this cell line, a statistically significant increase of chromosomes 18 and 20 nullisomy ($p<0,01$, Fisher's exact test) was induced by E2 at each time point, as compared to control cells (Table 17).

Table 17. Nullisomy frequency of chromosomes 18 and 20 in MCF7 cells E2 treated at 24h, 48h and 96h

CHROMOSOME	Control n(%)	TREATMENTS E2. 0,01 µM			Fisher's test (p)		
		24h n(%)	48h n(%)	96h n(%)	24h	48h	96h
18	3(12)	12(71)	9(53)	3(27)	0.0002**	0.0052**	0.3351
20	8(30)	8(47)	15(88)	11(100)	0.3427	0.0004**	0.0001**

*p<0,05 **p<0,01
n(%) Total number and % of cells with nullisomy. The number of cells examined for chromosome counts was: control 26 metaphases; treated with E2: 17 after treatment for 24h, 17 after treatment for 48h and 11 after treatment for 96h.

On the other hand, in MCF7 after TAM treatment, eleven chromosomes (1, 2, 6, 7, 8, 10, 11, 17, 15, 19 and 20) had a variation on their copy number. Most of these alterations, with exception of +1, -6 and +8, were observed only in one of the treatment times, so these were considered as sporadic (Figure 26B and Annex 3). Six additional complex chromosomal aberrations - del(1)(p22), del(3)(p13), der(7)t(7;20)(p22;?)t(7;20)(?;?), add(8)(p23), der(9)t(9;21)(p24;?)t(8;21)(?;?) and der(11)t(4;11)(?;p15) (Tables 15 and 16, Figures 26B and 30) - were identified and constantly present at each time point of TAM treatment. An increase in the frequency of two pre-existing alterations, del(7)(q11.2) and del(12)(p11.2), was also observed after both E2 and TAM treatment (Annex 4).

4.3.3.2 T47D

T47D cells, after E2 treatment, presented a far more complex karyotype than control cells. The additional rearrangements were: +3, -7, -8, add(11)(q23), der(11)t(4;11)(?;p15), -14 +16, der(17)t(17;21)(q24;?) and +19, these were observed at two or three of the time points evaluated (Tables 15 and 16, Figures 27A and 31). While after TAM addition, as compared to control cells, only additional numerical alterations were identified (+6, -7, -8, +9, -14, -17 and +19) ($p<0,01$ Fisher's exact test) also observed at two or three of the time points evaluated (Annexes 5 and 6).

Altogether, TAM did not induce new structural chromosomal rearrangements (Tables 15 and 16, Figure 27B). Interestingly, five of the chromosomal rearrangements present in the control cells - del(3)(q22), del(7)(p21), del(7)(p13p14), del(7)(p13p15) and der(12)del(12)(p12)del(12)(q24) - were lost after E2 or TAM treatment (Annex 6). In analogy to MCF7, an increase in the frequency of some pre-existing numerical alterations was observed after both treatments in T47D (Annex 5).

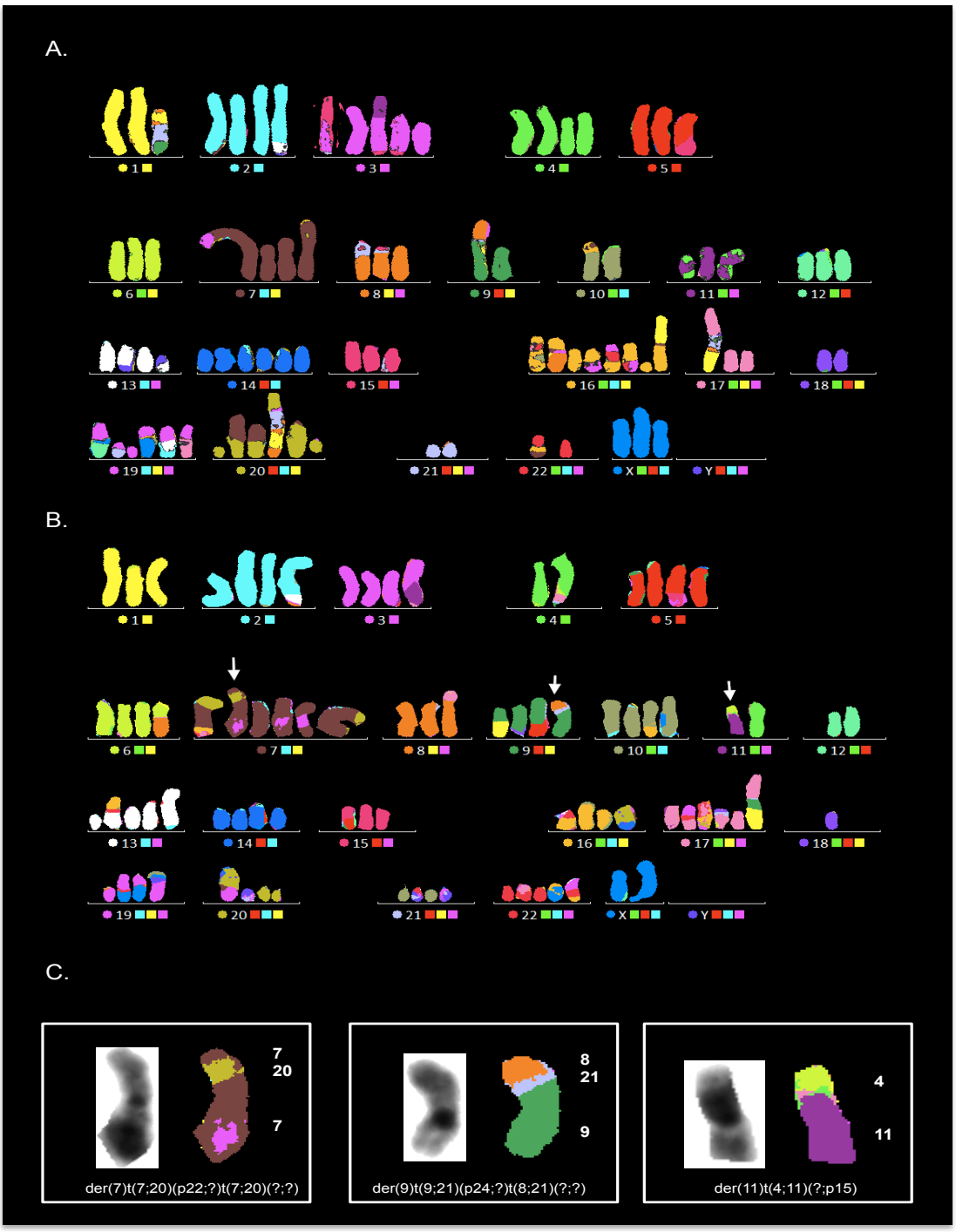


Figure 30. Conventional and Molecular cytogenetic results of MCF7 cell line. A) M-FISH karyotype of a representative metaphase of the control cell line. B) M-FISH karyotype of a representative metaphase of the TAM treated cell line C) G-Banding and M-FISH of some of the chromosomal alterations found in at least two of the three times of TAM treatment.

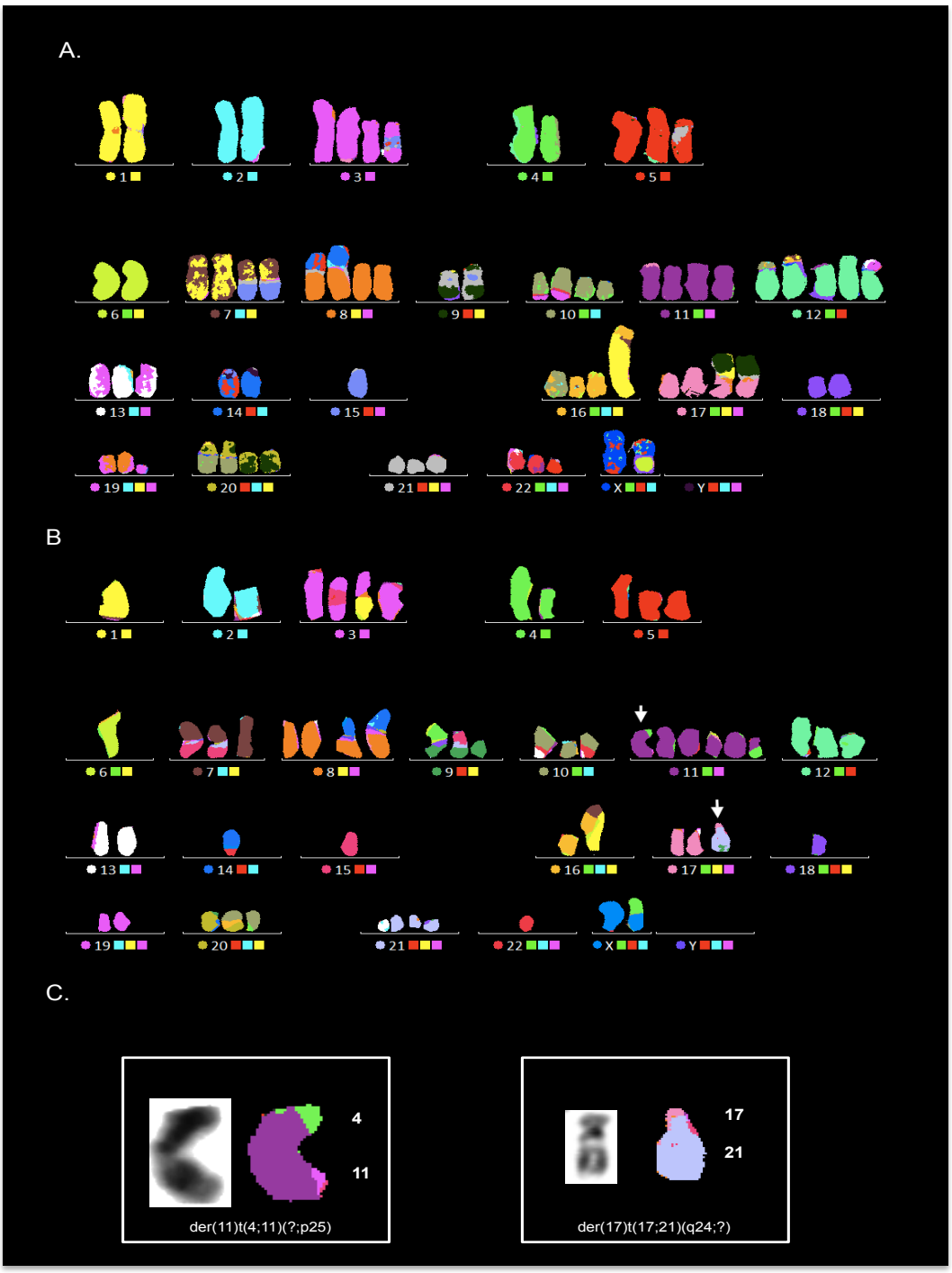


Figure 31. Conventional and Molecular cytogenetic results of T47D cell line. A) M-FISH karyotype of a representative metaphase of the control cell line. B) M-FISH karyotype of a representative metaphase of the E2 treated cell line C) G-Banding and M-FISH of some of the chromosomal alterations found in at least two of the three times of E2 treatment.

4.3.3.3 BT474

G-Banding and M-FISH karyotypes after E2 or TAM treatment in the BT474 cell line showed the same chromosomal complex rearrangements der(3)t(3;8)(p14;?), der(8)t(8;17)(p23;?)t(6;17)(?;?) and der(15;15)(q10;q10)) at each time point (Tables 15 and 16, Figure 28). Additional rearrangements were observed in at least two of the three times of E2 treatment - add(3)(q13), del(11)(p15) and -12 (Figure 28A and Annexes 7 and 8) - and after TAM treatment - del(1)(p22), del(3)(p13), dic(7;?)(q36;?), der(9)t(7;9)(?;p24), del(11)(p11.2), -12, -14 and der(22)t(20;22)(?;q13)t(16;20)(?;?) (Figures 28B and 32).

Three chromosomal rearrangements - der(X)t(X;18;X;12), del(1)(p36.1) and der(12)del(12)(p12)del(12)(q24) - present in control cells were lost after E2 and TAM treatment. An increase in the frequency of some preexisting chromosomal alterations was identified ($p \leq 0,01$. Fisher's exact test) that included: del(X)(q22), +3, del(3)(q11.2), del(3)(q13), +11, der(11)t(8;11)(q21.1;p15), +17 and +19, and observed after E2 or TAM treatment (Annexes 7 and 8).

4.3.3.4 SKBR3

Lastly, the SKBR3 control cell line displayed a complex karyotype with a particularly high frequency of chromosome 1 aberrations such as: del(1)(p13), del(1)(p22), del(1)(p34) and der(1)t(1;4)(q12;q12).

After E2 or TAM treatment the karyotype became even more complex with the appearance of new chromosome 1 abnormalities - dic(1;19)(p11;q13) and i(1)(q10) - as well as other chromosomal aberrations including der(6)dup(6)(p25p?), del(7)(q11.2), +19 and +20 ($p<0,05$. Fisher's exact test) (Annexes 9 and 10) (Tables 15 and 16, Figures 29A, 29B and 33). However, some specific additional rearrangements were observed at each time point of E2 treatment - del(1)(q11), +9, der(19)t(13;19)(q?;q13) and +21 (Tables 15 and 16, Figure 29A) - and of TAM treatment - der(7)t(7;14)(p11;q11.2) and der(19)t(10;19)(q11.2;q13.1) ($p<0,05$. Fisher's exact test) (Table 15 and 16, Figure 29B and 33).

A statistically significant increase in the frequency of some preexisting chromosomal abnormalities was also observed in SKBR3 cells (Annexes 9 and 10).

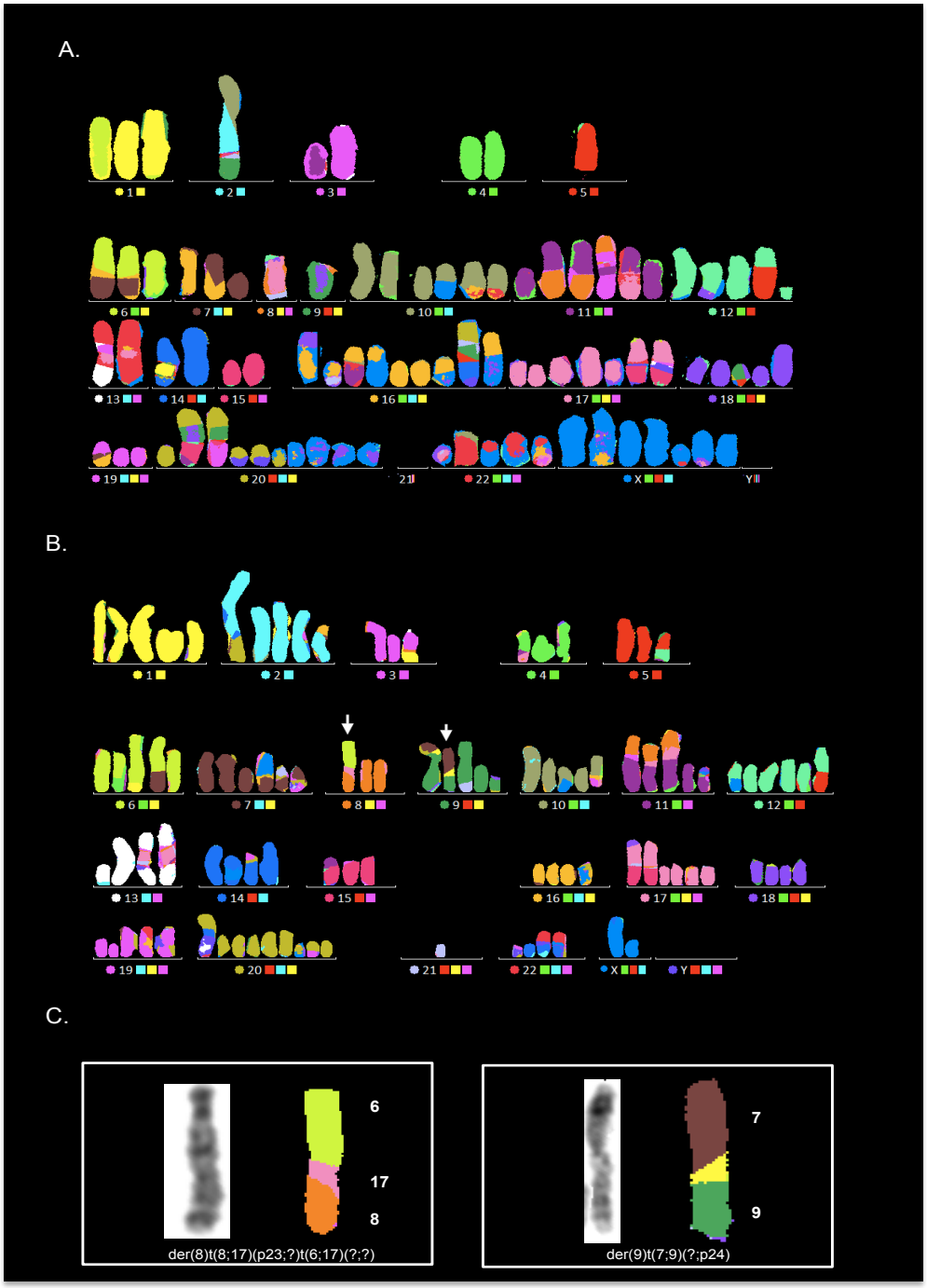


Figure 32. Conventional and Molecular cytogenetic results of BT474 cell line. A) M-FISH karyotype of a representative metaphase of the control cell line. B) M-FISH karyotype of a representative metaphase of the TAM treated cell line C) G-Banding and M-FISH of some of the chromosomal alterations found in at least two of the three times of TAM treatment.

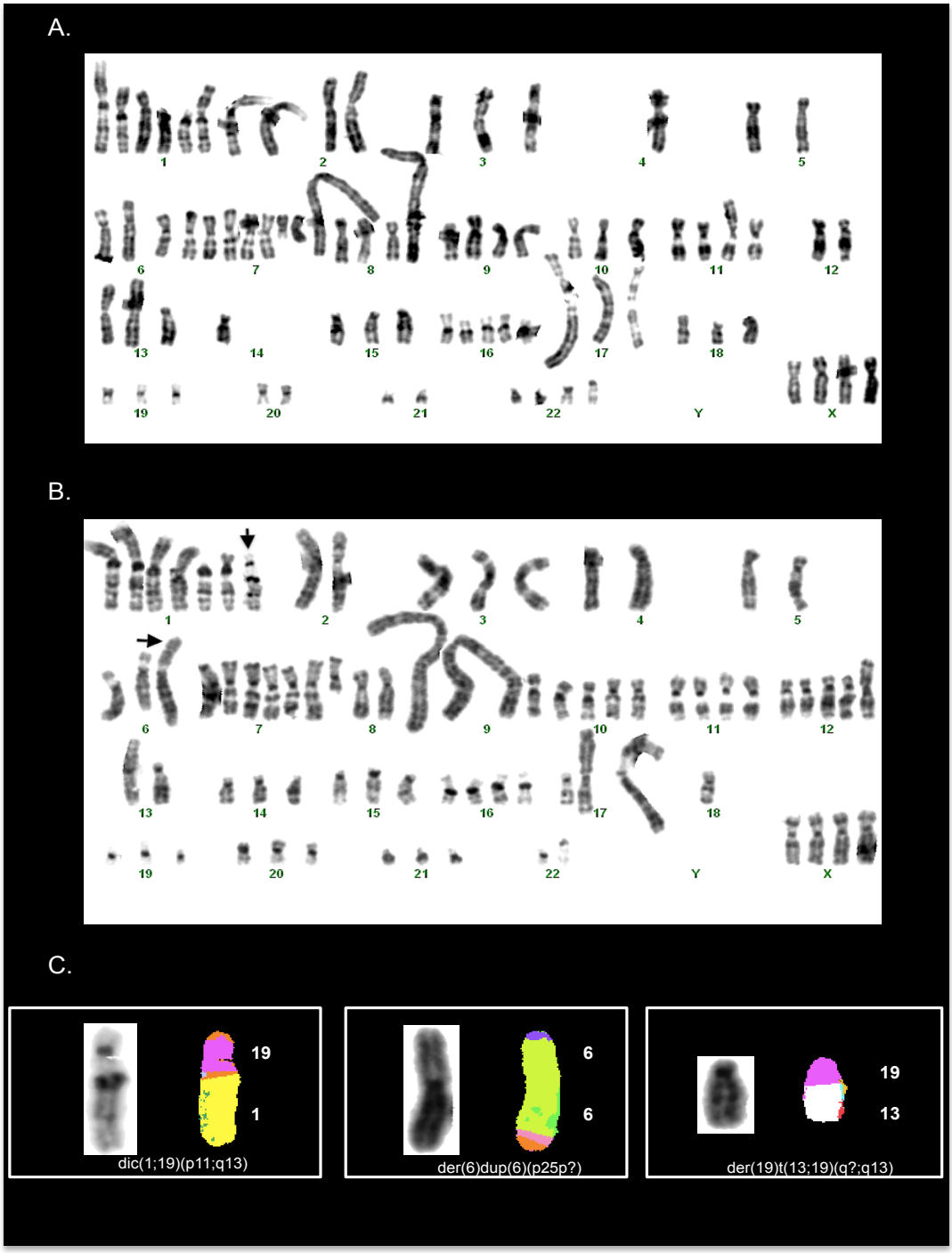


Figure 33. Conventional and Molecular cytogenetic results of SKBR3 cell line. A) G-Banding karyotype of a representative metaphase of the control cell line. B) G-Banding karyotype of a representative metaphase of the TAM treated cell line C) G-Banding and M-FISH of some of the chromosomal alterations found in at least two of the three times of TAM treatment.

4.3.4 Comparison of the effects of E2 and TAM in the karyotype of four breast cancer cell lines

As summarized in figure 34, G-Banding and M-FISH karyotype analyses showed that chromosome gains were lower in all cell lines after low dose TAM treatment as compared to E2. Chromosome losses were typical of TAM treatment for ER+ cells, while dicentric chromosomes were seen as an alteration of HER2+ cells after TAM and E2 treatment, but not of HER2- cells. Isochromosomes were only observed in SKBR3 cells. Additional material of unknown origin was not a typical alteration induced by TAM treatment of T47D as compared to E2 treatment. After exposure to E2, SKBR3 showed the highest frequency of aberrations, followed by the cell lines MCF7, BT474 and T47D respectively. Also observed in SKBR3 was the highest induction of different types of structural aberrations - breaks, translocations, derivatives chromosomes, isochromosomes, dicentric and deletions - while only some of these were observed in the other cell lines.

Only MCF7 showed reduction in the number of chromosomal aberrations after 48h and 96h of E2 treatment, probably by selection of more stable clones with less chromosomal alterations (Figure 34A)**.** Also, TAM induced new chromosomal aberrations in all cell lines. Again, SKBR3 (ER-) was the most sensitive to treatment, exhibiting high frequency of aberrations. Strangely, in T47D, TAM induced numerical, but not structural, abnormalities (Figure 34B). It is noteworthy that after E2 and TAM treatment, the induction of structural chromosomal abnormalities was higher in HER2+ than HER2- cells, with more numerical than structural abnormalities (Figures 34A and 34B).

4.3.5 FISH analysis with *HER2* (17q11.2-q12)/CEP17 and *SMS* (17p11.2)/ *RARA* (17q21.1) probes

FISH analyses with *HER2* (17q11.2-q12)/CEP17 and *SMS* (17p11.2)/ *RARA* (17q21.1) probes for MCF7, T47D, BT474 and SKBR3 cells have not evidenced a pattern alteration in all metaphases and nuclei analyzed after E2 and TAM treatment. Conversely, all metaphases and nuclei analyzed in these cell lines before and after treatments displayed the same pattern of hybridization for these genes. No differences were observed in the number of *HER2* gene copies between control and treated cell lines (Figure 35). The specific FISH pattern for these cell lines is indicated below.

In MCF7 cells, the FISH pattern before and after E2 and TAM treatment showed 4 green signals (centromere of chromosome 17 - CEP 17) and 2 red signals for the *HER2* gene (Figure 35A). The FISH pattern for *SMS/RARA* partly confirms the pattern with *HER2*/CEP17: two chromosomes with one green signal (in 17q21.1) and one red signal (in 17p11.2).
T47D FISH pattern showed 4 signals for CEP 17 and 2 signals for the *HER2* gene (Figure 35B). This was confirmed by *SMS (17p11.2)/RARA (17q21.1)* probes.

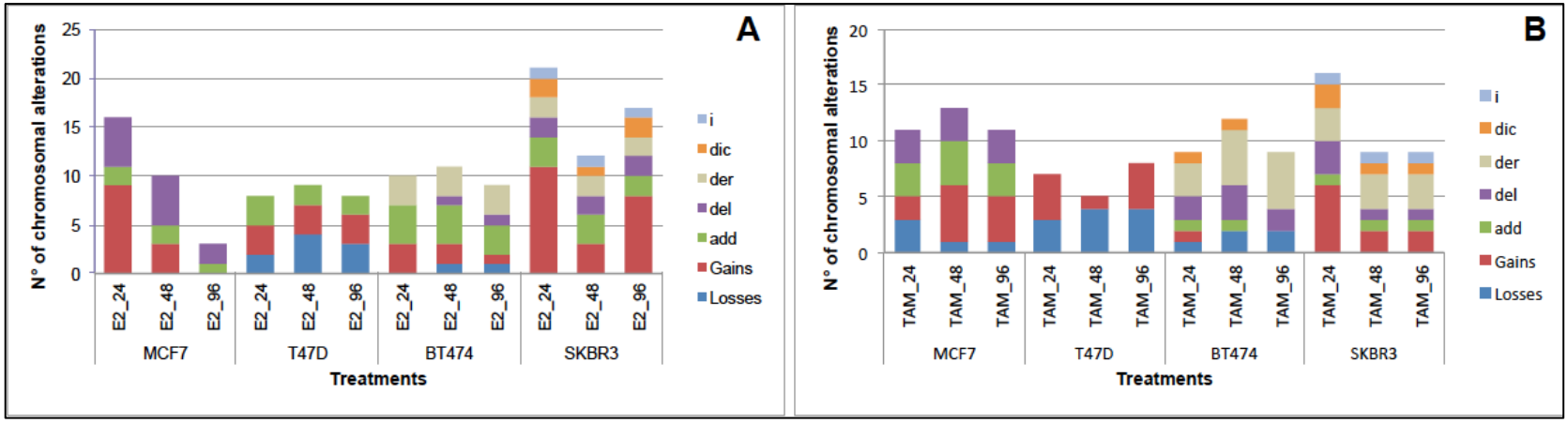


Figure 34. Total number of chromosomal aberrations induced by E2 (A) and TAM (B) treatment at 24h, 48h and 96h in MCF7, T47D, BT474 and SKBR3 cell lines. i= isochromosome; dic= dicentric chromosome; der= derivative chromosome; del= deletion; add= additional material of unknown origin.

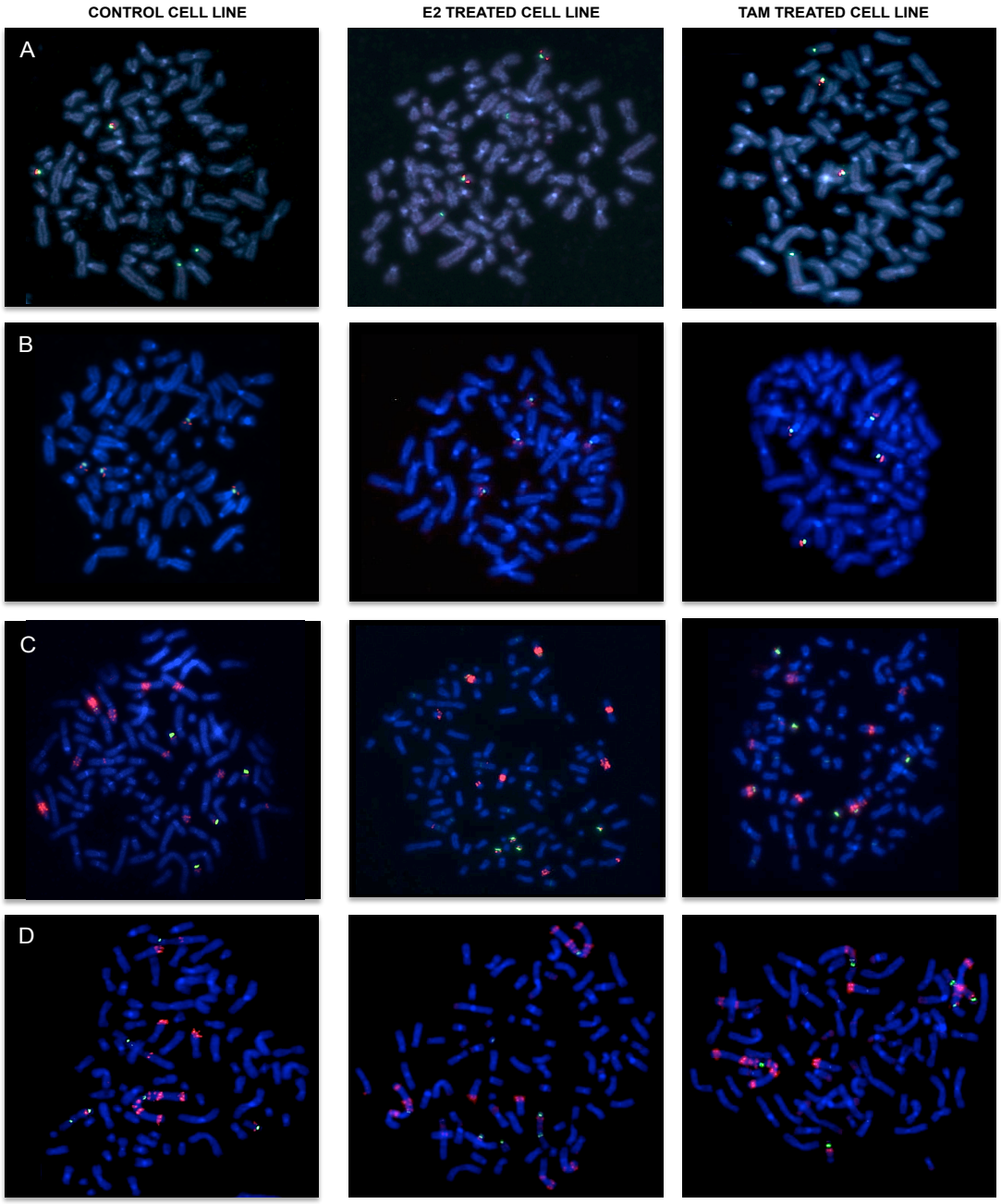


Figure 35. **FISH results with HER2/CEP17 probe on MCF7, T47D, BT474 and SKBR3 cells, control and treated with E2 and TAM**. A) FISH pattern of MCF7 cells indicating no *HER2* gene amplification. B) FISH pattern of T47D cells indicating no *HER2* gene amplification. C) FISH pattern of BT474 cells indicating *HER2* gene amplification. D) FISH pattern of SKBR3 cells indicating *HER2* gene amplification. No differences were observed in the number of *HER2* gene copies between control vs E2 and TAM treated cell lines.

FISH analysis for BT474 cells showed 6 green (CEP 17) signals and multiple red signals, which are indicative of *HER2* gene amplification (Figure 35C). These findings indicate that the amplification of the *HER2* gene was intrachromosomal and distributed in different rearranged chromosomes. With *SMS* (17p11.2)/ *RARA* (17q21.1) probes, only the 4 normal Chrs17 were shown.

Finally, the FISH pattern for SKBR3 cells showed 7 green signals (CEP 17) and multiple red signals (*HER2* gene). This pattern is indicative of *HER2* gene amplification, which was more intense than this observed in BT474 cells (Figure 35D). Also, in this cell line, the amplification of the *HER2* gene was intrachromosomal and distributed on different rearranged chromosomes. FISH with *SMS* and *RARA* genes showed co-amplification of *RARA* with *HER2* and only 3 SMS signals - 2 of these on chromosomes with CEP17, none on chromosome with *HER2* gene amplification.

4.4 DISCUSSION

Epidemiological studies and clinical evidence suggest that increased risk of developing breast cancer could be associated with increasing and constant exposure to estrogens. However, the mechanisms through which estrogens play a role in breast cancer are not fully understood yet. TAM has been the main hormonal therapy for both early and advanced breast cancer patients with hormone dependent tumours (ER+). Paradoxically, it also has been reported that TAM possesses a high mutagenic potential and causes chromosomal alterations. Nevertheless, type and frequency of chromosomal abnormalities as well as the mechanisms by which E2 and TAM induces chromosomal aberrations are only partially understood.

We performed this study in order to determine if E2 and TAM at low dose (0.01 μM and 1 μM respectively) have a role on cell proliferation and if they can induce genomic instability and new chromosomal aberrations in MCF7 and T47D (both are ER+/HER2-), BT474 (ER+/HER2+) and SKBR3 (ER-/HER2+) breast cancer cell lines. Finally, we investigated if ER and HER2 status are involved in the induction of them. Our results showed that all cell lines treated with E2 and TAM possess a more complex karyotype than control cells, displaying numerical changes (endoreduplications, aneuploidy, polyploidy) as well as more complex chromosomal rearrangements. These results are indicative of a possible karyotypic evolution - of the tumor cells with stable chromosomal alterations - toward a state of high proliferative capacity and cell survival. These stable chromosomal alterations, apparently provide to the cells a selective advantage for growth over control cells, and could eventually lead to the selection of tumor cells with high proliferative capacity, thus promoting clonal expansion.

Our results confirm that the induction of cell proliferation is dependent on the presence of ER, because ER+ cell lines showed an increase in cell proliferation after E2 treatment and cell decrease after TAM treatment (Figures 36 and 37).

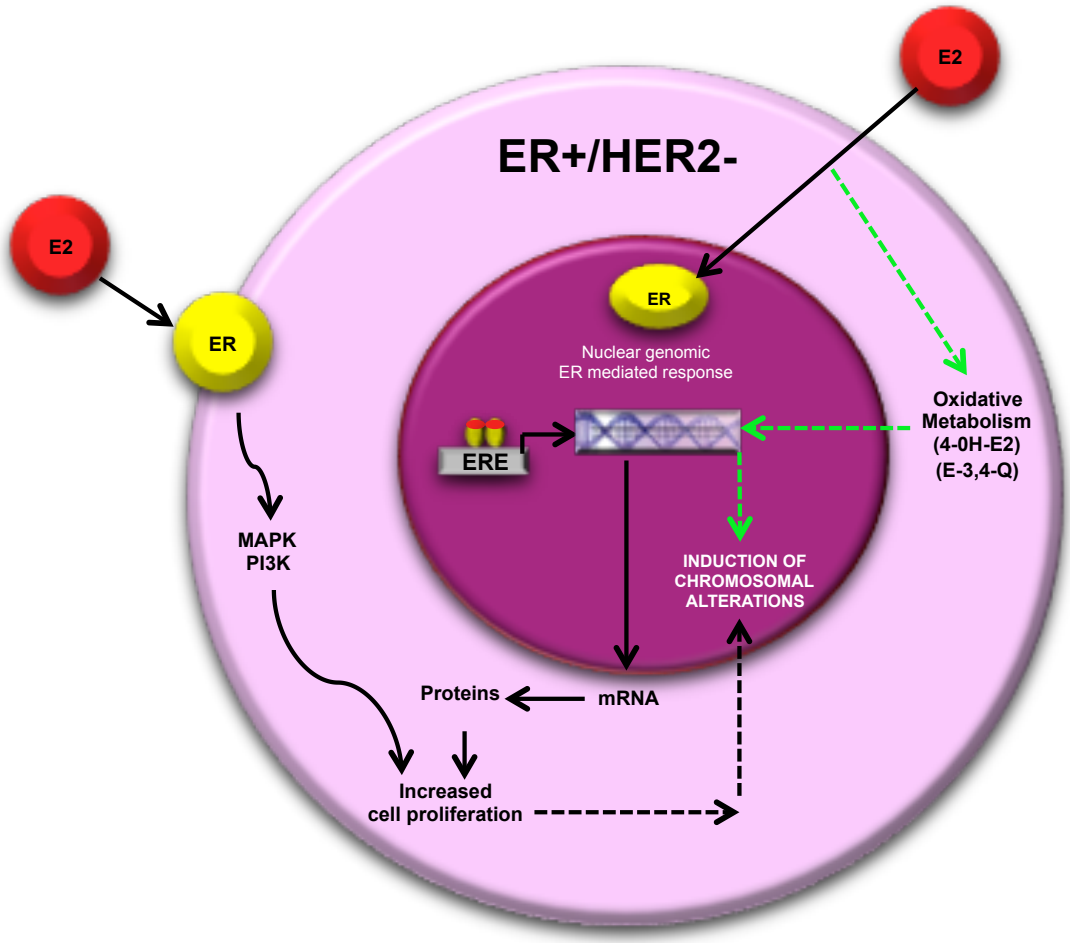


Figure 36. Proposed model of cell proliferation and induction of chromosomal alterations mediated by E2 in ER+/HER2- cells. In MCF7 and T47D cells (ER+/HER-), E2 induces an increase in cell proliferation and chromosomal damage possibly by at least three mechanisms, two of them are associated with E2 binding to their specific receptors (ER), either nuclear or plasma membrane ERs, stimulating cell proliferation and increasing the risk of causing direct damage to DNA during each cell division. A third mechanism is associated with the oxidative metabolism of E2 to form primary (4-OH-E2) and secondary metabolites (E-3,4-quinones), which can generate DNA adducts (unstable) and lead to the formation of chromosomal aberrations. ERE: estrogen receptor elements.

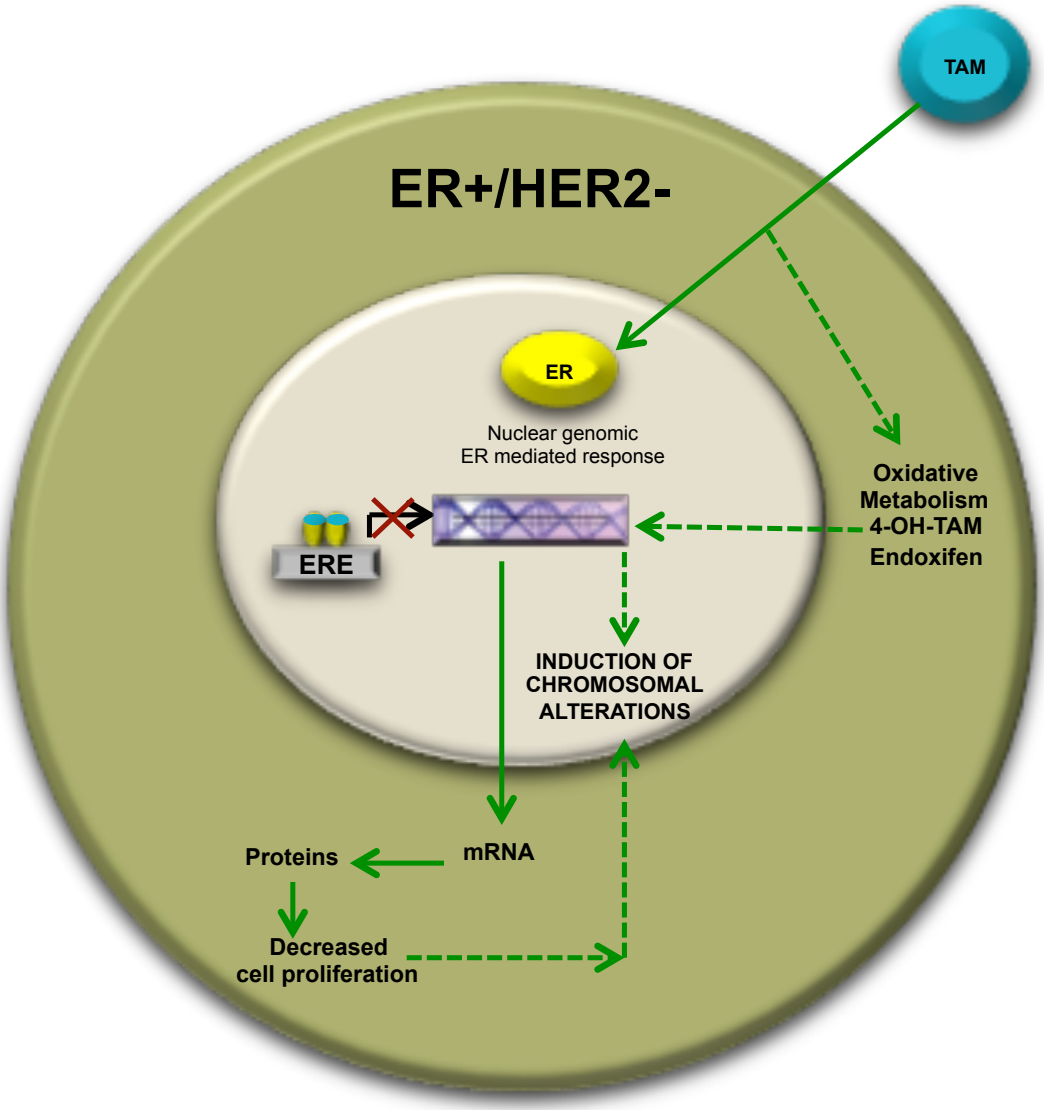


Figure 37. Proposed model of cell proliferation and induction of chromosomal alterations mediated by TAM in ER+/HER2- cells. In MCF7 and T47D cells (ER+/HER-), Tamoxifen (TAM) induces a decrease in cell proliferation by blocking the binding of E2 to its receptors (ER). In addition, in these cells, TAM induces chromosomal damage possibly by oxidative metabolism, which generates primary (4-OH-TAM) and secondary metabolites (Endoxifen). These metabolites can form DNA adducts and subsequently lead to the formation of chromosomal alterations. ERE: estrogen receptor elements.

BT474 (ER+/HER2+) showed the highest increase in cell proliferation after both E2 and TAM treatment. However, it should be noted, that after 48h of TAM treatment, cell numbers decreased to have a final increase at 96h. This phenomenon is probably due to clonal selection of subpopulations cells with chromosomal alterations beneficial and selectively advantageous for the survival and proliferation of tumor cells. The increased cell growth observed after TAM treatment could be indicative of the estrogen agonist activity exerted by the TAM in these cells (Figure 38). In addition, have been reported that the estrogen agonist activity of TAM is enhanced by the presence of ER and HER2 receptors in breast tumor cells (47, 48, 51). Indeed, the cross-communication between ER pathways and growth factor receptors pathways (EGFR, IGF-1, HER2) has been involved in cell proliferation, survival and resistance to endocrine therapy (TAM) in breast cancer (40, 47, 48).

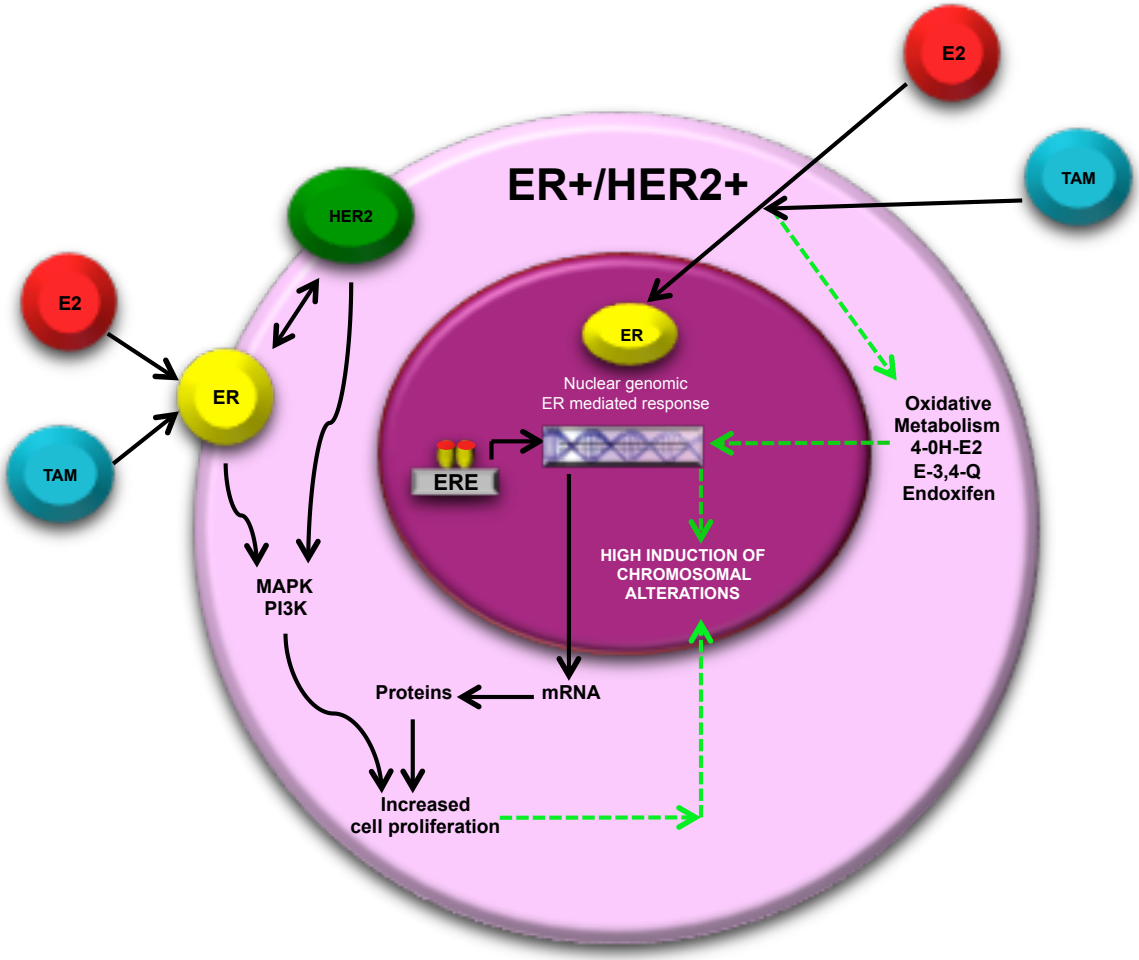


Figure 38. Proposed model of cell proliferation and induction of chromosomal alterations mediated by E2 and TAM in ER+/HER2+ cells. In

BT474 cells (ER+/HER+), E2 and TAM induce an increase in cell proliferation and chromosomal damage possibly by at least three mechanisms, two of them are associated with E2 binding to their specific receptors (ER), stimulating cell proliferation and increasing the risk of causing direct damage to DNA during each cell division. In addition, the non-genomic function of membrane-associated ER receptors and cross-talk to growth factors - including over-expression of the factor receptor HER2 - can all contribute to TAM agonist resistance and promote survival of the breast cancer cells. It is also to be noted that TAM can activate the membrane-associated ERs in a manner analogous to E2 ligands, thus accounting for its agonistic effects, including drug resistance. A third mechanism is associated with the oxidative metabolism of E2 and TAM to form primary (4-OH-E2, 4-OH-TAM) and secondary metabolites (E-3,4-quinones, endoxifen), which can generate DNA adducts (unstable) and lead to the formation of chromosomal aberrations.

In SKBR3 (ER-/HER2+), no alterations in cell proliferation were observed after treatment with E2 and TAM, except for a small increase at 96h; however, these agents induced a high frequency of chromosomal abnormalities. This could mean that the absence of ER does not allow E2 and TAM to exert their effects on cell proliferation/death, but either E2 or TAM may exert a direct effect on the increase of genomic instability, which causes chromosomal abnormalities (Figure 39). Likewise, the small proliferation increase at 96h in SKBR3 could be attributed to the presence and late response of the novel functional estrogen transmembrane receptor, G protein-coupled receptor 30 (GPCR30), which is expressed in this cell line (166) and modulates both non-genomic events and genomic transcriptional events of estrogen (52, 166). Effects mediated by GPCR30 are maintained when classic ERs are absent or blocked (167).

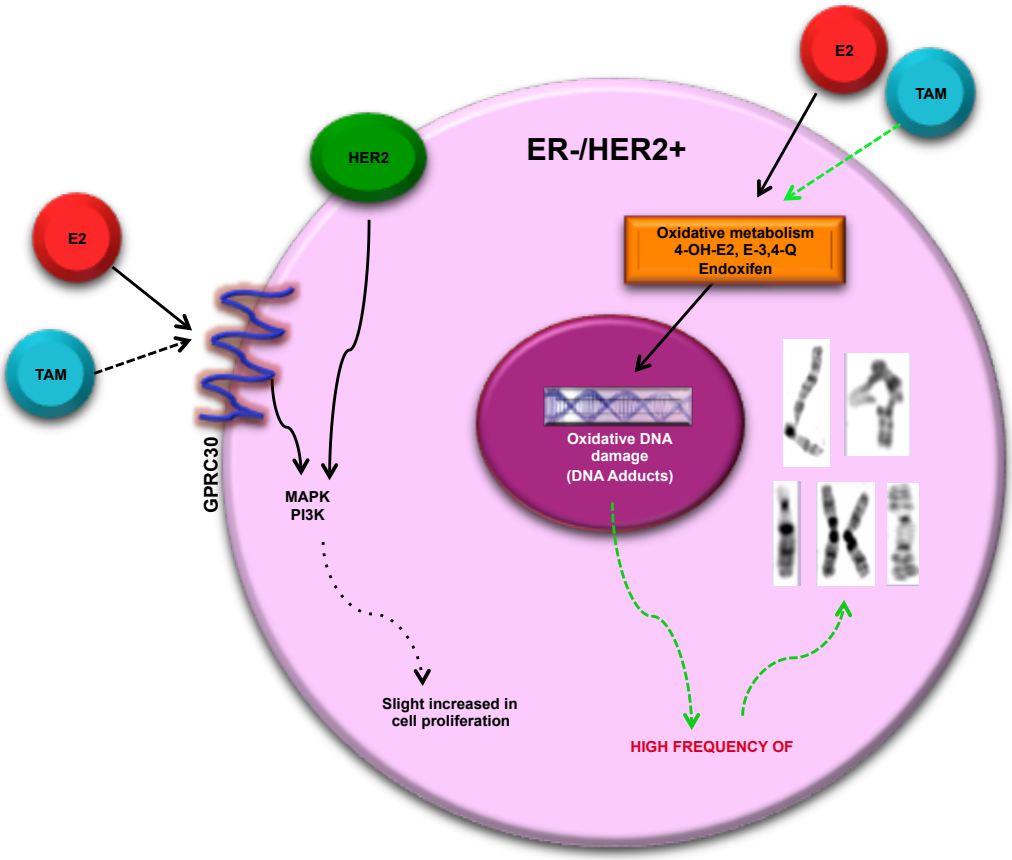


Figure 39. Proposed model of cell proliferation and induction of chromosomal alterations mediated by E2 and TAM in ER-/HER2+ cells. In SKBR3 cells (ER-/HER2+), E2 and TAM induce a small increase in cell proliferation and chromosomal damage, which could be attributed to the presence of the estrogen transmembrane receptor, GPCR30, which is expressed in this cell line. This suggests a role for GPCR30 in non-classical steroid hormone actions. Additionally, the chromosomal damage observed in these cells, could also be attributed to the oxidative metabolism of E2 and TAM.

E2 and TAM induce numerical chromosomal alterations in breast cancer cell lines

After E2 and TAM treatment, induction of polyploidy was observed in ER positive and negative cell lines; however, this was higher in the SKBR3 cell line (ER-). These results could indicate that E2 and TAM induce polyploidy through ER dependent and independent mechanisms and cause cell cycle arrest in G2/M, thus generating chromosomal instability (168). Polyploidy has been correlated with short survival, drug resistance and metastasis (124).

In the same way, our results indicate that E2 and TAM at low doses induce aneuploidy of any chromosome but in different frequencies in all cell lines. These results agree with previous studies where E2, but at high doses, exhibits microtubule disrupting activity both in estrogen receptor-positive and receptor-negative human breast cancer cell lines, which suggests that E2 itself induces microtubule disruption independent of its binding to estrogen receptor (2, 7-9, 56).

The most likely explanation for the aneugenic activity of E2 is that it disturbs the fidelity of assembly of the spindle apparatus (microtubules and centrioles) or disrupts polymerization of microtubules and is capable of generating alterations in the DNA, regulating proteins, and centromeres. This may happen directly via covalent binding (quinone metabolites of E2 bind covalently to the C-terminal regions of β-tubulin) or indirectly by free radical generation, which results in anaphase abnormalities and nondisjunction of chromosomes (2, 7-9, 56, 169). Subsequently, aneuploidy may induce cell transformation. As for TAM, this is the first report, which indicates its aneugenic ability in ER+ and ER- breast cancer cell lines.

The aneuploidy could have some effect on dosage of genes with a role in the control of cell proliferation/selection during tumor progression. In addition, it has been reported that the stress caused by aneuploidy precipitates an increase in mutation rate and/or increased genomic instability. This instability would further not only aid in the evolution of the tumor cells toward a state of high proliferative capacity but also could provide mechanisms of adaptation in a particular environment. Aneuploidy has been associated with disease and tumor initiation, promotion and progression (170), tumorigenesis (95, 171, 172), resistance to therapy and the possible emergence of new drug-resistant cell species with specific aneuploidies (3, 97, 173-175).

In detail, a statistically significant increase in the losses of chromosomes 18 and 20 in MCF7 cells after E2 treatment at all time points evaluated was observed, which is a frequent event in breast cancer and is believed to result in the loss of some tumour suppressor genes - *SMAD4* (97, 176), *DCC*, *RBBP8, SERPINB5* and *EPB41L3* on chromosome 18, which are being implicated in breast cancer development - and the loss of the *AURKA* (20q13.31), *E2F1* (20q11.22) and *MAPRE1* (20q11.1-11.23) genes, which are involved in the regulation of the mitotic cell division process, regulation of microtubule dynamic instability and cell cycle control, among others (Table 18).

In T47D cells, after E2 and TAM addition, a direct correlation between increased losses of chromosomes 8 and 14 and gains of chromosome 19 with increased cell proliferation at all time points evaluated was observed. These results are indicative of a possible selection of numerical alterations advantageous for cell proliferation and survival.

We observed that, in the SKBR3 cells, both E2 and TAM treatment induce gains of chromosomes 9, 19, 20 and 21. Interestingly, aneuploidies of chromosomes 9, 19 and 20 increased at 96h, time in which a significant difference in cell proliferation was observed, which was greater than control cells. These results indicate that these alterations could be playing a key role in cell proliferation. Aneuploidies of chromosomes 19 and 20 have been associated with poor prognosis and with the involvement of genes in development and tumor progression (Table 18) (11, 28, 61, 97, 177, 178).

E2 and TAM induce structural chromosomal alterations in breast cancer cell lines

We find that E2 and TAM are able to induce chromosomal aberrations (deletion, isochromosomes, translocations, chromosomal exchanges, chromosomal breaks and dicentric chromosomes) in all cell lines. These aberrations can result in chromosomal changes that lead to deregulated expression of genes, such as the loss of tumor suppressor genes, gains of oncogenes, fusion proteins with enhanced or aberrant transcriptional activity, and are all related to cancer progression and therapy resistance.

Table 18. Selected breast cancer oncogenes and tumor suppressor genes present in the chromosomal region affected by chromosomal abnormalities in MCF7, T47D, BT474 and SKBR3 cell lines after treatment with E2 or TAM for 24h, 48h and 96h.

Chromosomal Region	Genes	Cell line				Function	References
		MCF7	T47D	BT474	SKBR3		
1p13.3	CSF1				X	Induce cell proliferation.	(133)
1p22	BCL10	X		X		Oncogene, promote apoptosis.	(179)
1p22	BCAR3	X		X		Induce cell proliferation, gene involved in the development of estrogen resistance.	(180-182)
1p32p31	JUN				X	Oncogen.	(133)
1p36.21	PRDM2				X	Tumor suppressor gen binds to ER. Transcriptional regulation, E2 effector action.	(133)
1q11	MUC1				X	Plays a role in intracellular signaling. Upregulated in breast cancer.	(183)
1q21.1	CA14				X	Participates in a variety of biological processes, including respiration, calcification and bone	(184-186)

						resorption.	
1q21.3	PIP5K1A				X	Cell proliferation, breast cancer.	(184-186)
1q25.2-q25.3	COX2				X	Induces inflammation and mitogenesis.	(133)
1q32	KISS				X	Oncogene induces cell motility.	(184-186)
1q31	PTGS2				X	Induces inflammation and mitogenesis.	(187)
1q41	CENP-F				X	Kinetochore assembly.	(180-182)
1q42.12	ENAH				X	Cell shape and movement.	(180-182)
1q44	AKT3				X	Induces proliferation, cell survival, and tumorigenesis.	(180-182)
3p14	FHIT			X		Tumor suppressor gene. Resistance to tamoxifen in MCF-7 cells.	(188, 189)
3p14	FOXP1			X		Tumor suppressor gene, lost in several tumor types.	(190)
3p14	LRIG1			X		Inhibitor of receptor tyrosine kinases, breast cancer.	(191)
6p25	TFAP2A				X	Tumor suppressor gene, breast cancer.	(192)
6p25	DUSP22				X	Its related super-pathways are MAPK signaling pathways.	(176)
7p22	GPR30	X				G protein- coupled receptor 30, drug resistance.	(167)
7p22	SDK1	X				Cell adhesion protein, breast cancer.	(176)
7q11.2	LIMK1				X	Organization of actin cytoskeleton.	(193)
7q11.2	HSPB1				X	Oncogenesis and resistance to various anti-cancer therapies.	(193)
7q11.2	AUTS2				X	This gene is expressed in in breast cancer tissues.	(133)
7q21	AKAP9	X				P That assembles protein kinases on the centrosome.	(194)

7q21	DMTF1	X				Transcriptional activator. Promotes p53/TP53-dependent growth arrest.	(195)
7q32	HIPK2	X				Tumor suppressor gene, breast cancer.	(196)
7q36	MNX1	X				Putative transcription factor involved in pancreas development and function.	(197)
7q36	MLL3	X				MLL3, in coordination with ERs, play critical roles in transcriptional regulation of *HOXC10* in the presence of estrogen.	(197)
8p22	MTUS1	X		X		Tumor suppressor gene.	(198)
8p23	CTSB	X		X		Induces angiogenesis, invasion and metastasis in breast cancer.	(199)
8p23	CSMD1	X		X		Tumor suppressor gene, poor survival in breast cancer.	(176); (200)
8p23	FEZ1	X		X		Tumor suppressor gene, lost in breast tumors.	(201)
8p23	DLC1	X		X		Tumor suppressor gene, breast cancer.	(201)
9p24	JAK2	X		X		Non-receptor protein tyrosine kinase.	(176)
9p24	RLN2	X		X		Development of mammary gland. Invasion in breast cancer.	(202)
9p24	KANK1	X		X		Tumor suppressor gene, breast cancer.	(176)
9p24	JMJD2C	X		X		Demethylase, breast cancer.	(176)
10p11.2	ABI1	X				Plays a role in the progression of breast cancer.	(203)
11p15	HRAS	X	X			Signal transduction, tumor aggressiveness in breast cancer.	(204)
11p15	CTSD	X	X			Over-expressed by breast cancer cells and involved in invasion and metastasis.	(133)
11p15	CD151	X	X			Implicated in motility, invasion, and metastasis of cancer cells.	(205)
11p15	RRM1	X	X			Tumor suppressor gene, DNA repair.	(206)
11p15	MMP26	X	X			Involved in the induction of migration and angiogenesis.	(176)

11p15	CDKN1C	X	X			Negative regulator of cell cycle.	NCBI.(133)
11q23	ATM		X			Tumor suppressor gene, DNA repair.	(207)
11q23	CRYAB		X			Key component in the activation of the intracellular autocrine VEGF pathway.	(208)
11q23	ETS1		X			Oncogene. Transcription factor that regulates the expression of genes involved in tumor progression and metastasis.	(209)
11q23	CCND1		X			Cell cycle G1/S transition, tumorigenesis in various carcinomas.	(210)
11q23	PGR		X			Hormone receptor. Estrogen signal transduction.	(133)
15q10	BUB1B			X		Mitotic spindle checkpoint, chromosomal instability in breast cancer.	(211)
15q15	THBS1			X		Invasion, metastasis, angiogenesis.	(133)
15q26.3	IGF1R			X		Involved in the induction of cell growth and survival control.	(212)
17q24	BIRC5		X			Apoptosis inhibition	(133)
18q21.1	SMAD4	X				Tumor suppressor gene. Plays a pivotal role in mediating antimitogenic and proapoptotic effects of TGF-β.	(176); (197)
18q21.1	BCL2	X				Antiapoptotic gene. Over-expressed in breast cancer.	(176)
18q21.2	DCC	X				Tumor suppressor gene. Frequently mutated or downregulated in cancer.	(213)
19q13	ATF5				X	Cell cycle progression, breast cancer.	(214, 215)
19q13	LILRA6				X	Receptor for class I MHC antigens, breast cancer.	(176)
19q13	CYP2A6				X	Metabolism of pharmaceutical drugs, directly induced by estradiol.	(216)
19q13	TGF-β1				X	Involved in tamoxifen resistance in breast cancer.	(201, 217-219)
19q13	CEACAM1				X	Suppresses the tumorigenicity of breast cancer cells. It is down-regulated in around 30% of breast cancers.	(220, 221)

20q11.22	E2F1	X				Tumor-suppressor gene.	(222)
20q13.1	CDH4	X				Cell adhesion proteins, breast cancer.	(176)
20q13.1	MMP9	X				Matastasis and cancer cell invasion, breast cancer.	(223)
20q13.31	AURKA	X				Induces cell proliferation, plays a role in tumor development and progression.	(224)
22q13	ATF4			X		Adaptation of cells to stress factors, multidrug resistant gene.	(225)
22q13	XRCC6			X		Apoptosis induction.	(197)

In MCF7 (ER+/HER2-) after E2 treatment, a high induction of chromosomal alterations was observed; however, only some were stable throughout the time, whereas those that were unstable disappeared at 96 hours. These results are indicative of a clonal selection of cells that have acquired chromosomal abnormalities advantageous for cell proliferation and cell survival. Chromosome 7 was frequently the most altered; the affected regions include add(7)(q36), del(7)(p22), del(7)(q21) and del(7)(q32). Alterations of chromosome 7 are among the most frequent cytogenetic abnormalities found in primary human breast tumors (226). Located on 7q21 are genes with key roles in the assembly of protein kinases to the centrosome (*AKAP9*) or in the growth arrest (*DMTF1*). Deletions in 7q32 have been detected in 2–5% of breast carcinomas; in this locus is located the *HIPK2* gene, a potential tumor suppressor involved in breast cancer development. Other genes located in these chromosomal region, are *ABI1* (10p11.2), which encodes a protein that inhibits cell growth and is deleted in the majority of solid tumors, including breast and gastric cancer (203); *HRAS*, *CDKN1C, RRM1, MMP26, CD151, CTSD* are located on 11p15. *CTSD* takes part in invasion and metastasis processes of breast cancer (125-129). *CD151* has been postulated as a potential prognostic marker and therapeutic target in the treatment of estrogen-dependent breast cancer patients (205) (Table 18)

Furthermore, TAM in MCF7 induces different abnormalities in respect to those observed after E2 treatment. These chromosomal abnormalities could be affecting cell viability considering the reduction in cell proliferation compared to control cells. As further noted, only some alterations persist at all time points evaluated during TAM treatment. The altered chromosomal region, 7p22, contains important genes associated with breast and other cancers, including: *ACTB, FSCN1, SDK1* and *GPR30* on 7p22, which have a role in cell migration, invasion, metastasis and drug resistance (167, 176). Others, such as *CTSB, CSMD1, FEZ1* and *DLC1* on 8p23, have a role in angiogenesis, metastasis and poor survival in

breast cancer (176, 199-201). Additionally, *JAK2, RLN2, KANK1* and *JMJD2C* on 9p24 have been previously correlated with invasion in breast cancer (176, 202).

In T47D (ER+/HER2-) after E2 treatment, few new alterations were observed, some of which were constant in the time and present at high frequency while others were causal and disappeared in the late time evaluated. In summary, the new chromosomal alterations do not affect cell viability but may provide selective advantages for cell proliferation and survival. All of these altered regions have genes, such as *ATM, CRYAB, ETS1* and *CCND1* on 11q23 and *BIRC5* on 17q24 (Table 18), involved with the development of breast cancer (207-210). An alteration on 11p15 was also observed in MCF7 cells. TAM treatment did not induce new structural chromosomal abnormalities in T47D cells.

E2 and TAM in BT474 (ER+/HER2+) caused stable chromosomal abnormalities at all time points studied, some of which were significantly different while others had low frequency and disappeared at 96h. These stable chromosomal alterations apparently provide to the cells a selective advantage for growth over control cells and could eventually lead to the selection of tumor cells with high proliferative capacity, thus promoting clonal expansion. On the other hand, the chromosomal instability observed early (24h) could have influenced the emergence of new favorable subpopulations, in comparison to control cells, with additional karyotype alterations, including del(11)(p15) and -12 (after E2 treatment) and del(3)(p13) and der(9)t(7;9)(?;p24) (after TAM treatment). Chromosome 3 was the most affected by structural abnormalities after E2 and TAM treatments, with alterations that include: add(3)(p21), add(3)(q13), del(3)(p13) and der(3)t(3;8)(p14;?). Interstitial deletions of the short arm of this chromosome have been described as a recurrent change in breast carcinomas by Pandis et al. 1993 (227) and have since turned out to be the single most common structural rearrangement. Several genes involved in breast carcinomas have been identified at this chromosome arm (3p14), including: *FOXP1*, *LRIG1* and *FHIT* implicated in the resistance to TAM (188-191). Another chromosomal alteration after TAM treatment is der(22)t(20;22)(?;q13)t(16;20)(?;?). The chromosomal region 22q13 contains numerous genes of potential interest in cancer development, among which are: *ATF4, MMP9, AURKA, SERHL2, LARGE* and *XRCC6* (176, 197, 225) (Table 18).

In SKBR3 (ER-/HER2+) cells, after E2 and TAM, was observed the induction and selection of cell populations with stable chromosomal alterations that were seemingly, beneficial to the clonal evolution of the cell population. The karyotype became more complex with the appearance of new chromosome 1 abnormalities - dic(1;19)(p11;q13) and i(1)(q10) - at all time points. The effect was hardest on chromosome 1, leading to a complete loss of integrity of its short arm and subsequent translocation to other chromosomal regions. Chromosome 1 is recurrently altered in a number of human malignancies (228). Gains at 1q is one of the most common copy number alterations seen in breast cancer (184, 185) and

has been involved in early stages of disease development and high-grade tumors (229, 230). In the affected regions are located the genes *BCAR3* (1p22), *AKT3* (1q44), *CENPF* (1q41) and *ENAH* (1q42), which have been related to aneuploidy, chromosomal instability, larger tumor size and anti-estrogen resistance; these genes are being postulated as good biomarkers to determine increased cell proliferation of breast cancer (180-182). Additional alterations detected were on chromosomal regions 7q11.2 and 19q13. The 7q11.2 region contains the *LIMK1* and *HSPB1* genes, which are implicated in cancer development, oncogenesis and resistance to various anti-cancer therapies (193, 197). The 19q13 region contains the genes *TGF-β1*, associated with TAM resistance (201, 217, 218) and *CYP2A6,* which is implicated in the metabolism of pharmaceutical drugs (Table 18). The chromosomal regions observed altered after treatments in SKBR3 cells, have genes reported to be expressed at a higher level in ER- breast cancer and have been related with the presence of aneuploidy (231, 232).

These results suggest that E2 and TAM induce in all cell lines analyzed chromosomal abnormalities that could lead to the activation of important genes implicated in the acquisition of proliferation advantages, cell survival, resistance to anti-cancer therapy (TAM) and DNA damage repair.

Nevertheless, the most widely acknowledged mechanism of E2 carcinogenicity is mediated by its specific nuclear receptors (ERα, ERß) (233). However, we have seen that, in the ER- SKBR3 cell line, many chromosomal abnormalities were induced after E2 and TAM treatment. This chromosomal damage could be attributed to the presence of the estrogen transmembrane receptor, GPCR30, which is expressed in this cell line (166). In fact, recent research has reported that TAM has high binding affinities to this receptor (GPCR30) and mimics the actions of E2 (234); thus, the chromosomal damage observed after TAM addition could be related to it (Figure 39). The identification of this distinct class of GPCR-like steroid membrane receptors suggests a role for GPCR30 in non-classical steroid hormone actions. These results could indicate that E2 and TAM may have a mutagenic action through a genotoxic, non ER-mediated mechanism (2, 5, 47, 52, 55, 57).

On the other hand, the observed increase in the frequency of some preexisting chromosomal abnormalities after E2 and TAM treatment, demonstrated that the cells, which contain these rearrangements had a proliferation and survival advantage over the other ones, as it increased after treatments.

We observed that HER2+ cells (BT474) showed a similar response when were treated with E2 or TAM, as since some from the same chromosomal aberrations were induced after the addition of these agents. A similar behavior was also observed in SKBR3 cells. In *HER2+* cells, the number of chromosomal abnormalities induced after E2 and TAM treatment was higher than HER2- cells. This behavior could indicate not only that the response of these cell lines to E2 and

TAM is modified by the presence of HER2 but also that other signaling pathways different from those mediated by ER could be involved.

TAM induced similar abnormalities in MCF7 and BT474 cells, including del(1)(p22) and del(3)(p13); however, the frequency of these aberrations was different for them. The induction of the same chromosomal alteration in more than one cell line could indicate that E2 and TAM cause chromosomal damage through the same signaling pathway but triggers multiple cellular responses that lead to heterogeneous gene alteration. This behavior can explain the high intratumoral and intertumoral heterogeneity observed in breast cancer characterized by a high diversity of clonal abnormalities, which gives specific properties to cells.

The induction of chromosomal abnormalities by E2 and TAM, observed in this study, could be explained by two mechanisms. First, ER-mediated DNA replication blocks may cause chromosomal breaks and lead to translocation or large deletion (2-4). Second, the oxidative metabolism of E2 and TAM can produce DNA adducts, which generate mutations and chromosomal aberrations (2, 54-57) (Figures 36 – 39)

In summary, E2 and TAM exposure could lead to the acceleration of the emergence of chromosomal instability and selection of new cell populations with stable chromosomal abnormalities. Such alterations could provide to cells proliferative and survival advantages and could allow the cell population to evolve and become resistant to therapy. These results are consistent with the perception that responsiveness in breast cancer is multifaceted, involving ER dependent and independent pathways. In fact, E2 and TAM induce genomic alterations that are similar to those observed in primary breast cancer, as reported recently (176, 197) (Annex 11).

The induction of chromosomal alterations by E2 and TAM indicates that a careful assessment of the risk and the benefit of E2 and TAM administration should be considered.

4.5 CONCLUSIONS

Our results demonstrated that the exposure at low dose to E2 and TAM increases chromosomal aberrations and produces specific chromosomal abnormalities independent from ER status, and causes alterations in cell proliferation in ERα+ cell lines. This is a genotoxic effect is higher in those cell lines with *HER2* gene amplification/overexpression than HER2- cells. SKBR3 cells (ER-) were found to be sensitive to TAM, exhibiting an increase in chromosomal aberrations. Thus, these results could provide insight into the mutagenesis that may be induced by E2 or TAM associated with clinical treatments. Breast cancer cell lines should provide a useful model, not only to discover and characterize chromosomal abnormalities

induced by drug exposure, but also to study the mechanisms underlying genomic instability.