

Short-term anastrozole therapy reduces Ki-67 and progesterone receptor expression in invasive breast cancer: a prospective, placebo-controlled, double-blind trial

Andre Mattar · Angela Flávia Logullo · Gil Facina ·
Suely Nonogaki · Fernando Augusto Soares ·
Luiz Henrique Gebrim

Received: 26 June 2010 / Accepted: 16 September 2010 / Published online: 1 October 2010
© Springer-Verlag 2010

Abstract

Purpose The objective of this study was to compare Ki-67, Bcl-2, Bax, Bak, ER, and PgR expression in post-menopausal women with ER-positive invasive breast cancer (IBC) before and after short-term hormone therapy (HT) with either tamoxifen or anastrozole in order to identify a possible biomarker profile associated with hormone resistance.

Methods Fifty-eight patients with palpable IBC were assigned to receive neoadjuvant therapy with either anastrozole, placebo, or tamoxifen for 26 days prior to surgery. Tissue microarray blocks were constructed from pre- and post-treatment biopsy samples and used for immunohistochemical analysis. Biomarker (Ki-67, Bcl-2, Bax, Bak, ER, and PgR) levels were assessed semiquantitatively using the Allred score. A statistical analysis was performed using general estimating equations (GEE) and analysis of variance (ANOVA) with a significance level of 0.05.

Results There was a significant reduction in PgR scores from baseline (mean, 4.22) to post-treatment (mean, 1.94) in the anastrozole group, but only a non-significant trend toward an increase in PgR scores was found in the tamoxifen group. There was a significant reduction in Ki-67 scores from baseline (mean, 3.61) to post-treatment (mean, 2.56) in the anastrozole group ($P = 0.01$), but only a non-significant trend toward a reduction in Ki-67 scores was found in the tamoxifen group.

Conclusions There was a significant reduction in PgR and Ki-67 expression in the group treated with anastrozole. In the present study, the short-term HT was not associated with changes in apoptosis-related protein levels, regardless the type of drug used.

Keywords Anastrozole · Breast cancer · Ki-67 · Estrogen receptor · Progesterone receptor · Short-term hormone therapy

A. Mattar (✉) · G. Facina · L. H. Gebrim
Department of Gynecology, Federal University of São Paulo (UNIFESP), Rua Napoleão de Barros, 715 - 7°.andar, CEP 04024-002 São Paulo, SP, Brazil
e-mail: mattar.andre@gmail.com

A. F. Logullo
Department of Pathology, Federal University of São Paulo (UNIFESP), São Paulo, SP, Brazil

S. Nonogaki
Laboratory of Immunohistochemistry, Adolfo Lutz Institute, São Paulo, SP, Brazil

F. A. Soares
Department of Pathology, Antonio Prudente Foundation, São Paulo, SP, Brazil

Abbreviations

ANOVA	Analysis of variance
ER	Estrogen receptor
GEE	General estimating equations
HT	Hormone therapy
IMPACT trial	Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen trial
IBC	Invasive breast cancer
PgR	Progesterone receptor
SD	Standard deviation
SERM	Selective estrogen-receptor modulator
UNIFESP	Universidade Federal de São Paulo
WHO	World Health Organization

Introduction

With the advent of hormone therapy (HT), the assessment of prognostic factors including estrogen-receptor (ER) and progesterone-receptor (PgR) levels has been associated with about a 30% reduction in breast cancer mortality among tamoxifen users (EBCTCG 1998). Primary or neoadjuvant chemotherapy and HT have been used not only with the purpose of increasing eligibility for breast-conserving therapy with tumor downstaging, but especially as a predictive tool for clinical response (Dowsett 2003). The major limitations of adjuvant systemic therapy are progressive, functional, and genetic alterations induced by drugs in tumor cells that may lead to hormone resistance. Usually, hormone resistance is identified only in the event of distant metastases or regional recurrence, since the primary tumor is no longer present for the monitoring of responses to treatment (Macaskill et al. 2006).

Sustained exposure to endogenous or exogenous estrogen is a well-established cause of breast cancer (Colditz 1998; Hankinson et al. 2004). At least 70% of breast cancers are classified as ER-positive breast cancers (Harvey et al. 1999), and interfering with estrogen action has been a mainstay of breast cancer treatment for more than a century. Early therapies included surgical removal of the ovaries, but the synthesis of competitive inhibitors of estrogen-ER binding during the 1970s led to the first, and to date most successful, targeted cancer therapy: the selective ER modulator (SERM) tamoxifen (Jensen and Jordan 2003). Subsequently, other new, effective endocrine therapies have been developed that target estrogen synthesis, such as aromatase inhibitors (Johnston and Dowsett 2003) including anastrozole.

It is now clear that the third generation of aromatase inhibitors is more effective than tamoxifen in both the neoadjuvant and adjuvant settings for hormone receptor-positive breast cancers (Smith and Dowsett 2003). Anastrozole provides a better clinical response than tamoxifen in neoadjuvant therapy (Dixon et al. 2000). Correlative studies on neoadjuvant therapy provide an opportunity to better understand the molecular determinants of this differential efficacy, and to assess the possibility that there may be identifiable subgroups that derive similar efficacy from tamoxifen, despite aromatase inhibitors have been shown to be more effective than tamoxifen (Dowsett et al. 2005a).

However, tumors do not always respond to drug treatment according to tumor stage and clinical or morphological prediction (Harvey et al. 1999). One-third of women treated with tamoxifen for 5 years will have recurrent disease within 15 years (EBCTCG 2005) and so endocrine-resistant disease may represent up to one-quarter of all breast cancers (Musgrove and Sutherland

2009). Therefore, the major challenge for the successful treatment of breast cancer is the development of more specific biomarkers that predict therapeutic response to endocrine therapy.

Primary HT has the potential benefit of interfering with tumor cell kinetics, inhibiting uncontrolled proliferation and increasing apoptosis. Therefore, primary HT may provide satisfactory clinical response and facilitate surgery, and have the ability to predict response to adjuvant therapy (Dowsett et al. 2007).

Biomarkers of cell proliferation and apoptosis have been studied in the search for indicators that may allow early prediction of outcome in breast cancer after a short-term drug exposure (neoadjuvant HT) (Dowsett et al. 2007). Patients with breast cancer who received short-term therapy with aromatase inhibitors or tamoxifen showed reduced levels of Ki-67 (Dowsett et al. 2006). This reflects an early change in cell kinetics mediated by modulation of estrogen pathway, although its clinical significance is not clear yet (Hengartner 2000).

Bcl-2 is one of most studied apoptosis biomarkers and has been described as an anti-apoptotic protein (Hengartner 2000). Recent reviews point out that Bcl-2 overexpression is correlated with tumor differentiation, higher positivity for ER/PgR, and longer survival time (Jager et al. 2002; Konstantinidou et al. 2002). However, it remains unclear whether short-term HT results in loss of apoptosis control or even whether ER modulation significantly affects apoptotic genes. Therefore, early changes in ER genes could be a predictive tool to assess the effectiveness of short-term HT. Bax and Bak are proteins from the Bcl-2 family with pro-apoptotic functions that may act as tumor suppressors (Krajewski et al. 1999). In patients with metastatic breast cancer, a low expression of these proteins was found to be associated with a worse prognosis (Konstantinidou et al. 2002). Nevertheless, there are no reports on the predictive value of endocrine response and molecular biomarkers of apoptosis (Jager et al. 2002).

A prospective double-blind, placebo-controlled study was conducted to address the role of these biomarkers in the primary breast cancer treatment. The study was designed to evaluate the effect of anastrozole and tamoxifen on primary infiltrating breast carcinomas for 26 days by immunohistochemical assessment of ER, PgR, Ki-67, Bcl-2, Bax and Bak expressions pre- and post-treatment.

Materials and methods

The study was approved by the Human Investigation Committee of Universidade Federal de São Paulo (UNIFESP), Brazil, under the process number CEP 1153/05, and registered with ClinicalTrials.gov, number NCT01016665.

It was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Written informed consent was obtained from all patients prior to their inclusion in the study, and anonymity was assured.

Fifty-eight patients with invasive breast carcinomas were enrolled at Pérola Byington Hospital and São Paulo Hospital between April 2005 and June 2008. All patients had ER- and/or PR-positive tumors and underwent definitive surgical treatment (radical mastectomy or quadrantectomy with axillary dissection) in a mean period of 26 days after the incisional biopsy, which confirmed the diagnosis.

The exclusion criteria were patients with endocrine disease, negative expression for estrogen and/or progesterone receptors, history of thromboembolism, and those who were currently receiving HT or had previously received any treatment for breast cancer (surgery, radio- and/or chemotherapy), had been pregnant in the last 12 months before diagnosis, did not comply with the prescribed medication regimen, or postponed surgery.

The patients were randomized into three groups to receive a daily dose of either tamoxifen 22 mg (tamoxifen group, $n = 15$) or anastrozole 1 mg (anastrozole group, $n = 18$), or placebo (placebo group, $n = 25$) for 26 consecutive days prior surgery (Fig. 1).

The first tumor sample was obtained at the time of diagnosis by incisional biopsy performed in an outpatient facility, using local anesthetic without epinephrine (2% lidocaine). Only one biopsy fragment was obtained per patient. A second tumor specimen was obtained from all patients during definitive surgery under general anesthesia. Both tumor samples were processed using the same paraffin-embedding technique described in the next paragraph.

Histology and immunohistochemistry

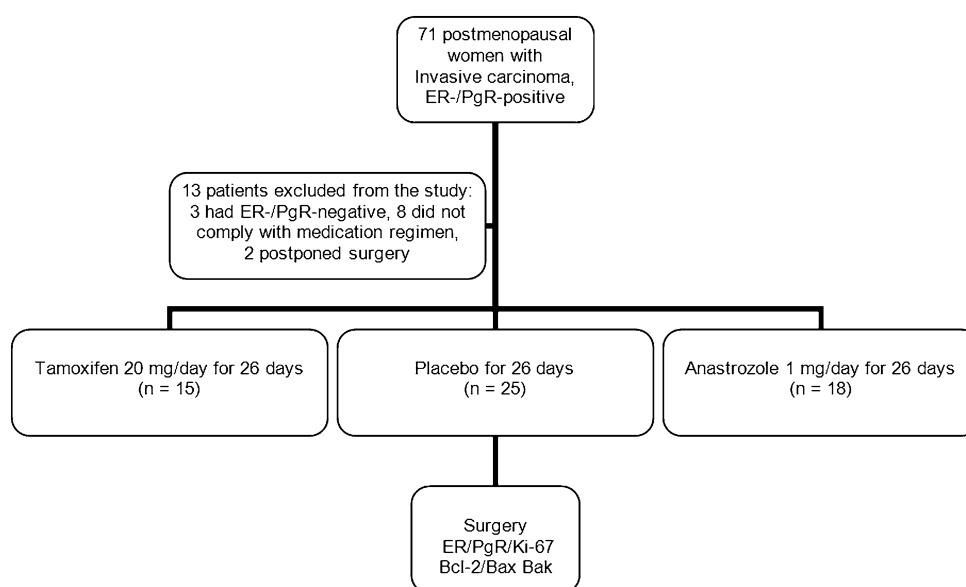
Formalin-fixed, paraffin-embedded tumor biopsy samples obtained at the time of diagnosis and during definitive surgery from 33 patients treated with either tamoxifen or anastrozole and of 25 placebo patients were retrieved from the pathology files of our institution.

Samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Specimen pairs were cut into 4- μ m sections, mounted onto lysine-coated slides, stained with hematoxylin-eosin, and examined to confirm the diagnosis of carcinoma, according to the World Health Organization (WHO) criteria (1981).

Sections were dewaxed by heating at 60°C for 10 min and washing 3 times in xylene, and rehydrated in graded alcohol solutions. Next, the sections were submitted to heat-induced antigen retrieval by pressure cooking in 10 mM/l citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with four 5-min immersions in 3% hydrogen peroxide, followed by washing with distilled water and 0.01 M phosphate-buffered saline (PBS) at pH 7.4.

All biomarkers were analyzed after immunohistochemical staining with the antibodies. The sections were incubated with specific antibodies against the biomarkers in a humid chamber for 2 h at room temperature and then were washed with PBS (pH 7.4). The reaction was amplified with the addition of a secondary antibody (LSAB + System-HRP kit, Code K0690, DakoCytomation) and incubated for 20 min, after which streptavidin-peroxidase complex was added and incubation was continued for another 20 min at room temperature. Following incubation, the sections were washed with PBS (pH 7.4). After

Fig. 1 Study design



incubation with diaminobenzidine for 5 min at 37°C, the sections were counterstained with Harris hematoxylin for 2 min. Lymph node sections known to be positive for each marker were used as positive controls. Sections used as negative controls were incubated with PBS instead of primary antibody. All cells stained brown were considered positive.

Biomarker scoring

All slides were examined by two investigators (AFLW and AM) and scored semi-quantitatively, according to Allred's criteria, using two parameters: the number of stained cells and color intensity of the stain. With regard to the intensity of staining, the scores ranged from 0 to 3. A scale from 0 to 5 was used to represent the proportion of stained cells with respect to the total number of cells. These parameters were used to evaluate each immunoreaction. The sum of these two partial scores resulted in an overall score, where 0 and 2 indicate that no cell was stained. Scores ranging from 3 to 8 corresponded to positive cases, meaning that all cases with an overall score greater than 2 were considered positive.

The percentage of staining was also used in this study, and for that we used a digital image analysis system, which consists in a microscope NIKON JAPAN LABOPHOT-2, Serial No. 440 021, with lens NIKON 40X, coupled to a digital color video camera SDC-312ND POWER: DC12 V 3 W brand Samsung Techwin CO. LTD Serial No. R4602662, which transmits the image to a computer with Intel processor with 4 GB RAM, and 500 GB hard drive, equipped with an Ethernet scanner, with software WinTV32 and Paint in Windows XP environment. The immunoreaction was assessed by a 40× objective (increased end-400×) considering the positive cell whose nucleus exhibited brown color characteristic.

Statistical analysis

Descriptive statistics was used to summarize the sample characteristics at baseline (mean, standard deviation (SD), minimum, median, maximum, frequency and percentage). The Kruskal–Wallis test was used to evaluate clinical characteristics.

Changes over time and differences between groups were evaluated with repeated-measures analysis of variance using rank transformation.

The Student's *t*-test was used to compare mean scores for the control group with scores for the tamoxifen and anastrozole groups.

Fisher's exact test was used to compare differences in ER/PgR, Ki-67, Bcl-2, Bax, and Bak expressions (negative expression, score = zero; positive expression, score ≠ zero).

All tests were performed at a significance level of 0.05 ($P \leq 0.05$).

Results

The mean patient age was 66 years at diagnosis and 48 years at menopause. The mean tumor size was 4 cm, and 76% of the tumors were classified as grade II invasive ductal carcinoma. Most of the patients had stage II (stage IIA, 60%; stage IIB 25%) tumors with ER-positive ($n = 98\%$) and PgR-positive ($n = 69\%$) status (Table 1). There were no significant differences among groups regarding clinical characteristics, and therefore the sample was considered homogeneous.

Mean pre- and post-treatment Allred scores for ER were, respectively, 6.60 (82%) and 6.76 (84%) for the placebo group, 7.22 (90%) and 6.44 (80%) for the anastrozole group, and 7.00 (87%) and 6.76 (84%) for the tamoxifen group (Table 2). Despite the slight reduction in post-treatment ER scores compared to baseline in patients treated with anastrozole for 26 days, no significant differences were found ($P = 0.52$).

Post-treatment Allred scores for PgR were significantly lower in the anastrozole group than in the other groups ($P = 0.01$). Mean pre- and post-treatment PgR scores were, respectively, 5.12 (64%) and 5.00 (62%) in the placebo group, 4.22 (52%) and 1.94 (24%) in the anastrozole group, and 5.40 (67%) and 6.73 (84%) in the tamoxifen group (Table 3). Variation in mean PgR scores is best represented in Fig. 2. The anastrozole group showed a significant post-treatment reduction in PgR scores compared to baseline. The tamoxifen group showed a slight increase in PgR expression after treatment, but not statistically significant.

The anastrozole group showed a significant reduction ($P = 0.01$) in post-treatment Allred scores for Ki-67 compared to baseline (Fig. 3), while the tamoxifen group showed a trend toward significance ($P = 0.06$). The mean pre- and post-treatment Ki-67 scores were, respectively, 2.68 (33%) and 2.92 (36%) in the placebo group, 3.61 (45%) and 2.56 (32%) in the anastrozole group, and 3.53 (44%) and 2.20 (27%) in the tamoxifen group (Table 4).

There was no significant relationship among pre- and post-treatment Allred scores for Bcl-2, Bax, and Bak between or within groups.

Discussion

Primary or neoadjuvant therapy is increasingly used as a predictor of outcome. However, there are few studies on

Table 1 Patient characteristic

Drug	Age	Tumor size	Nodal status	Pathological stage	HER-2 status	Grading SBR	Nuclear grading	Vessel invasion	Necrosis	Desmoplasia
Anastrozole	42	2,5	1/10	T2N1a	2	2	2	0	0	2
	69	7	5/18	T3N2a	0	1	1	1	0	2
	71	3,5	0/15	T2N0	0	2	1	0	0	1
	73	3,8	1/6	T2N1a	1	1	2	1	0	2
	66	5,5	7/17	T3N2a	2	1	1	1	1	2
	64	3	0/12	T2N0	0	2	1	0	0	2
	54	6	5/17	T3N2a	0	1	1	1	0	2
	71	2,8	1/11	T2N1a	0	2	1	0	0	2
	67	3	11/18	T2N3a	0	2	2	0	0	2
	60	6	12/28	T3N3a	0	1	1	0	0	0
	69	3,2	1/11	T2N1a	2	2	1	0	0	2
	49	4,6	8/18	T2N2a	3	2	1	1	0	0
	54	5,5	0/11	T3N0	2	2	2	0	0	1
	68	5,8	2/16	T3N2a	0	3	3	0	2	0
	64	1,8	0/2	T1cN0	0	2	2	0	0	1
	67	5,5	13/23	T3N3a	0	2	2	0	0	2
	54	3,5	0/13	T2N0	0	3	2	0	0	1
	63	4	0/9	T2N0	0	3	2	0	0	0
Placebo	77	6	5/15	T3N2a	3	3	3	0	1	2
	64	4	1/20	T2N1a	1	2	2	0	0	2
	65	3,5	0/1	T2N0	1	3	2	0	0	1
	83	5	1/17	T2N1a	3	2	1	0	0	1
	66	8	3/13	T3N1a	3	2	2	0	0	0
	74	4	0/14	T2N0	0	3	2	1	1	0
	67	4,5	6/14	T2N2a	0	2	2	0	0	1
	79	3,7	2/14	T2N1a	0	2	1	1	0	1
	85	3,8	1/8	T2N1a	0	1	1	0	0	0
	56	6,5	0/24	T3N0	0	2	2	0	0	2
	70	2,5	0/14	T2N0	0	2	3	0	0	2
	70	1,8	0/18	T1cN0	0	2	2	0	0	1
	73	4,5	6/15	T2N2a	0	2	2	1	0	1
	63	4,1	1/17	T2N1a	1	2	1	0	1	1
	59	3,3	0/18	T2N0	1	2	2	0	0	0
	63	3	0/10	T2N0	2	1	2	0	0	0
	71	3,5	3/10	T2N1a	0	2	2	0	0	1
	68	3	0/11	T2N0	0	2	2	1	2	2
	66	1,5	3/13	T1cN1a	2	2	2	0	0	1
	63	2	0/6	T2N0	2	2	2	1	0	1
	62	3	4/13	T2N2a	0	2	2	0	0	1
	72	3,5	2/30	T2N1a	0	2	2	0	0	1
	56	2,5	0/20	T2N0	0	1	2	0	0	2
	76	2	0/17	T1cN0	0	2	2	0	0	2
	76	5	11/15	T2N3a	0	2	2	0	2	1

Table 1 continued

Drug	Age	Tumor size	Nodal status	Pathological stage	HER-2 status	Grading SBR	Nuclear grading	Vessel invasion	Necrosis	Desmoplasia
Tamoxifen	43	3	0/13	T2N0	0	1	1	1	0	1
	82	4	0/11	T2N0	1	2	2	0	0	1
	87	6,4	1/19	T3N1a	2	2	2	0	0	2
	55	3,2	1/15	T2N1a	0	1	2	0	0	0
	76	4	0/13	T2No	0	1	1	1	0	2
	72	5,2	7/15	T3N2a	3	1	1	0	0	2
	70	5,5	1/16	T3N1a	0	2	2	0	0	2
	85	3,2	0/14	T2N0	2	2	1	1	0	1
	61	2	2/17	T1cN1a	1	2	2	0	2	1
	56	2,8	0/19	T2N0	0	3	2	0	2	1
	73	3	8/17	T2N2a	2	2	2	0	0	0
	50	7,5	5/27	T3N2a	0	2	2	0	2	0
	48	2	0/12	T1cN0	2	3	2	0	0	1
	64	2	0/16	T1cN0	0	2	2	0	0	2
	60	3,7	8/21	T2N2a	0	3	3	1	0	2

hormone therapy and outcome in the literature (Ellis et al. 2008). The sole evaluation of clinical response to neoadjuvant therapy has proven inadequate to determine tumor stage and predict response and prognosis in breast cancer after a short-term exposure to drugs due to the formation of tissue edema and hematoma following biopsy; in this situation, a period of time of about 2–3 months is required before clinical response can be assessed.

There is a growing interest in the study of biomarkers as early predictors of hormone resistance, and of clinical and pathological response (Colozza et al. 2005; Dixon et al. 2000; Dowsett 2004; Dowsett et al. 2001; Dowsett et al. 2007; Fisher et al. 1988; Geisler et al. 2001; Gompel et al. 2004; Harvey et al. 1999; Hayes 2008; Macaskill et al. 2006; Molino et al. 1997; Musgrove and Sutherland 2009; Pinto et al. 2001; Rose et al. 2003; Trihia et al. 2003; Whitfield et al. 2006; Pankaj and Alastair 2006). However, the optimum time to evaluate biomarkers for tumor response (ER, PgR, apoptosis, and proliferation) is yet to be defined. Although cellular changes have been described in vitro after 24 h of drug exposure, Dowsett et al. (2005c) reported that after two weeks of hormone therapy, cellular changes are similar to those observed after 12 weeks of treatment. A treatment period of 26 days was chosen for this study because this is the average time needed to complete routine preoperative testing in most Brazilian institutions, justifying the use of placebo without ill consequences to the patients.

The effects of short-term hormone therapy on breast cancers were evaluated by comparing the expression of the biomarkers ER, PgR, Ki-67, Bcl-2, Bak, and Bax in the

treatment groups with that in the placebo group using the Allred scores. This scoring system has the advantage of providing an objective quantification of biomarker expression. Allred scores have been used in different studies on neoadjuvant endocrine therapy (Ellis et al. 2008; Harvey et al. 1999), permitting the direct comparison of our results with those from other studies.

In the present study, post-treatment ER expression did not vary significantly among the three groups, which is in agreement with the finds reported in the Immediate Pre-operative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) trial (Dowsett 2004). Possibly, ER-alpha alone is not an early predictor of response to short-term anastrozole and tamoxifen therapies. Further studies on ER-beta as an early predictor of response to short-term hormone therapy may be of great value (Dowsett et al. 2005a). Only patients treated with anastrozole had a significant reduction in PgR expression, which is in agreement with the literature (Dowsett 2004; Dowsett et al. 2005a; Ellis et al. 2008). Probably, the production of estrogen was consistently blocked and the expression of PgR was significantly reduced due to the mechanism of action of aromatase inhibitors (Miller et al. 2003). No significant reduction in PgR expression was found in patients treated with tamoxifen. Some patients showed an increase in PgR expression after treatment, which is in agreement with the findings of Dowsett et al. in the IMPACT trial (Dowsett 2004; Dowsett et al. 2005a; Dowsett et al. 2005c) and may be justified by the partial agonist effect of tamoxifen (a phenomenon called tamoxifen flare) in this initial phase of treatment.

Table 2 Mean pre- and post-treatment Allred scores for estrogen receptor (ER) in the placebo, anastrozole, and tamoxifen groups (in parenthesis the percentage of staining)

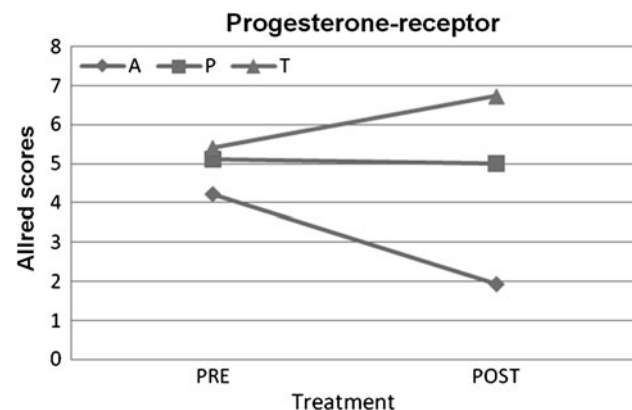
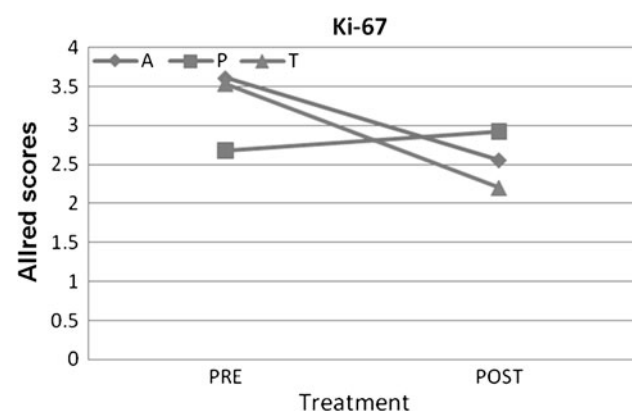
	Placebo	Anastrozole	Tamoxifen
ER pre-treatment	6.60 (82%)	7.22 (90%)	7.00 (87%)
ER post-treatment	6.76 (84%)	6.44 (80%)	6.76 (84%)

$P = 0.52$, analysis of variance (ANOVA)

Table 3 Mean pre- and post-treatment Allred scores for progesterone receptor (PgR) in the placebo, anastrozole, and tamoxifen groups (in parenthesis the percentage of staining)

	Placebo	Anastrozole ^a	Tamoxifen
PgR pre-treatment	5.12 (64%)	4.22 (52%)	5.40 (67%)
PgR post-treatment	5.00 (62%)	1.94 (24%)	6.73 (84%)

^a $P = 0.01$, analysis of variance (ANOVA)

**Fig. 2** Mean pre- and post-treatment Allred scores for progesterone-receptor expression in the anastrozole (A), placebo (P), and tamoxifen (T) groups**Fig. 3** Mean pre- and post-treatment Allred scores for Ki-67 expression in the anastrozole (A), placebo (P), and tamoxifen (T) groups

There was a significant reduction in Ki-67 expression in the anastrozole group, which is in agreement with the findings of Dowsett et al. (Dowsett et al. 2005c) who

Table 4 Mean pre- and post-treatment Allred scores for Ki-67 in the placebo, anastrozole, and tamoxifen groups (in parenthesis the percentage of staining)

	Placebo	Anastrozole	Tamoxifen
Ki-67 Pre-treatment	2.68 (33%)	3.61 (45%)	3.53 (44%)
Ki-67 Post-treatment	2.92 (36%)	2.56 (32%)	2.20 (27%)
P -value ^a	1.00	0.01	0.06

Pre- vs. Post-treatment

^a Bonferroni test

studied changes in Ki-67 expression in patients undergoing short-term therapy with anastrozole, tamoxifen or a combination of both, at 2 and 12 weeks of treatment (IMPACT trial). In the present study, tamoxifen therapy was not associated with a significant reduction in Ki-67 expression; only a non-significant trend toward a reduction was observed ($P = 0.06$). Curiously, although there was a slight increase in PgR expression in the tamoxifen group, cell proliferation measured by Ki-67 did not vary significantly, suggesting that proliferation and PgR expression are not part of the same pathway. Changes in Ki-67 expression after hormone therapy seems to be more strongly related to prognosis than to clinical response (Dowsett 2004; Dowsett et al. 2005a; Dowsett et al. 2005c), especially in node-negative patients (Dowsett and Dunbier 2008).

Inhibition of proliferation, detected by Ki-67 expression, may not result in regression of the primary tumor. Russo suggested that a reduction in the size of the tumor may not be related only to the number of epithelial cells destroyed but also to a reduction in the stromal edema (Dowsett et al. 2005c), although the effects of tamoxifen and anastrozole on stromal cells are not defined.

In the IMPACT and P024 trials, there was a linear correlation between PgR expression and cell proliferation (Ki-67) with reduction in Ki-67 expression in groups treated with tamoxifen and aromatase inhibitors (Dowsett 2004; Ellis and Ma 2007). In both studies, there was a significantly greater reduction in Ki-67 expression in the groups treated with aromatase inhibitor (Dowsett et al. 2005a; Ellis and Ma 2007). In the P024 trial, letrozole was more effective than tamoxifen regardless of the level of ER expression. In the IMPACT trial, there was a positive significant correlation between ER expression and degree of Ki67 suppression, which was more evident when comparing Ki-67 and ER expressions at 2 weeks of treatment; the suppression of Ki-67 was particularly lower in the tamoxifen group than in the anastrozole group for the lowest quartile of ER expression and for PgR-negative tumors. Our data suggest that anastrozole is more efficient than tamoxifen in suppressing Ki-67, regardless of the Allred score for ER and/or PR.

Stendahl et al. (Stendahl et al. 2004) reported that adjuvant tamoxifen therapy provided greater benefit to patients with tumors that were strongly positive for PgR than to patients with PgR-negative tumors. PgR negativity may be indicative of tamoxifen resistance in short-term drug response, and non-genomic mechanisms may be involved in the long-term outcome of patients with breast cancer. Tumor response to aromatase inhibitors is maintained even in PgR-negative tumors. In the present study, anastrozole, which blocks the genomic pathway, reduced Ki-67 expression after 26 days of treatment.

PgR expression is dependent upon the action of estrogen, or in another words, the expression of PgR can be used as a marker for estrogen-dependent cancers with a functional ER (Gruber et al. 2002). Based on this information, it can be inferred that a reduction in PgR and Ki-67 scores after 26 days of adjuvant treatment suggests a good response. The hormone-receptor scores obtained in this study after a short treatment period may lead to different interpretations.

Treatment with tamoxifen resulted in a decrease in Ki-67 expression, but differently from anastrozole, tamoxifen behaves as a partial estrogen agonist. Tamoxifen treatment increased PgR expression. The initial antiproliferative effect may be mediated by ER-beta or a different cellular mechanism mediated by the stroma (Dowsett et al. 2001; Dowsett et al. 2005b). Controversy exists as to whether PgR has an indirect effect on cell kinetics or is blocked by tamoxifen, since the conformation of this receptor allows binding (Jordan 1994).

No significant differences were found for the apoptotic proteins Bak, Bax, Bcl-2. Overexpression of anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L, has been related to chemoresistance in cancer, while a high level of proapoptotic proteins, such as Bax, promotes apoptosis and may be used as a predictor of response to chemotherapy (Krajewski et al. 1999).

Bcl-2 expression was positively correlated with ER and PgR expression in several studies (Leek et al. 1994; Silvestrini et al. 1994). However, this correlation was not observed in the present study. Since this is a complex phenomenon regulated by a large number of molecular mechanisms, controversial results have been reported regarding apoptosis biomarkers after hormone therapy for breast cancer (Dowsett et al. 2005a). No data on Bcl2, Bak, and Bax expression in breast cancer before and after neoadjuvant HT have been reported in the literature. Apoptosis in cancer cells is a late phenomenon, and the use of hormone therapy for 26 days is not a long enough period to regulate apoptosis.

Nevertheless, the estrogen pathway involving ERs is probably not the only pathway that controls apoptosis in breast carcinomas, since ER-negative tumors may be

positive for Bcl-2 and more sensitive to chemotherapy (Silvestrini et al. 1996).

In conclusion, our results showed the different effects of both the anastrozole and tamoxifen therapies on the epithelial cells. A reduction in PgR and Ki-67 positivity after neoadjuvant therapy with either anastrozole or tamoxifen suggests that these biomarkers are predictors of short-term response to HT (especially to anastrozole therapy) and may contribute to therapeutic specificity in the treatment of breast cancer. Apoptotic proteins (Bcl-2, Bak, and Bax) were not influenced by short-term HT.

Acknowledgments The authors acknowledge all the patients who gave their consent to participate in this trial. The authors declare that they have no competing interests. The authors also declare that they have no financial and/or non-financial competing interests (political, personal, religious, ideological, academic, intellectual, commercial, or any other) in relation to this manuscript. There was no external funding for this study.

Conflict of interest We declare that we have no conflict of interest.

References

- Colditz G (1998) Relationship between estrogen levels, use of hormone replacement therapy, and breast cancer. *J Natl Cancer Inst* 90(11):814–823. doi:10.1093/jnci/90.11.814
- Colozza M, Azambuja E, Cardoso F, Sotiriou C, Larsimont D, Piccart MJ (2005) Proliferative markers as prognostic and predictive tools in early breast cancer: where are we now? *Ann Oncol* 16(11):1723–1739. doi:10.1093/annonc/mdi352
- Dixon JM, Renshaw L, Bellamy C, Stuart M, Hoctin-Boes G, Miller WR (2000) The effects of neoadjuvant anastrozole (arimidex) on tumor volume in postmenopausal women with breast cancer: a randomized, double-blind, single-center study. *Clin Cancer Res* 6(6):2229–2235
- Dowsett M (2003) Preoperative models to evaluate endocrine strategies for breast cancer. *Clin Cancer Res* 9(1):502s–510s
- Dowsett M (2004) Biomarker investigations from the atac trial: the role of ta01. *Breast Cancer Res Treat* 87(1):11–18
- Dowsett M, Dunbier AK (2008) Emerging biomarkers and new understanding of traditional markers in personalized therapy for breast cancer. *Clin Cancer Res* 14(24):8019–8026. doi:10.1158/1078-0432.ccr-08-0974
- Dowsett M, Bundred NJ, Decensi A, Sainsbury RC, Lu Y, Hills MJ, Cohen FJ, Veronesi P, O'Brien MER, Scott T, Muchmore DB (2001) Effect of raloxifene on breast cancer cell ki67 and apoptosis. *Cancer Epidemiol Biomarkers Prev* 10(9):961–966
- Dowsett M, Ebbs SR, Dixon JM, Skene A, Griffith C, Boeddinghaus I, Salter J, Detre S, Hills M, Ashley S, Francis S, Walsh G, Smith IE (2005a) Biomarker changes during neoadjuvant anastrozole, tamoxifen, or the combination: influence of hormonal status and her-2 in breast cancer—a study from the impact trialists. *J Clin Oncol* 23(11):2477–2492. doi:10.1200/jco.2005.07.559
- Dowsett M, Elizabeth F, Deborah D, Ben H (2005b) The biology of steroid hormones and endocrine treatment of breast cancer. *Breast (Edinburgh, Scotland)* 14(6):452–457
- Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, Griffith C, Boeddinghaus I, Salter J, Detre S, Hills M, Ashley S, Francis S, Walsh G (2005c) Short-term changes in ki-67 during

- neoadjuvant treatment of primary breast cancer with anastrozole or tamoxifen alone or combined correlate with recurrence-free survival. *Clin Cancer Res* 11(2):951s–958s
- Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, Griffith C, Boeddinghaus I, Salter J, Detre S, Hills M, Ashley S, Francis S, Walsh G, A'Hern R (2006) Proliferation and apoptosis as markers of benefit in neoadjuvant endocrine therapy of breast cancer. *Clin Cancer Res* 12(3):1024s–1030s. doi:[10.1158/1078-0432.ccr-05-2127](https://doi.org/10.1158/1078-0432.ccr-05-2127)
- Dowsett M, Salter J, Hills M, Detre S, A'Hern R, Smith I (2007) Short-term presurgical treatment for all? *Breast Cancer Res* 9(Suppl 2):S13
- EBCTCG (1998) Tamoxifen for early breast cancer: An overview of the randomised trials. *The Lancet* 351(9114):1451–1467
- EBCTCG (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. *The Lancet* 365(9472):1687–1717
- Ellis M, Ma C (2007) Letrozole in the neoadjuvant setting: The p024 trial. *Breast Cancer Res Treat* 105:33–43
- Ellis MJ, Tao Y, Luo J, A'Hern R, Evans DB, Bhatnagar AS, Chaudri Ross HA, von Kameke A, Miller WR, Smith I, Eiermann W, Dowsett M (2008) Outcome prediction for estrogen receptor-positive breast cancer based on postneoadjuvant endocrine therapy tumor characteristics. *J Natl Cancer Inst* 100(19):1380–1388. doi:[10.1093/jnci/djn309](https://doi.org/10.1093/jnci/djn309)
- Fisher B, Redmond C, Fisher E, Caplan R (1988) Relative worth of estrogen or progesterone receptor and pathologic characteristics of differentiation as indicators of prognosis in node negative breast cancer patients: findings from national surgical adjuvant breast and bowel project protocol b-06. *J Clin Oncol* 6(7):1076–1087
- Geisler J, Detre S, Berntsens H, Ottestad L, Lindtjörn B, Dowsett M, Lønning PE (2001) Influence of neoadjuvant anastrozole (arimidex) on intratumoral estrogen levels and proliferation markers in patients with locally advanced breast cancer. *Clin Cancer Res* 7(5):1230–1236
- Gompel A, Chaouat M, Hugol D, Forgez P (2004) Steroidal hormones and proliferation, differentiation and apoptosis in breast cells. *Maturitas* 49(1):16–24
- Gruber CJ, Tschugguel W, Schneeberger C, Huber JC (2002) Production and actions of estrogens. *N Engl J Med* 346(5):340–352. doi:[10.1056/NEJMra000471](https://doi.org/10.1056/NEJMra000471)
- Hankinson S, Colditz G, Willett W (2004) Towards an integrated model for breast cancer etiology: the lifelong interplay of genes, lifestyle, and hormones. *Breast Cancer Res* 6(5):213–218
- Harvey JM, Clark GM, Osborne CK, Allred DC (1999) Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol* 17(5):1474
- Hayes D (2008) Markers of endocrine sensitivity. *Breast Cancer Res* 10(Suppl 4):S18
- Hengartner MO (2000) The biochemistry of apoptosis. *Nature* 407(6805):770–776
- Jager JJ, Jansen RLH, Arends JW (2002) Clinical relevance of apoptotic markers in breast cancer not yet clear. *Apoptosis* 7(4):361–365
- Jensen EV, Jordan VC (2003) The estrogen receptor. *Clin Cancer Res* 9(6):1980–1989
- Johnston SRD, Dowsett M (2003) Aromatase inhibitors for breast cancer: lessons from the laboratory. *Nat Rev Cancer* 3(11):821–831
- Jordan VC (1994) Molecular mechanisms of antiestrogen action in breast cancer. *Breast Cancer Res Treat* 31(1):41–52
- Konstantinidou AE, Korkolopoulou P, Patsouris E (2002) Apoptotic markers for tumor recurrence: a minireview. *Apoptosis* 7(5):461–470
- Krajewski S, Krajewska M, Turner B, Pratt C, Howard B, Zapata J, Frenkel V, Robertson S, Ionov Y, Yamamoto H, Perucho M, Takayama S, Reed J (1999) Prognostic significance of apoptosis regulators in breast cancer. *Endocr Relat Cancer* 6(1):29–40. doi:[10.1677/erc.0.0060029](https://doi.org/10.1677/erc.0.0060029)
- Leek RD, Kaklamani L, Pezzella F, Gatter KC, Harris AL (1994) Bcl-2 in normal human breast and carcinoma, association with oestrogen receptor-positive, epidermal growth factor receptor-negative tumours and in situ cancer. *Br J Cancer* 69(1):135–139
- Macaskill EJ, Renshaw L, Dixon JM (2006) Neoadjuvant use of hormonal therapy in elderly patients with early or locally advanced hormone receptor-positive breast cancer. *Oncologist* 11(10):1081–1088. doi:[10.1634/theoncologist.11-10-1081](https://doi.org/10.1634/theoncologist.11-10-1081)
- Miller WR, Anderson TJ, Evans DB, Krause A, Hampton G, Dixon JM (2003) An integrated view of aromatase and its inhibition. *J Steroid Biochem Mol Biol* 86(3–5):413–421
- Molino A, Micciolo R, Turazza M, Bonetti F, Piubello Q, Bonetti A, Nortilli R, Pelosi G, Cetto GL (1997) Ki-67 immunostaining in 322 primary breast cancers: associations with clinical and pathological variables and prognosis. *Int J Cancer* 74(4):433–437
- Musgrove EA, Sutherland RL (2009) Biological determinants of endocrine resistance in breast cancer. *Nat Rev Cancer* 9(9):631–643
- Pankaj GR, Alastair MT (2006) Cyclin d1 and breast cancer. *Breast (Edinburgh, Scotland)* 15(6):718–727
- Pinto AE, André S, Pereira T, Nóbrega S, Soares J (2001) Prognostic comparative study of s-phase fraction and ki-67 index in breast carcinoma. *J Clin Pathol* 54(7):543–549. doi:[10.1136/jcp.54.7.543](https://doi.org/10.1136/jcp.54.7.543)
- Rose C, Vtoraya O, Pluzanska A, Davidson N, Gershanovich M, Thomas R, Johnson S, Caicedo JJ, Gervasio H, Manikhas G, Ayed FB, Burdette-Radoux S, Chaudri-Ross HA, Lang R (2003) An open randomised trial of second-line endocrine therapy in advanced breast cancer: comparison of the aromatase inhibitors letrozole and anastrozole. *European journal of cancer (Oxford, England: 1990)* 39(16):2318–2327
- Silvestrini R, Veneroni S, Daidone MG, Benini E, Boracchi P, Mezzetti M, Di Fronzo G, Rilke F, Veronesi U (1994) The bcl-2 protein: a prognostic indicator strongly related to p53 protein in lymph node-negative breast cancer patients. *J Natl Cancer Inst* 86(7):499–504. doi:[10.1093/jnci/86.7.499](https://doi.org/10.1093/jnci/86.7.499)
- Silvestrini R, Benini E, Veneroni S, Daidone M, Tomasic G, Squicciarini P, Salvadori B (1996) P53 and bcl-2 expression correlates with clinical outcome in a series of node-positive breast cancer patients. *J Clin Oncol* 14(5):1604–1610
- Smith IE, Dowsett M (2003) Aromatase inhibitors in breast cancer. *N Engl J Med* 348(24):2431–2442. doi:[10.1056/NEJMra023246](https://doi.org/10.1056/NEJMra023246)
- Stendahl M, Kronblad A, Ryden L, Emdin S, Bengtsson NO, Landberg G (2004) Cyclin d1 overexpression is a negative predictive factor for tamoxifen response in postmenopausal breast cancer patients. *Br J Cancer* 90(10):1942–1948
- Trihia H, Murray S, Price K, Gelber RD, Golouh R, Goldhirsch A, Coates AS, Collins J, Castiglione-Gertsch M, Gusterson BA (2003) Ki-67 expression in breast carcinoma. *Cancer* 97(5):1321–1331
- Whitfield ML, George LK, Grant GD, Perou CM (2006) Common markers of proliferation. *Nat Rev Cancer* 6 (2):99–106. doi:http://www.nature.com/nrc/journal/v6/n2/supinfo/nrc1802_S1.html