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RESEARCH

Treatment Response to Preoperative Anthracycline-Based Chemotherapy in Locally Advanced Breast Cancer: The Relevance of Proliferation and Apoptosis Rates

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Abstract Objectives were to evaluate the relevance of proliferating fraction (Ki-67) along with apoptotic index (AI) which denoted growth index (Ki-67/AI ratio, GI) to predict pathological response to preoperative chemotherapy, and the pattern of their modifications following chemotherapy in women with locally advanced breast cancer. Archival material of diagnostic biopsies and surgical specimens from 106 patients were examined. Response rate to chemotherapy in this group was 95 %, eight (8 %) patients achieved a pathological complete remission (pCR) and five (5 %) had a progressive/stable disease (PD/SD). The expression of Ki-67 and AI were assessed using immunohistochemistry and in situ DNA nick labeling assay respectively. Higher baseline level of Ki-67 and GI were associated with an improved pathological response (p=0.0001 and p=0.008), but the degree of correlation with GI was no greater than that with Ki-67 alone. Ki-67 below 1 % highly indicated a lack of tumor response. High AI which characterized the opposite chemo-sensitive tumors, pCR vs. PD/SD (p=0.72) implied that treatment response was not influenced by the "presence" or "absence" of apoptosis. A significant decrease in Ki-67 (p<0.001), AI (p=0.035) and GI (p=0.008) was found following chemotherapy, but percentage change in biomarker values revealed an increase in a number of cases. Higher initial Ki-67 and AI was associated with profound reduction of GI and raising value of GI after treatment, respectively. Such a variance of a given parameter elicited by chemotherapy may have various impact on disease outcome.

Keywords Breast cancer · Primary chemotherapy · Ki-67 · AI · Growth index

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Introduction

Predictive marker can be defined as a factor that indicates sensitivity or resistance to a specific treatment [1]. Hormonal receptors and c-erbB2 expressions aid in selecting the breast cancer therapies, such as, tamoxifen and trastuzumab [2], but predictive molecular determinants for conventionally dosed chemotherapy responsenses are only emerging [3–5]. Neoadjuvant clinical setting or preoperative (primary) systemic therapy of breast cancer has been proposed as an ideal in vivo model for studying the tumor biological features that might become reliable markers for the assessment of tumor response to therapy and/or valuable indices for long–term disease outcome [6, 7]. In addition, the use of neoadjuvant chemotherapy offers the opportunity to test clinical relevance of the pattern of modifications in the cell phenotype induced by therapy with the tumor remaining in situ throughout treatment as an in vivo measure of response.

The proliferation kinetics and apoptosis pathway are considered as the most relevant phenomena that are associated with cellular effects induced in vivo by chemotherapy [8]. The

impossibility of performing an apoptotic program is considered as an important factor in the appearance of resistance to cytotoxic drugs [8–10]. Nevertheless, evidence for the importance of this mechanism of sensitivity / resistance in clinical conditions has not yet led to unequivocal attitude. At present the multi-parameter signatures have been derived with the aim of improving the accuracy of prediction of susceptibility to cytotoxic drugs. Given that changes in apoptosis and proliferation are ultimately involved in the chemoresponsiveness, these parameters of cell growth are suitable as single candidate of predictors of tumor response.

The balance between proliferation and apoptosis is crucial in determining the overall growth or regression of the tumor [8, 11]. A complication in the use of proliferative indices as single markers of response is the undeniable contribution of apoptosis to tumor growth dynamics. Therefore, it was suggested that an index based on the Ki-67/apoptosis ratio, a parameter which was described as a growth index - GI [12], would be more appropriate for the prediction of response to drug therapy [13]. Since only a few studies evaluated the clinical utility of growth index in neoadjuvant chemotherapy setting [14], the question remains whether the growth index itself may be a better indicator of tumor response rather than each of the two parameters individually.

In this paper the predictive value of GI, together with the estimation of relevance of its two constitutive parameters, Ki-67 antigen as an indicator of proliferating fraction and the apoptosis phenomenon, are considered to be of main importance. The objective was to determine which of the parameters provides the accurate prediction of the pathological remission of breast cancer to anthracycline-based chemotherapy. In addition, we assessed the pattern of their modifications following chemotherapy.

Materials and Methods

Patients

Patients with primary breast cancer who received neoadjuvant, anthracycline-based chemotherapy at our institution between September 1999 and September 2003 have been chosen. Criteria for the selection of patients were as follows:1) an incisional biopsy of the primary breast cancer confirming invasive carcinoma before commencing the treatment; 2) patients who had primary locally advanced breast cancer that was not strictly operable; 3) available and sufficient sample of tumor tissue for the accurate measurement of apoptosis and proliferation, preserved in paraffin blocks in both cases, setting up the initial diagnosis and evaluation of resected surgical material. Patients with bilateral or metastatic disease were not included in the analysis. Women with inflammatory breast cancer were included (5 cases). Data on age and time of the last menstrual cycle histories were taken from patients' medical history. Tumors were classified according to the WHO scheme [15] and graded using modified histological Scarf-Bloom-Richardson score [16].

Treatment and Efficiency Assessment

Prior to surgery, all patients were treated with standard anthracycline-based chemotherapy (5-fluorouracil 500 mg/m2, doxorubicin 50 mg/m2 and cyclophosphamide 500 mg/m2 intravenously), repeatedly every 3 weeks for three to four cycles, according to the institutional protocol at that time. After the clinical assessment of objective response of the primary tumor at the end of cycle III, chemotherapy continued to cycle IV, unless the disease progression (or stable disease) was established in the course of administration. The patients were considered for surgery within 3–4 weeks following the last cycles of chemotherapy, which was usually modified radical mastectomy or quandrantectomy with full axillary dissection. Objective pathological responses were determined by macro/microscopic examination of the resected surgical material after completion of therapy. Pathological complete remission (pCR) was defined as the absence of histological evidence of invasive cancer cells in breast and lymph nodes, but it includes the presence of in situ lesions according to the recommendations of International Expert Panel [17]; histologically determined residual, invasive disease of any degree (in the breast and / or lymph nodes) included the cases with pathological partial response (pPR) [17, 18]. The cases for which there was clinical evidence of progression (stable) disease during treatment (PD/SD) were not considered due to unsuitability for surgical treatment. The local research and ethics committee approved the study prior to patient recruitment.

Ki-67 Immunohistochemistry

Ki-67 immunostaining was performed on histological sections prepared from a biopsy sample taken before treatment and from the surgical specimens removed after treatment. Four-μm sections from formalin-fixed, paraffin-embedded tissues were cut and mounted on SuperFrost® Plus slides. Tissue sections were deparaffinized, rehydrated, and treated with 3 % hydrogen peroxide for 10 min to neutralize endogenous peroxidase activity. The slides were subjected to heat-induced epitope retrieval by immersing them in 10 mM boiling citrate buffer (pH 6) in microwave oven for 15 min (400 W), followed by a 20-min cooling-off period, and then rinsed in Tris-buffered saline (TBS, pH 7.4). To reduce nonspecific staining, sections were preincubated at room temperature first in 0.1 M Tris–HCl pH 7.5 buffer containing 3 % BSA and then in TBS containing 0.02 % biotin (15 min each). Sections were then incubated for 30 min with an anti - Ki-67 mAb (clone MIB-1, Dako, Denmark) at 1: 80 dilutions. After an additional three washes in

TBS, the staining was revealed using the streptavidin-biotin-peroxidase method (LSAB+kit, DAKO, Denmark) and diamino-benzidine (DAB) as a chromogen, according to the manufacturer's directions. Slides were slightly counterstained with haematoxylin, dehydrated and mounted. Positive control (cases with known imunoreactivity) and negative control (omission of primary antibody) were performed in each staining procedure. Paired incisional biopsies and surgical specimens from the same patients were stained in the same run.

In situ Apoptosis Assay

The method of TdT-mediated dURT-biotin nick end labeling (TUNEL) is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to exposed 3'- OH ends of DNA fragments, ensuring a synthesis of a polydeoxynucleotide polymer [19]. TdT was used to incorporate labeled nucleotides at sites of DNA strand breaks in a template-independent manner. The signal was amplified by immunoenzyme complex, enabling conventional histochemical identification by light microscopy. In situ detection of cleaved, apoptotic fragments was performed using a commercial kit („In situ Cell Death Detection Kit, POD" Roche, Basel, Switzerland) according to the manufacturer's instructions for paraffin-embedded tissues. Tissue sections were deparaffinized, rehydrated, and treated with 3 % hydrogen peroxide for 10 min to neutralize endogenous peroxidase activity. The nuclei of tissue sections were stripped of proteins by microwave irradiation for 5 min (400 W) using 10 mM citrate buffer, pH 6.0, followed by rapid cooling with addition of doubly deionized water. After rinsing twice with TBS for 5 min, to reduce nonspecific staining, sections were preincubated at room temperature first in 0.1 M Tris–HCl pH 7.5 buffer containing 3 % BSA and then in TBS containing 0.02 % biotin (15 min each) The next step included the application of TUNEL reaction mixture on section, for 1 h at 37 C in humidified chamber. This reaction mixture was composed of enzyme TdT and the fluorescein labeled nucleotides. The according concentration of TdT was reduced by diluting it 1:5 up to 1: 7 with TUNEL Dilution Buffer pursuant to instructions. Converter-POD solution (anti-fluoroscein antibody conjugated with horse-radish peroxidase) was added, and the sample was kept for 30 min at 37 C in a humidified chamber. After rinsing (3 times in TBS, for 5 min), DAB chromogen was added to generate an insoluble colored substrate at the site of DNA fragmentation. Slides were counterstained with haematoxylin, dehydrated and mounted.

Scoring

The stained slides were evaluated independently by two of the authors (KK, ST) using a standard light microscope (Olympus Bx51, Olympus Optical, Tokyo, Japan). Sections were examined at low magnification (x 20) to identify „hot spot" areas with the most intense and frequent staining. Successive counts, performed by individuals blinded to the groups, were made within at least five selected „hot spot" areas in each case at magnification of x 40. Referring to MIB-1 positivity, only clear nuclear staining in malignant cells was considered positive. Stained apoptotic cells were recorded, and evan unstained cells displaying classic apoptotic morphology were also incorporated in the AI, avoiding areas of necrosis. The proliferation index (Ki-67 index) was defined as the number of Ki-67 – positive nuclei per 1000 tumor cells. The apoptosis index (AI) was defined as the number of TUNEL-positive cells per 3000 malignant cells counted. Growth index (GI) was calculated as the ratio of Ki-67 index and AI.

Statistical Analysis

Descriptive statistics were reported as proportions and medians. Pathological responses to the regimen were reported as the proportion of patients responding to therapy out of the total number of patients enrolled in the study. Differences between specified patient groups have been analyzed using the Mann–Whitney U test. Correlation analyses were done using Spearman's non parametric correlation coefficient. Comparison of pre- and post-treatment samples within matched cohort of patients with surgical specimens was performed using Wilcoxon matched-pairs signed-rank test. Proportional change in Ki-67 score, AI and growth index were calculated as the ratio of on-treatment and pretreatment values, to examine whether the proportional change differs significantly from unity. Categorical data were compared using Fisher's exact and χ^2 test. P values<0.05 were considered statistically significant.

Results

Clinical Data, Tumor Pathology and Treatment Response

During the study period 106 patients were eligible for the analysis. The median age of the study group was 49 years - interquartile (IQ range - 25–75 %, 31–69 years) with 54 (51 %) of the patients being premenopausal. The patients and tumor characteristics and pathological tumor responses are summarized in Table 1. Ninety three (87 %) patients had residual disease in the post-chemotherapy specimen (pPR), and 8 patients (8 %) achieved pathological complete remission (pCR). In five (5 %) patients during primary chemotherapy a PD/SD was clinically confirmed and their post-therapy surgical tissue has not been available. Comparisons of pre and post-treatment specimens were available only in subgroup of patients with pPR; patients with a clinical assessment of PD/SD during therapy were not suitable for

Table 1 Patient and tumor characteristics and treatment response

Characteristics	No. of patients (%)
No.of patients	106 (100)
Age (years)	
Median	49
[a]IQ range	31–69
Menopausal status	
Pre	54 (51)
Post	52 (49)
Clinical staging	
T0	2 (2)
T1	4 (4)
T2	43 (40)
T3	13 (12)
T4	7 (7)
T4b	32 (30)
T4d	2 (2)
Unknown	3 (3)
Nodal stage	
Node negative	6 (5)
Node positive	98 (93)
Unknown	2 (2)
Histological type	
IDC	36 (34)
ILC	27 (25)
Others	2 (2)
Unclassiffied	25 (24)
Unknown	16 (15)
Histological grade	
I	3 (3)
II	46 (43)
III	16 (15)
Unclassified	25 (24)
Unknown	16 (15)
ER status	91 (86)
Negative	44 (48)
Positive	47 (52)
PR status	91 (86)
Negative	67 (74)
Positive	24 (26)
Unknown	15 (14)
Pathological response	
Complete remission (pCR)	8 (8)
Partial remission (pPR)	93 (87)
Progressive/Stable disease (PD/SD)	5 (5)

[a] IQ range 25–75 % range

surgical treatment, and invasive malignant breast cancer cells were not presented in residual tissue samples of patients with pCR.

Biomarkers before Chemotherapy: Association with Pathological Tumor Response

Baseline Values of Biomarkers Table 2 lists the median and IQ ranges of the baseline values of biomarkers for the entire group and subgroups of patients related to treatment outcome. Median baseline values of Ki-67 index and AI for all of the study participants were 14.68 (IQ range, 5.18–24.72) and 1.314 (IQ range, 0.734–1.818), respectively. For patients who achieved a pPR, median baseline values of Ki-67 index and AI were 14.68 (IQ range, 5.25–23.86) and 1.207 (IQ range, 0.674–1.736) respectively. For patients who had pCR, median Ki-67 index and AI were 34.77 (IQ range, 18.24–59.94) and 2.239 (IQ range, 1.621–2.744) respectively. For the patients displaying disease progression during therapy (PD/SD), the median baseline value for Ki-67 index was 0.70 (IQ range, 0.46–4.13) and median value of AI was 1.901 (IQ range, 1.575–2.391).

Correlations between Biomarkers at a Baseline and their Association with Pathological Tumor Response We observed a significant positive correlation between Ki-67 index and AI in pretreatment samples within the entire group (ρ=0.201, p=0.041), but not in a predominant subgroup of patients with pPR (ρ=0.182, p=0.08). No significant correlation between Ki-67 and AI, at a baseline, was observed in patients with pCR or in patients with PD/SD.

Higher basal Ki-67 values were associated with better pathological response (ρ=0.362, p=0.0001), but association of AI values with pathological tumor response was not found (ρ=0.103, p=0.29). The GI which summarizes the opposing effects of apoptosis and proliferation was also

Table 2 Median and [a]IQ range of baseline values of Ki-67 index, AI and [b]GI according to pathological response

Biomarker		Ki-67 index	AI	[b]GI
Patient subgroups i.e., response categories	Entire group			
	Median	14.68	1.314	10.20
	[a]IQ range	5,18–24,72	0,734–1,818	3,84–24,88
	pCR			
	Median	34.77	2.239	14.84
	IQ range	18.24–59.94	1,621–2,744	8,25–27,84
	pPR			
	Median	14.68	1.207	10.39
	IQ range	5,25–23,86	0,674–1,736	3,90–25,21
	PD/SD			
	Median	0.70	1.901	0.37
	IQ range	0,46–4,13	1,575–2,391	0,28–1,75

[a] IQ range - interquartile (25–75 %) range

[b] GI - growth index (Ki-67 index / AI)

significantly associated with better treatment outcome (ρ=0.260, p=0.008).

Status of Biomarkers in Relation to Pathological Tumor Response Based on cut-offs for biomolecular assessments defined according to the median values, the association of marker' status (positive/negative) with treatment response was analyzed, and results are presented in Tables 3 and 4.

A baseline status of Ki-67 index was associated with improved pathological remission (p=0.029, Table 3). A clear statistical significance of Ki-67 status for the prediction of response to cytotoxic drugs was found only for tumors with pCR compared to those with PD/SD (p=0.021, Table 4). It is worthy of notice that there was only the negative status of Ki-67 in patients in whom the disease progression during therapy (PD/SD) was observed, Table 3.

Baseline status of AI was associated with better pathological remission (p=0.005, Table 3). Further analysis showed that the predictive significance of baseline status of AI was observed in patients with pPR compared to both, the subgroup of patients with pCR (p=0.027, Table 4) and that with PD/SD (p=0.021, Table 4). However, baseline status of AI did not show significant difference (p=1.0, Table 4) between the extremes in terms of tumor chemo-sensitivity, i.e., tumors with pCR compared to tumors with PD/SD.

A strong trend for predicting pathological response was found in relation to the status of GI (p=0.059, Table 3). Due to lack of significant differences in the baseline status of GI between different response categories (Table 4), status of GI was not predictive for breast cancer pathological response to the effects of chemotherapy.

Scattered Plot Diagrams of Baseline Values of Biomarkers Related to Different Response Categories Scattered plot diagrams of baseline values of biomarkers in relation to tumor response categories are presented on Fig. 1a-c. Values of markers are presented on a logarithmic scale to allow the lower values to be visually separated. Figure 1a shows that there is opposite distribution of Ki-67 values in tumors with opposed sensitivity to the effects of chemotherapy (pCR vs. PD/SD). Approximately, the Ki-67 level below the threshold of 10 % ($\log_{10}$ (10)=1) may indicate a low proliferative tumors in which the resistance to the cytotoxic therapy is likely to occur. Moreover, the rigorous value as a threshold for Ki-67 of about 1 % ($\log_{10}$(1)=0) strongly indicates a lack of tumor response to chemotherapy. Conversely, the value of Ki-67≥10 % points to the more proliferative tumors with the possibility of pathological complete remission induced by chemotherapy. The baseline values of AI show similar distribution in tumors with the opposite chemo-sensitivity (pCR vs. PD/SD), Fig. 1b. The graphical distribution of GI values (Fig. 1c) resembles the distribution of Ki-67 values, suggesting that the observed difference in baseline GI between extra sensitive (pCR) and resistant (PD/SD) tumors may be primarily due to the proliferative activity of malignant cells.

Table 3 The distribution (frequency) of the baseline status of Ki-67 index, AI and [a]GI according to pathological response, and correlation of biomarker status with response

Biomarker	Patient subgroups with different treatment outcome			
	pCR No.of cases (%)	pPR No.of cases (%)	PD/SD No.of cases (%)	p
Ki-67 index				
Positive	6 (75)	48 (52)	0	0.029
Negative	2 (25)	45 (48)	5 (100)	
AI				
Positive	7 (88)	41 (45)	5 (100)	0.005
Negative	1 (12)	51 (55)	0	
[a]GI				
Positive	48 (52)	5 (63)	0	0.059
Negative	44 (48)	3 (37)	5 (100)	

[a] GI - growth index (Ki-67 index / AI)

Table 4 Correlation between the different response categories in relation to the baseline status of biomarkers

Response categories	Biomarker		
	Ki-67 index p	AI p	[a]GI p
pPR vs. pCR	0.37	0.027	0.72
pPR vs. PD/SD	0.056	0.021	0.056
pCR vs. PD/SD	0.021	1.0	0.08

[a] GI - growth index (Ki-67 index / AI)

Analysis of Variability (Heterogeneity) of Biomarkers Between Patients' Subgroups with Different Treatment Outcome Differences in the distribution of biomarker baseline values between patient's subgroups with different response to chemotherapy are summarized in Table 5, showing that the baseline values of Ki-67 were significantly different between all three subgroups of patients. Taking into consideration the median and IQ ranges for biomarkers that are shown in Table 2, baseline Ki-67 values (Med 34.77, IQ range, 18.24–59.94) were significantly higher in patients who achieved pCR , compared to both, those with pPR (p=0.008) and those with PD/SD (p=0.003), Table 5.

The lowest values of Ki-67 (Med 0.70, IQ range 0.46–4.13, Table 2) found in tumors that progressed or remained stable during chemotherapy imply that these tumors could be construed as extremely chemo-resistant breast cancers as opposed to highly chemo-sensitive tumors with pCR. In patients with pPR the values of Ki-67 were of "intermediate level" (Med 14.68, IQ range, 5.23–23.86), but

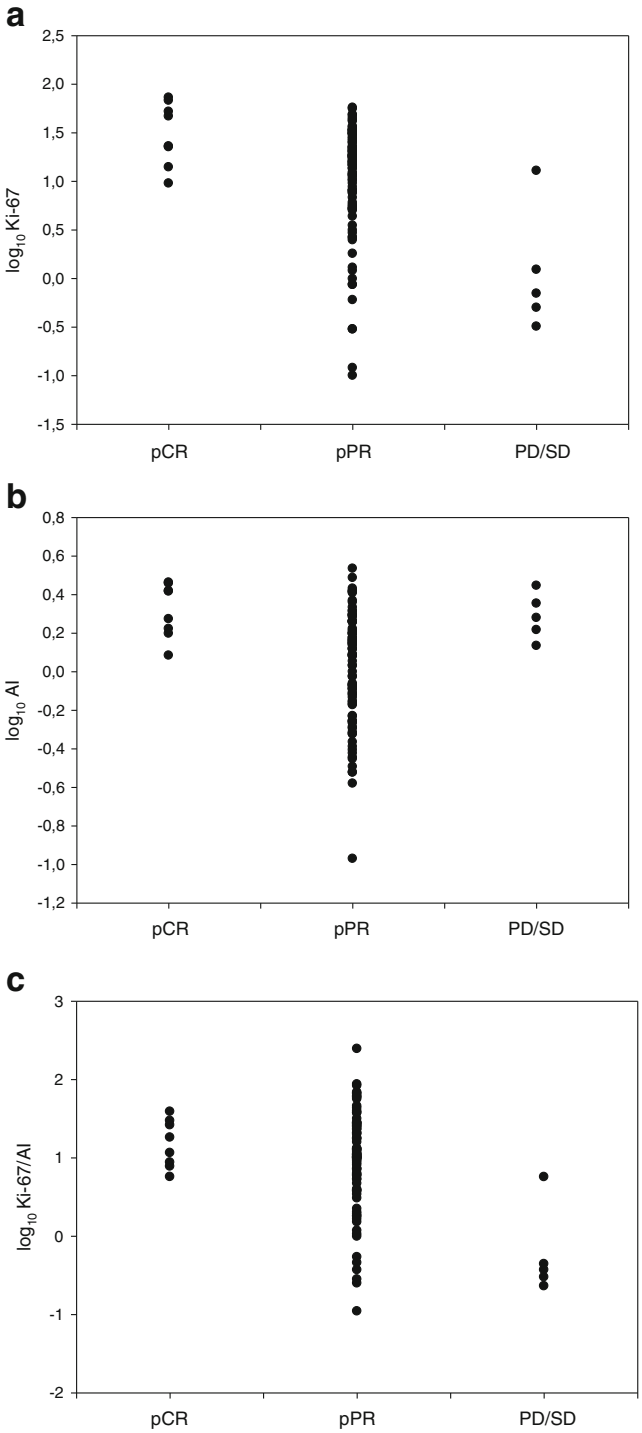

Fig. 1 Scattered plot diagrams of baseline values of biomarkers related to different response categories. **a** Ki-67 values related to treatment outcome, **b** AI values related to treatment outcome, **c** Growth index (GI) values related to treatment outcome

still significantly different compared to both, the patient subgroup with pCR (p=0.002) and the patient subgroup with PD/SD (p=0.025), Table 5.

The baseline AI values were significantly lower in patients with pPR (Med 1.207; IQ range, 0.674–1.736; Table 2) compared to both, those in patients with pCR (p=0.002.) and those in patients with PD/DS (p=0.025), Table 5. There was no significant difference in AI values between patients with pCR and those with PD/SD (p=0.72, Table 5).

Table 5 Differences in the distribution of baseline values of Ki-67 index, AI and [a]GI between different response categories

Biomarker	Compared response categories	p
Ki-67	pPR vs. pCR	0.008
	pPR vs. PD/SD	0.010
	pCR vs. PD/SD	0.003
AI	pPR vs. pCR	0.002
	pPR vs. PD/SD	0.025
	pCR vs. PD/SD	0.72
[a]GI	pPR vs. pCR	0.38
	pPR vs. PD/SD	0.003
	pCR vs. PD/SD	0.002

[a] GI - growth index (Ki-67 index / AI)

The baseline GI values, were significantly lower in patients with PD/SD (Med 0.37; IQ range, 0.28–1.75; Table 2) compared to both, those in patients with pPR (p=0.003) and those in patients with pCR (p=0.002), Table 5. No difference in GI values was found between patients with pPR and those with pCR (p=0.38, Table 5). A very low values of GI in patients with PD/SD are likely due to very low values of Ki-67 index in these patients (Table 2).

Effects of Chemotherapy on Biomarkers within Matched Paired Samples (Patients with pPR)

Changes in Biomarkers after Chemotherapy Changes in biomarkers after chemotherapy are presented in Table 6. The percentage changes in biomarker values that represent on-treatment (residual) value as the proportion of pretreatment (initial) value (Post-/Pre- value) are presented in Table 7.

Table 6 Change in Ki-67 index, AI and [a]GI in matched paired breast samples (patients with pPR) after chemotherapy

Biomarker	Pre-th	Post-th	p
Ki-67			
Median	14.68	5.21	p<0,001
[b]IQ range	5,23–23,86	1,60–13,34	
AI			
Median	1.207	0.827	0.035
IQ range	0,674–1,736	0,504–1,263	
[a]GI			
Median	10.39	8.22	0.008
IQ range	3,90–25,21	2.29–14.94	

[a] GI - growth index (Ki-67 index / AI)

[b] IQ range - 25–75 % range

Table 7 Percentage change (on-treatment value as proportion of pre-treatment value) in Ki-67 index, AI and [a]GI in matched paired breast samples (patients with pPR) after chemotherapy

Biomarker		No. of cases (%)	Median	[b]IQ range
Ki-67 index	c↑	25 (27)	2.3	1,50–4,17
	d↓	67 (73)	0.28	0,10–0,50
AI	↑	38 (43)	1.47	1,20–1,83
	↓	51 (57)	0.52	0,30–0,72
[a]GI	↑	32 (36)	2.35	1,60–5,81
	↓	57 (64)	0.25	0,05–0,51

[a] GI - growth index (Ki-67 / AI)

[b] IQ range - 25–75 % range

[c] ↑ - an increase of value in breast residual specimens

[d] ↓ - a dicrease of value in breast residual specimens

Ki-67 Index The median Ki-67 index in pre-chemotherapy samples was 14.68 (IQ range 5.23–23.86) and following chemotherapy it has decreased significantly to 5.21 (IQ range 1.60–13.34; $p<0.001$, Table 6). The percentage change in Ki-67 values (Table 7) showed that there was reduction in 67 (73 %) cases and that median of decreased residual values corresponded to 0.28 part of the analogous basal value (IQ range 0.10–0.50). It means that median of decreased Ki-67 values was 28 % (IQ range 10–50 %) of the analogous pretreatment values. A decrease in Ki-67 index after chemotherapy, greater than 75 % prevailed in 46 % of these samples (not shown). In contrast, 25 (27 %) patients showed an increase after chemotherapy, whereby the median of increased residual Ki-67 index was 2.3 (IQ range 1.50–4.17). In other words, increased residual values of Ki-67 index were on average 2.3 times higher than the corresponding values before treatment. Of the 25 tumors that showed an increase in Ki-67 index after chemotherapy, half (56 %) of them displayed an increase greater than 100 %.

AI There was a significant decrease in AI after chemotherapy relative to the pretreatment samples ($p=0.035$, Table 6). Median AI pre-chemotherapy was 1.207 (IQ range 0.674–1.736), and after treatment it has decreased to 0.827 (IQ range 0.504–1.263), Table 6. Percentage change in AI (Table 7) showed decrease in 51 (57 %) cases, wherein the median of decreased values was 0.52 (IQ range 0.30–0.72), showing that lowered residual values of AI corresponded to an average of 0.52 part of the basal values. Reduction of AI that was greater than 50 % was observed in 48 % of these specimens. Conversely, there was an increase in AI in 38 (43 %) patients, where the median of increased values was 1.47 (IQ range 1.20–1.83). The rise in AI greater than 50 % was found in 17 (45 %) of these samples, related to the analogous initial values. In addition, a significant, even stronger positive correlation between Ki-67 index and AI was found in residual tumor specimens after chemotherapy ($\rho=0.25$, $p=0.016$).

Growth Index There was significant fall in GI following chemotherapy, from 10.39 (IQ range 3.90–25.21) to 8.22 (IQ range 2.29–14.94, $p=0.008$), Table 6. GI decreased in 57 (64 %) cases (Table 7) to the average of quarter of a value that was detected prior to the therapy (Med 0.25, IQ range 0.05–0.51), while GI increased in 32 (36 %) cases with an average rise of 2.35 times its initial value (Med 2.35, IQ range 1.60–5.81).

Correlations of Biomarkers with their Percentage Change Values Induced by Chemotherapy (Data Not Shown) A trend of positive correlation was obtained between pretreatment Ki-67 index and the percentage change in AI induced by chemotherapy ($\rho=0.209$, $p=0.051$, $n=88$) which means that higher proliferation at baseline tends to induce higher increase in the apoptotic activity of residual malignant cell population after the exposure to chemotherapy. A significant positive correlation was found between pre-therapy AI and percentage change of Ki-67 index after chemotherapy ($\rho=0.262$, $p=0.012$, n=91), suggesting that higher apoptosis before therapy was highly associated with raising proliferation in residual tumor samples. A statistically significant negative correlation between the pre-therapy Ki-67 values and percentage change in GI was found ($\rho=-0.260$, $p=0.015$, $n=88$). This finding implys that higher Ki-67 at a baseline was associated with profound reduction of GI after therapy. Furthermore, statistically significant positive correlation between pre-therapy AI and percentage change in GI was found ($\rho=0.402$, $p=0.0001$, $n=89$) indicating that the higher apoptotic activity in tumors before treatment was associated with raising value of tumor GI after treatment.

Discussion

The induction of pathological complete remission (pCR), have been consistently found to be associated with improved disease outcome [20–22]. For that reason the pCR may be regarded as a surrogate marker for indetifying the chemo-sensitive subpopulation in primary breast cancer, that is an extremely-sensitive tumor [23]. Concerning the efficiency, the current analysis demonstrates that the pCR rate (8 %) following neoadjuvant FAC in breast cancer patients is somewhat lower than that reported in other studies. One of the plausible reasons for the lower rates of pCR may be that combination of cytotoxic drugs, doses and schedule of administration to the patients were different. Also, numerous published studies for evaluation of the

relevance of biomarkers in neoadjuvant clinical setting, increasingly include the patients with operable breast cancer, meaning that our patients'cohort had selection bias for biologically aggressive cancers. The patients selected for this study all had to be candidates for neoadjuvant chemotherapy. The high pathological response rates as seen in this cohort (95 %) were similar to those reported elsewhere for neoadjuvant chemotherapy, limiting the assessment of markers for their relationship with tumor response. The relatively small size of the patient subgroup with opposed chemosensitivity (pCR vs. PD/SD) would also allow only very strong predictors of response to be revealed. Our current study, although retrospective, is strengthened by the analysis of a relatively large paired cohort and this was a single center study in which standardised therapeutic approaches were employed.

In this analysis, a positive correlation between Ki-67 index and AI was detected in all analyzed specimens prior to chemotherapy, implying that an elevated proliferation of malignant cells was associated with their more pronounced apoptotic activity, which is consistent with previous reports [14, 24, 25]. Therefore, the malignant cell population of highly proliferative tumors also disappears very quickly. The statistical significance was, to some extent weak and probably, an absence of association between the two in prevailing patient subgroup (pPR), could contribute to this. A high and low baseline level of Ki-67 characterized tumors with a diametrically different chemo-sensitivity i.e., pCR and PD/SD respectively, both of which were associated with similar higher values of AI (Fig. 1b) yielding a weak, but significant positive correlation between proliferation and apoptosis in the entire group. Nevertheless, in residual malignant cells which could be considered as resistant to chemotherapy, an even stronger relationship between these two variables was found, suggesting that the regulatory controlling mechanism common to both apoptosis and proliferation continues to maintain a delicate balance between the two, even after treatment [26]. Since predictive factor is a measure - statistical variable that is associated with a contribution or lack of benefit to therapy [1], it was expected that baseline level of Ki-67 but no AI in the pre-treatment samples was associated with better pathological tumor response in our patients' cohort. Moreover, regarding the distribution of the Ki-67 values related to treatment outcome (Fig. 1a), threshold level below 1 % that relates to low proliferation, highly indicates a lack of tumor response to chemotherapy. Considered in a similar way, the level of Ki-67 above 10 % points to the more proliferative tumors with the possibility of pathological complete remission induced by chemotherapy. These observations are consistent with widely reported positive relationship between level of cell proliferation and response to cytotoxic drugs [27], including the effect of doxorubicin on breast cancer cell lines [28]. This is consistent with previous neoadjuvant studies, which have shown the predictive value of baseline Ki-67 in relation to the pathological [29–37] or clinical response [14, 38–40] of breast cancer to the effects of chemotherapy. Our findings are also comparable to the reports which have revealed that Ki-67 index is a valuable marker for the effectiveness of anthracycline-based chemotherapy shedules [28–30, 32, 33, 35, 38, 40].

Furthemore, many studies have confirmed that proliferation is a dominant feature of multigene signatures in breast cancer. For example, study that was based on gene set enrichment analysis indicated that higher expression of proliferation-related genes characterised tumors with complete response among both ER- and ER + breast cancers, although measuring Ki-67 mRNA expression levels alone was not sufficient to separate cases with complete remission from those with residual cancer after chemotherapy [41]. However, taking into account the notable number of studies which did not confirm a value of Ki-67 antigen as a marker of breast cancer chemo-responsiveness [42–48], most often stated reasons for the discrepancy of published results were different patient's cohort, definition of response - „pathological versus clinical", and differences in chemotherapy regimens that were evaluated in different studies. In particular, arbitrary chosen „cut-offs" for Ki-67 status in terms of low versus high proliferation varied (range of 5–40 %), thus preventing a comparison of results between different studies. It has been suggested that values of Ki-67 index would be considered as a continuous variable [25], which was done in our study.

Although the apoptosis levels (AI) before therapy was not predictive for chemotherapy response, the initial status of AI displayed predictive value. However, the fact that baseline status of AI between the opposed tumors with respect to chemo-responsiveness i.e., pCR vs. PD/SD, did not show meaningful difference (Fig. 1b), considerably diminished the relevance of AI. Consequently, we found that treatment resistance was not associated with „absence" of apoptosis and that treatment response was not related to the „presence" of apoptosis. Therefore it is unlikely, that the apoptosis pathway of cell death is not the only one determinant of chemotherapy response. Mitotic catastrophe and senescence have been marked as relevant mechanism of cell death due to the effects of chemotherapy [49, 50]. Our findings are consistent with previous studies reporting that AI before chemotherapy is not related to breast cancer regression induced by chemotherapy that is assessed clinically [14, 26, 47, 51] or to a lesser extent using pathological response criteria [46, 52–54]. In this regard, our results suggest that the very low proliferation which may be accompanied with high apoptosis, favors the failure of anthracycline-based chemotherapy applied on locally advanced breast cancer in neoadjuvant setting.

It is important to note that the isolated comparision of the apoptotic and proliferative indices has some limitations, because each index reflects both the number of times that each event takes place in unit time and the proportion of cells in the population capable of undergoing each event [55]. Thus, growth index allow us to measure the frequency of occurrence of proliferation relative to apoptosis. Given that apoptosis provides the major counterbalancing determinant of tumor growth, an index based on the Ki-67 – apoptosis ratio (growth index, GI) has been proposed to approximate the contribution that these two factors may make in tumor growth. In the present analysis, a very low values of GI in a situation of PD/SD compared to higher values for tumors with pCR, suggest that rather rare occurrence of malignant cell proliferation relative to apoptosis contributes to the occurence of tumor resistance to cytotoxic drugs. The negative predictive value of low proliferation was thus even more convincing, than the positive predictive value of high proliferation. It is worthwhile to notice that the scattered plot diagrams of GI and Ki-67 values that correspond to tumors with opposed chemo-responsiveness i.e., pCR vs. PD/SD, are of a similar configuration, despite the different configuration of Ki-67 values between them. This finding suggests that the Ki-67 values have a more marked influence on values of GI. There was a significant association of GI and pathologic response of breast cancer to chemotherapy albeit the lack of relationship between pretreatment AI values and tumor response. The degree of correlation with growth index was, however, no greater than that with Ki-67 alone. Besides, the association between GI analyzed as a status (positive / negative) and pathological response showed a trend of significance remaining under significance level in our patients' cohort. Archer. et al. (2003). reported the same results regarding the predictive relevance of these three parameters in relation to clinical tumor response [14]. The assessment of predictive value of growth index with the estimation of relevance of the two main parameters, Ki-67 index and AI, related to breast cancer pathologic response induced by chemotherapy in neoadjuvant setting, has not been published yet.

Contrary to the results from previous studies, there was no difference in pathological response rate according to the steroid receptor status [32, 35, 40, 42], although there was finding similar to ours [48]. Different methodological determination of steroid receptors in the tumor samples analyzed in this study (biochemical methods, predominantly), unlike the IHC method applied in other studies, may be one reason for the discrepancy of results.

Residual malignant cells can be considered as a relatively resistant population of cells to the cytotoxic effects of chemotherapy, allowing the assessment of possible variations of cell phenotype elicited by therapy [7, 26]. In our study, we found that Ki-67 index, AI and GI were significantly reduced after chemotherapy in a group of the surgical specimens (matched cohort of 93 patients). In residual malignant cell population at the cessation of chemotherapy there was a shift of both, the apoptosis and the proliferation towards lowered levels wherein the balance between apoptosis and proliferation was even more pronounced and maintained. This raises the questions of whether the decrease in proliferation is the result of down regulation in the entire cell population by triggering "switching off" of proliferative regulators by therapy, or it reflects a selection of residual, less proliferative cells that are intrinsically less sensitive to chemotherapy and have been preserved throughout treatment [13]. The decreased proliferation found here following chemotherapy compared to its baseline pre-treatment values confirms previous studies [26, 32, 34, 39, 43, 45, 56]. In view of the fact that apoptosis is one of the main pathways of malignant cell death undergoing clinically effective doses of cytotoxic drugs [57], comparing pretherapy with post-therapy specimens might be useful, because it could distinguish the endogenous AI in patients' tumors from their AI induced by the chemotherapy [60]. Ellis et al. (1998) also showed decline in AI along with Ki-67 index in the post-treatment samples, which was particularly significant in those with partial clinical response, in contrast to the smallest with a complete clinical remission [26]. In our analysis the overall statistically significant increase in AI elicited by chemotherapy in post-therapy samples was not observed, unlike some other studies that have shown it [51, 58, 59]. This discrepancy could stem from the fact that in the present study, changes in biomarkers (AI e.g.) elicited by chemotherapy were analyzed only in patients with pathological partial response in which malignant tissue samples were available in both cases, before and after chemotherapy (matched paired breast samples). In contrast to our analysis, the previous studies took into consideration a change in apoptotic malignant cell activity elicited by chemotherapy in regards to breast cancer response assessed by clinical criteria, allowing access to tumor specimens for evaluation in all response categories. Besides, it has been considered that the timing of sampling after commencing treatment might be determinative in quantifying the increase in AI [14]; the Ellis pilot study sampled at 24 h after chemotherapy, and the later published reports corroborated this findings of early induction of apoptosis within 24–48 h after drug administration [14, 53] or even later, after several days [47, 51].

Dowsett et al. have suggested that if the response of the tumor to a particular treatment is being evaluated, then it is the change between the baseline and on-treatment value that is relevant [61]. Only a few of the published studies have assessed the change in the biomarker expression to the effect of chemotherapy in an approach that was expressed as a relative / percentage change in biomarkers' value [26, 34, 44, 62]. This method of calculating the change pattern of residual (post-therapy) values over their pretherapy ones, gives an information about the direction of changes (decrease / increase) and the contribution of the residual value

compared to the baseline. Percentage change analysis in biomarkers of individual patient revealed an apparent increase in residual values in a number of cases, although an overall statistically significant reduction of a given biomarker after chemotherapy was obtained. Thus, in our analysis Ki-67 was reduced in 73 % of the residual tumor samples (the median of decreased Ki-67 was 28 % of analogous pretreatment values), while in 27 % of the cases the Ki-67 was increased after therapy to an average of 2.3 times its initial values at baseline. Similarly, AI was decreased in 57 % of the samples after chemotherapy, while AI increased in 43 % patients, yeilding the statistical lowering of apoptosis in general. It is possible that the reduction in apoptosis may represent a selection of apoptotic resistant cells in the tumor specimens, or it is a reaction to the profound reduction of proliferation. The „shift pattern“ of both the Ki-67 and AI towards lower values may reflect this. Given the association of higher AI values with higher Ki-67 after therapy, the finding of tumor samples with elevated residual apoptotic activity may be considered as an indirect evidence of unfavorable clinical course of these tumors [13], although the prognostic value of AI has not been found as an independent prognostic parameter [24]. A higher proliferation at baseline tends to manifest higher rise in apoptotic activity of residual malignant cell population after exposure to chemotherapy. This might be related to the pronounced effect of chemotherapy on rapidly proliferative tumors. Given that tumors with higher proliferation are more sensitive to the effects of chemotherapy, a higher Ki-67 at baseline indicates a greater chance of higher residual value of AI as an indicator of apoptosis induction to the effects of cytotoxic drugs.

In addition, higher apoptosis before therapy was highly associated with raising proliferation in residual tumor samples. It is possible that regression of the tumors in response to cytotoxic therapy occurs as a result of markedly reduced proliferation alongside the largely maintained rate of apoptosis. Given that tumors with a higher proliferation rate before treatment are associated with generally higher apoptosis rate, on average a decrease from a high Ki-67 level to a lower value would be associated in the on-treatment sample with a higher apoptosis level than a tumor that has decreased to the same low Ki-67 level from a moderate level [63]. This implies that the tumor growth dynamics of two tumors with similar Ki-67 levels on-treatment may be quite different depending on their initial proliferation / apoptosis balance. Considering this finding, together with an undoubted contribution of apoptosis to tumor growth dynamics, analysis of growth index is highly advantageous.

The overall reduction of GI includes the increase in GI in 36 % cases with a median of increased value of 2.35 parts of the initial values, and lowering of GI in 64 % cases to approximately one quarter of the value that was detected prior to therapy. Higher proliferation of untreated tumors was associated with a more pronounced reduction in tumor growth index after treatment. But, the higher apoptotic activity in tumors before treatment was associated with raising value of tumor growth index after treatment. Due to an association of higher initial apoptosis with raising proliferation in residual tumor samples, it can be indirectly speculated that the finding of raising value of growth index after therapy in tumors with a higher initial apoptotic activity could be due to the simultaneous increase in proliferation.

A disadvantage of using only surgical samples to assess changes in biomarkers' expression elicited by chemotherapy is that observed changes cannot be assessed as determinants of tumor response. It is not known whether the observed changes and the characteristics of tumor cells after chemotherapy represent just a variation of the phenotype of cancer cells that are exposed to the influence of cytotoxic drugs or suggest the appearance of new neoplastic clones [7, 13]. Patients with residual disease after primary chemotherapy constituting a predominant population in other studies (70–90 %), integrate breast cancers which are heterogeneous with respect to the degree of regression as well as clinical outcome [20]. A well known paradoxical feature is that higher Ki-67 at baseline suggests a high chance of achieving pathological complete remission of breast cancer as an effect of primary chemotherapy, whereas in cases where it does not occur and highly proliferative disease is maintained, the clinical outcome is unfavorable. There are reports stating that the low post–treatment Ki-67 proliferation index for patients not achieving a pathological complete response can be a meaningful prognostic marker for better disease outcome [37, 39, 43, 56]. An increase in median Ki-67 following therapy was observed in the relapsed subgroup and a decrease in the sugroup that did not relapse in patients with residual disease after chemotherapy [63]. Likewise, it has been reported that high apoptosis increase and proliferation decrease after chemotherapy along with lower proliferation/apoptosis ratio showed significantly favourable prognosis [59]. It has been stated that post-treatment Ki-67 index would presumably represent a combined value of „original prognostic value“ and „therapeutic prognostic value“, while post-treatment apoptotic status would represent more „therapeutic prognostic value“ [60, 59].

In view of that, attention should be paid to whether the value of a given biomarker increases or decreases in individual cases, since this may have various impact on disease outcome, pointing to a different patient prognosis. Although the predictive relevance of biomarkers, in the present study, should be confirmed in a larger study that could be supported by the clinical parameters of response, relapse-free disease, overall survival etc. this pilot study aims to promote a consideration of varied outcome of a given parameter elicited by chemotherapy in the domain of clinical research. Finally, the type of modification by neoadjuvant therapy could also assist in the choice of drugs for adjuvant setting.

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Conflict of Interest The authors declare that there is no conflict of interest.

References

1. Hayes DF (2005) Primary therapy of early breast cancer: 9th International St. Gallen consensus conference. Prognostic and predictive factors revisited. Breast 14:493–499
2. Goldhirsch A, Glick JH, Gelber RD, Coates AS, Thűrlimann B, Senn HJ, Panel Members (2005) Meeting highlights: international expert consensus on the primary therapy of early breast cancer 2005. Ann Oncol 16:1569–1583
3. Tewari M, Krishnamurthy A, Shukla H (2008) Predictive markers of response to neoadjuvant chemotherapy in breast cancer. Surg Oncol 17:301–311
4. Hugh J, Hanson J, Cheang MCU, Nielsen TO, Perou CM, Dumontet C et al (2009) Breast cancer subtypes and response to docetaxel in node-positive breast cancer: use of an immunohistochemical definition in the BCIRG 001 Trial. J Clin Oncol 27:1168–1176
5. Albain KS, Barlow WE, Shak S, Hortobagyi GN, Livingston RB, Yeh IT et al (2010) Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal women with node-positive, oestrogen-receptor-positive breast cancer on chemotherapy: a retrospective analysis of a randomized trial. Lancet Oncol 11:55–65
6. Fisher B, Mamounas E (1995) Preoperative chemotherapy: a model for studying the biology and therapy of primary breast cancer. J Clin Oncol 13:537–540
7. Loønning PE (2003) Study of suboptimum treatment response: lessons from breast cancer. Lancet Oncol 4:177–185
8. Pomier Y, Sordet O, Antony S, Hayward RL, Kohn KW (2004) Apoptosis defects and chemotherapy resistance: molecular interaction maps and networks. Oncogene 23:2934–2949
9. Johnstone RW, Ruefli AA, Lowe SW (2002) Apoptosis: a link between cancer genetics and chemotherapy. Cell 108:153–164
10. Fulda S, Debatin KM (2006) Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25:4798–4811
11. Tamm I, Schriever F, Dörken B (2001) Apoptosis: implications of basis research for clinical oncology. Lancet Oncol 2:33–42
12. Harper-Wynne CL, Sacks NP, Shenton K, MacNeill FA, Sauven P, Laidlaw I et al (2002) Comparision of the systemic and intratumoral effects of tamoxifen and the aromatase inhibitor vorozole in postmenopausal patients with primary breast cancer. J Clin Oncol 20:1026–1035
13. Cleator S, Parton M, Dowsett M (2002) The biology of neoadjuvant therapy for breast cancer. Endoc Rel Cancer 9:183–195
14. Archer CD, Parton M, Smith IE, Ellis PA, Salter J, Ashley S et al (2003) Early changes in apoptosis and proliferation following primary chemotherapy for breast cancer. Br J Cancer 89:1035–1041
15. Scarff RF, Torloni H (1981) Histological Typing of Breast Tumors. International Histological Classification of Tumors, No 2. Geneva, WHO
16. Bloom HJG, Richardson WW (1957) Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years. Br J Cancer 11:337–359
17. Kaufmann M, Hortobagyi GN, Goldhirsch A, Scholl S, Makris A, Valaqussa P et al (2006) Recommendations from an international expert panel on the use of neoajuvant (primary) systemic treatment of operable breast cancer: an update. J Clin Oncol 24:1940–1949
18. Sneige N, Kemp B, Pusztai L, Asmar L, Hortobagyi GN (2001) Chemotherapy-induced histologic changes in mastectomy specimens and their potential significance. Breast 10:492–500
19. Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501
20. Bonadonna G, Valagussa P, Brambilla C, Ferrari L, Moliterni A, Terenziani M et al (1998) Primary chemotherapy in operable breast cancer: eight year experiance at the Milan Cancer Institutte. J Clin Oncol 16:93–100
21. Guarneri V, Broglio K, Kau SW, Cristofanilli M, Buzdar AU, Valero V et al (2006) Prognostic value of pathologic complete response after primary chemotherapy in relation to hormone receptor status and other factors. J Clin Oncol 24:1037–1044
22. Bertheau P, Lerebours F, Mounier N, de Roquancourt A, Espie M, Clot P et al (2005) Prognostic significance of a combined clinicopathologic score for response to primary systemic therapy in locally advanced breast cancer. Oncol Rep 14:513–520
23. Smith IE, Lipton L (2001) Preoperative/neoadjuvant medical therapy for early breast cancer. Lancet Oncol 2:561–570
24. Lipponen P (1999) Apoptosis in breast cancer: relationship with other pathological parameters. Endoc Relat Cancer 6:13–16
25. Urruticoechea A, Smith IS, Dowset M (2005) Proliferation marker Ki-67 in early breast cancer. J Cli Oncol 23:7212–7220
26. Ellis PA, Smith IE, Detre S (1998) Reduced apoptosis and proliferation and increased bcl-2 levels in residual breast cancer following preoperative chemotherapy. Breast Cancer Res Treat 48:107–116
27. Remvikos Y, Beuzeboc P, Zajdela A, Voillemot N, Magdalenat H, Pouillart P (1989) Correlation of pretreatment proliferative activity of breast cancer with the response to cytotoxic chemotherapy. J Nat Cancer Inst 20:1383–1387
28. Campiglio M, Somenzi G, Olgiati C, Beretta G, Balsari A et al (2003) Role of proliferation in HER-2 status predicted response to doxorubicin. Int J Cancer 105:568–573
29. Collecchi P, Baldini E, Giannessi P, Naccarato AG, Passoni A, Gardin G et al (1998) Primary chemotherapy in locally advanced breast cancer (LABC): effects on tumor proliferative activity, bcl-2 expression and the relationship between tumor regression and biological markers. Eur J Cancer 34:1701–1703
30. Wang J, Buchholz TA, Middleton LP, Allred DC, Tucker SL, Kuerer HM et al (2002) Assessment of histologic features and expression of biomarkers in predicting pathologic response to anthracycline-based neoadjuvant chemotherapy in patients with breast carcinoma. Cancer 94:3107–3114
31. Pohl G, Rudas M, Taucher S, Stranzl T, Steger GG, Jakesz R et al (2003) Expression of cell cycle regulatory proteins in breast carcinomas before and after preoperative chemotherapy. Breast Cnacer Res Treat 78:97–103
32. Faneyte IF, Schrama JG, Peterse JL, Remijnse PL, Rodenhuis S, van de Vijver MJ (2003) Breast cancer response to neoadjuvant therapy: predictive markers and relation with outcome. Br J Cancer 88:406–412
33. Ogston KN, Miller ID, Schofield AC, Spyrantis A, Pavlidou E, Sarkar TK et al (2004) Can patients to benefit from primary chemotherapy for breast cancer be predicted before commencement of treatment? Breast Cancer Res Treat 86:181–189
34. Vincent–Salomon A, Rousseau A, Jouve M, Beuzeboc P, Sigal-Zafrani B, Fréneaux P et al (2004) Proliferation markers predictive of the pathological response and disease outcome of patients with breast carcinomas treated by antracycline-based preoperative chemotherapy. Eur J Cancer 40:1502–1508
35. Prisack HB, Karreman C, Modlich O, Audretsch W, Danae M, Rezai M et al (2005) Predictive biological markers for response of invasive breast cancer to anthracycline /cyclophosphamide-based primary (radio-)chemotherapy. Anticancer Res 25:4615–4621
36. Fasching PA, Heusinger L, Niklos M, Hein A, Bayer CM, Rauh C et al (2011) Ki-67, chemotherapy response, and prognosis in breast

cancer patients receiving neoadjuvant treatment. BMC Cancer 11:486–498
37. Penault-Llorca F, Abrial C, Raoelfils I, Chollet P, Cayre A, Mouret-Reynier M-A et al (2008) Changes and predictive and prognostic value of the mitotic index, Ki-67, Cyclin D1, and Cyclo-oxigenase-2 in 710 operable breast cancer patients treated with neoadjuvant chemotherapy. Oncologist 13:1235–1245
38. Bozzetti C, Musolino A, Camisa R, Bisagni G, Flora M, Bassano C et al (2006) Evaluation of HER-2/Neu Amplification and other biological markers as predictors of response to neoadjuvant anthracycline-based chemotherapy in primary breast cancer: the role of anthracycline dose intensity. Am J Clin Oncol 2:171–177
39. Bottini A, Berruti A, Bersiga A, Brizzi MP, Bruzzi P, Aguggini S et al (2001) Relationship between tumour shrinkage and reduction in Ki-67 expression after primary chemotherapy in human breast cancer. Br J Cancer 85:1106–1112
40. Petit T, Wilt M, Millon R, Rodier JF, Borel C, Mors R et al (2004) Comparative value of tumor grade, hormonal receptors, Ki-67, HER-2 and topoisomerase II alpha status as predictive markers in breast cancer patients treated with neoadjuvant anthracycline-based chemotherapy. Eur J Cancer 40:205–211
41. Tordai A, Wang J, Andre F, Liedtke C, Yan K, Sotiriou C et al (2008) Evaluation of biological pathways involved in chemotherapy response in breast cancer. Breast Cancer Res 10:R37
42. von Minckwitz G, Sinn HP, Raab G, Loibl S, Blohmer JU, Eidtmann H et al (2008) Clinical response after two cycles compared to HER2, Ki-67, p53, and bcl-2 in independently predicting a pathological complete response after preoperative chemotherapy in patients with operable carcinoma of the breast. Breast Cancer Res 10:R30
43. Lee J, Im YH, Lee SH, Cho EY, Choi YL, Ko YH et al (2008) Evaluation of ER and Ki-67 proliferation index as prognostic factors for survival following neoadjuvant chemotherapy with doxorubicin/docetaxel for locally advanced breast cancer. Cancer Chemother Pharmacol 61:569–577
44. Burcombe RJ, Makris A, Richman PI, Daley FM, Noble S, Pittam M et al (2005) Evaluation of ER, PR, HER-2 and Ki-67 as predictors of response to neoadjuvant antracycline chemotherapy for operable breast cancer. Br J Cancer 92:147–155
45. Burcombe RJ, Wilson GD, Dowsett M, Khan I, Richman PI, Daley F et al (2006) Evaluation of Ki-67 proliferation and apoptotic index before, during and after neoadjuvant chemotherapy for primary breast cancer. Breast Cancer Res 8:R31
46. Arpino G, Ciocca DR, Weiss H, Allred DC, Daguerre P, Vargas-Roig L et al (2005) Predictive value of apoptosis, proliferation, HER-2, and topoisomerase IIα for anthracycline chemotherapy in locally advanced breast cancer. Breast Cancer Res Treat 92:69–75
47. Kariya S, Ogawa Y, Nishioka A, Moriki T, Ohnishi T, Ito S et al (2005) Relationship between hormonal receptors, HER-2, p53 protein, bcl-2, and MIB-1 status and the antitumor effects of neoadjuvant anthracycline-based chemotherapy in invasive breast cancer patients. Radiation Med 23:189–194
48. Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G (2004) Cell death by mitotic catastrophe: a molecular definition. Oncogene 23:2825–2837
49. Shay JW, Roninson IB (2004) Hallmarks of senensce in carcinogenesis and cancer therapy. Oncogene 23:2919–2933
50. Tiezzi DG, De Andrade JM, Candido Dos Reis FJ, Cosiski Marrana HR, Ribeiro-Silva A, Tiezzi MG et al (2006) Apoptosis induced by neoadjuvant chemotherapy in breast cancer. Pathology 38:21–27
51. Rein DT, Schondorf T, Breidenbach M, Janat MM, Weikelt A, Gohring UJ et al (2000) Lack of correlation between p53 expression, bcl-2 expression, apoptosis and ex vivo chemosensitivity in advanced human breast cancer. Anticancer Res 20:5069–5072
52. Buchholz TA, Davis DW, McConkey DJ, Symmans WF, Valero V, Jhingran A et al (2003) Chemotherapy-induced apoptosis and bcl-2 levels correlate with breast cancer response to chemotherapy. Cancer J 9:33–41
53. Vargas-Roig LM, Cuello-Carrión FD, Fernandez-Eskobar N, Daguerre P, Leuzzi M, Ibarra J et al (2008) Prognostic value of bcl-2 in breast cancer patients treated with neoadjuvant anthracycline based chemotherapy. Molecular Oncol 2:102–111
54. Navarrete MAH, Maier CM, Falzoni R, de Azavedo Quadros LG, Lima GR, Baracat EC, Nazario ACP (2005) Assessment of the proliferative, apoptotic and cellular renovation indices of the human mammary epithelium during the follicular and lutheal phases of the menstrual cycle. Breast Cancer Res 7:306–313
55. Aas T, Geisler S, Helle H, Børrensen-Dale AL, Lønning PE, Akslen LA (2005) Prognostic and predictive value of changes in tumor cell proliferation in locally advanced breast cancer primarily treated with doxorubicin. Oncol Reports 13:525–530
56. Zunino F, Perego P, Pilotti S, Pratesi G, Supino R, Arcamone F (1997) Role of apoptotic response in cellular resistance to cytotoxic agents. Pharmacol Ther 76:177–185
57. Frassoldati A, Adami F, Banzi C, Criscuolo M, Piccinini L, Silingardi V (1997) Changes of biological features in breast cancer cells determined by primary chemotherapy. Breast Cancer Res Treat 44:185–192
58. Takada M, Kataoka A, Masazaku T, Bando H, Toyama K, Horiguchi S et al (2004) A close association between alteration in growth kinetics by neoadjuvant therapy and survival outcome in primary breast cancer. Int J Oncol 25:397–406
59. Shao ZM, Li J, Wu J, Qi-Xia H, Zhen-Zoi S, Fontana JA, Barsky SH (1999) Neo-adjuvant chemotherapy for operable breast cancer induces apoptosis. Breast Cancer Res Treat 53:263–269
60. Dowsett M, A'Hern RA, Salter J, Zabaglo L, Smith IE (2009) Who would thought a single Ki-67 measurement would be predict long-term outcome? Breast Cancer Res 11:S15
61. Assersohn LA, Salter J, Powles TJ, A'hern R, Makris A, Gregory RK et al (2003) Studies of potential utility of Ki-67 as a predictive molecular marker of clinical response in primary breast cancer. Breast Cancer Res Treat 82:113–123
62. Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, Griffith C et al (2006) Proliferation and apoptosis as marker of benefit in neoadjuvant endocrine therapy of breast cancer. Clin Cancer Res 12:1024–1029
63. Jones RL, Salter J, A'Hern R, Nerurkar A, Parton M, Reis-Filho JS et al (2009) The prognostic significance of Ki-67 before and after neoadjuvant chemotherapy in breast cancer. Breast Cancer Res Treat 116:53–68