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Predictive Biological Markers for Response of Invasive Breast Cancer to Anthracycline / Cyclophosphamide-based Primary (Radio-)Chemotherapy

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Abstract. *Background: The role of biological markers for the prediction of neoadjuvant chemotherapy and radio-chemotherapy may be evaluated using pathological complete response [pCR] in patients with invasive breast cancer. Materials and Methods: To investigate this, pre-treatment biopsies from 517 patients with locally advanced breast cancer were analyzed for expression of estrogen receptor [ER], progesterone receptor [PgR], Her-2/neu, epidermal growth factor receptor [EGF-R], p53, Bcl-2 and MIB-1 by immunohistochemistry [IHC], and these data were compared to the pathological response after preoperative epirubicine/cyclophosphamide [EC] chemotherapy (+/– radiotherapy). Results: pCR was more frequent (28.3%, 56/198) in tumors that received radio-chemotherapy compared to chemotherapy alone (11.9%, 38/319, $p<0.0001$). Patients with high grading, lower ER, PgR, Bcl-2 or a higher proliferation had a significantly greater benefit from chemotherapy. The overexpressions of Her2/neu or EGF-R were weakly correlated to pCR, while p53 staining did not have any predictive value. Younger patients, with negative PgR and high proliferation index, had the highest benefit from EC therapy (56% pCR). The different multivariate indices of binary regression, PLS-DA and SIMCA, had similar predictive quality and were slightly superior to univariate factors. Conclusion: This study emphasizes the value of traditional biological markers and Bcl-2 for use in the individual selection of a primary therapy regimen.*

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"Neoadjuvant" chemotherapy or, technically more correct, "preoperative" chemotherapy is increasingly used in patients with advanced local breast cancer. The reduction of tumor volume diminishes the extension of surgical resection. The aim of this study was to evaluate the prediction of immunohistochemical markers for epirubicine/cyclophosphamide [EC] therapy success. Neoadjuvant chemotherapy provides the opportunity of early identification of treatment failures or therapy success. There are ongoing trials for final confirmation that there is a close correlation between clinical [cCR], or better a pathological confirmed response [pCR] and long-term survival (1, 2). Although it should be without question that evaluation of the long-term benefit of primary chemotherapy is the most worthwhile aim, it is difficult to estimate the contribution of the primary therapy after different downstream surgical strategies and additional post-surgical procedures (XRT and/or further chemotherapy regimes) have been carried out. Neoadjuvant therapy provides the opportunity to evaluate the correlated, but up-to-now insufficient, biological markers for prediction of chemotherapy. Estrogen receptor and progesterone receptor are the primary markers available today and, although widely accepted for selection of patients for endocrine therapy, their role in primary chemotherapy remains controversial (3). In addition to the steroid receptors, the EGF-receptor family (predominantly HER-2/*neu* (4) and EGF-R), the tumor suppressor gene *p53* (5) and the antiapoptotic Bcl-2 (6) are also under investigation. An ever increasing number of studies has screened for differential gene expression, recently with gene-array techniques, in order to identify factors that can predict response to chemotherapy (7).

The aim of this study was to evaluate the role of the immunohistochemically determined markers in the prediction of pathological response to neoadjuvant chemotherapy and

to compare it to predictive values of clinical parameters. The analysis of the correlation to pathological complete response was regarded for each univariate factor. Different multivariate models were tested, and the factors included were combined respectively with coefficients to give a regression score. These scores are regarded as predictive indices and were compared with the univariate prediction.

Materials and Methods

Patients and therapy. This study was performed as a co-operation between the Institute of Chemical Oncology, University of Düsseldorf, and the interdisciplinary Breast Center IBC, City Hospital Düsseldorf. All patients (n=517) were recruited between 1994 and 2004, Germany. The patients' ages ranged from 32 to 78 with a median of 57±10 years. Patients provided informed consent to all procedures. All tumor samples were collected as jet needle biopsies of primary invasive breast cancer before immunohistochemicals EC treatment. For validation of the predictive value of the different [IHC] parameters, all patients were examined prospectively. Patients received 4 (96%) to 6 courses of epirubicine 90 mg/m^2 and cyclophosphamide 600 mg/m^2 as up-front chemotherapy. In 217, cases this EC scheme was accompanied or followed with tamoxifen until surgery. Additionally, 50 Gy local radiation therapy was administered to 198 patients prior to surgery. These 198 irradiated patients [EC XRT(+)] and the 319 non-irradiated patients [EC XRT(–)] were considered as two separate cohorts. Election for the additional irradiation depended on the later oncologic surgery design and was not randomized. Patients underwent definitive mastectomy or breast conserving surgery 4.3±1.3 months after primary tumor biopsy. Tumors were graded and classified from hematoxylin and eosin (H&E)-stained sections of the first jet-biopsy, and histopathological evidence of response was scored on a surgical resection specimen obtained after chemotherapy. As neither disease-free survival nor overall survival were in the focus of this study, post-surgery therapy schemes were not further considered for interpretation.

Histopathological response to EC. Pre-therapeutic tumor size was determined by MRT and/or ultrasonography. The size of the residual tumor at the time of surgery was determined by a pathologist. A pathological complete response (pCR) was defined as absence of any residual invasive tumor (pT0N0). The intermediate partial response (pPR) was defined as at least 50% reduction of the initial tumor diameter (including DCIS), and NC (non-response) was defined either as marginal or absence of tumor reduction or observation of progressive disease. Pathological response was the target variable for each model in receiver operation characteristic (ROC) analysis with dichotomization of pCR *versus* the combined worse group of partial responder and nonresponder. Thus, the subdivision of partial response and nochange information was not regarded for the multivariate model calculations, but was used for multivariate model validation.

Immunohistochemistry [IHC]. Five-μm-thick sections of paraffin-embedded tissue areas were used for IHC. Paraffin was removed by xylene and a decreasing series of ethanol. Epitope retrieval was performed by heat induction in Target Retreival Solution pH 6.1 (DAKO, Cytomation, Hamburg, Germany) and steam. Tissues were blocked for endogenous peroxidase in a 0.3% H_20_2 solution for 15 min. Monoclonal antibodies (ERα: ER1D5, DAKO 1:35, *p53*: DO-7 1:200, PgR: PGR636 DAKO 1:50, Bcl-2: Clone 124 1:200 DAKO, EGFR: 31G7 (Zymed, San Francisco, CA, USA) 1:20, Her-2/*neu* polyclonal DAKO 1:250, and Mib-1 DAKO 1:200) were used for specific epitope detection. The ChemMate DAKO Peroxidase/DAB detection kit was used for linking and staining.

Table I. *Clinical and pathological characteristics of patients.*

	Number (% of known)	
	EC XRT (–)	EC XRT (+)
Number	319	198
Age		
<50 years	72 (22.6%)	52 (26.3%)
≥50 years	247 (77.4%)	146 (73.7%)
Nodal status		
N0	48 (36.1%)	77 (60.6%)
N+	85 (63.9%)	50 (39.4%)
Pathol. response to EC		
NC	128 (40.1%)	52 (26.3%)
PR	153 (48.0%)	90 (45.5%)
CR	38 (11.9%)	56 (28.3%)
Histological characteristics		
Histological type		
ductal invasive	147 (51.4%)	103(59.9%)
lobular invasive	105 (36.7%)	48(27.9%)
others	34 (11.9%)	21(12.2%)
Grade		
G1	25 (8.4%)	23 (12.9%)
G2	218 (73.6%)	115 (64.6%)
G3	53 (17.9%)	39 (21.9%)
Immunohistochemical scores		
ER score <10	80 (25.2%)	35 (17.8%)
ER score ≥10	238 (74.8%)	162 (82.2%)
PgR score <10	107 (33.6%)	44 (22.4%)
PgR score ≥10	211 (66.4%)	152 (77.6%)
Bcl-2 score <100	118 (48.8%)	47 (39.2%)
Bcl-2 score ≥100	124 (51.2%)	73 (60.8%)
p53 score <180	269 (87.6%)	173 (91.1%)
p53 score ≥180	38 (12.4%)	17 (8.9%)
Mib-1 ≤30%	185 (58.9%)	139 (71.3%)
Mib-1 >30%	129 (41.1%)	56 (28.7%)
EGFR = 0 to 2+	143 (87.7%)	29 (87.9%)
EGFR >2+	30 (17.3%)	4 (12.1%)
HER-2 = 0 to 2+	257 (80.6%)	171 (87.2%)
HER-2 > 2+	62 (19.4%)	25 (12.8%)

Table II. *Predictive values of tumor characteristics based on receiver operating characteristic. EC XRT(–).*

	correlation	pCR *vs.* (PR+NC)		(pCR+PR) *vs.* NC		
		AUC	*p*-value	AUC	*p*-value	n
ER score	(–)	0.757	0.000	0.617	0.000	318
PgR score	(–)	0.746	0.000	0.648	0.000	318
Bcl-2 score	(–)	0.702	0.000	0.621	0.000	242
p53 score	0	0.525	0.624	0.503	0.201	307
Mib-1 score	(+)	0.712	0.000	0.643	0.000	314
EGF-R score	(+)	0.614	0.080	0.516	0.748	173
Her2/*neu* score	(+)	0.567	0.181	0.619	0.000	319
Age	(–)	0.603	0.039	0.502	0.945	319
Size	0	0.501	0.981	0.553	0.169	229
Grade	(+)	0.614	0.038	0.583	0.015	296
ductal *vs.* lobular	0	-	0.276[a]	-	0.247[a]	252
nodal (N0 *vs.* N+)	(–)	-	0.096[a]	-	0.046[a]	133

[a]Fisher's exact test; (+) correlation , (–) inverse corr., (0) no corr.

Table III. *Predictive value of tumor characteristics based on receiver operating characteristic. EC XRT(+).*

	correlation	pCR *vs.* (PR+NC)		(pCR+PR) *vs.* NC		
		AUC	*p*-value	AUC	*p*-value	n
ER score	(–)	0.613	0.013	0.585	0.078	197
PgR score	(–)	0.665	0.000	0.582	0.081	196
Bcl-2 score	(–)	0.595	0.098	0.526	0.678	120
P53 score	0	0.516	0.734	0.563	0.188	190
Mib-1 score	(+)	0.598	0.032	0.528	0.546	195
EGF-R score	(+)	0.693	0.081	0.565	0.763	33
Her2/*neu score*	0	0.524	0.598	0.522	0.635	196
Age	0	0.503	0.940	0.536	0.445	198
Size	0	0.555	0.275	0.533	0.514	162
Grade	0	0.581	0.093	0.504	0.937	178
ductal *vs.* lobular	0	-	0.575[a]	-	0.326[a]	151
nodal (N0 *vs.* N+)	0	-	0.552[a]	-	0.846[a]	127

[a]Fisher's exact test; (+) correlation , (–) inverse corr., (0) no corr.

Slides were counterstained with methyl green and coverslipped with Entelan.

Histological scores were calculated by multiplication of a six-point scaled color intensity (0 to 5) and the proportion of positive cells for ERα, PgR, Bcl-2 and *p53*. The proliferation marker Mib-1 was scored quantitatively by the percentage of positively-stained nuclei. Circumferential membrane-bound staining for Her2/*neu* and EGF-R were scored on a modified 3+ scale: "0"=No staining ; "1"=1+ (>10% cells weakly positive); "3"= 2+(moderate homogeneous staining) and "5"= 3+(strong homogenous staining). The scores "2" and "4" were intermediates for 1+/2+ and 2+/3+, respectively. The patients' clinical characteristics and the results of immunohistochemical findings are summarized in Table I.

Statistical analysis. To account for the potential influence of presurgical irradiation or short-term tamoxifen treatment on the pathological CR, cross tables and the Fisher's exact test were used prior to further analysis. Analyses were done using SPSS for Windows, Release:11.0.1. For univariate analysis, test variables are considered primary as continuous, because dichotomization by cut-offs may reduce potentially important information for prediction (8). We therefore investigated the ROC curves of the different classifiers, which summarize the sensitivities and specificities over all possible thresholds (9). Grading and size were used as trend categories.

Multivariate analysis was performed using a bivariate logistic regression analysis with the tested markers included as continuous variables. Three different logistic strategies were compared: Multi-1: multivariate inclusion of all factors with an univariate significance of $p<0.05$ without consideration of possible variable interactions. Multi-2: stepwise forward conditioned multivariate analysis with criteria for inclusion $p<0.05$ and limit for exclusion $p>0.20$, also without considering interactions. Multi-3: The same strategy as Multi-2 but additionally considering all possible paired interactions. Furthermore, two multivariate statistical projection methods were used for classification: soft independent modelling of class analogy [SIMCA] (10) and the partial least squares discriminant analysis [PLS-DA] (11). Both are classification strategies based on reduction of input dimensions on the basis of principal component analysis [PCA] and were calculated with SIMCA-P 10.0.4.0 software, (Umetrics, Umea, Sweden). For both models the first two principal components were calculated. The results of class prediction probabilities were comparatively evaluated in ROC analysis.

Results

Five hundred and seventeen patients with operable invasive breast cancer were treated with neoadjuvant EC-based chemotherapy and were analyzed for predictive molecular markers for pathologically scored presurgical response; 18.2% of all the patients had a pathological complete response, 47.0% had a tumor reduction of more than half of the initial diameter and 34.8% had progress or no pathological response after 4 to 6 EC cycles. Since about 38% of the patients were also locally irradiated, we compared the response rate for irradiated [EC XRT(+)] and non-irradiated patients [EC XRT(–)] cohorts. In the radio-chemotherapy cohort 28.3% (56/198) complete tumor remission (pCR) was recorded, while we observed 11.9% pCR (38/319) in patients treated with chemotherapy alone ($p<0.0001$). Based on these results, both cohorts were further analyzed independently. The additional treatment with tamoxifen until surgery did not have any significant effects on pCR in any cohort.

Table IV. *Pathological response and predictive markers to neoadjuvant EC chemotherapy +/– XRT. Biological markers were regarded as dichotomized classes. Significant classification were marked with asterisks.*

Dichotomized characteristics	EC XRT(–) pCR n(%)	*p*-value	EC XRT(+) pCR n(%)	*p*-value
<50 years	* 15 (20.8%)	0.012	15 (28.8%)	0.525
≥50 years	23 (9.3%)		41 (28.0%)	
N0	9 (18.8%)	0.096	25 (32.5%)	0.552
N+	7 (8.2%)		13 (26.0%)	
ductal invasive	17 (11.6%)	0.278	33 (32.0%)	0.575
lobular invasive	7 (6.7 %)		13 (27.0%)	
G1	* 1 (4.0%)	0.008	4(17.4%)	0.060
G2	19 (8.7%)		31 (27.0%)	
G3	11 (20.8%)		16 (41.8%)	
ER score <10	* 23 (28.8%)	0.000	* 19 (54.3%)	0.000
ER score ≥10	15 (6.3%)		37 (22.8%)	
PgR score <10	* 28 (26.2%)	0.000	* 20 (45.5%)	0.008
PgR score ≥10	10 (4.7%)		36 (23.7%)	
Bcl-2 score <100	* 25 (21.2%)	0.000	18 (38.3%)	0.164
Bcl-2 score ≥100	5 (4.0%)		19 (26.0%)	
p53 score <180	30 (11.2%)	0.110	50 (28.9%)	1.000
p53 score ≥180	8 (21.1%)		5 (29.4%)	
Mib-1 ≤30 %	* 11 (5.9%)	0.000	31 (22.3%)	0.003
Mib-1 >30 %	27 (20.9%)		25 (44.6%)	
EGFR = 0 to 2+	* 15 (10.5%)	0.033	7 (24.1%)	0.073
EGFR >2+	8 (26.7%)		3 (75.0%)	
HER-2 = 0 to 2+	* 26 (10.1%)	0.051	47 (27.5%)	0.477
HER-2 >2+	12 (19.4%)		9 (36.0%)	

Correlating tumor characteristics with response to EC. The preoperative chemotherapy characteristics of patients and tumors were tested for correlation with pathological response. Patients who received an additional radiation therapy showed a significantly higher response rate, but generally the prechemotherapeutic characteristics had lower predictive values if irradiation was additionally applied (Table II *vs.* Table III). In both cohorts, the tumor size at diagnosis was not correlated to response, while higher grading clearly correlated with pCR. However, in the EC XRT(+) group this observation was not significant. The probability of complete response to EC alone was 2-fold higher in patients below 50 years than in older patients (20.8% *vs.* 9.8%, Table IV). In particular, 7 out of 13 young patients (<37 years) developed a complete response under EC therapy. The response was not significantly correlated to ductal or lobular tumor type. The ROC area under the curve analysis showed that tumors with a high Mib-1 staining had a better rate of response. The ER and PgR contents were negatively correlated to response. On a histological score threshold equal to 10, the ER- or PgR-negative tumors displayed a 4.5-fold and 5.5-fold higher benefit, respectively, than the ER- or PgR-positive tumors. In the EC XRT(+) group the pCR proportion increased, so that 45.5% of the ER-negative and 54.3% of the PgR-negative tumors did not show any residual tumor after therapy.

In the EC XRT(–) group (Table II), it can be seen that tumors with lower Bcl-2 scores have a better prognosis. Using a histological score threshold of 100, the group of tumors with low Bcl-2 expression had a 5.5-fold higher chance of having a complete response (Table IV). In the EC XRT(+) cohort this advantage was diminished and lost significance (Table III). Continuously scaled EGF-R and Her2/*neu* correlated positively with response in the EC XRT(–) group only. Both EGF-R and Her2/*neu* displayed a higher value of prediction after being dichotomized by separation on a >2+ cut-off level. EGF-R overexpressing tumors showed a 2.6-fold higher proportion of pCR than low or weak expressing tumors.

It is obvious that biological factors have a higher predictive value for pCR than for NC (Table II). The areas under the ROC curves for NC prediction are definitively smaller than those for pCR calculation. Although significance is reduced, all variables indicate the same direction of correlation regarding the prediction of the NC class. We presume that NCs are not separated restrictively by pathological definition from partial responder (pPR), as the pCR can be defined. The only exception is the Her2/*neu* expression, which became significant in prediction of cases that would not respond to EC therapy alone (NC), while pCR prediction by Her-2/*neu* was not significant. We found EGF-R to be correlated to p53 and Mib-1 expression, while the inverse correlation to ER and PgR were also of high significance (data not shown).

Multivariate models of correlated characteristics. Multivariate analysis was used in order to improve the combination of different conventional prognostic markers into a prognostic index for prediction of success of EC XRT(–) therapy.

In the first binary logistic regression model, all univariate significant markers were included as continuous covariates. The response was defined as dichotomized pCR *vs.* (PR & NC) and used as a dependent variable. In the Multi-1 model, all factors were included without condition. Only Mib-1 was independently associated with response ($p=0.011$). This model showed higher AUC values in ROC analysis than each of the univariate factors (see Table V for

Table V. *Multivariate analysis – Comparison of five different models (Multi 1-3 binominal regressions, PLS-DA, SIMCA) calculated on prediction of pathological complete response in the EC-XRT(–) therapy group. The predictive value of the models was tested in the NC (no change) vs. pPR (partial response) classification and in the EC XRT(+) collective. Factors with significant multivariate influence p<0.05 were marked with an asterisk. The ranking of included factors displays their degree of contribution to the model.*

EC		XRT(–)					XRT(+)		
		pCR *vs.* (PR + NC) used for model calculation		NC *vs*. PR used for model validation			pCR *vs*. (PR + NC) used for model validation		
Model	included factors	AUC	*p*-value	AUC	*p*-value	n	AUC	*p*-value	n
Multi-1	Mib-1*, PgR, grading, ER, age, Bcl-2	0.796	0.000	0.678	0.000	226	0.652	0.000	108
Multi-2	Age*, PgR*, Mib-1*	0.783	0.000	0.664	0.000	313	0.663	0.000	194
Multi-3	age*, Mib-1 by age*, PgR by Mib-1*	0.809	0.000	0.608	0.002	313	0.665	0.000	194
PLS-DA	ER, Mib-1, PgR, Bcl-2, age, grading	0.780	0.000	0.644	0.000	319	0.617	0.000	198
SIMCA	PgR, Mib-1, grading, ER, Age, Bcl-2	0.761	0.000	0.666	0.000	319	0.640	0.000	198

ROC curve comparison). In the second step, factors below a univariate *p*-value <0.2 were included into the model stepwise. The resulting model was determined by a combination of age, Mib-1 and PgR, giving a slightly better resolution between pCR and (PR+NC). In the third conditional model, all paired interactions were additionally included into the Multi-2 model. The resulting Multi-3 model comprises the covariates age, Mib-1 corrected for age and PgR corrected for Mib-1. The prediction of Multi-3 was not superior to the models without considering interactions.

Nevertheless, patient ages, which univariately had a lower impact on prediction than receptors and proliferation, were included with significance into the conditional Multi-2 and Multi-3 models in combination with PgR and Mib-1. We found that the younger patients with missing PgR-expression had a better profit (48.1% pCR *vs.* 4.4%) from these predictors than patients over 50 years old (18.5% pCR *vs.* 4.8%). Further, younger patients profited more from a higher proliferation (31.7% pCR *vs.* 7.4%) than did the elder group (15.9% pCR *vs.* 5.7%).

Neither PLS-DA nor SIMCA could improve the multivariate prediction more than the binary logistic models, when significant univariate biological factors and clinical information were integrated into these models.

We validated the 5 models acquired by the efficacy in separating pCR, by application of the regressions to separate pPR *vs.* NC in the same patient collective and to their prediction of pCR in the EC XRT(+) group (Table V). The AUC data, achieved in the validation sets, were expectedly lower than in the AUC of model calculation, but all models were still very significant $p<0.01$ in both validation strategies. The univariate predictive values of the established biological factors, like ER, PgR or Mib-1 in the validation sets, were in the same range of size (Table II and Table III) as the multivariate models (Table V). Thus, the profit of multivariate indices is obvious in the data set used for model calculation, but has limited the worth of improvement towards univariate factors, when tested with the validation data. The multivariate binary logistic analysis of patients with additional radiation therapy EC XRT(+) resulted in a function of regression that contained only the PgR expression as an independent variable.

Discussion

Primary chemotherapy provides an ideal scenario for the investigation of predictive factors of response to chemotherapy in breast cancer. However, considering that complete pathological response is a relatively uncommon scenario, it requires a huge cohort as was used in this study. We had to correct our data set for patients who received only EC therapy without additional irradiation. The high local effects that were achieved by irradiation of tumors seem to overwhelm the effects of accompanying systemic chemotherapy and the influence of predictive markers, when using the pCR as a surrogate marker for the success of therapy. All analyzed biological markers that we found significantly correlated to response in the EC XRT(–) subset, displayed the same direction of association in the EC XRT(+) cohort, but with lower AUC values. The increased proportion of pCR in the EC XRT(+) group *vs.* chemotherapy alone has been previously reported by Aryus *et al.* (12) in a small number-based protocol, which we could verify on this extended data-set. While XRT had intensive influence on pCR rates, the preoperative influence of additional tamoxifen was not superior to the epirubicin-cyclophosphamide combination without hormonal support. Tamoxifen was administered to 50.5% of the ER-positive patients, but not included as a consequent concomitant preoperative agent. Bottiny and colleagues (13) studied the

concomitant effect of tamoxifen on 211 patients and concluded that the addition of tamoxifen to epirubicin primary chemotherapy did not significantly improve the response rate.

We confirm previous and recent observations that low ER and low PgR expressions are predictive for positive chemotherapy response. In ROC analysis, we could show that there is an inverse quantitative relationship between ER/PgR expression and probability of pCR. The high correlation that we found was confirmed by Colleoni *et al.* (14), who actually observed a high correlation of ER- or PgR-negative tumors and positive Ki-67 expression with response to primary chemotherapy. Ring *et al.* also found a higher proportion of pCRs in ER-negative tumors. In their extensive analysis of 435 tumors, they confirmed that overall survival (OS) was better in those patients who achieved a pCR compared to those who did not (15).

The two most studied regulators of apoptosis, p53 and bcl-2, were also analyzed, but the results were in contrast to those of Bottini *et al.* (16), who found that p53 expression was a significant predictor for poor clinical CR in patients submitted to epirubicine. We could not observe an association of pCR to p53 in our data. Bottini summarized four additional p53 studies on adjuvant therapy. In three CMF studies, no effect of p53 staining on responsiveness was shown, whereas the fourth study on anthracycline-based FAC therapy revealed an association between p53 accumulation and poor response.

This is the second publication to report that the lack of Bcl-2 can predict a better response to neoadjuvant chemotherapy. Since we found a positive correlation of Bcl-2 to steroid hormone receptor expression and an inverse correlation to proliferation, it is surprising why this association is rarely reported. Although we could not estimate an independent contribution to response, as Bcl-2 did not have a great influence in multivariate regression, Ogston *et al.* (17) identified the absence of Bcl-2 to be the only marker for better pathological response in a multivariate model. It is untypical in their set of 104 patients that MIB-1 and PgR were only marginally correlated and did not have any significance on pCR. Thus, we presume that their results are distorted by the low number of patients and a very complex neoadjuvant regime combining CVAP (cyclophosphamide-vincristine-doxorubicine-prednisonole) in conjunction with docetaxel or additional cycles of anthracycline. Fanyete *et al.* also failed to show Bcl-2 or Ki-67 influence on pCR, but their data comprised only 3 pCRs (3%) (18). Better and even multivariate correlations of steroid receptors and Ki-67 to pCR were described by Petit *et al.* (19) in their analysis of 119 FEC-treated patients. They found the highest univariate correlation of pCR to tumor grade that was also predictive in both our patient cohorts. The role of Her-2/*neu* as a predictor for adjuvant anthacycline therapy is discussed controversially. According to our observations and as repeatedly reported (13, 20, 21), Her-2/*neu* overexpression is associated with increased proportions of pCR, but this tendency failed to show significance.

The proportion of EGF-R overexpression was more rare than that of Her2/*neu*. Neither factors was correlated, and overexpression of EGF-R had higher predictive value for response than that of Her-2/*neu*. If proliferation, or one of the steroid receptors was included in multivariate analysis, EGF-R lost its predictive contribution. It was reported that patients with positive EGF-R had a significantly worse postrelapse survival than those with negative EGF-R (22).

We found interesting interactions of age, proliferation and progesterone receptor status in multivariate analysis. In the group of patients where these three factors are in the favorable status, 56% of complete tumor remission was achieved. If only one of these factors was unfavorable, this property dropped to 16.7% pCR *versus* 4.9% pCR in cases where all three factors were unfavorable. Therefore, the relative risk gradually increased as the number of poor findings increased. The same pattern was reported for a combination of EGF-R, ER and ploidy (23).

We also observed a higher value of prediction of significant variables in patients aged below 50, except for Ki-67. Since different rankings of univariate correlation were observed in comparable studies and, consequently, multivariate stepwise regression highlighted different, independent parameters, we decided to test more integrative methods without stepwise inclusion, such as the multivariate projection-based strategies SIMCA and PLS-DA. The use of PLS-DA regression and SIMCA models are described as useful methods when the number of subjects is small compared to the number of explanatory variables and when there is a high collinearity between these variables (24). These methods were actually successfully used for array data and also for the evaluation of conventional putative prognostic factors (25). We presume that, because of the high internal correlation between our factors, these models were not superior to binary regression.

In conclusion, the present study indicated the predictive significance of grading, ER, PgR, Bcl-2, proliferation and EGF-R for primary anthracycline therapy. Since there is a high internal correlation between these factors, regression of combined variables delivered only a slight improvement in prediction. We observed a higher impact of biological factors on response in younger patients and in patients without additional local radiotherapy, which itself resulted in higher pCR rates. Additional presurgical tamoxifen application did not improve the response and did not have any influence on the predictive value of variables. The combination of more or less dependent factors may result in a profiling, which reinsures tumor classification and

improves diagnostic quality. However, for further refinement, we expect a large contribution from the arising gene-array profiling, which should be screened for proliferation- and ER- independent variables.

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