

# Evaluation of prognostic factors following flow-cytometric DNA analysis after cytokeratin labelling: I. Breast cancer

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**Abstract.** In gynecologic oncology valid prognostic factors are necessary to estimate the course of disease and to define biologically similar subgroups for analysis of therapeutic efficacy. The presented study is a prospective study concerning prognostic significance of DNA ploidy and S-phase fraction in breast cancer following enrichment of tumor cells by cytokeratin labelling. Epithelial cells were labeled by FITC-conjugated cytokeratin antibody (CK 5, 6, 8, and CK 17) prior to flow cytometric cell cycle analysis in 327 fresh specimens of primary breast cancer.

Univariate analysis in breast cancer detected the prognostic significance of DNA-ploidy, S-phase fraction and CV (coefficient of variation) of G<sub>0</sub>G<sub>1</sub>-peak of tumor cells for clinical outcome, especially for nodal-negative patients. Multivariate analysis could not confirm prognostic evidence of DNA-ploidy and S-phase fraction.

In conclusion, in breast cancer no clinical significance for determination of DNA-parameters was found.

## 1. Introduction

Prognostic factors are an important basis for optimal choice of therapeutic strategy. Prognostic factors are important to decide whether additional adjuvant therapeutic modalities are necessary. They render an individual care for patients with gynecologic malignancies. In addition to known classic prognostic factors like clinical stage, operability and histopathologic results many so called “prognostic factors” of the morphologic and molecular genetic domain were investigated in the last decades. New studies showed that grading and classic histopathological parameters are no reliable predictors of outcome in individual patients for example in oral leukoplakia and oral carcinoma [1].

Chromosomal rearrangements represent an early step in the initiation of tumorigenesis. The regulation of growth's control mechanisms is changed following alteration of the expression of certain genes. Alteration of genetic factors is often accompanied by quantitative changes of DNA content such as the p53 gene alteration, which is associated with DNA aneuploidy [2].

All studies investigating DNA content by flow cytometry were performed without identification of tumor cells prior to DNA analysis. Several studies indicate that mutations in genes controlling chromosome segregation during mitosis and centrosome abnormalities play a critical role in causing chromosome instability in cancer [3]. And chromosomal aberrations seem to occur exclusively in aneuploid tumor cell lines [3]. It has been shown for different carcinomas that cytokeratin staining is able to detect epithelial tumor cells [4,5]. For breast carcinoma [4,6] and cervical carcinoma [7], about 20% of DNA-aneuploid tumor sub-

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populations are missed without enrichment for cytokeratin positive cells. In addition, S-phase fraction can be determined more accurately [6–10]. Multiparameter analysis has been recommended for future flow cytometric DNA analysis [11,12]. The presented prospective study investigated the prognostic significance of DNA ploidy and S-phase fraction in breast cancer following enrichment of tumor cells by cytokeratin labelling.

## 2. Materials and methods

Fresh tumor tissue of carcinomas of the breast ( $n = 327$ ) were dissociated by combined mechanical/enzymatic method [13] as described previously in detail [14]. In short, disaggregation of tissue was performed in a prewarmed enzyme mixture in Dulbecco's phosphate buffered saline (PBS) consisting of 2 mg/ml trypsin (type III, 10,000 U/mg protein, Sigma chemicals), 2 mg/ml collagenase (type I-S 180 U/mg solid, Sigma chemicals) and 0.2 mg/ml DNase (type I, 1548 U/mg protein, Sigma chemicals) at 37°C for 10 minutes. Cell number and viability of the single cell suspension was determined by cell counter and trypan blue exclusion prior to fixation in 70% methanol (–20°C). Following resuspension of  $5 \times 10^4$  to  $2 \times 10^5$  cells in 0.5 ml PBS/5% fetal calf serum (FCS) cells were labeled for cytokeratins 5, 6, 8 and 17 using FITC conjugated monoclonal mouse anti-human cytokeratin antibodies (Dako CK1/DAKO-CK (Dakopatts A/S); 100 µg IgG1/ml, dilution 1 : 20, at room temperature, 30 min, dark). Following centrifugation and washing steps cells were stained for DNA with 0.5 ml PBS

containing propidium iodide (50 µg/ml) and RNase (Sigma, type I-AS, 1 mg/ml) at 37°C for 10 minutes. Negative controls were performed identically using a FITC conjugated mouse IgG1 isoantibody. Female human lymphocytes and HeLa cells processed in the same way served as controls for ploidy, cytokeratin staining and coefficient of variation. 10,000 to 40,000 cells per sample were measured in a FACScan flowcytometer 488 nm argon laser, Beckton Dickinson (BD) equipped with Hewlett Packard hardware and a pulse processor for doublet discrimination. Data acquisition and analysis was performed with the Cellfit software of BD. DNA ploidy was determined according to Hidemann et al. [15]. Tumor proliferation was estimated by calculating percentages of G<sub>0</sub>G<sub>1</sub>-, S-, and G<sub>2</sub>M-phase of each individual analyzable stemline using the SOBR method (sum of broadened rectangles). For details of quality control compare Kimmig et al. 1994 [6].

### 2.1. Patients

#### 2.1.1. Breast cancer

The clinical course of breast cancer was analyzed in 240/327 patients with primary breast cancer, thereby 319 were of ductal and 8 of lobular type. For prospective analysis of recurrence free survival (RFS) and cancer specific survival (CSS) we excluded breast cancer patients with only local excision ( $n = 1$ ), preoperative chemotherapy ( $n = 2$ ), primary metastases ( $n = 39$ ), contralateral breast cancer ( $n = 15$ ), patients with secondary malignancies, independent on time interval to breast cancer diagnosis ( $n = 18$ ), ductal carcinoma *in situ* ( $n = 4$ ) and invasive carcinoma lobulare ( $n = 8$ ).

Table 1

Distribution of clinical histopathological prognostic factors in patients with primary breast cancer ( $n = 327$ ). The distribution of patients with prospective analysis are documented in brackets

Tumor size number ( $n$ )	pTis 4 {0}	pT1 79 {60}	pT2 162 {129}	pT3 23 {18}	pT4 59 {33}
Lymph node involvement number ( $n$ )	pN0 138 {108}	pN1(1–3Ln) 78 {63}		pN1(>3Ln) 97 {70}	nd 14 {0}
Grading number ( $n$ )	1 8 {7}	2 175 {131}		3 142 {100}	4 2 {2}
Estrogen receptors number ( $n$ )	negative 128 {100}	positive 199 {140}			
Progestagen receptors number ( $n$ )	negative 134 {100}	positive 193 {140}			
Metastasis number ( $n$ )	M0 288 {240}		M1 39 {0}		

Table 2

Distribution of therapy modalities in patients with primary breast cancer. Only patients analyzed prospectively were determined ( $n = 240$ ).

Therapy of breast cancer	Number ( $n$ )
Breast conserving therapy and radiation	73
Mastectomy and lymphonodectomy	166
Ablatio simplex	1
Adjuvant radiation	21/166
Adjuvant chemotherapy	92
Adjuvant hormone therapy	64

Prospective analysis was, therefore, performed in 240 patients with median age of 57 years (range 33.4 to 86.9 years).

The distribution of five clinico-histopathological parameters for the 327 patients is shown in Table 1; thereby patients were analyzed by univariate and multivariate analysis (noticed in brackets). Therapy modalities for the last mentioned group was demonstrated in Table 2. Cut off for positive or negative steroid receptors were 10 fmol/mg protein.

The median follow up was 1413 days (25th percentile 778 days / 75th percentile 2114 days). 240 patients had a complete follow up and 76 (32%) of these patients showed locoregionary relapse or metastasis, whereas intramammarian relapse after breast preserving therapy was not valued. 57 patients died, 48 (20%) as result of their disease status.

### 2.1.2. Statistics

The experimental data were analyzed with using SAS (SAS Institute Inc., Cary NC) on a UNIX work station. The different proportions of DNA-aneuploid tumors found with gating for cytokeratin positive cells compared to those without gating were described using a two-by-two table. As no definitive reference test for determination of ploidy was available, the results obtained with gating could only be described in relation to our results without gating. The McNemar test was used to test the null hypothesis that the results of the two methods are distinguishable. In order to compare proportions from cell counts obtained by cell cycle analysis with the statistical test they were transformed to the angular scale using an arcsin transformation. The transformed proportions were compared with the use of a paired  $t$ -test [16]. Calculation of recurrence free survival (RFS) and tumor dependent overall survival (CSS) was estimated according to the Kaplan–Meier method. The independent prognostic significance of each factor was evaluated by Cox regression model. Two-tailed  $p$ -values are given.  $P$ -values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Breast cancer

In 327 patients with breast cancer, single cell suspensions of tumors were investigated.

The analysis of all cell suspensions showed that 202 of the tumor specimens (62%) were DNA-aneuploid without cytokeratin labelling of tumor cells, whereas 250 (76.5%) were detected as DNA-aneuploid following cytokeratin staining. The number of tumor specimens with more than one DNA-subpopulation increased from 46 (14% of  $n = 327$ ) to 74 (23%).

The distribution of the DNA-indices of cytokeratin-labeled tumor cells is presented in detail elsewhere [14].

#### 3.1.1. Correlation of DNA-ploidy, DNA-index, CV and cell cycle fraction with classic clinical-histopathological prognostic factors in breast cancer

Patients with DNA-aneuploid tumors show more often worse differentiated tumors (G III,  $p < 0.0001$ ) and axillary lymph node involvement ( $p = 0.01$ ). The same results were found for DNA-index. Tumors with a higher CV were significantly more often estrogen- or progesterone receptor negative ( $p = 0.05$ ,  $p = 0.003$ ). The S-phase fraction correlated significantly with tumor size ( $p = 0.02$ ), lymph node status ( $p = 0.02$ ), estrogen- ( $p = 0.006$ ), progesterone receptor ( $p < 0.0001$ ) and grading ( $p < 0.0001$ ). No correlation for G<sub>2</sub>M-phase fraction to clinical-histopathologic parameters was assessed.

#### 3.2. Prognostic significance of DNA-ploidy and S-phase fraction in breast cancer in comparison to classic clinical-histopathologic prognostic factors

##### 3.2.1. Prognostic significance of classic clinical-histopathologic prognostic factors

Recurrence-free survival (RFS) and cancer specific overall survival (CSS) were performed in 240 patients with primary breast carcinoma. The axillary lymph node involvement and the histopathologic grading showed the highest discrimination capacity and statistical significance for RFS and CSS. Nearly all patients with metastatic disease of breast cancer will die, so the results for RFS are predictors for CSS because of only limited follow-up. After a median follow-up time of 46.5 months RFS of patients without axillary lymph

node involvement was 79.6% and in case of grading of G1 or G2 76.8%, but in case of lymph node involvement RFS was only 59.1%, respectively 58.0% for G3 ( $p = 0.0003$ ;  $p = 0.0002$ ). The corresponding data for CSS were 90.7% and 88.4% versus 71.2% and 69% ( $p = 0.0001$ ). But also age, tumor size, estrogen- and progesterone receptors were univariate significant prognostic factors.

For estimation of time course and relation of censored patients to patients with relapse or tumor dependent death Kaplan–Meier curves were shown for the parameters tumor size, lymph node status, grading and progesterone receptor for RFS and CSS.

### 3.2.2. Prognostic significance of classic clinical-histopathologic prognostic factors in dependence on lymph node status

The high prognostic significance of axillary lymph node status is known. But unfortunately, also lymph node negative patients show relapse in 30%. These patients did not get obligatory any adjuvant treatment in the past, so it is very important to identify further prognostic factors for these patients. Thereby patients with and without lymph node involvement were analyzed separately.

In case of negative nodes ( $n = 108$ ) all mentioned factors, especially histopathologic grading and hormone receptors, were of prognostic significance in univariate analysis apart from age.

Also for nodal negative patients Kaplan–Meier curves were shown for estimation of time course and relation of censored patients to patients with relapse or tumor dependent death for the parameters tumor size, grading and progesterone receptor for RFS and CSS. Whereas tumor size ( $p = 0.004$ ) seems to be the predominant parameter concerning RFS in addition to histopathologic grading ( $p = 0.002$ ), the predominant parameter in CSS was the progesterone receptor ( $p = 0.008$ ).

In contrast, for lymph node positive patients ( $n = 132$ ) concerning RFS no significant prognostic factor ( $p > 0.05$ ) was detected, but the significance for most of the prognostic factors concerning CSS was maintained. The histopathologic grading was the most important factor, too ( $p = 0.02$ ). But thereby in contrast to the nodal negative patients, also age showed a borderline significant prognostic significance.

### 3.3. Prognostic significance of DNA-ploidy, DNA-index and CV

The prognostic significance of DNA-ploidy for RFS and CSS was investigated with respect to three parameters:

1. DNA-ploidy differentiated as DNA-diploid with inclusion of near peridiploid tumors (DNA-content  $> 1.8c$  and  $< 2.2c$ ) and DNA-aneuploid (each other DNA-content). With cytokeratin labelling of tumor cells a higher detection rate of DNA-aneuploid subpopulation was present. For estimation of clinical relevance the prognostic significance of DNA-ploidy was analyzed with and without cytokeratin labelling.
2. DNA-index (relation of DNA-content of the tumor cell to DNA-content of normal DNA-diploid somatic cells of the same organism): Tumors with a DNA-index  $\leq 1.3$  were compared to tumors with a DNA-index  $> 1.3$ .
3. The CV as index for “microvariance” of DNA-content was stratified at median.

Univariate analysis found a prognostic relevance for DNA-ploidy for CSS with 91.2% versus 76.5% for DNA-diploid and DNA-aneuploid tumors ( $p = 0.038$ ). The difference of 77.2% to 65.6% in RFS was not statistically significant. Without cytokeratin labelling similar results were detected. DNA-index discriminated worse and reached no significance, in contrast to the CV, that showed the highest significance for RFS (76.9% versus 59.8%;  $p = 0.019$ ) and also for CSS (87.6% versus 72.6%;  $p = 0.018$ ).

For estimation of time course and relation of censored patients to patients with relapse or tumor dependent death Kaplan–Meier curves were shown for the parameters DNA-ploidy with and without cytokeratin labelling, CV and S-phase fraction for RFS (Fig. 1) and CSS (data not shown).

Although after an observation time of 30 months a trend of higher RFS for patients with DNA-diploid tumors was detected, this trend had no statistic significance, but after cytokeratin labelling differentiation seemed to be somewhat better. Whereas a significant better RFS for patients with lower CV was observed ( $p = 0.0019$ ), no difference was found for patients with respect to high or low proliferation of tumor. Concerning CSS similar results were detected for CV and S-phase fraction, whereas DNA-ploidy is a significant prognostic factor ( $p = 0.03$ ). And the discrimination after cytokeratin labelling at similar significance is also somewhat better.

### 3.4. Prognostic significance of DNA-ploidy, DNA-index and CV in dependence on lymph node status

In nodal negative patients recurrence-free survival after cytokeratin labelling in DNA-aneuploid tumors

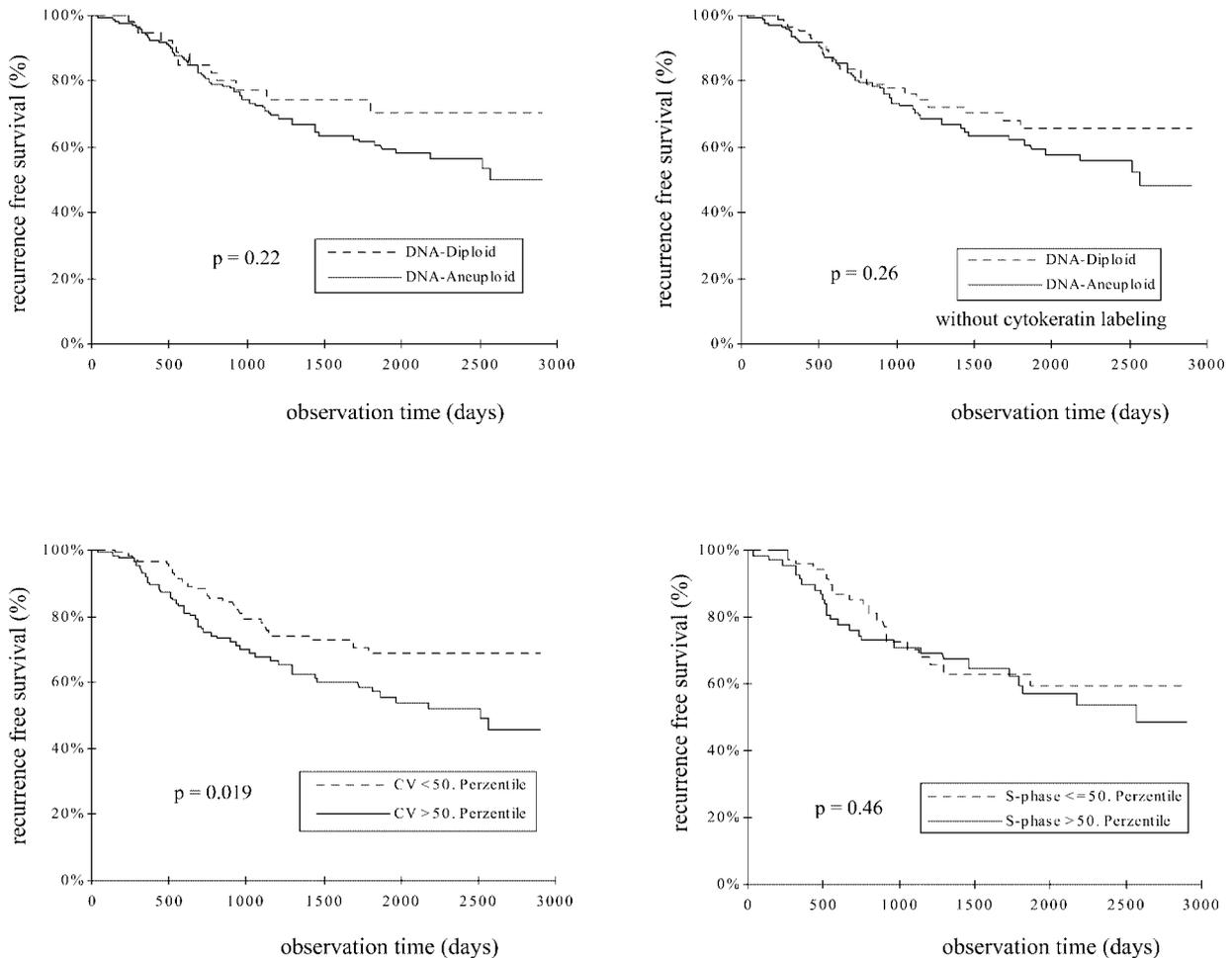


Fig. 1. Primary breast cancer ( $n = 240$ ). Kaplan–Meier curves for RFS in dependence on DNA-ploidy (with and without cytokeratin labelling), CV and S-phase fraction were performed. The significance level ( $p$ ) was determined by univariate analysis with the log-rank test.

decreased to 74.3% from 91.2% in DNA-diploid tumors ( $p = 0.052$ ). Without cytokeratin labelling the difference is lower (86.8% versus 72.3%) and is not significant ( $p = 0.096$ ). DNA-index has no prognostic significance.

For presentation of time course and relation of censored patients to patients with relapse or tumor dependent death Kaplan–Meier curves were shown and a better RFS for patients with DNA-diploid tumors and low CV of  $G_0G_1$ -peak was demonstrated (Fig. 2) and the same was found for CSS (data not shown). But for CSS DNA-ploidy has no statistic significance. Tumors with low S-phase fraction tend to have a better RFS and CSS, but the differences were not significant.

In contrast to lymph node negative patients, lymph node positive patients show no prognostic significance concerning the mentioned parameters. Whereas

DNA-ploidy and DNA-index show no differences in subgroups concerning RFS, worse data for DNA-aneuploid tumors for CSS were found, but without any statistic significance. The greatest difference was present for CV, but again without any significance in log-rank test ( $p = 0.08$ ).

### 3.5. Prognostic significance of S-phase fraction, $G_2M$ -phase fraction with and without identification of tumor cells by cytokeratin labelling

The prognostic significance of S-phase fraction,  $G_2M$ -phase fraction were evaluated separately for results with and without cytokeratin labelling.

For stratification in low and high proliferated tumors, the known parameters were divided into quar-

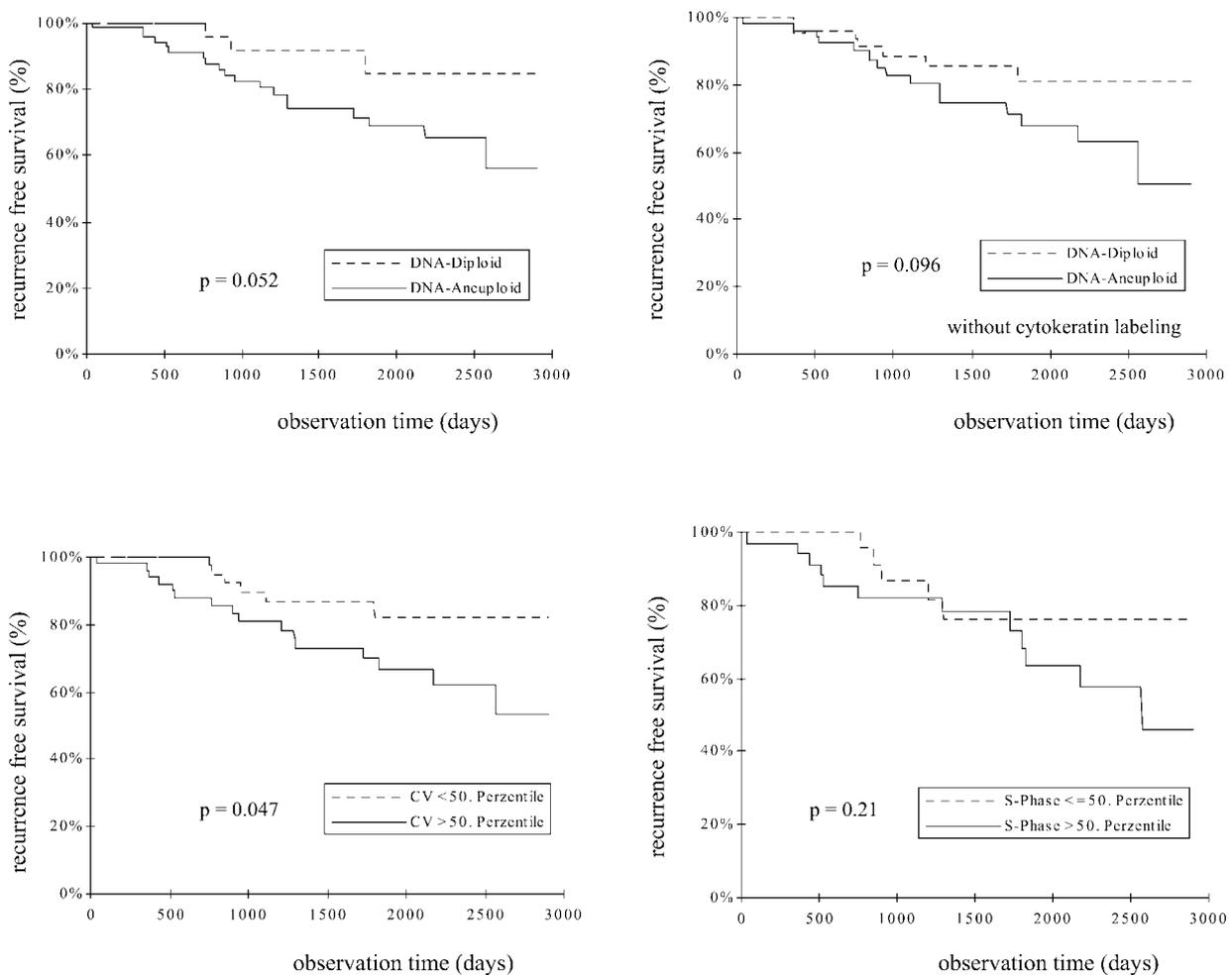


Fig. 2. Node-negative breast cancer ( $n = 108$ ). Kaplan–Meier curves for RFS in dependence on DNA-ploidy (with and without cyokeratin labelling), CV and S-phase fraction were performed. The significance level ( $p$ ) was determined by univariate analysis with the log-rank test.

tiles. For all parameters an analysis was performed with division at median and the inferior 3 quartiles versus the highest quartile; in addition, the lowest quartile versus the 3 upper quartiles were analyzed for the S-phase fraction of cyokeratin positive cells.

A trend of lower RFS and CSS was detected for higher proliferating tumors. The trend was only of borderline significance for RFS in S-phase fraction with a cut-off at the lowest quartile versus the rest (81.6% versus 61.7%;  $p = 0.058$ ), and median of G<sub>2</sub>M-phase fraction (76% versus 57%;  $p = 0.056$ ). Whereas 81.6% of the patients with a S-phase fraction in the lowest quartile survive recurrence-free, only 52.9% were found with S-phase fraction in the uppermost quartile. This was only present for cell cycle parameters of cyokeratin-positive cells. The analysis of all cells detected no significance.

### 3.6. Prognostic significance of S-phase fraction, G<sub>2</sub>M-phase fraction with and without identification of tumor cells by cyokeratin labelling in dependence on lymph node status

Nodal negative patients showed a decrease of RFS from 88.2% to 50.0% and CSS from 100% to 75% from the lowest to highest quartile of S-phase fraction in dependence on lymph node status. However, only the proliferation fraction S + G<sub>2</sub>M reached statistic significance after cyokeratin labelling for the highest quartile versus the rest with an RFS of only 47.1% for high proliferating tumors versus 84.3% for medium and low proliferating tumors ( $p = 0.03$ ). No statistic significance was found for all parameters in case of no cyokeratin labelling.

Lymph node positive patients show a trend for better prognosis for RFS and CSS in lower proliferating tumors. RFS decreased from 70% in case of low S + G<sub>2</sub>M-phase fraction to 48.8% in case of higher S + G<sub>2</sub>M-phase fraction. A similar result was detected for G<sub>2</sub>M-phase fraction (71.8% versus 47.4%), whereas in S-phase fraction a difference was observed only for the first quartile versus the rest (70% versus 56.1%). This was not statistically significant. An analogue situation was present for CSS.

### 3.7. Prognostic significance of DNA-ploidy and S-phase fraction in breast cancer in comparison to classic prognostic factors: multivariate analysis

#### 3.7.1. All patients

Because of close relationship between prognostic factors, a multivariate analysis was necessary for evaluation of independent prognostic significance.

We therefore performed COX-multivariate analysis for all statistically significant parameters in univariate analysis. Analyzed were age of patients, tumor size (pT), lymph node status (pN), grading, estrogen- and progesterone receptor, DNA-ploidy and CV of G<sub>0</sub>G<sub>1</sub> peak.

Variables of 236 patients with primary breast cancer were presented in Table 3.

For RFS tumor size, axillary lymph node status, progesterone receptor and CV of G<sub>0</sub>G<sub>1</sub> peak of tumor cells were significant with a relative risk (RR) between 1.6 (CV) and 2.6 (progesterone receptor). For patients younger than 52 years, CSS was significantly worse with a relative risk of 2.1 and the RR in case of lymph node positivity is even 2.4. The grading is identified also as an independent prognostic factor with an RR of 1.9. The highest relative risk showed progesterone receptor, with an RR of 2.7.

#### 3.7.2. Patients with nodal negative or nodal positive breast cancer

In nodal negative patients, tumor size, grading, progesterone receptors and CV of G<sub>0</sub>G<sub>1</sub> peak were of independent significance for RFS with a RR between 3.8 (progesterone receptor) and 4.0 (tumor size). For CSS only the grading showed a significant correlation after multivariate analysis with an RR of 6.5 ( $p = 0.007$ ).

Multivariate analysis in nodal positive patients detected no significant prognostic factor for RFS. In CSS a worse tumor differentiation showed an RR of 2.1 ( $p = 0.03$ ).

## 4. Discussion

### 4.1. Prognosis of breast cancer

Breast cancer is the most common malignancy of women. One of nine women gets this tumor [17]. With respect to health and business it is very important which therapy will be performed and when. Therefore, a correct estimation of tumor biology and course of disease is necessary. Half of the women with primary operable breast cancer could be cured by operation only. These patients would not have any benefit by adjuvant treatment. The most important predictor of outcome for these patients is the lymph node involvement [18, 19]. In case of negative lymph nodes only 30% of patient show relapse within 10 years [20]. Tumor size is an important prognostic factor in node negative patients; the five year survival decreases from >99% in case of tumor diameter of <0.5 cm to 82% for tumors > 5 cm [21]. The recurrence-free survival within 20 years decreases in tumors of 1.7 to 2 cm diameter to

Table 3

Primary breast cancer ( $n = 236$ ). Cox-multivariate analysis of univariate prognostic relevant parameters for recurrence-free survival (RFS) and tumor-dependent overall survival (CSS). For all parameters multivariate analysis were performed. For all parameters the value of effect of covariable estimation ( $\beta$ ), standard deviation ( $\sigma$ ), relative risk (RR) and the belonging 95% confidence interval (95%CI) was determined

Breast cancer $n = 236$	RFS					CSS				
	$\beta$	$\sigma$	$p$	RR	95%CI	$\beta$	$\sigma$	$p$	RR	95%CI
Age	-0.38	0.24	0.19			-0.76	0.31	0.01	2.13	1.17-3.91
Tumor size (pT)	0.58	0.19	0.002	1.79	1.23-2.58	0.58	0.24	0.02	1.79	1.11-2.87
Lymphnode status	0.59	0.27	0.03	1.81	1.07-3.05	0.88	0.38	0.02	2.42	1.16-5.04
Grading	0.33	0.27	0.22			0.64	0.34	0.06	1.90	0.98-3.71
Estrogen receptor	0.14	0.36	0.70			0.95	0.38	0.92		
Gestagen receptor	-0.77	0.24	0.001	2.15	1.35-3.43	-0.99	0.31	0.001	2.70	1.48-4.93
DNA-ploidy	-0.03	0.33	0.91			0.33	0.49	0.51		
CV G <sub>0</sub> G <sub>1</sub> -peak	0.48	0.25	0.05	1.62	0.99-2.63	0.61	0.32	0.06	1.84	0.98-3.46

59% compared to 88% in tumors smaller than 1 cm diameter [22]. Also the histopathological grading according to Bloom and Richardson [23] correlates with risk for relapse. Risk for metastasis within 5 years increases from 2.3% (grading I) to 15.2% (grading II) [24]. The expression of steroid receptors showed an advantage of 8–10% in recurrence-free and overall survival within 5 years [17]. Up to now no parameter was found, also no “new prognostic factor” like HER-2 oncogene, cathepsin D and others, that result in a definitive categorization in relapse risk versus no relapse risk [17,25]. Our investigation confirmed significant prognostic factors like lymph node status, tumor size, grading and progesterone receptor in multivariate analysis.

#### 4.2. Prognostic significance of DNA-parameters in comparison to clinical histopathological prognostic factors

##### 4.2.1. DNA-ploidy

The published data concerning prognostic significance of DNA-ploidy are numerous, but controversial. As early as 1980 it was shown that patients with DNA-diploid breast cancer survive longer than DNA-aneuploid ones [26]. The first euphoria decreased as other research groups could not reproduce these results. Cause for controversial results could be the varying methods of tissue processing (paraffin versus frozen sections versus fresh specimen), varying tissue dissociation and acquisition of data. Another reason for controversial results are often small patient numbers and because of this very different patient collectives concerning tumor histology, stage, steroid receptor expression and age.

Of great relevance is also good quality of follow-up, statistics and correct documentation. In addition, it is crucial to perform not only univariate but also multivariate analysis.

Most studies could not evaluate any multivariate significant prognostic significance for DNA-ploidy [27], but others found especially for nodal negative patients a multivariate significance [28,29]. In contrast, Canizares et al. declared DNA-ploidy as independent prognostic factor for node-positive and also node-negative patients [30]. A prognostic factor that is dependent on method and chosen collective is not useful for clinical routine. Thus it does not seem understandable that DNA-ploidy was declared as established prognostic factor for breast cancer [17] and as important factor for estimation of relapse risk for

nodal negative patients [25]. Our investigation in 327 patients with breast cancer and 240 patients is one of the largest collective concerning prospectively performed DNA-analysis. It is the largest study with the use of cytokeratin antibodies for identification of tumor cells. Univariate analysis found a decrease in RFS from 77.2% to 64.6% (n.s.) and for CSS from 91.2% to 76.5% ( $p = 0.03$ ) for the whole collective. Whereas for nodal positive patients no significant influence of DNA-ploidy for course was detected, for nodal negative patients the RFS decreased from 91.2% to 74.3% ( $p = 0.05$ ) and CSS decreased from 97.1% to 87.8% (n.s.). Multivariate analysis found no prognostic significance for DNA-ploidy in both groups. The high correlation of DNA-ploidy with the histopathologic grading ( $p < 0.0001$ ) and with the axillary lymph node state ( $p = 0.01$ ) explains the loss of prognostic significance in multivariate analysis.

##### 4.2.2. Variation coefficient of $G_0G_1$ -peak of tumor cells

Human solid tumors develop different DNA-alterations, that could lead to changes in genetic informations and biologic behavior. At least two different mechanisms for development of genetic instability were identified. On the one hand, the abnormalities of the p53 gene, that lead to an alteration in DNA-content of cells, that could be diagnosed as DNA-aneuploidy by flow-cytometry. On the other hand, mechanisms were identified that cause broad microsatellite instability in near diploid cells [2]. The last mentioned mechanisms result only in small changes of DNA-content. Cytogenetic examinations showed that this part of aneuploid clones in breast cancer, that has only a small deviation of DNA-content of stem cell population, could not be diagnosed by flow cytometry [31]. These subpopulations could not be assessed in a shared manner because of the limited resolution of  $G_0G_1$ -peak. Because of different DNA-contents in comparison to stem cell line and DNA-diploid cell lines a broadening of  $G_0G_1$ -peak and thereby an increase in variation coefficient results. If the genetic alteration leads to a biologic advantage in tumor growth, the CV of tumor cells should have prognostic significance. For estimation of influence of method to CV parallel determinations of the CV in tumor cells (3.8%) and also of contaminating normal cells (3.2%) were performed. In univariate analysis concerning the prognostic significance of CV of tumor cells, we found a significant decrease of RFS in total collective from 76.9% to 59.8% ( $p = 0.02$ ) and a decrease of CSS from 87.6% to 72.6% ( $p = 0.02$ ). Thus, in univariate analysis the CV was a stronger

prognostic factor compared to DNA-ploidy. Also in multivariate analysis it was of borderline significance with a relative risk (RR) of 1.6 for RFS ( $p = 0.05$ ) and 1.8 for CSS ( $p = 0.06$ ). The CV showed a correlation of expression of estrogen to progesterone receptors ( $p = 0.05 \pm 0.003$ ), but no correlation for the other examined parameters. Up to now the prognostic significance of this parameter has not been described by other groups.

#### 4.2.3. S-phase fraction

The proliferation rate could be measured in mitotic activity as important parameter of tumor behavior about 100 years ago [32]. The thymidin labelling index (TLI) as proliferation marker was examined and his prognostic significance could be shown, especially low TLI results in good prognosis [33]. In clinical routine the TLI could not be established because fresh tissue is necessary and the method is very time intensive. The determination of S-phase fraction, as marker of tumor cell proliferation, also in fixed specimen is methodologically easier.

In most studies S-phase fraction was detected as significant prognostic factor. However, one study found a significance only for DNA-diploid tumors. 2 other studies included DNA-ploidy and G<sub>2</sub>M-phase for reaching significance [34]. A further observation was that in 3 additional studies for determination of independent prognostic significance of S-phase fraction the histopathologic grading was not included in multivariate analysis [35]. However, grading is a very important prognostic factor especially for node negative patients [17]. Various authors found a significant correlation between grading and S-phase fraction [36]. Therefore the inclusion of the grading in multivariate analysis is absolutely necessary for valid statements. One large study ( $n = 802$ ) with a long time of follow-up (>8 years) included the most important histopathologic prognostic factors like tumor size, lymph node status and grading in a multivariate analysis [37]. In this study S-phase fraction was a high significant prognostic factor also in multivariate analysis (RR 2.9;  $p < 0.0001$ ). Since this is the only study showing independent prognostic significance of S-phase fraction; it is questionable, whether therapeutic consequences of a high S-phase fraction may be drawn [38]. Our investigation confirmed the trend of a worse recurrence-free and tumor dependent overall survival. The recurrence-free survival decreased in the whole collective from 81.6% for the lowest quartile to 52.9% for the uppermost quartile and the tumor dependent overall survival decreased from 94.7 to 69.4%. This difference was of

borderline significance in univariate analysis; with cut off at median we found no significance. The difference in RFS of nodal negative carcinoma was 88.2% versus 50% and 100% versus 75% for tumor dependent overall survival. But the difference does not reach significance at the 0.05 level.

#### 4.3. Conclusion for breast cancer

The prognostic significance of DNA-ploidy and S-phase fraction in univariate analysis is evident and plausible in view of molecular biology. However, in multivariate analysis no independent prognostic significance could be found for both factors. Even if a prognostic significance could be demonstrated in larger trials, the expected survival advantage of the prognostic better group would be too small to justify a change of therapeutic strategy. So we have no indication for determination of DNA-ploidy and S-phase fraction for clinical routine diagnostic. The parameters should only be determined to investigate scientific aspects concerning a probable predictive significance for estimation of therapy success in case of adjuvant chemotherapeutic or hormone therapy.

## 5. Summary

An ideal prognostic factor should predict the course of disease exactly. At present the well-known "classic" prognostic factors, like tumor size, lymph node involvement and grading are the most important factors.

In addition, for breast cancer for example the hormone receptor stage is an excellent prognostic factor. The flow cytometric DNA-cell cycle analysis got importance as an additional diagnostic factor for a better estimation of prognosis in different malignant tumors.

The discrepancy of results of various studies, however, is confusing concerning risk estimation.

In a prospective study we studied the influence on tumor cell enrichment by using cytokeratin antibodies by flow cytometric data of DNA-ploidy and cell cycle distribution and as second step the prognostic significance of data in correlation to classic histopathological prognostic parameters.

In 240 breast cancers we determined all the parameters with a median observation period of nearly 4 years.

The identification of DNA-aneuploid subpopulations was improved by cytokeratin labelling. Hereby 15% of former DNA-diploid classified subpopulations

were identified as DNA-aneuploid ones after cytokeratin gating.

The S-phase fraction increased after cytokeratin labelling for 30%. A significant correlation was found for DNA-ploidy, lymph node involvement and grading, correlation between S-phase fraction and tumor size the lymph node involvement, grading and steroid hormone receptor status.

Univariately, the prognostic significance of DNA-ploidy, S-phase fraction and variation coefficient of G<sub>0</sub>G<sub>1</sub>-peak of tumor cells, especially for node negative patients was assessed.

Multivariately, neither DNA-ploidy nor S-phase fraction was of prognostic significance, although an advantage in growth of tumors with higher proliferation rate and higher mutation was evident. The high correlation with classic prognostic factors may be the reason. Our results showed that there is no indication for determination of DNA-ploidy or S-phase fraction in breast cancer in clinical routine.

In breast cancer a DNA-analysis for prognostic purposes should not be performed, because neither a prognostic significance for DNA-parameters in multivariate analysis could be assessed, nor a clinical consequence will result. However, cytokeratin labelling for enrichment of tumor cells led to methodological convincing advantages for detection of DNA-aneuploid subpopulations and for detection of cell cycle fractions. Concerning prognostic statement of the determined parameters marginal advantages were detected in comparison to the analysis without cytokeratin labelling. The higher costs of cytokeratin labelling by flow-cytometric DNA-analysis therefore are not justified for clinical routine.

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