Eur J Cancer, Vol. 28, No. 2/3, pp. 711–716, 1992.
Printed in Great Britain

0964-1947/92 $5.00 + 0.00

DNA Ploidy and Other Results of DNA Flow Cytometry as Prognostic Factors in Operable Breast Cancer: 10 Year Results of a Randomised Study

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We evaluated breast cancer specimens from 241 patients of a controlled clinical trial by means of DNA flow cytometry. We report the correlations between DNA index (DNI) and fraction of cells in S-phase (SPF) and other prognostic parameters. Both univariately and in a Cox model, the predictive power of these factors is evaluated after a follow-up of more than 10 years. There are strong correlations between DNI and SPF ($P = 0.0001$) and between flow cytometry parameters and clinical and histopathological factors such as axillary lymph node involvement, tumour size and histological grade. In univariate analysis DNI fails to provide prognostic information, whereas SPF turns out to be able to differentiate between patients at high and low risk for relapse and death ($P = 0.002$). In the multivariate Cox model, too, SPF is an important prognostic parameter with respect to patient survival (relative risk: + 86%), only surpassed by nodal involvement. DNI, however, turns out to be an independent predictor of relapse free survival and distant recurrence free survival. By combination of DNI and SPF, patients can be divided into three prognostic subgroups. We conclude that data from DNA flow cytometry can be of great importance for the decision on the level of aggressiveness of adjuvant therapy for an individual patient and therefore may help to avoid overtreatment and toxicity.
Eur J Cancer, Vol. 28, No. 2/3, pp. 711–716, 1992.

INTRODUCTION

BECAUSE BREAST cancer is the most common malignancy in women worldwide, it has been the subject of numerous studies trying to establish the relative values of different prognostic indicators. Treatment strategies are variable and the decision as to which approach to take can frequently depend on information regarding the nature of the biological behaviour of an individual tumour. Several prognostic indicators have been examined for their ability to predict the outcome of the disease in a given patient, in order to differentiate between patients at high and low risk of relapse of the disease after surgery. Undoubtedly the best prognostic indicator to date is the presence or absence of malignant cells in the axillary lymph nodes [1]. Among other conventional clinicopathological characteristics, tumour size [2], histological and nuclear grades [3], and hormone receptor status [3–5] have been assessed as potential prognostic factors in patients with breast cancer.

More recently, new techniques have permitted access to new cytological features, such as the DNA content of tumour cells. Recently, the thymidine labeling index [6], cathepsin D production [7], growth-factor receptors such as the protein product of the HER-2/*neu* (also known as the *erb*-B2) [8] and the epidermal growth-factor receptor (EGFR) genes [9], the anti-metastatic gene nm23 [10], and stress-response-protein-27 [11] have been shown to have predictive power in breast cancer patients. These factors could therefore be used to help decide what intensity of therapy is appropriate for an individual patient.

Flow cytometry, which can measure both ploidy and proliferative activity, has also been shown to give useful prognostic information. Several studies have been published within the last years, comparing ploidy and cell kinetic measurements to important clinical parameters both in node-positive and node-negative disease. However, there is great variability in these data, probably due to methodological or technical differences and/or short-time follow-up. The most important question, whether data derived from tumour DNA provides independent prognostic information in terms of survival or recurrences, is still under debate.

The purpose of our study was to investigate the correlations between DNA parameters and other prognostic parameters in breast cancer, including lymph node involvement, tumour size, hormone receptor status, histopathological features, and menstrual status. We also investigated the long term prognostic impact of tumour DNA flow cytometry information on patient overall survival and relapse-free-survival after surgery for stage I and stage II breast cancer with or without chemotherapy administered in a randomised fashion. Finally we tried to combine the results of DNA flow cytometry in an attempt to identify groups of patient with high and low risk of relapse or death from breast cancer.

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Revised and accepted 25 Oct. 1991.

PATIENTS AND METHODS

Patients and study design

241 consecutive patients with stage I and II breast carcinoma (T1-3, N0-1, M0) entered a prospective clinical trial between 1977 and 1982. Details of the protocol have been reported elsewhere [12]. In short, patients were stratified by T-, N- and menstrual status and intraoperatively randomised on the basis of frozen section of axillary lymph nodes using the method of Pocock and Simon [13]. Surgical treatment and adjuvant therapy were determined simultaneously. Surgical treatment consisted either of conservative breast surgery with axillary dissection (n = 34), of modified radical mastectomy with axillary dissection (n = 142), or of radical mastectomy (n = 65). Patients were thereafter randomly allocated into three groups: (A) untreated surgical control; (B) chemotherapy consisting of cyclophosphamide (100 mg daily for 10 days orally), 750 mg 5-fluorouracil (5-FU), 5 mg vinblastine, and 25 mg methotrexate administered intravenously on days 1 and 8. Four such cycles were administered in the first year and two cycles in the second and third year, respectively; (C) chemoimmunotherapy consisting of the chemotherapy as in group B and additional administration of azimexon, a nonspecific immunostimulant. No radiation therapy was given postoperatively to any patient. All patients were regularly followed at least every 3 months for the first 3 years and at intervals of 6 months thereafter. The routine evaluation of the patients included clinical examination and laboratory analyses, mammography at least every 6 months, and chest radiography, liver ultrasound, and bone scanning whenever clinically indicated. The missing information on survival of a few patients lost for follow-up was obtained from the Central Population Registry of Austria.

Histological examination and laboratory assays

Tumour size was defined from the pathological size immediately after surgery. The histological grade was determined by the method of Bloom and Richardson [14]. Oestrogen-receptor (ER) and progesterone-receptor (PgR) content were assayed with the dextran-coated charcoal method and Scatchard analysis as described previously [15]. A level of at least 10 fmol/mg cytosol protein was considered as receptor positive. Flow cytometry determination of DNA ploidy levels were performed in nuclei isolated from paraffin-embedded tissue [16]. DNA content was measured by the method of Vindelov *et al* [17]. A tumour with a single G1 peak was considered to be diploid, whereas evidence of an additional G1 peak indicated aneuploidy. The proliferative activity—percentage of cells in S-phase (SPF)—was calculated by counting the number of cells between the inclination points of the descending G1 peak and the ascending G2-M peak [18].

The DNA index (DNI), a value that expresses the amount of DNA content relative to normal, was calculated as the ratio of the peak channel number of the aneuploid G0G1 peak to the peak channel number of the diploid G0G1 peak, which by definition was considered to be the left-most peak. By definition, the DNI for a diploid population is 1.0.

After separate analysis the cut-off for SPF was set to 11% in order to define two groups, with low and high SPF, respectively.

When ploidy status could not be determined because of poor quality of the sample (excess debris, too few cells) or insufficient resolution to distinguish two peaks, the histograms were considered uninterpretable. In this analysis, histograms with coefficients of variation of < 8% were considered to be of good quality.

Table 1. *Cofactors for univariate and multivariate analyses*

		n (%)
Tumour size	< 2 cm	103 (53.9)
	≥ 2 cm	88 (46.1)
Nodal status	Negative	97 (50.8)
	Positive	94 (49.2)
Grading	I and II	132 (75.4)
	III	43 (24.6)
ER	Positive	102 (67.1)
	Negative	50 (32.9)
PgR	Positive	70 (46.7)
	Negative	80 (53.3)
DNI	1.0 (diploid tumours)	71 (37.2)
	More than 1.0 (aneuploid tumours)	120 (62.8)
SPF	< 11%	69 (51.1)
	≥ 11%	66 (48.9)
Menstrual status	Premenopausal	76 (39.8)
	Postmenopausal	115 (60.2)
Adjuvant therapy	Untreated surgical control	61 (31.9)
	Chemotherapy and chemoimmunotherapy	130 (68.1)
Age	< 50 years	68 (35.6)
	≥ 50 years	123 (64.4)

Statistical analysis

Data were maintained on an IBM 3270 computer using a SAS-based database (SAS Institute, Cary, North Carolina) and all analyses were performed with SAS and BMDP statistical packages (BMDP Statistical Software, Los Angeles, California).

The value of the prognosis factors (covariates) was determined according to statistical models, with the use of BMDP. Survival was expressed as the number of months from the date of primary treatment of breast cancer to the occurrence of an event and was analysed in three ways: as overall survival (OS), as relapse-free-survival (RFS), and as distant-recurrence-free-survival (DRFS). RFS was defined as the interval between the date of operation and the first recurrence of breast cancer. Patients who died due to reasons other than breast cancer, without any signs of breast cancer recurrence, were considered censored for all analyses (n = 6, 3.1%). Curves for all analyses were calculated according to the method of Kaplan and Meier [19]. Differences between curves were assessed with Mantel's log rank test for censored data on survival [20].

Multivariate analysis included 10 covariates, all of which—after appropriate testing—were considered as fixed (not time-dependent). For optimal categorisation of continuous covariates, univariate comparison was performed with χ^2 values. The prognostic value of the different covariates was evaluated with the use of the product-limit estimate of survival function.

All covariates listed in Table 1 were entered into a Cox proportional hazards model [21]. Covariates were selected in a stepwise fashion (backward to forward), with use of the maximum likelihood ratio. A P value of 0.15 was adopted as the limit for the inclusion of a covariate. DNI and SPF were then added to the model to assess their ability to provide incremental prognostic information.

Several subgroup analyses were performed to exclude interpretation bias. The flow cytometry factors then were combined in an attempt to define prognostic subgroups.

Table 2. Patient characteristics (n = 191)

	Median (S.E.) (range)
Patient age (years)	55.6 (10.7) (28.9–70.1)
Axillary lymph nodes examined (n)	11.7 (0.4) (2–29)*
DNA-index:	1.48 (0.03) (1.0–3.5)
SPF (n = 135):	12.88 (0.54) (3.2–29.2)
Length of follow-up—all patients (months)	89.0 (13.0) (1.6–156.9)
Length of follow-up—survivors only (months)	123.7 (16.5) (91.3–156.9)
Total no. of events	n (%)
Dead, due to advanced breast cancer	73 (38.2)
Dead, without any signs of cancer recurrence	6 (3.1)
Dead, unknown breast cancer	13 (6.8)
Alive, without symptoms	84 (44.0)
Alive, with recurrence	11 (5.8)
Alive, unknown breast cancer	4 (2.1)
Recurrences (n = 84)	
Local recurrence	15 (17.9)
Distant recurrence	48 (57.1)
Multiple distant recurrences	14 (16.7)
Local and distant recurrences	7 (8.3)

* 5.5% less than 8.

Qualitative and quantitative comparisons of groups with respect to pretreatment, treatment and follow-up variables were performed with the χ^2 statistic and the t-test (two-tailed) for independent variables, respectively. Correlations are expressed as Spearman rank-correlation coefficients (rs), group data are expressed as mean (S.E.) (range).

RESULTS

Flow cytometry was performed successfully in 191 (79.3%) out of the 241 tumour specimens. In order to avoid selection bias, we evaluated the main characteristics of the 50 cases with uninterpretable histograms and found out that they did not differ significantly from those of the other 191. OS and RFS of these 50 patients did not differ from that of the other patients. (Mantel P = 0.7, details not shown). The following analyses were therefore confined to the 191 patients with known DNA characteristics. Out of these, it was possible to determine the SPF in 135 (72.3%) patients.

The median duration of follow-up was 124 months (range 91–157); as of their last recorded follow-up evaluation, 99 patients (52%) were alive and 84 (44%) had had no recurrence. Patients characteristics, DNA flow cytometry results, and observed events are summarised in Tables 1 and 2.

In diploid tumours (DNI = 1), the mean SPF was significantly lower than in tumours with DNI of more than 1:8.6 (0.51)vs. 17.6 (0.54) ($P < 0.0001$, Fig. 1).

Correlation of covariates

Complete correlation analysis is given in Table 3. Tumour size correlated with nodal involvement (0.20, P = 0.0026) and inversely with oestrogen receptor content (rs = −0.22, P = 0.003). Grading correlated inversely with oestrogen receptor content (rs = −0.35, $P < 0.0001$). Oestrogen receptor positivity correlated strongly with progesterone receptor positivity (rs = 0.43, $P < 0.0001$). S-phase-fraction correlated with DNI (rs = 0.66, P = 0.0001), nodal involvement (rs = 0.27, $P < 0.002$), Grading (rs = 0.27, $P < 0.003$), and weakly with tumour size (rs = 0.16, P = 0.055). DNI correlated with tumour size (rs = 0.18, P = 0.013) and Grading (rs = 0.19, P = 0.015).

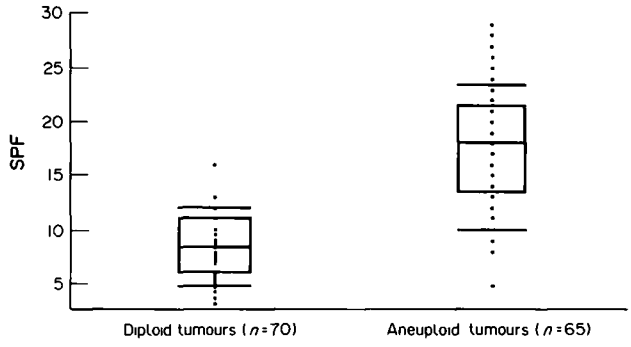

Fig. 1. SPF in diploid vs. non-diploid tumours (n = 135). t-test: $P < 0.0001$, quantiles: 90, 75, 50, 25, 10%.

Univariate analysis

The OS, RFS, and DRFS for all covariates is shown in Table 4. For all patients, it was 57, 56, and 60%, respectively, at 10 years. Group comparison performed with all the covariates listed in Table 1 revealed that nodal status, grading tumour size, and SPF were able to distinguish between patients groups at high and low risk for death, relapse and distant metastases.

As expected, nodal involvement was the best predictor of relapse and death (Fig. 2): Whereas 72% of all node negative patients were alive 10 years after operation, only 37% of the node positive patients survived ($P < 0.0001$). The estimated figures for RFS and DRFS are 78% and 81% in the node negative group vs. 37% and 37% in the node positive group.

DNI failed to distinguish between patients with poor and with good prognosis (OS: 61 vs. 50%, P = n.s; RFS: 63 vs. 51%, P = n.s; DRFS: 68 vs. 53%, P = n.s.), whereas SPF was of high prognostic impact with respect to all analyses: OS: 66 vs. 45% (P = 0.0021); RFS: 66 vs. 46% (P = 0.011); DRFS 69 vs. 50% (P = 0.023). As shown in Table 4, the significance of SPF as a prognostic factor was similar to grading or tumour size. Only the prognostic strength of nodal status was greater.

Oestrogen receptor status, progesterone receptor status, age group, adjuvant therapy, and menopausal status did not bear any prognostic significance in this analyses.

Multivariate analysis

Since there are strong correlations between some of the variables (see above), in order to find out the individual prognostic strength of the covariates, they were entered into a Cox' proportional hazards model (List of covariates: Table 1). Results are shown in Table 5.

With respect to OS, nodal status [β = 1.22, P = 0.0001, relative risk (RR) = 3.39], grading (β = 0.55, P = 0.09, RR = 1.73) and tumour size (β = 0.51, P = 0.082, RR = 1.66) remained as independent prognostic variables. SPF (β = 0.64, P = 0.036, RR = 1.84), too, could be shown to provide independent prognostic information, whereas DNI could not.

For RFS, nodal status (β = 1.63, $P < 0.0001$, RR = 5.11) and grading (β = 0.61, P = 0.062, RR = 1.90) are the established prognostic factors. In contrast to SPF, DNI has independent prognostic strength (β = 0.74, P = 0.011, RR = 2.09), which is slightly more than grading.

*Table 3. Correlations of covariates** (n = 191)

	T	G	N	ER	PgR	DNI	SPF	Age
T	—	NS	0.20, 0.0026	−0.22, 0.0031	NS	0.18, 0.013	0.16, 0.055	NS
G		—	NS	−0.35, <0.0001	NS	0.19, 0.015	0.27, 0.0025	NS
N			—	NS	NS	NS	0.27, 0.0015	NS
ER				—	0.43, <0.0001	NS	NS	NS
PgR					—	NS	NS	NS
DNI						—	0.66, 0.0001	NS
SPF							—	NS
Age								—

* Spearman coefficients, *t*-test.

*Table 4. Univariate analysis**

		OS			RFS			DRFS		
	n	Group 1†	Group 2	Mantel	Group 1	Group 2	Mantel	Group 1	Group 2	Mantel
T	191	61	46	0.02	64	43	0.005	69	45	0.0004
N	191	72	37	<0.0001	78	37	<0.0001	81	37	<0.0001
G	175	60	37	0.003	59	41	0.01	63	41	0.01
ER	152	53	55	0.7	59	52	0.9	57	57	0.9
PgR	150	56	54	0.9	59	49	0.7	61	54	0.6
DNI	191	61	50	0.1	63	51	0.1	68	53	0.08
SPF	135	66	45	0.002	66	46	0.01	69	50	0.02
Meno	191	59	51	0.2	52	57	0.8	58	61	0.9
Th	191	48	58	0.06	58	55	0.7	62	58	0.8
Age	191	53	55	0.9	51	58	0.4	57	59	0.8

* Actuarial survival after 10 years.
† Groups as shown in Table 1.

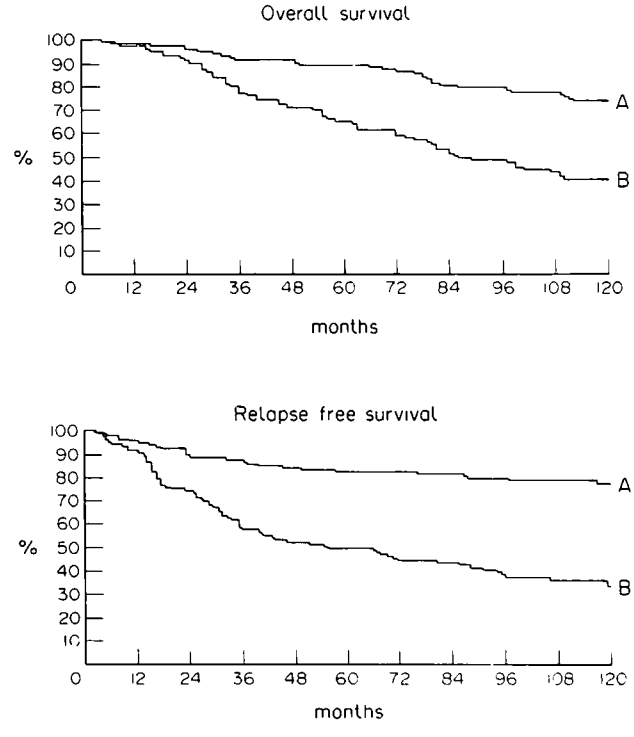

Fig. 2. Node negative vs. node positive. (A) N negative (*n* = 97). (B) N positive (*n* = 94). Mantel-Test: *P* < 0.0001 (OS), *P* < 0.0001 (RFS).

With respect to DRFS, nodal status (β = 1.40, $P < 0.0001$, RR = 4.07), Grading (β = 0.75, $P = 0.026$, relative risk (RR) = 2.11), tumour size (β = 0.62, $P = 0.043$, RR = 1.85) and DNI (β = 0.67, $P = 0.031$, RR = 1.96) turned out to provide useful prognostic information.

Subgroup analyses

Because of the overwhelming influence of the factor nodal status we performed separate subgroup analyses in the node negative (*n* = 97) and in the node positive (*n* = 94) subgroup. Because of smaller sample size the levels of significance were changed, but trends were similar to the results of the whole study population. In general, effects were more pronounced in the node positive subgroup (data not shown).

Finally, we combined DNI and SPF in order to define three prognostic subgroups: (A) diploid tumours (*n* = 71); (B) aneuploid tumours with low SPF (*n* = 11); and (C) aneuploid tumours with high SPF (*n* = 54). The 10 year survival rates for these subgroups were: With respect to OS: (A) 65% vs. B: 61% vs C: 46% ($P = 0.02$); RFS: A: 63% vs. B: 50% vs. C: 41% ($P = 0.047$) (Fig. 3).

DISCUSSION

Our findings confirm previously published results that S-phase-fraction values are significantly higher in aneuploid tumours [22]. As previously shown in node negative cohorts [23], we could confirm that SPF adds prognostic information to the ploidy status in all patients with operable breast cancer, at

Table 5. Multivariate analysis (Cox proportional hazards regression) of prognostic covariates of survival in 118 patients followed-up for a median of 10 years

	Overall Survival			Relapse-free-survival			Distant-disease-free-survival		
Covariate	β	P	RR	β	P	RR	β	P	RR
G	0.55	0.10	1.73	0.61	0.062	1.84	0.75	0.026	2.11
T	0.51	0.082	1.66	—	0.28	—	0.62	0.043	1.85
N	1.22	0.0001	3.39	1.63	<0.0001	5.11	1.40	<0.0001	4.07
DNI	—	0.43	—	0.74	0.011	2.09	0.67	0.031	1.96
SPF	0.64	0.036	1.84	—	0.95	—	—	0.93	—

β = standard coefficient, P = probability, RR = relative risk.

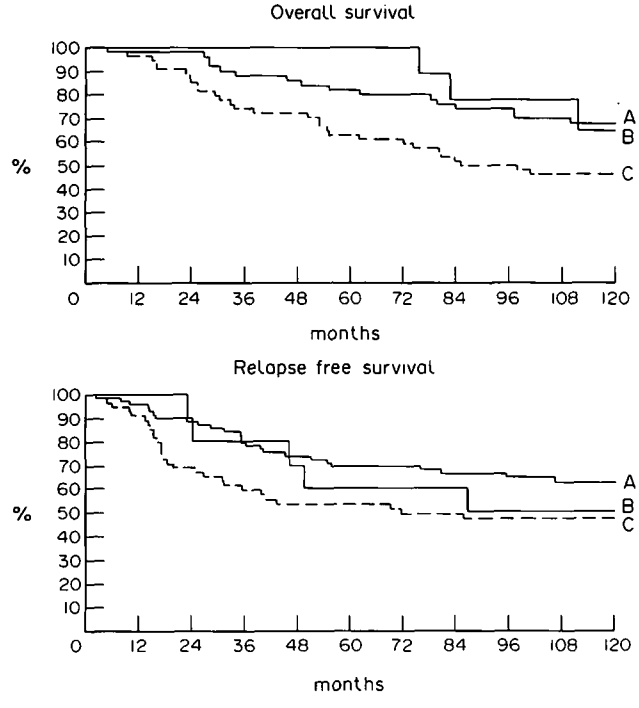

Fig. 3. Three groups by DNA Index and SPF (A) DNA Index = 1 (n = 71) (B) DNA Index > 1 and SPF <= 11 (n = 11) (C) DNA Index > 1 and SPF > 11 (n = 54). Mantel-Test: P = 0.022 (OS), P = 0.049 (RFS).

least with respect to OS. This was also found by Kalloniemi *et al.* in patients with stage III disease [24].

Oestrogen and progesterone receptor status did not provide prognostic information in the present study, neither in univariate nor in multivariate analysis. Previous early evaluation of data from the same study population suggested a prognostic impact of hormone receptor status [25], showing that this effect is lost with time, which is in accordance with Thorpe *et al.* [26].

Lymph node status is generally accepted as the most valuable predictor of recurrence in breast cancer [27]. Tumour size is also a predictor of recurrence, but these two factors are strongly correlated, and therefore the relative strength of tumour size as an independent predictor of relapse is still under debate [28]. We were able to confirm the findings of Sigurdsson *et al.* [23] that tumour size is associated with higher aneuploidy and proliferation rates, suggesting tumour size as a parameter for biological aggressiveness, and indeed, in the multivariate analysis, tumour size was shown to be an independent predictor of OS (RR = 1.66) and DRFS (RR = 1.85), however, the probabilities are borderline and the effect is lost with respect to RFS.

The question of whether flow cytometry results can serve as an independent predictor of outcome in breast cancer, is not answered: Sigurdsson *et al.* [23] demonstrated an independent impact of SPF, but not of DNA Index in node negative patients; Hedley confirmed this for node positive patients [29]. Several study groups were not able to find an independent prognostic impact of ploidy status [23, 29–32], whereas such an impact was proven by others [33–35]. In our study, we were able to prove an independent prognostic value for SPF with respect to overall survival, with a relative risk of 1.84 the second strongest after nodal status. However, with respect to RFS and DRFS, DNI turned out to be the more valuable predictor of outcome.

Thus, though it does not seem possible to rely on one flow cytometry parameter alone, their combination can be useful to differentiate between patient groups at high and low recurrence risk, as we were able to show by subgroup analysis. To date, the decision about the aggressiveness of an adjuvant chemotherapy scheme for a given patient seems to be based on the synopsis of clinical, histopathological and flow cytometry data, at least until more precise information about the biological behaviour of an individual tumour is available, obtained by flow cytometry or other methods. Nevertheless, our study demonstrates that data derived from DNA flow cytometry can provide helpful information about the recurrence risk in an individual patient. However, these data have to be interpreted in context with clinical and histopathological measurements.

1. Fisher B, Bauer M, Wickerham DL, *et al.* Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. *Cancer* 1983, **542**, 1551–1557.
2. Valagussa P, Bonadonna G, Veronesi U. Patterns of relapse and survival following radical mastectomy. *Cancer* 1978, **41**, 1170–1178.
3. Fisher B, Redmond C, Fisher ER, *et al.* Relative worth of estrogen or progesterone receptor and pathologic characteristics of differentiation as indicators of prognosis in node negative breast cancer patients: Findings from National Surgical Adjuvant Breast and Bowel Project protocol B-06. *J Clin Oncol* 1988, **6**, 1076–1087.
4. Knight WA, Livingston RB, Gregory EJ, *et al.* Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. *Cancer Res* 1977, **37**, 4669–4671.
5. Clark GM, McGuire WL, Hubay CA, *et al.* Progesterone receptors as a prognostic factor in stage II breast cancer. *N Engl J Med* 1983, **309**, 1343–1347.
6. Tubiana M, Pejovic MH, Chavaudra N, Contesso G, Malaise EP. The long-term prognostic significance of the thymidine labelling index in breast cancer. *Int J Cancer* 1984, **33**, 441–445.

7. Tandon AK, Clark GM, Chamness GC, *et al.* Cathepsin D and prognosis in breast cancer. *N Engl J Med* 1990, **322**, 297–302.
8. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* 1987, **235**, 177–182.
9. Sainsbury JR, Farndon JR, Needham GK, *et al.* Epidermal-growth-factor receptor status as predictor for early recurrence of and death from breast cancer. *Lancet* 1987, **1**, 1398–1402.
10. Colm H, Henry JA, May FEB, Westley BR, Angus B, Lennard TWJ. Expression of the antimetastatic gene nm23 in human breast cancer: An association with good prognosis. *J Natl Cancer Inst* 1991, **83**, 281–285.
11. Thor A, Benz C, Moore D, *et al.* Stress response protein (srp-27) determination in primary human breast carcinomas: clinical, histologic, and prognostic correlations. *J Natl Cancer Inst* 1990, **83**, 170–178.
12. Reiner G, Jakesz R, Schemper M, *et al.* Prognostic impact of flow cytometry and interaction with adjuvant chemotherapy in patients with operable breast cancer. In: Salmon SE, ed. *Adjuvant Therapy of Cancer*; Philadelphia, W.B. Saunders Company. 1990, **6**, 333–338.
13. Pocock GI, Simon R. Sequential treatment assignment with balancing for prognostic factors in the controlled clinical trial. *Biometrics* 1975, **31**, 103–115.
14. Bloom HJG, Richardson WW. Histologic grading and prognosis in breast cancer. *Br J Cancer* 1957, **11**, 359–377.
15. Jakesz R, Dittrich O, Hanusch J, *et al.* Simultaneous and sequential determinations of steroid hormone receptors in human breast cancer. *Ann Surg* 1985, **3**, 305–310.
16. Schutte B, Reynders MMJ, Bosman FTG, *et al.* Flow cytometric determination of DNA ploidy level in isolated nuclei from paraffin embedded tissue. *Cytometry* 1986, **6**, 26–30.
17. Vindelov LL, Christensen U, Nissen NI. A detergent trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* 1983, **3**, 323–327.
18. Hiddeman W, Schumann J, Andreeff M, *et al.* Convention of nomenclature for DNA cytometry. *Cytometry* 1984, **5**, 445–446.
19. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1987, **53**, 457–481.
20. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep* 1966, **50**, 163–170.
21. Cox DR. Regression models and life-tables. *J R Stat Soc (B)* 1972, **34**, 187–220.
22. Merkel DE, Dressler LG, McGuire WL. Flow cytometry, cellular DNA content, and prognosis in human malignancy. *J Clin Oncol* 1987, **5**, 1690–1703.
23. Sigurdsson H, Baldetorp B, Borg A, *et al.* Indicators of prognosis in node negative breast cancer. *N Engl J Med* 1990, **322**, 1045–1053.
24. Kalloniemi OP, Blanco G, Alavaikko M, *et al.* Improving the prognostic value of DNA flow cytometry in breast cancer by combining DNA-index and S-phase. *Cancer* 1988, **62**, 2183–2190.
25. Jakesz R, Reiner G, Schemper M, *et al.* Significant survival benefit of node-negative breast cancer patients treated with adjuvant chemotherapy: Seven-year results. *Recent Results in Cancer Research* 1988, **115**, 180–184.
26. Thorpe SM, Rose C, Rasmussen BB, Mouridsen HT, Bayer T, Keiding N. Prognostic value of steroid hormone receptors: multivariate analysis of systemically untreated patients with node negative primary breast cancer. *Cancer Res* 1987, **47**, 6126–6133.
27. Fisher B, Slack NH. Number of lymph nodes examined and the prognosis of breast carcinoma. *Surg Gynecol Obstet* 1970, **131**, 79–88.
28. McGuire WL, Tandon AK, Allred DC, Chamness GC, Clark GM. How to use prognostic factors in axillary node-negative breast cancer patients. *J Natl Cancer Inst* 1990, **12**, 6–14.
29. Hedley DW, Rugg CA. Association of DNA index and S-phase fraction with prognosis of N+ early breast cancer. *Cancer Res* 1987, **47**, 4729–4735.
30. Keyhani-Rofagha S, O'Toole RV, Farrar WB, *et al.* Is DNA ploidy an independent prognostic indicator in infiltrative node-negative breast adenocarcinoma? *Cancer* 1990, **65**, 1577–1582.
31. Owainati AA, Robins RA, Hinton C, *et al.* Tumour aneuploidy, prognostic parameters and survival in primary breast cancer. *Br J Cancer* 1987, **55**, 449–454.
32. Clark GM, Dressler LG, Owens MA, Pounds G, Oldaker T, McGuire WL. Prediction of relapse or survival in patients with node negative breast cancer by DNA flow cytometry. *N Engl J Med* 1989, **320**, 627–633.
33. Ewers SB, Baldetorp B, Killander D, Langstrom E. Flow cytometry, DNA ploidy and number of cell populations in the primary breast cancer and their correlation to the prognosis. *Acta Oncol* 1989, **28**, 913–918.
34. Cornelisse CJ, van de Velde CJ, Caspers RJ, Moolenaar AJ, Hermans J. DNA ploidy and survival in breast cancer patients. *Cytometry* 1987, **8**, 225–234.
35. Kalloniemi OP, Hietanen T, Mattila J, Lehtinen M, Lauslahti K, Koivula T. Aneuploid DNA content and high S-phase fraction of tumour cells are related to poor prognosis in patients with primary breast cancer. *Eur J Cancer Clin Oncol* 1987, **23**, 277–282.