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Prediction of relapse in patients with breast cancer by DNA cytometry

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Abstract

Nuclear DNA content was quantified for 193 ductal invasive breast carcinomas in a prospective study. The results were correlated with various clinical parameters. Of these tumours 66.2% were non-diploid and the incidence of non-diploid tumours is significantly higher in later stages of the disease. The median DNA values distribution tend to be bimodal, although in stage III a large number of cases showed DNA values between 2C and 4C. No association was observed between ploidy and menopause, lymph node status or incidence of recurrence. The mean times of relapse were 22.4 and 18.8 months in diploid and non-diploid groups, respectively. The present data suggest association between non-diploidy and tumour aggressiveness. Using Bayesian statistical analysis, the probability of the mean time of relapse in diploid group to be longer than in non-diploid group, given the data, is 0.875.

Keywords: Breast cancer; DNA ploidy; Recurrrence

1. Introduction

The accurate determination of prognosis is extremely important for therapy optimization at the level of the individual cancer patient. In breast cancer this is a challenging problem, due to its heterogeneous histopathology and clinical course.

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Extensive investigations have been directed toward the identification of reliable prognostic factors that enable us to define more homogeneous sub-groups within those of tumours with the same histopathological features and at same stage.

It has been suggested that patients with small [14] and well-differentiated [4] primary tumours, hormone receptors positive [1], and with no axillary lymph node involved at presentation [5], have longer survival time. The TNM classification system, based on primary tumour size (T), lymph node involvement (N) and distant metastases (M), has so far been the best guideline for choosing primary treatment. However, some of these factors may reflect the age of tumour rather than its biological potential [11]. Several other morphological (tumour necrosis, mitotic rate, cytological and histological grade, border contour, etc.) or clinical (menstrual status, age, pregnancy number, age at first pregnancy) parameters have also been investigated as predictors of probability of relapse [4,5,20]. None of them seems to greatly improve the predictive power of the TNM classification system.

Although many of these factors are considered of prognostic value, they are not able to explain the variability in the observed survival and replapse-free interval of breast cancer patients. Some of them have been combined to establish prognostic indices (stage, age at menarche, menstrual status and age, by Caleffi et al. [9]; mitotic index tumour size and lymph node status, by van der Linden et al. [33]) that seem to be more powerful predictors than the independent factors.

Abnormal DNA content is a primary tumour characteristic that has been found to be of prognostic value for an increasing number of solid tumours. In breast cancer DNA ploidy could be correlated with the clinical course of the tumour [10,13,17,18], although its exact meaning is controversial. Independent prognostic value has been shown for nuclear content DNA [21,22], estrogen receptor [19,29] and *erb*-B2 oncogene amplification [30].

The aim of the present study was to evaluate the importance of the primary breast tumour DNA ploidy in relation to their clinical behavior, prospectively. Normally, survival analysis of breast cancer patients to define the prognostic factors requires a large number of individuals and long follow-up periods due to the heterogeneous progress of the disease. Prospective studies hardly fulfill these requirements, especially in Brazil, where there is no centralization of cancer patient information. Bayesian statistical methods, which were used to analyze our data, allowed us to determine that the relapse-free time will be shorter in non-diploid than diploid tumours, with a probability of 0.875.

2. Materials and methods

The study group consisted of 198 patients with ductal invasive carcinoma (DIC) referred to the Fundação Oncocentro de São Paulo (São Paulo, Brazil) from 1984 to 1986. The patients were staged according to the post-surgical TNM classification for breast cancer of the International Union Against Cancer (UICC) [32].

Clinicopathological, staging, treatment and follow-up information could be readily accessed from the computerized register, maintained by that Foundation, of all women who had been diagnosed as breast cancer patients, until 1989.

In accordance with the WHO recommendations [32], recurrence of disease (reappearance of known lesions or development of new lesions) was accepted if microscopically confirmed, or if a combination of clinical, scintigraphic, radiological and biochemical data were conclusive.

Representative tumour specimens were collected and transferred to the laboratory in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 μg/ml and streptomycin, 100 μg/ml). Cytological preparations were made by imprinting of fresh cut surface of the tumour specimens. These preparations were fixed in ethanol:acetic acid:chloroform (3:1:1) for 3–4 min and then stained by Feulgen reaction under the following conditions: hydrolysis by HCl for 12 min at 60°C, followed by Schiffs reagent for 60 min at room temperature.

Cytophotometric measurements of the stained cells were made with a Zeiss microspectrophotometer equipped with a 0.5-μm step size scanning stage (Zeiss, Oberkochen, Germany) which was interfaced to a minicomputer.

The measurements were made at 570 nm, using a 100 × 1.30 oil immersion objective. Sixty morphologically identified tumour cells per case were measured randomly. Normal diploid human fibroblast (primary cultures) preparations were included in each staining batch. The median DNA value of the measured tumour cells was calculated in relation to the DNA content of the diploid control (2C) and expressed as C. Median DNA values ranging from 2.0 to 2.4C were defined as diploid.

Association between DNA ploidy and clinicopathological parameters was assessed by means of chi-square tests in contingency tables. Relapse time intervals were analyzed using Bayesian statistical methods [2]. Many patients abandoned the study for different reasons. We have to consider that it may be impossible to do a complete follow-up of patients who will take a long time to relapse or die. This led to a large number of censured data. It could be assumed, however, that as both diploid and non-diploid groups are similarly influenced by these factors direct conclusions are difficult. Evidently samples with many censored events exhibit considerable data variability which makes it difficult to apply standard statistical methods. Bayesian statistical analyses are based on probability calculations. In the present study we calculated the probability of the expected value of relapse-free time of a group (diploid) to be longer than the expected value of relapse-free time of the other group (non-diploid). The probabilities were calculated using the survival exponential model, that permit inclusion of the censored data. Actually, we used the information $\{X > t\}$ when the element to abandon the study in time t with X being the time necessary to relapse (metastasis or local recurrence). Note that with the exponential model the probability of $\{X > t\}$ can be expressed and included in the likelihood in order to increase the estimator's precision. To perform the Bayesian analysis we considered uniform prior densities on the positive real line. Although not strictly correct, these prior densities were used to avoid any personal influence on the final results. Hence, for the 2 groups, we calculated the posterior distributions and their averages. The distribution of the ratio of the 2 averages happens to be the F of Snedecor. Using this fact we calculated the posterior probability that the mean time of relapse of the first group is longer than that of the second group. The result, 0.875

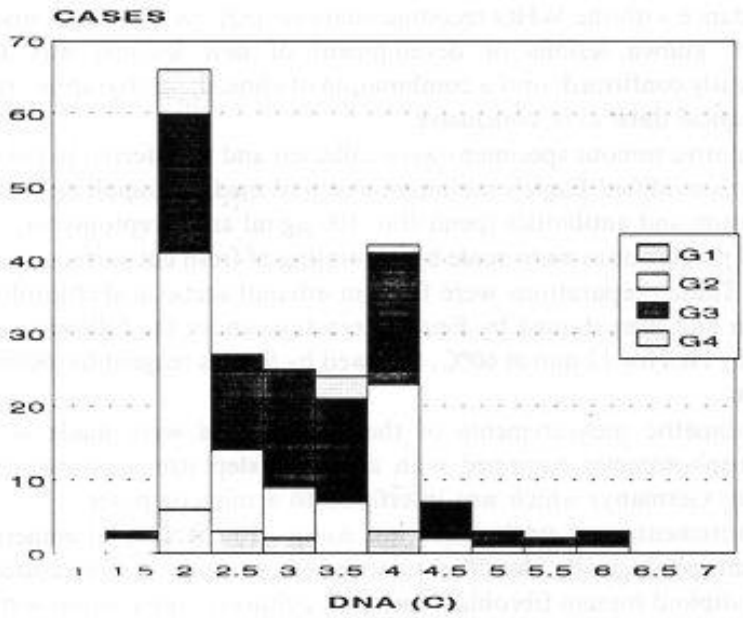

Fig. 1. Distribution median DNA values of ductal invasive carcinomas: G1-stage I (n = 14), G2-stage II (n = 83), G3-stage III (n = 91),and G4-stage IV (n = 11).

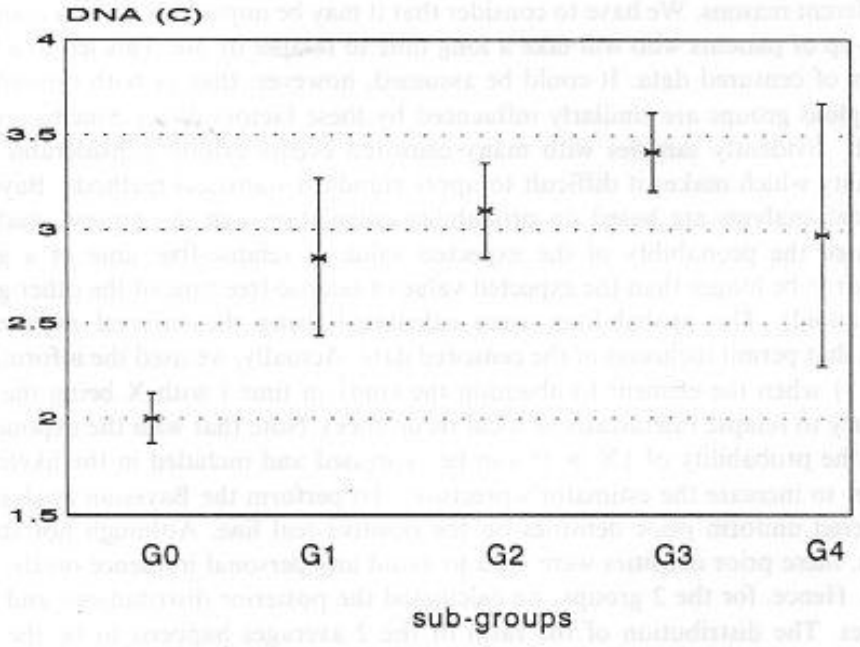

Fig. 2. Comparison of mean DNA values observed in fibroadenomas (G0) and in stages I (G1), II (G2), III (G3) and IV (G4) ductal invasive carcinoma.

(a high probability), indicates that this event is most unlikely to have occured by chance.

3. Results

Nuclear DNA content was quantified in cytological preparations of 198 ductal invasive carcinomas. The incidence of diploid tumours was 33.8%, whereas all 15 fibroadenomas (benign lesions), analyzed as additional control, were diploid. In contrast with fibroadenomas (G0), carcinomas showed greatly abnormal median DNA values, reaching very high values (> 6C). Median DNA values of ductal invasive carcinomas, grouped according to TNM stage I, II, III, and IV in GI, G2, G3 and G4, respectively, are presented in Fig. 1. The distribution of these values tends to be bimodal, with increasing incidence of non-diploid tumours in later stages when compared to earlier stages, and predominance of values between 2C and 4C in stage III.

The mean DNA value of each group increases with the disease progression, being lower in stage IV, probably as a consequence of their heterogeneity and small number of cases (Fig. 2).

Defining the ploidy as diploid (1.8 to 2.4C), hyperploid (2.5 to 3.5C), tetraploid (3.6 to 4.4C) and hypertetraploid (< 4.4C), the majority of carcinomas were diploid or tetraploid (33.8% each), and almost all of the remaining tumours were hyperdiploid (24.8% of the total). Only a few, 7.5%, were hypertetraploid (Table 1). The overall data show that the majority of tumours were non-diploid (66.2%).

From the initial 198 patients with ductal invasive carcinoma, only 183 were followed up. Some of their clinicopathological characteristics are shown in Table 2. The association relationship between the different parameters, important to the characterization of studied tumours, was obtained by contingency table analysis.

According to the TNM system classification, the majority of the tumours were stage II (41.9%) and III (45.9%) tumours, while the others fell into stage I (7.1%) and stage IV (5.5%). For the purpose of this study, we decided to group them in Early (I+II) and Late (III+IV). A significant association was found: patients with diploid tumours were more likely to be in early (E) stages of the disease (Table 3).

Table 1
Distribution of tumours according to the DNA ploidy

Ploidy	No. of cases (%)
Diploid	67 (33.8)
Hyperdiploid	49 (24.8)
Tetraploid	67 (33.8)
Hypertetraploid	15 (7.5)
Total	198

Table 2
Clinicopathological characteristics of the 183 patients with ductal invasive carcinoma

Age	No. of cases (%)
<40 years	24 (13)
40 to 49 years	38 (21)
50 to 59 years	73 (40)
>60 years	48 (26)
Total	183
Menopause	
(NM) pre-menopause	60 (33)
(PM) post-menopause	123 (67)
Total	183
Stage (TNM)	
I	14 (7.7)
II	76 (41.5)
III	82 (44.8)
IV	11 (6.0)
Total	183
Lymph nodes	
0	50 (29.1)
1 to 3	66 (38.4)
>3	56 (32.5)
Total	172

Post-menopausal patients amounted to 67% and this percentage showed no significant alteration when the patients were stratified according to the disease stage (E and L), or to the ploidy of the tumour (diploid and non-diploid). Post-menopausal (PM) women represented 68.48% of the total number of patients, 75% of the non-

Table 3
Tumour distributions according to ploidy and staging

DNA	Stage		
	E (TNM I+II) (%)	L (TNM III+IV) (%)	Total
Diploid	37 (68.5)	17 (31.5)	54
Aneuploid	59 (45.7)	70 (54.3)	129
Total ($P = 0.008$)	96 (52.5)	87 (47.5)	183

Table 4
Ploidy and nodal status

DNA	No. of positive lymph nodes (%)		
	0	1 – 3	+ de 3
Diploid	20 (40)	18 (27.3)	20 (27.7)
Non-diploid	30 (60)	48 (72.7)	52 (72.2)
Total	50 (100)	66 (100)	72 (100)

Table 5
Recurrence in the total group, according to staging

Recurrence	Stage		
	E (TNM I+II) (%)	L (TNM III+IV) (%)	Total
e1 (+)	25 (39.1)	39 (60.9)	64
e0 (–)	71 (59.7)	48 (40.3)	119
Total ($P = 0.0122$)	96 (52.5%)	87 (47.5%)	183

Table 6
Recurrence in the non-diploid tumours, according to staging

Recurrence	Stage		
	E (TNM I+II) (%)	L (TNM III+IV) (%)	Total
e1 (+)	16 (32.6)	33 (67.4)	49
e0 (–)	43 (53.8)	37 (46.2)	80
Total ($P = 0.0314$)	59 (45.7)	70 (54.3)	129

Table 7
Recurrence in the diploid tumours according to staging

Recurrence	Stage		
	E (TNM I+II) (%)	L (TNM III+IV) (%)	Total
e1 (+)	9 (60.0)	6 (40.0)	15
e0 (–)	28 (71.8)	11 (28.2)	39
Total ($P = 0.6109$)	37 (68.5)	17 (31.5)	54

diploid/early and 63.33% of the non-diploid/late. PM patients also predominate within diploid tumours, 69.44% and 64.71% of those in early and late stages, respectively. There was a tendency to a slightly higher percentage of PM in earlier stages of the disease.

Despite being one of the breast tumour's TNM classification system parameters, lymph node status had its association with the tumour ploidy level evaluated independently. Three groups were then defined based on the lymph node status: with 0, 1 to 3 and more than 3 positive lymph nodes. Most of the patients were in the second and third group, 35.1% and 38.3%, respectively. Table 4 shows the diploid and non-diploid tumour distribution according to these sub-groups. Twenty out of

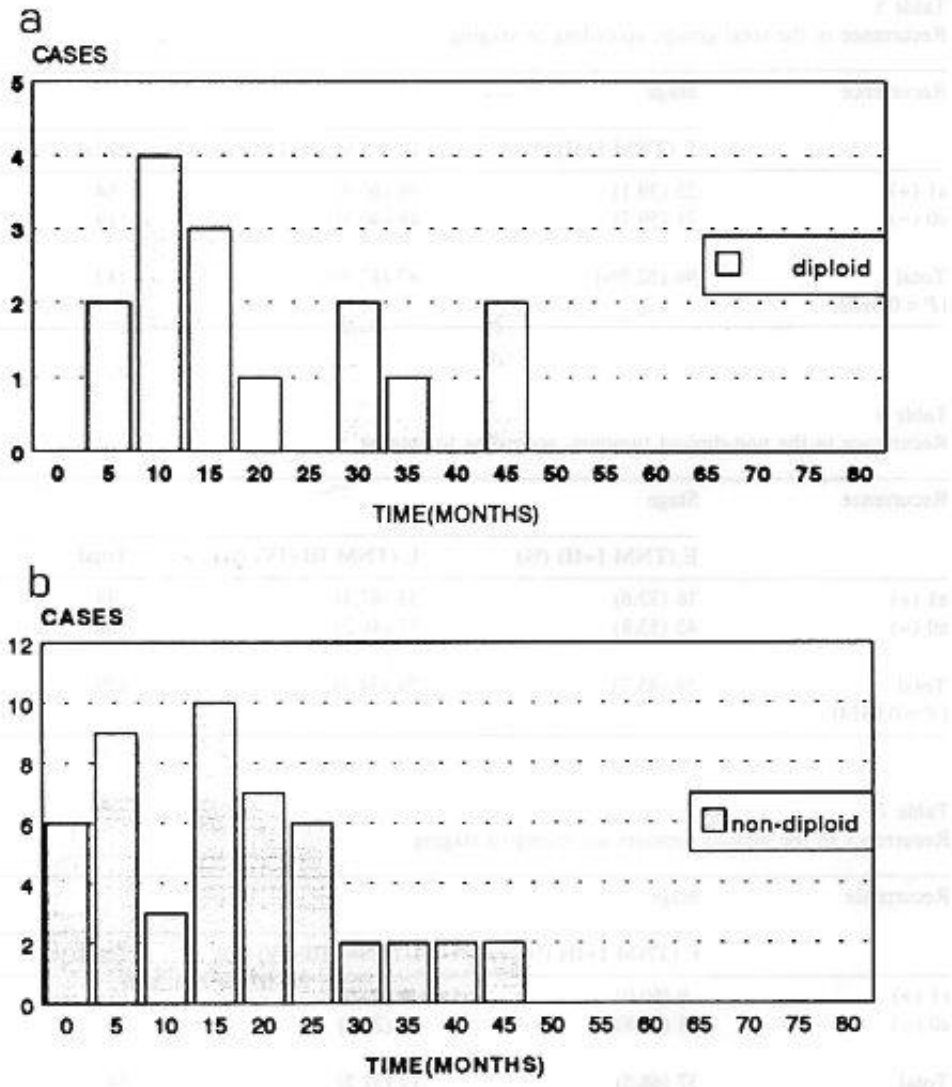

Fig. 3. Distribution of cases according to interval of relapse: (a) diploid, and (b) non-diploid tumours.

50 (40%) lymph node-negative patients were diploid, whereas only 38 out of 132 (28%) of those showing axillary metastasis were diploid.

Time of relapse was considered an indicator of disease progression. It was defined as the time interval between the patient presentation and a new disease manifestation: local recurrence and distant metastases. Exceptionally, in the case of the small number of stage IV tumours, metastases in a new site or cancer death were established as indicators of disease progression. According to these criteria, patients were divided into 2 sub-groups: e0, did not relapse and e1, relapsed, during the period of observation.

When all patients were considered, the number of late stage tumours is significantly higher in sub-group e1 ($P = 0.0122$), as expected (Table 5). Late stage tumours (L)

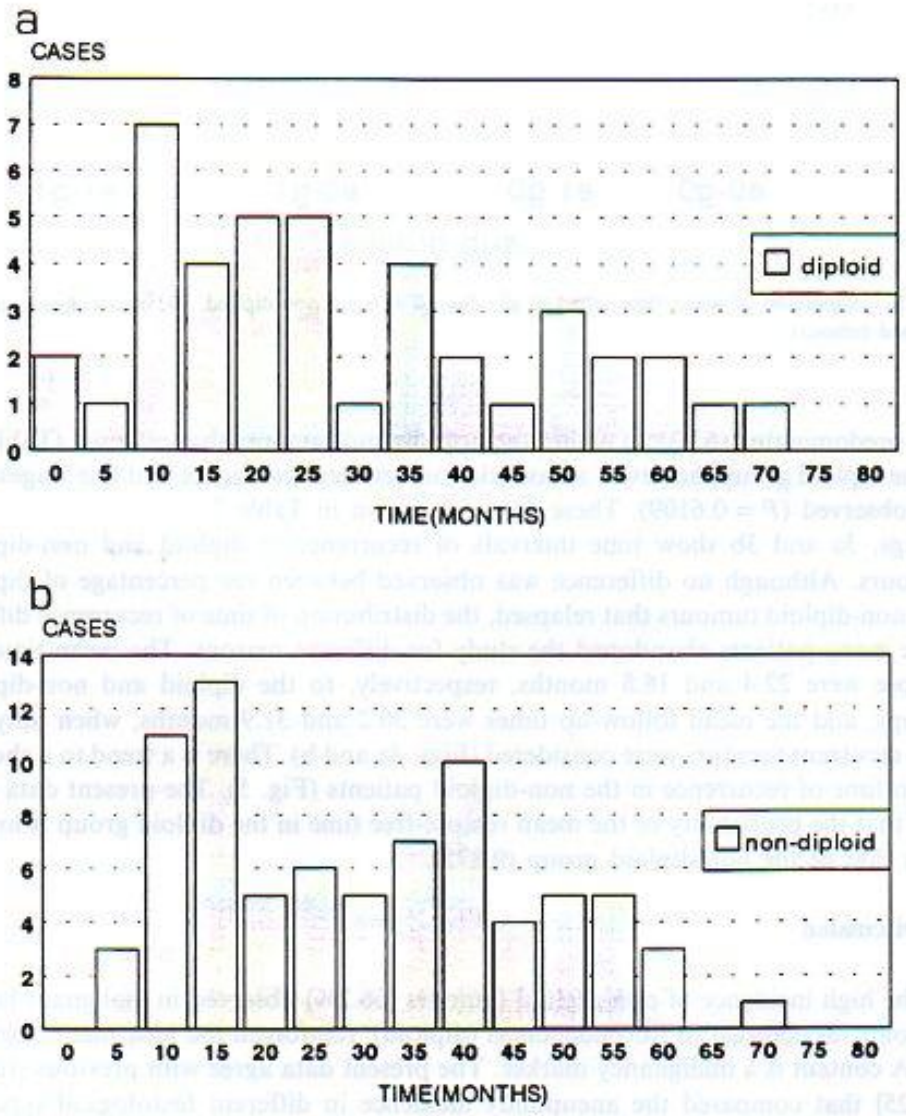

Fig. 4. Distribution of cases according to time of follow-up: (a) diploid, and (b) non-diploid tumours.

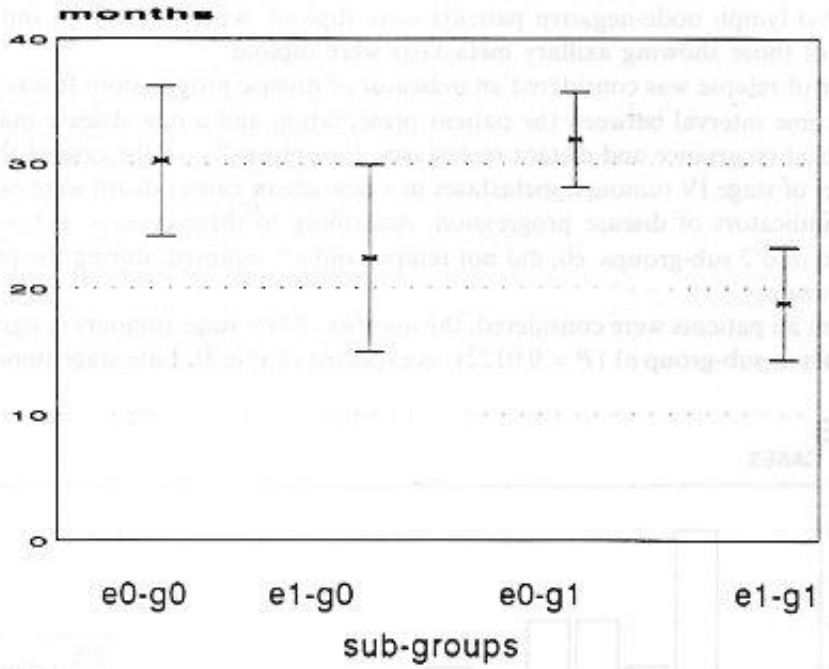

Fig. 5. Comparison of mean time interval: g0, diploid and g1, non-diploid, e0, non-relapsed and e1, relapsed tumours.

also predominated (67.35%) within the non-diploid tumours that relapsed (Table 6). In the diploid group, however, an association between recurrence and late stages was not observed ($P = 0.6109$). These data are shown in Table 7.

Figs. 3a and 3b show time intervals of recurrence in diploid and non-diploid tumours. Although no difference was observed between the percentage of diploid and non-diploid tumours that relapsed, the distribution of time of recurrence differs, since many patients abandoned the study for different reasons. The mean times of relapse were 22.4 and 18.8 months, respectively, to the diploid and non-diploid groups, and the mean follow-up times were 30.2 and 31.9 months, when only the non-recurrent tumours were considered (Figs. 4a and b). There is a trend to a shorter mean time of recurrence in the non-diploid patients (Fig. 5). The present data suggest that the probability of the mean relapse-free time in the diploid group is longer than that of the non-diploid group (0.875).

4. Discussion

The high incidence of non-diploid tumours (66.2%) observed in malignant breast tumours, in contrast to fibroadenomas (diploid), reinforced the idea that abnormal DNA content is a malignancy marker. The present data agree with previous studies [23,25] that compared the aneuploidy incidence in different histological types of breast tumours. According to McDivitt et al. [20] non-diploidy would always be

associated with more aggressive tumours, belonging or not to the same histological type.

Feichter et al. [13] presented a table reviewing the flow cytometry data related to incidence of aneuploidy in mammary cancer. Thirteen of 21 citations referred to aneuploid percentage higher than 60%. Other studies using different methods of DNA quantification [3], showed a similar range of incidence of non-diploid tumours. However for direct comparison of different studies, we have to keep in mind that several factors could induce variability in the ploidy analysis such as fixation of specimens, method of staining and quantification. Apparently there is a good agreement between different quantification methods such as image analysis and flow cytometry [10] and between the latter and scanning cytometry [15]. Retrospective studies using material from paraffin-embedded block tissues might introduce errors in DNA quantification. Tumour specimens have to be dewaxed and enzymatically dissociated to obtain a single cell suspension prior to staining, and these procedures may introduce a high level of cell debris in the preparations. Therefore, prospective studies using fresh specimens are very important reliable data.

The role of aneuploidy in tumour biology is still unknown and being such a global phenomenon it probably involves structural chromosomal aberrations besides the numerical ones. Consequently it could mediate gene dosage imbalance and gene deregulation, and so determine a cell growth promotor effect. Further information on the role of DNA alterations in early phases of transformation processes (tumour initiation and progression) is important for understanding their involvement in the etiology of cancer. Aneuploidy has been widely studied during tumour progression phase.

The distribution of median DNA values of the ductal invasive carcinomas tends to be bimodal, in agreement with previous reports [12,15,23]. Tumour classification in ploidy sub-groups showed that the diploid and tetraploid are dominant subgroups, supporting the bimodal distribution. Since the mean DNA value should correspond to the modal chromosomal number in these cells [31], we could infer that our data agree with the Ewers et al. [12] hypothesis. These authors pointed out that the evolution of hypotetraploid stemlines may have occurred via a polyploidization process of normal cells, followed by gradual loss of chromosomes. Consequently the probability of tumours to be tetraploid would be higher than that of hypotetraploid.

Higher DNA ploidy values were associated to late stages of disease, suggesting that DNA abnormalities increase with the progression of the disease. These data could be explained by Nowell's theory [24] which states that the process of malignant transformation would lead to a genetic instability, allowing successive new tumour cell clones to arise, some of them being more prone to proliferate and metastasize.

The significant association observed between diploid tumours and early stages of disease is important mainly if we consider that initial and late phases are balanced in our sample, as a result of the large number of cases in stage III (G3). This is an unusual characteristic from other studies since more efficient programs of cancer prevention in developed countries lead stage II, or even I, to predominate. Sharma et al. [28] who analyzed nuclear DNA in Indian patients point to some of their pecu-

liar features such as lower age, high number of parities, and early age at first childbirth. The patients we analyzed were older and consequently most of them were post-menopausal. Additional and conclusive information on prognostic factors may be obtained from studies of tumours representing different geographic regions and genetic backgrounds. This was previously suggested by Paterson et al. [26], when they analyzed the oncogene c-*erb*B-2 amplification in a well-controlled female population in northern Alberta, Canada. One subset of node-negative patients with breast cancer who are at high risk of disease recurrence and poor overall survival could be identified.

We observed no association between ploidy and axillary lymph node status. The correlation between these parameters has been controversial being positive [15] or negative [12,23] in different studies. Cornelisse et al. [11] showed a significant positive association only with tumours presenting more than 10 positive lymph nodes.

In summary, our data suggest the association of non-diploidy and tumour aggressiveness. Hypotetraploid tumour cells may represent those with high aggressive potential [3]. However, we can not exclude an additional effect of DNA abnormalities in earlier phases of tumoral development or the very existence of tumour subgroups where aneuploidy is already present from initial stages, being a specific biological characteristic. In neuroblastomas for example, the oncogene N-*myc* amplification is correlated to the stage of the disease, but high numbers of gene copies indicate a poor prognosis, independent of the stage of the disease [8].

The percentage of relapse is similar in non-diploid and diploid tumours. Previous studies reported a high percentage of recurrence [12,28] or death [18] in patients with non-diploid tumours. Others showed no effect of ploidy on the survival prediction after metastization [6,16,17]. The time of recurrence (local or distant) after mastectomy or first therapy reflects the tumour progression and is an important parameter to be evaluated. Shorter times of recurrence were consistently observed in non-diploid tumours. Based on our data, the probability of a shorter disease-free interval in patients with non-diploid than in those with diploid tumours is very high: 0.875. This result suggests that the tumour ploidy is a relevant factor in clinical evaluation of breast cancer patients. The determination of its exact dimension still depends on additional information, mainly in relation to the response to adjuvant therapy. The viabilization of sequential analysis of different parameters such as estrogen receptor and nuclear DNA quantification in single cytological preparation from fine-needle biopsies [27], increases the possibilities of performing prospective studies.

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