

# Proliferation in non-Hodgkin's lymphomas and its prognostic value related to staging parameters

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Received 4 November 2003

Accepted 29 December 2003

**Abstract.** In malignant lymphomas, cell kinetics has shown to be related with histologic type as well as with the clinical behaviour. The aim of our study was to investigate the relevance of cell proliferation parameters on overall survival in non-Hodgkin's lymphomas as well as their relationship with prognostic factors such as International Prognostic Index (IPI). We performed DNA-flow-cytometry (S-phase fraction and detection of DNA-aneuploidy) as well as cytologic examination and the AgNOR technique in material obtained by fine needle aspiration of lymph nodes at diagnosis. The majority of the patients were stage IV by Ann Arbor and intermediate risk by IPI (42/55). When analyzing all patients together, histologic type by the WHO classification, IPI and the presence of a DNA-aneuploid clone could not separate well patients with a different survival. For all patients, univariate Cox analysis revealed S-phase (SPF) and AgNOR parameters to be of prognostic value. In the multivariate analysis, however, only SPF remained in the final model. Yet, when stratifying for DNA-ploidy, only the total number of AgNORs/nucleus was an independent parameter. Looking only at the DNA-diploid cases, the AgNOR pattern remained the most important parameter, whereas for the DNA-aneuploid cases this was true for SPF. When studying patients with B large cell lymphoma separately, only DNA-ploidy was a prognostic factor. In summary, cell kinetic parameters reveal important prognostic information in NHL patients. Furthermore, DNA-aneuploidy seems to interfere with the analysis of the AgNOR pattern.

Keywords: Lymphoma, cell proliferation, DNA-aneuploidy, prognosis, AgNORs

## 1. Introduction

Non-Hodgkin's lymphomas (NHL) are a heterogeneous group of neoplastic disorders derived from the lymphoid system. In recently described classifications such as the REAL and the WHO proposal [10], the different entities have been defined by morphology, phenotype and genetic features. The concept of "grade of malignancy", established by the Kiel classification and the Working Formulation, has been excluded from these classification systems. In recent years, cell kinetics of NHLs has been extensively studied, providing

a better understanding of the molecular abnormalities leading to alterations in cell proliferation and apoptosis observed in the different types [28,34]. In NHLs with a small growth fraction, cell accumulation is due to a prolonged cell survival with inhibition of apoptosis [15,24,28]. Therefore, most cells are in G<sub>0</sub>. Several studies have demonstrated the prognostic impact of the proliferation rate within each type of NHL, measured by different markers such as Ki-67, S-phase fraction determined by flow cytometry, or AgNOR analysis [5, 12,15,16,25,33].

On the other hand, clinical studies have added parameters of tumour mass and cell turnover (LDH) to the Ann Arbor staging, establishing the International Prognostic Index [37]. The proliferative index has not been used for patient evaluation. There is controversy

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in the literature about the relative prognostic impact of clinical and cell kinetic parameters (proliferation and apoptosis) on survival in NHLs [11,15,19,25,30,33].

Our aim was to examine the relationship among the proliferation pattern, clinical staging, LDH levels and survival in a group of NHLs. Cell proliferation was analysed by DNA-analysis in flow-cytometry, and by the AgNOR technique.

## 2. Material and methods

We studied adult patients treated at the Hematology Center of the State University of Campinas, Brazil, during the last 5 years. Diagnosis was made by routine lymph node biopsy before treatment. Simultaneously, cytological material was obtained by fine needle aspiration for preparation of cytological smears and cell suspensions in PBS for DNA flow cytometry. From each patient we collected clinical and survival data. Staging procedures included physical examination, chest radiography, abdominal ultrasound, bilateral bone marrow biopsy and a complete biochemical profile, including determination of LDH. Patients were staged according to the Ann Arbor criteria and the International Prognostic Index (IPI) [37].

Patients with follicular and lymphoplasmacytic lymphoma and age above 60 years were treated by chlorambucil (0.3 mg/kg for 5 days every 30 days, 6–12 cycles). Those with B large cell lymphoma, follicular lymphoma and age below 60 years as well as patients with mantle cell lymphoma and T-cell lymphoma were treated by VACOP-B [36]. Lymphoblastic lymphoma and Burkitt's lymphoma were treated according to the BFM protocols [29] for these diseases. Follow-up was evaluated from the day of start of treatment until death (overall survival).

### 2.1. Lymph node morphology

Biopsies were paraffin embedded and sections were stained by HE, Giemsa and Gomori's silver impregnation for reticulin fibres. Immunohistochemical study included CD19, CD3, CD5, CD42 and expression of surface immunoglobulin (immunostaining for kappa and lambda light chains). Diagnosis was made according to the WHO classification [10]. Lymph node smears were stained by the May-Grünwald-Giemsa stain and the AgNOR technique.

### 2.2. AgNOR technique

Smears were fixed for 7 minutes in acetone. Then they were incubated for 10 min in silver nitrate/gelatine solution (2 vol : 1 vol), washed with deionized water and counterstained with hematoxylin [14,15].

A differential count of 100 consecutive cells was made, counting separately cells with compact nucleoli and those with AgNOR clusters. A compact nucleolus was defined as a large compact silver precipitation with the form of a nucleolus, in which no internal structure could be individualized. A cluster was defined as an area with large heterogeneous confluent silver precipitations within a light matrix inside the nucleolus (Fig. 1). Small silver dots spread throughout the nucleus were not included in the counts. For each case, we made a differential count of cells with only compact nucleoli (cells in G0) and those with at least one AgNOR cluster (% cells with AgNOR clusters). We computed also the mean number of AgNOR clusters per cell (mean AgNOR clusters/nucleus) as well as the total number of AgNORs/nucleus, irrespective of their configuration (mean AgNORs/nucleus).

### 2.3. DNA by flow-cytometry

Cells were suspended in PBS (at least  $1 \times 10^6$  cells/ml) and washed three times. Then they were incubated with propidium iodide according to the manufacturer's instructions (Cycle Test Plus DNA Reagent Kit, Becton Dickinson San Jose, CA). The samples were analysed by a FACScalibur flow cytometry equipment (Becton - Dickinson, San Jose, CA, USA). DNA analysis was made by the Mod Fit Software to calculate the percentage of cells in S-phase (SPF). The program also detects the presence of aneuploid cell populations, the percentage of the aneuploid cells among the whole cells as well as the S-phase of this population. The cases where the software separated one individualized population with a DNA peak different from 2c was considered as presenting a DNA-aneuploid clone.

### 2.4. Statistical analysis

First a descriptive statistics was made. Proliferation parameters of different diagnostic categories were compared by the Kruskal-Wallis test. Correlations between the variables were calculated by the Spearman rank order test.

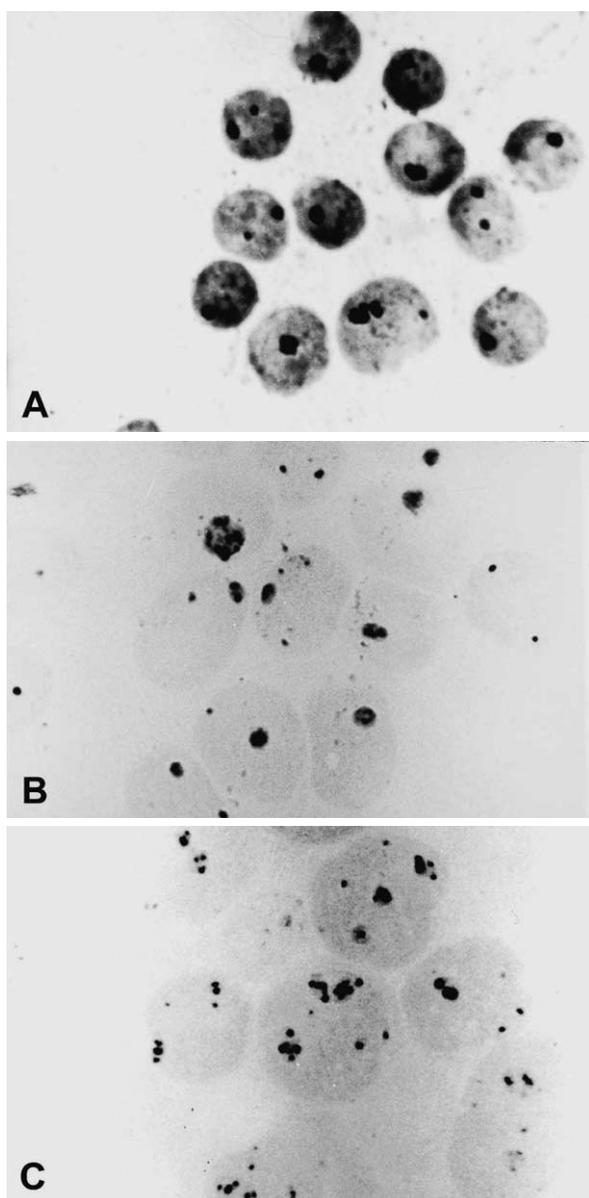


Fig. 1. Different AgNOR patterns in the cases: A – Follicular lymphoma. Many of the cells present one compact nucleolus. One large cell presenting 2 clusters of AgNORs on the middle left. B – Mantle cell lymphoma. All cells exhibit one AgNOR cluster. C – B large cell lymphoma. All cells show AgNOR clusters.

Survival analysis, stratified according to histologic diagnosis, grade according to the Kiel classification, IPI, DNA-aneuploidy, presence of bulky disease or bone marrow infiltration, was done according to the Kaplan–Meier method followed by the log-rank test.

Finally, in order to show the clinical applicability, statistically significant independent prognostic factors

were submitted to a cluster analysis (Ward algorithm). This was followed by the log-rank test, in order to suggest cut-off-points and to test the relevance of the newly created subgroups for survival.

The relations between survival and age, LDH, SPF and the three different AgNOR counts were analyzed by univariate Cox regressions. In a second step, all variables with a  $p < 0.1$  were included in a multivariate Cox-regression ( $p = 0.05$  for input and  $p = 0.1$  for output, backward conditional step-wise selection), in order to get a predictive model for the overall survival time. SPSS 8.0 and WinStat 3.1 softwares were used for the calculations.

Finally, we repeated this procedure only for the subgroup of large cell B lymphomas. In the same way, lymphomas with and without aneuploidy were examined separately.

### 3. Results

We studied 55 patients. Median age was 57 years (17–81); 39 were male and 16 female. Histologic types are listed in Table 1. B large cell and follicular lymphomas were the most frequent ones. Only 5 cases were of T-cell origin. The clinical and staging data are listed in Table 2. Most patients had advanced stage by the Ann Arbor criteria (44/55 cases stage IV). Regarding the IPI criteria, 42 patients had an intermediate and 8 had a high risk. Bulky disease was found in 25 patients (45%). LDH levels were elevated in 33 patients (60%). Bone marrow was involved in 29 patients (52%).

#### 3.1. Cell kinetic parameters

DNA-aneuploid cell populations were found in 11 cases, mostly in B large cell NHLs (Table 3). Concerning the S-phase fraction (SPF), variability was found within each type of NHL (Fig. 2), but this was more accentuated in B large cell, Burkitt and lymphoblastic lymphomas. SPF separated well between follicular and B large cell lymphoma.

Table 1

Classification of the cases by the WHO classification			
Lymphoblastic	2 cases	Follicular	10 cases
Burkitt	4 cases	Mantle cell	4 cases
B large cell	27 cases	Immunocytoma	5 cases
Anaplastic	2 cases	T cell peripheric	1 case

All three AgNOR parameters showed very similar differences between the diagnostic groups, similar to those described for SPF (Figs 2 and 3), except for mantle cell lymphoma. Lymphomas with a low proliferative fraction revealed in general most cells containing only one compact nucleolus.

Comparing all patients, SPF correlated well with “% cells with AgNOR clusters” ( $r = 0.71$ ), “mean AgNORs/nucleus” ( $r = 0.88$ ) and “mean AgNOR clusters/nucleus” ( $r = 0.87$ ). Since patients with anaplastic lymphoma, the single patient with T-peri-

Table 2  
Clinical features of the patients

	High grade	Low grade	Total	<i>p</i>
Age (median)	61	47	57	>0.05
	(17–81)	(25–85)	(17–85)	
Ann Arbor				>0.05
I	1	0	1	
II	4	0	4	
III	4	2	6	
IV	27	17	44	
IPI				>0.05
low risk	4	1	5	
low intermediate	14	11	25	
high intermediate	11	6	17	
high risk	7	1	8	
LDH				0.006
median	541	450		
	(286–3432)	(175–731)		
normal	12	10	22	
increased	24	9	33	
Bulky disease				>0.05
yes	18	7	25	
no	18	12	30	
Bone marrow				>0.05
involved	15	14	29	
not involved	21	5	27	

Table 3  
Cell kinetic parameters

	DNA-aneuploid cases	% S-phase*	% of cells with AgNOR clusters*	Median number of AgNOR clusters per cell*	Median AgNORs/cell*
Lymphoplasmacytic L.	2/5	1.4 (0.6–5.9)	59 (24–66)	1.34 (1.02–1.89)	0.67 (0.24–1.25)
Follicular L.	1/10	2.8 (0.6–8.2)	33 (16–54)	1.50 (1.20–1.80)	0.49 (0.21–0.90)
Mantle cell L.	1/4	2.3 (0.4–5.3)	100 (64–100)	1.22 (1.10–1.34)	1.15 (0.82–1.24)
B large cell L.**	6/30	13.8 (6.2–46.0)	100 (58–100)	2.46 (1.00–4.70)	2.46 (0.7–4.70)
Burkitt/lymphoblastic L.	1/6	34.7 (19.7–43.9)	100	3.83 (2.30–4.07)	3.8 (2.3–4.0)

\*Median (range).

\*\*Including the cases of anaplastic and T peripheric lymphoma (3 cases).

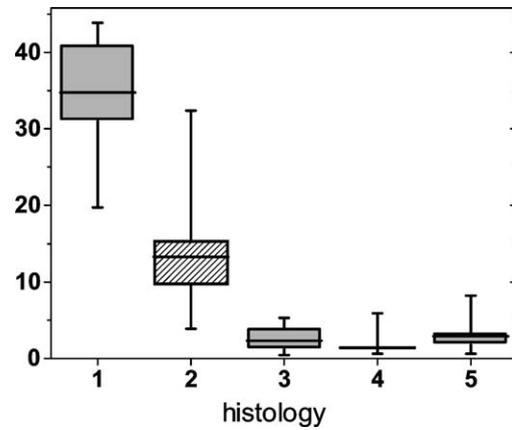


Fig. 2. SPF of the patients according to the WHO type: 1 – Burkitt and lymphoblastic lymphoma; 2 – B large cell lymphoma; 3 – mantle cell lymphoma; 4 – lymphoplasmacytic lymphoma; 5 – follicular lymphoma.

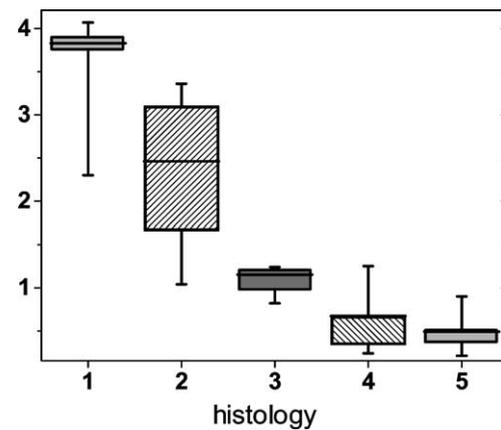


Fig. 3. Mean “total number of AgNORs/nucleus” of the patients according to the WHO type: 1 – Burkitt and lymphoblastic lymphoma; 2 – B large cell lymphoma; 3 – mantle cell lymphoma; 4 – lymphoplasmacytic lymphoma; 5 – follicular lymphoma.

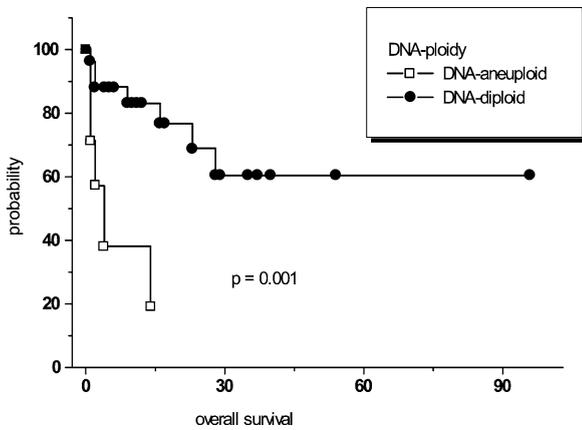


Fig. 4. Overall survival curve of the patients with B large cell NHL according to the presence of a DNA-aneuploid clone.

pheric lymphoma and patients with large cell lymphoma showed all very similar cell kinetic parameters, were joined them together for further statistical analyses. Mantle cell lymphoma showed a peculiar cell kinetic pattern: although its SPF was very low, the AgNOR count was higher than in lymphoplasmacytic and follicular lymphoma, since all cells presented at least one AgNOR-cluster (Fig. 1b).

### 3.2. Survival analysis

In the Kaplan-Meier analysis, histologic type according to the WHO classification ( $p = 0.07$ ), grade according to the Kiel classification ( $p = 0.051$ ) IPI ( $p = 0.30$ ) and the presence of a DNA-aneuploid clone ( $p = 0.20$ ) were not able to separate well patients with different survival. Yet analyzing separately patients with B large cell, Burkitt and lymphoblastic lymphomas, the presence of a DNA-aneuploid clone predicted a shorter survival (Fig. 4).

In the Cox model, no influence on survival was found for: age, histologic type, Ann Arbor stage and IPI, LDH levels, presence of bulky disease or bone marrow infiltration. However, there was significant influence of SPF ( $r = 0.18$ ;  $p = 0.008$ ) and the three AgNOR parameters: “% cells with AgNOR clusters” ( $r = 0.122$ ;  $p = 0.05$ ), “mean AgNORs/nucleus” ( $r = 0.166$ ;  $p = 0.023$ ) and “mean AgNOR clusters/nucleus” ( $r = 0.145$ ;  $p = 0.037$ ). Since the three different AgNOR variables showed a high covariance among them, we decided to select for the multivariate model only the variable with the highest  $r^2$  value.

In the multivariate analysis, only SPF remained as an independent prognostic factor in the final model

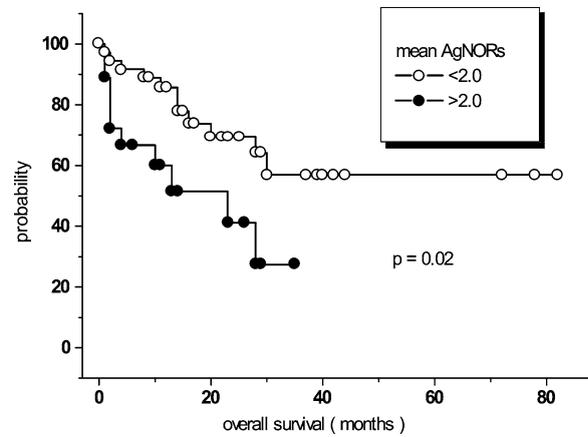


Fig. 5. Overall survival of the patients grouped by “total number of AgNORs/nucleus” below and above 2.0. This threshold was able to separate patients with a significantly different outcome.

( $r = 0.21$ ;  $p = 0.007$ ). Yet, when patients were stratified according to diploid and aneuploid cases, only the variable “mean AgNORs/nucleus” entered the final multivariate model ( $r = 0.18$ ;  $p = 0.02$ ).

Therefore we repeated the multivariate Cox-regression separately for the two subgroups according to the ploidy. For patients with diploid neoplasias only the AgNOR parameter entered the final model ( $r = 0.15$ ;  $p = 0.05$ ), and for those with aneuploidy only the SPF ( $r = 0.24$ ;  $p = 0.08$ ).

When looking separately at the patients with B large cell lymphoma (as well as Burkitt and lymphoblastic lymphomas) the only significant prognostic factor was the presence of aneuploidy ( $p = 0.008$ ;  $r = 0.27$ ;  $\text{ExB} = 5.45$ ), whereas all other variables showed  $p$  values above 0.2.

Applying a cluster analysis with the Ward algorithm to “mean AgNORs/nucleus”, the dendrogram obtained suggested a separation into 2 groups: group 1 with AgNOR values lower than 1.93 and group 2 with AgNOR values above 2.18 suggesting a cut-point value of about 2.05. In the log-rank test, the difference of survival between the two groups was statistically significant ( $p = 0.02$ ) (Fig. 5). The dendrogram of a cluster analysis of the S-phase values also suggested the creation of two clusters, but in the log-rank test it was not possible to demonstrate significant differences between the two groups. When creating three or more groups, the number of patients in some of them was as low as three. Therefore no cut-off-point was suggested for SPF. The Cox-regression showed that there is a continuously increasing risk for the patients with increasing S-phase values.

Looking at all other lymphomas together, SPF ( $p = 0.006$ ) and all three AgNOR parameters (“% cells with AgNOR clusters” = 0.022; “mean AgNORs/nucleus” = 0.0068; “mean AgNOR clusters/nucleus” = 0.020) as well as the grade of malignancy ( $p = 0.0068$ ) were significant in univariate analysis, but not ploidy ( $p > 0.2$ ). In the multivariate Cox-regression only the SPF remained as prognostic factor ( $p = 0.005$ ;  $r = 0.35$ ;  $\text{ExB} = 1.094$ ), even when stratified for DNA-ploidy.

#### 4. Discussion

We examined the influence of clinical features, stage as well as proliferation markers on survival in patients with NHLs. Previous works have shown that the proliferation rate might be an important prognostic marker for these neoplasias [2,3,5,11,16,25,32,33]. Nevertheless, this parameter has never been included in prognostic scores [35,37]. When IPI was introduced, LDH levels, considered as an indirect marker of tumour cell turnover, were included in a categorical way (normal vs. increased) into the score system. In the present study, however, no significant influence on survival was found for neither for LDH levels nor for the IPI score.

There is much debate on the importance of clinical and cell kinetic features for survival in NHLs [5, 11,26,34]. Our study shows, however, that there is a difference between looking for prognostic parameters valid for all cases together, or when focusing on different subtypes. When examining all lymphomas together, the proliferation rate measured by the SPF was the most important predictor of survival.

The concept of “grade of malignancy” had been introduced by the Kiel classification, based on type of the tumour cells as well as the mitosis rate. This is no longer used by the WHO classification [10] as each entity was considered to have its own biological behaviour. In our study, the WHO classification itself had borderline prognostic impact only in the univariate analysis. However, in the multivariate Cox model, only parameters of cell kinetics were important. Cluster analysis permitted the creation of two subgroups for “mean total number of AgNORs per cell” (mean AgNORs) with a cut-point of about 2.05. Yet this was not possible for the variable S-phase. This can be explained by the fact, that the S-phase values are more homogeneously distributed over the whole range without any clear-cut formation of clusters, whereas the “mean AgNORs” were mainly concentrated in two

groups, however not overlapping low and high grade NHLs. Therefore, “grade of malignancy” as a general concept was no more important. Recently, treatment strategies have been developed specifically for each WHO entity, rather than for groups of low or high grade NHLs.

Standardization of the AgNOR technique is surrounded by many controversies. However, it is the only technique that is able to provide an estimate of the cell duplication rate, by measuring the silver-stained area per nucleus [4,14]. Yet counting the total number of silver stained spots per nucleus has been more frequently used in the study of cell kinetics in neoplasias [13,14,20–23,26,27,38,39]. This parameter has shown a good correlation with SPF and its prognostic relevance has been established in a wide variety of tumours [1,13,14,20–23,26,27,30,38,39]. The AgNOR technique is also useful to discriminate cells in G0 from those in cell cycle [14–19,24]. This is especially useful in cases with a low SPF, where this parameter shows a low ability to discriminate the aggressiveness of the neoplasia.

Previous studies on the relation between the AgNOR technique and other cell kinetic parameters have shown that AgNOR clusters correspond to cells in the proliferative cell cycle [8,14,15,17,18]. It has also been shown in acute leukemias that SPF is best correlated with the number of AgNOR clusters/nucleus [14].

In the present study, AgNOR analysis was performed examining 3 parameters: percentage of cells with AgNOR “clusters” (% clusters), mean AgNOR “clusters” per cell (mean clusters) and mean total number of AgNORs per cell (mean AgNORs). NHLs with a low SPF presented few cells containing AgNOR clusters, mainly one. Together with an increase in SPF, the percentage of cells with AgNOR clusters rose and more than one cluster could be found. The exception was mantle cell lymphoma where a peculiar cell kinetic pattern could be disclosed by the AgNOR technique analysed together with the SPF. Although most cells presented AgNOR clusters, SPF was low, suggesting that cells enter the cell cycle, having therefore a proliferative potential. However, they are unable to complete it. Recent molecular studies have confirmed deregulation of the cell cycle machinery in mantle cell lymphoma [28,31]. This fact could lead to the high resistance to chemotherapy found in this NHL.

Each WHO category presented its own cell kinetic pattern with a variability within each entity. Although few overlap was found in SPF between follicular lymphoma and B large cell lymphoma, no cut-off value

could be suggested to separate low and high grade of malignity.

In the present study, the AgNOR parameter that showed the highest correlation with SPF as well as with survival was "mean total AgNORs/nucleus", as it has been found in solid tumours. Moreover, when looking at the  $r^2$  values in the Cox regression, this parameter correlated best with the survival curve. The parameter "percentage of cells with AgNOR clusters" that is important in chronic lymphocytic leukemia [15, 17–19], was less useful in NHLs.

When examining all cases together, the AgNOR pattern did not enter the final multivariate Cox-model. But when stratifying for DNA-ploidy, the mean number of AgNORs/nucleus was the only variable remaining in the final multivariate model. The same result was obtained when examining separately DNA-diploid NHLs. This finding clearly indicates that the number of silver precipitations per nucleus is a result of both proliferative activity and DNA-ploidy. Similar results have already been shown for lymphomas or other tissues [6,7,12,23]. Therefore DNA-aneuploidy may interfere in the AgNOR analysis. In DNA-diploid cases, this AgNOR parameter could well substitute SPF. Therefore, the AgNOR approach, which is fast and cheap, and can be used as a surrogate for SPF, even in places where flow cytometry or image analysis are available. Nevertheless one must into account that DNA-aneuploidy may interfere in this result.

DNA-aneuploidy is a well known prognostic factor in solid tumours [1,7,27]. Its influence on prognosis of NHLs has also been subject to controversies [2,3,6,31]. Some studies associated DNA-aneuploidy with a shorter survival. Böcking et al. [2,3] using image-analysis, introduced a DNA-grade of malignancy based on the variance of nuclear DNA-contents around the normal  $2c$  value. This parameter correlated with several clinical features and with survival in NHLs. However, Grace et al. [6] found no influence of the incidence and extent of DNA-aneuploidy on response to treatment in NHLs. A DNA-aneuploid clone could be found only in 20% of our patients, more frequently in large B cell lymphomas. In the whole group, ploidy was not a significant prognostic parameter. But when only patients with large B cell lymphomas, Burkitt and lymphoblastic lymphomas were considered, DNA-ploidy was the only prognostic parameter. This was not the case in the remaining patients, where SPF remained the most important prognostic factor.

In conclusion, our study shows the importance of the proliferation rate and the DNA-aneuploidy in patients with NHL, specially in aggressive and advanced cases, as has already been proposed [35]. The association of clinical and cell kinetic parameters has been proposed to evaluate the prognosis in many types of neoplasias [1,13,14,20–23,26,27,30,38,39]. This approach could also help to stratify treatment protocols for NHLs.

### Acknowledgements

Flavia P.S. Costa received a student's fellowship from FAPESP (Fund for Research Development, State of São Paulo). Prof. Dr. Irene Lorand-Metze and Prof. Dr. Konradin Metzger are researchers of the National Research Council (CNPq).

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