

## DNA-Ploidy - A Prognostic Factor of Acute Lymphoblastic Leukemia (ALL) in Childhood



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**Abstract :** A number of studies have shown that the DNA-ploidy of mononucleated blood cells is a prognostic factor of acute lymphoblastic leukaemia in childhood, patients with an hyperploid DNA index showing a significantly better response to chemotherapy than those with a euploid DNA index. The aim of the present study was to re-evaluate this question for a subset of patients in the German-Austrian-Swiss ALL-BFM study, treated between 1984 and 1996. The DNA index was flow cytometrically determined in mononucleated blood cells from the bone marrow and/or peripheral blood of 104 children with acute lymphoblastic leukaemia. High-risk patients receiving more intensive treatment were excluded from the study. The recurrence-free survival of patients with hyperploid and euploid tumours was analyzed according to Kaplan and Meier, and the results were compared with those for other possible prognostic factors. Data analysis, incl. multivariate analysis, showed that DNA ploidy was indeed of significant prognostic value, with a risk ratio similar the initial leukocyte count. The appropriate cut-off point between diploid/near-diploid and hyperdiploid cases seems to be a DNA index of 1.10 rather than the most widely used 1.16. Overall, we are of the opinion that future studies should continue to include the DNA ploidy as a possible prognostic factor for acute lymphoblastic leukaemia in childhood.

**Key words :** Childhood leukaemia, Predictive assay, Risk factor, Ploidy, Flow cytometry.

### Introduction

Improvement and intensification of cytostatic therapy for children with acute lymphoblastic leukaemia (ALL) have led to a significant increase in success rates over the last three decades. More than 80% of the patients are currently cured with the first course of chemotherapy (Kager and Evans, 2006). Part of the increase is due to risk adapted treatment, which allows for the selection of patients in need of a more aggressive therapy, thus keeping the overall rate of unwarranted side effects within acceptable limits. A number of prognostic factors have been identified so far, but the search goes on and there may be room for further individualisation.

Factors known for their unfavourable prognosis include age under 2 or above 10 years, haemoglobin concentration >10g/dl, thrombocyte count <100/nl, L2 or L3 morphology, and lymphadenopathia (Gaynon *et al.*, 1997; Moricke *et al.*, 2005). Before 1995, the ALL-BFM study group used a combination of initial lymphoblast count and liver and spleen size as a risk factor measuring tumour mass (Schrappe *et al.*, 1987). Thereafter, initial leukocyte count was considered more relevant (Schrappe *et al.*, 2000). Since 2000, therapy decisions are based mainly on the presence or absence of certain cytogenetic markers and their molecular equivalents, as well as an insufficient response

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to prednisone, which was introduced as an additional criterion as early as 1990 (Reiter *et al.*, 1994).

For 30 years now, ploidy is known as a prognostic factor in childhood leukaemia. Secker-Walker *et al.* (1978) used chromosomal analysis to classify patients as hyperdiploid or diploid, and found that relapse-free survival was significantly longer in the former than in the latter group (Secker *et al.*, 1978). Look *et al.* (1985) confirmed this result with flow cytometric measurements (Look *et al.*, 1985), as did later studies (Smets *et al.*, 1987; Trueworthy *et al.*, 1992). The aim of the present study was to re-evaluate this question for a subset of patients in the ALL-BFM study treated between 1984 and 1996,

## Patients and Methods

### Patients

Between January 1984 and July 1996, 159 children with acute lymphoblastic leukaemia (first diagnosis) were studied with respect to the DNA content of their leukaemic cells. The diagnosis was confirmed cytologically and immunologically. Samples of bone marrow and/or peripheral blood were obtained immediately before the start of chemotherapy. Treatment was carried out at the Department of Paediatric Oncology of the University Hospital in Essen Germany, according to the respective BFM guidelines (ALL-BFM 83: 13 patients, ALL-BFM 86: 25 patients, ALL-BFM 90: 45 patients, ALL-BFM 95: 21 patients). A risk factor (RF) was calculated from the initial blast counts as well as liver and spleen size. This was used as the main determinant of risk adapted treatment until 1995 (Schrappe *et al.*, 1987). Although later decisions were based on the initial leukocyte count (Schrappe *et al.*, 2000), continued use of the RF in 1995/1996 would have identified the same high-risk patients. From 1990 onwards, an insufficient initial response to Prednisone treatment served

as an additional criterion for adjustment (peripheral blast count  $\geq 1,000/\mu\text{l}$  on day 8 after start of the treatment) (Reiter *et al.*, 1994). 35 patients were categorized as belonging to the high risk group and therefore received more intensive treatment; they were excluded from the present study. For another 20 patients, the DNA content could not be reliably established so that they, too, had to be excluded. With 53 of the remaining 104 patients, it was possible to determine the DNA content of cells from both bone marrow and peripheral blood. As there was a good agreement between the results from these two kinds of analysis, we also included in our study data from children of whom we had only one or the other sample (34 with bone marrow only; 17 with peripheral blood only). The age of the patients ranged from 0.2 to 16.5 years (median 4 years). The follow-up was 0.2 to 14 years (median 6 years).

### Methods

Mononucleated cells were obtained by sucrose gradient centrifugation (Lymphodex, density 1.077-1.080 g/ml) of heparinized bone marrow or peripheral blood. The cells were washed twice in Tris buffer and fixed in 96% ethanol. For flow cytometric analysis, part of the cell suspension was centrifuged, nuclei were isolated in pepsin solution (0.5% in 0.55 N HCl, pH 1.8, 10 min, 37°C), treated with RNase (0.1% in 0.1 M Tris buffer, pH 7.4, 10 min, room temperature) and stained with ethidium bromide ( $2.5 \times 10^{-5}$  M in 0.1 M Tris, 0.1 M NaCl, pH 7.5). Red fluorescence after blue excitation was measured for 3000–5000 cells in an flow cytometer (ICP 22, Phywe, Germany). The DNA histograms were analysed with a computer program designed by Dr. O. Ahrens (Laboratory for Technical Measurement Consultation and Development, Bargteheide, Germany). The DNA-index was determined with the help of control lymphocytes from healthy adult donors. A DNA-index of  $<1.10$  was defined as diploid,

whereas an index of  $\approx 1.10$  was considered hyperdiploid.

### Statistics

Different groups of patients were compared using the Mantel-Haenzel chi square test. The recurrence-free survival was analyzed according to Kaplan and Meier, the influence of certain parameters was tested with the proportional hazard model of Cox, using the PHREG procedure in the SAS/STAT software.

### Results

Altogether, 18 of the 104 patients had a recurrence at the time of analysis. As is evident from Table 1, these were almost exclusively patients with high initial leukocyte count ( $\approx 20,000$  /nl) and a diploid DNA index.

A more detailed analysis of the data was carried out according to Kaplan-Meier. Results

are shown for two parameters (initial leukocyte count and ploidy) in Figures 1 (a) and (b). The event free survival did not differ significantly between male and female patients, but it did depend on initial leukocyte count and ploidy. Five-year event free survival is given in Table 2.

There were two patients with a low initial leukocyte count that had a recurrence; these patients both had a diploid DNA index. On the other hand, the one patient with a hyperdiploid DNA index who had a recurrence showed a high initial leukocyte count. Therefore, within the group with low initial leukocyte count as well as hyperdiploid DNA index there was not a single recurrence. However, as the group was even smaller than that of the patients with a hyperdiploid tumour (25 instead of 28), the level of significance was not improved with

**Table 1 : Comparison of relevant parameters in patients with and without recurrence. Note that the percentages (%) next to the absolute numbers of patients (n) always relate to the overall number of patients in that column (18 with, 86 without recurrence)**

	With Recurrence			Without Recurrence			p
	n	%	Mean $\pm$ Standard Deviation (Median)	n	%	Mean $\pm$ Standard Deviation (Median)	
Age (y)	18		5.5 $\pm$ 4.6 (3.5)	86		5.7 $\pm$ 4.1 (4.1)	
Male	13	72.20%		47	54.70%		
Female	5	27.80%		39	45.30%		
Initial Leukocyte Count /nl	18		84,220 $\pm$ 118,838 (48,050)	86		31,156 $\pm$ 53,990 (9,400)	p<0.0001
< 20,000 /nl	2	11.10%		55	64.00%		
? 20,000 /nl	16	88.90%		31	36.00%		
<i>Immunology:</i>							
n.d.	0	0.00%		4	4.70%		
pre-B-ALL	5	27.80%		17	19.80%		
c-ALL	10	55.70%		46	53.60%		
T-ALL	2	11.10%		5	5.80%		
null-ALL	1	5.60%		2	2.30%		
% G <sub>1</sub> -Phase	18		91.7 $\pm$ 5.2 (92.7)	86		91.6 $\pm$ 9.0 (93.9)	
% S-Phase	18		4.8 $\pm$ 4.6 (3.3)	86		4.1 $\pm$ 3.8 (2.7)	
% G <sub>2</sub> -Phase	18		3.3 $\pm$ 2.6 (2.6)	86		4.3 $\pm$ 8.3 (2.6)	
DNA Index	18		0.98 $\pm$ 0.08 (0.97)	86		1.04 $\pm$ 0.12 (1.00)	p=0.0075
diploid	17	94.40%		59	68.60%		
hyperdiploid	1	5.60%		27	31.40%		

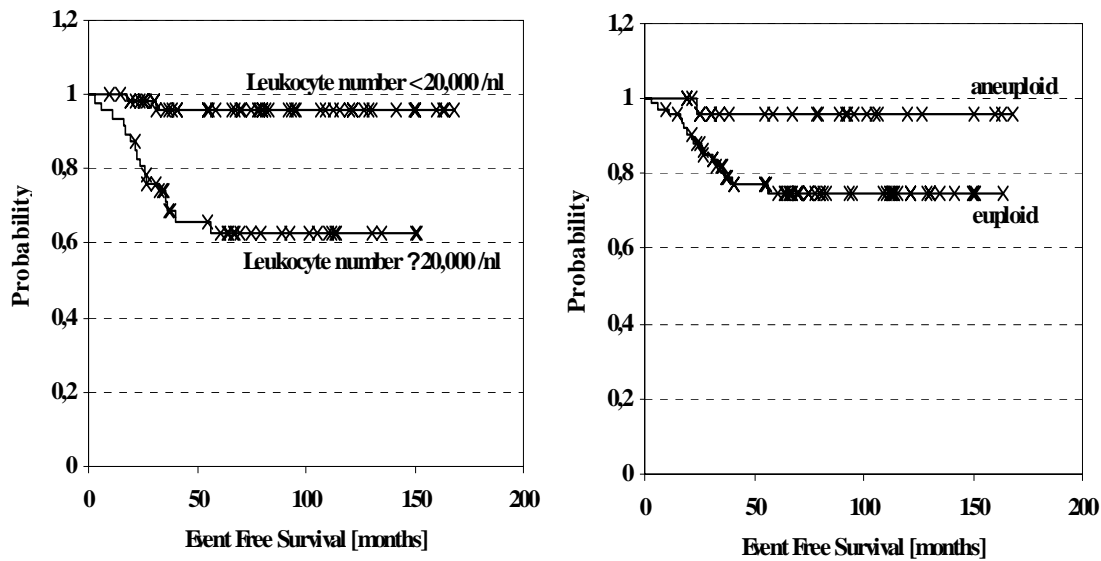


Fig. 1 : Kaplan-Meier plots for the event-free survival of ALL patients according initial leukocyte count (left) and ploidy (right)

Table 2 : Comparison of five-year event free survival for different groups of patients

	n	EFS		n	EFS	
Leukocytes < 20,000 /nl	57	0.96 + 0.03	Leukocytes ≥ 20 000 /nl	47	0.63 + 0.08	p<0.0001
hyperdiploid	76	0.96 + 0.04	diploid	28	0.75 + 0.05	p=0.035

the two criteria combined (p=0.037 instead of p=0.035).

The initial leukocyte count did not seem to be completely independent of ploidy. It was  $47,014 \pm 79,212$  for patients with a diploid tumour and  $19,103 \pm 30,035$  for patients with a hyperdiploid tumour, the difference being highly significant (p=0.0051).

Multivariate analysis confirmed, nevertheless, that initial leukocyte count and ploidy were both of prognostic value, and among all the parameters considered they were the only ones. Age, sex, immune status, and S- or G<sub>2</sub>-fraction did not reach significance. The analysis of maximum likelihood estimates showed that risk ratio was higher for ploidy (RR=6.9) than for the initial leukocyte count (RR=4.4).

### Discussion

Our study, based on a subset of patients in the ALL-BFM study treated between 1984 and 1996, is in line with evidence presented by others that ploidy predicts chemotherapy response in children with acute lymphoblastic leukaemia. It significantly correlates with relapse-free survival, the risk ratio being similar to that observed with other prognostic factors used at the time, in particular the initial leukocyte count.

It has been suggested by Look *et al.* (1985) that the cut-off between diploid/near-diploid and hyperdiploid cases should not be set at a DNA-index of 1.10 as done here, but rather at 1.16 (Look *et al.*, 1985). Others have followed this suggestion (Smets *et al.*, 1995;

Coustan *et al.*, 2006). Among our patients there were 7 with a DNA index between 1.10 and 1.16. They all had clearly distinguishable hyperdiploid cell lines. No recurrence was observed in any of these patients (median follow-up 78 months). It seems to us, therefore, that it is not unreasonable to include them with the hyperdiploid group. At least one chromosomal study has proposed, however, that the prognosis of patients with 51 – 55 chromosomes (roughly equivalent to a DNA-index between 1.12 and 1.20) may be worse than that of patients with > 56 chromosomes (Raimondi *et al.*, 1992). Further clarification is obviously needed here.

In general, chromosomal studies have come to the same conclusion as we have, namely that for diploid /near-diploid cases of acute lymphoblastic leukaemia prognosis is worse than for hyperdiploid cases (Secker *et al.*, 1978; Dastugue *et al.*, 1992; Raimondi *et al.*, 1996). An advantage of karyotype analysis of course is that it can further distinguish between diploidy and pseudo-diploidy. The latter seems to be a prognostic factor in its own right, pointing to the importance of chromosomal translocations (Hyakuna *et al.*, 2000; Heerema *et al.*, 2000).

Chromosomal studies do of course provide more detailed data than flow cytometric analysis, but they are very tedious and not always easy to integrate into the daily routine of a paediatric clinic. We are therefore of the opinion that there is still a place for flow cytometric analysis of DNA ploidy, especially as the question of the most appropriate cut-off between diploid/near-diploid and hyperdiploid cases seems to be unsettled.

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