Hospital for Children and Adolescents
University of Helsinki
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PROGNOSTIC FACTORS

IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

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ACADEMIC DISSERTATION

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1. Summary

The prognosis of children with acute lymphoblastic leukemia (ALL) has improved remarkably during the past four decades. The main reasons for this development are intensive multiagent chemotherapy and effective prophylactic treatment for central nervous system leukemia. Risk-adjusted therapy according to recognized prognostic factors has also played an important role. The aim of this study was to find and characterize new prognostic factors and at the same time to analyze the value of established factors in the setting of contemporary intensive therapy.

Our study population comprised several cohorts of Finnish children with ALL from 1975 to 2000. The studies of morphology (n=251) and P-glycoprotein (P-gp, n=118) were population-based. In the biological tempo and *TEL-AML1* study, the cohort from 1975-81 (n=100) was from the Children's Hospital, University of Helsinki, but the cohort from 1989-1991 (n=102) covered the whole country. The patients (n=79) in the comparative genomic hybridization (CGH) study were from three oncology centers: Helsinki, Kuopio, and Tampere. The minimal residual disease (MRD) study was a single center project (n=41). Blast cell morphology and early response to treatment were studied blindly in a centralized fashion. P-gp expression was detected by flow cytometry with the monoclonal antibody JSB1. *TEL-AML1* expression was investigated with fluorescent *in situ* hybridization (FISH), and the changes in DNA copy number with CGH. In the MRD study metaphase FISH was used. The main statistical methods used were Kaplan-Meier analysis with the log-rank test for survival analysis, and the Mann-Whitney U test and chi-square test for comparing the different study groups.

In the group of children with white blood cell count (WBC) $<50x10^9$/L, L2 morphology was associated with an unfavorable outcome. P-gp expression was associated with T-lineage ALL, but not with other unfavorable features. Nor was it a prognostic factor for early response to treatment or for the ultimate outcome.

According to our study, children with "very-low-risk" ALL (WBC $<10x10^9$/L, Hemoglobin <90g/L, age 2 to <10 years) already had a favorable outcome, with event-free survival (EFS) of 76% in the 1970s, before anthracyclines were employed in their treatment. *TEL-AML1* fusion was more common in the very-low-risk group than in the other patients. The fusion was associated with a better outcome in the whole study population and especially in the non-low-risk subgroup.

CGH, when added to standard G-banding, increased the number of patients with detectable genetic aberrations. Notably, the frequency of losses at 12p was higher than previously described, nine of 79 patients. This loss was associated with favorable clinical and laboratory features, as well as with the *TEL-AML1* fusion.

The main finding in the MRD study was that, of 41 children, 11 had detectable leukemic cells in the bone marrow at some point post induction, and, in five cases, even after completion of therapy. In spite of the persistence of the malignant clone, nine of these children remain in continuous complete remission (CCR) and none of the five who were still positive after completion of therapy has experienced a relapse.

2. List of original publications

This thesis is based on the following articles, referred to in the text by Roman numerals

I Kanerva J, Saarinen-Pihkala UM, Riikonen P, Mäkipernaa A, Möttönen M, Salmi TT. Reemphasis on lymphoblast L2 morphology as a poor prognostic factor in childhood acute lymphoblastic leukemia. Med Pediatr Oncol 33: 388-394, 1999

II Kanerva J, Tiirikainen M, Mäkipernaa A, Riikonen P, Möttönen M, Salmi TT, Krusius T, Saarinen-Pihkala UM. Multiple drug resistance mediated by P-glycoprotein is not a major factor in a slow response to therapy in childhood ALL. Pediatr Hematol Oncol 15: 11-21, 1998

III Kanerva J, Tiirikainen MI, Mäkipernaa A, Riikonen P, Möttönen M, Salmi TT, Krusius T, Saarinen-Pihkala UM. Initial P-glycoprotein expression in childhood acute lymphoblastic leukemia: no evidence of prognostic impact in follow-up. Pediatr Hematol Oncol 18: 27-36, 2001

IV Kanerva J, Saarinen-Pihkala UM, Niini T, Riikonen P, Möttönen M, Mäkipernaa A, Salmi TT, Vettenranta K, Knuutila S. Favorable outcome in 20-year follow-up of children with very-low-risk ALL and minimal standard therapy, with special reference to *TEL-AML1* fusion. Submitted

V Kanerva J, Niini T, Vettenranta K, Riikonen P, Mäkipernaa A, Karhu R, Knuutila S, Saarinen-Pihkala UM. Loss at 12p detected by comparative genomic hybridization (CGH): Association with *TEL-AML1* fusion and favorable prognostic features in childhood acute lymphoblastic leukemia (ALL). A multi-institutional study. Med Pediatr Oncol 37: 419-425, 2001

VI Kanerva J, Vettenranta K, Autio K, Knuutila S, Saarinen-Pihkala UM: Minimal residual disease by metaphase FISH in the marrow of children with ALL: clonal cells during or after chemotherapy may not predict relapse. Leukemia Research (in press)

3. Abbreviations

AIEOP	Italian Association of Pediatric Hematology and Oncology
ALL	acute lymphoblastic leukemia
AML	acute myelogenous leukemia
ARA-C	cytarabine
BFM	Berlin-Frankfurt-Münster
BM	bone marrow
BMT	bone marrow transplantation
CCG	Children's Cancer Group
CCR	continuous complete remission
CD	cluster of differentiation
CGH	comparative genomic hybridization
CLCG	Children Leukemia Cooperative Group
CML	chronic myelogenous leukemia
CNS	central nervous system
COALL	Co-operative study group for childhood acute lymphoblastic leukemia
CR	complete remission
DAPI	4', 6-diamidino-2-phenylindole-dihydrochloride
DCLSG	Dutch Childhood Leukemia Study Group
DFCI	Dana-Farber Cancer Institute
EFS	event-free survival
EGIL	European group for the immunological characterization of leukemias
EORTC	European Organisation for Research and Treatment of Cancer
FAB	French-American-British
FC	flow cytometry
FISH	fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate
GST	glutathione-S-transferase
Hb	hemoglobin
HD	high dose
HR	high risk
IR	intermediate risk
iv	intravenous
L-asp	L-asparaginase
LRP	lung resistance protein
MDR	multiple drug resistance
MLL	mixed lineage leukemia
MP	mercaptopurine
MRC	Medical Research Council
MRD	minimal residual disease
MRP	multiple drug resistance related protein
MTX	methotrexate
NOPHO	Nordic Society of Pediatric Hematology and Oncology
OS	overall survival
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
POG	Pediatric Oncology Group
SD	standard deviation
SJCRH	St Jude Children's Research Hospital
SR	standard risk
TCCSG	Tokyo Children's Cancer Study Group
TdT	terminal deoxynucleotidyl transferase
VCR	vincristine
vs.	versus
WBC	white blood cell count

4. Introduction

The first attempts to treat childhood acute lymphoblastic leukemia (ALL) with chemotherapeutic agents were made in the 1940s. With combination chemotherapy, permanent cures for more than single exceptional patients became possible in the 1960s. It was also realized that certain clinical features were associated with an inferior outcome, while other features were more common in survivors. These findings have lead to the concept of risk-adapted therapy according to the prognostic factors: children at high risk of relapse receive more intensive treatment than those at lower risk.

With the improved treatment results and universal intensification of therapy, some of the earlier prognostic factors have lost their significance as predictors of treatment outcome. At the same time, developments in laboratory methods (immunophenotyping, cytogenetics, and molecular cytogenetics) have enabled the employment of new factors for prognostic classification. These include specific immunophenotypic subgroups, genetic aberrations, and monitoring of the treatment response by molecular methods (=minimal residual disease). However, the treatment itself is the most important factor influencing the outcome of children with ALL.

The aim of this study was to evaluate potential new prognostic factors in childhood ALL. The value of established factors was also investigated in the context of current intensive therapies. Blast cell morphology did not have prognostic significance in the patient population as a whole, but, in the group with a white blood cell count (WBC) below $50x10^9$/L, L2 morphology was associated with an inferior outcome. *TEL-AML1* fusion and DNA loss in the short arm of chromosome 12 were associated with a favorable outcome. After completion of therapy, minimal residual disease (MRD) was detected by metaphase fluorescent *in situ* hybridization (FISH) in some patients who were in clinical and hematological long-term remission. The outcome of very-low-risk ALL was already good in the 1970s and, despite intensified treatment, has not improved substantially in the last 20 years.

5. Review of the literature

5.1. Short overview of changes in the prognosis and treatment of ALL through six decades

The history of drug therapy for childhood ALL dates back to the 1940s, when the first agents for cancer chemotherapy became available. In 1948, Farber and co-workers described temporary remissions in childhood acute leukemia treated with a folic acid antagonist, aminopterin (Farber et al. 1948). In the next decade (the 1950s), investigators realized that it was also possible to induce remission with steroids (Pearson and Eliel 1950). However, with both of these treatments, lasting remissions were quite exceptional.

In the 1960s, the idea of combining several chemotherapeutic agents with different mechanisms of action (although the mechanisms are still not entirely clear) totally changed the long-term prognosis of childhood leukemia, especially that of ALL. Induction therapy with vincristine (VCR), prednisone, and L-asparaginase, followed by maintenance therapy with 6-mercaptopurine (6-MP) and methotrexate (MTX) in children with ALL made permanent cure possible. With this approach, roughly 35% of children had a five-year survival by the end of the1960s (Pinkel et al. 1972, Baum et al. 1983, Baum et al. 1979). Prophylactic treatment for central nervous system (CNS) leukemia, started in the 1970s, produced further improvements in outcome. Cranial or craniospinal irradiation and intrathecal methotrexate (either alone or combined with other agents) improved the survival rates remarkably (Nesbit et al. 1981). Employment of systemic high-dose (HD) methotrexate therapy still further improved the outcome (Gustafsson et al. 2000).

In the 1980s, the delayed intensification phase (second induction) with multi-agent chemotherapy proved effective in preventing relapses (Reiter et al. 1994, Tubergen et al. 1993). In the treatment of relapses, allogeneic stem cell transplantation has provided a chance of cure for a number of children (Barrett et al. 1994). In the 90s, even unrelated donors of stem cells have been used with good results in children with ALL (Lausen et al. 1998, Saarinen-Pihkala et al. 2001). In carefully selected cases, bone marrow transplantation (BMT) in first remission is regarded as the treatment of choice. These patients include children with Philadelphia-chromosome-positive ALL, and those with a slow or a poor response to induction therapy (Forman et al. 1987, Saarinen et al. 1996).

In the treatment of childhood ALL, the novel antibody-based or other targeted therapeutic modalities are not yet widely adopted in clinical practice.

5.2. Prognostic factors

Childhood ALL is not a single disease, but a group of diseases with a variety of genetic aberrations in the leukemic blasts, leading to a wide range of clinical presentations and outcomes. The first features used in risk classification were the white blood cell count (=leukemic burden) at diagnosis and the age of the patient. These features are still included in the modern risk assessment. In the future, WBC and age may possibly be replaced by immunologic and genetic markers of the disease.

Great efforts have been made to define uniform prognostic criteria for childhood ALL. These criteria are essential when comparing the results of different treatment regimens in different study groups. The Rome Workshop in 1985 was the first serious attempt to make recommendations for categorizing ALL (Mastrangelo et al. 1986). The patient population was divided into two groups, standard risk (SR) and high risk (HR), according to the WBC and the age at diagnosis. The other aims of the Workshop were to create guidelines for data collection and statistical analysis. A follow-up workshop by the US National Cancer Institute (NCI) in 1993 agreed with the Rome risk grouping and did not include other elements in the risk classification (Table 1) (Smith et al. 1996).

Table 1. Rome/NCI risk classification of childhood ALL.

Risk	Definition
Standard	WBC < $50x10^9$/L and age 1 - <10 years
High	WBC ≥ $50x10^9$/L or ≥ 10 years

In recently published studies from different groups, various methods of risk grouping have been employed. The basis is still age and WBC, but in many protocols, important roles in the classification are played by extramedullary leukemia, immunophenotype, cytogenetics, and response to treatment. Several groups have adopted a calculated risk factor [RF = 0.2 x log (number of blasts/µL + 1) + 0.06 x liver size + 0.04 x spleen size (cm below costal margin)] suggested by the Berlin-Frankfurt-Münster (BFM) group (Schrappe et al. 2000b). The Medical Research Council (MRC) in the United Kingdom uses the Oxford hazard score (0.22 x log (WBC+1) + 0.0043 x age^2 – 0.39 x sex; boy =1, girl = 2) (Chessells et al. 1995).

White blood cell count (WBC)

The WBC at diagnosis is a crucial variable for describing the nature of the patient's leukemia and especially the tumor burden. The other measures of the tumor burden are the size of a mediastinal mass, hepatosplenomegaly, and enlargement of lymph nodes. Children with WBC of more than $50x10^9$/L are commonly considered to be at high risk of relapse and receive intensive treatment. In the Nordic material, the 5-year event-free survival (EFS) for children with WBC 50-$100x10^9$/L is 67.3%. The EFS continues to decline with the elevation of the lower cut-off point to WBC $100x10^9$/L (41.3%) or $200x10^9$/L (30.2%) (Gustafsson et al. 2000). Similar findings

are the rule in reports from various study groups. The cut-off point between the low- and high-risk groups differs in relation to the treatment protocol and its intensity. Some groups include children with WBC >25x10^9/L in the HR group (St Jude Children's Research Hospital, SJCRH; Co-operative Study Group for Childhood Acute Lymphoblastic Leukemia, COALL; Tokyo Children's Cancer Study Group, TCCSG), while others employ WBC >50x10^9/L (Nordic Society of Pediatric Hematology and Oncology, NOPHO; Children's Cancer Group, CCG) or 100x10^9/L (Pediatric Oncology Group, POG; Dana-Farber Cancer Institute, DFCI) as the criterion for intensive HR treatment. The cytogenetic features are closely linked to the WBC and at least partly explain the prognostic impact of WBC, although there is evidence that children with similar cytogenetic aberrations may have very different WBCs, and their prognostic value is related partly to the WBC and partly to the response to treatment (Pui and Evans 1998, Ribeiro et al. 1997, Schrappe et al. 1998).

The tumor burden of the leukemia is also a marker of its biological characteristics. Children with high WBC at the first presentation have a "rapid disease" with a high proliferation rate of the leukemic blasts. These patients often have near normal hemoglobin and platelet levels. In contrast, children with low WBC at diagnosis may have low hemoglobin and platelet levels, reflecting the slow development of their disease, which has for a long time interfered with the production of normal precursors of blood cells (Hirt et al. 1997a, Hirt et al. 1997b, Pyesmany et al. 1999). No thorough clinical evaluation of the features of "slow disease" is available.

Age

Infants and adolescents still have a less favorable prognosis than children between 1 and 10 years of age. However, contemporary intensive treatment regimens have decreased the effect of age. In the Nordic series (Gustafsson et al. 2000) infants fare worse than older children, but the difference between adolescents (>10 years of age) and other children older than 1 year is very slight. The inferior prognosis for infants is due, to a large extent, to typical cytogenetic aberrations, as 70-80% of infants have rearrangements of the mixed lineage leukemia (MLL) gene (Pui et al. 1995, Pui and Evans 1998). The cytogenetic and morphologic distribution of adult leukemia differs significantly from childhood ALL, which appears to be one of the main reasons for the better outcome of children. However, even with the same genetic aberrations, adults have an inferior outcome, which is at least partly due to their greater susceptibility to the toxic complications of therapy (Pui and Evans 1998). In the MRC UKALL X and Xa trials, age was an independent prognostic factor in a mixed population of children (excluding infants under 1 year of age) and adults (Chessells et al. 1998). To summarize, at least children over 10 years of age benefit from intensification of treatment (Chessells 2000). In the BFM-90 protocol, children aged 1-9 years had a favorable outcome (Schrappe et al. 2000a). The CCG also had a worse prognosis in children aged 10 or more years as compared with younger non-infants (Gaynon et al. 2000). The same trend has been shown in studies performed by many major co-operative study groups (POG, MRC, SJCRH, Italian Association of Pediatric Hematology and Oncology (AIEOP), Dutch Childhood Leukemia Study Group (DCLSG), COALL, Children Leukemia Cooperative Group (CLCG-EORTC) and TCCSG). These findings may be interpreted to mean that employment of the relatively intensive intermediate-risk (IR) protocol for children over 10 years of age by the NOPHO group has benefited these patients (Gustafsson et al. 2000).

Infant ALL is associated with a high WBC, hepatosplenomegaly, and CNS involvement. CD10 negativity and co-expression of myeloid markers are also common. Approximately 75% of the patients have the ALL1/MLL/HRX gene rearrangement (Biondi et al. 2000). The BFM group analyzed the outcome of 106 infants in three consecutive studies (BFM 83, 86, and 90) (Dordelmann et al. 1999). Half of the patients harbored the 11q23 rearrangement, which was associated with an inferior outcome. However, even in this infant population, the strongest prognostic factor turned out to be the poor response to prednisone, the other important unfavorable factors being age < 6 months, WBC $\geq 50x10^9$/L, and a pro-B immunophenotype without CD10. The findings concerning prognostic factors were very similar in the two CCG studies (CCG-107 and CG-1883) reported in 1999 (Reaman et al. 1999). Independent adverse prognostic factors were age <3 months, WBC $>50x10^9$/L, CD10 negativity, a slow induction response, and t(4;11). There are also substantial differences in pharmacokinetics between infants and older children (Biondi et al. 2000).

Sex

In earlier decades the patient's gender was a clear prognostic feature in childhood ALL. With the intensification of therapy the impact has decreased, but has not totally disappeared. Pui and co-workers (Pui et al. 1999) describe the changes in their institution during the past three decades. In the 60s, the difference in survival was more pronounced (10-year EFS for girls and boys 43.1% vs. 31.5%, respectively), but during their last completed study XIIIA (1991-1994) there was still a gender difference (84% vs. 71%) (Pui et al. 2000). This finding is partly explained by immunophenotype and DNA index; T-lineage ALL was more common in the boys and a favorable DNA index in the girls. In a POG study of precursor-B ALL patients from 1986-1994 (Shuster et al. 1998), boys also fared worse than girls, and the other prognostic factors did not differ between boys and girls. The gender difference was most pronounced in the group with WBC between 10 and 50. In our Nordic patients, the difference in the outcome between boys and girls has virtually disappeared with intensification of treatment (Gustafsson et al. 1998). During 1981-1986, the EFS for boys was 48% vs. 59% for girls, and with the contemporary protocols started in 1992 the EFS is 76% vs. 79%, respectively. However, in the recently published results of the BFM-90 study, female gender was associated with a favorable outcome (EFS 82% vs. 75%) (Schrappe et al. 2000a). In the United Kingdom, the MRC UKALL trials from the 70s to 1990 were analyzed, and even the results of the latest protocols show a prognostic advantage of female gender (Chessells et al. 1995). The negative impact of male gender is also evident in the trials conducted by the AIEOP, DCLSG and COALL groups.

In some treatment protocols, gender has influenced the treatment stratification. CCG-1881 for good risk, CCG-1891 for intermediate risk, and CCG-1901 and 1882 for high-risk patients employed a 2-year maintenance for girls and 3 years for boys (Nachman et al. 1998b, Lange et al. 1997, Hutchinson et al. 1996). In risk classification, the ALL97 protocol in the UK used the Oxford hazard score, which includes gender (Hann et al. 2000). DFCI 91-01 treated the SR boys with 18 Gy cranial irradiation, while the girls received intrathecal methotrexate + cytarabine instead (Silverman et al. 2001).

Immunophenotype

Immunophenotyping of leukemias has been possible since the 1970s, and at present, the diagnosis of ALL depends on immunophenotyping. Roughly 85% of children with ALL show the B-lineage phenotype, and the remaining 15% have the T-lineage. Such myeloid markers as CD13, CD33, and CD65 may be expressed on ALL blasts (Pui et al. 1993).

B-lineage ALL, especially, has been classified in different ways by various study groups. In the EGIL (European group for the immunological characterization of leukemias) classification (Bene et al. 1995), at least two of the three markers (CD19, CD79a, CD22) are required to be positive for the B-lineage, which is furthermore divided into four subgroups in order of maturation. Pro-B ALL has no expression of other B-lineage antigens, common ALL is CD10-positive, pre-B ALL has cytoplasmic IgM, and mature B-ALL has cytoplasmic or surface kappa or lambda. Pro-B ALL is also called pre-pre ALL in some classifications (Rothe and Schmitz 1996). The term B-cell precursor ALL is commonly used for the B-lineage without surface immunoglobulin expression (Margolin and Poplack 1997). However, Jennings and co-workers (Jennings and Foon 1997) use the term B-precursor ALL for the B-lineage without cytoplasmic IgM. Pui and co-workers (Pui et al. 1993) call this same immunophenotype "early pre-B ALL".

B-lineage ALL has been considered to have a more favorable prognosis than T-ALL. There are some differences in MTX metabolism between T-lineage and B-lineage ALL (Zhang et al. 1998). There is less synthesis of mtx-polyglutamates in T-ALL because of low folylpolyglutamate synthetase (FPGS) activity (Rots et al. 1999b). Lower dihydrofolate reductase (DHFR) levels also help to explain the better sensitivity of B-lineage ALL to MTX (Galpin et al. 1997). However, with intensification of therapy, the outcome of children with T-ALL is today almost equal to that of the B-lineage (Gaynon et al. 2000, Reiter et al. 1994). It has also become evident that the NCI/Rome risk criteria (Mastrangelo et al. 1986, Smith et al. 1996) are not valid in T-ALL. Many study groups subject those with T-ALL to more intensive therapy (NOPHO, SJCRH, BFM, COALL, TCCSG, AIEOP, DFCI). POG employs separate protocols for T-ALL.
Recent results indicating poor prognostic impact of T-ALL come from BFM-90, SJCRH, NOPHO, AIEOP, and DCLSG. In the SJCRH series, boys were more likely than girls to have T-ALL (20.9% vs. 10.7%) (Pui et al. 1999).

Mature B-cell leukemia is an aggressive disease with a high rate of proliferation, and previously it had a dismal prognosis. However, the introduction of brief treatment with high intensity regimens has improved the outcome substantially (Reiter et al. 1999, Reiter et al. 1992, Patte et al. 2001).

In several studies, mixed lineage ALL has been associated with a poor outcome (Fink et al. 1993a, Fink et al. 1993b, Wiersma et al. 1991), but recent reports suggest that no treatment modifications should be based on myeloid antigen co-expression. Only patients with CD2 and CD7 expression combined with myeloperoxidase may possibly benefit from treatment directed toward both lineages (Pui et al. 1993, Pui et al. 1998b, Pui et al. 1991, Uckun et al. 1997). There is also an association between *TEL-AML1* fusion and myeloid antigen expression, and these patients appear to have a favorable prognosis (Baruchel et al. 1997).

It is not easy to decide exactly, when a patient with ALL has a leukemia with truly mixed lineage. Several proposals have been made, based on the fluorescence intensity of myeloid markers and the percentage of myeloid antigen-positive cells. EGIL has established a scoring system for biphenotypic acute leukemias. A marker is considered positive if 20% or more cells are stained with the monoclonal antibody. Because of their high specificity, 10% is considered enough for anti-myeloperoxidase, CD3, CD79a, and terminal deoxynucleotidyl transferase (TdT) (Table 2) (Bene et al. 1995).

Table 2. Scoring system for the definition of biphenotypic acute leukemias (BAL)[a].

Points[b]	B-lineage	T-lineage	Myeloid lineage
2	CD79a cyt[c] IgM cyt CD22	CD3 (cyt/m)[d] anti-TCRα/β[e] anti-TCRγ/δ	anti-MPO[f] (anti-lysozyme)[g]
1	CD19 CD10 CD20	CD2 CD5 CD8 CD10	CD13 CD33 CDw65
0.5	TdT CD24	TdT CD7 CD1a	CD14 CD15 CD64 CD117

[a]BAL is defined when scores are over 2 points for the myeloid lineage and for one of the two lymphoid lineages. [b]Each marker scores the corresponding point. [c]cyt = cytoplasmic. [d]m = membrane. [e]TCR = T-cell receptor. [f]MPO = myeloperoxidase. [g]Specificity being assessed

There are major technical problems in measuring antigen expression. According to the sources of the reagents and the fluorochromes, the results may differ significantly (Howard et al. 1994).

Although the immunophenotype of leukemic blasts does not appear to have a very strong prognostic value in the setting of current therapy, the leukemia specific immunophenotype enables monitoring of MRD by flow cytometry (FC) with high sensitivity of detection.

Cytogenetics and molecular genetics

Karyotyping and G-banding of bone marrow (BM) samples has been possible since 1970 (Yunis and Sanchez 1973, Sanchez et al. 1973). Various structural and numerical abnormalities have been used for risk classification of childhood ALL. With the development of technology, characterization of the leukemic clone has reached the molecular level (Martinez-Climent 1997). Characterization of the molecular genetic abnormalities associated with leukemia has also increased our understanding of the pathogenesis and possible treatment options (Pui 2000). One important mechanism is a translocation involving antigen receptor genes, as in the t(8;14), when MYC and immunoglobulin gene enhancer genes fuse, leading to errors in the regulation of MYC transcription (Kersey 1997). Many of the aberrations in childhood ALL deregulate signal transduction pathways and transcription of genes critical to cell proliferation and differentiation (Kersey 1997). In many cases, no structural aberrations can be detected with the current methodology, but the number of chromosomes in the leukemic cells is abnormal. Hyperdiploidy is a more common finding than hypodiploidy. (Table 3)

Table 3. Recurrent numerical and structural chromosomal aberrations in childhood ALL (Ma et al. 1999).

Aberrations		Fusion genes	Lineage	Frequency %
Numerical changes	Hypodiploidy			7
	Diploidy			8
	Pseudodiploidy			42
	Hyperdiploidy 47-50			15
	Hyperdiploidy >50			27
	Triploidy or tetraploidy			1
Translocations	t(12;21)(p13;q22)	*TEL/AML1*	B	20-25
	t(9;22)(q34;q11)	*BCR-ABL*	B	4
	t(1;19)(q23;p13)	*PBX1/E2A*	B	5
	t(17;19)(q22;p13)	*HLF/E2A*	B	<1
	t(4;11)(q21;q23)	*MLL-AF4*	B	3
	t(11;19)(q23;13.3)	*MLL/ENL*	B	<1
	t(8;14)(q24;q32)	*myc-IgH*	B (mature)	2-5
	t(8;22)(q24;q11)	*myc-IgL*	B (mature)	<1
	t(2;8)(p12;q24)	*myc-IgK*	B (mature)	<1
	t(1;14)(p32;q11)	*TAL1/TCRδ*	T	<1
	t(1;7)(p32;q34)	*TAL1/TCRβ*	T	<1
	t(1;7)(p34;q34)	*LCK/TCRβ*	T	<1
	t(7;9)(q34;q32)	*TAL2/TCRβ*	T	<1
	t(7;9)(q34;q34)	*TAN1/TCRβ*	T	<1
	t(8;14)(q24;q11)	*myc-TCRα/δ*	T	<1
	t(11;14)(p15;q11)	*LMO1/TCRδ*	T	<1
	t(11;14)(p13;q11)	*LMO2/TCRδ*	T	<1
	t(7;10)(q24;q24)	*HOX11/TCRβ*	T	<1
	t(7;11)(q34;p13)	*RHOM2/TCRδ*	T	<1
	t(7;19)(q34;p13)	*TCRB/LYL1*	T	<1
	t(10;14)(q24;q11)	*HOX11/TCRδ*	T	<1

In the 1980s, it was discovered that patients with hyperdiploid ALL have a favorable outcome (Look et al. 1985). One explanation for this finding is the increased accumulation of MTX polyglutamates in hyperdiploid blasts (Whitehead et al. 1992, Belkov et al. 1999). In studies by CCG and POG, trisomies of chromosomes 4, 10, 17, and 18 have been linked with a favorable prognosis (Harris et al. 1992, Jackson et al. 1990). A hypodiploid karyotype, conversely, predicts a poor prognosis, and children with a near-haploid karyotype are at especially high risk of relapse, and should therefore be considered as candidates for BMT in first complete remission (CR) (Martinez-Climent 1997).

With current methodology, at least 90% of children have detectable numerical or structural chromosomal aberrations at diagnosis. G-banding is still the basic method for cytogenetic analysis of the leukemic clone. The employment of various molecular methods has had a major impact on our understanding of the biology of ALL, on treatment stratification, and on monitoring the response to treatment. With comparative genomic hybridization (CGH), it is possible to detect many more changes in DNA copy number than with G-banding. However, balanced translocations are not detectable with this method (Larramendy et al. 1998a, Larramendy et al. 1998b). With conventional FISH, it is possible to search for specific aberrations such as t(9;22) or t(12;21). The introduction of spectral karyotyping (SKY) and multicolor-FISH (M-FISH) has increased the possibilities for detecting both numerical and structural changes in chromosomes. Intrachromosomal aberrations such as inversions, small duplications, and deletions cannot be detected (Schröck et al. 1996, Speicher et al. 1996). The polymerase chain reaction (PCR) is widely used in the diagnostic work-up of childhood ALL, both for detection of leukemia-specific genetic aberrations and of the antigen receptor gene rearrangements used for monitoring of MRD (Pongers-Willemse et al. 1999, Drunat et al. 2001). The clinical value of micro-array technology is not yet clear. With this method, it is possible to study the expression of thousands of genes simultaneously. In the future, we shall probably gain much new knowledge of the pathogenesis of leukemias and other malignancies with the help of these gene expression studies (DeRisi et al. 1996, DeRisi and Iyer 1999).

The detected aberrations are of great importance, not only for diagnostic and stratification purposes, but also for monitoring the response to treatment. With such sensitive methods as FC or PCR, it is possible to detect a single cell amongst 10^4 - 10^6 cells in the BM sample. This MRD has been shown to be of prognostic importance (Cave et al. 1998, Coustan-Smith et al. 2000, van Dongen et al. 1998).

Of the chromosomal translocations, t(4;11) leading to 11q23 rearrangements is associated with a poor prognosis, although not in all age groups (Dordelmann et al. 1999, Johansson et al. 1998, Reaman et al. 1999). Johansson and co-workers reported an EFS of 72% in children aged 2-9 years with this translocation (Johansson et al. 1998). Cells with 11q23 rearrangements grow better on stromal layers than cells without this aberration (Kumagai et al. 1996). Moreover, in vitro drug resistance to prednisolone and L-asparaginase is common in these cells (Pieters et al. 1998). The other important translocation associated with a poor prognosis is the Philadelphia translocation, t(9;22). In ALL, the resulting fusion protein is of 190 kilodaltons, in contrast to the 210 kilodaltons in chronic myelogenous leukemia

(CML). The breakpoint within the *BCR* gene on chromosome 22 is closer to the centromere in ALL than in CML (Hermans et al. 1987). T(1;19) has been considered a poor prognostic factor in some protocols and has been an indication for more intensive therapy (Pui et al. 2000). CCG analyzed retrospectively the prognostic impact of t(1;19) and reported that only the balanced form of the translocation leads to an inferior outcome (Uckun et al. 1998).

The revolution of molecular cytogenetics has revealed aberrations that were not detectable with traditional karyotyping. The most common translocation detected in ALL is the t(12;21) leading to the *TEL-AML1* fusion gene. Roughly, 25% of children with ALL have this aberration, which has been associated with a favorable outcome, although no consensus has been reached of the prognostic value of this translocation (Baruchel et al. 1997, McLean et al. 1996, Maloney K. et al. 1999, Rubnitz et al. 1999, Seeger et al. 1998, Seeger et al. 2001, Loh et al. 1998). In the CLCG-EORTC 58881 study, the patients with *TEL-AML1* fusion suffered from late relapses. The investigators speculated that this phenomenon might have been caused by intravenous mercaptopurine (MP) during maintenance therapy, because no relapses had occurred in children not randomized to iv MP; the 6-year EFS in the iv group was only 57%. Why iv MP should increase the risk of relapse is not clear (Vilmer et al. 2000).

Extramedullary leukemia (CNS and testes)

CNS involvement is still important for the prognosis, although the prognostic value has decreased with employment of CNS-directed therapy including irradiation and chemotherapy (MTX, cytarabine) (Smith et al. 1996). A CNS-3 status ($\geq 5 \times 10^6$ blasts/L in the CSF) is invariably considered a poor prognostic factor, but the impact of CNS-2 status ($<5 \times 10^6$ blasts/L) is not clear. There is also evidence that a traumatic lumbar puncture at diagnosis may worsen the outcome (Gajjar et al. 2000). In the latest studies reported, the following groups still find CNS involvement a poor prognostic sign: BFM 90, TCCSG, DFCI, COALL, NOPHO, AIEOP, SJCRH, POG (good-risk B-lineage ALL), and CCG. In many treatment protocols, CNS involvement at diagnosis leads to intensified CNS-directed therapy.

Of the boys, 1.9% have clinically evident testicular involvement of leukemia at diagnosis. Bulky testicular involvement is mostly associated with a high tumor burden and other HR features (Gajjar et al. 1996). At diagnosis, occult testicular leukemia has no prognostic significance (Trigg et al. 2000). In the NOPHO and POG risk classifications, testicular leukemia is a criterion for poor outcome and stratifies boys with testicular ALL to the HR group (Gustafsson et al. 2000, Maloney et al. 2000). In the Nordic series the percentage of isolated testicular relapses is 4% of all relapses. Before the era of intensive systemic chemotherapy and the use of HD-MTX, the rate of testicular relapse was as high as 20% (Niemeyer et al. 1985). In the treatment of testicular relapses, irradiation with 18-24 Gy is employed (Uderzo et al. 1990, Wofford et al. 1992).

Lymphomatous features

Lymphomatous features (mediastinal mass, enlarged spleen, enlarged lymph nodes) lead to an increased risk of relapse. In these patients, the tumor load is usually high and the T-lineage immunophenotype common. In the CCG-123 study for lymphomatous leukemia, 65% of the children had the T-cell immunophenotype

(Steinherz et al. 1998). In most protocols, lymphomatous features stratify patients to more intensive treatment either by the employment of the BFM risk factor (BFM, AIEOP, DCLSG) or by including children with a mediastinal mass, enlarged spleen, or lymph nodes in the HR group (NOPHO, DFCI, CCG, POG). The independent prognostic value of clinical lymphomatous features is not easy to assess, because of the combination of these features with a high WBC and a T-cell immunophenotype in the same patients.

Morphology

The French-American-British (FAB) morphologic classification was introduced in 1976 by Bennett and co-workers (Bennett et al. 1976). According to morphology, 80% of childhood leukemias are classed as ALL and 20% as acute myolegenous leukemia (AML). ALL is further divided into three groups: L1, L2 and L3.

L3-ALL, also called Burkitt's leukemia, has nearly always a mature B-cell immunophenotype and is clinically characterized by rapid progression and often by prominent tumor lysis. The majority of children with L3-ALL also have a typical cytogenetic aberration; t(8;14), t(8;22), or t(2;8). Treatment differs markedly from other ALL subtypes and is extremely intensive, but of short duration. With this strategy, the outcome of children with L3-ALL is favorable (Patte et al. 2001, Reiter et al. 1999, Reiter et al. 1992).

The prognostic significance of L1/L2 morphology is not clear. Primarily, when centralized assessment of morphology was applied, Miller and co-workers in the Children's Cancer Group (CCG) demonstrated an association between L2 morphology and a poor prognosis (Miller et al. 1981, Miller et al. 1985, Miller et al. 1980). The role of morphology as an independent prognostic factor has not been confirmed by others (Hammond et al. 1986, Kalwinsky et al. 1985, Lilleyman et al. 1992, van Eys et al. 1986). A correlation between L2 morphology and a slow early response to treatment has also been described (Lilleyman et al. 1992). This raises the question of whether patients with L2 morphology require an induction therapy different from others, because of the known great prognostic importance of early cytoreduction, as assessed from day 8 or day 15 BM status (Lilleyman et al. 1997, Schultz et al. 1997, Steinherz et al. 1996).

In the contemporary ALL protocols lymphoblast L1/L2 morphology is not used for treatment stratification.

Drug resistance

The main reason for unfavorable treatment results is the drug resistance of the leukemic blasts. With intensive induction therapy, roughly 95% of the patients achieve CR by morphologic criteria, but in approximately 20% a relapse occurs later, during or after treatment (Margolin and Poplack 1997).

One major mechanism of drug resistance is the P-glycoprotein-mediated multiple drug resistance (MDR). This is caused by cross-resistance to several anti-cancer agents such as anthracyclines, vinca alkaloids, and epipodophyllotoxins (Arceci 1993). P-glycoprotein (P-gp) is a membrane-associated pump that actively reduces drug accumulation in the cells (Gottesman and Pastan 1993). Because the targets of

MDR are widely used in the treatment of childhood ALL, P-gp expression might be assumed to be an important prognostic factor.

In adult leukemias, MDR is associated with a poor induction response and a poor outcome (Legrand et al. 1999, Leith et al. 1999, Del Poeta et al. 1996). The clinical significance of MDR in childhood ALL is still uncertain. Some reports have shown a higher relapse rate and poorer survival in P-gp-positive patients (Dhooge et al. 1999, Goasguen et al. 1993, Sauerbrey et al. 1994). Other studies have not found P-gp expression to be clinically significant in childhood ALL (Pieters et al. 1992, Pieters et al. 1994, Ubezio et al. 1990, den Boer et al. 1998).

The effect of P-gp can be modified with various drugs (e.g. verapamil, quinine, cyclosporine, and PSC833) and the positive therapeutic effect of such modifications has been shown in adult AML (List et al. 1998, Dorr et al. 2001). In childhood AML, however, the outcome was not improved by cyclosporine (Dahl et al. 2000). No clinical trials of resistance modifiers have been published in childhood ALL.

Other mediators of drug resistance include multiple drug resistance related protein (MRP), lung resistance protein (LRP), and glutathione-S-transferase (GST). Of these, the one most investigated is MRP (den Boer et al. 1998, Grant et al. 1994, Michieli et al. 1999). Den Boer and co-workers studied MRP in childhood ALL and AML. There were no differences in MRP expression between de novo and relapsed patients, nor was MRP expression higher in patients with unfavorable clinical features (den Boer et al. 1998). There are no data concerning the prognostic value of MRP in childhood ALL.

In the study by den Boer et al (den Boer et al. 1998), the LRP level was higher in patients with multiple relapses and with AML than in those with ALL. No association with the prognosis was reported.

GST has been studied less in childhood ALL. The mu class of GST has been found to be associated with an unfavorable outcome (Hall et al. 1994). Stanulla and co-workers studied the GST genotype in a case-control setting in ALL. The null genotype of GST theta and mu, and the Val105/Val105 genotype of GST pi were associated with lower risks of relapse (Stanulla et al. 2000). However, many other studies have indicated that the role of GST does not appear to be important in childhood ALL (Koberda and Hellmann 1991, Russo et al. 1994, Sauerbrey et al. 1994, Tew 1994, Tiirikainen et al. 1994, Chen et al. 1997). In childhood AML, the type of GST genotype might be associated with the outcome. Davies and co-workers showed increased death in remission and inferior survival in patients with the GST theta-null genotype (Davies et al. 2001). This finding can be interpreted as the result of deficient GST function leading to decreased detoxifying capacity of normal cells.

Other mechanisms of drug resistance include low levels and activity of topoisomerase II, which reduces the effect of anthracyclines and epipodophyllotoxins. Over-expression of *bcl-2* and other genes regulating apoptosis can also lead to increased drug resistance. In addition to impaired drug transport and uptake, impaired intracellular activation, and increased inactivation, increased levels of target enzymes, increased repair of DNA damage, and mutations in mismatch

repair genes may also lead to increased resistance (Adamson et al. 1990, Chauncey 2001).

One way to study drug resistance is to employ various *in vitro* assays. Recently, the one mostly used has been the methyl-thiazole-tetrazolium (MTT) assay. With this assay, investigators have found the resistance profiles of various drugs to be of prognostic value in childhood ALL. An even better predictive value could be achieved by combining the results of certain drugs (Pieters et al. 1994, Kaspers et al. 1995, Kaspers et al. 1997, den Boer et al. 1998, Hongo and Fujii 1991, Pieters et al. 1991, Pieters et al. 1993, Hongo et al. 1997). The COALL study group has used the results of *in vitro* resistance determinations in treatment stratification (Janka-Schaub et al. 1999).

Pharmacokinetics

Genetic enzymatic variations in drug metabolism can alter the treatment effect substantially. An important example is the enzyme thiopurine S-methyltransferase. A deficiency of this enzyme leads to impaired inactivation of mercaptopurine, thioguanine, and azathioprine. Approximately 10% of people are heterozygous for the gene of this enzyme and thus need smaller drug doses. One in 300 have a homozygous deletion and are extremely sensitive to these drugs, but can tolerate small doses without toxic effects (Pui and Evans 1998, Yates et al. 1997, Evans et al. 1991, Evans et al. 2001).

The dose intensity of 6-MP has been shown to be a prognostic factor. In the study by Relling and co-workers, children receiving a lower treatment intensity (=more weeks of missed therapy due to neutropenia) had a worse outcome (Relling et al. 1999). Schmiegelow and co-workers measured concentrations of the erythrocyte mercaptopurine metabolite (6-tioguanine nucleotides), and erythrocyte methotrexate during maintenance therapy and showed higher concentrations to be associated with a favorable outcome (Schmiegelow et al. 1995).

6-MP and MTX have been detected to have a circadian effect. According to Schmiegelow and co-workers, evening dosing of both 6-MP and MTX during maintenance therapy leads to a better outcome (Schmiegelow et al. 1997).
The ability to excrete drugs from the body varies substantially between individuals. Therefore, to avoid toxic effects, it is important to monitor serum levels of MTX during high-dose therapy. With too low concentrations, on the other hand, the therapeutic effect can be hampered. Evans and co-workers studied standard vs. pharmacokinetically guided dosing of MTX, teniposide, and cytarabine. In B-lineage ALL, the patients with individualized MTX dosing (and higher concentrations) had a better outcome (Evans et al. 1998).

Treatment

The impact of prognostic factors is strongly influenced by the treatment given. By intensification of the treatment protocol, certain factors may lose their significance and the importance of other factors may change. This is clearly illustrated in the successive studies of various working groups from the 1980s to the 1990s. For example, the immunophenotype lost its prognostic value in COALL, CCG, and TCCSG protocols, gender in CCG, NOPHO, and DFCI, age (non-infants) in NOPHO and DFCI, and even WBC in DFCI. In contrast, the immunophenotype gained

significance in BFM, CNS involvement in BFM, CCG, and AIEOP, and gender in the AIEOP and CLCG-EORTC protocols. These alterations illustrate the complexity of assessing the prognostic significance of various factors. It is even more difficult to study the roles of individual drugs in this context. The only way is to conduct randomized studies with simple modifications of treatment. Several trials have shown that intensification of treatment leads to a better outcome.

Employment of intensive induction therapy with 4 to 6 drugs, including anthracyclines, has proved beneficial for most patients in many studies (Reiter et al. 1994, Rivera et al. 1993, Rivera et al. 1991, Niemeyer et al. 1991, Gaynon et al. 1993). On the other hand, protocols with a 3-drug induction without anthracyclines have also led to good outcomes (Kamps et al. 2000, Maloney et al. 2000).

Treatment intensification shortly after induction has been shown, in many studies, to improve the outcome (Reiter et al. 1994, Mahoney et al. 1998, Niemeyer et al. 1991, Rivera et al. 1991, Veerman et al. 1996, Gaynon and Lustig 1995, Chessells et al. 1995). Even children with standard or low-risk leukemia have benefited from intensification of their treatment (Chessells et al. 1995, Hann et al. 2000, Tubergen et al. 1993). Reinduction treatment after week 20 has also been shown to improve the outcome (Hann et al. 2000, Reiter et al. 1994, Nachman et al. 1998b, Tubergen et al. 1993).

Dexamethasone has been shown to be more potent than prednisolone in preventing CNS leukemia (Veerman et al. 1996, Gaynon and Lustig 1995). Early and intensive intrathecal medication prevents clinical CNS disease and CNS relapses (Veerman et al. 1996, Reiter et al. 1994, Pui et al. 1998a, Nachman et al. 1998a). Because of differences in the metabolism of methotrexate, HD-MTX is especially important in T-ALL (Reiter et al. 1994).

AIEOP studied the effect of increased doses of L-asparaginase during and after the second induction period (20 weekly doses of 20000 IU/m^2 vs. 4 weekly doses of 10000 IU/ m^2. No difference in outcome was observed (Rizzari et al. 2001).

Response to treatment

The BFM study group has, for a long time, employed the response to a 1-week single-agent steroid-treatment as a prognostic factor. This method has proved valuable in detecting a very-high-risk subgroup of children, who need intensive treatment to be permanently cured (Reiter et al. 1994, Schrappe et al. 2000a). The steroid response is important even in infant ALL with t(4;11) (Dordelmann et al. 1999). However, it is possible that treatment intensification decreases the prognostic importance of the steroid response (Nachman et al. 1998b).

By increasing the corticosteroid dose before a four-drug induction treatment, it was possible to improve the early (day 3 BM) response to treatment, but no effect on EFS could be shown in a DFCI study (Schwartz et al. 2001).

Other methods for evaluating the morphologic response to treatment are monitoring the peripheral blood blast count (Gajjar et al. 1995, Gaynon et al. 1997, Rautonen et al. 1988) or BM cytoreduction during multiagent induction treatment (Lilleyman et al. 1997, Miller DR et al. 1989, Schultz et al. 1997, Steinherz et al. 1996). Both these

methods have shown strong prognostic significance and are used to guide therapeutic decisions in various protocols. The treatment response in the BM on either day 8 or day 15 is of prognostic value and both of these time points are widely used.

Minimal residual disease

During the 1990s, it was shown by various methods that MRD during and after chemotherapy is a factor for a poor prognostic. Even the MRD in day 15 BM may be of prognostic value (Panzer-Grumayer et al. 2000). Many studies have shown that MRD detection during and after induction, during further therapy, and after completion of treatment, is a marker of inferior prognosis (Cave et al. 1998, Coustan-Smith et al. 2000, van Dongen et al. 1998). In the interpretation of the MRD results, the treatment protocol must be taken into account, especially during early time points such as the end of induction (zur Stadt et al. 2001). Most MRD studies have been performed with the PCR and flow cytometry (FC), which are sensitive to a level of 10^{-5} to 10^{-6}. A problem with these methods, especially PCR, is that it is impossible to tell whether the signal comes from living or dead cells (Campana and Pui 1995). The employment of standardized triple-stainings and ”empty spaces” analysis has increased the sensitivity and specificity of flow cytometric MRD monitoring (Lucio et al. 2001). Contamination is a major problem with PCR methodology, but modern real-time PCR techniques have helped with this problem because of the more closed analysis system. With these PCR methods, it is also possible to quantify MRD (Eckert et al. 2000, Drunat et al. 2001, Donovan et al. 2000). Another source of false results is clonal evolution, which can change the immunophenotype or the pattern of antigen receptor gene rearrangements. This problem may be less important when the MRD analysis is performed in the early phase of treatment. Oligoclonality may also cause false-negative results, if the resistant or relapsing clone is not the major clone chosen for MRD monitoring. With metaphase FISH, it is possible to study mitotically active proliferating cells. The sensitivity of metaphase FISH, on the other hand, is only 10^{-3}, which may be considered too low, especially at late time points during or after completion of therapy (El-Rifai et al. 1997). A common pitfall of all MRD detection methods is the probable patchy distribution of MRD in the BM. This could be overcome by detecting MRD in the peripheral blood, but thus far sensitivity has not been good enough. Because the blast content of blood is roughly one log lower than that of marrow, one would need to study many more cells to reach acceptable sensitivity. This makes MRD studies of peripheral blood too laborious, at least by FC (Brisco et al. 1997, Lal et al. 2001). Furthermore, in about 10% of acute leukemias, blasts never circulate, even at diagnosis and in overt disease.

5.3. Stratification of treatment according to prognostic factors

The goal in the search for prognostic factors is to individualize the treatment in order to avoid both over- and under-treatment of children with ALL. There is some evidence that low-risk patients might not need anthracyclines in induction (Harris et al. 1998, Mahoney et al. 1998, Veerman et al. 1996). However, even children with a standard-risk disease have been shown to benefit from treatment intensification (Tubergen et al. 1993, Hann et al. 2000, Chessells et al. 1995). The upfront prognostic factors serve as a valuable guide to treatment stratification, but the importance of response monitoring with both morphological, immunological, and molecular methods is increasing with the growing knowledge of leukemia cell kinetics during treatment (Campana and Pui 1995, Pui and Campana 2000). The Rome/NCI criteria for standard and high-risk ALL form a good basis for comparing the treatment results of different protocols, but they are not sufficiently specific for modern stratification of treatment. Both molecular and immunological criteria are widely used in contemporary treatment protocols.

Very-high-risk group

Children with Philadelphia chromosome-positive ALL may need stem cell transplantation in first CR. Both an HLA-identical sibling and a matched unrelated donor is a possible option (Arico et al. 2000, Chessells 2000). However, there is some evidence that a subset of Philadelphia-positive children presenting with WBC below $25x10^9$/L or a good response to steroids have a relatively favorable outcome, even with chemotherapy alone (Ribeiro et al. 1997, Schrappe et al. 1998). Other children who might benefit from very intensive therapy include those with a poor response to induction treatment, a very high WBC ($\geq 200x10^9$/L), hypodiploidy of <45 chromosomes, t(4;11), and infants (Chessels et al. 1997, Gustafsson et al. 2000, Heerema et al. 1999, Reaman et al. 1999, Steinherz et al. 1996).

Low-risk group

In almost every treatment protocol, factors associated with a low risk of relapse include low WBC ($<10\text{-}50x10^9$/L), age 1-9 years, absence of extramedullary leukemia, and absence of unfavorable cytogenetic aberrations. In the treatment of these children, avoidance of late effects is important, but at the same time the survival rate must not be endangered. If the treatment of low-risk children is decreased in intensity, one must monitor the treatment response extremely carefully. In addition to the morphologic response in the BM, MRD monitoring serves as a valuable tool for this purpose. With current sensitivity, it is possible to find those children who might need treatment intensification. To decrease the treatment intensity of children with a good response according to MRD monitoring is not yet possible. To date, there are no data indicating that treatment intensification based on MRD results leads to better survival.

5.4. Late effects

CNS preventive therapy has a major impact on increased survival rates in childhood ALL. However, cranial irradiation, intrathecal chemotherapy and systemic MTX cause significant long-term side effects (Margolin and Poplack 1997, Raymond-Speden et al. 2000, Shusterman and Meadows 2000). Cranial irradiation also affects the hypothalamic pituitary axis, leading to decreased growth hormone excretion and also impaired gonadotropin production. Chemotherapy, especially with alkylating agents, impairs gonadal function, leading to decreased fertility (Humpl et al. 1999, Margolin and Poplack 1997). Anthracyclines cause cardiomyopathy, which may lead to cardiac failure years after completion of treatment. The cumulative anthracycline dose is the major determinant of the risk of cardiomyopathy (Margolin and Poplack 1997, Nysom et al. 1998, Sorensen et al. 1997). Secondary neoplasms are a substantial risk after ALL therapy. The risk of AML after treatment with epipodophyllotoxins and other topoisomerase inhibitors is well recognized (Kimball Dalton et al. 1998). Brain tumors are the other major group of secondary malignancies in ALL survivors. The risk is especially associated with cranial radiotherapy (Relling et al. 1999). The BFM group analyzed secondary malignancies in 5006 children with ALL. They found 52 cases, of which 16 were AML, and 13 CNS tumors (Loning et al. 2000). Other late effects include skeletal problems, defects in the immune system, and psychosocial problems (Margolin and Poplack 1997).

Stratification of treatment aims at finding those children who need intensive treatment, but on the other hand, also those whose treatment could be less intensive and thus lead to less of adverse effects.

6. Aims of the study

General aims of the study were to evaluate new possible prognostic factors in childhood ALL, and, in the context of contemporary intensive treatment, to assess the value of established prognostic factors.

The specific aims were as follows.

1. To study lymphoblast morphology and its value in predicting the early response to treatment and survival in childhood ALL.
2. To study multiple drug resistance mediated by P-glycoprotein and its prognostic value in childhood ALL.
3. To study the prognostic value of age, WBC, and hemoglobin level in childhood ALL.
4. To study the incidence and prognostic value of *TEL-AML1* fusion in childhood ALL.
5. To study changes in DNA copy number and their clinical impact in childhood ALL.
6. To study minimal residual disease in childhood ALL by FISH.

7. Patients and methods

7.1. Patients

Certain parts of the study were population-based, whereas other parts comprised representative selected groups of ALL patients.

In the population-based morphology study (I), a total of 251 children with newly diagnosed ALL, 123 males and 128 females, were studied. Their median age was 4.6 (range 0.5-15.7) years. This series comprised all cases of childhood ALL in Finland from January 1, 1990, through June 30, 1995. All patients were followed up until December 31, 1998. The median follow-up for the survivors was 72 months (range 43-109).

The drug resistance study (II, III) comprised 84% of newly diagnosed childhood ALL cases in Finland between January 1, 1993, and June 30, 1995. A total of 118 children with ALL (103 at diagnosis and 15 at relapse) were investigated. Fifty-two were males and 66 were females. Their median age was 4.9 (range 1.1-16.7) years. The 19 children excluded from the study (because of no samples for P-glycoprotein measurements) did not differ from the study population. The median follow-up after diagnosis was 56 months (range 42-71 months).

In the biological tempo and *TEL-AML1* study (IV), two cohorts of Finnish children with ALL were retrospectively analyzed. Cohort 1 comprised 100 consecutive children diagnosed and treated at the Children's Hospital, University of Helsinki, during 1975 to 1981. During that period, 42% of Finnish children with ALL received their induction therapy in Helsinki and their data were uniformly recorded. Cohort 2 comprised 102 consecutive children diagnosed between 1989 and 1991 and covering the whole country. The median follow-up time was 19.6 years in cohort 1 and 9.2 years in cohort 2.

In the CGH study (V), 79 children with ALL were investigated, 33 males and 46 females, who represented 43% of all the children (79/185) diagnosed between January 1990 and June 1995 in three pediatric oncology centers in Finland (Helsinki, Kuopio, and Tampere). Their median age was 4.7 (range 0.6 - 15.7) years. During the same period, an additional 106 children were treated for ALL in these centers, but were not included in the study because no samples were available for retrospective CGH analysis. The median follow-up in this cohort was 74 months (range 51-121 months)

In the MRD study (VI), 41 children (20 males and 21 females) with ALL were prospectively studied from January 1994 to August 1998. All these children were diagnosed and treated at the Hospital for Children and Adolescents, University of Helsinki. Their median age was 4.4 years (range 0.6-14.5). The median follow-up was 41 months (range 17-85) from diagnosis. During the same period, an additional 44 children were treated for ALL at our center, but were not included in the study because of the absence of a suitable marker for metaphase-FISH, or on account of

incomplete monitoring. This group did not differ from the study group regarding such key factors as age, WBC at diagnosis or immunophenotype.

7.2. Treatment

From 1975 to 1981, patients were treated as follows. Induction included prednisone (40-60 mg/m^2/day for 6 weeks, + taper), weekly vincristine (1.5 mg/m^2 x7, max. single dose 2 mg), a 7-day course of L-asparaginase (L-asp) (10000 IU/dose), and intrathecal methotrexate (it MTX) (12 mg/m^2 x1-3). Cranial irradiation of 24 Gy was used for CNS prophylaxis in the majority (90/100). Twenty-three boys out of 50 received prophylactic testicular irradiation. Maintenance therapy consisted of MP (50 mg/m^2) daily, oral MTX (20 mg/m^2) weekly, and pulses of VCR (1.5 mg/m^2) and prednisone (40 mg/m^2/day for 5 days) monthly up to a total of 3 years. Fourteen children received rotational high-risk maintenance with cytarabine (ARA-C) (75mg/m^2), cyclophosphamide (30 mg/kg), and doxorubicin (30mg/m^2). One child with extremely poor presenting features underwent allogeneic BMT in first remission.

Between January 1, 1989, and December 31, 1991, patients were treated according to Nordic treatment protocols, which are very close to the contemporary NOPHO-ALL 92 protocol. Systemic MTX doses were lower in the SR and IR groups and cranial irradiation was also given to the IR group (Table 4) (Gustafsson et al. 1998)

All the children diagnosed between January 1, 1992, and December 31, 2000, were treated according to the NOPHO-ALL 92 protocol (Table 5) (Gustafsson et al. 2000).

In all risk categories, the start of induction was identical. Induction therapy consisted of prednisone (60 mg/m^2/day on days 1-35), vincristine (2 mg/m^2 weekly x6), and doxorubicin (40 mg/m^2 on days 1, 22, and 36, and, in the HR group only, on day 8). Intrathecal methotrexate (8-12 mg/dose according to age) was given on days 1, 8, 15, and 29. After day 35, a 10-day course of L-asparaginase was given. The second part of induction, given in the HR and IR protocols, consisted of cyclophosphamide, cytarabine, and 6-MP. The total duration of therapy was 2 years (IR and HR) or 2.5 years (SR), including CNS consolidation, delayed intensification (IR and HR), and maintenance.

Table 4. Treatment protocol for patients diagnosed between January 1, 1989, and December 31, 1991.

	Induction		Consolidation	Interim maintenance	Delayed intensification		Maintenance	Total duration from dg
	1	2			1	2		
HR	Predn VCR Adr 40mg/m^2 x 4 MTX it L-asp	Cyc Ara-C 6-MP po	HD-MTX 8g/m^2 x 2 or 4 [a] iv MTX it x 2 or 4 HD-Ara-C x 2 or 4[a] >5 years: 18Gy CNS irradiation	VCR+Predn pulses 6-MP po MTX po	Dexa VCR Adr MTX it L-asp	Cyc Ara-C 6-TG po	LSA_2L_2 [b]	2 years
IR	Predn VCR DNR 30mg/m^2 x 4 MTX it L-asp	Cyc Ara-C 6-MP po MTX it	ID-MTX 0.5g/m^2 x 4 iv MTX it x 4 6-MP po		Dexa VCR Adr L-asp	Cyc Ara-C 6-TG po MTX it >4 years: CNS irradiation	6-MP po MTX po	3 years
SR	Predn VCR Adr 40mg/m^2 x 3 MTX it L-asp		HD-MTX 1g/m^2 x 3 iv MTX it x 3				HD-MTX + MTX it pulses VCR + Predn pulses 6-MP po MTX po	3 years

Abbreviations: HR, high risk; IR, intermediate risk; SR, standard risk; Predn, prednisolone; VCR, vincristine; Adr, doxorubicin; DNR, Daunorubicin; L-asp, L-asparaginase; MTX, methotrexate; Ara-C, cytarabine; Cyc, cyclophosphamide;
6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; Gy, gray; it, intrathecal; po, oral; HD, high-dose; dg, diagnosis.

a) x2: children >5 years of age, x4: children <5 years of age, b) (Anderson et al. 1983)

In the HR group, five children were treated according to CCG-106, one according to CCG-1883, and one according to a special infant protocol.

Table 5. Treatment protocol for patients diagnosed between January 1, 1992, and December 31, 2000.

	Induction		Consolidation	Interim maintenance	Delayed intensification		Maintenance	Total duration from dg
	1	2			1	2		
HR	Predn VCR Adr MTX it L-asp	Cyc Ara-C 6-MP po	HD-MTX x 2 or 4 [a] MTX it HD-Ara-C x 2 or 4[a] >5years: 18Gy CNS irradiation	VCR+Predn pulses 6-MP po MTX po	Dexa VCR Adr MTX it L-asp	Cyc Ara-C 6-TG po	LSA_2L_2 [b]	2 years
IR	Predn VCR Adr MTX it L-asp	Cyc Ara-C 6-MP po	HD-MTX x4 MTX it 6-MP po		Dexa VCR DNR MTX it L-asp	Cyc Ara-C 6-TG po	HD-MTX + MTX it. pulses VCR + Predn pulses 6-MP po MTX po	2 years
SR	Predn VCR Adr MTX it L-asp		HD-MTX x 3 MTX it				HD-MTX + MTX it. pulses VCR + Predn pulses 6-MP po MTX po	2.5 years

Abbreviations: HR, high risk; IR, intermediate risk; SR, standard risk; Predn, prednisolone; VCR, vincristine; Adr, doxorubicin; DNR, daunorubicin; L-asp, L-asparaginase; MTX, methotrexate; Ara-C, cytarabine; Cyc, cyclophosphamide; 6-MP, 6-mercaptopurine; 6-TG, 6-thioguanine; Gy, gray; it, intrathecal; po, oral; HD, high-dose; dg, diagnosis.

[a)] x2: children >5 years of age, x4: children <5 years of age, [b)] (Anderson et al. 1983)

7.3. Risk classification

From 1979-1981, children were divided, at diagnosis, into HR and SR groups. The HR criteria were WBC >50 $x10^9$/L, mediastinal mass, T-cell immunophenotype, or age less than 1 year. No risk classification was employed for children from 1975-1978.

The cohort from 1989-1991 was divided into SR, IR and HR groups according to age, WBC, presence of extramedullary leukemia, cytogenetic aberrations, and immunophenotype (Table 6). Stratification of treatment was based on the risk grouping.

Table 6. Risk classification between January 1, 1989, and December 31, 1991.

SR
-age 2 to <10 years
-WBC ≤ 10 $x10^9$/L
-no HR criteria
IR
-age 2 to <10 years and WBC >10 to <50 $x10^9$/L
-age 1 to <2 or ≥10 years and WBC <50 $x10^9$/L
-not fulfilling SR criteria and no HR criteria
HR
-WBC ≥50 x 10^9/L
-T-ALL
-mediastinal mass
-lymphomatous leukemia[a]
-CNS ALL at diagnosis
-testicular ALL at diagnosis
-t(4;11), t(9;22)

a) requires at least one clinical and one laboratory criterion
clinical: mediastinal mass, enlarged lymph nodes (diameter at least 3 cm), splenomegaly (to the level of the umbilicus)
laboratory: WBC >50 x 10^9/L, T-ALL, hemoglobin >100 g/L

The NOPHO risk classification was used in children treated with the NOPHO-ALL 92 protocol. The classification was based on the previous one. Response to treatment and 22q- were added to the criteria for the HR group (Table 7).

Table 7: Risk classification between January 1, 1992 and December 31, 2000

SR
-age 2 to <10 years
-WBC ≤10 x10^9/L
-no HR criteria
IR
-age 2 to <10 years and WBC >10 to <50 x10^9/L
-age 1 to <2 or ≥10 years and WBC <50 x10^9/L
-no HR criteria
HR
-WBC ≥50 x 10^9/L
-T-ALL
-mediastinal mass
-lymphomatous leukemia
-CNS ALL at diagnosis
-testicular ALL at diagnosis
-slow response (M3 on day 15 marrow or M2/M3 on day 29)
-t(4;11), t(9;22), 22q-

7.4. Methods

Morphology (I)

The primary morphological diagnosis of leukemia was assessed from May-Grünwald-Giemsa-stained BM aspirate slides. The blast cell morphology was analyzed systematically and blindly in a centralized review by Jukka Kanerva and Ulla Saarinen-Pihkala together. We employed the FAB criteria with the CCG modification (Miller et al. 1981, Miller et al. 1985). Individual lymphoblasts were classified as L1, L2, or L3. Specifically, L1 cells were small, with scanty cytoplasm (<20% of surface area), a round or cleft nuclear membrane, and one (0-1) small nucleolus. L2 cells were large (≥2 red blood cell diameters), with more abundant cytoplasm (≥20% of surface area), round or irregular nuclear membrane contours, and one or more prominent nucleoli. L3 cells were characterized by deep basophilia of the cytoplasm and prominent vacuolization (Miller et al. 1981). If a patient had more than 90% of L1 blasts in the marrow, the leukemia was classified as L1-ALL. If there were ≥10% L2 blasts, the classification was L2-ALL. In cases with ≥25% L3 blasts, the morphological classification was L3-ALL.

Early treatment response (I, II, III, V)

The response to treatment was analyzed blindly in a centralized review of the routine BM aspirate and biopsy samples scheduled on day 15. The proportion of lymphoblasts was assessed, and the response was allocated to one of three categories: M1 (<5% blasts), M2 (5-25% blasts), or M3 (>25% blasts). Only those

patients whose BM aspirate was actually taken between days 13 and 18 from the start of treatment were included in the final analysis.

Drug resistance (II, III)

P-gp expression of the fresh BM samples was measured immunologically by a method described by Tiirikainen and co-workers (Tiirikainen et al. 1992, Tiirikainen 1995). Briefly, red cells were lysed and leukocytes were permeabilized with 1X solution of FACS Brand Lysing Solution 10X concentrate (Becton Dickinson, San Jose, CA, USA). For detection of P-gp, a monoclonal antibody JSB1 (Monosan, Sandio bv, Am Uden, The Netherlands), which detects an intracellular epitope of P-gp, was used. Labeled cells were analyzed on a FACScan flow cytometer (Becton Dickinson). For most of the samples, a fixed cut-off line was set to discriminate cells with elevated P-gp expression from those without. The cut-off line was determined, using non malignant BM samples (Tiirikainen et al. 1992). However, every analysis included a sample with an idiotypic control antibody, and if the fluorescence intensity of the cells in the control tube approached the limit, the cut-off was raised. Samples containing more positive cells than the mean + 3SD of the samples from control children were classified as positive.

Biological tempo of ALL (IV)

In the study for analyzing the biological tempo of ALL, we retrospectively reclassified every patient from cohort 1 (1975-1981) and cohort 2 (1989-1991) into one of two groups: very-low-risk or non-low-risk (= the remainder). The very-low-risk group had all of the following features: 1) WBC $<10x10^9$/L, 2) Hb <90 g/L, 3) age 2 to <10 years, and 4) no lymphomatous features (mediastinal mass, lymph nodes 3 cm, spleen down to umbilicus). These criteria were set not only on an established basis (WBC, age), but also, with Hb <90 g/L, in an attempt to exclude conditions with a high proliferation rate (“rapid disease“) (Hirt et al. 1997b). The lymphomatous features had been well recorded in the patients’ charts, a chest x-ray was taken routinely, and the spleen size and enlarged lymph nodes were well recorded already in the 1970s. The duration of symptoms was analyzed retrospectively from the patients’ charts. The cytogenetics and the immunophenotype were not analyzed, because data for these features were very scanty in cohort 1.

Fluorescent in situ *hybridization (FISH) for TEL and AML1 genes (IV, V)*

The FISH analyses were performed on smears or fresh preparations. Spectrum green- and orange-conjugated dual color DNA probes specific for *TEL* and *AML1* genes (Vysis, Downers Grove, IL, USA) were used. Hybridization and washes were performed according to the supplier's instructions. Smears were pretreated before hybridization. Briefly, the coverslips were removed with xylene and the slides were dehydrated in alcohol series (70%, 85%, and 100%). The slides were then fixed in a methanol/acetic acid fixative (3:1) at +4°C overnight followed by treatment with 1 M Na-thiocyanate at +65°C for 10 minutes and washing in 2XSSC at room temperature for 5 minutes. Next, the preparations were treated with 0.01N HCl at +37°C for 10 minutes and then transferred to 0.05N HCl with pepsin (0.05 mg/ml) at +37°C for 8 minutes. Finally, the slides were washed in cold running tap water for 5 minutes and dehydrated in alcohol series (70%, 85% and 100%).

Analysis of FISH (IV, V, VI)

The hybridizations were analyzed from images acquired with a Zeiss fluorescence microscope and the ISIS digital image analysis system (Metasystems, Altlussheim, Germany) based on an integrated high-sensitivity monochrome charge-coupled device camera and automated CGH analysis software. Three-color images were acquired, using three filters from Chroma (Chroma Technology Corp., Brattleboro, VT, USA) specific to fluorescein isothiocyanate (FITC), Texas-Red, and 4', 6-diamidino-2-phenylindole-dihydrochloride (DAPI).

Comparative genomic hybridization (V)

Diagnostic BM aspirates including at least 70% of lymphoblasts were studied by CGH and digital image analysis, as described previously (Larramendy et al. 1998b, Karhu et al. 1997). Briefly, tumor DNA and reference DNA from normal lymphocytes were labeled with FITC conjugated dCTP and dUTP, and Texas red conjugated dCTP and dUTP by nick translation to obtain fragments ranging from 600 to 2000 base pairs. Four hundred ng tumor DNA, 400 ng reference DNA, and 10 μg of unlabeled Cot-1 DNA) were then hybridized with normal metaphase spreads. Hybridization was performed at 37°C for 48 h. The hybridizations were analyzed with the ISIS digital image analysis system (MetaSystems, Altlussheim, Germany) based on an integrated high-sensitivity monochrome charge-coupled device camera and automated CGH analysis software. Three-color images (red for reference DNA, green for tumor DNA, and blue for counterstaining) were acquired from 8-12 metaphases per sample. In each CGH experiment, a negative (peripheral blood DNA from normal controls) and a positive (tumor DNA with known copy number changes) control were included and run simultaneously with the tumor samples

Minimal residual disease (VI)

At diagnosis, all children were karyotyped by standard G-banding. The diagnostic work-up also included CGH analysis of the marrow to increase the number of patients with a marker for the FISH studies. For the metaphase-FISH studies, standard cytogenetic preparations were used, as previously described (El-Rifai et al. 1996, El-Rifai et al. 1997). Only unambiguously analyzable diploid metaphase plates with no overlapping metaphases/nuclei were studied. Polyploid cells were excluded and all aberrations were checked by DAPI banding to confirm the morphology and identity of the chromosomal groups.
Chromosome painting was performed at diagnosis, to confirm the chromosomal aberrations chosen for MRD monitoring with specific library probes (American Type Culture Collection, Rockville, MD, USA) labeled with biotin-14-dATP by nick translation (Nick Translation Kit; Bethesda Research Clinical Laboratories, Gaithersburg, MD, USA). The cells were pretreated with pepsin (0.01 mg/ml, Sigma, St Louis MO, USA) and dehydrated in 70%, 90%, and 96% ethanol. Hybridization signals were detected with avidin-conjugated FITC (Vector Laboratories, Burlingame, CA, USA) and the cells were counterstained with DAPI (Sigma) and propidium iodide (Sigma). After mounting with Vectashield (Vector), the cells were analyzed, using a Zeiss fluorescence photomicroscope (Zeiss, Oberkochen, Germany) with Zeiss filters 02 (FITC) and 09 (DAPI) (El-Rifai et al. 1997).

One thousand metaphase cells were analyzed when possible. The success rate of metaphase FISH was 94%, with a median of 1000 cells/sample (range 2-2115). Only in 72 (=25%) cases did the number of analyzed cells amount to less than 500.

The BM samples were prospectively collected on days 15 and 29 of induction and at the cessation of therapy in all patients, before delayed intensification in the IR and HR groups, and before the second phase of induction in the HR group. If blood cell counts or clinical signs raised suspicion of relapse, additional sampling was performed. Patients with FISH-positivity were sampled more frequently, and even after completion of therapy.

Statistical methods

The Mann-Whitney U test (I-VI), the chi-square test, and Fisher's exact test (with expected values of less than five)(I, IV-VI), and analysis of variance and linear regression (II-III) were used to compare clinical and laboratory variables between the study groups. Logistic regression (I) was used to compare the early treatment response between the morphological groups L1 and L2. The Kaplan-Meier method with the log-rank test (I, III-V), and the Breslow test (I) were used to analyze outcome. Standard errors of survival were calculated according to Greenwood's formula (V). Cox regression was used to evaluate the impact of different prognostic factors on outcome (I). EFS was calculated from the date of diagnosis to the date of an adverse event or the time of last contact. Relapse, death in remission, and second malignancy were considered as events. Patients without events were censored at the time of last contact or at the date of BMT in first CR. The data were analyzed with SPSS for Windows software (versions 7.5 and 8.0.1.).

8. Results

8.1. Lymphoblast morphology (I)

At diagnosis, 197 (80%) of the 251 patients studied had less than 10%, 29 (12%) had 10-25%, and 20 (8%) had more than 25% of L2 blasts in the marrow. Accordingly, 80% were classified as L1-ALL, and 20% as L2-ALL. Five patients were excluded from the analysis; one patient had L3 morphology, and the BM slides of four patients were not available for centralized review. The distribution of L1 and L2 morphology was similar within all three risk categories.

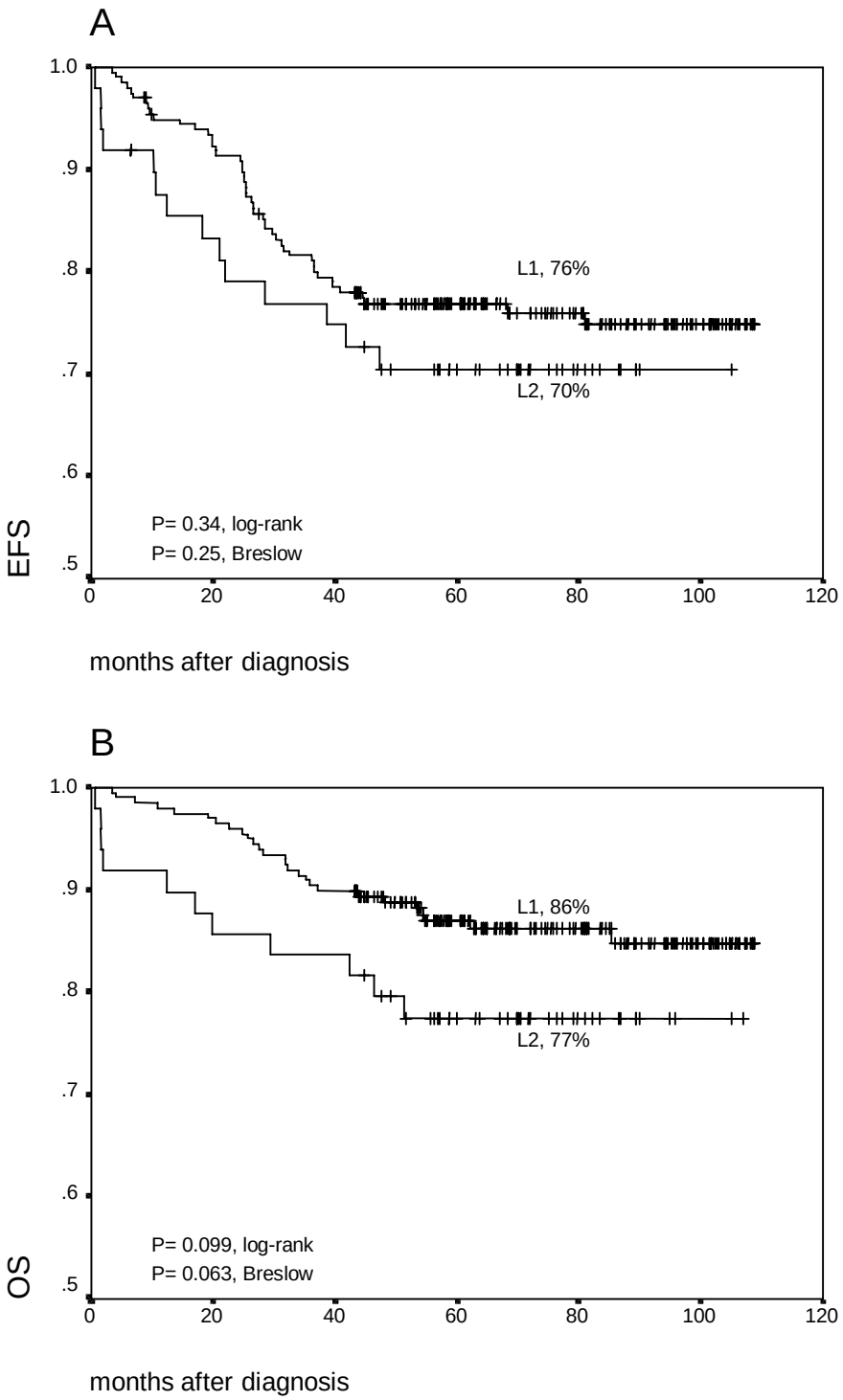
L2-ALL patients were more often slow responders, with 5% or more blasts in the marrow on day 15, than L1-ALL patients (27% vs. 13%; P= 0.048, chi-square test). There was a trend for a similar difference in response when the 25% criterion (M3 marrow) for a slow response was employed, but the groups were much smaller and statistical significance was not reached (12% vs. 6%, P= 0.24, Fisher's exact test) (Table 8).

Table 8. Early treatment response according to initial blast cell morphology.

	L1-ALL	L2-ALL	
number of patients	143	33	
treatment response			
>5% blasts	19 (13%)	9 (27%)	P= 0.048
>25%blasts	8 (6%)	4 (12%)	P= 0.24

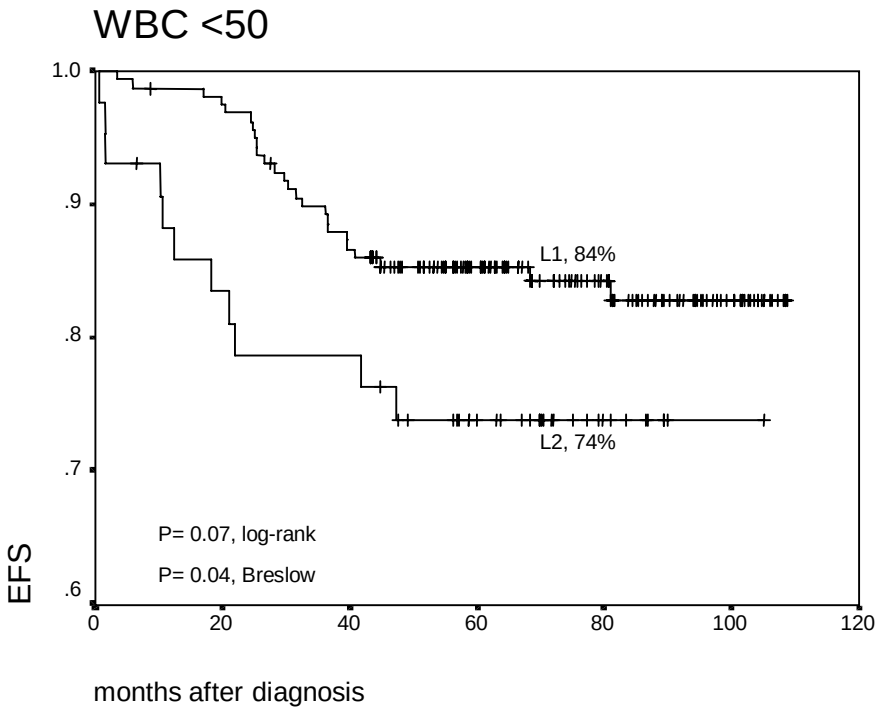
In the whole study population, the 6-year EFS was 75%, being 76% in L1 and 70% in L2 (P= 0.34, log-rank; P= 0.25, Breslow). The 6-year overall survival (OS) was 84%, being 86% in L1 and 77% in L2 (P= 0.099, log-rank; P= 0.063, Breslow) (Fig 1).

Figure 1. *EFS (A) and OS (B) according to morphology in 246 children with ALL.*



There was no difference in outcome between the children with 10-25% of L2 blasts and those with >25% of L2. Because a high WBC, reflecting the tumor burden, is a crucial factor for poor prognostic in childhood ALL, we analyzed outcome separately for patients with WBC below and above $50x10^9$/L at diagnosis. In the group with WBC <50, the 6-year EFS was 84% for L1- vs. 74% in L2-ALL patients (P= 0.07, log-rank; P= 0.042, Breslow) (Fig 2). OS was 91% in L1 and 81% in L2 (P= 0.035, log-rank; P= 0.023, Breslow).

Figure 2. *EFS according to morphology in children with WBC <50 $x10^9$/L.*



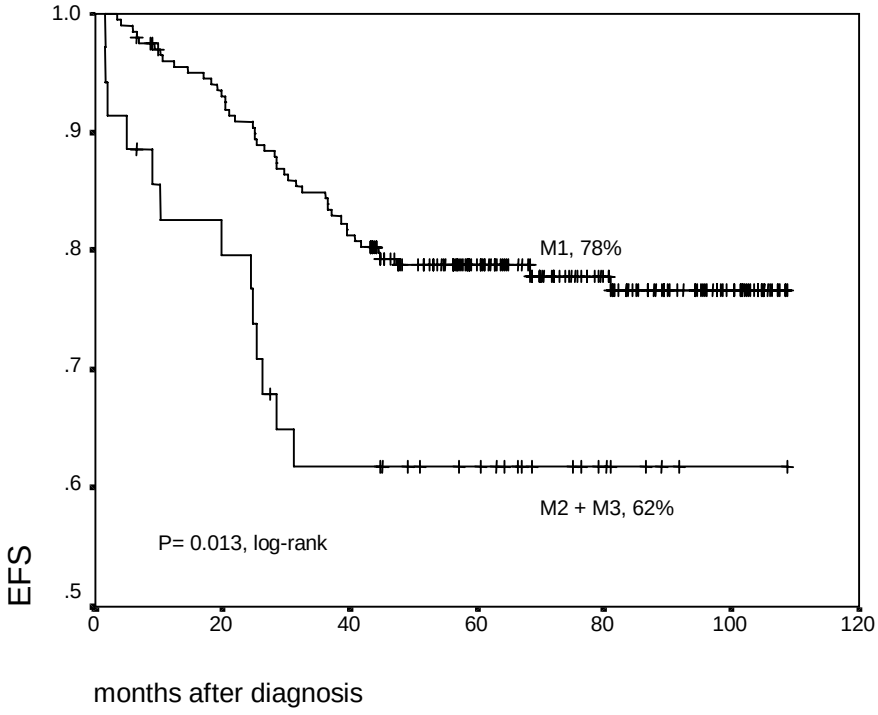
There was no difference in outcome between L1 and L2 among children with WBC>50. In multivariate analysis (Cox regression), morphology retained its prognostic value in the low-WBC group, when adjusted for age, sex, mediastinal mass, immunophenotype, and cytogenetics (Table 9).

Table 9. Multivariate analysis (Cox regression) of prognostic factors in the group with WBC below 50 x10^9/L.

Feature	P-value	Relative risk
FAB morphology L2 vs. L1	0.042	2.25
age (years)	0.98	
<2 vs. 2-10	0.045	3.19
>10 vs. 2-10	0.68	0.81
sex M vs. F	0.53	1.27
immunophenotype T vs. B	0.078	6.16
mediastinal mass	0.23	0.23
cytogenetics	0.050	
not available vs. normal	0.69	1.39
hyper 47-50 vs. normal	0.89	4.21
hyper >50 vs. normal	0.96	0.97
hypo vs. normal	0.98	
structural aberrations vs. normal	0.0072	3.81

Response to treatment (day 15 BM) was associated with outcome. The 6-year EFS was 78% for M1 patients and 62% for M2 + M3 patients (P = 0.013, log-rank) (Fig 3).

Figure 3. *EFS according to day 15 BM response in children with ALL.*



In the M1 subgroup, the difference in EFS between L1 and L2 was not significant (78% vs. 73%).

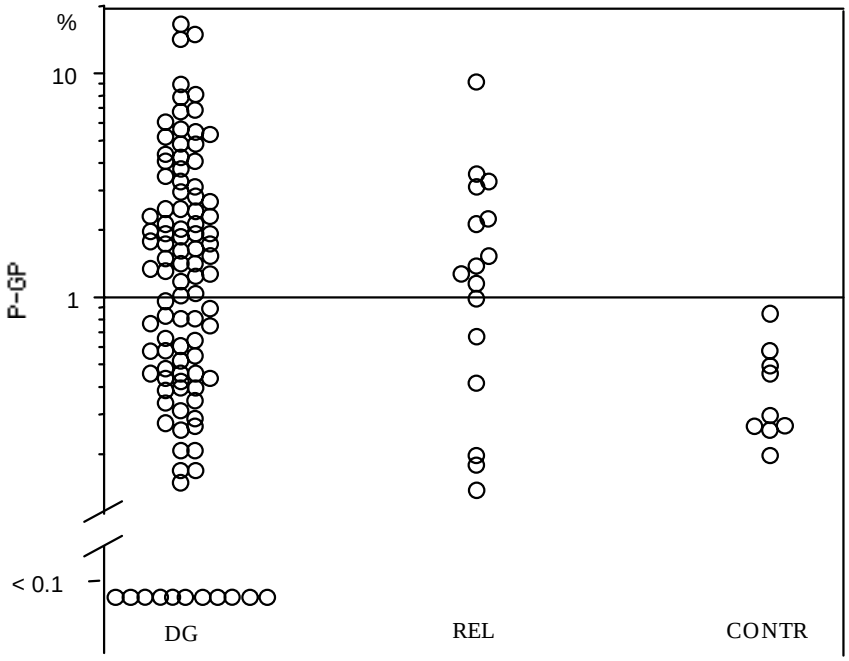
Lymphoblast morphology was not associated with immunophenotype, age, or sex, but there was an association with WBC; unexpectedly, the L1 group had a higher WBC at diagnosis. On the other hand, hyperdiploidy was more common in L2-ALL.

8.2. Multiple drug resistance (II, III)

P-glycoprotein expression

P-gp expression was successfully measured at diagnosis or at relapse in the lymphoblasts of 118 patients and nine control subjects. The mean proportion of P-gp-positive cells in the control samples was 0.41% (SD 0.21%). Patients with more than 1% (= mean + 3SD of the control subjects) of P-gp-positive lymphoblasts were classified as positive. At diagnosis, the median P-gp expression was 1.3% of the blasts (range, 0-16.9%) and at relapse 1.4% (range, 0.14-9.2%). At diagnosis, 55/103 (53%) expressed P-gp and at relapse 11/15 (73%) were positive (Fig 4).

Figure 4. *P-glycoprotein expression of lymphoblasts in controls and in children with ALL, 103 at initial diagnosis (DG) and 15 at relapse (REL) (one patient was studied both at diagnosis and at relapse). The upper normal limit (1%) is indicated by a horizontal line.*

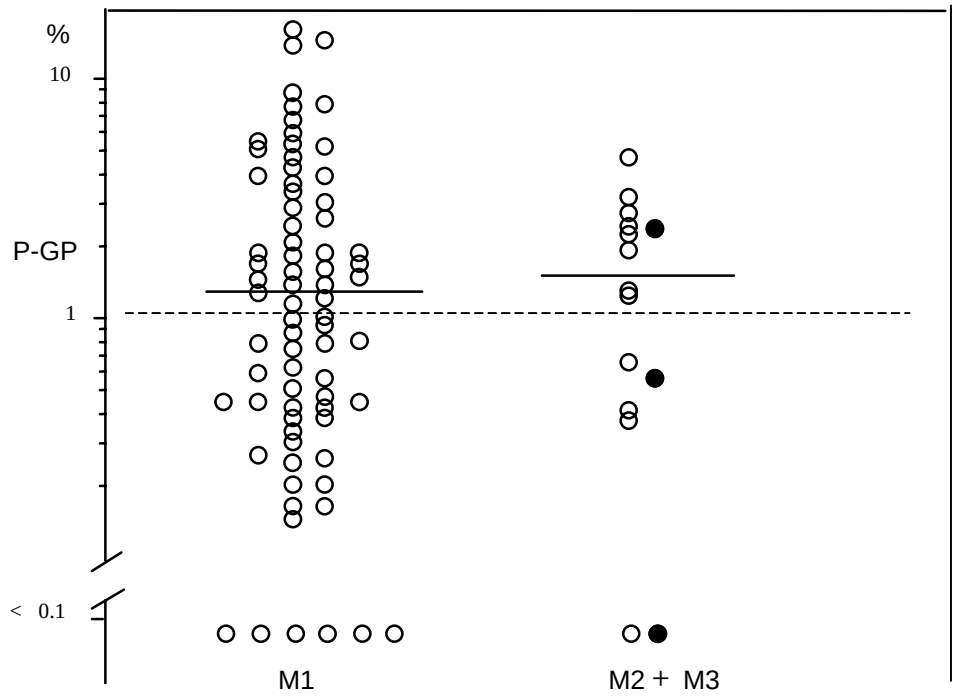


The median P-gp expression level was higher in T-ALL than in B-lineage ALL: 5.3% (range, 3.3-16.9%) vs. 1.0% (range, 0-14.9%) (P= 0.0015). The patients with a mediastinal mass had higher P-gp expression than the others, 5.3% vs. 1.1% (P= 0.0295). There was a strong correlation between T-lineage and mediastinal mass. P-gp expression did not correlate with the coexpression of myeloid antigens, nor was it related to age, sex, or initial leukocyte count. The P-gp level in the whole gated blast population correlated well with the P-gp expression of the proportion of gated blasts expressing the characteristic CD antigen for that blast population.

Early response and P-gp

The early response was evaluated as the proportion of lymphoblasts in the slides of day 15 BM aspirates. This analysis was restricted to those 100 patients whose samples were taken on days 13-18. The individual P-gp expression in the different response categories M1, M2, and M3 are shown as percentages in Figure 5, which demonstrates no positive correlation between P-gp expression and a slow response.

Figure 5. *P-glycoprotein expression in lymphoblasts of children with ALL at initial diagnosis, correlated with early response to therapy evaluated from the day 15 BM samples and categorized as M1 (<5% blasts), M2 (5-25% blasts), or M3 (>25% blasts). The results of M2 (open circles) and M3 (closed circles) are combined. The upper normal limit (1%) is indicated by a dotted horizontal line. The median proportion of P-gp positive blasts was 1.2% in M1, and 1.3% in M2+M3.*



FAB morphology and P-gp

At diagnosis, 84 patients (82%) had L1 morphology (>90 L1 cells), 18 (17%) had L2 (≥10% L2 cells), and one patient had L3 morphology. In the relapse group, 12 patients (80%) had L1 morphology and three patients (20%) had L2. The P-gp expression did not correlate with the morphological FAB type.

Karyotype and P-gp

Karyotyping was successful in 98 patients at diagnosis. Thirty-nine were normal, 32 were hyperdiploid, and one was hypodiploid. Eight had a translocation. In addition, 18 patients had multiple or unclassified chromosomal aberrations. The P-gp expression did not correlate with any of the karyotype aberrations.

Outcome and P-gp

Four-year EFS in the whole study population was 77%, and 4-year OS 89%. There was no difference in outcome between the initially P-gp-positive and P-gp-negative patients.

8.3. Biological tempo of ALL: "slow disease" (IV)

Two patient cohorts with very long-term follow-up were studied to characterize "slow disease" ALL. Cohort 1 (n=100) was from 1975-81 and cohort 2 (n=102) from 1989-91. The patient characteristics in cohorts 1 and 2 were comparable; the only difference was in the sex distribution (Table 10).

Table 10. Clinical features of the non-low-risk and very-low-risk groups in cohorts 1 (yrs 1975-81) and 2 (yrs 1989-91).

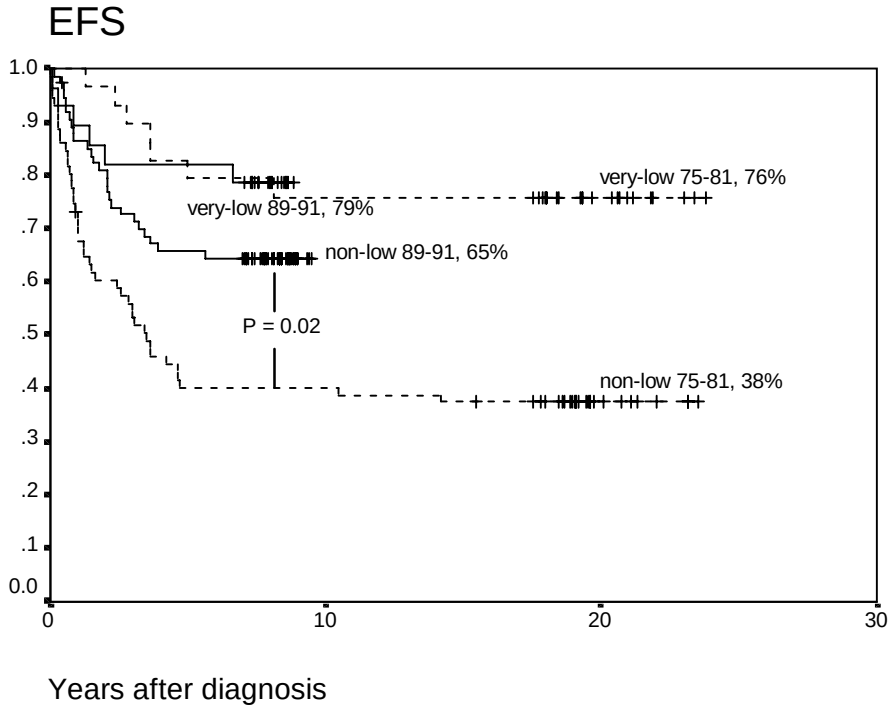
Group	**non-low-risk**		**very-low-risk**	
dg period	1975-81	1989-91	1975-81	1989-91
n	71	74	29	28
WBC (x10^9/l); median (range)	21.9 (1.9-220)	19.1 (0.7-1350)	4.9 (1.2-9.2)	4.0 (1.5-9.4)
age (yrs); median (range)	4.6 (0.1-15.9)	5.4 (0.1-15.8)	4.3 (2.1-9.6)	4.0 (2.2-9.8)
sex (M/F) %	58%/42%	49%/51%	31%/69%	57%/43%
number	41/30	36/38	9/20	16/12
mediastinal mass	8	10	0	0
CNS ALL at dg	1	2	0	0
duration of symptoms; days, median (range)	19.0 (0-120)	14.0 (0-240)	30.0 (0-100)	21.0 (3-150)

In cohort 1, there were 10 toxic deaths (8 infections, 1 gastrointestinal hemorrhage, 1 hepatic failure), of which 8 occurred in the non-low-risk group, and 2 in the very-low-risk group. Furthermore, one child received HR treatment for standard-risk disease for unknown reasons. In cohort 2, three toxic deaths occurred, all infection-related (1 in the non-low-risk group and 2 in the very-low-risk group). The very-low-risk group numbered 29 in cohort 1 and 28 in cohort 2, representing one-fourth of the patient population.

In the very-low-risk group, there was no difference in survival between the cohorts; EFS was 76% in cohort 1 and 79% in cohort 2. The group with non-low-risk disease

had a better outcome in cohort 2 than in cohort 1, EFS being 65% vs. 38% (P = 0.02, log-rank), respectively (Fig 6).

Figure 6. *EFS in very-low-risk and non-low-risk patients in cohort 1 (75-81) and cohort 2 (89-91).*

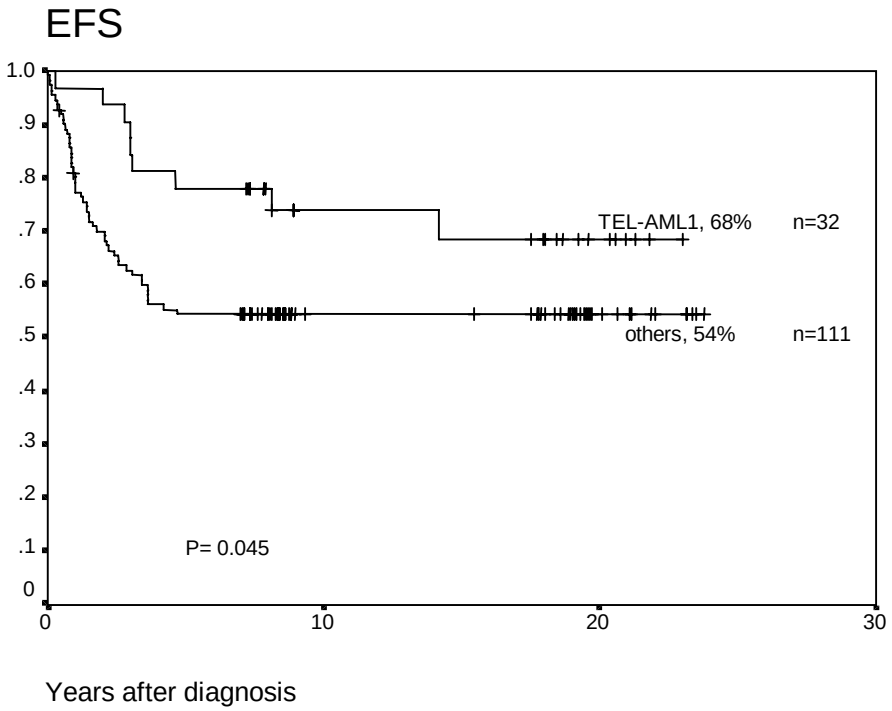


In both cohorts, the median duration of symptoms before diagnosis appeared longer in the very-low-risk group than in the non-low-risk group: 30.0 vs. 19.0 days in cohort 1 (P = 0.81, Mann-Whitney U), and 21.0 vs. 14.0 days in cohort 2 (P = 0.31, Mann-Whitney U), but the difference did not reach statistical significance (Table 10). The duration of symptom in the *TEL-AML1* positive children also appeared longer than in those without the fusion: 21.0 (0-110) vs. 14.0 (0-120) days, respectively (P = 0.48). The main symptoms with long duration (>30 days) were fatigue, loss of appetite, and arthralgia.

8.4. TEL-AML1 fusion (IV)

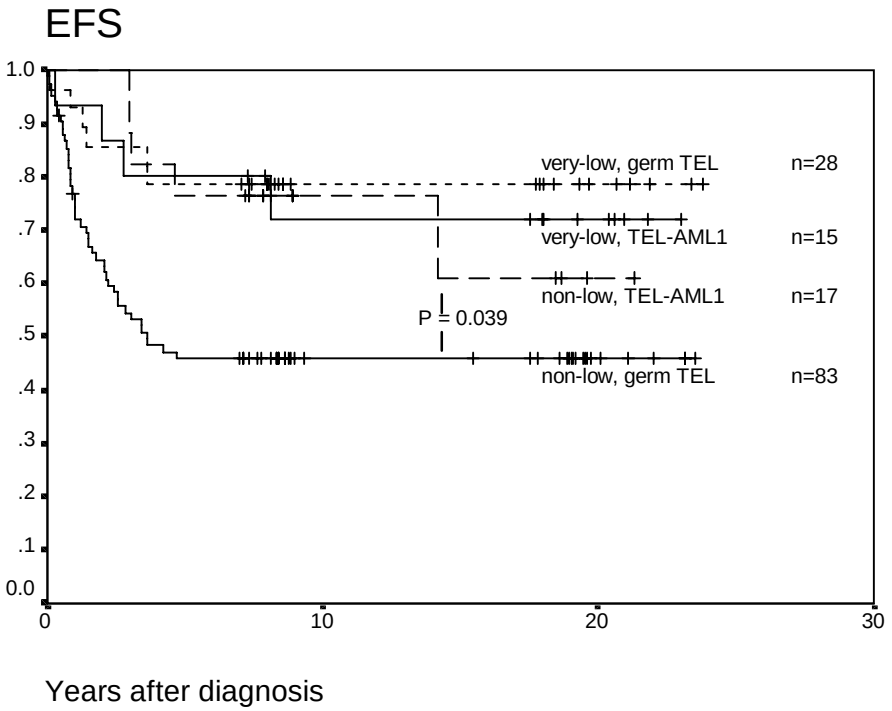
In the biological tempo study, stored diagnostic BM smears of 188 children were available for FISH studies of the *TEL-AML1* fusion. The result was technically reliable in 143 cases. Of these, 32 (22%) were positive for the fusion. *TEL-AML1* was more prevalent in the very-low-risk group (15/43 =35%) than in the non-low-risk group (17/94 =18%) (P = 0.03, chi-square test). Furthermore, the EFS of children with *TEL-AML1* fusion was better than that of those without, 68% vs. 54% (P = 0.045, log-rank) (Fig 7).

Figure 7. *EFS according to TEL-AML1 expression. Uncensored data, both cohorts included.*



However, the impact of the risk group on outcome was clearly more important than that of *TEL-AML1* fusion (Fig 8).

Figure 8. *EFS estimated in 143 children with ALL with successful FISH analysis of the TEL-AML1 fusion. The EFS is shown according to TEL-AML1 expression (germ TEL = no TEL-AML1 fusion) and risk group. P = 0.039 (log-rank) between TEL-AML1 -positive and -negative children in the non-low-risk group.*



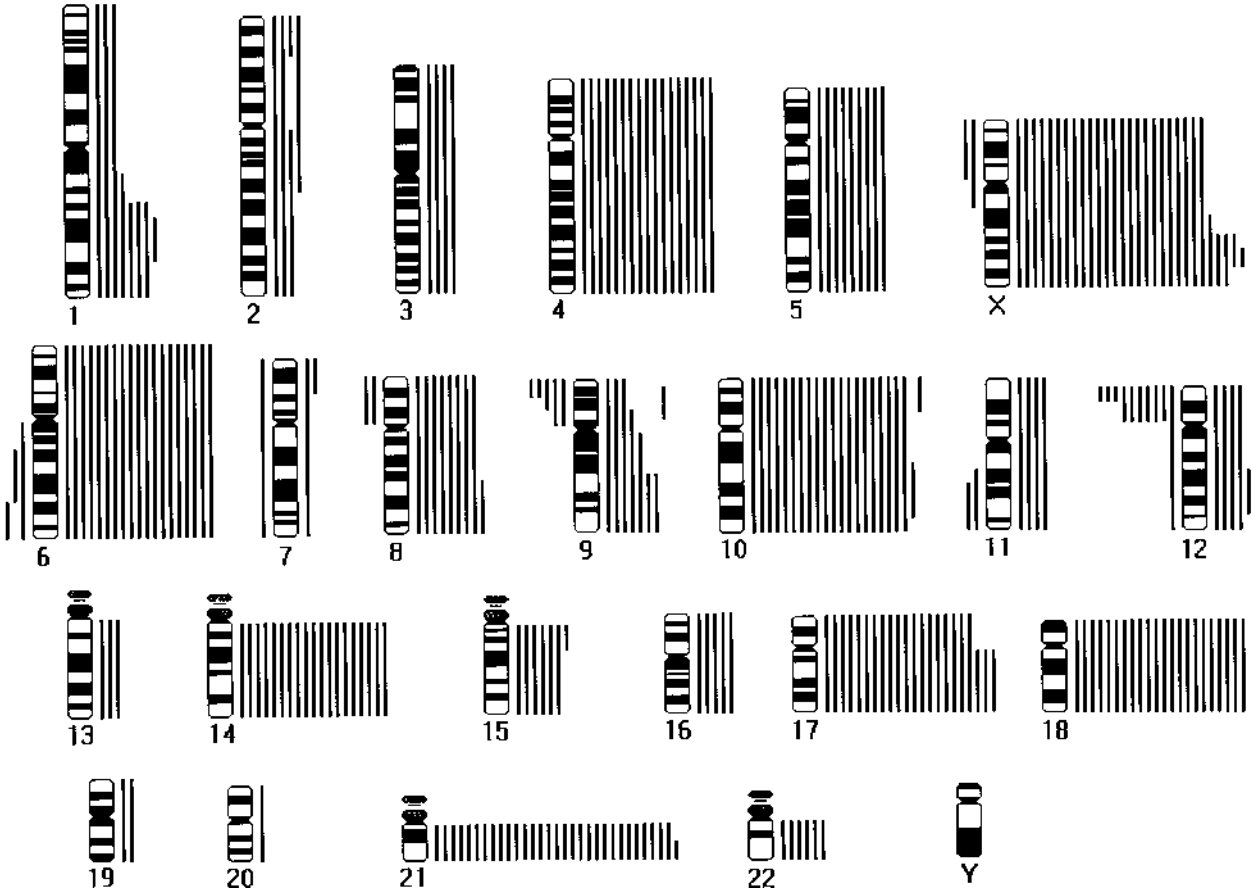
The 59 cases without FISH results, because of unavailable or poor samples, did not differ from the rest, as 14 were very-low-risk and 45 were non-low-risk patients. Of the 59, 37 survive in 1 CR, three in 2 CR, two in 3 CR, and 17 have died (4 toxic deaths).

8.5. Comparative genomic hybridization in childhood ALL (V)

In our CGH study of 79 children with ALL, CGH revealed DNA copy number changes in 57 (72%). In nine of these 57, standard cytogenetics failed to recognize any aberrations, and in an additional 22 CGH gave more detailed information about the chromosomal aberrations present. These findings increased the number of patients with a suitable marker for MRD monitoring from 48 to 57. In three of the 22 with normal CGH results, standard cytogenetics detected aberrations, but these did not affect the DNA copy number and were therefore not detectable by CGH (2 balanced translocations of t(4;11), and 1 inversion in chromosome 11). In two other cases, deletions [del(2), del(9)] were missed by CGH.

Gains were more frequent than losses, and changes of whole chromosomes more frequent than partial aberrations. The findings are summarized in Figure 9. Two partial losses were frequently found: losses in the short arms of chromosomes 9 and 12 (5 and 9 patients, respectively).

Figure 9. *Summary of gains and losses of DNA copy number in 79 children with ALL analyzed by CGH at diagnosis. Gains are shown on the right side and losses on the left of each chromosome. Each line represents a loss or gain in one patient.*



Nine of the 57 children, in whom changes in the DNA copy number were detected by CGH, had a partial deletion in 12p. The deletion was detected by standard cytogenetics in only one of these (Table 11).

Table 11. Clinical features, karyotype, CGH findings, and FISH findings in the nine patients with loss in 12p in the CGH analysis. All nine patients had the B-lineage immunophenotype, L1 morphology, and a good response to induction chemotherapy.

Patient No. (sex/age)*	WBC ($x10^9$/L)	Risk category	Karyotype in bone marrow cells	DNA copy number changes		TEL-AML1 fusion	No. of normal TEL-alleles	Outcome
				losses	gains			
1 (M, 10.3)	4.9	IR	45-46,+mar,inc[cp3]/46,XY[3]	11q21-qter, 12p13-pter	7p15-pter	+	0	CCR
2 (M, 6.6)	6.3	SR	45-46,XY,-12,+1-2mar,inc[cp3] /46,XY[2]	6q24-qter, 12p	Xq25-q26	+	0	CCR
3 (F, 1.8)	99.2	HR	47-48,+2-4 mar,inc[cp5]/46,XX[7]	12p	10p12-pter	+	0	Rel (4.5y), CR2
4 (F, 11.7)	5.8	IR	44-46,-C,+G,inc[cp4]/47,XX,+21c[3]	9p, 12p	8q22-qter, 21	-	0-1	CCR
5 (M, 5.7)	5.5	SR	46, XY[7]	6q, 8p, 12p, Xp	Xq23-q26	+	0-1	CCR
6 (M, 11.2)	5.8	IR	46, XY	12p13-pter	-	+	0-1	Rel (3.7y), CR2
7 (F, 2.2)	21.6	IR	46,inc,?del(12)(p12p13)/46,XX	12p13-pter	-	+	0	CCR
8 (F, 6.0)	8.1	SR	46,XX	12p	-	+	0	CCR
9 (F, 3.2)	1.9	SR	47,XX,+mar/46,XX	12p	-	+	0-1	CCR

SR = standard risk, IR = intermediate risk, HR = high risk, CCR = continuous complete remission, CR2 = second complete remission, Rel = relapse
* = age in years

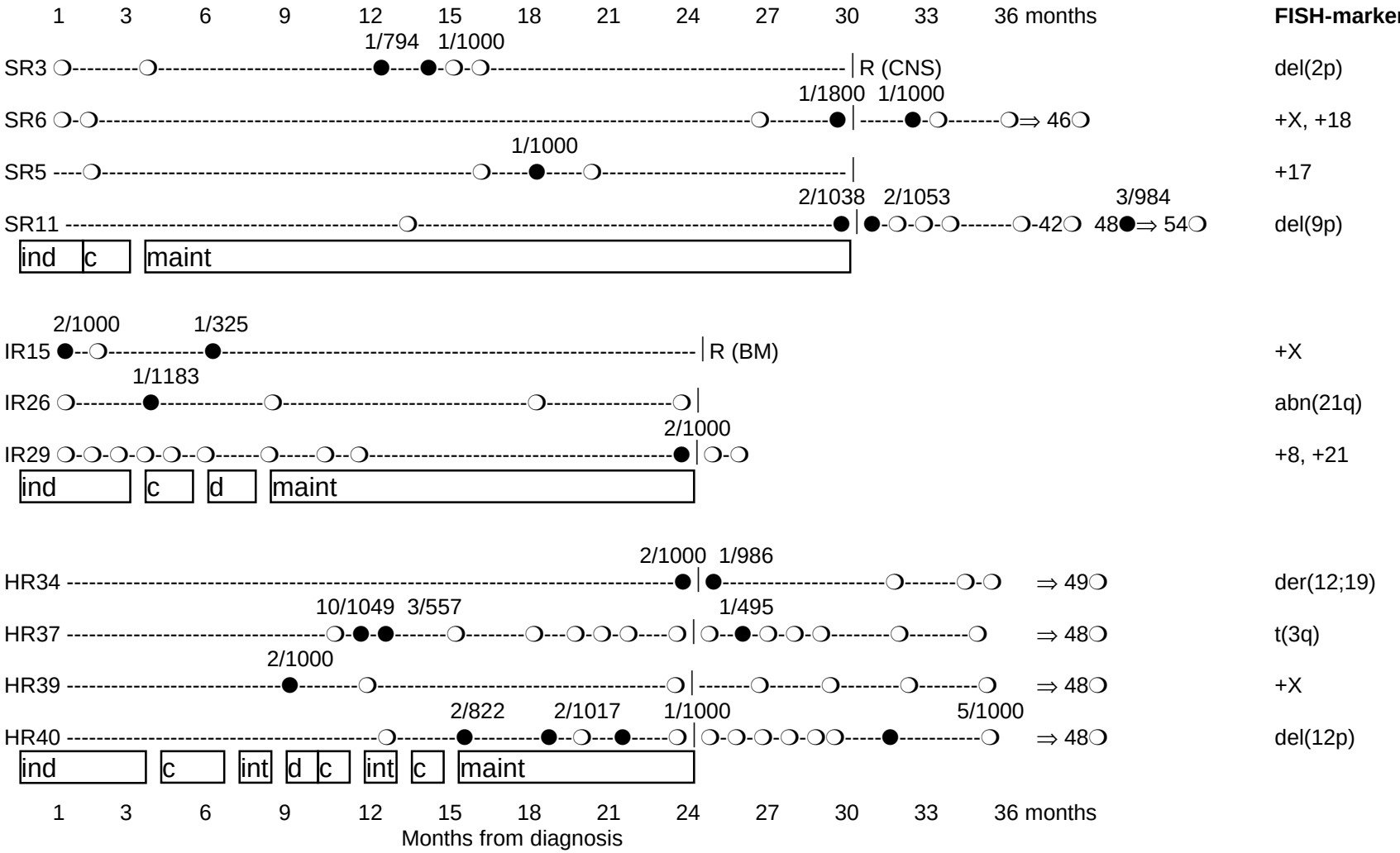
Seven of the nine survive in continuous complete remission (CCR) with a median follow-up of 74 months (range 51-121 months). Furthermore, the early response to treatment (day 15 BM) was optimal (= M1 BM) in all nine patients with the loss in 12p, and all nine had a B-lineage phenotype and L1 morphology, but none was hyperdiploid, and only one of nine had high (>50x10^9/L) WBC at diagnosis.

The nine children with 12p loss were analyzed with FISH for the *TEL-AML1* fusion. The fusion was present in eight out of nine patients. In seven patients, the fusion was present in most (74%-100%), and in one patient (No. 6) in 27% of the interphase cells. In addition, the non-translocated *TEL* allele was deleted in all eight patients. In five patients (Nos. 1-3, 7, and 8) the *TEL* allele was deleted in all cells, and in three patients (Nos. 5, 6, and 9) in 63-64% of the cells with the fusion. One patient (No. 4) did not have the fusion, but had deletion of the other *TEL* allele in 86% of the cells. (Table 11)

8.6. Minimal residual disease (VI)

Eleven of the 41 children studied were FISH-positive at some point after induction, and five of these even after completion of therapy (Figure 10). The proportion of positive cells ranged from 0.1% to 1.0%. Of the 11 children who were FISH-positive after induction, only one had a marrow relapse, detected 24 months after diagnosis. Another patient experienced a CNS relapse, with morphological and cytogenetic remission in the marrow, at 30 months. The other nine remain in CCR, being off therapy for a median time of 30 months (range 3-51 months).
Of the five children who were FISH-positive after cessation of therapy, with a median follow-up of 30 months (range 3-51 months) off therapy, none has relapsed. No action was taken, other than frequent BM sampling, on account of the detected presence of clonal cells.
Of the other 30 children with negative FISH results after induction, two have relapsed in the BM: one on therapy (at 5 months, FISH-negative on days 29 and 45), and one off therapy (at 3 months, FISH-negative at completion of therapy). One additional isolated testicular relapse with a FISH-negative BM occurred after 5 months off therapy. The pre-relapse BM samples of all these four children were reanalyzed after the relapse, but no evidence of the leukemic clone was detected. Of the six children transplanted in 1 CR, two were FISH-positive at transplant. All six are in CCR, with a median follow-up of 32 months (range 18-66) after transplantation.

***Figure 10**. Metaphase-FISH results in 11 patients with MRD detectable post-induction.*



❍, negative; ●, positive; |, end of therapy; R, relapse; BM, bone marrow; CNS, central nervous system; SR, standard risk; IR, intermediate risk; HR, high risk; ind, induction; c, consolidation; maint, maintenance; d, delayed intensification; in, interim maintenance

9. Discussion

9.1. Lymphoblast morphology (I)

In our series of childhood ALL treated with intensive Nordic protocols, lymphoblast L2 morphology was an unfavorable prognostic factor for both early cytoreduction and, in the low-risk group with WBC <50, for ultimate outcome. In this group, multivariate analysis revealed that morphology was an independent prognostic factor, being as strong as cytogenetics and stronger than immunophenotype, sex, age, or mediastinal mass. Examining the group with WBC below 50 $x10^9$/L is particularly important, because this group includes certain patients who need more intensive therapy than they are receiving according to the current risk classification. Our finding could be used as a tool for selecting poor-risk patients from this "standard bulk" or "favorable" group. However, the difference in survival between L1 and L2 may not be considered sufficiently large to justify treatment stratification; use of L1/L2 morphology in stratification would also necessitate centralized morphological analysis, at least on a national basis. It seems, however, that L2-ALL patients with WBC <50 $x10^9$/L do not all do well with minimal standard therapy.

Our series is population-based, comprising all cases of childhood ALL in Finland during a 5-year period. The results of treatment with the Nordic ALL protocols have given a 10-year OS of 78% and an EFS of 70% for those diagnosed in 1986-1991, and a 5-year OS of 86% and an EFS of 78% for those diagnosed in 1992-1996, respectively. The figures for Finland separately are almost identical with NOPHOs overall results (Gustafsson et al. 1998). Stratification of therapy into three risk categories, according to known prognostic factors, is an obstacle regarding analysis of factors not included in the classification. Nevertheless, as L1/L2 morphology was not associated with age, chromosomal aberrations, or the presence of extramedullary leukemia (testicular ALL, CNS ALL), this study enables us to draw certain conclusions. On the other hand, early induction treatment in the three risk groups was similar and of standard type, which is an advantage in the evaluation of the early response. Although the distribution of L2 morphology was similar in all risk categories, the ultimate outcome is certainly treatment-dependent, a fact that has to be taken into account when evaluating the role of L1/L2 morphology in survival. The NOPHO figures for EFS in the SR group and the IR group are 86.6% and 82.1%, respectively. Keeping this in mind, it is worrying that, for L2 children with WBC below 50 $x10^9$/L, the EFS was only 74%, i.e., quite low for SR or IR ALL.

It was Miller and his co-workers in the Children's Cancer Group who first suggested the value of L1/L2 morphology as a prognostic factor. Their results were based on a blinded central review (Miller et al. 1980, Miller et al. 1981, Miller et al. 1985). We have confirmed the finding of Miller and coworkers, the FAB morphology remaining an important prognostic factor even with the intensive therapies used in the 1990s. In 1980, Miller et al suggested that L2 morphology was an adverse prognostic factor in the groups with WBC below 10 and 10-50 $x10^9$/L, but not in the HR group with high WBC, and this matches our findings (Miller et al. 1980). Others have not found this difference in outcome and have been unable to repeat Miller's results (Hammond et

al. 1986, Lilleyman et al. 1992, Kalwinsky et al. 1985, van Eys et al. 1986), or have found that L2 morphology is not independent of other risk factors (Lilleyman et al. 1992). That we found a negative prognostic value of L2 morphology in the patients with WBC <50 but not in those with WBC >50 could be argued to depend on random variation rather than on a true interaction. However, our new data and Miller's old data support each other. Furthermore, a high WBC, over 50 $x10^9$/L, is such a strong indicator of an aggressive disease and high tumor burden that it clearly overrules the effect of other prognostic factors.

The proportion of L2-ALL in the above-mentioned studies ranged from 8 to 18%, which accords with our data, in which 20% were classified as L2-ALL. We wish to emphasize the importance of a central review, which is essential for a uniform morphological classification.

In our study, L2 morphology was not associated with any other adverse prognostic factor. In contrast, the median WBC was lower and hyperdiploidy was more common in the L2 group. T-ALL was slightly, but not significantly, over-represented in the L2 group. We do not know why L2 blasts seem to be more resistant to anticancer agents than L1 blasts. The biological basis of these morphological differences and their impact on the response to treatment still remains to be determined.

In adult ALL, in contrast to childhood ALL, L2 morphology is a dominant feature. In a German series of 471 patients, L2 comprised 68% of the cases, the proportions of L1 and L3 being 27% and 5%, respectively (Loffler et al. 1987). There is no consensus regarding the prognostic significance of L2 morphology in adult ALL (Hoelzer 1995).

In contemporary ALL protocols, L1/L2 morphology is not used for treatment stratification. The main reason for this is the conflicting results of studies assessing the prognostic value of morphology, and also the difficulties of organizing a centralized morphological review.

9.2. Multiple drug resistance (II, III)

According to our data, 53% of the children with newly diagnosed ALL had P-gp expression in more than 1% (= mean + 3SD of the control subjects) of their blasts. Even in these children, however, the median percentage of P-gp-positive blasts was low (2.4%), the upper range being of less than 20%. In the relapse group the proportion of P-gp-positive patients was not higher than among the newly diagnosed children. Grouping of patients as P-gp-positive (>1% cells positive) and P-gp-negative may be somewhat arbitrary, because the distribution of P-gp expression is continuous rather than bimodal.

P-gp expression has been demonstrated in several childhood cancers (leukemia, lymphoma, neuroblastoma, sarcoma, and retinoblastoma), and has been associated with a poor prognosis (Chan et al. 1995, Fojo et al. 1987, O'Meara et al. 1992). Studies have been performed at the DNA, mRNA, protein, and functional levels. These different methods produce results that are not easy to compare. Demonstrating *mdr1* expression at the nucleic acid level does not signify that P-gp is

actually produced. Detection of P-gp is not solid evidence for a functional protein, and there have been substantial problems in P-gp detection by immunocytochemistry and FC (Beck W.T. et al. 1996). Functional assays on clinical samples may detect other mechanisms of MDR, and have produced conflicting results. However, these assays may be helpful if used together with P-gp detection (Beck W.T. et al. 1996, Del Poeta et al. 1996, Tafuri et al. 1995, Xie et al. 1995, Ivy et al. 1996). We used flow cytometric detection of P-gp with the monoclonal antibody JSB1, directed to a highly conserved intracellular epitope of P-gp (Scheper et al. 1988); therefore the cells had to be permeabilized. Our novel permeabilization method does not alter the light-scattering characteristics of the lymphoblasts, thus allowing flow cytometric analysis of different cell populations. There are several other antibodies directed to either internal or external epitopes of P-gp, but none of these antibodies has been shown to be superior to the others (Lehne et al. 1995). External antibodies, such as MRK-16, may have the advantage of being more sensitive and allowing simultaneous measurement of P-gp function (Beck W.T. et al. 1996). We and other investigators have previously used JSB1, obtaining good concordance with other antibodies (Del Poeta et al. 1996, Goasguen et al. 1993, Tiirikainen et al. 1992). There is still a need for new studies of MDR in childhood ALL. In these future studies, the employment of the consensus guidelines for P-gp detection (Beck W.T. et al. 1996) will be extremely important for drawing conclusions from reliable and comparable results.

The level of P-gp expression has been found to be higher in adults than in children and higher in AML than in ALL (Leith et al. 1999, Legrand et al. 1999, Ludescher et al. 1995, Campos et al. 1992). In contrast, Beck (Beck J. et al. 1996) and den Boer (den Boer et al. 1998) detected lower P-gp expression in children with AML than with ALL. In the different studies of childhood ALL, the percentage of P-gp-expressing cells that is considered positive ranges from 1 to 20% (Sauerbrey et al. 1994, Brophy et al. 1994). We chose our level of 1% by extrapolation from the control series (mean + 3SD). With that choice, approximately 50% of our patients were classified as positive. A very similar cut-off limit has been obtained for adult control subjects (Tiirikainen M. I. et al. 1992). However, the level of P-gp expression that has clinical significance in ALL is so far unknown.

Using the 1% cut-off level, we found no difference between P-gp-positive and P-gp-negative patients either regarding the early response to therapy, or the ultimate outcome. Furthermore, the 15 children studied at relapse did not have higher P-gp expression than the newly diagnosed patients. Changing the cut-off to 2 or 5% did not alter our results. This is noteworthy, because our findings differ from those of some other reports. Goasguen and coworkers reported a series of 36 children, of whom 12 were P-gp positive at the >1% level. The P-gp-positive patients had a higher relapse rate, a shorter duration of remission, and a shorter median survival time (Goasguen et al. 1993). Sauerbrey and coworkers made a retrospective study of a series of 104 children with newly diagnosed ALL. Their P-gp-positive patients had a lower probability of remaining in first CCR than the P-gp-negative patients (Sauerbrey et al. 1994). Recently, Dhooge and coworkers reported a prospective study of 102 *de novo* ALL children. P-gp-negative children had significantly better EFS than those with P-gp expression (Dhooge et al. 1999). However, many studies have not been able to show that P-gp has prognostic value in childhood ALL (Pieters et al. 1992, Pieters et al. 1994, Ubezio et al. 1990, den Boer et al. 1998). Neither has

it been shown that other mediators of drug resistance, such as LRP or MRP, are important in childhood ALL.

ALL induction therapy was started with two agents, which are targets for P-gp-mediated drug resistance: vincristine and doxorubicin. However, even in our P-gp-positive group of patients, the proportion of lymphoblasts expressing P-gp was quite low (median 2.4%). Thus, even if all the P-gp-positive blasts had been left in the marrow on day 15, the morphological response might have been considered good (i.e. less than 5% blasts in the marrow). There may not have been enough time for the P-gp-positive clone to expand. Furthermore, the decisive factor may have been the response to prednisone, which was the third agent in the early induction therapy.

9.3. Biological tempo of ALL (IV)

With relatively simple criteria (WBC, age, Hb, and absence of lymphomatous features), we were able to identify a group of patients with ALL for whom the long-term prognosis is very good. Importantly, this favorable outcome was achieved already in the 70s without intensive therapy and, for example, without anthracyclines. The very-long-term follow-up of these patients, 20 years in cohort 1 and 9 years in cohort 2, indicates that the high EFS (76% and 79%, respectively) did not decrease with time because of late relapses, which are otherwise well-known to occur in low-risk ALL, also called “slow disease” (Margolin and Poplack 1997).

The number of toxic deaths was 10 in cohort 1, as opposed to only 3 in cohort 2. This is explained by the evident improvement of supportive care between the 1970s and the 1990s, including transfusion of blood components, broad-spectrum antibiotics, prophylactic cotrimoxazole, and myeloid growth factors.

For comparison, the current Nordic data from 1992-98 for SR ALL gives a 5-year EFS of 86.6% (Gustafsson et al. 2000). If the data are balanced (excluding the toxic deaths far more frequent in the earlier era), there is virtually no difference in EFS: 81% for cohort 1, 83% for cohort 2, and 86% for the most recent Nordic data.

One problem with the current ALL chemotherapy is certainly over-treatment of a proportion of standard-risk patients. The difficulty lies in identifying this “very-low-risk“ group of patients, which should not be unnecessarily exposed to potentially serious adverse effects. Acute toxicity due to anthracyclines in the induction therapy may give rise to more profound neutropenia and febrile infections, and, in the long run, to late cardiotoxicity. The present study suggests that a very favorable group can be selected using simple, well-established criteria. Today, we have additional tools for risk stratification; we are able to exclude cases with a poor prognosis on the basis of unfavorable cytogenetics or a slow response to therapy (Martinez-Climent 1997, Steinherz et al. 1996).

We were unable to find any bias or factor responsible for the very favorable outcome in our very-low-risk group from the earlier time period. Specifically, the higher proportion of females in this group was not the reason, because the girls did not do better than the boys in this series. The better outcome for boys in cohort 1 might be explained by the testicular irradiation; no explanation is available for the later cohort.

In the 70s, almost every patient with ALL (90%) received cranial irradiation. In the current Nordic protocols, most children receive high-dose MTX instead of cranial irradiation, and only HR patients over 5 years of age are prophylactically irradiated with 18 Gy. These patients also receive HD-MTX and HD-ARA-C. Yet the incidence of isolated CNS relapses has not been thereby increased (5% in cohort 1 vs. 2% in cohort 2).

In comparing the duration of symptoms in the very-low-risk and non-low-risk groups, we could discern a trend toward longer duration in the very-low-risk group. However, the retrospective nature of our study may have hampered this evaluation. The main symptoms of long duration (>30 days) were fatigue, loss of appetite, and arthralgia.

Our results indicate that, although major improvements have occurred in the outcome of childhood ALL in general and in the IR group in particular, not much improvement has occurred during the past 20 years among the children with very-low-risk ALL who, in the late 1970's, already had a very good prognosis with minimal standard therapy. Efforts should be made to identify this group and to keep their therapy at the minimum necessary, while increasing the therapeutic intensity in the other risk categories.

9.4. TEL-AML1 fusion

We had a unique opportunity to study *TEL-AML1* fusion with FISH on BM smears stored from the 1970s and early 1990s. The higher frequency of *TEL-AML1* fusion in the very-low-risk group is in accord with previous reports, suggesting the association of this translocation with a favorable outcome (Borkhardt et al. 1997, McLean et al. 1996, Rubnitz et al. 1999, Seeger et al. 1998). *TEL-AML1* fusion and its association with a good prognosis, although with late relapses ("slow disease"), appears as a promising supplementary feature in identifying children with low-risk disease (Shurtleff et al. 1995, McLean et al. 1996).

In our series, the prognostic impact of the *TEL-AML1* fusion gene was outweighed by traditional low-risk criteria (WBC, age, Hb). However, in contrast to the very-low-risk ALL and the "slow disease", *TEL-AML1* seemed to have prognostic importance in the non-low-risk "rapid disease". The more intensive therapy for cohort 2 has made this difference due to *TEL-AML1* apparent, as the OS of non-low-risk children has also improved from 41% to 75% through the 15 years. Our very-long-term follow-up data support the reported association of *TEL-AML1* fusion with a favorable prognosis, especially in non-low-risk ALL.

9.5. Comparative genomic hybridization in childhood ALL (V)

In our CGH study, loss of material in the short arm of chromosome 12 was detected by CGH in nine out of 79 ALL patients (11%). This frequency is much higher than the 2-3% reported in studies employing standard cytogenetics (Raimondi 1993, Chessels et al. 1997), but it also differs from a comprehensive CGH study, in which no losses

at 12p were detected in 72 children studied (Paszek-Vigier et al. 1997). Only one of our nine patients with loss at 12p was found to have a deletion by G-banding.

The resolution of CGH ranges from 3 to 10 megabases, suggesting that even the smallest losses detected may involve several genes. Because of the high frequency of *TEL-AML1* fusion in pediatric ALL and its association with deletion of the other *TEL* allele, we studied the patients with 12p loss by FISH. All nine cases had the deletion and eight of the nine harbored the *TEL-AML1* fusion. This does not exclude the possibility of loss of other important genes in the region. In fact, Takeuchi et al. (1996) have detected a frequent deletion at 12p very close to, but not including, the *TEL* gene (Takeuchi et al. 1996).

In the present study, loss in 12p seemed to be associated with established favorable prognostic features. The cases with 12p loss showed a trend toward a better overall survival, and also had a good early response to therapy and L1 morphology. All had the B-lineage immunophenotype.

Our novel finding of a trend toward a favorable outcome in cases with 12p loss is likely to be related to the cryptic translocation t(12;21) and the *TEL-AML1* gene fusion (Loh et al. 1998, Rubnitz et al. 1997, Borkhardt et al. 1997). However, the prognostic role of *TEL-AML1* fusion has not yet been elucidated. The discrepant findings of various studies may be related to different treatment protocols (Seeger et al. 1998). Our findings on the association of *TEL-AML1* fusion and deletion of the normal alleles support the suppressor role of the *TEL* gene (Cave et al. 1995, Takeuchi et al. 1996).

When employed together with other methods (G-banding, PCR, FISH), CGH appears to be a valuable tool in screening for chromosomal aberrations of stratificatory or prognostic value in childhood ALL as well as in screening for clonal genetic markers. The aberrations found with CGH can be used in MRD monitoring. In the present series, CGH increased the percentage of patients in whom markers were found for FISH-based MRD monitoring from 61% to 72%. Screening pediatric ALL patients for t(12;21) at diagnosis may be performed either with CGH or with specific FISH or PCR probes. We advocate CGH initially as a broad screen, and FISH specifically for the *TEL-AML1* fusion.

9.6. Minimal residual disease (VI)

Our FISH data indicate that, despite the presence of mitotically active blasts from the original leukemic clone during or after treatment, the vast majority of the children remained in hematological remission even though no action was taken to intensify or continue the treatment. Eleven of the 41 children in our series were FISH-positive at the level of 10^{-3} after induction, and only one of these has experienced a BM relapse. All five children with FISH-positive cells (at the level of 10^{-3}) in the BM after cessation of therapy, have remained in CCR. Our data are in agreement with those of Roberts and co-workers (Roberts et al. 1997), who reported a follow-up of 24 children studied by PCR and clonogenic blast-colony assay. Of the 17 children who stayed in remission, 15 had MRD detectable by PCR at the level of 10^{-3} - 10^{-5} and 5 MRD

detectable by both PCR and the blast-colony assay up to 35 months after completion of therapy.

Van Dongen and co-workers monitored 240 children with ALL by PCR analysis of immunoglobulin and T-cell receptor gene rearrangements and *TAL1* deletions at several time points during and after treatment. The MRD-positive ($\geq 10^{-6}$ level) children had relapse rates five- to ten-fold higher than the MRD-negative children. Yet, in their series, 9% of those remaining in CCR were MRD-positive after consolidation treatment, and two of the eight children positive after completion of therapy stayed in CCR (van Dongen et al. 1998).

As evidenced by the data of Roberts and co-workers, it appears that ”cure” may not be synonymous with eradication of the leukemic clone (Roberts et al. 1997). This is also supported by the limited data of Nizet and Wu, who studied ALL patients after completion of therapy and found some who remained in CCR (follow-up for up to 102 months) with MRD detectable (Nizet et al. 1993, Wu et al. 1996).

The sensitivity with which MRD can be detected varies from one method to another; being roughly 10^{-6} with PCR, 10^{-4} with FC, and 10^{-3} with FISH. However, patients in CCR but with detectable MRD have been demonstrated by all these methods.

Consequently, our data support the hypothesis that, in ALL patients, mechanisms other than the direct effects of chemotherapy may be involved in maintaining prolonged remissions. These could conceivably include alterations in the factors controlling the growth of the leukemic clone (Roberts et al. 1997), immunological effects (Montagna et al. 1995) or effects of the marrow microenvironment (Bradstock and Gottlieb 1995).

The presence of mitotic leukemic cell clones in the BM during or after ALL treatment does not invariably lead to a relapse. The presence of MRD during the course of childhood ALL, although an important parameter, needs to be interpreted with caution.

9.7. General discussion and future aspects

The use of various prognostic factors for treatment stratification has undoubtedly improved the treatment results and, moreover, decreased the late effects of therapy. However, in spite of the development of sophisticated laboratory methods, WBC at diagnosis and the tumor burden as a whole, as well the age of the patient, have retained their importance. Immunophenotyping and especially cytogenetics, both at the traditional G-banding and, more recently, molecular levels, provide valuable adjuncts to the risk classification. The morphological response monitoring, both after the steroid prephase in the peripheral blood and after multidrug induction in the BM, has proved useful for detecting patients with a high risk for relapse. It has also been possible to improve the outcome of the patients with a slow early response by intensification of treatment. The more recently employed submicroscopic minimal residual disease monitoring indicates that slow disappearance of the malignant cells, or their persistence, is a sign of an increased risk of relapse. However, it has also been shown that long-term remissions are possible despite the persistence of

submicroscopic levels of leukemia in the patient, indicating that host-disease interactions can regulate the emergence of overt hematological relapse.

As an example, in the new NOPHO-ALL 2000 protocols, the treatment response will be monitored more intensively than before. The morphological response will be employed for treatment stratification even within the “intensive therapy” group to sort out those children who, to be cured, need extremely intensive therapy. MRD monitoring by PCR and FC will be incorporated in the protocols systematically, and, in the future, the treatment of those with persistent or increasing MRD will possibly be intensified.

The enormous development in the field of molecular cytogenetics may offer another way for improving the treatment stratification. In the near future, we will be able to find genetic aberrations in every patient with leukemia, which will make it possible to assess the actual value of these aberrations as prognostic factors. Furthermore, the micro-array technology may enable us to recognize which genes are important for the pathogenesis and biology of leukemias. With this information, it will probably be possible to design novel therapeutic agents and strategies.

While MRD and certain genetic aberrations have emerged as new prognostic factors, the value of other new factors, such as drug resistance, has not yet been determined. The various mediators of drug resistance do not seem to be important in childhood ALL. The results of *in vitro* resistance assays have relatively good correlations with the clinical outcome, but it still remains to be seen whether treatment modifications according to resistance profiles lead to better outcomes.

In the field of MRD monitoring, the next important step is to study the effects of treatment modifications based on MRD findings. According to recent studies, it seems rational to intensify treatment in patients with persistent MRD, but we probably do not have enough data to justify decreasing the treatment intensity in patients with low or negative MRD levels. There is also a need for large prospective comparative studies of FC and PCR in MRD monitoring.

The most important factor influencing the ultimate outcome is the treatment itself. Intensification of treatment has already changed the importance of certain factors, such as age and immunophenotype. The adoption of new treatment modalities, including antisense oligonucleotides, for instance against antiapoptotic *BCL-2*, immunotherapy with cytotoxic T lymphocytes, and such monoclonal antibodies as Campath-1H against the CD52 antigen or rituximab, cell cycle checkpoint inhibitors, and inhibition of transcription factors may lead to even more changes in the risk classification. A recent example is the tyrosine kinase inhibitor STI571 (imatinib), which blocks the signal transduction of the BCR-ABL fusion protein. Good clinical responses have been observed in CML and Philadelphia chromosome-positive ALL. When targeted therapy becomes possible, the prognosis of certain subtypes of ALL may improve markedly.

10. Conclusions

The aim of this study was to find and characterize new prognostic factors and at the same time to analyze the value of established factors in the setting of contemporary intensive therapy. Our study population comprised several cohorts of Finnish children with ALL from 1975 to 2000. The studies of morphology (n=251) and P-glycoprotein (n=118) were population-based. In the biological tempo and *TEL-AML1* study, the cohort from 1975-81 (n=100) was from the Children's Hospital, University of Helsinki, but the cohort from 1989-1991 (n=102) covered the whole country. The patients (n=79) in the CGH study were from three oncology centers: Helsinki, Kuopio, and Tampere. The MRD study was a single center project (n=41). The main conclusions of this study are:

1. In the group of children with WBC <50x10^9/L, L2 morphology was associated with an unfavorable outcome, which may indicate that such children need more intensive treatment than they receive according to the contemporary Nordic risk classification. Difficulties in organizing a centralized morphological review have reduced the applicability of morphology in treatment stratification.
2. P-gp expression was associated with T-lineage ALL, but not with other unfavorable features. Nor was it a prognostic factor for early response to treatment or for the ultimate outcome. Our results support previous findings that mechanisms other than P-gp are more important for drug resistance in childhood ALL.
3. According to our study, children with "very-low-risk" ALL or "slow disease" (WBC <10x10^9/L, Hb <90g/L, age 2 to <10 years) already had a favorable outcome, with EFS of 76% in the 1970s, before anthracyclines were employed in their treatment. This indicates that, with contemporary protocols, a proportion of the low-risk children is certainly over-treated. However, we still need more reliable tools for identifying those children who would benefit from downgrading their treatment.
4. *TEL-AML1* fusion was more common in the very-low-risk group than in the other patients. The fusion was associated with a better outcome in the whole study population and especially in the non-low-risk subgroup.
5. CGH, when added to standard G-banding, increased the number of patients with detectable genetic aberrations. Notably, the frequency of losses at 12p was higher than previously described, nine of 79 patients. This loss was associated with favorable clinical and laboratory features, as well as with the *TEL-AML1* fusion. We advocate CGH as part of the screening of genetic aberrations in childhood ALL.
6. The main finding in the MRD study was that, of 41 children, 11 had detectable leukemic cells in the BM at some point post induction, and, in five cases, even after completion of therapy. In spite of the persistence of the malignant clone, nine of these children remain in CCR and none of the five who were still positive after completion of therapy has experienced a relapse. This finding indicates that permanent cure of children with ALL may not require eradication of all malignant cells from the body.

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