Optimizing Chemotherapy in Childhood Acute Myeloid Leukemia

JOSEFINE PALLE
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Abstract

Despite major advances in our understanding of the biology of childhood acute myeloid leukemia (AML) and the development of new cytotoxic drugs, the prognosis of long-term survival is still only 60-65 %.

In the present research, we studied the pharmacokinetics of drugs used in the induction therapy of childhood AML and performed in vitro drug sensitivity testing of leukemic cells from children with AML.

The aims of the studies were to correlate the results of the analysis to biological and clinical parameters and to identify subgroups of AML with specific drug sensitivity profiles in order to better understand why treatment fails in some patients and how therapy may be improved.

Blood samples were analysed to study the pharmacokinetics of doxorubicin (n=41), etoposide (n=45) and 6-thioguanine (n=50). Doxorubicin plasma concentration and total body clearance were correlated to the effect of induction therapy, and doxorubicin plasma concentration was an independent factor for complete remission, both in univariate and multivariate analysis including sex, age, and white blood cell count at diagnosis. For etoposide and 6-thioguanine no correlation was found between pharmacokinetics and clinical effect. Children with Down syndrome (DS) tended to reach higher blood concentrations of etoposide and thioguanine nucleotides, indicating that dose reduction may be reasonable to reach the same drug exposure as in children without DS.

Leukemic cells from 201 children with newly diagnosed AML, 15 of whom had DS, were successfully analysed for in vitro drug sensitivity by the fluorometric microculture cytotoxicity assay (FMCA). We found that samples from children with DS were highly sensitive to most drugs used in AML treatment. In non-DS children, the t(9;11) samples were significantly more sensitive to cytarabine (p=0.03) and doxorubicin (p=0.035) than other samples. The findings might explain the very favorable outcome reported in children with DS and t(9;11)-positive AML. A specific drug resistance profile was found for several other genetic subgroups as well. A detailed study of MLL-rearranged leukemia showed that cellular drug sensitivity is correlated both to partner genes and cell lineage, findings that support the strategy of contemporary protocols to include high-dose cytarabine in the treatment of patients with MLL-rearrangement, both in AML and acute lymphoblastic leukemia (ALL).

Our results indicate that drug resistance and pharmacokinetic studies may yield important information regarding drug response in different sub-groups of childhood AML, helping us to optimize future chemotherapy in childhood AML.

Keywords: childhood, acute myeloid leukemia, drug resistance, pharmacokinetics, chromosomal abnormalities, Down syndrome, MLL-rearrangement, t(9;11)

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To all the brave and admirable children who struggle with acute myeloid leukemia.
The Nordic centres that contributed to the studies.

The NOPHO centres of Paediatric Haematology and Oncology
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<th>Definition</th>
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<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
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<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BSA</td>
<td>Body surface area</td>
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<tr>
<td>CBF</td>
<td>Core binding factor</td>
</tr>
<tr>
<td>2-CdA</td>
<td>2-Chlorodeoxyadenosine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CR</td>
<td>Complete remission</td>
</tr>
<tr>
<td>CCR</td>
<td>Continuous complete remission</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DS</td>
<td>Down syndrome</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FAB</td>
<td>French-American-British (classification)</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in-situ hybridisation</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>GO</td>
<td>Gemtuzumab ozogamicin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>Inv</td>
<td>Inversion</td>
</tr>
<tr>
<td>ISCN</td>
<td>International System for Human Cytogenetic Nomenclature</td>
</tr>
<tr>
<td>6-MP</td>
<td>6-Mercaptopurine</td>
</tr>
<tr>
<td>ML-DS</td>
<td>Myeloid leukemia of Down syndrome</td>
</tr>
<tr>
<td>MLL</td>
<td>Mixed lineage leukemia</td>
</tr>
<tr>
<td>MRD</td>
<td>Minimal residual disease</td>
</tr>
<tr>
<td>MTT</td>
<td>Methyl-thiazol-tetrazolium</td>
</tr>
<tr>
<td>N</td>
<td>Number of patients</td>
</tr>
<tr>
<td>NOPHO</td>
<td>The Nordic Society of Pediatric Haematology and Oncology</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>p-EFS</td>
<td>Probability of event-free survival</td>
</tr>
<tr>
<td>p-DFS</td>
<td>Probability of disease-free survival</td>
</tr>
<tr>
<td>p-OS</td>
<td>Probability of overall survival</td>
</tr>
<tr>
<td>RD</td>
<td>Resistance disease</td>
</tr>
<tr>
<td>SI</td>
<td>Survival Index</td>
</tr>
<tr>
<td>SCT</td>
<td>Stem cell transplantation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SE</td>
<td>Standard error of the mean</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>t</td>
<td>Translocation</td>
</tr>
<tr>
<td>TGN</td>
<td>Thioguanine nucleotides</td>
</tr>
<tr>
<td>TCK</td>
<td>Total cell kill</td>
</tr>
<tr>
<td>6-TG</td>
<td>6-Thioguanine</td>
</tr>
<tr>
<td>TPMT</td>
<td>Thiopurine S-methyltransferase</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cell</td>
</tr>
<tr>
<td>WT 1</td>
<td>Wilms tumor gene 1</td>
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</table>
Cancer is rare in childhood but is still the second most common cause of death in this age-group next to accidents. Every year 250-300 children in Sweden, with a population of nine million inhabitants, are diagnosed with cancer and about one third of these have leukemia.\textsuperscript{1} The two most common forms of leukemia are acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). In children ALL is the most common form, comprising about 80 per cent of the cases, while AML comprises about 15 per cent. The contrary is true for adults.

The prognosis for childhood AML has improved significantly during the recent decades. Many treatment groups now achieve complete remission (CR) rates of 80\% to 90\%, relapse rates of 30\% to 40\%, event-free survival (EFS) rates of 50\%, and overall survival (OS) rates of 60\%.\textsuperscript{2-10} However this can only be achieved by using very intensive chemotherapy, which might result in relatively high rates of treatment-related complications, especially infections and significant late effects. The improvement of the supportive care, including generous and intense treatment of infections has therefore been an important cornerstone in making the child cope with the treatment.\textsuperscript{11,12}

Efforts are also being made to identify new cytotoxic drugs with more specific anti-leukemic effects and several phase III studies for such drugs are ongoing.\textsuperscript{13} In addition, more precise risk-group stratification, which would allow more tailored and further refined sub-group directed treatment, is being discussed. The detection of AML-specific molecular abnormalities, minimal residual disease monitoring and the identification of pharmacogenomics are a few examples.

Hopefully the increase in understanding of the biology of childhood AML, together with the continuous development of new, more “AML-specific” drugs, and even better supportive care, will help us to improve the treatment and survival of the affected children.
Acute myeloid leukemia (AML)

Epidemiology and aetiology

The annual incidence of AML in the Nordic countries was 0.7/100 000 children aged 1-<15 years at diagnosis 1985-2005. There is an incidence peak during the first 2 years of life, after which there is a decrease to a minimum at 10 years of age followed by an increase in adolescence and adulthood. AML is a heterogeneous disease and may arise either de novo or secondary to underlying disease, most commonly congenital syndromes such as Down syndrome (DS) (Figure 1). AML may also arise as a second malignancy after treatment with chemotherapeutic agents and ionizing radiation.

![Figure 1. Incidence of de novo childhood AML in the Nordic countries 1985-2001.](image)

Classification

In 1976 the French-American-British (FAB) cooperative group produced the first systematic classification for AML, the FAB-classification. This system is based mainly on morphologic characteristics and cytochemical staining patterns of bone marrow blasts, and has been repeatedly revised to incorporate diagnostic advances in immunophenotyping and cytogenetics. It is still a well-known and widely used model for classification AML. In 2002 the World Health Organization (WHO) re-classified the hematopoietic and lymphoid malignancies. This classification divides AML in four different subgroups: 1) AML with recurrent genetic abnormalities 2) AML with multilineage dysplasia 3) Therapy-related AML and 4) AML not otherwise categorized.
Both the FAB- and WHO classifications are based on reviews of adult cases. The latter has not been fully accepted by pediatric hematologists because it does not mention the unique features of AML in children with DS and because the blast threshold to diagnose AML was lowered from 30 to 20\%.\textsuperscript{21}

Genetic abnormalities in AML
Available evidence indicates that AML is the consequence of collaboration between at least two different types of pathological genetic events.\textsuperscript{22,23} Type I abnormalities confer a proliferative and/or survival advantage of the leukemic cell while type II abnormalities/mutations primarily serve to impair hematopoietic differentiation.\textsuperscript{24,25} This two-hit process leads to maturation arrest, proliferation advantage and the development of leukemia. The type I abnormalities are traditionally detected by cytogenetics,\textsuperscript{26} and by newer techniques such as fluorescence in situ hybridization (FISH) and reverse transcription-polymerase chain reaction (RT-PCR). They produce oncogenic fusion proteins as a consequence of structural abnormalities such as t(8;21), t(15;17), t(9;11) or inv(16). The type II mutations, for example FLT3, c-KIT and RAS-oncogenes, have more recently been discovered,\textsuperscript{27-30} and they are usually detected with molecular methods such as PCR.

The type I abnormalities can be subdivided into numerical and structural karyotype chromosomal abnormalities. A normal karyotype is identified in 20-30 per cent of children with AML compared to 40-50 per cent in adults. Numerical abnormalities are rare in childhood AML, the most common being trisomy 8, trisomy 21, loss of a sex chromosome associated with t(8;21) and FAB M2, monosomy 7 and partial deletion of the long arm of chromosome 7(7q-).\textsuperscript{31,32} In total, these account for 10 per cent of cases.\textsuperscript{33} The structural abnormalities are frequent in childhood AML and often related to morphology and FAB types; t(8;21) is seen in M2, t(15;17) is seen in > 90\% of M3 cases, inv(16) or t(16;16) is seen in M4Eo and the11q23 rearrangements correlate to M4 and M5.\textsuperscript{34} Structural abnormalities involving chromosome band 11q23 are among the most common genetic abnormalities in acute leukemia and are often associated with diverse leukemia subtypes including ALL and AML. In most instances there is a reciprocal translocation involving the MLL gene, mapped in the 11q23 region, and one of over 30 genes located in distinct chromosomal loci.\textsuperscript{35} Rearrangements of MLL are seen in up to 20\% of cases of AML, although the frequency varies among studies.\textsuperscript{36,37} Patients with 11q23 abnormalities tend to be young and to have high WBC counts at diagnosis.
Down syndrome

Children with DS have a significantly increased risk of developing myelo-dysplastic syndrome (MDS) and AML, especially before 5 years of age. MDS and AML in DS children are biologically distinct and characterized by different clinical behavior compared to non-DS children and the unifying term myeloid leukemia of DS (ML-DS) has been used for this disorder. For ML-DS the age at diagnosis is low, about 50% of the patients are diagnosed at 1 year of age, about one third at 2 years of age and only 1-2% at more than 4 years of age. A period of thrombocytopenia often precedes the diagnosis and there is a predominance of megakaryoblast leukemia, M7. They have a high frequency of GATA 1 mutations and a superior outcome when treated on modified AML protocols. Most recently published NOPHO data on DS patients show a 10 year p-EFS of 0.83. The favorable outcome in ML-DS has been explained by the increased sensitivity to cytarabine and daunorubicin, and a higher susceptibility of DS cells to apoptosis. Very intensive therapy is associated with a significantly higher mortality in ML-DS, and chemotherapy doses have often been reduced in DS patients with various grades of reduction depending on the treating center or physician. There is little knowledge in the field of pharmacokinetics in children with DS but differences in drug metabolism may contribute to the increased toxicity as well as the superior treatment results seen when DS children are treated for AML. The NOPHO AML 2004 treatment protocol was adjusted for children with DS such that they received slightly less treatment and the in the current protocol, ML-DS 2006, they will be given 4 blocks of treatment instead of 6 and 6-thioguanine (6-TG) will be excluded.

Prognostic factors

Most collaborative groups use risk group stratified treatment for AML. The most commonly used method is a combination of cytogenetics/ FAB groups and early response to treatment. Most study groups use specific treatment recommendations for children with DS and acute promyelocyte leukemia. Other prognostic factors described in AML are displayed in Table 1.
Table 1. *Some prognostic factors in childhood AML*

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Favourable</th>
<th>Unfavourable</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>Infants</td>
<td>Older children</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>Girls</td>
<td>Boys</td>
</tr>
<tr>
<td><strong>White blood cell count</strong></td>
<td>&lt; $5 \times 10^9$ /L</td>
<td>&gt; $5 \times 10^9$ /L</td>
</tr>
<tr>
<td><strong>Morphology</strong></td>
<td>FAB M2 with Auer rods</td>
<td>FAB M 7 in non DS patients</td>
</tr>
<tr>
<td></td>
<td>FAB M4 Eo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FAB M3</td>
<td></td>
</tr>
<tr>
<td><strong>Cytogenetic abnormalities</strong></td>
<td>t(9;11)</td>
<td>11q23 abnormalities other than t(9;11)</td>
</tr>
<tr>
<td></td>
<td>t(8;21)</td>
<td>Monosomy 7/7q</td>
</tr>
<tr>
<td></td>
<td>t(15;17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inv(16)</td>
<td></td>
</tr>
<tr>
<td><strong>Early response to treatment</strong></td>
<td>BM blasts after first course low &lt;15%</td>
<td>BM blasts after first course &gt; 15 %</td>
</tr>
</tbody>
</table>

**Minimal residual disease (MRD)**

For ALL it has been shown in several studies that early response to therapy is a very important prognostic factor and MRD assays, mainly flow cytometry or PCR techniques provide objective and sensitive measurements of low levels of leukemic cells.\(^{47}\)

In AML early blast clearence in bone marrow based on morphological examination is an important predictor of treatment outcome. MRD analysis has not had the same clinical importance in AML as in ALL, but studies have been showing that detectable leukemic blasts at the time of morphologic remission were predictive of more rapid relapse.\(^{48,49}\) One large study of the Children’s Cancer Group, Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, USA, was able to show that detectable MRD levels of disease in patients in morphological remission after consolidation therapy gives a 4.8 times higher risk of relapse and a 3.1 times higher risk of death.\(^{50}\) Another study at St Jude Children’s Research Hospital, Memphis, USA showed that the 2-year survival estimate in patients with detectable MRD at the end of induction therapy was 33% compared to 72% for MRD-negative patients.\(^{51}\)

Still there is no uniform method to study MRD in AML patients. RT-PCR can be used only in 40% of the patients, those with identifiable fusion transcripts. For flow cytometry an aberrant phenotype has to be defined at diagnosis. There is always a risk of a clonal/immunophenotypic shift during treatment and relapse that may confuse the interpretation of the result. Another approach to MRD studies is the analysis of quantitative PCR of Wilms tumor gene (*WT1*). WT1 is expressed in most acute leukemias and its detec-
tion in bone marrow has been associated with the presence, persistence and reappearance of leukemia. It has been shown to be detectable in approximately 70% of the AML cases at diagnosis and is one of the possible methods of MRD monitoring in childhood AML.52,53

Treatment of childhood AML

The treatment of AML consists of two general phases, remission induction and postremission consolidation therapy. The aim of the induction course is to achieve remission and the response to the initial course of therapy is one of the most important prognostic factors. In recent years therapeutical trials have shown that intensified induction therapy improves outcome.10,54,55

The induction therapy is generally based on cytarabine in combination with anthracyclines, and in the NOPHO protocol both etoposide and 6-TG are included. Intrathecal treatment with methotrexate is administered to patients without blast cells in CNS while patients with CNS leukemia receive intrathecal methotrexate, cytarabine and prednisolone.

The combination of drugs and the doses used in NOPHO-AML 2004 are the result of the experience of three previous protocols, NOPHO-AML 84, 88 and 93. To strike the golden mean between side effects and anti-leukemic effect is very difficult and NOPHO-AML 2004 states that the major findings from the NOPHO studies to the international discussions on the therapy of the childhood AML can be summarized as follows:

- The induction regimen in NOPHO-AML 88 was too toxic when the second induction was given shortly after the first induction. Postponing the second induction course to hematological recovery (NOPHO-AML 93) resulted in reduced toxicity and improved outcome.
- The response to the initial course of therapy is the most important prognostic factor.
- Outcome is associated with specific cytogenetic groups; a very favorable outcome in t(9;11), a trend toward superior outcome in t(8;21) and in inv(16) and a poor outcome in 11q23 rearrangements other than t(9;11)
- The use of high-dose cytarabine as consolidation therapy is now well established and is part of most protocols. In NOPHO-AML 88 the consolidation therapy was strengthened by adding mitoxantrone and etoposide. As long as recovery was complete after the previous course, the toxicity was manageable.
- Children with DS represent a large proportion of the patients. The prognosis is very favorable when high-dose cytarabine-based therapy is given.

The consolidation therapy in AML is also based on very intensive treatment with four to six courses of intensive chemotherapy. The question of stem cell transplantation (SCT) has been thoroughly studied in all collaborative groups and most groups today recommend treatment of good-risk patients with chemotherapy alone, as no improvement in OS has been shown for SCT in
these patients.\textsuperscript{56} The trend in Europe is to include SCT in the consolidation therapy for intermediate-risk and high-risk patients but only for relapse treatment in good-risk patients,\textsuperscript{57} while in the United States SCT in CR 1 is more supported.\textsuperscript{57-60} In ALL, maintenance therapy is widely used but in AML several studies have not been able to show any improvement in overall survival (OS). In the LAME 89/91 study the OS was even better in the non-maintenance group with the explanation that there was a lower salvage rate following relapse due to drug resistance in the group exposed to maintenance therapy.\textsuperscript{61} The optimal consolidation therapy is still unknown and further studies will be needed.

**Cytotoxic drug resistance**

Failure of cytotoxic drug treatment can be due to many factors including 1) low cellular sensitivity to cytotoxic drugs – cellular drug resistance, 2) low systemic exposure to the cytotoxic drug – pharmacokinetic resistance, and 3) an increase in the proliferative potential in the tumor cells between courses of chemotherapy – regrowth resistance.\textsuperscript{62}

**Cellular drug resistance**

At the cellular level, resistance of the leukemic cells to chemotherapy may occur in different ways; cellular uptake of the drug, intracellular metabolism, intracellular retention, drug efflux mechanisms, quantitative and qualitative changes in the drug target, efficiency of DNA-repair pathways and differences in the apoptotic machinery. All these factors can be studied in detail but in the end their net effect will determine the fate of the cancer cell and this can be assessed using the in-vitro drug resistance assays.

**In vitro cytotoxicity assays**

Development of laboratory tests for measurement of sensitivity and resistance to cytotoxic drugs in tumor cells has been going on since the beginning of the twentieth century.\textsuperscript{62} Basically the leukemic cells are exposed \textit{in vitro} to different cytotoxic drugs and the effect of the drugs on the survival or proliferation of the cells is studied. The assays can be divided into three main groups: 1) clonogenic assays, 2) cell proliferation assays and 3) assays based on the concept of total tumor cell kill.\textsuperscript{62} The assays are potentially suitable for guiding the clinician, especially when patients have resistant leukemia and for guiding the design of clinical studies.
Total cell kill assays

The assay used in this work is a total cell kill (TCK) assay, the fluorometric microculture cytotoxicity assay (FMCA). This assay was developed in Uppsala in the late 1980s and is based on the uptake of fluorescein diacetate (FDA) by viable cells and its conversion to fluorescent fluorescein after exposure to cytotoxic drugs in primary cultures. The generated fluorescence is linearly related to the number of living cells and the assay shows good correlation with other TCK assays. The FMCA is a sensitive assay capable of detecting small numbers of cells, it is easy to perform and it has a high capacity. The intra- and inter-assay variations have been estimated to be less than 5 % and less than 10-15% respectively.

Another frequently used TCK assay is the methyl-thiazol-tetrazolinium (MTT) assay. The MTT assay is based on the ability of living cells to convert a soluble tetrazolium salt into a colored insoluble product. This colored product can easily be measured and quantified by spectrophotometry. In childhood leukemia the MTT assay has been used in most studies.

The MTT assay and the FMCA assay share great similarity. They differ in that FMCA is based on detection of membrane disintegration which is a late and irreversible step in the events leading to cell death, while the MTT assay depends on peroxidase activity to reduce MTT to colored formazan crystals. Theoretically the enzyme activity necessary for MTT reduction may be influenced by temporary and reversible energy depletion. The incubation time with the cytotoxic drugs differs, in the FMCA the time of exposure is 72 hours while the duration in MTT is 96 hours.

Drug exposure in the assay

Selection of drug concentrations for in vitro cytotoxicity drug resistance assays is an important issue. There are different ways to find the appropriate drug exposure under assay conditions. One alternative is to find concentrations that mimic the in vivo situation by comparison of intracellular uptake in vivo and in vitro. For some drugs this will result in only small differences in cell kill between resistant and sensitive samples and it is therefore not a method for studying drugs under development, before pharmacokinetic data become available.

Alternatively, a full concentration-effect curve can be aimed at, and the estimated IC 50 value, the concentration that results in 50% cell survival, can be used as the measure of activity. This will result in a better estimation of the difference of resistant and sensitive samples, but for appropriate testing, an increased number of cells will be required.

In the FMCA procedure, cell samples have been tested at a large range of different drug concentrations resulting in concentration-response curves for
Each drug. The concentrations giving rise to the largest scatter of cell survival have then been chosen for further testing.

Factors that influence drug response

The response to a specific drug is determined by many different factors. The pharmacokinetics of the drug depends on absorption, distribution, metabolism and excretion of the administered drug. This leads to a large inter-individual variability in drug exposure that changes with age, concomitant exposure to other drugs, other diseases, nutritional status, kidney and liver function. Another factor is the penetration of drugs into specific organs such as the testis and the central nervous system, which are protected by barriers to prevent toxicity to these organs. This gives a great difference in dose intensity of the given treatment, something that is known to be of great importance for the outcome in acute leukemia. For example, the systemic clearance of anticancer drugs typically differs by a factor of 3 to 10 among patients, such that those with rapid drug clearance may benefit less than those with slower clearance if the dose is determined only according to body-surface area.

In a study at St Jude Children's Research Hospital, children with AML received individualized dosing of etoposide and cytarabine according to target plasma drug concentrations, but the potential clinical efficacy of this approach was offset by substantial acute and long-term toxicity. In another study at the same hospital, children with newly diagnosed ALL were randomised to post-remission treatment with methotrexate, teniposide and cytarabine, where dosing was based on body surface area or the rate of clearance. This study showed better outcome in patients with B-lineage leukemia and clearance-based doses, with a mean rate of complete remission after five years of 76 ± 6 percent compared to 66 ± 7 in the other group (p = 0.02). Prospective clinical trials are needed to determine the clinical usefulness of pharmacokinetic monitoring and to define more precisely the integration of such monitoring to optimize the treatment of childhood leukemias.

Pharmacogenomics describes the inherited basis of differences in the individual response to chemotherapeutic agents. One example of that is the difference in metabolism of thiopurines by thiopurine S-methyltransferase (TMPT), where 0.3% of the population are homozygotes and approximately 10% have a heterozygote deficiency of TMPT requiring dose reductions to avoid toxicity. These differences have been shown to vary among ethnic groups, something that probably partially explains the differences in outcome for patients of African of Hispanic descent compared to descendants from other European countries.
Cellular drug resistance is probably one of the major limitations to the success in chemotherapy in childhood leukemia and can be conferred to the leukemia cell by one or more mechanisms as described above. The best way of evaluating the treatment response is still by in-vivo measures such as bone marrow blast count and MRD measurement, which has become an important factor for post-induction treatment stratification.

New drugs for AML

New formulations of established drugs such as liposomal daunorubicin, known to give less cardiotoxic side effects, 86, or pegylated L-asparaginase are being taken into clinical practice. New nucleoside analogs, for example clofarabine, are being tested as either single agents or in combination therapy. The targeted therapy of monoclonal antibodies that are either administered “alone” or conjugated to a cytotoxic drug, a radioactive particle or a toxin is a new, specific way of treating cancer. The current NOPHO AML-2004 protocol includes a randomised study of gemtuzumab ozogamicin (GO, Mylotarg), an anti-CD33 antibody which is linked to the cytotoxic compound calicheamin, an anti-cancer antibiotic considered too toxic for untargeted use.87

Tyrosine kinase receptor inhibitors are another new type of anti-cancer treatments where the most successful drug, imatinib mesylate (Glivec), is successfully being used in ALL patients with t(9;22).

Many of the drugs administered in pediatric oncology have been used for several years and the introduction of new drugs is a long process, especially in children.88,89 A great number of drugs that are being used in pediatric practice are not registered for use in children, especially not for children < 2 years of age.90-92 Both ethical and logistical issues make it a challenge to perform studies in children, for example pharmacokinetic studies necessary for registration of a drug.93 Young children cannot provide informed consent themselves and their parents must decide for their children. The number and volume of blood samples in children must be limited and highly sensitive methods for the measurement of drug concentrations and a limited sampling strategy for data analysis are required.

Still, in spite of all the obstacles, pediatric trials are needed to further optimize chemotherapy in childhood acute myeloid leukemia.
Aims of the present studies

Papers I and II

To study the cellular drug resistance of leukemic cells in samples from children with newly diagnosed AML and relapsed AML by the fluorometric microculture cytotoxicity assay (FMCA)

To correlate the results of the FMCA to biological and clinical parameters such as age, sex, WBC count at diagnosis, FAB type, chromosomal abnormalities, DS, response to induction therapy and long-term clinical outcome

Papers III, IV and V

To study the pharmacokinetics of the drugs used in children receiving induction therapy for AML by analysis of drug concentrations in blood samples

To correlate the results of the drug analyses to biological and clinical parameters such as dose, age, sex, renal and hepatic functions, DS, response to induction therapy and outcome
Material and methods

Patients
The cellular drug resistance study
The study of cellular drug resistance is the basis for papers I and II and it comprised children (aged 0–18 years) with newly diagnosed AML from Nordic centers for pediatric oncology. In Paper I we also included patients with a relapse of AML and in Paper II we also included children with acute lymphoblastic leukemia (ALL). The study was a collaboration within the Nordic Society of Paediatric Haematology and Oncology (NOPHO) and the patients’ samples were collected between 1993 and 2007. Paper I included samples from 201 patients of whom 15 had DS and it also included samples from 55 non-DS patients with a relapse. Paper II included samples from non-DS patients, 132 with AML and 178 with ALL.

The pharmacokinetic study
The pharmacokinetic study is the basis for papers III-V and was performed as a NOPHO study between March 1995 and October 2000. Children with newly diagnosed AML were treated according to the NOPHO AML-93 protocol, and studied during the first induction course. 41 children (4 with DS) were successfully included in the doxorubicin study, Paper III, 45 children (5 with DS) in the etoposide study, Paper IV, and 50 children (4 with DS) were included in the 6-thioguanine study, paper V. Data for all three drugs were available in 28 patients (3 with DS).

Diagnosis
The diagnosis was established at each pediatric oncology center by analysis of bone marrow aspirates including morphology, immunophenotype, and cytogenetics of the leukemic cells. For cytogenetic diagnosis chromosome G-banding analyses of bone marrow and/or peripheral blood samples were performed using standard methods in 15 cytogenetic laboratories in the Nordic countries. The definition and description of clonal abnormalities followed the recommendations of ISCN (1995). Since 1996 (Sweden) and 2000 (all five Nordic countries) the karyotypes have been centrally reviewed. Fluorescence in situ hybridization (FISH), Southern blot, and reverse-
transcriptase polymerase chain reaction (RT-PCR) analyses have been increasingly applied to verify or more precisely characterize the chromosomal abnormalities found.

Three different treatment protocols were used: NOHPO AML-93, NOPHO AML-2004 and ML-DS 2006. Some Nordic centers participated in the study during the last years of the study period only. Patient characteristics and clinical follow-up data were obtained from annual reports submitted from the treating clinicians to the Nordic registry at the Childhood Cancer Research Unit in Stockholm.

The induction course of the NOPHO AML–93 protocol

This course included an intrathecal injection of methotrexate, usually administered when the child was under anesthesia to establish a central venous line for administration of drugs and a peripheral venous catheter for blood sampling. Etoposide, 100 mg per m² body surface area (BSA) per 24 h, and cytarabine, 200 mg/m² BSA/24 h, were administered concomitantly by constant infusion pump over a 96-h period day 1-4. During the same 96-h period, 100 mg/m² of 6-thioguanine was administered orally every 12 h to a total dose of 800 mg/m² (Figure 2). On day 5, doxorubicin 75 mg/m² was given as an 8-h infusion. Data on other drugs administered, e.g. antiemetics, analgesics and antibiotics, were not available to us.

![Induction course of the NOPHO AML–93 protocol](image)

<table>
<thead>
<tr>
<th>Mtx it</th>
<th>Ara-C</th>
<th>DOXO</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ □ □ □ □ □ □</td>
<td>□ □ □ □</td>
<td>□</td>
</tr>
</tbody>
</table>

**Mtx it**, Methotrexate intrathecal injection day 1
Dose according to age, < 1 year: 6 mg, 1-2 years: 8 mg, > 3 years: 12 mg

□ **6-TG**, 6-thioguanine tabl 100 mg/m² twice daily for 4 days

**Ara-C**, cytarabine 200 mg/m²/day as continuous infusion for 4 days

**VP-16**, etoposide 100 mg/m²/day as continuous infusion for 4 days

**DOXO**, doxorubicin 75 mg/m² as an 8-h infusion

Children less than 2 years of age, dosing after weight where 30 kg equals 1 m²

*Figure 2. Induction course of the NOPHO AML–93 protocol*
Samples

The in vitro cellular drug resistance study

Blood and bone marrow samples were collected in heparinized glass tubes, kept at room temperature, and sent by mail or through international express delivery companies. As a rule they reached the laboratory in Uppsala, Sweden, for processing within 24-36 h. Most of the samples (about 90%) were analyzed freshly, but for practical reasons some were cryopreserved. This was made in culture medium containing 10% dimethyl sulfoxide (DMSO) and 50% fetal calf serum by initial freezing for 24 h at −70°C followed by storage in liquid nitrogen. The cells were later thawed and analyzed. Previous studies showed that cryopreservation does not affect the in vitro sensitivity, and it has also been shown that the source of the leukemic cells (bone marrow or peripheral blood) does not affect the in vitro drug resistance measured.66,94,95

Leukemic cells were prepared by 1.077 g/ml Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) density-gradient centrifugation. Viability was determined by trypan-blue exclusion test. The median viability was 95% and FMCA was performed only when the viability was ≥ 70%. An independent hematologist estimated the proportion of leukemic cells on May-Grunewald-Giemsa stained cytocentrifugate preparations, using light microscopy. The median proportion of myeloblasts after separation was 90% and FMCA was performed only when this proportion was ≥70%.

The pharmacokinetic study

Blood samples were drawn before treatment and on day 2, approximately 48 h after start, on day 3, approximately 72 h after start, and on day 4 approximately 1 h before termination of cytarabine and etoposide infusions. The last blood sample was drawn on day 5, 7 h after start of the doxorubicin infusion, i.e. one hr before the infusion was completed. Blood was drawn from a venous line not used for drug infusion and collected in tubes containing EDTA. The sample was immediately put into ice water and centrifuged within 60 minutes and plasma and erythrocytes were then stored separately at -70°C until analysis.

Patient data (body weight, height, actual dose administered) were noted as well as times for drug administration and for blood sampling.
Methods

FMCA procedure

FMCA is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) (Sigma, St. Louis, MO) to fluorescein by cells with intact plasma membranes and has been described in detail previously (Figure 3). FDA was dissolved in DMSO (Sigma) and kept frozen (-20°C) as a stock solution (10 mg/ml) protected from light. The culture medium RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin was used throughout. Fifty thousand leukemic cells in 180 μl culture medium were seeded per well in 96-well microtiter plates prepared in advance with the different drugs to be tested. The culture plates were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO₂ for 72 h continuous drug exposure.

![Figure 3. The FMCA procedure](image)

The plates were then centrifuged (200xg, 5 min) and the medium was removed by automatic pipetting. After one wash with phosphate buffered saline (PBS), 200 μl/well of PBS containing FDA (10mg/ml) was added. Subsequently, the plates were incubated for 1 h at 37°C and the fluorescence
was then read by a scanning fluorometer (Fluoroscan 2; Labsystems OY, Helsinki, Finland). Six wells without drugs served as controls and six wells containing culture medium only served as blanks. Quality criteria for a technically successful assay included: 1) a proportion of leukemic cells, identified by morphology, of ≥ 70% in control wells after 72 h of incubation 2) a fluorescence signal in control wells of ≥ 5 times the mean blank value and 3) a mean coefficient of variation (CV) in control wells of < 30%. The results are presented as survival index (SI), defined as fluorescence in test wells / fluorescence in control wells (blank values subtracted) x 100 or as percent surviving cells. Thus, a low numerical value indicates high sensitivity to the cytotoxic effect of the drug.

Cytotoxic drugs were obtained from commercial sources and tested at the concentrations shown in Table. Each drug and concentration was tested in triplicate wells. The plates were stored at -70°C pending further use. The drugs were used at empirically derived cut-off concentrations, chosen to produce a large scatter of SI values among the samples. These concentrations were adopted from previous studies of leukemic cells.66 FMCA has been performed at the Department of Medical Sciences, Section of Pharmacology, University Hospital, Uppsala, Sweden

Table 2. Drugs used for test of in vitro drug resistance

<table>
<thead>
<tr>
<th>Drug</th>
<th>Origin</th>
<th>Solvent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amsacrine</td>
<td>Bristol-Myers Squibb</td>
<td>SW</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>Sigma</td>
<td>PBS</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>MSD</td>
<td>PBS</td>
<td>1.4 μg/ml</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Pharmacia</td>
<td>PBS</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Bristol-Myers Squibb</td>
<td>PBS</td>
<td>5 μg/ml</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>Pfizer</td>
<td>SW</td>
<td>0.1 μg/ml</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Meda</td>
<td>PBS</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>Organon</td>
<td>PBS</td>
<td>50 μg/ml</td>
</tr>
<tr>
<td>Vincristine</td>
<td>Lilly</td>
<td>PBS</td>
<td>0.5 μg/ml</td>
</tr>
<tr>
<td>6-TG (6-thioguanine)</td>
<td>Sigma</td>
<td>NaOH/SW</td>
<td>10 μg/ml</td>
</tr>
<tr>
<td>4-HC (4-hydroperoxy-cyclophosphamide)</td>
<td>Duke university</td>
<td>PBS</td>
<td>2 μg/ml</td>
</tr>
<tr>
<td>2-CdA (2-chloro-deoxyadenosine)</td>
<td>Janssen-Cilag</td>
<td>PBS</td>
<td>2 μg/ml</td>
</tr>
</tbody>
</table>

SW, sterile water
PBS, phosphate buffered saline
NaOH, sodium hydroxide
Idarubicin was added to the panel of drugs during the last years of the study
Analysis of drug concentrations

Plasma concentrations of doxorubicin and doxorubicinol were determined by reversed phase isocratic high performance liquid chromatography (HPLC), adopted and modified from Paul et al.\textsuperscript{97} In brief, 100 μl of idarubicin (10 μM) was added to 1 ml plasma. 500 μl of 0.1 M borate buffer (pH 9.3) was added and extraction was performed after addition of 6 ml chloroform/methanol (4:1 v/v). The chloroform phase was collected and evaporated to dryness. Thereafter, the residue was reconstituted by the addition of 200 μl of mobile phase (0.2% ammonium formiate pH 4/acetonitrile, 73:27). 50 μl was injected and doxorubicin, doxorubicinol, and the internal standard idarubicin were separated on a micro Bondapack phenyl column at a flow rate of 1.5 ml/min. Quantitation was made by fluorescence (excitation and emission wavelengths 520/600 nm). The analysis of doxorubicin was performed at the Department of Clinical Pharmacology, University Hospital of Linköping, Sweden.

Etoposide concentrations were determined by HPLC. Plasma samples were thawed and 0.5 ml was used for analysis. After the addition of teniposide (5 μg) as internal standard and liquid extraction with chloroform the organic phase was evaporated under nitrogen. The residue was redissolved in 1 ml water/methanol (50/50). The extract was injected (25 μl) into the HPLC system. A reversed phase system with a Nucleosil® column 7 μm (150 x 4.6 mm) equipped with a NewGuard Phenyl precolumn eluted with methanol/water/acetonitrile/acetic acid (43/52/4/1) at a flow rate of 1.0 ml/min was used to separate etoposide from endogenous compounds. Quantitation was performed using electrochemical detection. The signal was integrated using peak area ratios.\textsuperscript{98}

Free concentrations of etoposide were determined after removal of plasma proteins by ultrafiltration on Millipore® Centrifree filters. Subsequently, 50 μl of the ultrafiltrate was injected directly into the HPLC system.\textsuperscript{99} The etoposide analysis was performed at the Department of Oncology-Pathology, Karolinska University Hospital, Stockholm, Sweden.

The thioguanine nucleotides (TGN) in erythrocytes were determined by reversed phase HPLC as purine bases after acid hydrolysis and an extraction procedure, as described by Lennard and Singleton.\textsuperscript{100} The metabolites were quantified by absorbance at 330 nm. Calibrators were prepared by adding 6-TG from a stock solution to drug-free red blood cell concentrates. The limit of quantification for TGN was 13.3 x 10^{-3} μmol/mmol Hb. At this concentration, the inter-assay coefficient of variation was 18% (n=11). The analysis was performed at the Department of Clinical Pharmacology, University Hospital of Linköping, Sweden.
Pharmacokinetic evaluation

Based on recorded data, BSA was recalculated for all patients by the formula
\[ m^2 = \sqrt{\text{height (cm)} \times \text{weight (kg)}} / 3600 \]. Body mass index (BMI) was cal-
culated as weight/(height)². For doxorubicin and etoposide plasma clearance
(Cl) was calculated according to the formula Cl = D/T/Css, where D/T is the
actual dose rate and Css is the observed steady state concentration of the
drug.

For doxorubicin it has been shown that 80% of the steady state concentration
is reached after 7 hr of infusion,¹⁰¹⁻¹⁰³ and this was compensated for in the
calculations of plasma clearance by dividing the observed 7 hr doxorubicin
concentration by 0.8.

For 6-thioguanine (6-TG) the dose-normalized TGN concentration was cal-
culated by the formula TGN concentration x (target dose/ administered dose)
where the target dose was set at 200 mg/m²/24 h.

Statistical analysis

Non-parametric methods were generally used. The Spearman rank test was
used to examine correlations, the Mann-Whitney U test to compare values
from two groups, the Kruskall-Wallis test to examine differences between
three or more groups, the Wilcoxon signed rank test to compare two related
samples, the Friedman test to examine several related samples, and logistic
regression analysis to test the probability of a defined event. For linear re-
gression analysis, a natural log transformation of one (univariate) or several
covariates (multivariate analyses) was performed. Survival curves were con-
structed using the Kaplan-Meier method, and differences in outcome be-
tween subgroups were tested with the log-rank test. In the analysis of event-
free survival (EFS) events included induction failure (early death, death in
aplasia and resistant disease), death in remission, relapse and second malign-
nancy. Disease-free survival (DFS) was defined as the time from diagnosis
to a leukemia-related event, relapse or resistant disease (RD). The SPSS
11.5–15.0 software packages (SPSS Inc. Chicago, IL) were used for the
calculations. All analyses were two-tailed and the level of statistical signifi-
cance was set at p < 0.05.

Local ethics committees approved the studies.
Results

Paper I
Cellular drug sensitivity in childhood acute myeloid leukemia

From January 1995 to December 2007 samples from 252 non-DS children and 29 DS with de novo AML were received for testing of vitro drug resistance by FMCA, bone marrow was used in 83% and peripheral blood in 17% of the cases. Successful analysis was accomplished in 186 samples from non-DS patients and 15 samples from DS patients. We also received 55 samples from non-DS patients at the relapse out of which 41 were successfully analyzed. The technical success rate for in vitro testing of samples with sufficient number of cells was 74% and the reasons for failure of FMCA in diagnostic samples were: total number of cells too small to test any drug (n=8), low viability of leukemic cells (n=1), transport problems (n=10), too low proportion of blasts in control wells after incubation (n=24), too low signal-to-noise ratio (n=7), high coefficient of variation in controls (n=3), other technical problems (n=10), and high proportion of erythrocytes after density-gradient centrifugation (n=17).

Patients with a successful analysis were compared to all children diagnosed with AML in the Nordic countries during the study period (n=406) (Table 3). Cytogenetic analyses were available for all but three patients with a successful FMCA analysis. Children with a successful FMCA were significantly older than non-included children and they had a significantly higher WBC count at diagnosis. No statistically significant difference was found for sex or for the distribution of cytogenetic and FAB (French American British) classification subgroups. The probability for EFS, DFS and overall survival (OS) did not differ between included and not included patients. Median follow-up time was 5.3 years.

Patients with Down syndrome
FMCA was successfully performed in samples from 15 patients with DS. When compared to DS patients with an unsuccessful test or patients not tested (n=44), patients with a successful analysis did not differ significantly in age, (median 2.0 years and 1.9 years, respectively) or sex, whereas WBC count
Table 3. *Characteristics of 186 non-DS children with AML and successful FMCA compared to children with unsuccessful FMCA or children not tested (n=220)*

<table>
<thead>
<tr>
<th></th>
<th>successfully tested</th>
<th>not successfully tested or not tested</th>
<th>total</th>
<th>p</th>
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<tr>
<td>n</td>
<td>186</td>
<td>220</td>
<td>406</td>
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<td>Age years, median</td>
<td>8.1</td>
<td>4.6</td>
<td>6.1</td>
<td>p&lt;0.001</td>
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<td>(p 25-75)</td>
<td>2.9-13.0</td>
<td>1.4-10.6</td>
<td>1.8-12.3</td>
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<tr>
<td>Sex (n)</td>
<td>n.s</td>
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<tr>
<td>male</td>
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<td>109</td>
<td>209</td>
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</tr>
<tr>
<td>female</td>
<td>86</td>
<td>111</td>
<td>197</td>
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<td>WBC 10^9/L, median</td>
<td>23.1</td>
<td>9.5</td>
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<td>(p 25-75)</td>
<td>5.2-66.2</td>
<td>4.8-38.3</td>
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<td>other clonal abnorm.</td>
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<td>80</td>
<td>127</td>
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<tr>
<td>p-EFS</td>
<td>0.49 (±0.04)</td>
<td>0.44 (±0.04)</td>
<td>0.45 (±0.03)</td>
<td>0.88</td>
</tr>
<tr>
<td>p-EFS 3 years</td>
<td>0.50 (±0.04)</td>
<td>0.52 (±0.04)</td>
<td>0.51 (±0.03)</td>
<td>0.54</td>
</tr>
<tr>
<td>p-DFS 5 years</td>
<td>0.52 (±0.04)</td>
<td>0.56 (±0.04)</td>
<td>0.54 (±0.03)</td>
<td>0.98</td>
</tr>
<tr>
<td>p-DFS 3 years</td>
<td>0.54 (±0.04)</td>
<td>0.58 (±0.04)</td>
<td>0.56 (±0.03)</td>
<td>0.98</td>
</tr>
<tr>
<td>p-OS</td>
<td>0.62 (±0.04)</td>
<td>0.59 (±0.03)</td>
<td>0.59 (±0.02)</td>
<td>0.98</td>
</tr>
</tbody>
</table>

FMCA, fluorometric microculture cytotoxicity assay
p25-p75, 25th-75th percentiles
EFS (event-free survival), DFS (disease-free survival) and OS (overall survival) values are shown with standard error of the mean (SE)
was significantly higher in patients with a successful analysis, 8.6 vs 5.9 x 10⁹/l (p=0.035). There were no significant differences in FAB group, p-EFS, p-DS and OS between the two groups. The DS patients were significantly more sensitive to most drugs tested compared to non-DS patients, (Table 4). For the drugs used in the NOPHO AML 1993 and 2004 protocols, there were highly significant differences for cytarabine, doxorubicin and etoposide with a trend toward a difference for mitoxantrone (fewer samples tested), but no significant difference for idarubicin (only 5 DS samples tested) or 6-TG.

Table 4. Cellular drug resistance in samples from children with AML, Down syndrome (DS) vs non-DS

<table>
<thead>
<tr>
<th>Drug</th>
<th>DS n</th>
<th>median (p25-p75)</th>
<th>non-DS n</th>
<th>median (p25-p75)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amsacrine</td>
<td>13</td>
<td>27 (17-40)</td>
<td>180</td>
<td>47 (32-63)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>13</td>
<td>31 (15-42)</td>
<td>176</td>
<td>54 (38-74)</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>14</td>
<td>60 (36-76)</td>
<td>170</td>
<td>76 (62-89)</td>
<td>p=0.007</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>13</td>
<td>26 (20-34)</td>
<td>177</td>
<td>49 (31-68)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Etoposide</td>
<td>13</td>
<td>38 (26-49)</td>
<td>169</td>
<td>61 (44-79)</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>5</td>
<td>23 (8-42)</td>
<td>57</td>
<td>34 (22-53)</td>
<td>p=0.16</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>9</td>
<td>19 (12-30)</td>
<td>133</td>
<td>27 (16-45)</td>
<td>p=0.099</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>13</td>
<td>55 (49-74)</td>
<td>179</td>
<td>70 (56-85)</td>
<td>p=0.031</td>
</tr>
<tr>
<td>6-TG</td>
<td>12</td>
<td>30 (20-42)</td>
<td>178</td>
<td>40 (22-57)</td>
<td>p=0.18</td>
</tr>
</tbody>
</table>

The median percentage of surviving cells after drug exposure (SI) is shown, with 25th-75th percentiles in brackets

**Cellular drug resistance versus patient characteristics in non-DS patients**

As there were large interindividual variations in SI values we studied correlations between SI values and basic clinical characteristics. There was no significant correlation between drug resistance and age for any drug neither when patients were divided into groups (below 2, 2-10, and above 10 years of age) nor when age was treated as a continuous variable. There were no differences between boys and girls. SI values for 6-TG correlated negatively to WBC count at diagnosis (rho-0.25; p=0.01) but for other drugs no significant correlation was found.

**Cellular drug resistance in samples from genetic subgroups**

Characteristics of patients in the various cytogenetic subgroups are displayed in (Table 5). It shows some expected differences between the cytogenetic
### Table 5. Characteristics of non-DS patients with different cytogenetic subgroups in childhood AML

<table>
<thead>
<tr>
<th>Karyotype Abnormality</th>
<th>n</th>
<th>t(9;11)</th>
<th>t(11;19)</th>
<th>t(10;11)</th>
<th>other11q23</th>
<th>t(8;21)</th>
<th>inv16</th>
<th>t(15;17)</th>
<th>normal karyotype</th>
<th>other clonal abnormality</th>
<th>monosomy 7</th>
<th>no result</th>
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</thead>
<tbody>
<tr>
<td>normal other</td>
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<td>20</td>
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<td>3</td>
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<tr>
<td>t(11;19)</td>
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<td>other11q23</td>
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<tr>
<td>t(8;21)</td>
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<tr>
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<td>WBC median</td>
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</tr>
<tr>
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<td>1</td>
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</tr>
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<td>1</td>
<td>14</td>
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<td>2</td>
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<td>7</td>
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<tr>
<td>M7</td>
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<td>1</td>
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<td></td>
</tr>
<tr>
<td>other</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>p-EFS</td>
<td>0.49 (0.04)</td>
<td>0.68 (0.14)</td>
<td>0.67 (0.19)</td>
<td>0.38 (0.17)</td>
<td>0.64 (0.17)</td>
<td>0.54 (0.12)</td>
<td>0.57 (0.13)</td>
<td>0.75 (0.15)</td>
<td>0.35 (0.08)</td>
<td>0.54 (0.08)</td>
<td>0</td>
<td>0.65 (0.20)</td>
</tr>
<tr>
<td>p-DFS</td>
<td>0.52 (0.04)</td>
<td>0.73 (0.14)</td>
<td>0.67 (0.19)</td>
<td>0.50 (0.20)</td>
<td>0.71 (0.17)</td>
<td>0.54 (0.12)</td>
<td>0.67 (0.14)</td>
<td>0.86 (0.13)</td>
<td>0.40 (0.09)</td>
<td>0.56 (0.08)</td>
<td>0</td>
<td>0.66 (0.19)</td>
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<tr>
<td>p-OS</td>
<td>0.62 (0.04)</td>
<td>0.86 (0.10)</td>
<td>0.83 (0.15)</td>
<td>0.38 (0.17)</td>
<td>0.77 (0.14)</td>
<td>0.82 (0.10)</td>
<td>0.71 (0.13)</td>
<td>0.75 (0.15)</td>
<td>0.45 (0.08)</td>
<td>0.69 (0.07)</td>
<td>0</td>
<td>0.67 (0.27)</td>
</tr>
</tbody>
</table>

EFS, DFS and OS values are shown with standard error of the mean (SE)
subgroups, e.g. young age in children with 11q23 rearrangements, and relatively high age in the t(8;21) and inv(16) subgroups. The correlation between cytogenetic aberrations and FAB type agree with previously published data. The p-EFS and p-OS values are similar to what has been reported for corresponding subgroups in 219 Nordic children treated according to the NOPHO AML 1993 protocol, with a few notable exceptions. Patients with MLL rearrangements other than t(9;11) had an EFS of only 0.36 and an OS of 0.44, and patients with t(15;17) had an EFS of 0.47 and an OS of 0.63 in the previous report.

Results from the FMCA analysis of patients from different cytogenetic subgroups are displayed as SI values in Table 6. As an example, Figure 4, shows data for cytarabine in some well defined cytogenetic subgroups. For statistical calculations we compared each particular subgroup with all other samples included in the study population as a complementary group. The t(9;11) samples were significantly more sensitive than the complementary samples to cytarabine (p=0.03) and doxorubicin (p=0.035), but more resistant to dexamethasone (p=0.031) and prednisolone (p=0.025). Data for t(11;19), t(10;11) and other 11q23 abnormalities are displayed in Table 7, but for statistical analysis all patients with MLL rearrangements other than t(9;11) were pooled into one group. They tended to be more sensitive to doxorubicin (p=0.066) and etoposide (p=0.072), and were more resistant to prednisolone (p=0.031).

Samples with t(8;21) showed a significantly more resistant profile compared to complementary samples for cytarabine (p=0.001), doxorubicin (p=0.02), etoposide (p=0.015), idarubicin (p=0.034), mitoxantrone (p=0.003) and 6-thioguanine (p<0.001).

Samples with inv(16) were significantly more resistant to doxorubicin than complementary samples (p=0.001), but more sensitive to 6-thioguanine, (p=0.034).

Samples with t(15;17) were significantly more resistant to cytarabine (p=0.002) and 6-thioguanine (p=0.006).

**In vitro drug sensitivity versus clinical outcome**

We compared 127 patients who achieved CR after the first treatment course with 51 patients who did not, excluding patients who died during induction (n=5). Patients who reached CR were significantly younger, median 7.0 versus 9.2 years of age (p=0.03), but there were no significant differences in WBC count or SI values for any of the drugs tested (not shown).
Table 6. *In vitro* drug resistance in samples from children with AML and various cytogenetic subgroups

<table>
<thead>
<tr>
<th>Drug</th>
<th>t(8;21) n median (p25-p75)</th>
<th>inv 16 n median (p25-p75)</th>
<th>t(15;17) n median (p25-p75)</th>
<th>normal n median (p25-p75)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amsacrine</td>
<td>20 61 (45-73)</td>
<td>15 59 (45-67)</td>
<td>8 50 (42-64)</td>
<td>43 49 (33-62)</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>18 75 (58-83)</td>
<td>15 62 (48-66)</td>
<td>8 86 (60-92)</td>
<td>43 48 (32-67)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>19 78 (66-85)</td>
<td>15 63 (57-75)</td>
<td>7 81 (75-113)</td>
<td>39 78 (67-98)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>19 63 (48-77)</td>
<td>15 69 (46-88)</td>
<td>8 58 (40-72)</td>
<td>43 49 (30-66)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>18 72 (64-87)</td>
<td>13 70 (46-82)</td>
<td>8 63 (46-96)</td>
<td>39 60 (38-84)</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>10 52 (33-62)</td>
<td>4 38 (10-57)</td>
<td>2 42</td>
<td>12 28 (19-38)</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>13 47 (32-57)</td>
<td>11 16 (9-38)</td>
<td>8 25 (13-42)</td>
<td>31 27 (19-49)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>20 66 (61-84)</td>
<td>15 56 (50-81)</td>
<td>8 70 (65-119)</td>
<td>43 71 (56-82)</td>
</tr>
<tr>
<td>6-TG</td>
<td>19 67 (52-70)</td>
<td>15 32 (10-37)</td>
<td>8 68 (46-76)</td>
<td>43 35 (14-46)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug</th>
<th>monosomy 7 n median (p25-p75)</th>
<th>3q21q26 n median (p25-p75)</th>
<th>other clonal abn n median (p25-p75)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amsacrine</td>
<td>5 40 (32-62)</td>
<td>46 40 (22-60)</td>
<td></td>
</tr>
<tr>
<td>Cytarabine</td>
<td>5 42 (22-51)</td>
<td>3 81</td>
<td>44 54 (34-70)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>5 46 (42-55)</td>
<td>2 97</td>
<td>44 68 (50-84)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>5 44 (32-60)</td>
<td>4 74 (33-84)</td>
<td>46 48 (34-59)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>4 46 (32-69)</td>
<td>4 73 (48-85)</td>
<td>44 58 (42-76)</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>2 32</td>
<td>2 67</td>
<td>9 27 (16-45)</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>4 20 (13-44)</td>
<td>3 56</td>
<td>31 29 (14-42)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>5 47 (40-56)</td>
<td>4 61 (56-113)</td>
<td>44 62 (52-83)</td>
</tr>
<tr>
<td>6-TG</td>
<td>5 22 (18-28)</td>
<td>3 68</td>
<td>46 34 (17-55)</td>
</tr>
</tbody>
</table>

The median percentage of surviving cells after drug exposure (SI values) are shown, with 25th-75th percentiles in brackets.
Table 7. *In vitro* drug resistance, in samples from children with MLL gene rearranged AML

<table>
<thead>
<tr>
<th>Drug</th>
<th>t(9;11)</th>
<th>t(11;19)</th>
<th>t(10;11)</th>
<th>other 11q23</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>median (p25-p75)</td>
<td>n</td>
<td>median (p25-p75)</td>
</tr>
<tr>
<td>Amsacrine</td>
<td>15</td>
<td>38 (25-59)</td>
<td>5</td>
<td>35 (25-78)</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>15</td>
<td>36 (25-55)</td>
<td>5</td>
<td>45 (28-54)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>15</td>
<td>85 (77-93)</td>
<td>4</td>
<td>68 (58-78)</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>14</td>
<td>38 (21-56)</td>
<td>5</td>
<td>35 (20-51)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>15</td>
<td>58 (52-79)</td>
<td>4</td>
<td>41 (28-78)</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>6</td>
<td>39 (23-56)</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>11</td>
<td>24 (18-41)</td>
<td>4</td>
<td>16 (8-24)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>15</td>
<td>84 (73-90)</td>
<td>5</td>
<td>71 (62-90)</td>
</tr>
<tr>
<td>6-TG</td>
<td>15</td>
<td>39 (33-54)</td>
<td>5</td>
<td>38 (20-57)</td>
</tr>
</tbody>
</table>

MLL, mixed lineage leukemia

The median percentage of surviving cells after drug exposure (SI values) are shown, with 25th-75th percentiles in brackets.
When patients in CCR at follow-up (n=98) were compared to those who experienced a leukemic event, RD or relapse (n=70), no significant difference was found for age, WBC count or SI values for any drug tested (not shown). The same was true when the 124 patients alive at follow-up were compared to the 62 deceased patients (death from any cause).

Since there were marked differences in the drug sensitivity profile between patients with different cytogenetic aberrations, we repeated the comparison of patients in CCR at follow-up with patients who experienced RD or relapse within each of the three major subgroups. In the t(8;21) subgroup, patients who remained in CCR were significantly more sensitive to doxorubicin at diagnosis than refractory/relapsed patients, median SI 51% and 67%, respectively (p=0.039). In patients with “other clonal abnormalities”, patients in CCR were more sensitive to cytarabine at diagnosis than refractory/relapsed patients, median SI 47% and 65%, respectively (p=0.044).

*Figure 4.* Survival index (SI) for cells from different cytogenetic subgroups after exposure to cytarabine 0.5 μg/ml
In individuals with normal karyotype, CCR patients were more resistant than refractory/relapsed patients to dexamethasone, SI 91% versus 75% (p=0.028), and prednisolone, SI 79% versus 67% (p=0.020). Other drugs tested, as well as age and WBC count, did not differ significantly within any of the cytogenetic subgroups. To explore the predictive potential of the FMCA, we dichotomized data for each drug into those with SI values below and above the median, respectively. Kaplan-Meier curves showing p-DFS were plotted, as shown for doxorubicin in Figure 5. At univariate Cox regression analysis, SI values were not found to be of prognostic significance for p-DFS for any single drug tested.

The combined data for doxorubicin and mitoxantrone showed some predictive potential, however. For each of these two drugs, those with a cell survival below median were given a score of 1, and those with a cell survival above median were given a score of 2. For each patient, a total score was calculated by adding up the individual scores, ranging from 2 to 4. The relative risk of RD or relapse was 2.2 times greater (95% CI 1.1-4.7) in patients resistant to both drugs (score 4) as compared to those sensitive to both drugs (score 2) (p=0.032).

**Figure 5.** Predicted disease-free (p-DFS) survival for patients with doxorubicin sensitivity above (upper curve) or below median (p=0.086). p-DFS was defined as no leukemia related event (relapse or resistant disease).
Patients with a relapse

When we studied diagnostic samples from children who had an early relapse, < 1 year in CR 1, (n=35) we could show that they were significantly more resistant to mitoxantrone than children who experienced a late relapse (n=30), (p=0.028).

Samples with a successful FMCA from both diagnosis and relapse were available in 18 non-DS patients. In a paired comparison with Wilcoxon signed rank test, none of the drugs included in the AML treatment protocols showed any significant difference. Prednisolone was the only drug tested that showed a significant difference, relapse samples being more sensitive than diagnostic samples (p=0.044). When SI data were compared with a paired t-test, no significant differences were found, but for several drugs the mean SI values tended to be slightly lower in relapse samples.
Paper II

Cellular drug resistance in MLL-rearranged childhood acute leukemia is correlated to partner genes and cell lineage

**AML**

Data on the presence of 11q23 translocations were available in 155 of the 158 samples sent to our laboratory for test of in vitro drug resistance. Since t(9;11) and other 11q23 translocations are known to have different prognostic impact, they were treated separately. Eleven patients with t(9;11) and 16 patients with other 11q23 rearrangements were identified, resulting in an overall prevalence of these rearrangements of 7.1 and 10.3 %, respectively. Testing of cellular drug resistance by FMCA was successfully accomplished in 132 out of the 155 samples. Clinical and laboratory characteristics of the 132 study patients showed that median age was lower in patients with t(9;11) and other 11q23 translocations than in patients without any 11q23 translocation and that the distribution to FAB groups differed markedly, where 9/10 of the t(9;11) patients were FAB group M5 and 9/14 of the other 11q23 group were FAB group M5, while 11/108 of patients with no MLL rearrangement were FAB group M5. The in vitro drug resistance of study patients is shown in Table 8. Patients with t(9;11) were significantly more sensitive to cytarabine and doxorubicin than patients without any 11q23 rearrangement. Patients with t(9;11) were, however, more resistant to dexamethasone and prednisolone. Patients with other 11q23 translocations were also more sensitive to doxorubicin and more resistant to the glucocorticoids than patients without any 11q23 rearrangement, but did not differ in sensitivity to cytarabine.

**ALL**

Data on 11q23 rearrangement status were available in 226 of the 690 samples from patients with ALL sent to our laboratory for test of in vitro drug resistance and twenty-four patients with 11q23 rearrangement were identified, an overall prevalence of 10%. Testing of cellular drug resistance by FMCA was successful in 178 out of the 226 samples. A large proportion of the 11q23 positive patients were infants (59 %), and their WBC count at diagnosis was high, median 171 x 10⁹/L. Translocation t(4;11) was the single most common 11q23 aberration (41 %). We compared the 178 study patients with the 332 patients with successful FMCA but without available 11q23 data and also with the 48 patients with 11q23 data but unsuccessful FMCA and there were no differences in baseline data.
Table 8. Cellular drug resistance in children with AML

<table>
<thead>
<tr>
<th>Drugs</th>
<th>t(9;11) n=10</th>
<th>No 11q23 rearr. n=108</th>
<th>Other 11q23 rearr. n=14</th>
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<tbody>
<tr>
<td>Amsacrine</td>
<td>36</td>
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<td>48</td>
</tr>
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<td>Cytarabine</td>
<td>27</td>
<td>&lt;0.001</td>
<td>53</td>
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<tr>
<td>Dexamethasone</td>
<td>83</td>
<td>0.015</td>
<td>69</td>
</tr>
<tr>
<td>Doxorubicin</td>
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<td>0.005</td>
<td>50</td>
</tr>
<tr>
<td>Etoposide</td>
<td>54</td>
<td>0.46</td>
<td>60</td>
</tr>
<tr>
<td>Prednisolone</td>
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<td>0.021</td>
<td>62</td>
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<tr>
<td>Vincristine</td>
<td>60</td>
<td>0.28</td>
<td>66</td>
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<td>2-CdA</td>
<td>28</td>
<td>0.85</td>
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</tr>
<tr>
<td>4-HC</td>
<td>20</td>
<td>§</td>
<td>58</td>
</tr>
<tr>
<td>6-TG</td>
<td>38</td>
<td>0.70</td>
<td>41</td>
</tr>
</tbody>
</table>

Median values of the survival index, where a low numerical value indicates high cellular sensitivity to the cytotoxic effect of the drug 2-CdA, 2-chlorodeoxyadenosine 4-HC, 4-hydroperoxy-cyclophosphamide 6-TG, 6-thioguanine §, only 2 cases tested

The lymphoblasts of children with 11q23 rearrangement were significantly more sensitive to cytarabine and 2-CdA than those of children without any 11q23 rearrangement, with a difference in the same direction for amsacrine, dexamethasone, doxorubicin, etoposide, and prednisolone Table 9. In groups with different 11q23 rearrangements no significant difference was found. We studied the influence of age and found no difference in sensitivity for children with 11q23 rearrangement who were below or above one year of age. We also compared the in vitro sensitivity in infants, 0-6 and 6-12 months old, respectively and found that blast cells of children in the youngest group were significantly more resistant to amsacrine (p=0.008) and doxorubicin (p=0.003), with a trend in the same direction for dexamethasone, etoposide, prednisolone, and vincristine. Cytarabine was the only drug, for which the two groups showed a similar in vitro sensitivity. There was an inverse correlation between WBC count at diagnosis and cell survival after drug exposure, i.e. blast cells tended to be more sensitive in patients with high WBC counts. The correlation was statistically significant for amsacrine (p=0.006), dexamethasone (p=0.004), etoposide (p=0.011), and prednisolone (p=0.011), with rho values ranging between -0.2 and -0.3, and there was a trend in the same direction for all drugs tested. To further explore the impact of WBC count, we compared patients with 11q23 rearrangement who all had WBC ≥16 x 10^9/L with patients without 11q23 rearrangement and WBC ≥16 x
$10^9$/L (n=61). The 11q23 positive patients were still more sensitive to cytarabine (p=0.026), while there was no statistically significant difference for the other drugs tested.

Table 9. Cellular drug resistance in children with ALL

<table>
<thead>
<tr>
<th>Drug</th>
<th>11q23 rearr n=22</th>
<th>p</th>
<th>No 11q23 rearr n=156</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amsacrine</td>
<td>22</td>
<td>0.043</td>
<td>34</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>41</td>
<td>0.008</td>
<td>57</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>35</td>
<td>0.048</td>
<td>53</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>20</td>
<td>0.035</td>
<td>34</td>
</tr>
<tr>
<td>Etoposide</td>
<td>34</td>
<td>0.12</td>
<td>44</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>34</td>
<td>0.022</td>
<td>48</td>
</tr>
<tr>
<td>Vincristine</td>
<td>36</td>
<td>0.076</td>
<td>51</td>
</tr>
<tr>
<td>2-CdA</td>
<td>19</td>
<td>0.010</td>
<td>40</td>
</tr>
<tr>
<td>4-HC</td>
<td>30</td>
<td>0.36</td>
<td>26</td>
</tr>
<tr>
<td>6-TG</td>
<td>39</td>
<td>0.88</td>
<td>38</td>
</tr>
</tbody>
</table>

Median values of the survival index
2-CdA, 2-chlorodeoxyadenosine
4-HC, 4-hydroperoxy-cyclophosphamide
6-TG, 6-thioguanine

Comparison between AML and ALL

When 11q23 negative patients were compared, ALL patients were considerably more sensitive to amsacrine, dexamethasone, doxorubicin, etoposide, prednisolone, vincristine, and 4-HC (p≤0.001 for all drugs). Cytarabine and 2-CdA were the only drugs for which AML cells tended to be more sensitive, but the difference was not statistically significant.

When patients with 11q23 rearrangement were compared, ALL patients were younger (p=0.031) and had much higher WBC counts at diagnosis (p<0.001). ALL patients were more sensitive to dexamethasone and prednisolone (p<0.001), and to amsacrine (p=0.003), etoposide (0.020), and vincristine (p=0.008). For cytarabine there was a non-significant trend in the other direction.

In the AML group the two t(11;19) cases were of interest, since this translocation also occurred in a number of ALL patients. The AML patients with t(11;19) showed an “AML-profile” with relatively high resistance to gluco-
corticoids and vincristine, and sensitivity to cytarabine. Thus, they differed clearly from patients with ALL and t(11;19).

One patient with ALL displayed a t(9;11) translocation. Although the observation is anecdotal, it is interesting to note that this patient was relatively sensitive to cytarabine, etoposide and vincristine, but resistant to glucocorticoids, i.e. showed a profile with characteristics of both AML and ALL.

Paper III

Doxorubicin pharmacokinetics is correlated to the effect of induction therapy in children with acute myeloid leukemia

The median doxorubicin dose received by non-DS children ≥ 2 years of age was 74.9 mg/m², and the median dose received by children < 2 years of age was 61.7 mg/m² (p=0.013; Table 10). The target dose was 75 mg/m². The median doxorubicin concentration in plasma was 232 and 238 ng/ml in children above and below 2 years of age, respectively, but there was a large inter-individual variation (Table 10). Median total body clearance was 538 ml/min/m² in children aged ≥ 2 years and 446 ml/min/m² in children < 2 years of age (NS). The four infants aged 0.6, 1.0, 1.3, and 1.8 years had clearance values of 298, 590, 1128, and 303 ml/min/m², respectively.

The median plasma concentration of the metabolite doxorubicinol was 29 and 41 ng/ml in children above and below 2 years of age, respectively (NS). Median values for doxorubicinol calculated as per cent of doxorubicin were 17 and 29% in these groups (NS). Higher concentrations of doxorubicinol were found in patients with high doxorubicin levels (rho 0.38; p=0.024).

Total body clearance of doxorubicin was used to explore the correlation between pharmacokinetics and background variables, and all non-DS children were included in this analysis. Boys had a higher clearance than girls, 591 and 427 ml/min/m², respectively (p=0.020), while age, weight, height, ALT, AST, creatinine, albumin, WBC count at diagnosis, and dosage in mg/m², were non-significant.

Children with DS

Four children with DS, aged 1.2, 1.8, 1.9, and 2.3 years, respectively, were studied. They received a median doxorubicin dose of 42.5 mg/m² (Table 9). The median doxorubicin clearance of the DS children was 523 ml/min/m², a value comparable to that of non-DS children. The median doxorubicin concentration in plasma was 151 ng/ml, which represented 65% of that found in non-DS children (p=0.11).
Table 10. *Summary of doxorubicin pharmacokinetic parameters in children with or without Down syndrome (DS)*

<table>
<thead>
<tr>
<th></th>
<th>Non-DS &lt; 2 years</th>
<th>p value #</th>
<th>Non-DS &gt; 2 years</th>
<th>p value §</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose mg/m²</td>
<td>4</td>
<td></td>
<td>33</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>median</td>
<td>61.7</td>
<td>(0.013)</td>
<td>74.9</td>
<td>(0.002)</td>
<td>42.5</td>
</tr>
<tr>
<td>range</td>
<td>55.0-74.6</td>
<td></td>
<td>61.7-79.0</td>
<td></td>
<td>26.7-63.3</td>
</tr>
<tr>
<td>Plasma conc ng/ml</td>
<td>238</td>
<td>(0.90)</td>
<td>232</td>
<td>(0.14)</td>
<td>151</td>
</tr>
<tr>
<td>range</td>
<td>110-348</td>
<td></td>
<td>29-510</td>
<td></td>
<td>87-238</td>
</tr>
<tr>
<td>Clearance ml/min/m²</td>
<td>446</td>
<td>(0.53)</td>
<td>538</td>
<td>(0.48)</td>
<td>523</td>
</tr>
<tr>
<td>range</td>
<td>298-1128</td>
<td></td>
<td>254-4211</td>
<td></td>
<td>348-551</td>
</tr>
<tr>
<td>Dox-ol conc ng/ml</td>
<td>41</td>
<td>(0.50)</td>
<td>29</td>
<td>(0.56)</td>
<td>29</td>
</tr>
<tr>
<td>range</td>
<td>29-110</td>
<td></td>
<td>29-116</td>
<td></td>
<td>29-52</td>
</tr>
<tr>
<td>Dox-ol %</td>
<td>29</td>
<td>(0.21)</td>
<td>17</td>
<td>(0.42)</td>
<td>24</td>
</tr>
<tr>
<td>range</td>
<td>8-36</td>
<td></td>
<td>7-45</td>
<td></td>
<td>12-47</td>
</tr>
</tbody>
</table>

# < 2 years vs. > 2 years old non-DS children  
§ DS vs. all non-DS children  
Dox-ol, doxorubicinol  
Dox-ol %, doxorubicinol conc/ doxorubicin conc x 100

Pharmacokinetics vs. effect in non-DS children

Twenty-six out of 37 patients (70%) went into complete remission (CR) after the first treatment course, whereas 11 did not. Patients who reached CR had a significantly higher median plasma concentration of doxorubicin than those who did not, 249 ng/ml (25th-75th percentiles 178 – 326) and 180 ng/ml (81 – 232), respectively (p=0.036;Figure 6). Figure 7 shows the predicted probability of CR as a function of doxorubicin concentration in a univariate analysis, indicating a concentration-effect relationship (p=0.031). In multivariate analysis including sex, age, and WBC count at diagnosis, doxorubicin concentration was the only independent factor for CR (p=0.021), with a trend value for age (p=0.082; less probability for CR with increasing age). Patients who reached CR had a significantly lower median doxorubicin clearance than those who did not, 513 ml/min/m² (25th-75th percentiles 364 –
603) and 657 ml/min/m² (538 – 1488), respectively (p=0.017). There was no difference in doxorubicinol concentrations between the groups (p=0.14).

Twenty patients were in continuous complete remission (CCR) at the latest follow-up (nine after allogeneic SCT in first CR), while 15 had relapsed (four after allogeneic SCT in first CR), with a median follow-up time of 7.3 years (range 4.3 – 9.7 years). Two patients died in CR after allogeneic SCT in first CR. Doxorubicin concentration was not an independent factor for CCR in univariate (p=0.14) or multivariate analysis including SCT (p=0.12). The two patients who died in CR had doxorubicin concentrations of 232 and 400 ng/ml, respectively.

Figure 6. Doxorubicin plasma concentration in children who went into complete remission after the first induction course (n=26) and those who did not (n=11). Children with DS are excluded.
Repeated courses
Repeated sampling was successful in thirteen patients (one with DS) receiving a second treatment course identical to the first one. The median interval between the start of the courses was 28 days (range 25-47 days). The correlation between the clearance values from the two courses was not statistically significant (rho 0.37; p=0.21). Median doxorubicinol as percentage of doxorubicin was 17 and 16% for the two courses (rho 0.38; p=0.20).

Figure 7. Predicted probability of complete remission (CR) after induction therapy as a function of doxorubicin concentration (n=37). Children with Down syndrome are excluded.
Paper IV
Etoposide pharmacokinetics in children treated for acute myeloid leukemia

There was no statistically significant difference, or any trend toward a difference, between etoposide concentrations measured 48, 72 and 95 hr after start of the infusion (p=0.54). The same was true for the concentrations of free etoposide and the percentage of free etoposide. For each individual, we used the mean value of these three observations as the steady state concentration of the drug in the subsequent calculations.

Children without DS
The median etoposide dose received by children ≥ 2 years of age was 99.9 mg/m²/24 hr day 1-4, which was very close to the target dose of 100 mg/m²/24 hr. The median dose received by children < 2 years of age was 75.8 mg/m²/24 hr (p=0.003) (Table 11) The median steady state concentration of etoposide was 4.00 µg/ml in children aged ≥ 2 years and 3.03 µg/ml in children < 2 years old (p=0.055; Table 11). The median concentration of free etoposide was 0.12 and 0.14 µg/ml in these groups, respectively. Median values for free etoposide calculated as per cent of total etoposide were 3.1 and 4.2%. Median total body clearance was very similar in the two age groups, 17.6 and 17.1 ml/min/m² in children above and below 2 years of age, respectively.

Total body clearance was used to explore the correlation between pharmacokinetics and background variables. There was no difference between boys and girls. In a monovariate analysis, clearance was significantly correlated to ALT (rho -0.33; p=0.038), while age, weight, height, BMI, AST, creatinine, albumin, WBC count at diagnosis, and dosage in mg/m² were non-significant. When tested in linear regression analysis after log transformation of the clearance and ALT values, no significant correlation was found (p=0.26), and the predictive value of ALT levels was low (R²=0.03).

Children with DS
Five children with DS, aged 1.2, 1.8, 1.9, 2.3, and 3.4 years, respectively, were studied (Table 11). They received a median etoposide dose of 66.2 mg/m²/24 hr The median etoposide clearance of the DS children was 13.6 ml/min/m², a value about 20% lower than in non-DS children (p=0.067). Free etoposide values were of the same magnitude as in non-DS children.

Repeated courses
Repeated sampling was successful in 18 patients (one with DS) receiving a second treatment course identical to the first one. The median interval between the start of the courses was 23 days (range 18-42 days). Median clear-
ance was 16.2 and 15.9 ml/min/m² for course 1 and 2, respectively. Most patients showed little variability from course-to-course and the correlation between the clearance values from the two courses was high (rho 0.56; p=0.017). The percentage of free etoposide was also similar, with median values of 2.8 and 3.3%, respectively (rho 0.82; p=0.001; n=13).

There was no significant correlation between etoposide clearance measured during course 2 and any of the background variables mentioned above (body composition and biochemical variables were re-tested before start of course 2).

Table 11. Summary of etoposide pharmacokinetic parameters in children with or without Down syndrome

<table>
<thead>
<tr>
<th></th>
<th>Non-DS &lt; 2 years</th>
<th>p #</th>
<th>Non-DS &gt; 2 years</th>
<th>p §</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Dose, mg/m²/24 hr</td>
<td>4</td>
<td>(0.003)</td>
<td>36</td>
<td>(0.001)</td>
<td>5</td>
</tr>
<tr>
<td>median range</td>
<td>75.8</td>
<td>68.1-86.5</td>
<td></td>
<td>99.9</td>
<td>49.4-107.5</td>
</tr>
<tr>
<td>Steady state conc, μg/ml</td>
<td>3.03</td>
<td>(0.055)</td>
<td>4.00</td>
<td>(0.27)</td>
<td>3.37</td>
</tr>
<tr>
<td>median range</td>
<td>2.75-3.25</td>
<td></td>
<td>1.65-10.6</td>
<td></td>
<td>2.51-4.25</td>
</tr>
<tr>
<td>Clearance, ml/min/m²</td>
<td>17.1</td>
<td>(0.96)</td>
<td>17.6</td>
<td>(0.067)</td>
<td>13.6</td>
</tr>
<tr>
<td>median range</td>
<td>16.4-19.8</td>
<td></td>
<td>5.2-41.7</td>
<td></td>
<td>7.6-19.7</td>
</tr>
<tr>
<td>Clearance, ml/min/kg</td>
<td>0.79</td>
<td>(0.12)</td>
<td>0.62</td>
<td>(0.81)</td>
<td>0.65</td>
</tr>
<tr>
<td>median range</td>
<td>0.72-0.87</td>
<td></td>
<td>0.14-1.30</td>
<td></td>
<td>0.36-0.84</td>
</tr>
<tr>
<td>Free etoposide, μg/ml</td>
<td>0.14</td>
<td></td>
<td>0.12</td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>median range</td>
<td>0.06-0.21</td>
<td></td>
<td>0.0-0.31</td>
<td></td>
<td>0.05-0.07</td>
</tr>
<tr>
<td>Free etoposide, %</td>
<td>4.2</td>
<td></td>
<td>3.1</td>
<td></td>
<td>1.49</td>
</tr>
<tr>
<td>median range</td>
<td>2.0-6.5</td>
<td></td>
<td>0.0-6.8</td>
<td></td>
<td>1.24-2.53</td>
</tr>
</tbody>
</table>

# Non-DS < 2 years s. > 2 years
§ Ds vs. all non-DS children
Free etoposide and etoposide % were only measured in 2 non-DS children < 2 years, 27 non-DS children > 2 years and 3 DS children

Pharmacokinetics vs. effect in non-DS children

We compared the 26 patients who went into CR after the first treatment course with the 13 patients who did not. They showed no significant difference, or any trend toward a difference, in etoposide steady state concentrations or clearance values (p=0.94 and 0.85, respectively). The same was true for the concentrations of free etoposide and the percentage of free etoposide
(tested in 18 CR and 10 non-CR patients). The etoposide steady state concentration was not an independent factor for CR in univariate (p=0.82) or multivariate regression analysis including sex, age, and WBC count (p=0.71). Fig 8 shows the predicted probability of CR as a function of etoposide concentration in a univariate analysis.

Twenty patients were in continuous CR at the latest follow-up (seven after allogeneic SCT in first CR), while 17 had relapsed (four after allogeneic SCT in first CR), with a median follow-up time of 7.6 years (range 4.5-9.8 years). Two patients died in CR after allogeneic SCT. There were no statistically significant differences between CR and relapse patients with respect to etoposide plasma concentration or total body clearance measured during the first induction course (p=0.68 and 0.94, respectively).

Figure 8. Predicted probability of (CR) after induction therapy as a function of etoposide concentration (n=39). Children with DS are excluded.
Paper V
Thioguanine pharmacokinetics in children treated for acute myeloid leukemia

Non-DS children
Erythrocyte TGN concentrations were measured at 48, 72, 95 and 106 h after treatment start. The concentration in the 48-h sample was significantly lower than in the 72, 95 and 106-h samples, \( p<0.001 \), while there was no significant difference (NS) between the other three samples. In the following calculations we used the mean TGN concentration of the 72, 95 and 106-h samples as a measure of drug exposure for each individual.

They received a median 6-TG dose of 199 mg/m\(^2\)/24 h, close to the target dose of 200 mg 6-TG /m\(^2\)/24 h and the median TGN concentration in children > 2 years of age was 2.30 μmol/mmol Hb. The five children < 2 years of age received a median dose of 143 mg 6-TG /m\(^2\)/24 h resulting in a median TGN concentration of 2.13 μmol/mmol Hb (Table 12). To allow a comparison between patients, irrespective of dose, the dose-normalized TGN concentration was calculated. We found a large inter-individual variation in erythrocyte TGN levels after dose normalization as well (30-fold). The dose-normalized concentration of TGN was used to explore the correlation to background variables. We did not find any significant correlation to age, weight, height, ALT, AST, albumin, WBC count, or Hb at diagnosis.

Children with Down syndrome
The four children in the study with DS were 1.1, 1.7, 1.9 and 3.3 years old, respectively. They received a median dose of 114 mg 6-TG /m\(^2\)/24 h and their median TGN concentration was 3.76 μmol/mmol Hb, respectively. Their median dose-normalized TGN concentration was 7.44 μmol/mmol Hb, which was significantly higher than for the non-DS children \( p=0.04 \).

Repeated courses
Thirty-six out of 50 patients went into complete remission (CR) after the first course and received a second treatment course identical to the first one. The median time interval between the start of the courses was 31 days (range 20–50 days). Patients with high TGN concentrations tended to have a longer interval between treatment courses (rho 0.31, \( p=0.088 \) for non-DS children). The three DS children that went into remission after the first course all had long time intervals between the courses (Fig 3).

Repeated sampling was successful in 18 of the 36 patients (one with DS) and there was a strong correlation in TGN concentrations between courses, rho 0.76, \( p<0.001 \).
Table 12. *Summary of 6-TG pharmacokinetic parameters in children with and without DS.*

<table>
<thead>
<tr>
<th></th>
<th>Non-DS &lt; 2 years</th>
<th>p #</th>
<th>Non-DS &gt; 2 years</th>
<th>p §</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TGN conc (μmol/mmol Hb)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>5</td>
<td>0.49</td>
<td>41</td>
<td>0.29</td>
<td>4</td>
</tr>
<tr>
<td>median</td>
<td>2.13</td>
<td></td>
<td>2.30</td>
<td></td>
<td>3.76</td>
</tr>
<tr>
<td>range</td>
<td>0.82-3.04</td>
<td></td>
<td>0.57-25.3</td>
<td></td>
<td>1.83-9.29</td>
</tr>
<tr>
<td><strong>Dose, mg/m²/24 h</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. *</td>
<td>5</td>
<td>0.001</td>
<td>35</td>
<td>0.002</td>
<td>4</td>
</tr>
<tr>
<td>median</td>
<td>143</td>
<td></td>
<td>199</td>
<td></td>
<td>114</td>
</tr>
<tr>
<td>range</td>
<td>109-157</td>
<td></td>
<td>113-233</td>
<td></td>
<td>83-169</td>
</tr>
<tr>
<td><strong>Dose normalised TGN conc (μmol/mmol Hb)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>5</td>
<td>1.0</td>
<td>35</td>
<td>0.04</td>
<td>4</td>
</tr>
<tr>
<td>median</td>
<td>2.97</td>
<td></td>
<td>2.85</td>
<td></td>
<td>7.44</td>
</tr>
<tr>
<td>range</td>
<td>1.11-5.58</td>
<td></td>
<td>0.79-23.4</td>
<td></td>
<td>4.17-11.0</td>
</tr>
</tbody>
</table>

# Non-DS < 2 years vs. > 2 years
§ DS vs. all non-DS children
* Data on the exact dose of 6-TG administered were missing in six cases.
¤ Dose-normalised TGN concentration was calculated by the formula TGN concentration x (target dose/administered dose) where the target dose was set at 200 mg/m²/24 h.

**TGN concentration correlated to response in non-DS patients**

We compared the 33 patients who went into CR after the first treatment course with the 12 patients who did not and there were no significant differences in TGN concentrations. The TGN concentration was not an independent factor for CR in univariate (p=0.92) or multivariate regression analysis including sex, age and WBC count (p=0.92).

Twenty-four patients were in continuous CR at the latest follow-up (eleven after allogeneic SCT in first CR), with a median follow-up time of 9.2 years (range 7.9-12.7 years), while 18 had relapsed (six after allogeneic SCT in first CR). The median TGN concentration for CR and relapse patients was 2.54 and 2.23 μmol/mmol Hb, respectively (NS).
Discussion

The cellular drug resistance study

In this study we could show that high cellular sensitivity to cytarabine and doxorubicin might explain the excellent treatment results in children with AML and t(9;11). In the same direction, cells from patients with DS, who also have a superior outcome in AML were significantly more sensitive to a number of drugs. There were clear differences in drug-resistance profiles in between other cytogenetic AML subgroups even if drug sensitivity and outcome did not always correlate.

In paper II where both AML and ALL were studied our findings indicate that cellular drug resistance is correlated to both the cell lineage and the type of 11q23 rearrangement. The study supports the strategy of contemporary protocols to include high-dose cytarabine in the treatment of 11q23 positive patients both in AML and ALL.

Since less than half of the total number of AML patients diagnosed in the Nordic countries were successfully tested in these studies, the representativeness of our material has to be considered. We received samples from 252 out of 406 non-DS children diagnosed with AML during the study period and 186 of them were successfully analyzed. A major reason for this incompleteness was that some centers participated only during the last years of the study period. Included patients were significantly older and had higher WBC counts at diagnosis than non-included patients. This might reflect difficulties in retrieving extra bone marrow for research purposes from the youngest children. A higher WBC count in successfully analyzed samples was also reported in other studies of AML samples in total cell kill assays. In ALL samples, we have reported that WBC count at diagnosis is negatively correlated to the SI for several drugs, but in the present study a similar correlation was found only for 6-TG. We have no explanation for this difference between ALL and AML samples. However, the great similarities in distribution of FAB type and cytogenetic subgroups as well as in p-EFS, p-DFS and p-OS, strongly argue for that our patient material in important respects is representative for the whole NOPHO cohort of children with
AML. For ALL, a previous report has presented detailed evidence that the samples sent to us for FMCA are representative of the whole population.\textsuperscript{107}

**Cellular drug resistance in cytogenetic subgroups in AML and ALL**

Cytogenetic subgroups are important prognostic factors. For AML the use of cytogenetic subgroups for determination of treatment strategies is quite new compared to ALL. One of the aims of this study was to explore if differences in drug resistance profiles for different cytogenetic subgroups could guide us in the way of understanding the prognostic significance of cytogenetics.

**MLL-gene rearrangements**

In paper II we studied samples from patients with MLL gene rearrangements in both ALL and AML. In paper I, the data for AML were confirmed in a somewhat larger population. MLL gene rearrangements arise from fusion of this gene at 11q23 with a great number of partner genes and is seen in up to 20\% of cases of AML and in 4-8\% in ALL although the frequency varies among studies.\textsuperscript{37} Patients with 11q23 abnormalities tend to be young and in ALL they have high WBC counts at diagnosis.\textsuperscript{108} In AML, patients with the specific 11q23 abnormality t(9;11) are associated with a favorable outcome in both the NOPHO report, where p-EFS at 7 years was 86\%, and the St Judes Children’s Research Hospital (Memphis, USA) report.\textsuperscript{37,106} Patients with t(9;11) are now classified as good risk in the current treatment protocols of these groups. As discussed in both paper I and II we could show that samples from these patients are significantly more sensitive to cytarabine and doxorubicin, two important drugs in the treatment of childhood AML. Thus, the in vitro data offer an explanation for the favorable clinical outcome in these patients. Our results confirm results reported by Zwaan et al 2002, who studied cellular drug resistance in childhood AML and found that t(9;11) samples were more sensitive to cytarabine, etoposide, anthracyclines, and 2-CdA than other AML samples.\textsuperscript{34}

“Other MLL-rearrangements” in AML, excluding t(9;11), have been associated with an average,\textsuperscript{109,110} or worse than average clinical outcome.\textsuperscript{37} We did not find increased cellular drug resistance to any drug for these samples. On the contrary there was a non-significant trend toward lower resistance to doxorubicin and etoposide than in other AML patients. The “other MLL”-group consists of several subgroups. In this study we tested 15 patients with t(9;11), 5 patients with t(11;19), 7 with t(10;11) and 10 undefined “other 11q23” (Table 6). There might be important differences between the sub-subgroups of children with non-t(9;11) MLL-rearrangements, but numbers were too small to allow statistical analysis.
Samples from children with ALL and MLL rearrangements were more sensitive to almost all drugs tested, including glucocorticoids, than ALL patients without MLL rearrangements. These data did not correlate to the well-known fact that ALL patients with 11q23 rearrangements have an unfavorable prognosis. Part of the explanation might be the correlation between cell proliferation and in vitro drug resistance in ALL described by Kaaijk et al. Cell survival after drug exposure correlated negatively to WBC count, i.e. high WBC count was correlated to high drug sensitivity in vitro in our patient material. As expected, the WBC count at diagnosis was high in our ALL patients with 11q23 rearrangements, ranging between 16 and 950 x 10^9/L. WBC count might act as a “confounding factor” when in vitro data are correlated to clinical outcome, especially if groups with large differences in WBC count are compared, as in ALL with or without 11q23 rearrangement. In the clinical setting, factors other than cellular drug sensitivity also come into play, such as the rate of tumor cell re-growth and stroma interactions, which might explain why high WBC count at diagnosis in childhood ALL strongly correlates with a worse clinical outcome. Still, the in vitro data might be clinically useful. In full agreement with the findings of Ramakers-van Woerden et al, we demonstrate that the 11q23 positive patients are particularly sensitive to cytarabine. This supports the idea of the ongoing clinical trials, where two large prospective international studies in infants (Children’s Oncology Group and Interfant) are testing the efficacy of intensified therapy that includes high-dose cytarabine.

**Comparison between ALL and AML**

That samples from children with AML and ALL have markedly different drug sensitivity profiles was an early finding, and has recently been described in detail by Zwaan et al. Our comparison of patients without 11q23 abnormalities fully supports previous data that AML samples are more resistant to a wide range of drugs including glucocorticoids, vincristine, anthracyclines, and etoposide, but equally sensitive to cytarabine and 6-thioguanine as ALL samples. These data are in good agreement with clinical practice and may contribute to the difference in prognosis between childhood ALL and AML.

When patients with 11q23 rearrangements were compared, much of the ALL and AML patterns described above were found. Thus, AML samples were much more resistant to typical “ALL-drugs” like glucocorticoids and vincristine, while there was a trend in the other direction for cytarabine. Our findings indicate that both cell lineage and the type of MLL rearrangement are of importance for the cellular drug resistance. This fits well with studies of the gene expression profile in MLL-rearranged pediatric ALL, and AML, where it was reported that the lineage of origin is of importance, but that a shared gene expression signature can be identified for cases with MLL chimeric fusion genes. This expression signature probably reflects a cellular
pathobiology which is of importance for the in vitro sensitivity to cytarabine, shared by MLL rearranged childhood leukemias. However, direct experimentation will be required to determine which of the identified genes play a mechanistic role for drug sensitivity. Our data support the strategy of contemporary protocols to include cytarabine in the treatment of 11q23-positive patients both in AML and ALL.

**Other cytogenetic subgroups in AML**

Leukemias characterized by t(8;21) and inv(16) are also known as core binding factor (CBF) leukemias. They are generally thought to carry a favorable prognosis, but some research groups have reported diverging results for the t(8;21) sub-group with 5 year p-EFS below 50%.\(^5,10,12,20,21\) For the t(8;21) patients included in the present study p-EFS and p-OS was 0.54 and 0.82, respectively. This indicates a relatively high relapse rate, with a high salvage rate after relapse, a finding also reported by other groups.\(^13,122\)

We found that the t(8;21) samples had a resistant profile, where the cells were significantly more resistant than other AML samples to all five drugs in the NOPHO AML treatment protocols: cytarabine, doxorubicin, etoposide, mitoxantrone, and 6-thioguanine. Patients with the karyotype inv(16), clearly connected to FAB M4, are considered as good-risk patients, and were found to have 60% EFS and 77% OS in the NOPHO AML 93 material.\(^106\) The samples from inv(16) patients had a specific drug sensitivity profile, and were more sensitive to 6-thioguanine but more resistant to doxorubicin than other AML samples.

A favorable outcome has been reported for patients with t(15;17), associated with FAB M3,\(^123\) and this was the case in our patient material as well. However, we did not find a sensitive drug profile in this patient group. On the contrary, they were significantly more resistant to cytarabine and 6-thioguanine than other AML-patients, and a previous report on three t(15;17) positive children indicated similar findings.\(^124\) This is not astonishing, since the favorable outcome mainly is achieved by addition of ATRA to regular chemotherapy,\(^123,125\) and with chemotherapy alone the clinical outcome in this subgroup is less favorable, with 30-50% survival.\(^33,126\)

High resistance to corticosteroids, as compared to ALL samples, is characteristic for most AML subgroups. In patients with “other clonal abnormalities” the resistance to dexamethasone and prednisolone was significantly less pronounced than in other AML subgroups. Since this group represents a mixed bag, it is not possible to make a detailed analysis of the background to these data, and any clinical implications remain uncertain. An interesting finding was that samples from children with monosomy 7 (n=5) were markedly sensitive to dexamethasone and prednisolone with median SI values of 46 % and 47%, respectively, values otherwise seen only for corticosteroids in ALL.
Cellular drug resistance in AML related to treatment response and outcome

To study the relation between initial treatment response and cellular drug resistance, we compared drug resistance in patients who went into remission after one treatment course with those who did not. In our material no significant differences in drug resistance were shown. Other studies in both children and adults have been able to show a relationship between initial response and drug resistance, \textsuperscript{73,127} and drug resistance and outcome.\textsuperscript{128} In a recent Nordic relapse study of AML children, Abrahamsson et al could show that length of first complete remission (CR1) was the strongest predictor of survival.\textsuperscript{129} Here we could show that children who had an early relapse (<1 year in CR1) were significantly more resistant to mitoxantrone than children with a late relapse. For all other drugs there was a lack of difference in drug resistance when we compared patients in CCR with patients who experienced a leukemia-related event, or when comparing data for patients who died with those who were alive at follow-up. Similar findings were reported by Zwaan et al 2002.\textsuperscript{34} and the authors suggested that factors other than cellular drug resistance must have played a more important role in determining clinical outcome in their cohort of childhood AML patients. They point out differences in pharmacokinetics or pharmacogenomics, clonal evolution, and/or relapse potential of residual disease. Another possibility was that the study of cellular drug resistance correctly predicted the cytotoxicity profile of the bulk of AML cells, but did not reflect a small but resistant subclone of leukemic stem cells that eventually caused relapse.

Since we found marked differences in the drug sensitivity profile between patients with different chromosomal aberrations, we also attempted to study the relation of SI data to long-term clinical outcome within the largest cytogenetic subgroups. In the t(8;21) subgroup, we found that patients who remained in CCR were significantly more sensitive to doxorubicin at diagnosis than relapse patients, and the same was true for cytarabine in patients with “other clonal abnormalities”. These findings underscore the fact that childhood AML is a heterogeneous disease and that analysis of pooled data might obscure biologically relevant findings. Ideally, the relation between cellular drug resistance and clinical outcome should be further studied in larger cohorts of well-defined cytogenetic subgroups.

Successful FMCA in paired samples from patients with samples both from diagnosis and relapse was achievable in only 18 patients. There were no indications of higher resistance in samples collected at relapse than in samples from diagnosis. This somewhat unexpected finding needs to be confirmed in larger groups of patients, and it might also be fruitful to study patients who suffer repeated relapses and develop a disease with clinically overt chemoresistance. The NOPHO experience is that many relapse patients
can be salvaged, as indicated by rather marked differences between DFS and OS data in some subgroups. Our findings that the bulk of leukemic cells collected at first relapse might be as chemosensitive as those in diagnostic samples are in agreement with these clinical observations.

The pharmacokinetic study

We performed a pharmacokinetic study of the drugs used in children with AML receiving their first and for some also a second identical treatment course. Our main findings were that we could show that doxorubicin plasma concentration and total body clearance during up-front treatment correlated to the effect of induction therapy. We could for etoposide and 6-TG show that DS children might be candidates for dose reduction as they end up with equal or higher concentrations than non-DS children in spite of markedly reduced doses.

Our original plan was to measure the plasma levels of all four drugs administered during induction therapy: cytarabine, etoposide, 6-TG and doxorubicin. Cytarabine measurements failed due to lack of a specific and reproducible method for analysis of the drug. The other drugs were successfully analyzed, but for practical reasons results for all three drugs were not available in all patients (a major factor was a transport accident). Considering that complete data were available in only 25 patients the drugs were in practice analyzed separately.

Doxorubicin

The median total body clearance of doxorubicin found here in 41 children is similar to that reported by us for children treated for acute lymphoblastic leukemia (ALL). In the ALL study, doxorubicin was infused after injection of vincristine and during concomitant treatment with oral prednisolone. Here, doxorubicin was given on day 5 of an intense induction course, preceded by a 4-day continuous infusion of etoposide and cytarabine with concomitant administration of oral 6-thioguanine. Apparently, the pharmacokinetics of doxorubicin was not significantly influenced by any of these drugs. In agreement with previous studies, we found a large inter-individual variation, with more than 10-fold differences in steady state concentrations and clearance values. Correlation to known background variables could explain these differences only to a limited extent.
Published data regarding the age dependency of doxorubicin pharmacokinetics in children are equivocal.\textsuperscript{130-133} In the present study, we found no statistically significant correlation between pharmacokinetic parameters and age. Thus, the study did not yield data that could improve doxorubicin dosage compared to that based on BSA.

In adults, a lower plasma clearance of doxorubicin has been observed in females as compared to males.\textsuperscript{134} It has also been reported that nausea associated with anthracycline-containing regimens is more severe in girls than in boys,\textsuperscript{135} and that girls run a higher risk of abnormalities in cardiac function.\textsuperscript{136,137} In the present study, we found that boys had higher clearance values than girls giving a possible explanation for the clinical symptoms.

A relationship between plasma concentrations of doxorubicin and the outcome of induction therapy has been reported for AML in adults,\textsuperscript{114} but to our knowledge there are no studies of the correlation between doxorubicin plasma levels and therapeutic effect in childhood leukemia or any other pediatric malignancy.\textsuperscript{138} We found a statistically significant correlation between doxorubicin pharmacokinetics and the effect of remission induction therapy. Patients who reached CR had significantly higher plasma levels and lower total body clearance of doxorubicin than those who did not. The doxorubicin concentration was an independent factor for CR in univariate (p=0.031) and multivariate analysis including sex, age, and WBC count at diagnosis (p=0.021).

At long-term follow-up, patients who remained in CCR tended to have higher plasma levels of doxorubicin than those who relapsed, but the difference was not statistically significant. Doxorubicin concentration was not an independent factor for CCR in univariate or multivariate analysis. This discrepancy between short and long-term clinical effect might partly be due to the fact that doxorubicin was given during induction therapy only, while the four consolidation courses consisted of cytarabine, etoposide and mitoxantrone. The fact that 41% of the non-DS patients received allogeneic stem cell transplantation (SCT) in first remission is also an obvious confounding factor in the evaluation of long-term effects of doxorubicin. The number of patients treated without SCT was too small for meaningful analysis.

Even when a relationship between plasma concentrations and effect can be established, pharmacokinetically guided therapy is only feasible if there is a limited intra-individual variability from course to course. Thirteen of the children in this study, who went into CR after the first induction course, received a second identical course 3-4 weeks later. Although some patients showed very little course-to-course variability, the results were quite unpredictable in others.
Etoposide

We studied 45 patients for etoposide pharmacokinetics and found that steady state levels of etoposide were reached before 48 h, as evidenced by very stable plasma levels throughout the sampling period. This was expected, since the terminal half-life of etoposide in plasma is short, ranging between 2 and 6 h in children.139-142 Etoposide is known to be highly bound to plasma proteins, and our finding that only 3-4% of total etoposide was in free form agrees with previous reports.143,144

The median steady state concentration in the infants was 76% of that found in children ≥ 2 years of age, indicating that they received a less intense treatment. Median total body clearance of etoposide was very similar in the two age groups, and all four infants had clearance values close to the median clearance of older children confirming earlier data.139,145

Many contemporary protocols, for example the Interfant 99 and Interfant 06, international collaborative treatment protocols for infants under one year of age with ALL, recommend dose reduction of etoposide for this age group, sometimes with additional reduction for infants < 6 months old. Dose reduction results in low plasma levels, and this might be one of the reasons why infants treated for ALL have an inferior prognosis, especially those below 6 months of age. We think available data support the idea that infants > 3 months old should receive etoposide in doses calculated from body surface area as in children > 1 year of age. Children < 3 months of age represent a special problem, since renal function is immature at birth, with a gradual maturation during the first weeks and months.146

Previous publications have reported that etoposide elimination was decreased by cyclosporin and nephrotoxic drugs such as cisplatin and carboplatin (see reviews).138,147 Prednisone, on the other hand, strongly induced etoposide clearance, probably by its effect on CYP3A4-mediated metabolism of etoposide.148 The clearance values found in our patient material were similar to those reported in a number of previous studies,139-142 indicating that etoposide pharmacokinetics were not significantly influenced by the concomitant administration of cytarabine and 6-thioguanine.

Renal excretion accounts for about 45% of systemic etoposide clearance and renal impairment affects etoposide pharmacokinetics.142 Hepatic metabolism also plays an important role.140 We found no correlation between etoposide clearance and creatinine or aminotransferase levels, but this was probably due to the fact that few patients had values outside the reference intervals. Still, the small number of children with clearly elevated creatinine or aminotransferase levels tended to have lower than average etoposide clearance values. We found a limited course-to-course variability, indicating that pharmacokinetically guided dosing of etoposide might be clinically relevant, if it can be demonstrated that this approach increases response or decreases toxicity without jeopardizing the anti-tumor effect. We were, however, un-
able to demonstrate any correlation between etoposide pharmacokinetics and clinical response.

6-TG
In the study of 6-TG pharmacokinetics in 50 patients we measured the levels of TGN metabolites in erythrocytes as TGN gradually accumulates in the erythrocytes after repeated oral intake. This method circumvents the problem of widely fluctuating plasma levels of the parent drug during dose intervals. A previous study showed that patients receiving 40 mg 6-TG/m² once daily during maintenance therapy reached steady state concentrations of 0.1–0.3 μmol/mmol Hb after 1-2 weeks. Our patients received 100 mg 6-TG /m² twice daily, and their TGN levels reached a plateau already after 72 h of treatment, with a median concentration of 2.3 μmol/mmol Hb. Thus, a five-fold increase in dose resulted in a rapid accumulation of TGN in erythrocytes and an approximately 10-fold increase in TGN concentration. The TGN levels measured at 72, 95 and 106 h were very similar, with little intra-individual variation, but it is not clear if this represents a true steady state. In any case, we judged that the mean value of these three samples was the best available measure of drug exposure in our patients.

The present data confirm previous findings of large inter-individual variations in 6-TG pharmacokinetics. We compared the TGN concentrations with baseline data such as age, weight, length, BMI, ALT, AST, albumin, WBC count and Hb at diagnosis, but found no significant correlations. The extensive first pass metabolism in intestinal mucosa and liver after oral administration leads to a low and highly variable bioavailability, which appears to be the most probable explanation for the inter-individual differences in erythrocyte TGN concentration. To allow a comparison between patients irrespective of dose, as patients below 2 years of age receive reduced doses, the dose-normalized TGN concentration was calculated. No significant differences were found between children below and above 2 years of age. Thus, our data give no support to dose reduction of 6-TG in infants. However, it must be kept in mind that only two children were < 1 year and none < 6 months of age.

The children with DS received reduced doses of 6-TG compared to non-DS children. After dose normalization they had significantly higher TGN concentrations, indicating that dose reduction might be considered to reach the same drug exposure as in non-DS children. A correlation between TGN levels and post-treatment neutropenia and thrombocytopenia has been reported in patients receiving maintenance therapy. We found a non-significant (p=0.088) correlation between TGN concentrations and time interval to the next treatment course in non-DS children. We did not, however, find any correlation between TGN concentration
and our pre-defined endpoints for clinical effect, i.e. bone marrow morphology after induction therapy and long-term clinical follow-up. Several factors might have contributed: 6-TG was administered as one of four drugs during the induction course and was followed by consolidation blocks without 6-TG, which might “dilute” or obscure any effect of the drug. Furthermore, it has been reported that TGN concentrations in neutrophils can differ from those in erythrocytes, and therefore erythrocyte TGN may not reflect drug exposure of the target cells as well as has been presumed.\textsuperscript{157,158}

**Correlations between drugs**

As mentioned above, the doxorubicin plasma concentration was an independent factor for CR in children with AML treated according to NOPHO AML-93, but we found no correlation between etoposide pharmacokinetics and remission or relapse rate. Children with high TGN concentrations tended to have longer treatment intervals to the next course, but we found no correlation to our predefined parameters for clinical response, i.e. remission and relapse rate. There was no correlation, or any trend toward a correlation, between TGN and etoposide steady state concentrations (n=35), or between TGN and doxorubicin concentrations (n=36). This was not unexpected, since the drugs are metabolized and eliminated by different pathways. In a multivariate analysis, including TGN, doxorubicin and etoposide concentrations, none of the drugs was an independent factor for CR. However, complete data were available for only 25 non-DS patients.

**Patients with AML and Down syndrome**

Children with ML-DS have a superior prognosis compared to non-DS children. The favorable outcome in ML-DS has been explained by the increased sensitivity to chemotherapy, especially cytarabine and anthracyclines,\textsuperscript{34,40,41} and a higher susceptibility of DS cells to apoptosis.\textsuperscript{42,43} The mechanism of increased drug sensitivity and toxicity in ML-DS is not fully understood, but differences in pharmacokinetics and pharmacodynamics, as well as trisomy 21-related genetic changes, might influence the response to therapy.\textsuperscript{159} The technical success rate for FMCA in samples from DS children was 52%, which is considerably lower than for non-DS samples. A major reason was spontaneous cell death in control wells, a finding which might reflect that DS cells are prone to apoptosis.\textsuperscript{42,43} However, the results in the 15 children with successful FMCA were very clear-cut, and our data confirm the previously reported high sensitivity to cytarabine and doxorubicin.\textsuperscript{124} ML-DS cells were also significantly more sensitive to etoposide than non-DS samples, with a trend in the same direction for mitoxantrone.
In the pharmacokinetic study, we observed that though the NOPHO AML-93 protocol had no recommendations for dosage in DS children, the doses were often reduced compared to doses of non-DS children. There were large differences in practice between centers, probably reflecting an uncertainty caused by the almost complete lack of pharmacokinetic data for anticancer drugs in DS children. To our knowledge, etoposide pharmacokinetics has been reported for two DS patients only, and for doxorubicin and 6-TG no reports are known to us. Our patient material is admittedly small, but hopefully adds some knowledge that might be useful. For doxorubicin, DS children had a total body clearance comparable to that of non-DS children, suggesting that dose reduction is unnecessary from a strictly pharmacokinetic point of view. On the other hand, etoposide and 6-TG data indicated that DS children might be candidates for dose reduction to reach drug exposure levels similar to non-DS children.

For children with AML treatment has changed markedly over the past decades. Until fairly recently, DS children received no treatment at all, due to the misconception that they were not curable. Then study reports showed that they had a favorable outcome when treated according to regular AML protocols though they experienced more toxic side effects of the treatment.39,120,160 The outcome is now improving even more by reducing doses of treatment.120 The clinical observations of young age, low WBC count, predominance of FAB M7, and presence of GATA-1 mutations, together with the finding of a specific drug sensitivity profiles, clearly indicate that ML-DS AML is a unique leukemia.21,44 It has been suggested that the high drug sensitivity is primarily a gene-dosage effect involving specific chromosome 21-localized genes that influence the metabolism of certain drugs.40,159 However, this is contradicted by the fact that samples from DS children with ALL do not show the sensitive profile seen in AML patients. On the contrary they are significantly more resistant to dexamethasone than samples from non-DS ALL patients, with trends in the same direction for several other drugs.41
Conclusions

Paper I

The cellular drug sensitivity study confirms our earlier findings (Paper II) that high cellular sensitivity to cytarabine and doxorubicin might explain the excellent treatment results in children with AML and t(9;11). In the same direction, cells from patients with DS, who also have a superior outcome in AML, were significantly more sensitive to a number of drugs. There were clear differences in drug-sensitivity profiles between cytogenetic subgroups, indicating future possibility of more subgroup-directed chemotherapy.

Paper II

The study of cellular drug sensitivity in MLL-rearranged leukemia indicate that sensitivity is correlated to both the cell lineage and the type of 11q23 rearrangement. High cellular sensitivity to cytarabine and doxorubicin might explain the excellent treatment results in children with AML and t(9;11). The present study supports the strategy of contemporary protocols to include high-dose cytarabine in the treatment of 11q23 positive patients both in AML and ALL.

Paper III

The doxorubicin pharmacokinetic study showed that doxorubicin plasma concentration and total body clearance during up-front treatment correlated to the effect of induction therapy. DS children received reduced doses, had an equal clearance and achieved lower plasma concentrations than non-DS children.

Paper IV

The etoposide pharmacokinetic study indicates that special dose-calculation guidelines for infants > 3 months of age are not substantiated by age-dependent pharmacokinetics. DS children might be candidates for dose reduction if our data are confirmed in larger numbers of patients. Low course-to-course variability indicates that pharmacokinetically guided dosing of
etoposide might be clinically relevant, if larger studies can demonstrate that this approach decreases toxicity or increases response rates.

Paper V

The thioguanine pharmacokinetic study showed that children with high TGN concentrations tended to have longer treatment intervals to the next course, but we found no correlation to our predefined parameters for clinical response, i.e. remission and relapse rate. Children with DS had significantly higher TGN concentrations, indicating that dose reduction might be considered to reach the same drug exposure as in non-DS children.
Future directions

Test of *in vitro* drug sensitivity, by different methods, has this far been able to show specific drug sensitivity profiles in certain subgroups of patients. For patients with Down syndrome and patients with t(9;11), who have an excellent prognosis, we and others have shown that the leukemic cells are significantly more sensitive to several drugs used in AML therapy than cells from other AML patients.

For other groups of AML patients, it has been more difficult to show relations between cellular drug sensitivity and initial treatment response or long-term clinical effect and the reason for that is to a large extent unknown.

One way of improvement will be to more detailed identify the chromosomal abnormalities. Today some sub-groups are wellknown but a large group of childhood AML leukemias are labeled “other abnormalities”, a group which probably consists of several different subgroups with varying prognosis. “Normal karyotype” is also one large group, which certainly will be subdivided into several. To be able to do this, large and well defined patient materials have to be examined in controlled studies.

Modern high throughput techniques give a possibility to correlate *in vitro* sensitivity data to more detailed information on the genetics of the leukemic cells. Such studies of gene expression, copy number changes, and DNA methylation are now discussed in NOPHO’s AML group.

Another reason for lack of correlation between cellular drug sensitivity and treatment response can be that the study material was not representative for the resistant subclone of leukemic stem cells that eventually caused relapse. New techniques for improving the precision of the *in vitro* drug sensitivity method should be applied, and one way can be to further purify the cells to be analysed by techniques like flow cytometry or separation by magnetic beads. This would ascertain the representativity of the cell sample being studied.

Factors other than cellular drug resistance play important roles in determining clinical outcome in childhood AML patients such as differences in pharmacokinetics or pharmacogenomics, clonal evolution, and/or relapse potential of residual disease. The study of pharmacogenomics might be one way of improving anti-cancer drug dosing. Today, the thiopurine methyl-
transferase activity can be measured to guide the dosing of 6-mercaptopurine and 6-thioguanine. Finding similar pharmacogenomic factors for other drugs could simplify future pharmacological studies and dosing by the use of high throughput techniques, e.g. SNP arrays or micro arrays for gene expression profiling.

Many of the drugs administered in pediatric oncology have been used for several years and the introduction of new drugs is a long process, especially in children. To perform a pharmacokinetic study is a challenge because of both ethical and logistical issues. For some drugs, pharmacokinetic monitoring could be of great importance, since receiving inadequate drug concentrations can lead to an ineffective treatment and/or undesired side-effects. The dosing of anticancer drugs in infants and children with Down syndrome is presently based on assumptions rather than solid facts. Our findings give some indications, but patient numbers are small, and ideally large cohorts of children should be studied. However, the paucity of such reports in the literature illustrates how difficult these studies are to perform, and the need to find new methods.

Another of our findings, that doxorubicin concentration and total body clearance during up-front treatment were correlated to the effect of induction therapy, is the first report of such a relationship in childhood leukemia. If the findings are validated in prospective studies, therapeutic monitoring might be clinically useful.
Cancer i barnaåren är ovanligt men ändå näst efter olyckor den vanligaste dödsorsaken i denna åldersgrupp. Av de olika cancerformerna hos barn utgör leukemi ungefär en tredjedel och de vanligaste typerna av leukemi är akut lymfatisk leukemi, ALL, ca 80 procent och akut myeloisk leukemi, AML, ca 15 procent. För både ALL och AML hos barn har överlevnadssiffrorna dramatiskt förbättrats under de senaste decennierna och i dag överlever drygt 80 procent med ALL och ca 60% med AML. Alla barn med leukemi i Norden behandlas och följs upp i enlighet med NOPHOs (Nordic Society of Pediatric Haematology and Oncology) rekommendationer, något som lett till välkontrollerade behandlingar och goda behandlingsresultat med internationella mått mått.

De delarbeten som ingår i avhandlingen, I-V, baseras på två studier där prover och epidemiologiska data kommer från hela Norden. Delarbete I och II baseras på en studie av leukemicells känslighet för cytostatika (cellgift). Benmärks-och blod prover tagna på barn som nyligen insjuknat i AML har skickats för analys med fluorometric microculture cytotoxicity assay (FMCA) vid avdelningen för klinisk farmakologi i Uppsala. Vid denna metod inkuberas levande leukemiceller i analys-brunnar innehållande olika typer av cytostatika i 72 timmar varefter andelen överlevande celler mäts.

I de två första delarbetena har cytostatikakänslighet undersöks på prover från ca 200 barn som nyinsjuknat i AML. I delarbete I görs en genomgång av cytostatikakänslighet hos barn med AML. I delarbete II studeras cytostatikakänslighet hos barn med cytogenetiska förändringar av typen MLL-rearrangemang både vid AML och ALL. I båda delarbetena kan vi visa att celler från gruppen t(9;11), en typ av MLL-rearrangemang som visat mycket goda överlevnadssiffror, uppvisar en uttalad cytostatikakänslighet jämfört med andra grupper. Vi kan i delarbete II också visa att patienter med MLL-rearrangemang, både vid ALL och AML är mer känsliga för cytarabin, något som stöder pågående studier där man behandlar dessa patienter med cytarabin.

I delarbete I kan vi visa en uttalad cytostatikakänslighet hos leukemiceller från barn med Down syndrom (DS), något som väl korrelerar med den kliniska effekten av behandlingen. Barn med DS har en kraftigt ökad risk att insjukna i AML under de första levnadsåren men samtidigt en god överlevnad.

Delarbetena III-V beskriver farmakokinetiken för tre av de cytostatika som ingår i AML behandlingen samt korrelationen mellan farmakokinetik och klinisk effekt. Analys av doxorubicin utfördes på prover från 41 barn, etoposid från 45 barn och 6-thioguanin (6-TG) från 50 barn. För doxorubicin sågs en korrelation mellan koncentration, clearence och effekt av den första behandlingskuren. Dessutom var doxorubicin koncentrationen en oberoende faktor för komplett remission. För etoposid och 6-TG fanns ingen korrelation mellan farmakokinetik och effekt av behandlingen. Vid jämföbara doser hade barn med DS högre koncentrationer av både etoposid och 6-TG talande för att en dosreducering för dessa patienter sannolikt behövs för att de inte skall uppnå högre koncentrationer än barn utan DS.

Sammanfattningsvis tyder våra resultat på att studier både av cytostatikakänslighet och farmakokinetik kan tillföra viktig information om den individuella patientens svar på behandlingen, något som kan hjälpa oss i vår strävan att alltmer kunna skräddarsy behandlingen för barn med AML.
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