Session I:
BPCA – Study Proposals

- Daunomycin
- Methotrexate

Impact of body composition on pharmacokinetics of doxorubicin in pediatric patients: A Glaser Pediatric Research Network study. *S. L. Berg, L. Bomgaars, C. Twist, K. Matthay, T. Moore, D. Wypij; Texas Children's Cancer Ctr, Houston, TX; Stanford Univ Sch of Medicine, Palo Alto, CA; Univ of CA at San Francisco, San Francisco, CA; Univ of CA at Los Angeles, Los Angeles, CA; Harvard Medcl Sch, Boston, MA*

Background Body composition affects various physiologic processes involved in the distribution, metabolism, and elimination of drugs. Therefore body composition might be related to important pharmacokinetic (PK) parameters like clearance or half-life. These relationships have never been systematically studied for doxorubicin (dox) and its metabolite doxorubicinol (doxol). In addition, the PK behavior of dox and doxol have not been thoroughly described in children. In this study we evaluated the relationship between body composition and doxorubicin PK in children. **Methods** Eligible subjects were ≥ 1 and ≤ 21 years old; weighed ≥ 12 kg; received dox administered as an infusion of any duration < 24 hours, on either a 1-day or 2-day schedule; had ALT/AST ≤ 3 times the upper limit of normal and bilirubin $\leq$ upper limit of normal tested within 14 days prior to dox; and provided informed consent/assent. Blood samples were drawn prior to dox administration, at the midpoint of the infusion and at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 48 hours after the infusion for 1-day schedules, or at 0, 0.5, 1, 2, 4, and 6, hours after the day 1 infusion and immediately prior to the day 2 infusion, then at 0, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, and 48 hours after the day 2 infusion for 2-day schedules. Body composition was determined using dual-energy x-ray absorptiometry (DXA). Body mass index (BMI) was calculated from height and weight. Dox and doxol concentrations were analyzed by reverse-phase high-pressure liquid chromatography with fluorescence detection. Dox plasma PK were analyzed with maximum likelihood estimation as implemented in ADAPT II. **Results** Data are available for 16 subjects (12 male; 8 Hispanic, 6 Caucasian, 2 Asian). The median age is 15 years (range 3–21). The median % body fat by DXA is 22 % (range 16–36). The median BMI is 20 (range 14–30). The median dox clearance is 382 ml/m^2/min (range 141–768). The median terminal half-life is 30.6 hr (range 18.1–146.6). Dox clearance decreases with increasing BMI ($r=-0.56$, $P=0.02$) and tends to decrease with increasing % body fat ($r=-0.43$, $P=0.10$). **Conclusions** Potential relationships between increasing BMI, decreasing dox clearance, and dox toxicity should be explored.

CHILDREN'S ONCOLOGY GROUP

***ABTR06C1* Pharmacokinetics of Daunomycin in Children**
Study Chair: Stacey Berg, MD

Abstract:

Daunomycin is an anthracycline antineoplastic drug widely used in the treatment of acute lymphocytic leukemia and other malignancies in childhood. There are no data on the pharmacokinetics of daunomycin in obese children, and only scanty data on daunomycin pharmacokinetics in children in general. Like many anticancer agents, daunomycin has a narrow therapeutic index. Doses of anticancer drugs are usually calculated based on body surface area (BSA) or body weight as a uniform standard. This practice is based on the concept that hepatic and renal function are proportionate to BSA. In most studies, however, variability in overall drug clearance is only partially accounted for by variability in BSA. There is a growing list of drugs for which clearance has been poorly correlated with BSA. In addition, after equivalent BSA-based doses, some patients experience little toxicity while others may show severe toxic side effects. Therefore some have questioned whether normalizing anticancer drug dose to BSA is the optimal method for selecting a dosing regimen in adults. Furthermore, the appropriate dosing of anticancer drugs in patients who are very large or who are obese presents a major therapeutic challenge. Better understanding of the relationships among body size, body composition, and pharmacokinetics could provide a rational approach to the problem of appropriate drug dosing

Hypothesis:

We hypothesize that obesity and body composition, as well as age, gender, and ethnic background, will have an impact on the pharmacokinetic behavior of daunomycin, a widely used anticancer agent, in patients 21 years of age and younger.

Specific Aims:

Primary: To determine the pharmacokinetics of daunomycin in children.

Secondary:

1. To evaluate the relationship between body composition (percent body fat) and daunomycin pharmacokinetics in children.

2. To determine whether daunomycin pharmacokinetics are correlated with gender, age, or ethnic background in children.

Background and Significance:

Daunomycin is an anthracycline antineoplastic drug widely used in the treatment of acute lymphocytic leukemia and other malignancies in childhood. Like many anticancer agents, daunomycin has a narrow therapeutic index. Myelosuppression and stomatitis are common acute toxicities. Cardiomyopathy is an important dose-dependent late effect that is being recognized with increasing frequency. Despite its frequent use, however, daunomycin's pharmacokinetics and pharmacodynamics have not been studied systematically in children and very little is known about the relationship between pharmacokinetic parameters and covariates like obesity, body composition, age, gender, or ethnicity. Dosing is empiric, and a rational basis for dose modifications in children

who are overweight or obese, in particular, is lacking. This represents a significant gap in our knowledge of the safe and appropriate use of this important agent.

Doses of anticancer drugs are usually calculated based on body surface area (BSA) or body weight as a uniform standard. This practice is based on the concept that hepatic and renal function are proportionate to BSA. In most studies, however, variability in overall drug clearance is only partially accounted for by variability in BSA. There is a growing list of drugs for which clearance has been poorly correlated with BSA. In addition, after equivalent BSA-based doses, some patients experience little toxicity while others may show severe toxic side effects. (1) Therefore some have questioned whether normalizing anticancer drug dose to BSA is the optimal method for selecting a dosing regimen in adults. (2) Furthermore, the appropriate dosing of anticancer drugs in patients who are very large or who are obese presents a major therapeutic challenge.

Better understanding of the relationships among body size, body composition, and pharmacokinetics could provide a rational approach to the problem of appropriate drug dosing. The major factors affecting distribution of drugs in the tissues are body composition, regional blood flow, and the affinity of the drug for plasma proteins and/or tissue components. Obese people have larger absolute lean body masses as well as fat masses than non-obese individuals; however, their body fat percentage is much more markedly increased. (3) There are data supporting the hypothesis that many physiologic processes involved in the distribution, metabolism, and elimination of drugs may be altered in obese individuals. Obesity has been reported to alter the pharmacokinetics of several anticancer agents. (3-7) For most drugs, however, there are limited data evaluating the potential relationship between body composition and pharmacokinetics or toxicity of specific agents. Furthermore, it is unclear whether it is better to dose obese patients based on actual weight, ideal weight, or some compromise value. In obese patients, calculated drug doses can be as much as 25 to 30% higher if total body weight is used to determine BSA than if ideal body weight is used. An upper limit or "cap" of dosing based on a BSA of 2 - 2.2 m^2 is often empirically recommended. (1) Conversely, however, there is concern that patients who are given reduced doses may have a decreased dose intensity of treatment and a worse disease outcome. (8)

Obesity is a increasingly common problem. (9) Obesity is currently defined by body mass index (BMI), which is expressed by the equation BMI = weight (in kg)/height^2 (in m^2). Children with a BMI greater than the 85th and 95th percentiles of the second National Health and Nutrition Examination Survey (NHANES II) are considered overweight and obese respectively. (10-12) Recent statistics in pediatrics revealed that 24% of children have a BMI greater than the 85th percentile for age and 13% of children have a BMI greater than the 95th percentile for age. Thus it is likely that an increasing number of children diagnosed with leukemia and other cancers are likely to be overweight or obese at presentation. In order to treat these patients optimally it will be critical to understand appropriate dosing of the commonly used anticancer drugs in obese patients.

While BMI is the most common measure currently utilized to identify overweight or obese individuals, relatively few studies have been done to evaluate the accuracy of BMI in the assessment of the actual body composition of an individual patient. (13-16) In children, it may be important to consider maturation stage, race, gender, and distribution of body fat as well as BMI in determining whether children should be considered obese. (13) Therefore an exploration of the relationship between body composition and BMI in children with cancer is warranted.

There is very little data on the effect of gender, age, liver function, or ethnic background on anthracycline pharmacokinetics in children. One report noted variation of peak doxorubicin concentration with age. (17) Others have noted gender related differences in clearance. (18) There are some suggestions that anthracycline clearance decreases in the presence of liver dysfunction, the data is not conclusive. (19-22) We will conduct exploratory studies to see whether these factors may correlate with daunomycin pharmacokinetics.

Preliminary Data:

Daunomycin assay

We have considerable experience in analyzing plasma concentrations of anthracyclines in our laboratory. We have recently revalidated a previously published HPLC assay for doxorubicin (23, 24), in which we use daunomycin as internal standard. In brief, plasma samples are spiked with daunomycin as internal standard, then undergo solid phase extraction using Nexus cartridges conditioned with 1 ml methanol followed by 1 ml water. After loading 0.5 mL of plasma mixed with 0.5 mL 1% phosphoric acid the sample is rinsed with water and 5% acetonitrile then eluted with acetonitrile. Eluates are evaporated to dryness under nitrogen at 37°C. Prior to injection onto the HPLC system samples are reconstituted in 0.5 m of 1% phosphoric acid.

100 µL of reconstituted sample is injected via a Model 717 Plus Autosampler (Waters, Inc) onto a Luna C18(2), 3µm, 4.6 mm X 150 mm analytic column with a Phenomenex C18, 2 mm X 3 mm, 3µ guard column and eluted with a gradient consisting of solvent A (75% 20 mM KH_2PO_4 with 1mL/L H_3PO_4/25% acetonitrile v/v) from 0-7 min followed by solvent B (60% 20 mM KH_2PO_4 with 1mL/L H_3PO_4/40% acetonitrile) from 7-11 min followed by solvent A from 11-20 min at an isocratic flow rate of 1 ml/min. Peaks are monitored on a Model 474 Scanning Fluorescence Detector (Waters, Inc) with an excitation wavelength of 480 nm and an emission cutoff of 550 nm. The retention times are 4 minutes for doxorubicinol, 8 minutes for doxorubicin, and 15 minutes for daunomycin. Recovery of doxorubicin and doxorubicinol is approximately 80%. The limit of quantitation of doxorubicin and doxorubicinol is 2 ng/ml, and the standard curve is linear from 0.01 – 20 µg/ml. The intraday and interday coefficients of variation are less than 7%. Daunomycinol is also well separated in this assay. We anticipate that this assay will be very suitable for quantifying daunomycin and daunomycinol. The primary difficulty is obtaining analytic grade daunomycinol, which must be made by a contract chemical laboratory.

As part of our work with doxorubicin, we performed in vitro experiments to determine whether pharmacokinetic (PK) samples can be drawn through the same central catheter used for drug administration. We found that pharmacokinetic samples too often became contaminated with residual drug from the line, even when large volumes of blood are drawn as a “discard” to flush the catheter. At present, therefore, use of a separate peripheral IV for pharmacokinetic sampling in patients is required. However, Drs. Barrett and Adamson at Children’s Hospital of Philadelphia have developed a method that clears the line adequately to permit it to be used for sampling other anticancer agents (Peter Adamson, personal communication). If we are able to validate this method for daunomycin, we will amend our protocol to permit sampling through the central line.

Daunomycin Clinical Pharmacology

There are few studies of daunomycin pharmacokinetics in children. In adults, daunomycin is relatively highly protein bound with a large volume of distribution and a prolonged terminal half-life. Daunomycin metabolism is incompletely understood, but parent drug is rapidly converted to the alcohol metabolite daunomycinol, which also has a long half-life (20-40 hr). After a dose of daunomycin plasma exposure to the metabolite exceeds that of the parent drug. (25, 26)The most serious adverse effect of the drug is irreversible, dose-dependent cardiotoxicity. that may have its onset many years after the completion of therapy. Therefore, the cumulative lifetime dose of daunomycin and the cardiac function of patients receiving the drug are monitored closely. Some data suggests that anthracycline induced cardiac toxicity may be mediated by the alcohol metabolites and not by parent drug. (27-30). Children treated at younger ages appear to be more vulnerable to this toxicity, possibly because subclinically damaged myocardium is unable to meet the demands of body growth. (31-34)

Daunomycin Pharmacokinetics in Obesity

There are no data on the pharmacokinetics of daunomycin in obese children. We are currently conducting a study on the relationship between BMI, body composition, and the pharmacokinetics of the closely related anthracycline doxorubicin. Analysis of the preliminary data shows that doxorubicin clearance decreases with increasing BMI (35). The effect of obesity seems similar in adults. Rodvold et al studied twenty-one obese (defined as ≥ 115% of ideal body weight) adults treated with doxorubicin. Obesity was associated with a prolonged elimination half-life and decreased clearance, and the percentage of ideal body weight correlated strongly with the decrease in clearance. (7). Although effects of body composition on daunomycin pharmacokinetics have not been reported, it is reasonable to speculate that they would be similar to that observed with doxorubicin.

Study Design and Methods:

The research described in this study is a protocol to determine the BMI and body composition of children under the age of 21 who are receiving daunomycin as part of prescribed chemotherapy, and to correlate those results with the pharmacokinetic behavior of daunomycin in the same patients. The administration of daunomycin itself is not part of this study, and the patients' clinical care will not be affected by participation.

Subjects:

Prospective subjects who meet the appropriate age and chemotherapy protocol will be recruited at participating institutions that have IRB approval for the study.

Eligibility

Age : Eligible patients will be < 21 years old

Chemotherapy protocol : All patients must be receiving chemotherapy that includes daunomycin administered as an infusion of any duration <24 hours. This includes bolus and all short infusion schedules.

Informed consent: All patients or their parents/legal guardians will provide informed consent/assent

Exclusion criteria

Women who are known to be pregnant or lactating

Patients with significant uncontrolled systemic illness

Pre-study evaluations

History and Physical Exam

A complete history and physical examination including height, weight and body surface area will be performed within the 14 days prior to the day of daunomycin administration and pharmacokinetic sampling.

Weight measurement

Patients should be weighed with only light clothing; shoes must be removed before weight is measured.

Height measurement

Patients should be measured using a stadiometer after removing shoes.

Laboratory evaluation

Pre-study laboratory evaluations will include: CBC with differential and platelet count, ALT, AST, bilirubin, BUN, creatinine, total protein, albumin, PT/PTT, alkaline phosphatase, and GGT. While the evaluations must be performed no greater than 14 days prior to daunomycin administration, if patients have had significant intercurrent illness or treatment that might affect organ function, laboratory work should be performed at an appropriately closer interval to daunomycin administration.

<u>On-Study Evaluations</u>

Prior Therapy

Standard Children's Oncology Group prior therapy data will be collected on all patients entered onto this study.

Concomitant Medications

Assessment of all concomitant medication administration will be made and recorded on the appropriate form. This list should include any medications the patient receives during the 7 days prior to daunomycin administration as well as all medications administered during the period of pharmacokinetic sampling.

Toxicity

Although daunomycin administration is not part of this protocol and therefore adverse events from daunomycin will not be related to participation on this protocol, we will collect standard CTCAE v.3 adverse events for the chemotherapy cycle associated with the daunomycin dose around which the PK samples are drawn. This will permit exploration of possible relationships between PK results and toxicity, although the analysis can only be preliminary since the concomitant chemotherapy will vary from patient to patient.

Body Composition (at participating institutions)

Body composition testing must be completed within 7 days before or after administration of the dose of daunomycin. Body composition will be assessed using dual-energy x-ray absorptiometry (DXA). The effective dose is less than 5-7 microSv.

Pharmacokinetic Sampling

Plasma pharmacokinetic sampling for daunomycin and daunomycinol levels will be performed with one cycle of daunomycin administered as per the patient's treatment protocol. Blood samples will be drawn from a location different from the infusion site according to the following schedule: prior to the drug infusion, at the midpoint of the infusion if infusion is ≥ 30 min in duration, and at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 (when feasible), 24, and 48 and 72 (when feasible) hours after the infusion. At each time point, 3 mL of blood should be collected into a green top tube (sodium heparin). For regimens with daunomycin dosing on two days, the samples will be drawn on day 1 prior to the drug infusion, and at end of infusion and at 0.5, 1, 2, 4, and 6, hours after the end of the day 1 infusion and immediately prior to the day 2 infusion, then at the end of infusion and at 0.5, 1, 1.5, 2, 4, 6, 8, and 12 (when feasible) hours after the end of the day 2 infusion. Samples will also be

collected at 24, 48, and 72 (when feasible) hours after the end of the day 2 infusion. The total amount of blood drawn will be approximately 35 to 40 mL; no greater than 3 mL/kg (< 5% of the blood volume) will be obtained.

Pharmacokinetic Methods

Samples will be immediately separated by centrifugation and plasma frozen until analysis. Daunomycin and daunomycinol concentrations in plasma will be measured as described above.

Feasibility testing for drawing PK samples through the central line will assessed using the method being developed by Dr. Adamson's group for actinomycin and vincristine. In brief, this method involves fitting a 3-way stopcock to the catheter hub, then pulling 5 mL of blood from the patient side of the catheter and returning it to the patient side. This is repeated 4 times, followed by the pulling one more 5 mL "waste," then the actual sample. We will test this procedure in vitro using normal donor blood after infusion of daunomycin through the catheter in order to ensure that PK samples are not contaminated with residual daunomycin. If the method can be validated, the protocol will be amended to permit drawing PK samples from the central line through which drug was administered.

Pharmacokinetic Modeling

Pharmacokinetic modeling will be performed using ADAPT II software. (36) Population modeling will be performed using mixed effect models in WinNonMix. (37, 38)

Statistical Considerations

The primary goal of this study is to evaluate the impact of obesity and body composition on the pharmacokinetic parameters of daunomycin in children. The concentration-time data will be analyzed by model dependent and model-independent means. Pharmacokinetic data will be analyzed using ADAPT II software (Biomedical Simulations Resource, University of Southern California). The model-derived pharmacokinetic parameters will be used for subsequent analysis unless there is significant evidence of lack of fit of the model to the data.

The pharmacokinetic parameters (with AUC as the primary parameter of interest) will then be correlated with the BMI. Linear regression will be performed to examine the relationship between daunomycin AUC and BMI and between daunomycin terminal half-life and BMI.

The DXA results will be analyzed with body composition software, version 5.56 (Hologic, Inc., Waltham, Massachusetts). DXA measurement of fat mass in children has been shown to be reliable when compared to hydrodensitometry estimates (16), with an in vivo precision of 2-4% for children. Body fatness will be based on the percentage of fat obtained by using DXA and is defined as (100)(fat mass)/body weight (14).

Based on the literature and the investigators' experience with doxorubicin pharmacokinetics, it is expected at least a moderate correlation between the pharmacokinetic parameters of daunomycin and body mass index. A sample size of 100 would be sufficient to detect a minimum correlation coefficient (between pharmacokinetic parameters and BMI) of 0.32 with an α of 0.05 and 90% power. For such a correlation, this would mean that

10% of the variability in the pharmacokinetic parameter being evaluated (e.g. clearance) could be explained by the effect of BMI on that parameter. The power to detect a higher correlation would be greater than 90%.

In addition to the univariate analysis, multivariate analysis of the data will also be performed. We plan to assess the significance of the relationship between the following characteristics: BMI, BSA, ALT, bilirubin, age, gender, and ethnicity, and daunomycin PK parameters. The table below outlines the increment in R^2 associated with any one variable that can be detected with high probability as a function of the statistical importance, in terms of R^2, of the remaining variables in the regression model.

Increment in R^2 Detectable with Large Probability When 100 Patients are Evaluated and Six Predictor Variables are Included in the Regression Model

R^2 Associated with Predictor Variables Incorporated Into the Extant Regression Model	Increment in R^2 Associated With addition of the 'New' Variable' to the Regression Model	Probability that the Increment in R^2 Will be Identified as Significant Using a Two-Sided Test of Size 0.05
0.50	0.04	0.83
0.40	0.05	0.85
0.30	0.06	0.86

The relationship between daunomycin pharmacokinetics and body composition (percent body fat) will also be examined in order to explore whether body composition is a more accurate predictor of altered daunomycin kinetics than is BMI.

An attempt will be made to include approximately equal numbers of male and female children and a broad range of ages and ethnic backgrounds as well as BMI. We intend to enroll at least 15 patients younger than 3 years of age in order to ensure that we have sufficient young patients to identify any important influence of age on daunomycin PK. On a similar study, for the first 16 patients enrolled, the average BMI is 19.6, with a standard deviation of 4.2, and a range of 16.5-30. We believe therefore that it is reasonable to expect that we will see a similar variation in BMI on this study. We will perform planned interim analyses to evaluate whether recruiting strategies or eligibility should be altered to address these issues. The enrollment of <3 year olds on study will be monitored half-way through meeting the study accrual goals (~50 patients). If there are too few patients in this age group, accrual may be restricted until the required minimum number of such patients is accrued on study.

Human Subjects

Gender/minority recruitment

The ethnic distribution varies between institutions. Each institution will recruit according to the populations served. The estimated distribution for each institution is described in the individual IRB applications. Subjects from both genders and all racial/ethnic groups are eligible for the currently proposed study if they meet the eligibility criteria.

Risks and Benefits

The primary risk to patients on this study is adverse events associated with blood drawing. These include the risks of pain and infection. All blood draws will be done under routine sterile precautions. Temporary peripheral intravenous lines will be placed for collection of blood for pharmacokinetic sampling. The study requires body composition testing by DXA. There are no discomforts associated with the DXA measurement except lying still for a few minutes. The technologist is at the side of the patient during the procedure. The DXA instrument uses an x-ray beam, similar to that for a chest x-ray, but the intensity of the DXA beam is very weak. The DXA scan would have to be repeated about 40-50 times to produce a dose similar to that for the chest x-ray. The dose from one DXA scan is about the same as one receives everyday from the natural background radiation sources (sun, air, Earth, building materials, other people). In addition to the physical risks listed above, patients will be informed of the results of their body composition testing. There is the potential for psychological stress associated with being told that one is obese. Subjects will receive no direct benefit from entering the study. However, the information gained from this study could be very important in developing dosing strategies for daunomycin in the obese patient population.

Time

The pre-study evaluations (history and physical exam and phlebotomy for laboratory analyses) will take approximately 30 minutes. The body composition testing by DXA will take approximately 30 minutes. Pharmacokinetic sampling will cover a 48 hour period after daunomycin administration. Subjects will be compensated appropriately for their time and effort.

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Activated:
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Version Date: 01/09/06

CHILDREN'S ONCOLOGY GROUP

AALL0434

Intensified Methotrexate, Nelarabine (Compound 506U78; IND # 52611) and Augmented BFM Therapy for Children and Young Adults with Newly Diagnosed T-cell Acute Lymphoblastic Leukemia

A Groupwide Phase III Study

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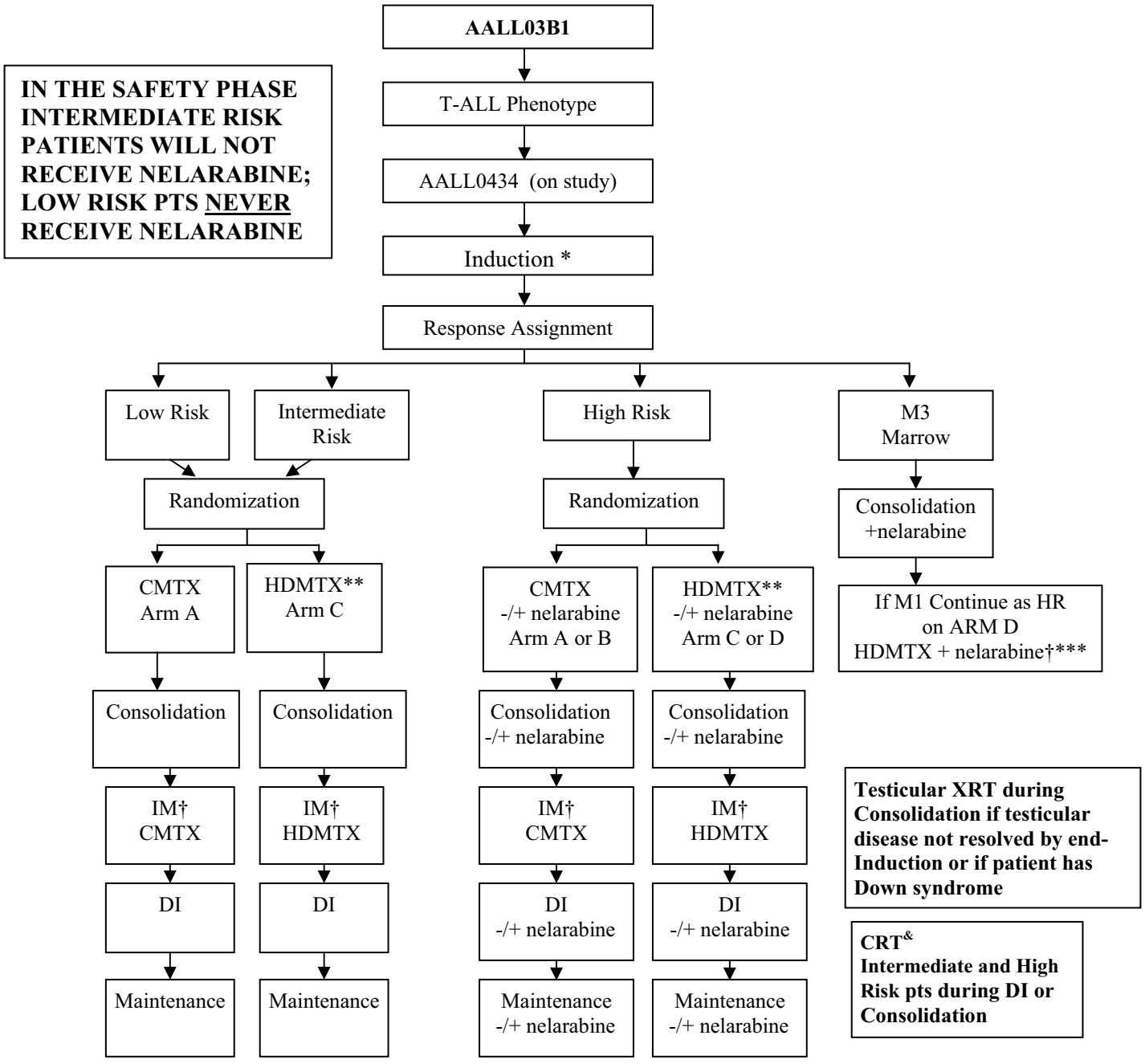
ABSTRACT

AALL0434 is a COG group-wide Phase III study designed for patients with T-lineage acute lymphoblastic leukemia (T-ALL) from 1-30 years of age. Although event free survival (EFS) and overall survival continue to increase for children and young adults with T-ALL, "On-Treatment" relapses in the central nervous system (CNS) and bone marrow compartments continue to be common causes of treatment failure. There is evidence that both nelarabine (Compound 506U78) and high dose methotrexate (HDMTX) are effective in preventing relapse in T-ALL. To specifically address the early treatment failures associated with T-ALL, this study will test the safety and efficacy of these two therapeutic interventions. The study utilizes a 2 x 2 factorial design with augmented intensity BFM backbone. After a Day 29 risk assignment has been determined, patients will become eligible for treatment assignment or randomization. Patients will be randomized to receive Capizzi style escalating dose IV methotrexate without leucovorin rescue (plus PEG Asparaginase) versus high dose methotrexate (5 gm/m^2) with leucovorin rescue during the two month interim maintenance phase of therapy. During the safety phase of the trial only the high risk patients will be randomized to either receive or not to receive nelarabine. During the efficacy phase of the study, intermediate risk patients will also be randomized to either receive or not to receive nelarabine at a dose of 650 mg/m^2/day for 5 days during the Consolidation, Delayed Intensification and Maintenance phases of therapy. All patients will receive only one Delayed Intensification course. All Intermediate and High Risk patients will receive prophylactic cranial radiation (1200 cGy) either during Consolidation (if randomized to treatment Arm A (CMTX) or Arm B (CMTX + Nel)) or Delayed Intensification (if randomized to treatment Arm C (HDMTX) or Arm D (HDMTX + Nel)). All patients classified as CNS3 will be non-randomly assigned to receive HDMTX and will receive cranial radiation therapy (1800 cGy) during Delayed Intensification. Patients with testicular leukemia will be non-randomly assigned to receive HDMTX and will receive testicular irradiation (2400 cGy) during Consolidation therapy, if testicular disease does not resolve by the end of Induction therapy. Low-risk patients, who are NCI standard risk by age and WBC, with no testicular disease at diagnosis, CNS1 and rapid early responders (RERs) with an M1 marrow on Day 8 or 15, and minimal residual disease (MRD) < 0.1% on Day 29, have an excellent outcome and therefore will not receive nelarabine in either the safety or randomized phases; nor will they receive cranial radiation.

EXPERIMENTAL DESIGN SCHEMA: SAFETY PHASE



* Induction evaluation = Day 8 BMA; if not M1 then repeat on Day 15.
Evaluation of BMA and MRD on Day 29.
** Patients with CNS3 and/or testicular disease at Dx will be assigned to HDMTX arms
***Patient may also be taken off study for alternate therapy, including BMT
†Patients must be M1 at end-Consolidation to continue on therapy
RER = M1 marrow on Day 8 and < 0.1% MRD on Day 29 OR
M2/M3 marrow on Day 8 and M1 marrow on Day 15 and
< 0.1% MRD on Day 29.
SER = M2/M3 on Day 15 OR positive MRD on Day 29.
Low Risk = NCI SR by age & WBC count; RER, M1 on Day 15 and MRD < 0.1% on Day 29; CNS 1 status; and no testicular disease at diagnosis.
Intermediate Risk = RER or SER with MRD < 1% on Day 29; any CNS status.
High Risk = M2 at end of Induction or MRD ≥ 1% on Day 29; any CNS status.

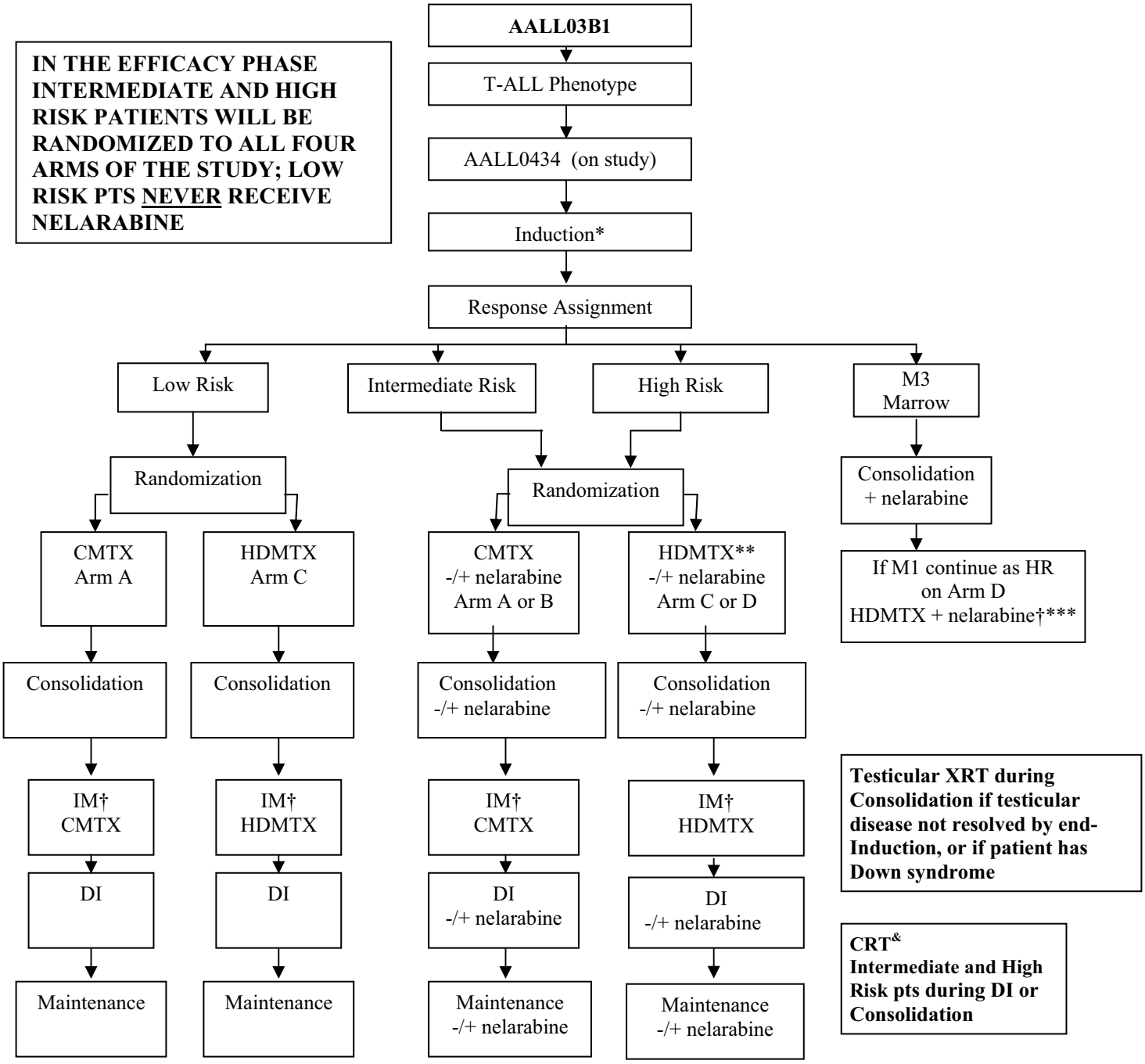
CMTX = Capizzi escalating MTX
HDMTX = High dose MTX
IM = Interim Maintenance
DI = Delayed Intensification

Patients with Down Syndrome will not Receive HDMTX
Patients with a prior seizure disorder will Not receive nelarabine

& CRT = cranial radiation (See Section 14.0 for details).

The safety phase ends when the 1st 20 HR pts to receive nelarabine have been evaluated per Section 10.2.

EXPERIMENTAL DESIGN SCHEMA: EFFICACY PHASE



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Evaluation of BMA and MRD on Day 29.
** Patients with CNS3 and/or testicular disease at Dx will be assigned to HDMTX arms
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Intermediate Risk = RER or SER with MRD < 1% on Day 29; any CNS status.
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& CRT = cranial radiation (See Section 14.0 for details).

1.0 GOALS AND OBJECTIVES (SCIENTIFIC AIMS)

1.1 Main Clinical Objectives

1.1.1
To determine, through randomization, the relative safety and efficacy of the addition of nelarabine (Compound 506U78) to augmented BFM therapy (Regimen C, CCG-1961).

1.1.2
To determine the relative safety and efficacy of high dose methotrexate (5gm/m^2) with leucovorin rescue compared to escalating methotrexate without leucovorin rescue plus PEG-Asparaginase (Capizzi I) delivered during Interim Maintenance.

1.2 Secondary Clinical Objective

1.2.1
To determine the relative safety and efficacy of withholding radiation in patients with low risk T-ALL, while treating intermediate and high-risk patients with 1200 cGy of prophylactic cranial radiation.

1.3. Secondary Correlative Objectives

1.3.1
Exploratory studies will be undertaken on the pharmacokinetics and intracellular pharmacology of nelarabine for consenting patients

2.0 BACKGROUND

T-cell lymphoid malignancies have distinct biochemical, immunologic and clinical features which set them apart from non-T-lymphoid malignancies. [1-6] Historically, the diagnosis of T-ALL portended a worse prognosis than other forms of non-T childhood ALL. [1,7,8] Over the past three decades, the introduction of intensive, high-dose, multi-agent pulse chemotherapy has significantly improved the EFS for patients with T-ALL from 15-20% to 40-73%.[9-12] Current trials have further improved outcomes for children with T-ALL, but have plateaued in the 70-75% EFS range, as shown by Dana Farber Cancer Institute (DFCI) 85-01, [13] DFCI 87-01 and Berlin-Frankfurt-Münster (BFM)-86.[14] The recent Pediatric Oncology Group (POG) T-cell ALL protocol, POG 9404, randomized the addition of high dose methotrexate (HDMTX) to the DFCI regimen and found a statistically significant improvement in 4-year EFS rates for NCI high-risk patients treated with and without HDMTX of 77.9% vs. 65.5%, respectively. The 76.9% EFS at 5 years for patients with T-ALL, treated on CCG 1961, regimen C, without HDMTX, is comparable to that seen on 9404 with HDMTX. The majority of patients with T-ALL and NCI standard-risk features clearly fare better with more aggressive therapy, as demonstrated by EFS rates of 71% on CCG 1952/1962 versus 87.4% on POG 9404 (89.2% within the HDMTX arm). Based on a projected 2 year EFS rate of 85% for T-cell patients compared to 94% for pre-B ALL patients treated on the CCG 1991 study (RHR of 3.12 {p = .002}), T-ALL patients will no longer be eligible for COG SR-ALL trials and will be treated on the T-cell specific AALL0434 trial.

Rarely does one find a drug with lineage-specific cytotoxicity as recently discovered for nelarabine. Nelarabine (2-amino-9-B-D-arabinofuranosyl-6-methoxy-9H-purine) is a water-soluble prodrug of araG (9-B-arabinofuranosylguanine), a synthetic deoxyguanosine derivative that is resistant to cleavage by endogenous purine nucleoside phosphorylase and is cytotoxic to T-lymphoblasts at micromolar concentrations. Cytotoxicity is mediated by the accumulation of araG nucleotides, especially d-araGTP, in T-cells greater than in B-cells, resulting in inhibition of ribonucleotide reductase and inhibition of DNA

Activated: December 29, 2003
Closed:
Version Date: February 14, 2005
Amendment Number: 2

CHILDREN'S ONCOLOGY GROUP

AALL0232

High Risk B-precursor Acute Lymphoblastic Leukemia

A Phase III Group-Wide Study

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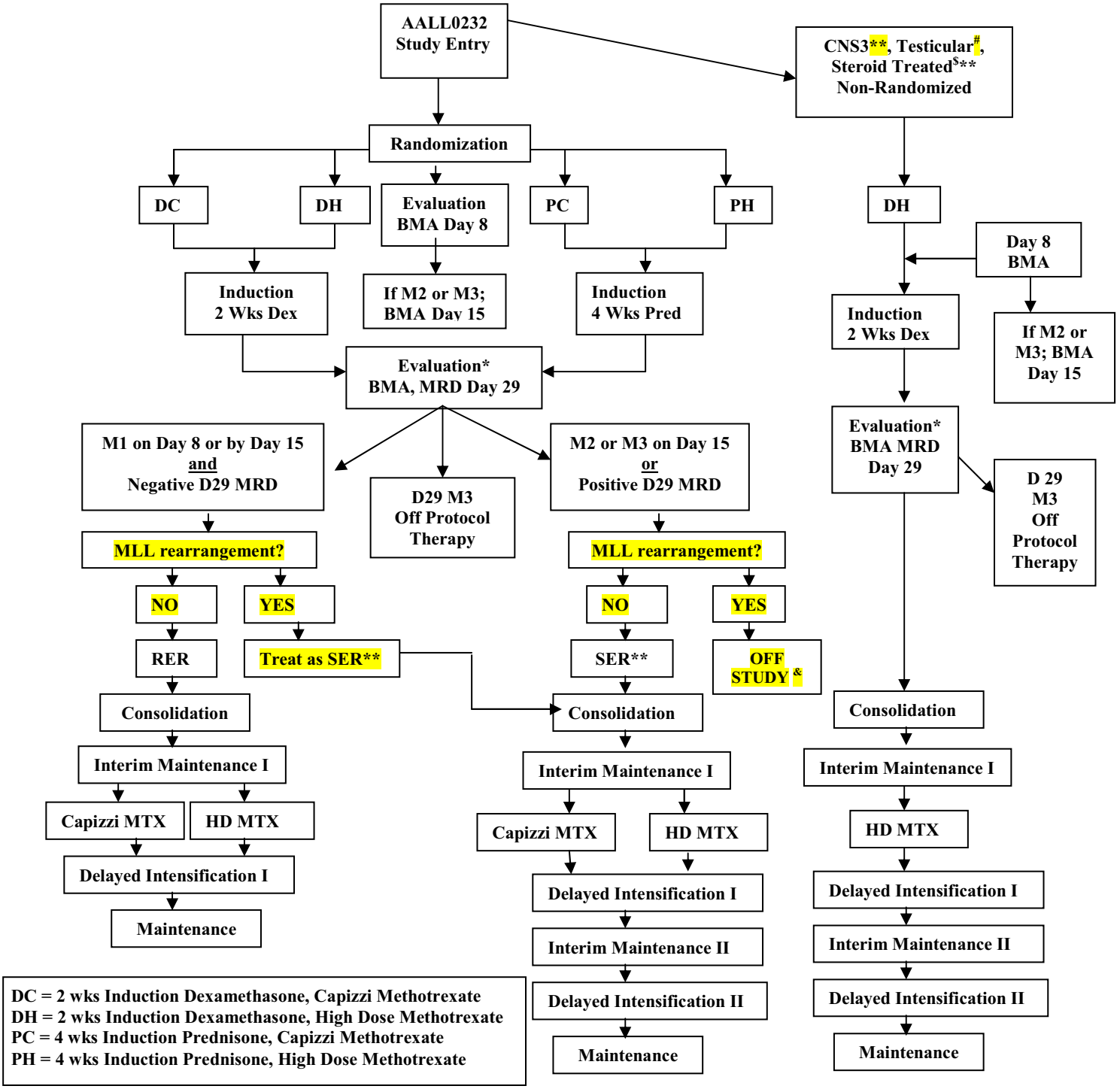
SHORT ABSTRACT

AALL0232 is a COG group-wide phase III study designed for NCI high risk patients with acute lymphoblastic leukemia (ALL) from 1-30 years of age. Although event free survival and overall survival continue to increase for children with high risk ALL, CNS disease has become an increasing cause of treatment failure. There is evidence that both dexamethasone and high dose methotrexate prevent CNS relapse. To specifically address the relative increase in CNS events this study will test safety and efficacy of these two therapeutic interventions. The study utilizes a 2 x 2 factorial design with an augmented intensity BFM backbone. Patients will be randomized upfront to receive dexamethasone 10 mg/m^2/day for 14 days versus prednisone 60 mg/m^2/day for 28 days during Induction and to receive high dose methotrexate (5 gm/m^2) versus Capizzi escalating methotrexate during Interim Maintenance I. Patients classified as rapid early responders (RER) will receive one Delayed Intensification course and those classified as slow early responders (SER) will receive two Delayed Intensification courses.

EXPERIMENTAL SCHEMA



*** If Day 29 BM is M2 or M1 with MRD ≥ 1% patient receives 2 weeks of additional Induction followed by re-evaluation of BM morphology and MRD status. If marrow is M2 or M3 or MRD ≥ 1% on Day 43 patient is Off Study and may be eligible for AALL0031.**

**** Patients receive cranial XRT during DI-2**

 # Patients receive testicular XRT during Consolidation

$ Steroid Treated: Patients who received more than 48 hours of steroids during the week immediately prior to diagnosis.

& May be eligible for AALL0031.

Note: Patients with Down syndrome (DS) will be blocked from randomization to the high dose methotrexate arms. The eligible arms for this group of patients are DC or PC, unless the patient has DS with CNS3 and/or testicular involvement. These DS patients will be non-randomly assigned to DC with DDI and the appropriate radiation therapy.

1.0 GOALS AND OBJECTIVES (SCIENTIFIC AIMS)

1.1
To improve the outcome of children with high risk acute lymphoblastic leukemia.

1.2
To determine the relative safety and efficacy of dexamethasone given for 14 days versus prednisone given for 28 days during Induction.

1.3
To determine the relative safety and efficacy of high dose methotrexate (5 gm/m^2) with leucovorin rescue compared to escalating methotrexate without leucovorin rescue (Capizzi I) delivered during Interim Maintenance I.

1.4
To correlate Day 29 Minimal Residual Disease (MRD) with Event Free Survival (EFS) and Overall Survival (OS).

1.5
To correlate early marrow response status with Day 29 MRD status.

1.6
To improve outcome by identifying additional high risk patients by Day 29 MRD for treatment with fully augmented BFM.

2.0 BACKGROUND

Sequential clinical trials in the Children's Cancer Group (CCG) and the Pediatric Oncology Group (POG) have resulted in incremental improvements in event free survival and overall survival for children with NCI high risk acute lymphoblastic leukemia (ALL). A critical component of these improved results has been the use of intensified post induction therapy. Previous CCG trials have implemented augmented post-induction therapy in both lower and high risk subgroups[1] while POG trials have focused on optimizing intermediate dose methotrexate during consolidation. [2]

In CCG-1882, patients showing a rapid early response received standard CCG-modified BFM therapy and were randomized to receive either IT methotrexate and cranial radiation or intensified IT methotrexate alone for pre-symptomatic CNS therapy. Overall, intensified IT methotrexate produced a slightly better EFS compared to IT methotrexate and cranial radiation. In patients greater than 10 years of age, intensified IT methotrexate resulted in a significant decrease in the incidence of bone marrow relapse. Patients showing a slow early response to induction therapy were randomized to receive CCG-modified standard BFM (IT methotrexate/cranial RT for pre-symptomatic CNS therapy) and "augmented" BFM which featured additional vincristine and L-asparaginase in consolidation and reconsolidation phases and Capizzi methotrexate (vincristine and IV methotrexate on Day 1 and L-asparaginase on Day 2) replaced oral 6-MP/methotrexate in interim maintenance. In addition, patients received a second intensified interim maintenance and reinduction reconsolidation (DI phase). Augmented therapy produced a marked improvement in EFS compared to standard CCG-modified BFM, particularly for patients less than 13 years of age. CCG-1961 evaluated the role of two components of the augmented regimen: increased early intensification, and longer duration of intensive therapy in the form of a second interim maintenance and DI phase. Recent analysis of the patients with rapid early response randomized on CCG-1961 demonstrates that augmenting the intensity of early therapy leads to a better

Review

Chemotherapy

Chemotherapy 2003;49:92–104
DOI: 10.1159/000069773

Received: August 5, 2002
Accepted after revision: January 2, 2003

Biochemical and Clinical Aspects of Methotrexate Neurotoxicity

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Key Words
Methotrexate · Neurotoxicity · Folates · Homocysteine · Adenosine · Biopterins · Excitatory amino acids · CNS · Cancer

Abstract
Acute, subacute and chronic neurotoxicity have been observed after the administration of high-dose and/or intrathecal methotrexate (MTX). Acute toxicity is usually transient without permanent damage. Subacute and chronic toxicity are associated with changes in the brain and/or the spinal cord which may be progressive and even lead to coma and death in severe cases. It is believed that MTX can induce direct toxic effects to the CNS by damaging the neuronal tissue. Moreover, MTX interferes with the metabolic pathways of folates, excitatory amino acids, homocysteine, S-adenosylmethionine/S-adenosylhomocysteine, adenosine and biopterins, inducing biochemical alterations which have been associated with neurotoxic symptoms. It has been suggested that acute toxicity is partly mediated by adenosine, whereas homocysteine, S-adenosylmethionine/S-adenosylhomocysteine, excitatory amino acids and biopterins may play an important role in the development of subacute and chronic toxicity. A better understanding of the pathogenesis of MTX neurotoxicity would offer the possibility of developing new therapeutic strategies for its treatment or prevention.

Introduction

Methotrexate (MTX, amethopterin) is a folate analogue that was introduced in clinical practice more than 50 years ago. Today, it is widely used in the treatment of patients with psoriasis, rheumatoid arthritis and other autoimmune diseases. Moreover, it plays a major role in the therapy of malignant diseases, such as acute lymphoblastic leukaemia (ALL), lymphoma, medulloblastoma and osteosarcoma, in which high doses are administered.

However, the introduction of intensified treatment protocols was associated with an increased risk of neurotoxicity which may be transient and reversible, but severe neurological disorders leading to coma or even death may occur as well. The exact pathophysiological mechanisms of neurotoxicity are still not understood although this issue has been widely discussed in the past decade. It has been postulated that the neurotoxicity associated with MTX is a consequence of its direct damage to the central nervous system (CNS). In addition, MTX induces metabolic alterations which could at least partly be responsible for the observed neurotoxicity.

In this article, the available scientific literature on MTX neurotoxicity is summarised focusing on its multifactorial pathogenesis. The impact of direct and indirect CNS effects of MTX on clinical symptoms is discussed. Furthermore, we review present and future therapeutic options for circumvention of this severe toxicity.

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Clinical Symptoms of Neurotoxicity

MTX-related neurotoxicity is usually classified into three forms as acute, subacute and chronic neurotoxicity [1, 2]. A summary of the clinical symptoms is shown in table 1.

Table 1. Classification of neurotoxicity (according to the time of appearance after administering methotrexate) and clinical symptoms

Neurotoxicity	Clinical symptoms
Acute (during or within hours)	somnolence, confusion, fatigue, disorientation, seizures chemical arachnoiditis: headache, nausea, vomiting, fever, back pain, dizziness
Subacute (after days to weeks)	encephalopathy: hemiparesis, ataxia, speech disorders, seizures, confusion, affective disturbances myelopathy: pain in the legs, sensory changes, paraplegia, bladder dysfunction
Chronic (after months to years)	learning disability, cognitive disturbances, decrease in intelligence leucoencephalopathy: confusion, somnolence or irritability, seizures, ataxia, dementia, dysphasia, quadriparesis, visual disturbances, slurred speech, coma, death

Acute Neurotoxicity

During or within hours after high-dose (≥ 1 g/m^2) MTX infusion, somnolence, confusion, fatigue, disorientation and seizures may occur [1]. Intrathecal MTX may be associated with acute arachnoiditis, also called chemical arachnoiditis [1]. This syndrome occurs in 5–40% of patients [1], usually 2–4 h after injection, and lasts for 12–72 h [3]. The most common symptoms of arachnoiditis are headache, nausea, vomiting, fever, back pain, and dizziness [1]. Pathologically, pleocytosis, increased protein levels in the CSF, and high intracranial pressure have been observed [1, 4, 5]. This form of neurotoxicity occurs more often when no cranial irradiation is concomitantly applied, probably due to the inhibition of the acute inflammatory response by ionising radiation [6]. The acute neurotoxicity is thought to be related to the administered dose and the peak MTX levels in the CSF [7, 8].

Subacute Neurotoxicity

Days to weeks following the exposure to MTX, an encephalopathy may occur that is characterised by hemiparesis, ataxia, speech disorders, seizures, confusion, and affective disturbances [9–11]. Due to these symptoms, the term 'stroke-like syndrome' is used. Patients usually recover spontaneously after 48–72 h, and subsequent MTX courses do not have an increased risk of recurrence of this syndrome [10].

Intrathecal MTX may be associated with a severe myelopathy followed by symptoms as pain in the legs, sensory changes, paraplegia and bladder dysfunction [12, 13]. Pathologic studies do not reveal any striking inflammation or vascular abnormalities [12, 13].

Rarely, both the brain and the spinal cord may be affected, resulting in severe neurological disorders with poor prognosis [14]. Highly intensive short treatment sequences [15] delayed elimination from the CSF [16] or long-term cumulative treatment [15] are considered to be risk factors for subacute toxicity.

Chronic Neurotoxicity

Chronic neurotoxicity may develop months to years following MTX therapy. The most characteristic syndrome is a leucoencephalopathy presenting with confusion, somnolence or irritability, impaired vision and speech, seizures, ataxia and dementia [1, 4, 17]. In more severe cases, quadriparesis, coma or even death may occur [4, 18]. However, partial recovery or stabilisation is possible, primarily in children [5, 17, 18]. Leucoencephalopathy primarily involves the white matter [19, 20], especially the periventricular regions and the centrum semiovale [4] and is characterised by demyelination, multifocal white matter necrosis, astrocytosis and axonal damage [4]. Intracerebral calcifications [21–23], cerebral atrophy [23, 24] and mineralising microangiopathy [13, 25] have been described. Inflammatory cellular reactions have not been observed [4].

Leucoencephalopathy occurs most frequently after a combination of high-dose MTX, intrathecal MTX and cranial irradiation (45%), whereas the incidence after high-dose MTX and/or intrathecal MTX alone is less than 2% [1, 4]. Enhanced toxicity is especially observed if ionising radiation is given before the administration of MTX. This may be due to synergistic toxic effects of MTX and irradiation and/or an altered distribution of MTX in the CNS which can lead to an accumulation of MTX in certain areas of the brain. Several mechanisms are considered to be responsible for this accumulation. First, irradiation increases the permeability of the blood-brain barrier [26]. Second, the clearance of MTX from the CNS may be disturbed by a decreased CSF turnover.

Third, the ependymal barrier may be disrupted, enabling MTX to enter the white matter. Finally, irradiation may induce changes at the cellular level [1].

Besides leucoencephalopathy, chronic toxicity may be associated with significant neuropsychological dysfunctions such as learning disability, cognitive disturbances and decrease in intelligence. Combined MTX and cranial irradiation is thought to be responsible for these sequelae [27–29]. Nevertheless, different authors showed that patients treated with MTX without cranial irradiation may also have learning disability and a decrease in intelligence quotient [24, 30, 31].

Attempts have been made to find an early marker of chronic toxicity. Increased levels of myelin basic protein [15] and tau protein [32] in the CSF, a decreased choline/water ratio [33] and prolonged motor-evoked potential latency [34] seem to correlate with demyelination.

Direct Toxic Effects of MTX on the CNS

Due to changes observed in leucoencephalopathic patients, such as coagulative necrosis of the white matter, axonal swelling, demyelination and astrocytosis, MTX may exert a direct toxic effect to the CNS. Furthermore, severe neurotoxicity occurring shortly after the administration of overdosed MTX [35, 36], seems to support this hypothesis.

Several experiments on animals and cultured cells were performed in order to elucidate the direct pathogenic mechanism of MTX to the CNS. Weller et al. [37] observed no short-term cytotoxic action of up to 2 m*M* MTX in cocultures of cerebellar and cortical neurones and astrocytes. In contrast, Gregorios et al. [38] showed that alterations of astrocytes were the earliest and primary pathologic finding in the rat brain after MTX exposure. An impaired function of the astrocytes could lead to a disturbance of neuronal activity or even neuronal damage since astrocytes have a supplementary function for neurons and play an important role in the uptake and metabolism of neurotransmitters. Oligodendroglial cells, known to build the myelin sheath, neurones and endothelial cells were relatively spared. Gilbert et al. [39] observed axonal loss and demyelination of neurons after exposure of cerebellar rat explants to 1 μ*M* MTX for 2 weeks. The demyelination was more pronounced after an exposure of 5 weeks. Oligodendroglial cells preserved their function indicating that the demyelination was a consequence of axonal loss. The pathological mechanism of the axonopathy is still unclear. Considering the results of Gregorios et al. [38] and the fact that neurons do not replicate and should therefore be insusceptible to MTX, it is possible that the axonopathy is a consequence of astrocytosis. Bruce-Gregorios et al. [40] suggested that astrocytosis occurs predominantly due to a MTX-induced inhibition of RNA and de novo purine synthesis. The explanation for the insusceptibility of other replicating cells, such as oligodendroglial and endothelial cells, to MTX may be that astrocytes seem to be the likely site for uptake and polyglutamation of MTX [41, 42]. However, due to the contradictory literature data, the effect of MTX on the CNS still remains unclear. Further studies with cultured cells and animal models over a time period of several weeks are required.

Biochemical Pathways Related to MTX Activity

MTX affects DNA and RNA synthesis by interfering with the biosynthesis of thymidine and purines. MTX and MTX polyglutamates, polyglutamated by the enzyme folylpolyglutamate synthetase [43], inhibit the enzyme dihydrofolate reductase (DHFR). As a result, the levels of dihydrofolate in the cell increase while the levels of tetrahydrofolates (THF) such as 5,10-methylenetetrahydrofolate (5,10-methylene-THF), required for the thymidylate synthesis and 10-formyltetrahydrofolate (10-formyl-THF), important for the purine synthesis (fig. 1) decrease. Moreover, MTX polyglutamates and dihydrofolate polyglutamates formed by the enzyme folylpolyglutamate synthetase directly inhibit the enzyme thymidylate synthase [44, 45] responsible for the conversion of uridine monophosphate (dUMP) to thymidine monophosphate (dTMP).

MTX strongly interferes with the metabolism of homocysteine by reducing the level of 5-methyltetrahydrofolate (5-methyl-THF) which serves as the donor of the methyl group for the methylation of homocysteine to methionine. This reaction is catalysed by the enzyme methionine synthase and requires vitamin B_{12} as a cofactor. In the kidney and the liver, homocysteine can be metabolised to methionine by betaine-homocysteine methyltransferase, but this enzyme has not been found in the brain of animals or man [46]. The reaction of methionine and adenosine triphosphate in the presence of the enzyme methionine adenosyltransferase yields S-adenosylmethionine (SAM), the most important methyl donor in cellular metabolism. SAM is converted to S-adenosylhomocysteine (SAH), which is degraded by S-adenosylhomocysteine hydrolase to homocysteine and adenosine (fig. 1).

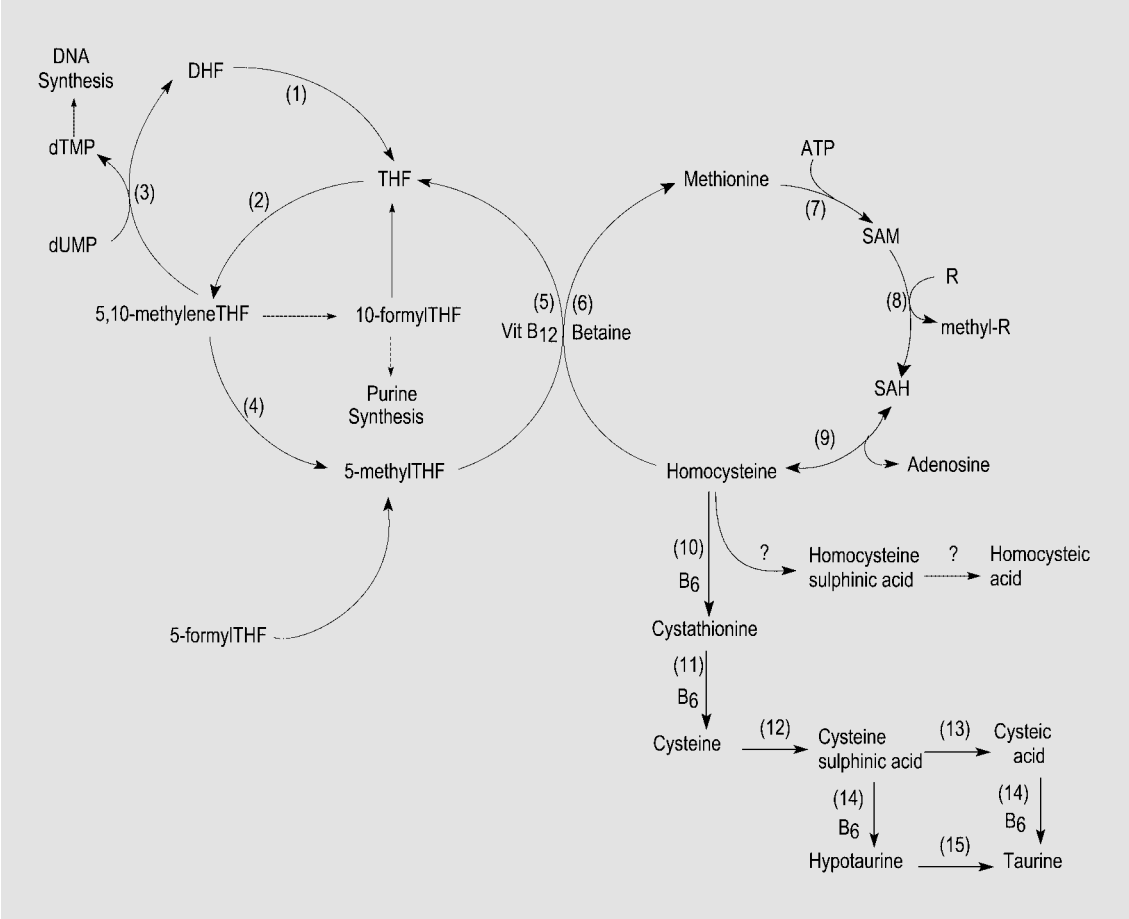

Fig. 1. Metabolism of folates and homocysteine. ATP = Adenosine triphosphate; DHF = dihydrofolate; SAH = S-adenosylhomocysteine; SAM = S-adenosylmethionine; THF = tetrahydrofolate; dTMP = thymidine monophosphate; dUMP = uridine monophosphate; 1 = dihydrofolate reductase; 2 = serine-hydroxymethyltransferase; 3 = thymidylate synthase; 4 = 5,10-methylenetetrahydrofolate reductase; 5 = methionine synthase; 6 = betaine-homocysteine methyltransferase; 7 = methionine adenosyltransferase; 8 = *R*-methyltransferase; 9 = SAH hydrolase; 10 = cystathionine-β-synthase; 11 = cystathionase; 12 = cysteine dioxygenase; 13 = cysteine sulphinic acid dehydrogenase; 14 = cysteine sulphinic acid decarboxylase; 15 = hypotaurine dehydrogenase.

The main route of catabolism of homocysteine is through entering the transsulfuration pathway starting with the conversion to cystathionine. Cystathionine is further converted to cysteine which is subsequently oxidised to cysteine sulphinic acid and cysteic acid. Cysteine sulphinic acid and cysteic acid can be decarboxylated to hypotaurine and taurine by the enzyme cysteine sulphinic acid decarboxylase (fig. 1). In analogy to the cysteine oxidation pathway, homocysteine can be oxidised to homocysteine sulphinic acid and homocysteic acid. As enzymes catalysing this metabolic route of homocysteine have not been described, a non-enzymatic conversion has been suggested [47]. The sulphur-containing amino acids cysteine sulphinic acid, cysteic acid, homocysteine sulphinic acid and homocysteic acid, structure analogues of aspartate and glutamate, are known to exhibit strong excitatory effects [48].

As an indirect consequence of MTX activity levels of homocysteine, SAH and sulphur-containing amino acids increase while the levels of methionine and SAM decrease.

MTX-polyglutamates and dihydrofolate polyglutamates also inhibit two enzymes of the purine synthesis glycineamide-ribonucleotide-transformylase (GART)

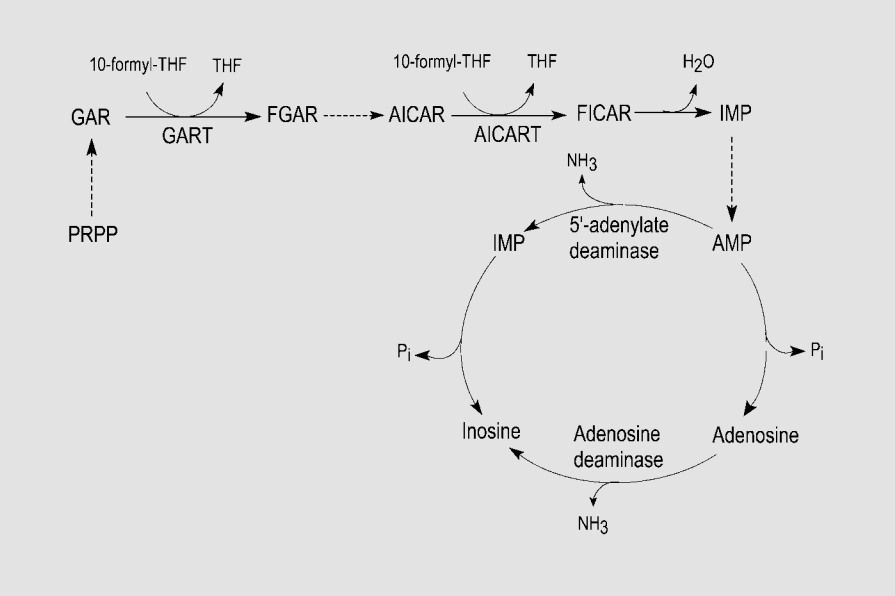

Fig. 2. Selected steps in purine synthesis and degradation. AICAR = 5-Aminoimidazole-4-carboxamide-ribonucleotide; AICART = 5-aminoimidazole-4-carboxamide-ribonucleotide-transformylase; AMP = adenosine monophosphate; FGAR = formyl-glycineamide-ribonucleotide; FICAR = 5-formamidoimidazole-4-carboxamide-ribonucleotide; GAR = glycineamide ribonucleotide; GART = glycineamide-ribonucleotide-transformylase; IMP = inosine monophosphate; P_i = inorganic phosphate; PRPP = 5-phosphoribosyl-1-pyrophosphate; THF = tetrahydrofolate.

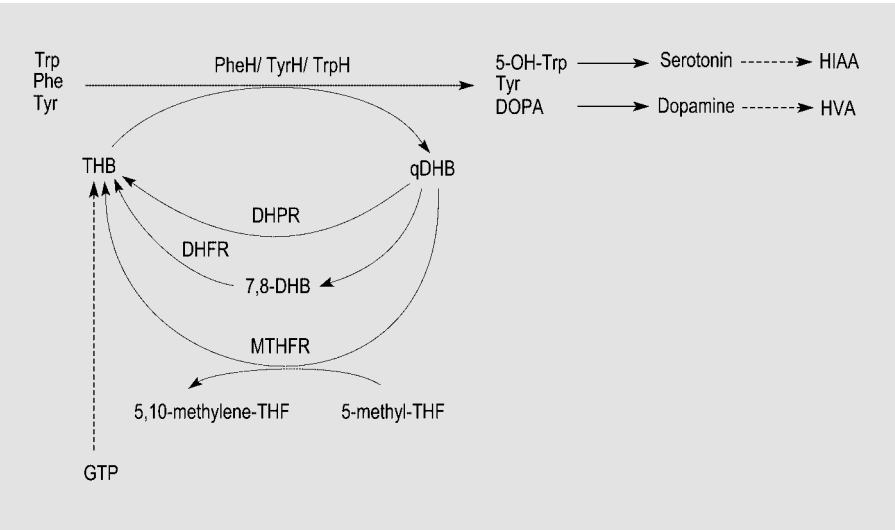

Fig. 3. Biosynthesis of bioamines and metabolism of biopterins. 7,8-DHB = 7,8-dihydrobiopterin; DHFR = dihydrofolate reductase; DHPR = dihydropteridine reductase; GTP = guanosine triphosphate; HIAA = 5-hydroxyindoleacetic acid; HVA = homovanillic acid; MTHFR = 5,10-methylenetetrahydrofolate reductase; 5-OH-Trp = 5-hydroxytryptophan; Phe = phenylalanine; PheH = phenylalanine hydroxylase; qDHB = quinoide dihydrobiopterin; THB = tetrahydrobiopterin; Trp = tryptophan; TrpH = tryptophan hydroxylase; Tyr = tyrosine; TyrH tyrosine hydroxylase.

and 5-aminoimidazole-4-carboxamide-ribonucleotide-transformylase (AICART) [49] leading to an accumulation of glycineamide-ribonucleotide (GAR) and 5-aminoimidazole-4-carboxamide-ribonucleotide (AICAR) (fig. 2). AICAR can inhibit 5′-adenylate deaminase and adenosine deaminase, resulting in elevated levels of adenylate and adenosine, respectively [50].

Finally, MTX affects the biopterin pathway by inhibiting the regeneration of tetrahydrobiopterin (THB) from dihydrobiopterin (fig. 3). THB is required for the hydroxylation of tyrosine, phenylalanine and tryptophan. THB can be either synthesised de novo from guanosine triphosphate (GTP), a pathway not affected by MTX, or it can be recycled from quinoide dihydrobiopterin (qDHB) in three ways all of which are affected directly or indirectly by MTX. MTX directly inhibits the enzyme dihydropteridine reductase (DHPR) [51], necessary for the reduction of qDHB to THB. This pathway is considered to be the main source of salvage. qDHB can be non-enzymatically converted into 7,8-DHB which is reduced to THB by dihydrofolate reductase.

Moreover, THB may be regenerated from qDHB in the presence of the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR), whereby 5-methyltetrahydrofo-

Table 2. MTX-induced metabolic alterations, pathogenic mechanisms and clinical consequences

Substance	CSF levels after MTX	Suggested pathogenic mechanisms	Clinical symptoms
SAM SAH	↓ ↑	methylation capacity ↓, demyelination	leucoencephalopathy, depression, dementia
Homocysteine	↑	direct toxic effects to the vascular endothelium, coagulation ↑, oxidative stress ↑	cerebrovascular ischemia mineralising microangiopathy, focal neurological deficits
SEAA	↑	excitability ↑, excitotoxicity, neurodegeneration	seizures, dementia
Adenosine	↑	altered cerebral blood flow, neuronal excitability	nausea, vomiting, lethargy, headache, seizures
THB	↓?	impaired biosynthesis of dopamine, serotonin	affective disturbances, hypokinesis, limb rigidity

SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; SEAA = sulphur-containing excitatory amino acids; THB = tetrahydrobiopterin.

late is converted to 5,10-methylene-THF (fig. 3). This salvage process can be affected by MTX due to depletion of 5-methyltetrahydrofolate.

After high-dose MTX, 5-formyltetrahydrofolate (leucovorin) is administered as rescue. Leucovorin antagonises the activity of MTX by several mechanisms. It competes with MTX for the entry into the cell and for the enzymes folylpolyglutamate synthetase and DHFR, leading to reduced levels of MTX polyglutamates and a diminished inhibition of DHFR. Moreover, leucovorin is rapidly converted into other reduced folates, thereby restoring the pool of reduced folates (see fig. 1).

MTX-Induced Metabolic Alterations and Possible Clinical Consequences

The MTX-induced metabolic alterations, their pathogenic mechanism and clinical symptoms are summarised in table 2.

Folates

Depletion of reduced folates has been observed in some metabolic disorders such as DHFR deficiency and folate malabsorption. Moreover, CSF levels of 5-methyltetrahydrofolate were shown to be decreased in patients treated with MTX [52]. The neurological impairment associated with folate deficiency is characterised by symptoms such as insomnia, forgetfulness, seizures, irritability, depression and schizoid psychosis [53].

However, since the folate metabolism is closely related to the homocysteine and biopterin metabolic pathways, it is difficult to assess whether the pathological findings are directly due to folate depletion. Patients with a deficiency of reduced folates were shown to have decreased levels of SAM [54, 55], THB, homovanillic acid and 5-hydroxyindoleacetic acid [56] as well as elevated levels of homocysteine [57, 58]. All of these metabolites were shown to be involved in the development of neurological disturbances.

S-Adenosylmethionine/S-Adenosylhomocysteine

SAM is the methyl donor of various molecules including catecholamines, indoleamines, choline, creatine, nucleic acids, proteins and phospholipids. Decreased levels in the brain can therefore lead to hypomethylation.

Significantly decreased levels of SAM in the CSF have been observed in various neuropsychiatric illnesses such as depression, Alzheimer's dementia and Parkinson's disease [59]. Moreover, treatment with SAM was shown to improve cognitive dysfunction as well as mood and speed of mental processing in patients with Alzheimer's dementia [60] and induce an antidepressant effect [61]. Furthermore, it was suggested that decreased levels of SAM were correlated with demyelination [56] and therefore with neurological disorders. Surtees et al. [55] found decreased

levels of SAM in the CSF of patients with inborn errors of the methyl-transfer pathway and demyelination of the brain and the spinal cord.

However, in order to determine the methylation capacity in the brain, SAH levels should also be considered since SAH acts as a strong inhibitor of methylation reactions. Therefore it has been suggested to assess the SAM/SAH ratio (methylation ratio).

Significantly decreased levels of SAM and increased levels of SAH were reported in the CSF of HIV-positive patients in whom myelopathy and subacute encephalomyelitis may occur [62]. Kishi et al. [63] reported a lower ratio of SAM/SAH in the CSF of 2 ALL patients with toxic leucoencephalopathy in comparison to ALL patients without leucoencephalopathy, treated by the same protocol, and a control group consisting of patients without suspicion of altered methyl transfer pathway. The authors suggested that MTX-induced hypomethylation is partly responsible for the demyelination observed in patients with leucoencephalopathy.

Homocysteine

Homocysteine, an amino acid containing a highly reactive sulphhydryl group, is known to be an independent risk factor for arteriosclerosis [64], myocardial infarction [65] and stroke [66, 67]. Patients suffering from homocystinuria, due to a deficiency of cystathionine β-synthase or methionine synthase, were shown to have a high risk of thromboembolism, seizures and mental retardation [68–70]. The pathophysiological mechanisms of homocysteine are different and include direct toxic effects to the vascular endothelium and intima [71–73], altered coagulant properties of the blood [74–76] and increased oxidative stress [73, 77].

It was demonstrated that high- and low-dose therapy with MTX increased plasma levels of homocysteine [78–81]. Due to the pathophysiological mechanisms and the fact that high-dose and intrathecal MTX can be associated with significantly increased levels of homocysteine in the CSF [82, 83], it has been suggested that homocysteine is at least partly responsible for ischaemic white matter changes, mineralising microangiopathy, and focal neurological deficits observed after MTX treatment [2]. Since the periventricular deep white matter is poorly vascularised, it is believed to be more vulnerable to ischaemia-induced lesions [31].

In contrast, Packer et al. [84] investigated the coagulation profile and cerebral angiograms in patients with subacute toxicity suffering from hemiparesis, speech disorders, unconsciousness, and seizures and detected no abnormalities. Moreover, Flott-Rahmel et al. [85] suggested that it is unlikely that neurological complications observed in patients with homocystinuria are caused by homocysteine-induced vascular alterations. Due to the absence of focal neurological symptoms and brain infarctions in patients with homocystinuria, it was proposed that homocysteine-related metabolites, especially homocysteine sulphinic acid and homocysteic acid, are primarily responsible for neurodegenerative effects, a hypothesis first proposed by Grieco [70].

Sulphur-Containing Excitatory Amino Acids

Sulphur-containing amino acids derived from homocysteine, especially homocysteic acid are known to accumulate in the plasma of patients with homocystinuria [86]. It has been shown that sulphur-containing amino acids are present in the mammalian brain, predominantly in glial cells [87] and activate excitatory amino acid receptors such as the NMDA receptor [88, 89].

An intensive stimulation of the NMDA receptor may lead to seizures [90–92] and an even more severe phenomenon called excitotoxicity, which is mainly mediated by a high influx of calcium ions through the NMDA receptor-linked calcium channel. Increased intracellular levels of calcium stimulate several catabolic enzymes such as phospholipases, proteases and endonucleases and can thereby lead to cell death [90, 93, 94]. The concept of excitotoxicity has been implicated in the pathogenesis of acute (hypoglycaemia and ischaemia) and chronic neuronal degeneration (Huntington's disease, amyotrophic lateral sclerosis and Alzheimer's disease) [90, 93, 95].

Besides the direct activation of excitatory amino acid receptors an increase in excitation may be mediated by several further mechanisms. First, sulphur-containing amino acids are capable of releasing the excitatory amino acids aspartate and glutamate [96, 97] and to inhibit the reuptake of these neurotransmitters in neuronal and glial plasma membranes [98–102] which is considered to be the most important mechanism to control levels of excitatory amino acids in the synaptic cleft and hence avoid toxicity. Moreover, damage to astrocytes induced by MTX [103, 104] or sulphur-containing amino acids [95] may contribute to an increased excitatory transmission since astrocytes manage the enzymatic inactivation of glutamate. Elevated excitotoxic levels of glutamate could further damage astrocytes leading to an increased vulnerability of neurones and glia cells [95].

Of particular interest is the fact that glutamate is massively released from neural structures during hypoxia and ischaemia [105, 106]. Therefore, ischaemic conditions

induced e.g. by homocysteine-mediated vascular obstructions could further contribute to neuronal damage.

In a retrospective study, Quinn et al. [107] determined sulphur-containing amino acids in CSF samples obtained from patients who received MTX for the treatment of cancer. In all patients, sulphur-containing amino acid levels were significantly increased and patients suffering from neurotoxicity at the time of lumbar puncture had the highest levels. In the control group, no sulphur-containing amino acids could be detected. In a following case report, Quinn et al. presented a 13-month-old boy who developed generalised tonic-clonic seizures 4 days after intrathecal administration of MTX for the treatment of ALL [108]. An analysis of the CSF performed on this day revealed remarkably elevated levels of cysteine sulphinic acid and homocysteic acid. At the time of diagnosis, no sulphur-containing amino acids could be detected in the CSF.

Recently, the use of dextromethorphan, a noncompetitive NMDA receptor antagonist, in 5 patients with severe symptoms of subacute neurotoxicity (e.g. dysarthria, hemiplegia, hemiparesis) was reported [109]. After administration of dextromethorphan, the neurological deficits were completely resolved. This finding confirms the role of excessive NMDA receptor stimulation in the pathogenesis of MTX neurotoxicity. Moreover, the authors suggest that dextromethorphan should be considered as an option to prevent and treat subacute neurotoxicity.

Since taurine, the final product of the metabolic pathway of sulphur-containing amino acids, is known to protect neural cells from excitotoxicity [106], it would be desirable to determine not only the levels of sulphur-containing amino acids but also the levels of taurine in the CSF of cancer patients in order to elucidate the ratio of sulphur-containing excitatory and inhibitory amino acids which are both expected to accumulate after MTX therapy.

Adenosine

Adenosine is known to regulate cerebral blood flow and neuronal excitability due to its activity on adenosine receptors. Trials with the adenosine deaminase inhibitor deoxycoformycine (pentostatin) in patients with refractory malignant diseases where high levels of adenosine were induced, revealed nausea, vomiting, headache, somnolence, and seizures as main adverse events [110].

It was shown in animal experiments that MTX can increase the levels of adenosine [111].

Bernini et al. [112] reported 6 children treated with MTX all having elevated levels of adenosine in the CSF and signs of acute neurotoxicity, including nausea, vomiting, lethargy, and headache. However, patients without neurotoxic symptoms had increased adenosine levels as well. Patients suffering from neurotoxicity were given the adenosine receptor antagonist aminophylline after conventional therapy with analgesics or antiemetics had failed. An amelioration of neurotoxic symptoms was observed in all patients; in 4 out of 6 patients, symptoms resolved completely within 30 min after administration. Peyriere et al. [113] reported a case treated with high-dose and intrathecal MTX who developed symptoms of neurotoxicity, including seizures, respiratory insufficiency and non-reactive coma, after the second therapy cycle. The signs improved after administering aminophylline.

These reports indicate that increased levels of adenosine may cause neurotoxicity, which is reversed by adenosine receptor antagonists.

Biopterins

Due to the cofactor role in the hydroxylation of tryptophan, phenylalanine and tyrosine decreased levels of tetrahydrobiopterin can affect the biosynthesis of important bioamines such as dopamine and serotonin. The defective metabolism of biopterin is characterised by dopamine and serotonin deficiency and symptoms such as extreme hypokinesis, trunk hypotonia, swallowing difficulty, limb rigidity, oculogyric crisis and recurrent hyperpyrexia [114, 115]. It was therefore suggested that MTX-induced THB deficiency is at least partly responsible for symptoms observed in subacute neurotoxicity [116].

Millot et al. [117] reported a 15-year-old boy who received MTX for the treatment of ALL and developed subacute toxicity with weakness, aphasia and anaesthesia 8 days after the third course. Homovanillic acid and 5-hydroxyindoleacetic acid, the major metabolites of dopamine and serotonin, respectively, determined in the CSF directly after the infusion of MTX, were decreased after the second and third course. Due to ethical reasons the metabolites were not determined during the neurological disorder. Two days after the spontaneous resolution of symptoms the values were in the normal range. Another case, a 3-year-old boy, developed neurotoxicity after the third course of systemic and intrathecal MTX. He presented with fever, confusion, aphasia, increased tone of limbs and coma. The CSF biopterin analysis revealed a decreased level of THB. Clinical improvement was not achieved with leucovorin rescue and methylprednisolone given for 3 days, but subsequent administration of *L*-dopa, carbidopa and 5-hydroxytryptophan (substitutive

Table 3. Therapeutic options to reverse MTX-induced neurotoxicity

Affected substance	Therapeutic option	Administered doses
SAM/SAH	SAM	n.a.
Homocysteine	betaine and vitamin B_6 vitamin B_{12}	10 g/day, 200–1,000 mg/day [129] n.a.
Sulphur-containing excitatory amino acids	dextromethorphan calcium channel blockers	1–2 mg/kg p.o. [109] n.a.
Adenosine	aminophylline	2.5 mg/kg [112, 113]
Tetrahydrobiopterin	tetrahydrobiopterin *L*-dopa, carbidopa, and 5-hydroxytryptophan	5–20 mg/kg/day [118, 119, 129] 10 mg/kg/day, 1 mg/kg/day, 7 mg/kg/day [118]

SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; n.a. = not yet administered in case of MTX neurotoxicity.

therapy for biopterin deficiency) ameliorated the comatose condition [118]. Blau et al. [119] reported cases of hyperphenylalaninaemia in ALL patients receiving MTX and suggested it was due to a deficiency of THB. However, the levels of THB in CSF were not determined. These patients were more depressed and apathetic than patients with normal levels of phenylalanine. After receiving THB, the levels of phenylalanine decreased significantly.

Other studies [120–123] revealed different results suggesting that after the administration of MTX the biosynthesis of serotonin and dopamine remains unaffected. Silverstein et al. [123] reported changes in the CSF levels of homovanillic acid and 5-hydroxyindoleacetic acid after administration of intrathecal MTX. Before treatment no significant differences were found compared to a control group. During the first 4–5 weeks, the values were stable with a modest decline of 5-hydroxyindoleacetic acid and homovanillic acid, but during weeks 6–9 the levels unexpectedly rose. Moreover, intrathecal cytosine arabinoside was shown to decrease levels of both metabolites significantly. In another study, Millot et al. [122] analysed biopterin levels as well as homovanillic acid and 5-hydroxyindoleacetic acid levels in the CSF of 57 children with ALL after high-dose MTX (5 g/m^2). They observed a significant increase in total biopterin levels after MTX compared to those before the infusion. The CSF levels of 5-hydroxyindoleacetic acid did not change significantly and a significant increase of homovanillic acid was detected after the second cycle compared to pre-treatment values. Only 6 children had a transient decrease in homovanillic acid and 5-hydroxyindoleacetic acid levels but none of them manifested neurotoxic symptoms.

Experiments with rats and neuroblastoma cell cultures, performed to induce a deficiency in THB with MTX, revealed no significant decrease in THB [120, 121] suggesting that MTX is unlikely to impair the hydroxylation of tyrosine and tryptophan [121].

Due to the contradictory literature data it still remains unclear whether MTX is capable of altering neurotransmitter biosynthesis by reducing the levels of THB.

Therapeutic Options

It has been suggested that the MTX/leucovorin ratio is important in preventing neurotoxicity [124]. However, significant neurotoxicity is observed in most high-dose chemotherapy protocols with MTX including leucovorin rescue, suggesting that the usual doses of leucovorin are not sufficient. Due to the fact that leucovorin rescues malignant cells as well, a more intensified leucovorin rescue does not seem to be judicious. Therefore, alternative therapeutic options are desired.

It has been shown that SAM may improve affective and cognitive disturbances in folate- and SAM-deficient patients [125, 126]. Furthermore, Surtees et al. [55] demonstrated that a demyelination of the brain and spinal cord can be reversed if SAM or methionine, the precursor of SAM, is administered. To our knowledge, SAM has not

yet been administered in case of MTX-induced neurotoxicity. Since a decreased SAM/SAH ratio has been described in neurotoxic patients a substitutive therapy with SAM may be a promising approach to prevent or reverse neurotoxic symptoms.

Elevated levels of plasma homocysteine can be decreased by vitamin B_6, vitamin B_{12} and/or betaine, cofactors involved in the metabolism of homocysteine [58, 127, 128]. Therefore, these cofactors could provide a therapeutic option to treat symptoms of neurotoxicity associated with high levels of homocysteine. It has been suggested to administer betaine (10 g/day) and vitamin B_6 (600 mg/day) during high-dose chemotherapy with MTX [129].

The NMDA receptor antagonist dextromethorphan (1–2 mg/kg p.o.) has been successfully used to treat toxic effects, probably induced by elevated levels of excitatory sulphur-containing amino acids [109]. CNS-permeable calcium channel blockers, e.g. nimodipine, could be an alternative for the prevention of excitotoxicity [130].

Symptoms of acute neurotoxicity can be reversed by the adenosine antagonist aminophylline given in a dose of 2.5 mg/kg [112, 113].

Alterations concerning the biopterin and bioamine pathway may be circumvented by administering THB and/or neurotransmitter precursors. THB at doses of 5–20 mg/kg [118, 119, 129] and the combination of *L*-dopa (10 mg/kg/day), carbidopa (1 mg/kg/day) and *DL*-5-hydroxytryptophan (7 mg/kg/day) [118] were shown to be effective in ameliorating symptoms of subacute neurotoxicity.

Since several pathways are simultaneously influenced by MTX, the most promising approach in preventing neurotoxicity should be a combination of the therapeutic options discussed above. It would be reasonable to give a substitutive therapy including SAM, betaine, vitamin B_6, vitamin B_{12} and THB concomitantly with MTX. Nevertheless, if toxic symptoms occur, NMDA receptor antagonists, calcium channel blockers, aminophylline or even neurotransmitter precursors may be attempted. The therapeutic options that may reverse MTX-induced neurotoxicity are summarised in table 3.

Concluding Remarks

There is some evidence that several biochemical pathways are affected by MTX. A decreased SAM/SAH ratio, elevated levels of homocysteine, sulphur-containing amino acids, adenosine and decreased levels of THB may account for individual symptoms in acute, subacute and chronic neurotoxicity of MTX. Moreover, direct toxic effects of MTX on neurons and astrocytes may contribute to neurotoxicity.

High levels of MTX in the CSF, delayed elimination from the CSF, frequent administration and cumulative effects have been shown to increase the risk of toxicity. Other risk factors are irradiation which may enhance neurotoxicity by a number of mechanisms and the concomitant use of other neurotoxic agents, e.g. cytosine arabinoside. Enzyme disorders affecting the metabolism of folates and homocysteine could potentiate neurological disorders. Predictive parameters in patients are desirable to discriminate between those who will benefit from treatment and those who will probably suffer from severe neurotoxicity.

Further studies are required to elucidate the impact of metabolic alterations on neurotoxic symptoms. The results will help to improve strategies for prophylaxis and therapy of MTX neurotoxicity.

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REVIEW

Pharmacogenetics of methotrexate

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Methotrexate (MTX) has proven efficient in the treatment of a number of malignancies, as well as non-malignant disorders characterized by a rapid cellular growth. Yet some patients might develop resistance, while others could have toxic side effects. MTX achieves its cytotoxicity through the inhibition of folate-dependent enzymes, suggesting that the genes controlling their activity or the levels of folate cofactors can modulate drug efficacy and, thus, the sensitivity of a patient to MTX. Indeed, several studies, conducted mostly in leukemia and rheumatoid arthritis patients, have addressed the potential for tailoring MTX therapy based on a patient's genetics. Several genetic variants have been shown to have a predictive role, among which the most frequently studied are those of methylenetetrahydrofolate reductase and thymidylate synthase genes. The other candidates, as well as gene–gene interactions, which may be even more important for the prediction of disease outcomes than the individual gene effects, are also briefly discussed.

Keywords: disease outcome, folate cycle, genetic variant, leukemia, methotrexate, pharmacogenetics, polymorphism, rheumatoid arthritis, therapeutic response, variability

future medicine

Introduction

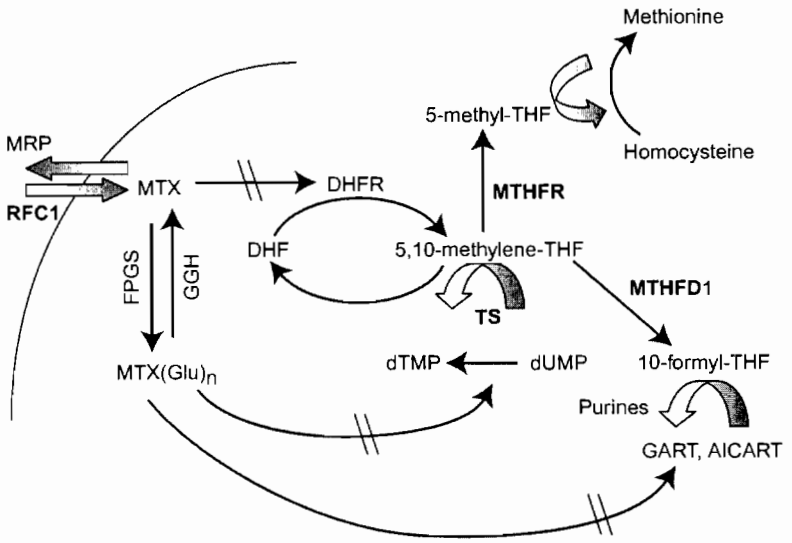
Interindividual diversity in drug effects is a major problem in clinical medicine and drug development. This indicates a need to identify the factors associated with variable drug sensitivity, which will allow for the better clinical management of patients resistant to treatment, as well as a reduction of toxicity for patients who respond well. A genetic contribution to variability in drug action has long been recognized [1] and, along with recent efforts to identify human genome variability, it opens up large possibilities for the development of pharmacogenetics. Pharmacogenetics, thus, establishes a link between individual genetic make-up and therapeutic response; analyzing, via the candidate gene approach, the functional polymorphisms of genes that code for pharmacokinetic and pharmacodynamic determinants of drug effects (drug disposition and resistance, and drug-associated toxicity). This knowledge is particularly important for diseases, such as cancer or other chronic disorders, whose treatment is based on long-term drug administration. One of the chemotherapeutics with a wide therapeutic spectrum is methotrexate (MTX). It is used in the treatment of number of malignancies and of several non-malignant diseases characterized by a rapid cellular growth [2]. MTX has been proven to be efficient in the treatment of childhood acute lymphoblastic leukemia (ALL) for relapse induction, the prevention of disease relapse in the CNS, and the maintenance of continuous complete remission [3,4].

Substitution of the amino group for the hydroxyl at the fourth position of the pteridine ring is the critical change in the MTX structure that leads to its capability to inhibit dihydrofolate reductase (DHFR) [2,5]. The importance of DHFR inhibition comes from the fact that folates are active as coenzymes only in their reduced form. Two specific tetrahydrofolates (THF) play an essential role in the folate cycle and in the synthesis of nucleic acid molecules: 5,10-methylenetetrahydrofolate (5,10-methylene-THF), which provides the carbon group for the reaction that converts deoxyuridylate (dUMP) to deoxythymidylate (dTMP); and 10-formyl-tetrahydrofolate (10-formyl-THF), which provides a single carbon group for the *de novo* synthesis of purines. MTX-induced inhibition of DFHR results in depletion of reduced tetrahydrofolate forms, thus contributing to the inhibition of nucleic acid synthesis and the resulting cellular death [5]. Lower levels of THF coenzymes are not sufficient for MTX cytotoxicity. Furthermore, it is dependent on the direct inhibition of the enzymes implicated in purine and thymidylate synthesis (thymidylate synthase [TS], glycinamide ribonucleotide transformylase [GART], and aminoimidazole carboxamide ribonucleotide transformylase [AICART]) performed by the glutamylated forms of MTX [2,5,6] (**Figure 1**).

MTX-induced folate depletion will also affect the homocysteine–methionine–polyamine pathway by increasing plasma homocysteine levels and by interfering with several methylation reactions, which are mediated by *S*-adenosyl-methionine [7].

 ISSN 1462-2416

Figure 1. Schematic representation of methotrexate action.



DHFR inhibition leads to the depletion of THF compounds, which, in turn, impairs purine and thymidine synthesis. The production of specific folate compounds requires the action of MTHFD1 and MTFHR to convert 5,10-methylene-THF into 10-formyl-THF and 5-methyl-THF, respectively. 5,10-Methylene-THF is needed as a cofactor for TS action; 10-formyl-THF is involved in purine synthesis; and 5-methyl-THF serves as a methyl donor for homocysteine methylation. The production of the glutamylated forms of MTX is enabled by the action of FPGS and GGH. $MTX(Glu)_n$ are an important contributor to MTX cytotoxicity by directly inhibiting TS, AICART, and GART.

The genes whose polymorphisms have been analyzed in several pharmacogenetics studies, as outlined in this review, are indicated in bold character.

AICART: Aminoimidazole carboxamide ribonucleotide transformylase; DHF: Dihydrofolate; DHFR: Dihydrofolate reductase; dTMP: Deoxythymidine monophosphate; dUMP: Deoxyuridine monophosphate; FPGS: Folylpolyglutamate synthase; GART: Glycinamide ribonucleotide transformylase; GGH: γ-Glutamyl hydrolase; MRP: Multi-drug resistance-associated protein; MTHFD1: 5,10-Methylenetetrahydrofolate dehydrogenase; MTHFR: 5,10-Methylenetetrahydrofolate reductase; MTX: Methotrexate; $MTX(Glu)_n$: MTX glutamates; RFC1: Reduced folate carrier; THF: Tetrahydrofolate; TS: Thymidylate synthase.

A number of enzymes are responsible for the maintenance of adequate levels of homocysteine. One of these is 5,10-methylenetetrahydrofolate reductase (MTHFR) that converts 5,10-methylene-THF into 5-methyl-THF. The latter provides the methyl group for homocysteine methylation [8].

Beside its role as an antitumor agent, MTX is also frequently used for the treatment of rheumatoid arthritis (RA) patients [5]. Low doses of MTX were initially introduced because of its antiproliferative properties. However, given that the administration of folic acid prevents much of the toxicity of MTX without interfering with its anti-inflammatory efficacy [9], it is quite likely that a different mechanism accounts for its therapeutic effect in RA patients [10]. Indeed, it has been shown that intracellular accumulation of purine biosynthesis intermediates, resulting from an inhibition of AICART by glutamylated forms of MTX, leads to a release of adenosine that exhibits anti-inflammatory properties (reviewed in [10,11]).

Interindividual variability in therapeutic response of diseases treated with methotrexate

Therapeutic improvements have led to remarkable cure rates, which today are in the range of 70–80% [4,12], for childhood ALL patients. However, these figures disguise varying success rates that are dependent on different features of the disease or those of the host [13]. A successful cure still remains elusive for certain patients because of drug resistance, rendering ALL the leading cause

of cancer-related death in children [12,14]. Intensive treatment also has significant long-term consequences, potentially causing secondary malignancies, growth retardation, bone abnormalities, or cognitive impairments [15,16]. Several lines of evidence have revealed variability in MTX response. For example, interindividual differences of MTX levels in serum and cerebrospinal fluid, as well as variations in plasma folate and homocysteine concentrations, were found after MTX treatment [17-19]. Accordingly, several groups reported that adjustment of the MTX dose based on drug plasma levels and drug clearance may improve treatment outcome in patients with ALL [20,21]. Likewise, side effects of MTX can occur in some patients but not in others. At least some of them, such as mucositis or hematological toxicity, appear to be directly related to MTX folate antagonisms, especially in tissues with a high cell turnover, which have a high requirement for purines and thymidine [22,23]. Likewise, in RA patients, the drug doses required to effectively suppress disease activity can differ widely between patients and, in some cases, MTX treatment can be almost ineffective, whereas in others it might result in the development of toxicities [24].

A number of genes that represent important candidates for MTX pharmacogenetics have emerged from studying the mechanism of MTX action. An additional reason for the selection of these genes is that variable levels of their protein products have already been reported to contribute to MTX resistance [25]. The most frequently involved mechanisms are the altered expression of target enzymes (mentioned briefly above), the decreased formation of MTX glutamates [MTX$(Glu)_n$] influenced by the action of folylpolyglutamate synthase (FPGS) and γ-glutamyl hydrolase (GGH) [25,26], and/or decreased cellular intake of the drug [25] due to the impaired reduced folate carrier function [25,27] (**Figure 1**). Given the different mechanisms of MTX action in inflammatory diseases, MTX efficacy and its side effects in RA patients can only partly be explained by folate antagonism and may also depend on other related metabolic pathways, such as the homocysteine–methionine–polyamine pathway and purine metabolism [28].

The functional variants of the majority of candidate genes have been reported and the roles of some of these polymorphisms have been assessed in several pharmacogenetic studies [29]. Most frequently analyzed were the variants of the *MTHFR* and *TS* genes.

5,10-Methylenetetrahydrofolate reductase

The MTHFR catalyzes the conversion of 5,10-methylene-THF into 5-methyl-THF, a major circulating form of folate that provides a methyl group for homocysteine methylation (**Figure 1**) [8,30]. MTHFR plays a key role in folate metabolism by channeling single-carbon units between nucleotide synthesis and methylation reactions [8]. Reduced 5-methyl-THF levels may decrease homocysteine methylation to methionine, resulting in hyperhomocysteinemia and DNA hypomethylation [31]. On the other hand, reduced levels of 5,10-methylene-THF, required for thymidylate synthesis, could lead to uracil misincorporation into DNA. This results in an increase in the frequency of chromosome damage [31,32], which is the effect that facilitates the action of certain chemotherapeutics, including that of MTX.

Two common polymorphisms, a *C677* to *T* transition causing an alanine to valine substitution at codon 222 (Ala222Val) [33], and an *A1298* to *C* transition causing a glutamic acid to alanine replacement at codon 429 (Glu429Ala) [34,35], have been described in the *MTHFR* gene. Both variants have an impact on enzyme function: *677T* affects the catalytic function of the enzyme; and *1298C* affects the regulatory MTHFR domain [33,34]. Reduced enzymatic activity has been observed in *TT677* or *CC1298* homozygotes, and, to a lesser extent, in heterozygous individuals [33,35,36]. The frequency of the *MTHFR 677T* allele varies substantially in different regions of the world. A *T677* allele is found with a frequency of 34% in Caucasians, 42% in Eastern Asians and 8% in Africans [37]. A north-to-south increase in allele frequency has also been observed in Europe [38]. Frequencies of 34, 21 and 9% of the *C1298* allele were reported for Caucasian, Japanese and African populations, respectively [37]. Both *MTHFR* polymorphisms are in linkage disequilibrium, appearing on three out of four possible haplotypes, with *T677-C1298* being virtually absent [39]. The role of *MTHFR* variants in a number of disorders involving disturbances in folate metabolism, such as neural tube defects (NTDs) [40], vascular diseases due to the increased homocysteine levels [41,42] and cancer susceptibility, is well documented [43-45]. Recently, several studies (summarized in **Table 1**) addressed the role of *MTHFR* polymorphisms, and particularly that of the *C677T* substitution, in response to MTX treatment or in the outcome of diseases treated with

Table 1. Summary of studies assessing the efficacy or toxicity of methotrexate treatment in the context of the indicated gene variants.

Gene	Polymorphism	Functional impact		Pharmacogenetic study					Ref.
		In vitro	*In vivo*	Disease	No. of patients	MTX treatment	Association		
							Efficacy	Toxicity	
MTHFR	*C677T*	+	+	Adult ALL	61	15–30 mg/m^2§	n.a.	+	[47]
				GVH prophylaxis in leukemia patients	220	4 x 10–15 mg/m^2	n.a.	+	[48]
				GVH prophylaxis in leukemia patients	53	4 x 10–15 mg/m^2	n.a.	-	[51]
				Childhood ALL	200	DFCI*	+	-¶	[50]
				Childhood ALL	53	St Jude*	n.a.	-#	[57]
				RA	106	Low‡	-	+	[49]
				RA	236	Low‡	-	+	[53]
				RA	105	Low‡	-	+	[54]
				RA	115	Low‡	-	-	[55]
				Ovarian cancer	43	2.5 mg/day for 21 days	-	+	[52]
MTHFR	*A1298C*	+	+	RA	106	Low‡	+	-	[49]
				RA	115	Low‡	-	-	[55]
				Childhood ALL	200	DFCI*	-	-¶	[50]
MTHFD1	*G1958A*	n.a.	+	Childhood ALL	200	DFCI*	-	-¶	[50]
TS	5′-UTR 28-bp repeat	+	+	Childhood ALL	200	DFCI*	+	n.a.	[71]
				Childhood ALL	80	BFM*	-	n.a.	[72]
				RA	115	Low‡	+	-	[55]
TS	3′-UTR 6-bp deletion	+	+/-	RA	115	Low‡	+	-	[55]
CCND1	*A870G*	+	+	ALL	200	DFCI*	+	-¶	[96]
RFC1	*G80A*	-	+	Childhood ALL	200	DFCI‡	+	n.a.	[110]
				Childhood ALL	53	St Jude*	n.a.	-	[57]

Pharmacogenetic studies of childhood ALL patients treated with DFCI protocols, as labeled in the column MTX treatment, are conducted in the same patient cohort. The reference number corresponds to the association study; the studies that documented functional significance of the indicated polymorphisms are cited in the corresponding sections of the text.

**Childhood ALL treatment protocols differ mostly by the number of high doses of MTX (HD-MTX). The DFCI protocol comprises single HD-MTX (4 mg/m^2) for relapse induction; the BFM protocol is composed of four HD-MTX (5 mg/m^2); and St Jude's Total XIV protocol includes seven HD-MTX (5g/m^2) given before induction (one dose), during consolidation (two doses) and during continuation (four doses) of treatment. HD-MTX are in all mentioned protocols followed by leucovorin rescue. ‡Low doses of MTX for the treatment of RA patients vary among studies but are generally in the 2.5 – 15 mg/week range and are administered for several months. §Refers to the weekly doses given during the maintenance phase of treatment during which period toxicity was recorded. ¶No difference in drug dose reduction or withdrawal due to toxic episodes; the analysis of toxic effects beyond the impact on MTX dose was not assessed. #The toxicity included neurotoxicity or thrombosis recorded until the 31st week of treatment.*

ALL: Acute lymphoblastic leukemia; BFM: Berlin-Frankfurt-Munster; CCND1: Cyclin D1; DFCI: Dana-Farber Cancer Institute; GVH: Graft versus host; MTHFD1: Methylenetetrahydrofolate dehydrogenase; MTHFR: Methylenetetrahydrofolate reductase; MTX: Methotrexate; n.a.: Not analyzed; RA: Rheumatoid arthritis; RFC1: Reduced folate carrier; TS: Thymidylate synthase; UTR: Untranslated region.

this drug, such as rheumatoid arthritis, ovarian cancer and hematological malignancies [46-54].

Due to the lower level of 5-methyl-THF, as in the case of *MTHFR* variants with reduced MTHFR activity, it was hypothesized that carriers of the variant genotype would be predisposed to adverse MTX drug effects [46]. Taub *et al.* [46] assessed the relationship between the *MTHFR* variant *677T* and *in vitro* MTX sensitivity using lymphoblasts originating from ALL

patients who suffered from MTX-related neurotoxicity or hepatotoxicity. Cells with the *TT* genotype exhibited greater MTX sensitivity when compared with other *MTHFR 677* genotypes. The relationship between the *MTHFR C677T* polymorphism and development of drug side effects was also assessed in a clinical setting. Among 61 adults with ALL, *T* homozygous patients experienced a higher frequency of MTX intolerance, requiring dose modification and temporary MTX withdrawal during the maintenance phase of treatment [47]. Toxicity affecting liver and bone marrow function was seen more often. In the case of childhood ALL, as observed by the St Justine Hospital group (Montreal, Canada) in a study of 200 children, no association between the *MTHFR* polymorphisms and MTX toxicity was noted, at least not to the extent that it would lead to MTX withdrawal or dose reduction [50]. Further analyses, beyond MTX dose, are needed to confirm this observation.

The impact of the *MTHFR C677T* polymorphism on the development of toxic side effects following MTX treatment has also been assessed in other malignancies. For example, the association between the *677T* variant and post-transplantation complications in 220 adult patients with chronic myeloid leukemia has been reported [48]. All patients were given a short course of MTX (four doses of 10–15 mg/m^2) for prevention of a graft-versus-host (GVH) disease. A significantly reduced oral mucositis index score and delayed hematological recovery was observed among patients with the *TT* genotype when compared to *CC* individuals. Another study [51] addressed the same questions by analyzing the Turkish leukemia patient population (n = 53) who received similar MTX doses for GVH prophylaxis as in the study by Ulrich *et al.* [48]. No differences have been seen in the severity of mucositis, hematopoietic recovery time and the increment in bilirubin levels between *MTHFR* genotypes. The different origin and the number of patients investigated in these two studies might explain the observed discrepancies. In the study of Toffoli *et al.* [52], 43 ovarian cancer patients that underwent a 21-day treatment with low doses of MTX (2.5 mg/day) were analyzed. The comparison of toxicity parameters showed that *TT* patients had a 40-fold higher relative risk than patients with other *MTHFR* genotypes of developing high-grade (III or IV) toxicity.

Finally, similar genotype–phenotype correlations have been provided by studies of RA patients. Urano *et al.* [49] analyzed 106 RA patients who were treated with weekly low doses of MTX (2.5–7 mg) for at least 3 months. A differing effect of the two *MTHFR* polymorphisms was observed. Patients with the *C677-C1298* haplotype received lower doses of MTX and responded better to the treatment than those without this haplotype, as estimated by the number of tender or swollen joints, the change in C-reactive protein (CRP) and erythrocyte sedimentation rate values. Subjects carrying *T677-A1298* had a higher frequency of MTX side effects, mostly confined to stomatitis and an increase in hepatic transaminase values [49]. Similar observations regarding drug side effects have been reported by van Ede *et al.* [53]. The carriers of the *T677* allele among 236 studied patients had an increased risk of discontinuing MTX treatment because of adverse events that were mainly due to elevated liver enzyme levels. Gastrointestinal toxicity correlated with the *T677* variant in another study conducted on 105 RA patients [54]. Contrary to these findings, Kumagai *et al.* [55] did not find any of the *MTHFR* polymorphisms to be associated with MTX-related toxicity or efficacy.

The toxic effects observed in several studies in individuals with the *MTHFR TT677* genotype seem to be mostly driven by decreased 5-methyl-THF levels, which might lead to a lower methylation of homocysteine, lower methionine levels, and, in turn, reduced DNA methylation. However, a reduced MTHFR activity associated with variant *MTHFR* genotypes also conserves the higher 5,10-methylene-THF levels that are needed for dUMP to dTMP conversion. Availability of 5,10-methylene-THF could, thus, facilitate TS action, reducing the rate of uracil misincorporation and the resulting chromosome damage, which might reduce MTX efficacy. Indeed, the increase (twofold) of the risk of an event associated with the *MTHFR T677* variant (corresponding essentially to the *MTHFR T677-A1298* haplotype) has been observed in childhood ALL patients treated at St Justine Hospital [50]. Further adding to this observation are recent *in vitro* chemosensitivity assays carried out in colon and breast cell lines in the context of the *MTHFR 677* genotypes. The cells transfected with *T677* cDNA showed decreased MTHFR activity, changed intracellular folate distribution, and accelerated cellular growth rate [56]. Importantly, increased TS activity and decreased chemosensitivity to MTX were also observed [56].

Homocysteinemia arises as a consequence of MTX-induced folate depletion and it is thought

to mediate MTX-associated toxicity, particularly neurotoxicity [7]. Regardless of the MTX treatment, the association of *MTHFR T677* and homocysteinemia due to a lower concentration of 5-methyl-THF, which is needed for homocysteine methylation, has been reported in numerous studies [41,42]. This association seems to be particularly true in instances of folate deficiency [8]. Therefore, it was reasonable to hypothesize that the same *MTHFR* variants might also correlate with increased homocysteine levels following MTX administration. However, not all studies addressing this issue are in agreement. Following MTX administration, plasma homocysteine levels were significantly higher in ovarian cancer patients with the *TT* genotype when compared with other genotype groups [52]. Similar observations have been made in at least one other study conducted in RA patients [54]. On the other hand, the homocysteine levels measured in 53 children with ALL, who were treated on a single clinical protocol at St Jude Children's Research Hospital (Memphis, TN, USA), which included two courses of high-dose MTX (HD-MTX) as consolidation therapy, did not find any significant correlation between the increase in homocysteine levels following HD-MTX and *MTHFR* genotypes [57]. The correlation of these genotypes with neurotoxicity or thrombosis was also not observed. Likewise, the absence of correlation with homocysteine levels was reported in another study of RA patients [58]. Different diseases or types of malignancies, variable folate status before initiation of therapy, and differences in the drug doses and duration of MTX treatment might account for the observed differences.

5,10-Methylenetetrahydrofolate dehydrogenase

In addition to MTHFR, maintenance of the folate pool is provided by the action of the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase (MTHFD1). MTHFD1 plays an important role in the generation of the 5,10-methylene-THF and 10-formyl-THF pools required for *de novo* purine and thymidylate synthesis [59] (**Figure 1**). The *G* to *A* substitution at position 1958 of the *MTHFD1* gene, which causes an arginine to glutamine substitution at codon 653 located within the 10-formyl-THF synthetase enzyme domain, has recently been reported [60]. The role of a *MTHFD1 A1958* variant in susceptibility to NTDs has been documented [61]. This, along with the conservation of an arginine residue at the corresponding position of the *MTHFD1* gene across numerous species, suggests that replacement with glutamine may have functional consequences [61]. Since MTHFD1 Arg653Gln replacement lies within the 10-formyl-THF synthetase domain of MTHFD1, it is possible that a reduction in the formation of 10-formyl-THF could shift the folate balance toward 5,10-methylene-THF pools. This would allow for more efficient TS synthesis contributing to MTX resistance and/or malignancy progression. The study conducted at St Justine Hospital in an ALL patient cohort did not support the independent effect of the *MTHFD1* variant on ALL outcome [50] (**Table 1**).

Thymidylate synthase

TS is a key enzyme in the nucleotide biosynthetic pathway that converts dUMP to dTMP [5] and, thus, provides the only source for *de novo* thymidylate production (**Figure 1**). The cytotoxic action of MTX comprises of both indirect inhibition of thymidylate synthesis due to folate cofactor depletion, as well as direct enzyme inhibition performed by glytamylated forms of MTX [2,5,6]. TS suppression leads to deoxythymidine triphosphate (dTTP) depletion and increased uracil misincorporation into nucleic acid. This in turn results in chromosomal damage and apoptotic cell death [62,63]. Recently, a tandem repeat sequence was identified in the enhancer element of the 5′-untranslated region (5′-UTR) region of the *TS* gene [64]. It has been shown to be polymorphic, containing a variable number of 28-bp repeat element. The triple repeat (*3R*) allele was found to be represented with frequencies of 50–60, 60 and 80% in Caucasians, Southwest Asians and Eastern Asians, respectively [65-67]. The repeat element appears to function as an enhancer because *in vitro* studies have shown that TS mRNA expression and TS enzyme activity are associated with an increasing number of repeat sequences [64,68-70]. These findings have been extended to the clinical setting where studies have documented higher TS mRNA levels in *3R* homozygous patients when compared to those that are homozygous for a double repeat (*2R*) allele [69,70]. Several studies have addressed the potential of this polymorphism to predict the outcome of diseases treated with MTX (summarized in **Table 1**).

A study assessing the impact of the *TS* repeat polymorphism on the outcome of childhood ALL was conducted at St Justine Hospital [71]. It

analyzed 200 individuals of European descent living in the Province of Quebec, Canada, and who underwent treatment with three consecutive treatments with the Dana-Farber Cancer Institute (DFCI) protocols. As part of the multiagent chemotherapy, ALL patients were given a single HD-MTX (4 mg/m^2) for relapse induction, and intrathecal treatment for the prevention of CNS disease followed by low doses (30 mg/m^2) administered weekly for up to 2 years of continuous complete remission. Individuals that were *3R* homozygous were significantly overrepresented in the event-positive group and had shorter event-free survival (EFS) when compared with other genotypes [71]. Lauten *et al.* [72] addressed the same issue, although, in this case, they applied a different study design to analyze ALL patients treated with Berlin-Frankfurt-Munster (BFM) protocols. A total of 40 patients with relapse were compared with the same number of successfully treated patients that were matched according to treatment and clinical prognostic features. No difference in the frequency of the *3R* variant has been found between cases with and without relapse. The discrepant results from these two studies may be due to differences in study designs or in the number and heterogeneity of patient groups, as well as to differences between treatment protocols. For example, the first study was carried out in a relatively diverse patient population, whereas the other is rather restricted to patients with particular characteristics. Taking into regard the different treatment protocols that were used in these studies, it is also possible that this polymorphism is important only in a specific therapeutic context. Higher doses of MTX were used in the BFM protocol (four times 5 mg/m^2) and it could be possible that this can overcome the adverse effect of the *TS 3R* variant [72,73]. The coexistence in the different ratio of the other *TS* polymorphisms (described further in the text) between patient groups may also account for the observed differences. Although the mechanism of MTX action might be different in RA than in ALL patients, and it may not necessarily be dependent on the efficacy of thymidylate synthesis, one study addressed the role of *TS* repeat polymorphism in 115 RA patients treated with low doses of MTX for > 2 months [55]. A significantly higher frequency of *3R* homozygotes was found among RA patients who required higher doses of MTX for successful treatment [55].

The more detailed molecular mechanism by which the tandem repeat polymorphism enhances transcription has been recently elucidated [74,75]. It appears that the impact of the *3R* genotype on TS transcriptional activity is related to the additional upstream stimulatory factor (USF) protein-binding site of the *3R* allele compared with the *2R* variant. Moreover, both studies discovered a *G* to *C* polymorphism at nucleotide position 12 within the second 28-bp repeat of the *3R* allele indicated additional variability of the *TS* gene and particularly that of the tandem repeat polymorphism. This base substitution appears to change a critical residue in the USF consensus element, abolishing its ability to complex with the USF protein. A *3RC* construct displayed a similar transcriptional activity to that of a *2R* allele.

A polymorphism within the *TS* gene, consisting of a 6-bp deletion of the sequence TTAAAG at nucleotide 1494 of the TS cDNA located in the 3′-UTR, has been discovered through searching the public expressed sequence tag database [76]. The 6-bp deletion allele was found to be represented with frequencies of 41, 26, 52 and 76% in Caucasian, Hispanic, African–American and Chinese populations, respectively [77]. In general, the 3′-UTR alteration may influence gene function through post-transcriptional mechanisms, such as control of mRNA stability and translational efficiency [78]. *In vitro* analysis, at least in one study, indicated that the 6-bp polymorphism is associated with decreased mRNA stability, as shown by a 70% decrease in luciferase activity and mRNA quantities [77]. Lower mRNA levels in homozygous 6-bp$^-$/6-bp$^-$ colon cancer patients, compared with 6-bp$^+$/6-bp$^+$ individuals, further supported this finding [77]. However, others reported no correlation between altered TS transcription and the 6-bp deletion variant [79]. The study of Kumagai *et al.* [55] found significant improvement of the serum CRP levels in RA patients that are homozygous for the 6-bp deletion allele (**Table 1**). Our preliminary data suggest the absence of an association between 6-bp deletion variant and relapse rate in ALL patients, at least if this variant is analyzed independently from other *TS* polymorphisms.

In the light of the data regarding the *G* to *C* polymorphism within the *3R* allele, as well as those implicating the functional significance of the 6-bp deletion polymorphism, it would be interesting to further analyze the tumoral mRNA levels in the function of *TS* haplotypes. Similarly, association studies, as based on the

simultaneous impact of these three polymorphisms, may provide useful and more comprehensive information with respect to both drug efficacy and toxicity. It is worth noting that the source of the tissue used for *TS* genotyping might also be important for the predictive value of this polymorphism [80]. A high frequency of the loss of heterozygosity affecting the *TS* gene was observed in the cohort of colorectal cancer patients. It remains to be defined if this observation holds true for tumors of different origin, which will help define in which instances *TS* polymorphism status should be determined in tumor tissue.

Cyclin D1

The cyclin D1 (*CCND1*) gene product is one of the key proteins regulating progression through the G1 phase of the cell cycle [81]. CCND1 overexpression, arising through various mechanisms, has been documented in a number of malignancies, implicating its role in both tumorigenesis and tumor progression [82-85]. Besides favoring cell proliferation, altered expression of cell cycle regulators might also directly interfere with the drug effect. This is well documented by the p53 protein whose mutated forms show an increased resistance to a wide range of chemotherapeutics [86]. CCND1 might have a similar effect. It mediates the phosphorylation and functional inactivation of the retinoblastoma protein (pRB). An increased CCND1 expression can affect the phosphorylation state of pRB, leading to higher levels of transcription factor E2 (E2F) [87]. This, in turn, may lead to an increased E2F-mediated transcription of major MTX targets, such as DFHR and TS, which would ultimately affect MTX sensitivity (Figure 2) [25,87,88]. Indeed, the study of a fibrosarcoma cell line transfected with complementary DNA sequence encoding human CCND1 demonstrated that CCND1 overexpression can induce an increase in DHFR transcription, rendering cells resistant to MTX [87].

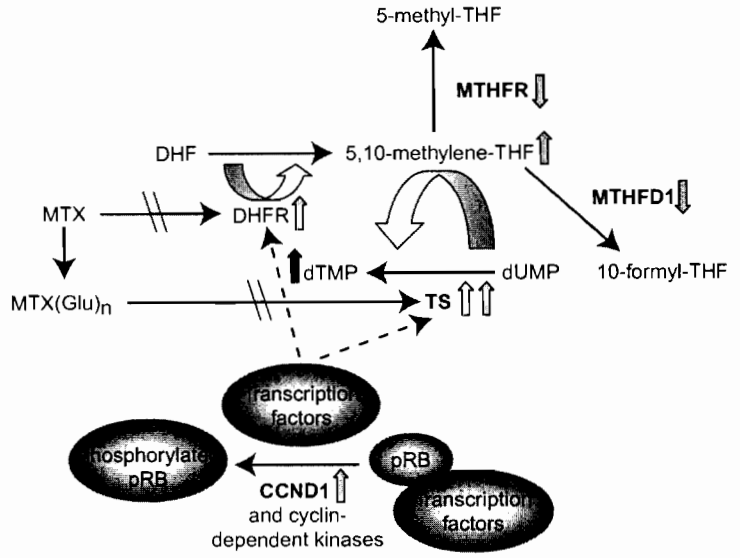
Different functional gene variants resulting in altered CCND1 expression can also arise as a consequence of the gene polymorphism. A recently described *CCND1 A870* to *G* substitution affects the splice donor site at the exon/intron boundary, modulating the ratio of CCND1 mRNA isoforms a and b [89-91]. Several studies showed the association of this polymorphism, and particularly that of allele *A*, with both tumor development and progression [92-94]. The observed impact was thought to be due to a higher level of transcript b resulting in a protein with a longer half-life that might affect transition through the G1/S checkpoint and cell proliferation [93,95]. In the case of diseases treated with MTX, interference with MTX sensitivity is an additional mechanism that can, beside interference with cell cycle progression, account for a worse outcome in the patients having a *CCND1 A* allele. In a study of 200 children with ALL conducted at St Justine Hospital, *AA* individuals were found at a frequency of 41.2 and 19.3% in the event and non-event groups, respectively (Table 1). Consequently, *AA* patients had a lower probability of 5-year post-treatment EFS compared to other *CCND1* genotype groups (66 versus 86%; p = 0.006) [96]. It should, however, be kept in mind that CCND1 might influence the responsiveness to multiple chemotherapeutics [97] and that the interference with the action of other components of the multi-agent ALL treatment regimen can also contribute to the observed association.

The altered function of other cell cycle proteins, such as pRB and E2F or other components of the cyclin-dependent kinase system involved in pRB phosphorylation, might also contribute to MTX resistance. In line with this are findings of several studies: osteosarcoma cells lacking an active *RB* gene were more resistant to MTX than cell lines with an active *RB* gene [98]; this gene's mutations were found more often in ALL patients who had relapsed [99]. To our knowledge, the analyses of the functional variants of these genes with disease outcome or MTX sensitivity have not yet been reported.

Reduced folate carrier

Reduced folate carrier (RFC1) is a major route for MTX delivery (Figure 1). Defective transport as a result of qualitative and quantitative alterations in cultured cells has frequently been documented as a reason for antifolate drug resistance. The predominant underlying mechanisms are inactivating *RFC1* mutations and the loss of *RFC1* gene expression due to the altered function of the transcription factors [100,101]. The mutations cluster primarily in the first transmembrane domain (TMD1), which results in a major loss of antifolate uptake while preserving sufficient reduced folate uptake to allow for cellular growth [102,103]. Likewise, alterations in RFC1 function leading to MTX resistance have been documented in a clinical setting [104,105]. However, inactivating mutations do not seem to

Figure 2. The interference of cyclin D1 with the folate cycle enzyme and the proposed mechanisms for gene–gene interactions.



CCND1-driven alteration in phosphorylation status of pRB can lead to the increased transcription of MTX targets, TS and DHFR (arrows with dotted lines).
The suggested enzyme activity, as presented by the arrows (pointing up for increased and down for decreased activity), are due to the presence of the described variants (*CCND1 A870, TS 3R, MTHFR T677* and *MTHFD1 A1958*). An increase in CCND1 expression can result in enhanced TS and DFHR expression (blue arrows). Decrease in MTHFR and MTHFD1 activity would lead to the increased levels of 5,10-methylene-THF needed as a cofactor for TS synthesis (pink arrows). The triple repeat *TS* polymorphism is associated with increased TS expression (yellow arrow). Gene–gene interactions (discussed in the text) that may lead to an enhanced resistance to MTX, can be schematically viewed as combinations of arrows; yellow and blue for CCND1 and TS interaction, and yellow and pink for TS and 5,10-methylene-THF, whose increase in concentrations result from altered MTHFR and MTHFD1 activities.
DHF: Dihydrofolate; DHFR: Dihydrofolate reductase; dTMP: Deoxythymidine monophosphate; dUMP: Deoxyuridine monophosphate; MTHFD1: Methylenetetrahydrofolate dehydrogenase; MTHFR: Methylenetetrahydrofolate reductase; MTX: Methotrexate; $MTX(Glu)_n$: MTX glutamates; pRB: Retinoblastoma protein; THF: Tetrahydrofolate; TS: Thymidylate synthase.

play a role, at least in ALL patients [103]. *RFC1* gene polymorphisms could also be partly responsible for the observed functional differences.

A *G80* to *A* polymorphism leading to a His27Arg replacement in the TMD1 of the RFC1 protein, has been described [106]. This polymorphism influences homocysteine levels and was found to play a role in the development of NTDs [106-108]. Since both folate and homocysteine homeostasis are affected by MTX action [5,109], it is plausible that the *RFC1 G80A* polymorphism may modulate the disease outcome in patients treated with this drug. In a study of 200 children affected with ALL (St Justine Hospital) [110], a modest influence of the *A* variant on the rate of relapse and on MTX plasma levels was observed. In a study of 53 ALL patients, conducted at St Jude Hospital, no relationship between neurotoxicity of thrombosis was observed based on the *RFC1 G80A* genotype [57] (Table 1). Given that *in vitro* assays measured similar MTX uptake for the *A* and *G* variants [111], the functional impact of this polymorphism remains unclear. The other variants, independently or in context of the *RFC1* haplotype, may also play a role in MTX response. For example, a 61-bp repeat polymorphism in the *RFC1* promoter associated with increased transcriptional activity of the gene, was recently reported [112]. Its relationship to the *G80A* polymorphism or its relevance for pharmacogenetics of MTX has not yet been assessed. Our preliminary results suggest little independent influence of this polymorphism on ALL outcome.

Gene–gene interactions

MTX-mediated inhibition of *de novo* purine and thymidylate synthesis consists of both substrate depletion and direct enzymatic inhibition [43,113-116]. Given that MTX glutamates directly inhibit TS, along with the fact that TS requires 5,10-methylene-THF for its activity, it is possible that combined effects of genes controlling TS activity and folate cofactor levels have a higher impact on MTX response than either of them regarded separately. Lower levels of TS and 5,10-methylene-THF would facilitate MTX action, while, in contrast, high levels would mediate MTX resistance. The latter would be expected in individuals that are homozygous for a triple repeat (*3R*) of the 5′-UTR *TS* polymorphism and who have MTHFR and/or MTHFD1 variants that shunt folate metabolism toward 5,10-methylene-THF conservation (Figure 2). Toward this end, the group at St Justine Hospital assessed the association of *MTHFR* and *MTHFD1* polymorphisms with childhood ALL outcome in the context of *TS 3R* homozygosity [50]. The association of both gene polymorphisms with ALL outcome seems more obvious when combined with that of the *TS* gene. Individuals with both *TS 3R3R* and *MTHFR T677A1298* haplotypes or *TS 3R3R* and *MTHFD1 A1958* genotypes had a remarkably lower probability of 5-year post-treatment EFS, compared with subjects with no event-predisposing genotypes (54 versus 93%, p = 0.0001, and 45 versus 95%, p = 0.0002, respectively).

Similarly, the combined effect can be observed for other genes participating in the folate cycle. The change in CCND1-mediated regulation of the pRB can lead to an increased expression of MTX targets and, thus, a reduction of sensitivity to MTX [25,87,88]. This would be particularly obvious in individuals with higher MTX target expression, such as in those that are homozygous for *TS 3R* (Figure 2). Indeed, the analysis of the combined impact of the *CCND1* and *TS* polymorphisms in childhood ALL patients showed that individuals with both *TS 3R3R* and *CCND1 AA870* genotypes were differently represented in the event and non-event group (26.5 versus 3.7%). Consequently, they had a significantly lower probability of 5-year post-treatment EFS, compared with those without these event-predisposing genotypes (37 versus 88%, p < 0.00005) [96].

Outlook and conclusion

Several studies showed that the variants of genes controlling the levels of folate cofactors or the activity of folate-dependent enzymes can affect the outcome of diseases treated with MTX. However, our understanding of the role of genetic variants in response to MTX has only just begun to be unravelled. Certain studies are still inconclusive and require further confirmations. Inconstancies that can be due to different treatment regimens and drug doses or insufficient sample sizes exist across the literature. Some studies care about the fact that gene variants vary in their frequencies across different subpopulations [117], whereas others might be confounded by these differences. Several mechanisms have been described to play a role in MTX resistance. There is, thus, no doubt that several genes and their polymorphisms have to be considered simultaneously to accurately predict the outcome of diseases treated with this drug. Most studies have addressed only one enzyme or one polymorphism at a time and are, hence, insufficient to fully predict MTX response, indicating a need for larger and collaborative studies. Further research will also need to focus on new candidate genes or on new polymorphisms in genes that have already been identified. In that regard, the polymorphisms of several genes whose variable products can modulate MTX response [26,27,118-122] are described in the literature. These include polymorphisms of the *DFHR* and *GGH* genes, as well as those encoding the family of multi-drug resistance protein, recently shown to participate in the MTX efflux [123-130]. No information is yet available regarding their role in MTX response.

An important step forward for pharmacogenetic studies is represented by the efforts of individual research groups and consortiums to identify SNP and make them publicly available through SNP databases. For example, the SNP500 Cancer Database [201] provides sequence and genotype assay information for candidate SNPs (including frequency in different populations, functional assays and the organization in haplotypes when available) that have been implicated in cancer development or progression [131]. Although the choice of candidate genes focuses primarily on the genes relevant for cancer etiology, there is a great overlap with those that might modulate differential response to chemotherapeutics. Important information for studies of antitumor agents is also provided by the Pharmacogenetics and Pharmacogenomics Knowledge Base [202], which collects and summarizes relevant

Highlights

- Due to their correlation with altered enzymatic activity, genetic polymorphisms can exert an influence on drug effects in many ways and, as such, contribute to interindividual variability in therapeutic response.
- This paper reviewed the current knowledge regarding the potential of the folate cycle polymorphisms to modulate the response to MTX.
- Larger studies are needed to analyze the whole spectra of relevant variants and to identify those with the highest predictive value, thus leading to individualized drug use and better disease outcomes.

information. This also includes the design of drug pathways that may assist researchers in the selection of pharmacogenetically relevant candidate genes.

The future holds the hope that the addition of new knowledge to that already acquired will allow individualized MTX treatment according to a patient's genetic profile by increasing drug efficacy while reducing its side effects.

Acknowledgments

Work in the author's laboratory was supported by the Canadian Institutes of Health Research, Leukemia Research Fund of Canada and Centre de Recherche, Hôpital Ste-Justine. M.K. is a scholar of the Fonds de la Recherche en Santé du Québec.

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AUGMENTED POST-INDUCTION THERAPY FOR CHILDREN WITH HIGH-RISK ACUTE LYMPHOBLASTIC LEUKEMIA AND A SLOW RESPONSE TO INITIAL THERAPY

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ABSTRACT

Background Children with high-risk acute lymphoblastic leukemia (ALL) who have a slow response to initial chemotherapy (more than 25 percent blasts in the bone marrow on day 7) have a poor outcome despite intensive therapy. We conducted a randomized trial in which such patients were treated with either an augmented intensive regimen of post-induction chemotherapy or a standard regimen of intensive post-induction chemotherapy.

Methods Between January 1991 and June 1995, 311 children with newly diagnosed ALL who were either 1 to 9 years of age with white-cell counts of at least 50,000 per cubic millimeter or 10 years of age or older, had a slow response to initial therapy, and entered remission at the end of induction chemotherapy were randomly assigned to receive standard therapy (156 children) or augmented therapy (155). Those with lymphomatous features were excluded. Event-free survival and overall survival were assessed from the end of induction treatment.

Results The outcome at five years was significantly better in the augmented-therapy group than in the standard-therapy group (Kaplan–Meier estimate of event-free survival [±SD]: 75.0±3.8 vs. 55.0±4.5 percent, $P<0.001$; overall survival: 78.4±3.7 vs. 66.7±4.2 percent, $P=0.02$). The difference between treatments was most pronounced among patients one to nine years of age, all of whom had white-cell counts of at least 50,000 per cubic millimeter ($P<0.001$). Risk factors for an adverse event in the entire cohort included a white-cell count of 200,000 per cubic millimeter or higher ($P=0.004$), race other than black or white ($P<0.001$), and the presence of a t(9;22) translocation ($P=0.007$). The toxic effects of augmented therapy were considerable but manageable.

Conclusions Augmented post-induction chemotherapy results in an excellent outcome for most patients with high-risk ALL and a slow response to initial therapy. (N Engl J Med 1998;338:1663-71.)

IN children with acute lymphoblastic leukemia (ALL) who are older than one year of age, certain presenting features, such as a white-cell count above 50,000 per cubic millimeter,[1-3] an age of 10 years or older,[4,5] the presence of bulky disease,[1,3,6] T-cell–lineage immunophenotype,[7-9] and various chromosomal translocations,[10-16] carry an increased risk of treatment failure. The outcome for most of these children has improved with the use of intensive chemotherapy after the induction of remission,[17-22] but approximately 30 percent of such high-risk patients eventually relapse.

Numerous studies have demonstrated that a rapid response to initial chemotherapy is an important prognostic factor in childhood ALL.[17,18,23-28] German investigators observed that patients with fewer than 1000 blasts per cubic millimeter in the peripheral blood after a seven-day course of prednisone had significantly better event-free survival than patients with 1000 or more blasts per cubic millimeter.[17,28,29] Similarly, we reported that children with 25 percent blasts or fewer in the bone marrow on day 7 had a better response to initial chemotherapy (three-year event-free survival, 77 percent) than those with more than 25 percent blasts (three-year event-free survival, 48 percent).[26] In an attempt to improve the outcome for children with a slow response to initial therapy, we developed a strategy of augmented, intensive post-induction chemotherapy that was based on previous successful regimens for ALL.[30-32] This approach appeared promising in a nonrandomized pilot study.[33] We now report on a randomized comparison of augmented therapy with standard intensive post-induction therapy in children with high-risk ALL who entered remission after a slow response to initial therapy.

METHODS

Patients

Children and adolescents with newly diagnosed ALL who were 1 to 9 years of age and had white-cell counts of at least 50,000 per cubic millimeter or who were 10 years of age or older were enrolled between January 1991 and June 1995. Those with lymphomatous features[6] were excluded. Diagnosis was based on morphologic, biochemical, and immunologic features of leukemic

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cells, including lymphoblast morphology as determined by Wright–Giemsa staining, negative staining for myeloperoxidase, and reactivity with monoclonal antibodies to lymphoid differentiation antigens associated with B-cell or T-cell lineage, as described previously.[34] Patients with slow initial responses (>25 percent marrow blasts on day 7) who had entered remission by day 28 were randomly assigned at the end of induction therapy to receive standard or augmented therapy.

Treatment Protocol

All patients received identical five-week courses of induction chemotherapy, as previously described.[33] The post-induction regimens are given in Table 1. During the first year of post-induction therapy, the augmented regimen included more vincristine, asparaginase, methotrexate, and dexamethasone than the standard regimen, although the standard regimen included more oral methotrexate, prednisone, and mercaptopurine. Therapy was continued for two years for girls and for three years for boys, beginning with the first interim maintenance period (Table 1)[35] (and unpublished data). Presymptomatic central nervous system therapy consisted of intrathecal methotrexate and cranial radiation. This protocol was approved by the National Cancer Institute and the institutional review boards of the participating institutions. Informed consent was obtained from the patients, their parents, or both, as deemed appropriate, according to Department of Health and Human Services guidelines.

Study Design and Statistical Analysis

Balanced block randomization was used to ensure that approximately equal numbers of patients were randomly assigned to each regimen. The study was monitored by an independent data-monitoring committee and followed a monitoring plan that was based on group sequential monitoring boundaries[36] that required analysis of results at six-month intervals for a maximum of 10 analyses. With a target enrollment of 296 randomized patients, we estimated that the study had a power of approximately 81 percent at the final analysis to detect a change in five-year event-free survival from 45 percent to 62 percent or more with a two-sided log-rank test (alpha level, 0.05). The monitoring boundary was crossed in July 1996 (the ninth planned data analysis), and at that time study results were released.

This analysis was performed in December 1997. Similarities between patients in the two groups were assessed with chi-square tests for homogeneity of proportions. Outcome analyses used life-table methods and associated statistics. The primary end point examined was event-free survival from the time of randomization. The events considered were relapse at any site, death during remission, or a second malignant neoplasm, whichever occurred first. Data on patients who had not had an event at the time of the analysis were censored in the analysis of event-free survival at the time of the last contact with them. Life-table estimates were calculated by the Kaplan–Meier procedure, and the standard deviation of the life-table estimate was obtained with Greenwood's formula.[37] The Kaplan–Meier estimates (±SD) are presented for either the first five years or the first three years after randomization, depending on the number of patients in the follow-up. Ninety-five percent confidence intervals can be approximated as the life-table estimates ±1.96 SD. The log-rank statistic was used to compare patterns of event-free survival and overall survival in the groups.[38,39] Comparisons of randomized treatment regimens were performed according to the intention-to-treat method. Stratified log-rank tests were also used to adjust for the possible modifying effect of other factors on the comparison of interest.[40] An adjusted Cox regression analysis was used to determine the influence of prognostic factors on the primary treatment effect. Life-table analyses of the effect of isolated central nervous system and marrow relapses on the results with each regimen were compared with the log-rank statistic. Life-table analysis of the relative risk of an adverse event was calculated with the log-rank ratio of observed events to expected events.[41]

RESULTS

Patients

A total of 1136 patients were enrolled. Three patients died before day 7, and marrow was not obtained on day 7 from 15 patients. Of the remaining 1118 patients, 360 (32 percent) had slow responses to initial therapy. Of these, 340 (94 percent) entered remission after induction therapy, 19 did not enter remission after induction therapy, and 1 received modified induction therapy and therefore was deemed ineligible. Of the 340 eligible patients, 317 (93 percent) underwent randomization. A subsequent review revealed that 6 of these patients did not have a slow response; thus, 311 patients were eligible for the study. Of these, 156 were assigned to standard therapy and 155 were assigned to augmented therapy.

The characteristics of the patients in the two groups are shown in Table 2. There were no significant differences between the groups. Most patients were at least 10 years of age, and approximately half had white-cell counts of at least 50,000 per cubic millimeter. Centrally reviewed cytogenetic data on translocations associated with a high risk of an adverse event were available for 91 of the patients: 3 patients had the t(4;11) translocation, 4 had t(1;19), and 7 had t(9;22). Among 209 patients with immunophenotypic data, 87.6 percent had ALL of B-cell lineage.

Study Violations

Thirteen patients (seven in the standard-therapy group and six in the augmented-therapy group) received a bone marrow transplant during their first remission but were included in the intention-to-treat analysis. Indications for transplantation included the presence of a t(9;22) translocation (four patients), a white-cell count of more than 200,000 per cubic millimeter (three patients), virus-associated hemophagocytic syndrome (one patient), the presence of myeloid antigen (two patients), and other reasons (three patients). Two patients in the standard-therapy group and one patient in the augmented-therapy group refused cranial radiotherapy. Five patients assigned to augmented therapy did not receive the second cycle of delayed intensification therapy. Major changes in treatment were required for three patients assigned to standard therapy (two patients had fungal infections, and one had an elevation in aminotransferases) and five patients assigned to augmented therapy (three patients had elevations in aminotransferases, one had leukoencephalopathy, and one was not compliant with oral therapy).

Outcome of Treatment

At the time the study data were released in July 1996, the four-year event-free survival rate was significantly better among patients in the augmented-therapy group than among those in the standard-therapy

TABLE 1. THE STANDARD-THERAPY AND AUGMENTED-THERAPY REGIMENS.*

STANDARD THERAPY			AUGMENTED THERAPY		
PHASE	TREATMENT	DOSE	PHASE	TREATMENT	DOSE
Consolidation (5 wk)	Prednisone	7.5 mg/m² day 0; 3.75 mg/m²/day days 1, 2	**Consolidation (9 wk)**	Cyclophosphamide	1000 mg/m²/day IV days 0, 28
	Cyclophosphamide	1000 mg/m²/day IV days 0, 14		Cytarabine	75 mg/m²/day SQ or IV days 1–4, 8–11, 29–32, 36–39
	Mercaptopurine	60 mg/m²/day PO days 0–27		Mercaptopurine	60 mg/m²/day PO days 0–13, 28–41
	Vincristine	1.5 mg/m²/day IV days 14, 21, 42, 49		Vincristine	1.5 mg/m²/day IV days 14, 21, 42, 49
	Cytarabine	75 mg/m²/day IV days 1–4, 8–11, 15–18, 22–25		Asparaginase	6000 U/m²/day IM days 14, 16, 18, 21, 23, 25, 42, 44, 46, 49, 51, 53
	Methotrexate†	IT days 1, 8, 15, 22		Methotrexate†	IT days 1, 8, 15, 22
	Radiotherapy‡	Cranial, 1800 cGy		Radiotherapy‡	Cranial, 1800 cGy
		Cranial, 2400 cGy, and spinal, 600 cGy			Cranial, 2400 cGy, and spinal, 600 cGy
					Testicular, 2400 cGy
Interim maintenance (8 wk)	Mercaptopurine	60 mg/m²/day PO days 0–41	**Interim maintenance I (8 wk)**	Vincristine	1.5 mg/m²/day IV days 0, 10, 20, 30, 40
	Methotrexate	15 mg/m²/day PO days 0, 7, 14, 21, 28, 35		Methotrexate	100 mg/m²/day IV days 0, 10, 20, 30, 40 (escalate by 50 mg/m²/dose)
				Asparaginase	15,000 U/m²/day IM days 1, 11, 21, 31, 41
Delayed intensification (7 wk)			**Delayed intensification I (8 wk)**		
Reinduction (4 wk)	Dexamethasone	10 mg/m²/day PO days 0–20, then taper for 7 days	Reinduction (4 wk)	Dexamethasone	10 mg/m²/day PO days 0–20, then taper for 7 days
	Vincristine	1.5 mg/m²/day IV days 0, 14, 21		Vincristine	1.5 mg/m²/day IV days 0, 14, 21
	Doxorubicin	25 mg/m²/day IV days 0, 7, 14		Doxorubicin	25 mg/m²/day IV days 0, 7, 14
	Asparaginase	6000 U/m²/day IM days 3, 5, 7, 10, 12, 14		Asparaginase	6000 U/m²/day IM days 3, 5, 7, 10, 12, 14
Reconsolidation (3 wk)	Vincristine	1.5 mg/m²/day IV days 42, 49	Reconsolidation (4 wk)	Vincristine	1.5 mg/m²/day IV days 42, 49
	Cyclophosphamide	1000 mg/m² IV day 28		Cyclophosphamide	1000 mg/m² IV day 28
	Thioguanine	60 mg/m²/day PO days 28–41		Thioguanine	60 mg/m²/day PO days 28–41
	Cytarabine	75 mg/m²/day SQ or IV days 29–32, 36–39		Cytarabine	75 mg/m²/day SQ or IV days 29–32, 36–39
	Methotrexate†	IT days 29, 36		Methotrexate‡	IT days 29, 36
				Asparaginase	6000 U/m²/day IM days 42, 44, 46, 49, 51, 53
Maintenance (12 wk)§	Vincristine	1.5 mg/m²/day IV days 0, 28, 56	**Interim maintenance II (8 wk)**	Vincristine	1.5 mg/m²/day IV days 0, 10, 20, 30, 40
	Prednisone	40 mg/m²/day PO days 0–4, 28–32, 56–60		Methotrexate	100 mg/m²/day IV days 0, 10, 20, 30, 40 (escalate by 50 mg/m²/dose)
	Mercaptopurine	75 mg/m²/day PO days 0–83		Asparaginase	15,000 U/m²/day IM days 1, 11, 21, 31, 41
	Methotrexate	20 mg/m²/day PO days 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77		Methotrexate†	IT days 0, 20, 40
	Methotrexate†	IT day 0	**Delayed intensification II (8 wk)**	Same as for delayed intensification I	
			Maintenance (12 wk)§	Vincristine	1.5 mg/m²/day IV days 0, 28, 56
				Prednisone	60 mg/m²/day PO days 0–4, 28–32, 56–60
				Mercaptopurine	75 mg/m²/day PO days 0–83
				Methotrexate	20 mg/m²/day PO days 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77
				Methotrexate†	IT day 0

*IV denotes intravenously, PO orally, IT intrathecally, SQ subcutaneously, and IM intramuscularly.

†The doses were age-adjusted as follows: age 1 to 1.9 years, 8 mg; age 2 to 2.9 years, 10 mg; age ≥3 years, 12 mg. Patients with central nervous system disease at diagnosis did not receive intrathecal methotrexate on days 15 and 22 of consolidation therapy.

‡During the first two weeks of consolidation therapy, patients without central nervous system disease at diagnosis received 1800 cGy of cranial radiotherapy in 10 fractions; patients with central nervous system disease at diagnosis received 2400 cGy to the cranial midplane in 12 fractions and 600 cGy to the spinal cord in 3 fractions. In the augmented-therapy group, patients with testiculomegaly at diagnosis received 2400 cGy bilateral testicular radiation in 8 fractions.

§The cycles of maintenance therapy were repeated until the total duration of therapy, beginning with the first interim maintenance period, reached two years for girls and three years for boys.

TABLE 2. CHARACTERISTICS OF THE PATIENTS AT DIAGNOSIS.

CHARACTERISTIC*	STANDARD THERAPY (N=156)	AUGMENTED THERAPY (N=155)	P VALUE†	CHARACTERISTIC*	STANDARD THERAPY (N=156)	AUGMENTED THERAPY (N=155)	P VALUE†
	no. (%)				no. (%)		
Age (yr)			0.85	Hemoglobin (g/dl)			0.94
1–9	50 (32.1)	54 (34.8)		1–7.9	82 (52.9)	78 (52.7)	
10–15	73 (46.8)	68 (43.9)		8.0–10.9	52 (33.5)	48 (32.4)	
≥16	33 (21.2)	33 (21.3)		≥11.0	21 (13.5)	22 (14.9)	
White cells ($\times 10^{-3}$/mm^3)			0.53	Platelets ($\times 10^{-3}$/mm^3)			0.23
<50	79 (50.6)	76 (49.0)		1–49	83 (54.2)	81 (54.0)	
50–199	59 (37.8)	66 (42.6)		50–149	41 (26.8)	50 (33.3)	
≥200	18 (11.5)	13 (8.4)		≥150	29 (19.0)	19 (12.7)	
Sex			0.61	CNS disease at diagnosis			0.69
Male	89 (57.1)	83 (53.5)		Yes	3 (1.9)	3 (2.0)	
Female	67 (42.9)	72 (46.5)		No	151 (98.1)	150 (98.0)	
Race			0.55	Morphology§			0.12
White	106 (67.9)	111 (71.6)		L1	121 (77.6)	105 (67.7)	
Black	7 (4.5)	9 (5.8)		Mixed L1/L2 or L2/L1	23 (14.7)	29 (18.7)	
Other	43 (27.6)	35 (22.6)		L2	12 (7.7)	21 (13.5)	
Down's syndrome			0.99	Immunophenotype¶			0.98
Yes	3 (1.9)	4 (2.6)		B-cell lineage	94 (87.0)	89 (88.1)	
No	153 (98.1)	151 (97.4)		T-cell lineage	14 (13.0)	12 (11.9)	
Liver‡			0.25	Karyotypic features‖			0.32
Normal	97 (62.2)	82 (52.9)		Number			
Moderately enlarged	56 (35.9)	70 (45.2)		Diploid (46)	18 (42.9)	11 (22.4)	
Markedly enlarged	3 (1.9)	3 (1.9)		Pseudodiploid (46)	3 (7.1)	4 (8.2)	
Spleen			0.49	Hypodiploid (<46)	12 (28.6)	19 (38.8)	
Normal	72 (46.2)	62 (40.0)		Hyperdiploid (47–50)	4 (9.5)	5 (10.2)	
Moderately enlarged	83 (53.2)	91 (58.7)		Hyperdiploid (>50)	5 (11.9)	10 (20.4)	
Markedly enlarged	1 (0.6)	2 (1.3)		Translocations			0.66**
Lymph nodes			0.17	t(4;11) present	1 (2.4)	2 (4.1)	
Normal	96 (61.5)	82 (52.9)		t(4;11) absent	41 (97.6)	47 (95.9)	
Moderately enlarged	56 (35.9)	71 (45.8)		t(1;19) present	3 (7.1)	1 (2.0)	
Markedly enlarged	4 (2.6)	2 (1.3)		t(1;19) absent	39 (92.9)	48 (98.0)	
Mediastinal mass			0.78	t(9;22) present	3 (7.1)	4 (8.2)	
Absent	150 (96.2)	149 (96.8)		t(9;22) absent	39 (92.9)	45 (91.8)	
Present	6 (3.8)	5 (3.2)					

*Because of rounding not all percentages total 100. Percentages were based on the number of patients for whom there were data on the various characteristics. CNS denotes central nervous system.

†The global chi-square test for homogeneity was used.

‡The degree of organomegaly was determined as described by Steinherz et al.[6]

§The French–American–British system of classification was used.

¶Data on the immunophenotype were available for a subgroup of 108 patients in the standard-therapy group and 101 patients in the augmented-therapy group.

‖Centrally reviewed and accepted cytogenetic data were available for a subgroup of 91 patients.

**The P value is for the overall comparison for the three translocations.

group (75.4±4.0 vs. 57.2±4.5 percent, P=0.009, adjusted for multiple evaluations of the data). At that time the median follow-up for patients with event-free survival was 31 months (range, 1 to 63). When we reanalyzed the data in December 1997 after an additional follow-up period of approximately 1.5 years, 5-year event-free survival remained significantly better in the augmented-therapy group than in the standard-therapy group (75.0±3.8 vs. 55.0±4.5 percent, P<0.001) (Fig. 1). The median follow-up for patients with event-free survival was 49 months (range, 2 to 82 months). The difference in event-free survival was maintained (P<0.001) when patients who received a bone marrow transplant were censored at the time of transplantation. Overall survival at five years was also better in the augmented-therapy group than in the standard-therapy group (78.4±3.7 vs. 66.7±4.2 percent, P=0.02).

There were 65 events in the standard-therapy group and 36 events in the augmented-therapy group (Table 3). Isolated marrow relapse was the main cause of treatment failure for both regimens, occurring in 43 patients in the standard-therapy group and 30 patients in the augmented-therapy group (P=0.004 by the log-rank test), whereas central nervous system relapses were more common among patients in the standard-therapy group (8 vs. 0, P=0.002 by the log-rank test). Seven patients in the standard-

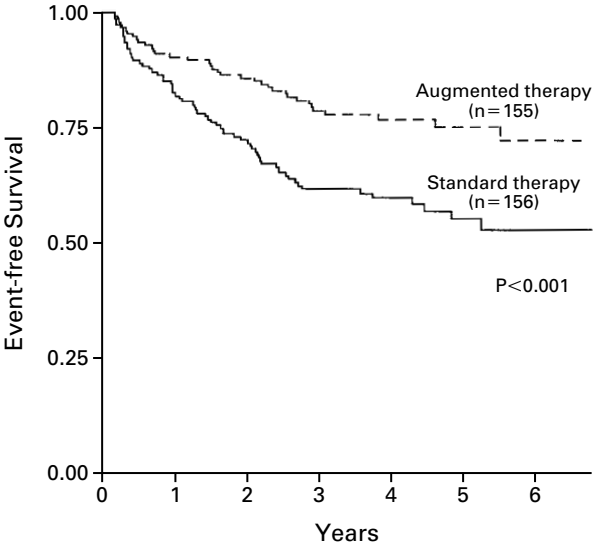
therapy group and four patients in the augmented-therapy group died while in remission.

In all subgroups analyzed, the results were better among patients who received augmented therapy than among those who received standard therapy. The difference in outcome between groups was most pronounced for patients who were one to nine years of age, all of whom had high white-cell counts as dictated by the eligibility criteria, with five-year event-free survival of 41.7±8.4 percent in the standard-therapy group and 84.6±5.0 percent in the augmented-therapy group ($P<0.001$) (Fig. 2A) and a relative risk of an adverse event in the standard-therapy group of 4.6. For patients who were 10 or more years old with white-cell counts of at least 50,000 per cubic millimeter, the outcome was better after augmented therapy than after standard therapy (three-year event-free survival, 66.7±9.7 vs. 47.9±9.7 percent) (Fig. 2B), with a relative risk of an adverse event of 1.7 in the standard-therapy group ($P=0.21$). Among patients who were 10 or more years old with white-cell counts below 50,000 per cubic millimeter, the five-year event-free survival rate was 73.3±5.7 percent in the augmented-therapy group and 66.2±5.8 percent in the standard-therapy group (relative risk of an adverse event, 1.26; $P=0.45$). Among 31 patients with white-cell counts of 200,000 per cubic millimeter or higher, event-free survival was better for those in the augmented-therapy group (relative risk of an adverse event in the standard-therapy group, 2.2; $P=0.14$).

Augmented therapy improved the outcome for patients with ALL of either B-cell lineage or T-cell lineage. Estimates of five-year event-free survival for patients with B-cell–lineage ALL were 74.7±5.1 percent with augmented therapy and 52.2±5.9 percent with standard therapy ($P=0.002$). For patients with T-cell–lineage ALL, event-free survival at three years was 91.7±8.0 percent in the augmented-therapy group and 71.4±12.1 percent in the standard-therapy group ($P=0.25$). Furthermore, the outcome for patients with ALL of T-cell lineage was similar to that for patients with ALL of B-cell lineage, regardless of regimen.

Prognostic Factors

An analysis of prognostic factors for the entire cohort of patients indicated that most base-line characteristics did not influence event-free survival. However, a white-cell count of 200,000 per cubic millimeter or higher, race other than black or white, and the presence of a t(9;22) translocation were prognostically important. For patients with white-cell counts of at least 200,000 per cubic millimeter, three-year event-free survival was 47.4±9.1 percent, as compared with 72.4±2.7 percent for those with white-cell counts below 200,000 per cubic millimeter ($P=0.004$). Patients who were neither black nor white had a significantly increased risk of an adverse



No. at Risk						
Augmented therapy	138	127	96	62	36	14
Standard therapy	127	110	77	53	27	9

Figure 1. Event-free Survival during Five Years of Follow-up in Patients with ALL, According to the Type of Post-Induction Chemotherapy.

Table 3. Frequency and Type of Events among Patients Assigned to Standard or Augmented Therapy.

Event	Standard Therapy (N=156)	Augmented Therapy (N=155)
	no. (%)	
Isolated marrow relapse	43 (27.6)	30 (19.4)
Central nervous system relapse	8 (5.1)	0
Marrow and central nervous system relapse	3 (1.9)	1 (0.6)
Testicular relapse	2 (1.3)	0
Marrow and testicular relapse	1 (0.6)	0
Relapse at other sites	1 (0.6)	0
Second cancer	0	1 (0.6)
Death in remission*	7 (4.5)	4 (2.6)
Total	65 (41.7)	36 (23.2)

*All but two deaths were related to the toxicity of treatment.

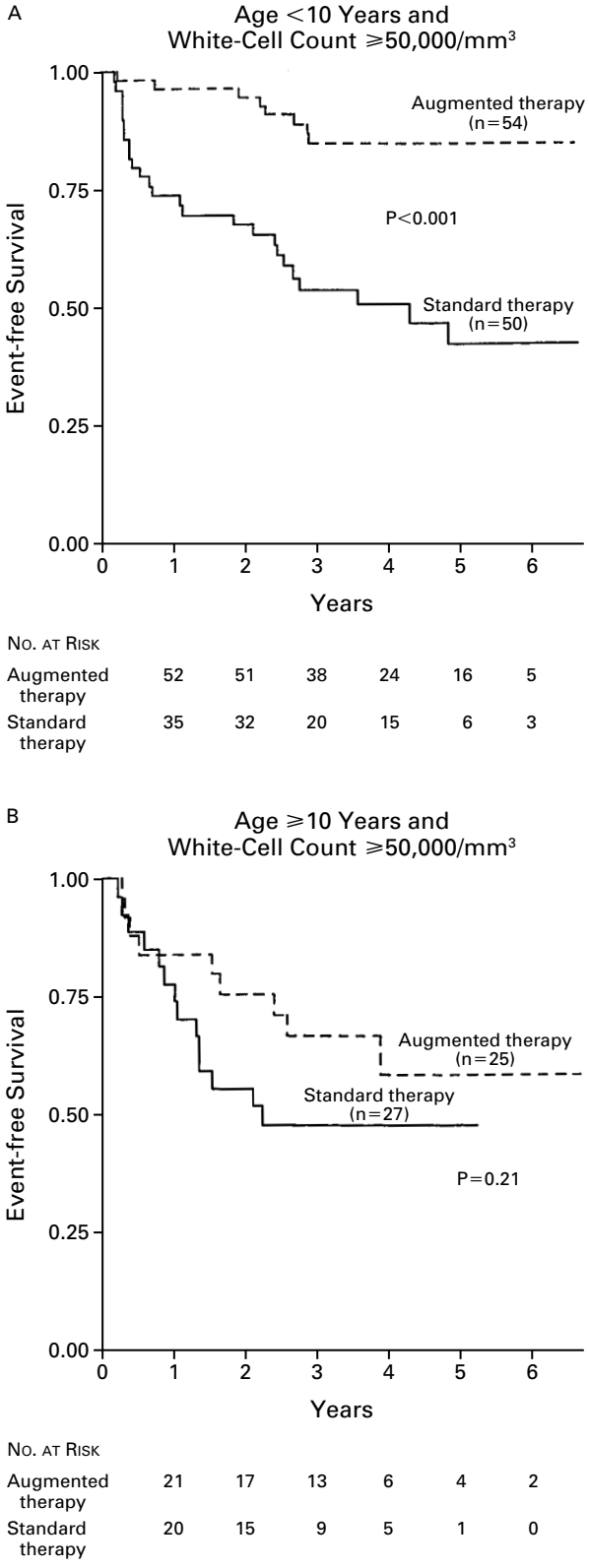


Figure 2. Event-free Survival during Five Years of Follow-up in Patients with ALL Who Received Standard Therapy or Augmented Therapy, According to Age and White-Cell Count at Diagnosis.

event, as compared with whites or blacks (five-year event-free survival, 51.2±6.0 percent vs. 69.4±3.4 percent; $P<0.001$). Patients with a t(9;22) translocation had a significantly increased risk of an adverse event, as compared with those without this translocation (three-year event-free survival, 28.6±17.1 percent vs. 73.6±4.8 percent; $P=0.007$).

Notably, of the seven patients with the Philadelphia chromosome, two of the three in the standard-therapy group and three of the four in the augmented-therapy group had events. Both patients with the Philadelphia chromosome who survived without an event (one in each group) received a bone marrow transplant while in first remission. A Cox regression analysis with adjustment for these and other common prognostic factors revealed no attenuation of the effect of treatment on the difference in outcome between the augmented-therapy and the standard-therapy groups ($P=0.001$).

Toxic Effects

The toxic effects of the two types of therapy are shown in Table 4. There was a higher frequency of allergic reactions to *Escherichia coli* asparaginase in the augmented-therapy group than in the standard-therapy group (64 vs. 4 reactions). The majority of the patients with allergic reactions (49 and 4, respectively) successfully continued asparaginase therapy after they were switched to erwinia asparaginase or polyethylene glycol asparaginase. Osteonecrosis developed in 20 patients in the augmented-therapy group and in 14 patients in the standard-therapy group; only 1 of these patients was under 10 years of age at the time of diagnosis. Life-table estimates for the occurrence of osteonecrosis at three years were 15.1 percent for the augmented-therapy group and 11.9 percent for the standard-therapy group ($P=0.44$). No cases had developed after three years of follow-up. The mean total duration of hospitalization was slightly longer for patients in the augmented-therapy group than in the standard-therapy group, primarily because of the additional time needed for the second cycles of interim maintenance and delayed intensification therapy (data not shown).

Three patients in the augmented-therapy group died in remission as a result of toxicity: one died of acute respiratory distress syndrome, one of pulmonary toxicity, and one of *Candida tropicalis* infection; one patient in remission was murdered. Seven patients in the standard-therapy group died in remission. Four of these deaths were due to documented infection: aspergillosis in one patient, clostridium septicemia in one, hepatosplenic candidiasis in one, and infection with an unspecified gram-negative bacteria in one. Of the remaining three deaths, one was due to pulmonary hemorrhage, one was due to acute respiratory distress syndrome after a presumed infection, and one was due to unknown causes.

TABLE 4. TOXIC EFFECTS OF STANDARD AND AUGMENTED THERAPY.

TOXIC EFFECT	STANDARD THERAPY (N=156)	AUGMENTED THERAPY (N=155)
	no. (%)	
Allergic reaction to asparaginase		
Escherichia coli asparaginase	4 (2.6)	64 (41.3)
Erwinia asparaginase	0	15 (9.7)*
Polyethylene glycol asparaginase	0	2 (1.3)*
Pancreatitis	2 (1.3)	5 (3.2)
Thrombotic events	0	4 (2.6)
Mucositis	0	38 (24.5)
Seizures	3 (1.9)	5 (3.2)
Leukoencephalopathy	1 (0.6)	2 (1.3)
Osteonecrosis	14 (9.0)	20 (12.9)
Stroke	1 (0.6)	0
Death†	6 (3.8)	3 (1.9)

*These allergic reactions occurred in patients after they had switched from *E. coli* asparaginase to erwinia asparaginase or polyethylene glycol asparaginase.

†The causes of death are given in the Results section.

DISCUSSION

We previously reported that among children with high-risk ALL, those with a rapid response to initial therapy (defined as the presence of no more than 25 percent blasts in the marrow on the seventh day of induction chemotherapy) had a better outcome than those with a slow response (more than 25 percent blasts).[24,26,27] Other investigators also reported poor outcomes for patients with a slow response to prednisone or multiagent induction therapy.[17,25,28,29] In this randomized trial of post-induction treatment of patients with a slow response, we found that the outcome with augmented treatment was superior to that with standard treatment (five-year event-free survival, 75 percent vs. 55 percent). In our nonrandomized pilot study of augmented therapy, the four-year event-free survival rate (±SD) was 70.8±4.6 percent.[33] Furthermore, subsequent analysis of the pilot study revealed a six-year event-free survival rate of 65.4±4.9 percent, suggesting that the results of the randomized trial are unlikely to change significantly with longer follow-up. Our results also suggest that the degree of cytoreduction achieved after one to two weeks of induction chemotherapy is a useful indicator of the susceptibility of leukemic cells to chemotherapeutic drugs.

Augmented treatment significantly improved event-free survival overall (75.0±3.8 percent, as compared with 55.0±4.5 percent in the standard-therapy group). In all subgroups analyzed, augmented therapy resulted in improved event-free survival. The difference was significant in the subgroup of patients who were one to nine years of age, all of whom had high white-cell counts. There was a trend toward a better outcome among older patients. There was also a trend toward improved outcomes with augmented therapy in patients with ALL of either B-cell lineage or T-cell lineage. This finding is in agreement with our analysis, which demonstrated improved outcome for the entire cohort of children with T-cell–lineage ALL who were treated with Children's Cancer Group protocols between 1989 and 1995.[34] Augmented therapy was ineffective for the seven patients with the Philadelphia chromosome. Five of these seven patients had events, and four of them ultimately died. The two patients who survived without events received a bone marrow transplant while in first remission. These data are consistent with recent data from European studies of children with ALL who have a poor response to initial prednisone therapy.[42]

The toxic effects of augmented therapy have been considerable, but they appear to be manageable. The most common long-term toxic effect was osteonecrosis, which occurred almost exclusively in adolescent patients.

We noted a significantly lower rate of central nervous system relapse in the augmented-therapy group than in the standard-therapy group. Since the patients assigned to each regimen received cranial radiotherapy and intrathecal therapy for presymptomatic treatment of the central nervous system, the benefit observed with augmented therapy may have been due to the use of intensified systemic therapy. Indeed, previous investigators have noted a similar effect with intensive systemic therapy.[43-45]

Although we do not know which components of augmented therapy were responsible for the improved outcome, we surmise that the effect is attributable to the increased dose intensities and prolonged duration of therapy. During the interim maintenance phase in the augmented-therapy regimen, repeated courses of vincristine, intravenous methotrexate, and asparaginase replaced the daily oral mercaptopurine and the weekly oral methotrexate used in the standard-therapy regimen. The augmented regimen also included an additional two weeks of nonmyelosuppressive therapy with vincristine and asparaginase during each consolidation or reconsolidation course and included both a second interim maintenance phase and a second course of delayed intensification.

A recent Children's Cancer Group study of intermediate-risk ALL showed that patients with a slow response had an improved outcome when treated with two courses of delayed intensification rather than one course,[45] suggesting that prolonged therapy was important to the improved outcome with augmented therapy in the current study. We are attempting to distinguish the relative contributions of

early increased dose intensity and a prolonged duration of therapy in a new therapeutic study of children with high-risk ALL.

Supported by grants from the National Institutes of Health (CA 13539, CA 02971, CA 17829, CA 05436, CA 10382, CA 20320, CA 03888, CA 02649, CA 03750, CA 03526, CA 36015, CA 26270, CA 26044, CA 07306, CA 11796, CA 42764, CA 13809, CA 10198, CA 29013, CA 26126, CA 14560, CA 27678, CA 29314, CA 28851, and CA 28882).

APPENDIX

The following institutions and principal investigators of the Children's Cancer Group participated in the study: Group Operations Center, Arcadia, Calif. — W. Bleyer, A. Khayat, H. Sather, M. Krailo, J. Buckley, D. Stram, R. Sposto; University of Michigan Medical Center, Ann Arbor — R. Hutchinson; University of California Medical Center, San Francisco — K. Matthay; University of Wisconsin Hospital, Madison — P. Gaynon; Children's Hospital and Medical Center, Seattle — R. Chard; Rainbow Babies and Children's Hospital, Cleveland — S. Shurin; Children's National Medical Center, Washington, D.C. — G. Reaman; Children's Hospital of Los Angeles, Los Angeles — J. Ortega; Children's Hospital of Columbus, Columbus, Ohio — F. Ruymann; Columbia Presbyterian College of Physicians and Surgeons, New York — S. Piomelli; Children's Hospital of Pittsburgh, Pittsburgh — J. Mirro; Vanderbilt University School of Medicine, Nashville — J. Lukens; Doernbecher Memorial Hospital for Children, Portland, Oreg. — L. Wolff; University of Minnesota Health Sciences Center, Minneapolis — W. Woods; Children's Hospital of Philadelphia, Philadelphia — A. Meadows; Memorial Sloan-Kettering Cancer Center, New York — P. Steinherz; James Whitcomb Riley Hospital for Children, Indianapolis — P. Breitfeld; University of Utah Medical Center, Salt Lake City — R. O'Brien; University of British Columbia, Vancouver — C. Fryer; Children's Hospital Medical Center, Cincinnati — R. Wells; Harbor–UCLA and Miller Children's Medical Center, Long Beach, Calif. — J. Finklestein; University of California Medical Center, Los Angeles — S. Feig; University of Iowa Hospitals and Clinics, Iowa City — R. Tannous; Children's Hospital of Denver, Denver — L. Odom; Mayo Clinic and Foundation, Rochester, Minn. — G. Gilchrist; Izaak Walton Killam Hospital for Children, Halifax, N.S. — D. Barnard; University of North Carolina, Chapel Hill — J. Wiley; University of Medicine and Dentistry of New Jersey, Camden — M. Donaldson; Children's Mercy Hospital, Kansas City, Mo. — M. Hetherington; University of Nebraska Medical Center, Omaha — P. Coccia; Wyler Children's Hospital, Chicago — J. Nachman; M.D. Anderson Cancer Center, Houston — B. Raney; Princess Margaret Hospital, Perth, Western Australia — D. Baker; New York University Medical Center, New York — A. Rausen; and Children's Hospital of Orange County, Orange, Calif. — M. Cairo.

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AUGMENTED POST-INDUCTION THERAPY FOR CHILDREN WITH HIGH-RISK ACUTE LYMPHOBLASTIC LEUKEMIA AND A SLOW RESPONSE TO INITIAL THERAPY

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ABSTRACT

Background Children with high-risk acute lymphoblastic leukemia (ALL) who have a slow response to initial chemotherapy (more than 25 percent blasts in the bone marrow on day 7) have a poor outcome despite intensive therapy. We conducted a randomized trial in which such patients were treated with either an augmented intensive regimen of post-induction chemotherapy or a standard regimen of intensive post-induction chemotherapy.

Methods Between January 1991 and June 1995, 311 children with newly diagnosed ALL who were either 1 to 9 years of age with white-cell counts of at least 50,000 per cubic millimeter or 10 years of age or older, had a slow response to initial therapy, and entered remission at the end of induction chemotherapy were randomly assigned to receive standard therapy (156 children) or augmented therapy (155). Those with lymphomatous features were excluded. Event-free survival and overall survival were assessed from the end of induction treatment.

Results The outcome at five years was significantly better in the augmented-therapy group than in the standard-therapy group (Kaplan–Meier estimate of event-free survival [±SD]: 75.0±3.8 vs. 55.0±4.5 percent, $P<0.001$; overall survival: 78.4±3.7 vs. 66.7±4.2 percent, $P=0.02$). The difference between treatments was most pronounced among patients one to nine years of age, all of whom had white-cell counts of at least 50,000 per cubic millimeter ($P<0.001$). Risk factors for an adverse event in the entire cohort included a white-cell count of 200,000 per cubic millimeter or higher ($P=0.004$), race other than black or white ($P<0.001$), and the presence of a t(9;22) translocation ($P=0.007$). The toxic effects of augmented therapy were considerable but manageable.

Conclusions Augmented post-induction chemotherapy results in an excellent outcome for most patients with high-risk ALL and a slow response to initial therapy. (N Engl J Med 1998;338:1663-71.)

IN children with acute lymphoblastic leukemia (ALL) who are older than one year of age, certain presenting features, such as a white-cell count above 50,000 per cubic millimeter,[1-3] an age of 10 years or older,[4,5] the presence of bulky disease,[1,3,6] T-cell–lineage immunophenotype,[7-9] and various chromosomal translocations,[10-16] carry an increased risk of treatment failure. The outcome for most of these children has improved with the use of intensive chemotherapy after the induction of remission,[17-22] but approximately 30 percent of such high-risk patients eventually relapse.

Numerous studies have demonstrated that a rapid response to initial chemotherapy is an important prognostic factor in childhood ALL.[17,18,23-28] German investigators observed that patients with fewer than 1000 blasts per cubic millimeter in the peripheral blood after a seven-day course of prednisone had significantly better event-free survival than patients with 1000 or more blasts per cubic millimeter.[17,28,29] Similarly, we reported that children with 25 percent blasts or fewer in the bone marrow on day 7 had a better response to initial chemotherapy (three-year event-free survival, 77 percent) than those with more than 25 percent blasts (three-year event-free survival, 48 percent).[26] In an attempt to improve the outcome for children with a slow response to initial therapy, we developed a strategy of augmented, intensive post-induction chemotherapy that was based on previous successful regimens for ALL.[30-32] This approach appeared promising in a nonrandomized pilot study.[33] We now report on a randomized comparison of augmented therapy with standard intensive post-induction therapy in children with high-risk ALL who entered remission after a slow response to initial therapy.

METHODS

Patients

Children and adolescents with newly diagnosed ALL who were 1 to 9 years of age and had white-cell counts of at least 50,000 per cubic millimeter or who were 10 years of age or older were enrolled between January 1991 and June 1995. Those with lymphomatous features[6] were excluded. Diagnosis was based on morphologic, biochemical, and immunologic features of leukemic

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cells, including lymphoblast morphology as determined by Wright–Giemsa staining, negative staining for myeloperoxidase, and reactivity with monoclonal antibodies to lymphoid differentiation antigens associated with B-cell or T-cell lineage, as described previously.[34] Patients with slow initial responses (>25 percent marrow blasts on day 7) who had entered remission by day 28 were randomly assigned at the end of induction therapy to receive standard or augmented therapy.

Treatment Protocol

All patients received identical five-week courses of induction chemotherapy, as previously described.[33] The post-induction regimens are given in Table 1. During the first year of post-induction therapy, the augmented regimen included more vincristine, asparaginase, methotrexate, and dexamethasone than the standard regimen, although the standard regimen included more oral methotrexate, prednisone, and mercaptopurine. Therapy was continued for two years for girls and for three years for boys, beginning with the first interim maintenance period (Table 1)[35] (and unpublished data). Presymptomatic central nervous system therapy consisted of intrathecal methotrexate and cranial radiation. This protocol was approved by the National Cancer Institute and the institutional review boards of the participating institutions. Informed consent was obtained from the patients, their parents, or both, as deemed appropriate, according to Department of Health and Human Services guidelines.

Study Design and Statistical Analysis

Balanced block randomization was used to ensure that approximately equal numbers of patients were randomly assigned to each regimen. The study was monitored by an independent data-monitoring committee and followed a monitoring plan that was based on group sequential monitoring boundaries[36] that required analysis of results at six-month intervals for a maximum of 10 analyses. With a target enrollment of 296 randomized patients, we estimated that the study had a power of approximately 81 percent at the final analysis to detect a change in five-year event-free survival from 45 percent to 62 percent or more with a two-sided log-rank test (alpha level, 0.05). The monitoring boundary was crossed in July 1996 (the ninth planned data analysis), and at that time study results were released.

This analysis was performed in December 1997. Similarities between patients in the two groups were assessed with chi-square tests for homogeneity of proportions. Outcome analyses used life-table methods and associated statistics. The primary end point examined was event-free survival from the time of randomization. The events considered were relapse at any site, death during remission, or a second malignant neoplasm, whichever occurred first. Data on patients who had not had an event at the time of the analysis were censored in the analysis of event-free survival at the time of the last contact with them. Life-table estimates were calculated by the Kaplan–Meier procedure, and the standard deviation of the life-table estimate was obtained with Greenwood's formula.[37] The Kaplan–Meier estimates (±SD) are presented for either the first five years or the first three years after randomization, depending on the number of patients in the follow-up. Ninety-five percent confidence intervals can be approximated as the life-table estimates ±1.96 SD. The log-rank statistic was used to compare patterns of event-free survival and overall survival in the groups.[38,39] Comparisons of randomized treatment regimens were performed according to the intention-to-treat method. Stratified log-rank tests were also used to adjust for the possible modifying effect of other factors on the comparison of interest.[40] An adjusted Cox regression analysis was used to determine the influence of prognostic factors on the primary treatment effect. Life-table analyses of the effect of isolated central nervous system and marrow relapses on the results with each regimen were compared with the log-rank statistic. Life-table analysis of the relative risk of an adverse event was calculated with the log-rank ratio of observed events to expected events.[41]

RESULTS

Patients

A total of 1136 patients were enrolled. Three patients died before day 7, and marrow was not obtained on day 7 from 15 patients. Of the remaining 1118 patients, 360 (32 percent) had slow responses to initial therapy. Of these, 340 (94 percent) entered remission after induction therapy, 19 did not enter remission after induction therapy, and 1 received modified induction therapy and therefore was deemed ineligible. Of the 340 eligible patients, 317 (93 percent) underwent randomization. A subsequent review revealed that 6 of these patients did not have a slow response; thus, 311 patients were eligible for the study. Of these, 156 were assigned to standard therapy and 155 were assigned to augmented therapy.

The characteristics of the patients in the two groups are shown in Table 2. There were no significant differences between the groups. Most patients were at least 10 years of age, and approximately half had white-cell counts of at least 50,000 per cubic millimeter. Centrally reviewed cytogenetic data on translocations associated with a high risk of an adverse event were available for 91 of the patients: 3 patients had the t(4;11) translocation, 4 had t(1;19), and 7 had t(9;22). Among 209 patients with immunophenotypic data, 87.6 percent had ALL of B-cell lineage.

Study Violations

Thirteen patients (seven in the standard-therapy group and six in the augmented-therapy group) received a bone marrow transplant during their first remission but were included in the intention-to-treat analysis. Indications for transplantation included the presence of a t(9;22) translocation (four patients), a white-cell count of more than 200,000 per cubic millimeter (three patients), virus-associated hemophagocytic syndrome (one patient), the presence of myeloid antigen (two patients), and other reasons (three patients). Two patients in the standard-therapy group and one patient in the augmented-therapy group refused cranial radiotherapy. Five patients assigned to augmented therapy did not receive the second cycle of delayed intensification therapy. Major changes in treatment were required for three patients assigned to standard therapy (two patients had fungal infections, and one had an elevation in aminotransferases) and five patients assigned to augmented therapy (three patients had elevations in aminotransferases, one had leukoencephalopathy, and one was not compliant with oral therapy).

Outcome of Treatment

At the time the study data were released in July 1996, the four-year event-free survival rate was significantly better among patients in the augmented-therapy group than among those in the standard-therapy

TABLE 1. THE STANDARD-THERAPY AND AUGMENTED-THERAPY REGIMENS.*

STANDARD THERAPY			AUGMENTED THERAPY		
PHASE	TREATMENT	DOSE	PHASE	TREATMENT	DOSE
Consolidation (5 wk)	Prednisone	7.5 mg/m² day 0; 3.75 mg/m²/day days 1, 2	**Consolidation (9 wk)**	Cyclophosphamide	1000 mg/m²/day IV days 0, 28
	Cyclophosphamide	1000 mg/m²/day IV days 0, 14		Cytarabine	75 mg/m²/day SQ or IV days 1–4, 8–11, 29–32, 36–39
	Mercaptopurine	60 mg/m²/day PO days 0–27		Mercaptopurine	60 mg/m²/day PO days 0–13, 28–41
	Vincristine	1.5 mg/m²/day IV days 14, 21, 42, 49		Vincristine	1.5 mg/m²/day IV days 14, 21, 42, 49
	Cytarabine	75 mg/m²/day IV days 1–4, 8–11, 15–18, 22–25		Asparaginase	6000 U/m²/day IM days 14, 16, 18, 21, 23, 25, 42, 44, 46, 49, 51, 53
	Methotrexate†	IT days 1, 8, 15, 22		Methotrexate†	IT days 1, 8, 15, 22
	Radiotherapy‡	Cranial, 1800 cGy		Radiotherapy‡	Cranial, 1800 cGy
		Cranial, 2400 cGy, and spinal, 600 cGy			Cranial, 2400 cGy, and spinal, 600 cGy
					Testicular, 2400 cGy
Interim maintenance (8 wk)	Mercaptopurine	60 mg/m²/day PO days 0–41	**Interim maintenance I (8 wk)**	Vincristine	1.5 mg/m²/day IV days 0, 10, 20, 30, 40
	Methotrexate	15 mg/m²/day PO days 0, 7, 14, 21, 28, 35		Methotrexate	100 mg/m²/day IV days 0, 10, 20, 30, 40 (escalate by 50 mg/m²/dose)
				Asparaginase	15,000 U/m²/day IM days 1, 11, 21, 31, 41
Delayed intensification (7 wk)			**Delayed intensification I (8 wk)**		
Reinduction (4 wk)	Dexamethasone	10 mg/m²/day PO days 0–20, then taper for 7 days	Reinduction (4 wk)	Dexamethasone	10 mg/m²/day PO days 0–20, then taper for 7 days
	Vincristine	1.5 mg/m²/day IV days 0, 14, 21		Vincristine	1.5 mg/m²/day IV days 0, 14, 21
	Doxorubicin	25 mg/m²/day IV days 0, 7, 14		Doxorubicin	25 mg/m²/day IV days 0, 7, 14
	Asparaginase	6000 U/m²/day IM days 3, 5, 7, 10, 12, 14		Asparaginase	6000 U/m²/day IM days 3, 5, 7, 10, 12, 14
Reconsolidation (3 wk)	Vincristine	1.5 mg/m²/day IV days 42, 49	Reconsolidation (4 wk)	Vincristine	1.5 mg/m²/day IV days 42, 49
	Cyclophosphamide	1000 mg/m² IV day 28		Cyclophosphamide	1000 mg/m² IV day 28
	Thioguanine	60 mg/m²/day PO days 28–41		Thioguanine	60 mg/m²/day PO days 28–41
	Cytarabine	75 mg/m²/day SQ or IV days 29–32, 36–39		Cytarabine	75 mg/m²/day SQ or IV days 29–32, 36–39
	Methotrexate†	IT days 29, 36		Methotrexate‡	IT days 29, 36
				Asparaginase	6000 U/m²/day IM days 42, 44, 46, 49, 51, 53
Maintenance (12 wk)§	Vincristine	1.5 mg/m²/day IV days 0, 28, 56	**Interim maintenance II (8 wk)**	Vincristine	1.5 mg/m²/day IV days 0, 10, 20, 30, 40
	Prednisone	40 mg/m²/day PO days 0–4, 28–32, 56–60		Methotrexate	100 mg/m²/day IV days 0, 10, 20, 30, 40 (escalate by 50 mg/m²/dose)
	Mercaptopurine	75 mg/m²/day PO days 0–83		Asparaginase	15,000 U/m²/day IM days 1, 11, 21, 31, 41
	Methotrexate	20 mg/m²/day PO days 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77		Methotrexate†	IT days 0, 20, 40
	Methotrexate†	IT day 0	**Delayed intensification II (8 wk)**	Same as for delayed intensification I	
			Maintenance (12 wk)§	Vincristine	1.5 mg/m²/day IV days 0, 28, 56
				Prednisone	60 mg/m²/day PO days 0–4, 28–32, 56–60
				Mercaptopurine	75 mg/m²/day PO days 0–83
				Methotrexate	20 mg/m²/day PO days 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77
				Methotrexate†	IT day 0

*IV denotes intravenously, PO orally, IT intrathecally, SQ subcutaneously, and IM intramuscularly.

†The doses were age-adjusted as follows: age 1 to 1.9 years, 8 mg; age 2 to 2.9 years, 10 mg; age ≥3 years, 12 mg. Patients with central nervous system disease at diagnosis did not receive intrathecal methotrexate on days 15 and 22 of consolidation therapy.

‡During the first two weeks of consolidation therapy, patients without central nervous system disease at diagnosis received 1800 cGy of cranial radiotherapy in 10 fractions; patients with central nervous system disease at diagnosis received 2400 cGy to the cranial midplane in 12 fractions and 600 cGy to the spinal cord in 3 fractions. In the augmented-therapy group, patients with testiculomegaly at diagnosis received 2400 cGy bilateral testicular radiation in 8 fractions.

§The cycles of maintenance therapy were repeated until the total duration of therapy, beginning with the first interim maintenance period, reached two years for girls and three years for boys.

TABLE 2. CHARACTERISTICS OF THE PATIENTS AT DIAGNOSIS.

CHARACTERISTIC*	STANDARD THERAPY (N=156)	AUGMENTED THERAPY (N=155)	P VALUE†	CHARACTERISTIC*	STANDARD THERAPY (N=156)	AUGMENTED THERAPY (N=155)	P VALUE†
	no. (%)				no. (%)		
Age (yr)			0.85	Hemoglobin (g/dl)			0.94
1–9	50 (32.1)	54 (34.8)		1–7.9	82 (52.9)	78 (52.7)	
10–15	73 (46.8)	68 (43.9)		8.0–10.9	52 (33.5)	48 (32.4)	
≥16	33 (21.2)	33 (21.3)		≥11.0	21 (13.5)	22 (14.9)	
White cells ($\times 10^{-3}$/mm^3)			0.53	Platelets ($\times 10^{-3}$/mm^3)			0.23
<50	79 (50.6)	76 (49.0)		1–49	83 (54.2)	81 (54.0)	
50–199	59 (37.8)	66 (42.6)		50–149	41 (26.8)	50 (33.3)	
≥200	18 (11.5)	13 (8.4)		≥150	29 (19.0)	19 (12.7)	
Sex			0.61	CNS disease at diagnosis			0.69
Male	89 (57.1)	83 (53.5)		Yes	3 (1.9)	3 (2.0)	
Female	67 (42.9)	72 (46.5)		No	151 (98.1)	150 (98.0)	
Race			0.55	Morphology§			0.12
White	106 (67.9)	111 (71.6)		L1	121 (77.6)	105 (67.7)	
Black	7 (4.5)	9 (5.8)		Mixed L1/L2 or L2/L1	23 (14.7)	29 (18.7)	
Other	43 (27.6)	35 (22.6)		L2	12 (7.7)	21 (13.5)	
Down's syndrome			0.99	Immunophenotype¶			0.98
Yes	3 (1.9)	4 (2.6)		B-cell lineage	94 (87.0)	89 (88.1)	
No	153 (98.1)	151 (97.4)		T-cell lineage	14 (13.0)	12 (11.9)	
Liver‡			0.25	Karyotypic features‖			0.32
Normal	97 (62.2)	82 (52.9)		Number			
Moderately enlarged	56 (35.9)	70 (45.2)		Diploid (46)	18 (42.9)	11 (22.4)	
Markedly enlarged	3 (1.9)	3 (1.9)		Pseudodiploid (46)	3 (7.1)	4 (8.2)	
Spleen			0.49	Hypodiploid (<46)	12 (28.6)	19 (38.8)	
Normal	72 (46.2)	62 (40.0)		Hyperdiploid (47–50)	4 (9.5)	5 (10.2)	
Moderately enlarged	83 (53.2)	91 (58.7)		Hyperdiploid (>50)	5 (11.9)	10 (20.4)	
Markedly enlarged	1 (0.6)	2 (1.3)		Translocations			0.66**
Lymph nodes			0.17	t(4;11) present	1 (2.4)	2 (4.1)	
Normal	96 (61.5)	82 (52.9)		t(4;11) absent	41 (97.6)	47 (95.9)	
Moderately enlarged	56 (35.9)	71 (45.8)		t(1;19) present	3 (7.1)	1 (2.0)	
Markedly enlarged	4 (2.6)	2 (1.3)		t(1;19) absent	39 (92.9)	48 (98.0)	
Mediastinal mass			0.78	t(9;22) present	3 (7.1)	4 (8.2)	
Absent	150 (96.2)	149 (96.8)		t(9;22) absent	39 (92.9)	45 (91.8)	
Present	6 (3.8)	5 (3.2)					

*Because of rounding not all percentages total 100. Percentages were based on the number of patients for whom there were data on the various characteristics. CNS denotes central nervous system.

†The global chi-square test for homogeneity was used.

‡The degree of organomegaly was determined as described by Steinherz et al.[6]

§The French–American–British system of classification was used.

¶Data on the immunophenotype were available for a subgroup of 108 patients in the standard-therapy group and 101 patients in the augmented-therapy group.

‖Centrally reviewed and accepted cytogenetic data were available for a subgroup of 91 patients.

**The P value is for the overall comparison for the three translocations.

group (75.4±4.0 vs. 57.2±4.5 percent, P=0.009, adjusted for multiple evaluations of the data). At that time the median follow-up for patients with event-free survival was 31 months (range, 1 to 63). When we reanalyzed the data in December 1997 after an additional follow-up period of approximately 1.5 years, 5-year event-free survival remained significantly better in the augmented-therapy group than in the standard-therapy group (75.0±3.8 vs. 55.0±4.5 percent, P<0.001) (Fig. 1). The median follow-up for patients with event-free survival was 49 months (range, 2 to 82 months). The difference in event-free survival was maintained (P<0.001) when patients who received a bone marrow transplant were censored at the time of transplantation. Overall survival at five years was also better in the augmented-therapy group than in the standard-therapy group (78.4±3.7 vs. 66.7±4.2 percent, P=0.02).

There were 65 events in the standard-therapy group and 36 events in the augmented-therapy group (Table 3). Isolated marrow relapse was the main cause of treatment failure for both regimens, occurring in 43 patients in the standard-therapy group and 30 patients in the augmented-therapy group (P=0.004 by the log-rank test), whereas central nervous system relapses were more common among patients in the standard-therapy group (8 vs. 0, P=0.002 by the log-rank test). Seven patients in the standard-

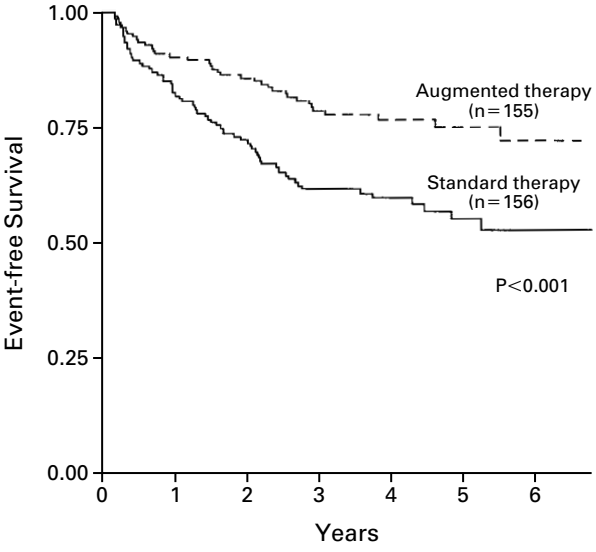
therapy group and four patients in the augmented-therapy group died while in remission.

In all subgroups analyzed, the results were better among patients who received augmented therapy than among those who received standard therapy. The difference in outcome between groups was most pronounced for patients who were one to nine years of age, all of whom had high white-cell counts as dictated by the eligibility criteria, with five-year event-free survival of 41.7±8.4 percent in the standard-therapy group and 84.6±5.0 percent in the augmented-therapy group ($P<0.001$) (Fig. 2A) and a relative risk of an adverse event in the standard-therapy group of 4.6. For patients who were 10 or more years old with white-cell counts of at least 50,000 per cubic millimeter, the outcome was better after augmented therapy than after standard therapy (three-year event-free survival, 66.7±9.7 vs. 47.9±9.7 percent) (Fig. 2B), with a relative risk of an adverse event of 1.7 in the standard-therapy group ($P=0.21$). Among patients who were 10 or more years old with white-cell counts below 50,000 per cubic millimeter, the five-year event-free survival rate was 73.3±5.7 percent in the augmented-therapy group and 66.2±5.8 percent in the standard-therapy group (relative risk of an adverse event, 1.26; $P=0.45$). Among 31 patients with white-cell counts of 200,000 per cubic millimeter or higher, event-free survival was better for those in the augmented-therapy group (relative risk of an adverse event in the standard-therapy group, 2.2; $P=0.14$).

Augmented therapy improved the outcome for patients with ALL of either B-cell lineage or T-cell lineage. Estimates of five-year event-free survival for patients with B-cell–lineage ALL were 74.7±5.1 percent with augmented therapy and 52.2±5.9 percent with standard therapy ($P=0.002$). For patients with T-cell–lineage ALL, event-free survival at three years was 91.7±8.0 percent in the augmented-therapy group and 71.4±12.1 percent in the standard-therapy group ($P=0.25$). Furthermore, the outcome for patients with ALL of T-cell lineage was similar to that for patients with ALL of B-cell lineage, regardless of regimen.

Prognostic Factors

An analysis of prognostic factors for the entire cohort of patients indicated that most base-line characteristics did not influence event-free survival. However, a white-cell count of 200,000 per cubic millimeter or higher, race other than black or white, and the presence of a t(9;22) translocation were prognostically important. For patients with white-cell counts of at least 200,000 per cubic millimeter, three-year event-free survival was 47.4±9.1 percent, as compared with 72.4±2.7 percent for those with white-cell counts below 200,000 per cubic millimeter ($P=0.004$). Patients who were neither black nor white had a significantly increased risk of an adverse



No. at Risk						
Augmented therapy	138	127	96	62	36	14
Standard therapy	127	110	77	53	27	9

Figure 1. Event-free Survival during Five Years of Follow-up in Patients with ALL, According to the Type of Post-Induction Chemotherapy.

Table 3. Frequency and Type of Events among Patients Assigned to Standard or Augmented Therapy.

Event	Standard Therapy (N=156)	Augmented Therapy (N=155)
	no. (%)	
Isolated marrow relapse	43 (27.6)	30 (19.4)
Central nervous system relapse	8 (5.1)	0
Marrow and central nervous system relapse	3 (1.9)	1 (0.6)
Testicular relapse	2 (1.3)	0
Marrow and testicular relapse	1 (0.6)	0
Relapse at other sites	1 (0.6)	0
Second cancer	0	1 (0.6)
Death in remission*	7 (4.5)	4 (2.6)
Total	65 (41.7)	36 (23.2)

*All but two deaths were related to the toxicity of treatment.

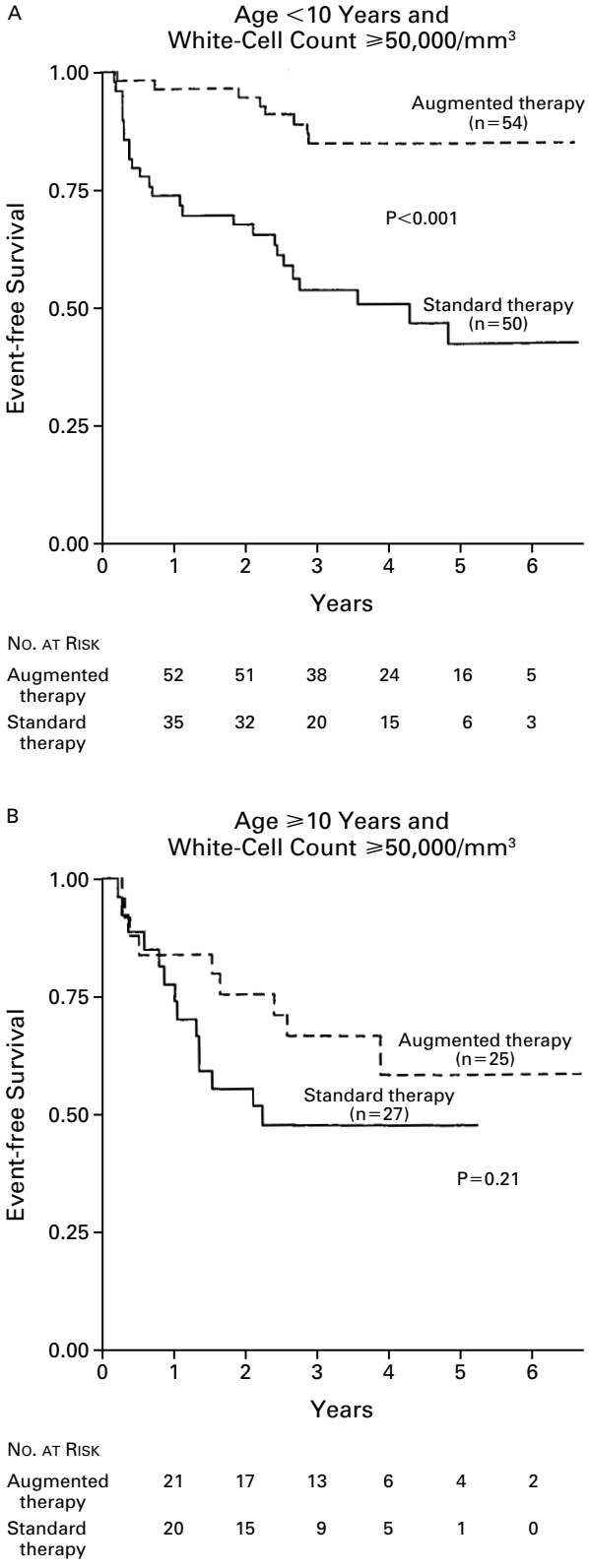


Figure 2. Event-free Survival during Five Years of Follow-up in Patients with ALL Who Received Standard Therapy or Augmented Therapy, According to Age and White-Cell Count at Diagnosis.

event, as compared with whites or blacks (five-year event-free survival, 51.2±6.0 percent vs. 69.4±3.4 percent; $P<0.001$). Patients with a t(9;22) translocation had a significantly increased risk of an adverse event, as compared with those without this translocation (three-year event-free survival, 28.6±17.1 percent vs. 73.6±4.8 percent; $P=0.007$).

Notably, of the seven patients with the Philadelphia chromosome, two of the three in the standard-therapy group and three of the four in the augmented-therapy group had events. Both patients with the Philadelphia chromosome who survived without an event (one in each group) received a bone marrow transplant while in first remission. A Cox regression analysis with adjustment for these and other common prognostic factors revealed no attenuation of the effect of treatment on the difference in outcome between the augmented-therapy and the standard-therapy groups ($P=0.001$).

Toxic Effects

The toxic effects of the two types of therapy are shown in Table 4. There was a higher frequency of allergic reactions to *Escherichia coli* asparaginase in the augmented-therapy group than in the standard-therapy group (64 vs. 4 reactions). The majority of the patients with allergic reactions (49 and 4, respectively) successfully continued asparaginase therapy after they were switched to erwinia asparaginase or polyethylene glycol asparaginase. Osteonecrosis developed in 20 patients in the augmented-therapy group and in 14 patients in the standard-therapy group; only 1 of these patients was under 10 years of age at the time of diagnosis. Life-table estimates for the occurrence of osteonecrosis at three years were 15.1 percent for the augmented-therapy group and 11.9 percent for the standard-therapy group ($P=0.44$). No cases had developed after three years of follow-up. The mean total duration of hospitalization was slightly longer for patients in the augmented-therapy group than in the standard-therapy group, primarily because of the additional time needed for the second cycles of interim maintenance and delayed intensification therapy (data not shown).

Three patients in the augmented-therapy group died in remission as a result of toxicity: one died of acute respiratory distress syndrome, one of pulmonary toxicity, and one of *Candida tropicalis* infection; one patient in remission was murdered. Seven patients in the standard-therapy group died in remission. Four of these deaths were due to documented infection: aspergillosis in one patient, clostridium septicemia in one, hepatosplenic candidiasis in one, and infection with an unspecified gram-negative bacteria in one. Of the remaining three deaths, one was due to pulmonary hemorrhage, one was due to acute respiratory distress syndrome after a presumed infection, and one was due to unknown causes.

TABLE 4. TOXIC EFFECTS OF STANDARD AND AUGMENTED THERAPY.

TOXIC EFFECT	STANDARD THERAPY (N=156)	AUGMENTED THERAPY (N=155)
	no. (%)	
Allergic reaction to asparaginase		
Escherichia coli asparaginase	4 (2.6)	64 (41.3)
Erwinia asparaginase	0	15 (9.7)*
Polyethylene glycol asparaginase	0	2 (1.3)*
Pancreatitis	2 (1.3)	5 (3.2)
Thrombotic events	0	4 (2.6)
Mucositis	0	38 (24.5)
Seizures	3 (1.9)	5 (3.2)
Leukoencephalopathy	1 (0.6)	2 (1.3)
Osteonecrosis	14 (9.0)	20 (12.9)
Stroke	1 (0.6)	0
Death†	6 (3.8)	3 (1.9)

*These allergic reactions occurred in patients after they had switched from *E. coli* asparaginase to erwinia asparaginase or polyethylene glycol asparaginase.

†The causes of death are given in the Results section.

DISCUSSION

We previously reported that among children with high-risk ALL, those with a rapid response to initial therapy (defined as the presence of no more than 25 percent blasts in the marrow on the seventh day of induction chemotherapy) had a better outcome than those with a slow response (more than 25 percent blasts).[24,26,27] Other investigators also reported poor outcomes for patients with a slow response to prednisone or multiagent induction therapy.[17,25,28,29] In this randomized trial of post-induction treatment of patients with a slow response, we found that the outcome with augmented treatment was superior to that with standard treatment (five-year event-free survival, 75 percent vs. 55 percent). In our nonrandomized pilot study of augmented therapy, the four-year event-free survival rate (±SD) was 70.8±4.6 percent.[33] Furthermore, subsequent analysis of the pilot study revealed a six-year event-free survival rate of 65.4±4.9 percent, suggesting that the results of the randomized trial are unlikely to change significantly with longer follow-up. Our results also suggest that the degree of cytoreduction achieved after one to two weeks of induction chemotherapy is a useful indicator of the susceptibility of leukemic cells to chemotherapeutic drugs.

Augmented treatment significantly improved event-free survival overall (75.0±3.8 percent, as compared with 55.0±4.5 percent in the standard-therapy group). In all subgroups analyzed, augmented therapy resulted in improved event-free survival. The difference was significant in the subgroup of patients who were one to nine years of age, all of whom had high white-cell counts. There was a trend toward a better outcome among older patients. There was also a trend toward improved outcomes with augmented therapy in patients with ALL of either B-cell lineage or T-cell lineage. This finding is in agreement with our analysis, which demonstrated improved outcome for the entire cohort of children with T-cell–lineage ALL who were treated with Children's Cancer Group protocols between 1989 and 1995.[34] Augmented therapy was ineffective for the seven patients with the Philadelphia chromosome. Five of these seven patients had events, and four of them ultimately died. The two patients who survived without events received a bone marrow transplant while in first remission. These data are consistent with recent data from European studies of children with ALL who have a poor response to initial prednisone therapy.[42]

The toxic effects of augmented therapy have been considerable, but they appear to be manageable. The most common long-term toxic effect was osteonecrosis, which occurred almost exclusively in adolescent patients.

We noted a significantly lower rate of central nervous system relapse in the augmented-therapy group than in the standard-therapy group. Since the patients assigned to each regimen received cranial radiotherapy and intrathecal therapy for presymptomatic treatment of the central nervous system, the benefit observed with augmented therapy may have been due to the use of intensified systemic therapy. Indeed, previous investigators have noted a similar effect with intensive systemic therapy.[43-45]

Although we do not know which components of augmented therapy were responsible for the improved outcome, we surmise that the effect is attributable to the increased dose intensities and prolonged duration of therapy. During the interim maintenance phase in the augmented-therapy regimen, repeated courses of vincristine, intravenous methotrexate, and asparaginase replaced the daily oral mercaptopurine and the weekly oral methotrexate used in the standard-therapy regimen. The augmented regimen also included an additional two weeks of nonmyelosuppressive therapy with vincristine and asparaginase during each consolidation or reconsolidation course and included both a second interim maintenance phase and a second course of delayed intensification.

A recent Children's Cancer Group study of intermediate-risk ALL showed that patients with a slow response had an improved outcome when treated with two courses of delayed intensification rather than one course,[45] suggesting that prolonged therapy was important to the improved outcome with augmented therapy in the current study. We are attempting to distinguish the relative contributions of

early increased dose intensity and a prolonged duration of therapy in a new therapeutic study of children with high-risk ALL.

Supported by grants from the National Institutes of Health (CA 13539, CA 02971, CA 17829, CA 05436, CA 10382, CA 20320, CA 03888, CA 02649, CA 03750, CA 03526, CA 36015, CA 26270, CA 26044, CA 07306, CA 11796, CA 42764, CA 13809, CA 10198, CA 29013, CA 26126, CA 14560, CA 27678, CA 29314, CA 28851, and CA 28882).

APPENDIX

The following institutions and principal investigators of the Children's Cancer Group participated in the study: Group Operations Center, Arcadia, Calif. — W. Bleyer, A. Khayat, H. Sather, M. Krailo, J. Buckley, D. Stram, R. Sposto; University of Michigan Medical Center, Ann Arbor — R. Hutchinson; University of California Medical Center, San Francisco — K. Matthay; University of Wisconsin Hospital, Madison — P. Gaynon; Children's Hospital and Medical Center, Seattle — R. Chard; Rainbow Babies and Children's Hospital, Cleveland — S. Shurin; Children's National Medical Center, Washington, D.C. — G. Reaman; Children's Hospital of Los Angeles, Los Angeles — J. Ortega; Children's Hospital of Columbus, Columbus, Ohio — F. Ruymann; Columbia Presbyterian College of Physicians and Surgeons, New York — S. Piomelli; Children's Hospital of Pittsburgh, Pittsburgh — J. Mirro; Vanderbilt University School of Medicine, Nashville — J. Lukens; Doernbecher Memorial Hospital for Children, Portland, Oreg. — L. Wolff; University of Minnesota Health Sciences Center, Minneapolis — W. Woods; Children's Hospital of Philadelphia, Philadelphia — A. Meadows; Memorial Sloan-Kettering Cancer Center, New York — P. Steinherz; James Whitcomb Riley Hospital for Children, Indianapolis — P. Breitfeld; University of Utah Medical Center, Salt Lake City — R. O'Brien; University of British Columbia, Vancouver — C. Fryer; Children's Hospital Medical Center, Cincinnati — R. Wells; Harbor–UCLA and Miller Children's Medical Center, Long Beach, Calif. — J. Finklestein; University of California Medical Center, Los Angeles — S. Feig; University of Iowa Hospitals and Clinics, Iowa City — R. Tannous; Children's Hospital of Denver, Denver — L. Odom; Mayo Clinic and Foundation, Rochester, Minn. — G. Gilchrist; Izaak Walton Killam Hospital for Children, Halifax, N.S. — D. Barnard; University of North Carolina, Chapel Hill — J. Wiley; University of Medicine and Dentistry of New Jersey, Camden — M. Donaldson; Children's Mercy Hospital, Kansas City, Mo. — M. Hetherington; University of Nebraska Medical Center, Omaha — P. Coccia; Wyler Children's Hospital, Chicago — J. Nachman; M.D. Anderson Cancer Center, Houston — B. Raney; Princess Margaret Hospital, Perth, Western Australia — D. Baker; New York University Medical Center, New York — A. Rausen; and Children's Hospital of Orange County, Orange, Calif. — M. Cairo.

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