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Plasma Methotrexate, Red Blood Cell Methotrexate and Red Blood Cell Folate Values and Outcome in Children with Precursor B Acute Lymphoblastic Leukemia: A Report from the Children's Oncology Group

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Abstract

Plasma steady state methotrexate (MTX) level and red blood cell (RBC) MTX and folate concentrations were evaluated in 1124 children with newly diagnosed acute lymphoblastic leukemia (ALL) enrolled on the Pediatric Oncology Group studies 9005 (lower risk; Regimens A and C) and 9006 (higher risk; Regimen A). These regimens included intermediate dose MTX (1 gram/m²) given as a 24 hour infusion every other week for 12 doses during intensification. Plasma MTX level was evaluated at the end of MTX infusions. RBC MTX and folate concentrations were measured at the end of intensification. The five year continuous complete remission (CCR) was 76 ± 1.4% versus 85 ± 3.0% for those patients with steady state MTX levels ≤ 14 μM, respectively (p=0.0125). Hispanic children had significantly reduced median steady state MTX levels, 8.7 μM, compared to non-Hispanic children, 9.95 μM (p=0.0015), but this did not correlate with a difference in outcome. Neither RBC MTX, RBC folate, nor the RBC MTX:folate ratio identified children at increased risk of failure.

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Keywords

Red Blood Cell Methotrexate and Folate; Childhood Acute Lymphoblastic Leukemia

INTRODUCTION

Methotrexate (MTX) is an important component of chemotherapy for children with acute lymphoblastic leukemia (ALL). As a single agent, MTX resulted in remission rates of 29–83%, depending on the dose and route utilized^{1–2}. Today, it is a component of all frontline therapies for children with ALL, given intrathecally, orally, intramuscularly, and/or intravenously. Despite its long history, however, the optimal route and dosage of MTX remain unknown.

After patients achieve remission, leukemic cell exposure to MTX cannot be measured. Therefore surrogate markers of exposure have been evaluated, including plasma MTX levels and red blood cell (RBC) MTX and folate concentrations^{3–9}. Plasma MTX levels correlated with outcome in some studies, but not in others^{10–13}, possibly because these levels do not reflect blast cell exposure/uptake or account for variation in duration of exposure or peak concentration. RBC MTX uptake occurs only during the erythroblast stage of development¹⁴, through the same mechanisms utilized by folate. MTX is then polyglutamated¹⁵ and retained throughout the life of the RBC. When MTX dosing is unchanged, steady state RBC MTX concentrations are reached after 6–12 weeks^{3–4}. Therefore, RBC MTX concentrations have been studied as a surrogate marker of overall drug exposure and of compliance, absorption and metabolism. The study reported here prospectively sought to determine whether RBC MTX and folate concentrations or steady state plasma MTX levels were related to treatment outcome.

MATERIALS AND METHODS

Patients

From January 1991 until September 1994, patients with newly diagnosed B-precursor ALL were enrolled on concurrent Pediatric Oncology Group (POG) studies 9005 and 9006 for patients with lower and higher risk of relapse, respectively. Patients were classified at diagnosis as having a lower or higher risk of relapse based on: (1) age, (2) white blood cell count (WBC), (3) DNA index (DNA content in leukemic cells/DNA content of normal cells), (4) presence of central nervous system (CNS) leukemia (blasts present on cytospin with WBC ≥ 5 cells/ μ L), and (5) presence of testicular disease (Table 1). Patients later found to have the Philadelphia chromosome [t(9;22)] or t(1;19) were classified as being at higher risk of relapse, regardless of other presenting features. Diagnosis was based on morphology and immunohistochemical staining. Lymphoblasts were Sudan black or esterase negative, lacked T-cell associated markers, and were of L₁ or L₂ FAB morphology or L₃ and lacked surface immunoglobulin. Patients who completed intensive continuation (excluding those with t(9;22) and t(4;11)) are included in this analysis. Studies were approved by the institutional review board at each participating center. Informed consent was obtained from patients and parents or legal guardians prior to study enrollment. The follow-up period was to September 1, 2005, when the study data were frozen.

Treatment

The treatment regimens for POG 9005 Regimens A and C and 9006 Regimen A have been previously reported^{16–17}. Each of these regimens included intermediate dose MTX (ID MTX) (1 gram/m²) as a 24 hour infusion every other week for 12 doses during the intensive

continuation of treatment. Forty-eight hours after the start of the methotrexate infusion, leucovorin 5 mg/m² IV/PO was administered every 6 hours × 5 doses or until the plasma methotrexate level was < 0.1 µM. The total duration of therapy was 2.5 years after completion of induction.

CNS negative patients received prophylaxis with 16 and 18 doses of age adjusted triple intrathecal therapy on POG 9005 and 9006, respectively. Patients with CNS leukemia were treated on POG 9006 and received 11 doses of age adjusted intrathecal therapy, followed by radiation therapy (2400 cGy cranial and 1500 cGy spinal) at the completion of intensive continuation. No additional intrathecal therapy was administered after craniospinal radiation. Patients with testicular disease received bilateral testicular radiation.

Initial marrow response was assessed on day 29 of induction. Complete response was defined as ≤ 5% blasts on bone marrow examination with hematologic recovery of peripheral counts and normal physical exam. If 6 – 25% blasts remained on the day 29 marrow, an additional two weeks of induction therapy with vincristine and prednisone was administered. Induction failure was defined as > 25% blasts on day 29 or > 5% blasts on day 43. Bone marrow relapse was defined as >25% marrow blasts. CNS relapse was defined as positive cytomorphology and ≥ 5 WBC/µl. Bilateral testicular biopsies were required to confirm testicular relapse.

Sample Collection and Processing

Steady state plasma MTX levels were obtained at the end of each 24 hour ID MTX infusion and were assayed at the local institutions. Samples for RBC MTX and folate were drawn at completion of intensive continuation (week 25). Intensive continuation for all regimens included administration of the final ID MTX on day 1 of week 23 with the last MTX administration prior to sample acquisition of 20 mg/m² intramuscularly on day 1 of week 24. Whole blood was collected into ethylenediaminetetraacetic acid (EDTA) containing tubes. Care was taken to ensure that the vacuum was maintained within the tube. Samples were shipped via regular mail at room temperature to Southwestern Medical Center, Dallas, Texas. The methods for RBC MTX and RBC folate assays have been previously published^{4, 18–20}.

Statistical Considerations

There were 1150 patients enrolled on POG 9005 Regimens A and C and POG 9006 Regimen A who lacked t(9;22) and t(4;11). The primary endpoint was continuous complete remission (CCR), and was computed as the time from the completion of intensive continuation to failure (relapse, secondary malignancy, or death) or last contact (non-failures). A Wilcoxon rank sum or Kruskal-Wallis test was used to evaluate the effects of NCI risk group, gender and race on RBC MTX, RBC folate, RBC MTX:folate ratio, median steady state MTX level (median of the 12 institutional post-infusion values). Univariate analyses were conducted to study the association of NCI risk group, DNA index, gender, median steady state MTX level, RBC MTX, RBC folate, and RBCMTX:folate with CCR. Survival curves were computed using the Kaplan-Meier method and the corresponding standard errors was computed using Greenwood's formula of Kalbfleisch and Prentice²¹. Results were given as estimate + standard error in this report. Multivariate analysis of the relative prognostic factors for CCR was performed using the Cox regression model²². Stepwise Cox regression was performed using a significance level of 0.05 for both entering and removing explanatory variables from the model.

RESULTS

Of the 1150 patients, 1124 patients completed intensive continuation (26 failures: 12 induction failures, 5 relapses, 5 induction deaths, 3 remission deaths, and 1 other). This report is based on these 1124 patients. Patient characteristics are presented in Table 2. Fifty three percent of patients were male and 71%, 9%, 13%, and 7% of patients self-identified as Caucasian, African-American, Hispanic, or other, respectively. Median age at diagnosis was 4.25 years (range 1.07–20.38). The demographic summary for the entire cohort of 1150 patients was similar to that for the 1124 patients included in this analysis.

Of the 1124 patients who completed intensive continuation, 286 patients experienced events (Table 3). Direct comparison of the RBC MTX, RBC folate, and RBC MTX:folate ratio with remission status (Figure 1 and Table 4), failed to show significant differences between the remission and event groups. The median steady state MTX levels were 9.93 and 9.45 μM for remission and event groups, respectively, $p = 0.0123$.

The effects of NCI risk group, gender, and race on RBC MTX, RBC folate, RBC MTX:folate ratio and median steady state MTX level are shown in Tables 5 and 6. Gender and NCI risk group did not result in a significant difference in values for any of the measured parameters. Race did not result in a significant difference in values for RBC MTX, RBC folate, or RBC MTX:folate ratio. Hispanic children had significantly lower median steady state plasma MTX levels (8.70 μM vs. 9.95 μM ; $p = 0.0015$), but similar outcomes with five year CCR rates of $72 \pm 3.8\%$, $79 \pm 1.4\%$, and $74 \pm 4.4\%$ for Hispanic, Caucasian, and African American children, respectively ($p=0.09$), Figure 2.

The univariate analyses of the association of NCI risk group, DNA index, gender, and median steady state MTX level, RBC MTX, RBC folate, and RBC MTX:folate with CCR revealed significant associations with NCI risk ($p < 0.0001$), DNA index ($p = 0.0025$), Gender ($p = 0.0031$), median steady state MTX level ($p = 0.0126$), and non-significant associations with RBC folate ($p = 0.7365$), RBC MTX ($p = 0.1284$) and RBC MTX:folate ratio ($p = 0.1512$). The receiver operator characteristic (ROC) curves²³ were utilized to discriminate the effect of MTX level on CCR. The optimal cutoff of MTX level was 14 μM and the univariate analyses of the association of the dichotomized median steady state MTX level with CCR revealed a p-value of 0.0051 at the cutoff of 14 μM . The five-year CCR was $76 \pm 1.4\%$ versus $85 \pm 3.0\%$ for those patients with median steady state MTX levels $\leq 14 \mu\text{M}$ and $> 14 \mu\text{M}$, respectively ($p=0.0125$), Figure 3. When analyzed by risk group, patients with lower risk of relapse had a trend towards improved outcome with a five-year CCR of $79 \pm 1.5\%$ versus $86 \pm 3.3\%$ for those with median steady state MTX levels $\leq 14 \mu\text{M}$ and $> 14 \mu\text{M}$, respectively, but this did not reach statistical significance ($p=0.1089$). However, patients with higher risk of relapse had a five-year CCR of $64 \pm 3.6\%$ versus $84 \pm 6.5\%$ for those with median steady state MTX levels $\leq 14 \mu\text{M}$ and $> 14 \mu\text{M}$, respectively ($p=0.0231$).

In the first Cox regression analysis, the relative prognostic importance of NCI risk group, DNA index, gender, and dichotomized median steady state MTX level were evaluated in the 1005 patients with complete data. The rank order of significance of each of the factors in the Cox regression was: (1) NCI risk group ($p<0.001$), (2) gender ($p=0.004$), (3) dichotomized median steady state MTX level ($p=0.006$), and (4) DNA index ($p=0.007$). Patients with median steady state MTX levels $\leq 14 \mu\text{M}$ (for a given NCI risk group, DNA index, and gender) had an increased instantaneous risk of failure of 1.84 (95% confidence interval, 1.19–2.84) relative to those with median steady state MTX levels $> 14 \mu\text{M}$.

In the second Cox regression analysis, the relative prognostic influence of NCI risk group, DNA index, gender, RBC MTX, RBC folate, and RBC MTX:folate ratio on CCR were evaluated in the 690 patients with complete data. Only NCI risk group reached significance

($p=0.005$). After adjustment for NCI risk group, the RBC MTX concentration was closest to significance ($p=0.066$).

DISCUSSION

MTX is an integral component of therapy for childhood ALL, but the optimal dosing schedule remains unknown. MTX has been administered in a wide range of dosages from 20 mg/m²/week by mouth during maintenance therapy to 33.6 grams/m² intravenously over 24 hours during consolidation²⁴. An intermediate dose of 1 gram/m² infusion over 24 hours every other week for 12 doses was chosen for the POG 9005/9006 based on pharmacological studies demonstrating that ID MTX infusions were capable of sustaining plasma MTX levels of approximately 7–10 μ M (believed to be cytotoxic for leukemic cells)^{25–27}. Twenty-four hour infusions of MTX bypassed the considerable variability in plasma drug levels following oral schedules and allowed increased time of exposure to the drug. Plasma concentrations at the end of the infusion and red cell folate and MTX concentrations were collected and analyzed so as to determine if they would serve as surrogate markers of leukemic blast MTX exposure and if they would correlate with CCR.

Higher plasma MTX levels and increased systemic clearance have correlated with a decreased risk of relapse^{11, 13, 28}, though significant inpatient variability may impact analyses¹². Investigators at St. Jude Children's Research Hospital previously reported that steady state plasma MTX levels > 16 μ M correlated with decreased risk of relapse, and the present study containing over 1000 patients demonstrated that median steady state plasma MTX levels > 14 μ M correlated with decreased risk of relapse. Consistent with the study conducted at St. Jude Children's Research Hospital, the plasma MTX level remained statistically significant for outcome only in higher risk patients²⁹. Although this did not reach statistical significance, there was a trend towards improved CCR in patients with lower risk of relapse and steady state plasma MTX levels > 14 μ M in our study. The differences in steady state MTX cutoff may be attributed to variation in patient characteristics, schedule of administration of MTX, or concomitant chemotherapy agents. Specifically, questions have been raised as to the role of leucovorin in modulating the safety and efficacy of methotrexate. Early preclinical studies utilizing leucovorin rescue in a mouse model of leukemia demonstrated the difficulty associated with the delivery of an anti-folate and a reduced folate rescue. Specifically, excessive delay or the use of "low doses" of leucovorin were associated with toxic deaths while large doses of leucovorin or the delivery of rescue shortly after the completion of MTX therapy interfered with efficacy^{30–31}. Clinically, Skarby et al³² found that higher doses of leucovorin, despite their correlation with higher plasma MTX concentrations, were associated with an increased risk of relapse and Sterba et al³³ found that leucovorin modulates the biologic impact of MTX with a fall in the area under the curve for plasma homocysteine concentrations with successive courses of MTX in response to a leucovorin mediated increase in plasma folate concentrations. In contrast, successful therapies developed by investigators at St Jude Childrens Research Hospital include early leucovorin dosing at hour 36 after the delivery of intrathecal methotrexate, emphasizing the complexities in understanding the impact of leucovorin and methotrexate on outcome in multi-agent trials. It is not known if a change in the dose or schedule of leucovorin delivery would have had a significant impact on the outcome of this trial.

Although Hispanic patients had significantly lower steady state MTX levels, this did not correlate with outcome, nor were these lower MTX levels clinically significant. It should be noted that this subset analysis was not in the original study design or power calculations. Additionally, the accuracy of self-reported ethnicity has been brought into question based upon results of genetic ancestry determined by genome-wide single nucleotide

polymorphisms. In a genetic ancestry study of pediatric ALL patients from the Children's Oncology Group and St. Jude Children's Research Hospital, American Indians as identified by genetic ancestry analysis often self-reported as having Hispanic ethnicity. This cohort of American Indians had a significantly increased risk of relapse³⁴. Further understanding of genetic ancestry may improve our ability to correlate outcome data with ethnicity.

A recent study of pediatric ALL patients from the Children's Oncology Group and St. Jude Children's Research Hospital demonstrated an association between germline single-nucleotide polymorphisms (SNPs) and end induction minimal residual disease (MRD)³⁵. This study identified 102 SNPs associated with MRD, 21 of which were also associated with drug metabolism. Fourteen of these SNPs were associated with either MTX clearance or accumulation of polyglutamates. Three of the SNPs associated with MTX metabolism were also associated with etoposide clearance. This suggests that SNPs that affect MTX metabolism may also affect the metabolism of other chemotherapeutic agents. It is possible that plasma MTX concentrations obtained on the current study may simply be a surrogate of altered metabolism of other chemotherapeutic agents.

RBC MTX concentration may parallel the cellular uptake and polyglutamation of MTX in lymphoblasts. RBC MTX and folate concentrations were monitored before and during treatment on the POG 8036 pilot. A weak correlation between improved CCR and higher RBC MTX concentrations after consolidation was observed⁴. However, the present study failed to detect correlations between CCR and RBC MTX, RBC folate, or RBC MTX:folate concentrations, indicating that these parameters could not prospectively identify patients at higher risk of an event. These data suggest that RBC concentrations may not be an accurate surrogate for blast cell MTX or folate status during consolidation. It may, however, be more critical during the maintenance phase of therapy where both compliance and metabolism are of greater concern due to chronic administration of low dose oral therapy. Clearly, killing of lymphoblasts by MTX is dependent on factors beyond MTX or MTX:folate concentrations such as folylpolyglutamate synthetase, dihydrofolate reductase, and thymidylate synthetase activity, endogenous folate pools and many other factors.

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REFERENCES

1. Perrin JC, Mauer AM, Sterling TD. Intravenous methotrexate (amethopterin) therapy in the treatment of acute leukemia. *Pediatrics*. 1963; 31:833–839. [PubMed: 13942585]
2. Frei E III, Freireich EJ, Gehan E, et al. Studies of Sequential and Combination Antimetabolite Therapy in Acute Leukemia: 6-Mercaptopurine and Methotrexate. *Blood*. 1961; 18:431–454.
3. Schröder H, Clausen N, Ostergaard E, et al. Pharmacokinetics of erythrocyte methotrexate in children with acute lymphoblastic leukemia during maintenance treatment. *Cancer chemotherapy and pharmacology*. 1986; 16:190–193. [PubMed: 3456283]
4. Graham ML, Shuster JJ, Kamen BA, et al. Red blood cell methotrexate and folate levels in children with acute lymphoblastic leukemia undergoing therapy: a Pediatric Oncology Group pilot study. *Cancer Chemother Pharmacol*. 1992; 31:217–222. [PubMed: 1464159]
5. Graham ML, Shuster JJ, Kamen BA, et al. Changes in red blood cell methotrexate pharmacology and their impact on outcome when cytarabine is infused with methotrexate in the treatment of acute lymphocytic leukemia in children: a pediatric oncology group study. *Clin Cancer Res*. 1996; 2:331–337. [PubMed: 9816176]

6. Cole PD, Zebala JA, Alcaraz MJ, et al. Pharmacodynamic properties of methotrexate and Aminotrexate during weekly therapy. *Cancer Chemother Pharmacol.* 2006; 57:826–834. [PubMed: 16170572]
7. Vijayanathan V, Smith AK, Zebala JA, et al. High-performance liquid chromatography separation of aminopterin-polyglutamates within red blood cells of children treated for acute lymphoblastic leukemia. *Transl Res.* 2007; 150:367–373. [PubMed: 18022599]
8. Kamen BA, Holcenberg JS, Turo K, et al. Methotrexate and folate content of erythrocytes in patients receiving oral vs intramuscular therapy with methotrexate. *The Journal of pediatrics.* 1984; 104:131–133. [PubMed: 6581287]
9. Kamen BA, Nylen PA, Camitta BM, et al. Methotrexate accumulation and folate depletion in cells as a possible mechanism of chronic toxicity to the drug. *British Journal of Haematology.* 1981; 49:355–360. [PubMed: 6170307]
10. Balis FM, Holcenberg JS, Poplack DG, et al. Pharmacokinetics and pharmacodynamics of oral methotrexate and mercaptopurine in children with lower risk acute lymphoblastic leukemia: a joint children's cancer group and pediatric oncology branch study. *Blood.* 1998; 92:3569–3577. [PubMed: 9808549]
11. Evans WE, Abromowitch M, Crom WR, et al. Clinical pharmacodynamic studies of high-dose methotrexate in acute lymphocytic leukemia. *NCI Monogr.* 1987; 5:81–85. [PubMed: 3481040]
12. Seidel H, Nygaard R, Moe PJ, et al. On the prognostic value of systemic methotrexate clearance in childhood acute lymphocytic leukemia. *Leuk Res.* 1997; 21:429–434. [PubMed: 9225071]
13. Camitta B, Mahoney D, Leventhal B, et al. Intensive intravenous methotrexate and mercaptopurine treatment of higher-risk non-T, non-B acute lymphocytic leukemia: A Pediatric Oncology Group study. *J Clin Oncol.* 1994; 12:1383–1389. [PubMed: 8021728]
14. da Costa M, Iqbal MP. The transport and accumulation of methotrexate in human erythrocytes. *Cancer.* 1981; 48:2427–2432. [PubMed: 7296492]
15. Baugh CM, Krumdieck CL, Nair MG. Polyglutamylation of methotrexate. *Biochem Biophys Res Commun.* 1973; 52:27–34. [PubMed: 4197190]
16. Mahoney DH Jr, Shuster JJ, Nitschke R, et al. Acute neurotoxicity in children with B-precursor acute lymphoid leukemia: an association with intermediate-dose intravenous methotrexate and intrathecal triple therapy--a Pediatric Oncology Group study. *J Clin Oncol.* 1998; 16:1712–1722. [PubMed: 9586883]
17. Lauer SJ, Shuster JJ, Mahoney DH Jr, et al. A comparison of early intensive methotrexate/mercaptopurine with early intensive alternating combination chemotherapy for high-risk B-precursor acute lymphoblastic leukemia: a Pediatric Oncology Group phase III randomized trial. *Leukemia.* 2001; 15:1038–1045. [PubMed: 11455971]
18. Kamen BA, Caston JD. Direct radiochemical assay for serum folate: competition between 3H-folic acid and 5-methyltetrahydrofolic acid for a folate binder. *The Journal of laboratory and clinical medicine.* 1974; 83:164–174. [PubMed: 4202673]
19. Kamen BA, Takach PL, Vatev R, et al. A rapid, radiochemical-ligand binding assay for methotrexate. *Analytical biochemistry.* 1976; 70:54–63. [PubMed: 1259156]
20. Kamen BA, Caston JD. Properties of a folate binding protein (FBP) isolated from porcine kidney. *Biochemical Pharmacology.* 1986; 35:2323–2329. [PubMed: 3729988]
21. Kalbfleisch, JD.; Prentice, RL. *The Statistical Analysis of Failure Time Data.* New York: John Wiley and Sons, Inc.; 1980.
22. Cox DR. Regression models and Life-Tables. *Journal of Royal Statistical Society.* 1972; 34:187–220.
23. Patrick J, Thomas L, Margaret S. Time-Dependent ROC curves for censored survival data and a diagnostic marker. *Biometrics.* 2000:337–344. [PubMed: 10877287]
24. Hilden JM, Dinndorf PA, Meerbaum SO, et al. Analysis of prognostic factors of acute lymphoblastic leukemia in infants: report on CCG 1953 from the Children's Oncology Group. *Blood.* 2006; 108:441–451. [PubMed: 16556894]
25. Keefe DA, Capizzi RL, Rudnick SA. Methotrexate Cytotoxicity for L5178Y/Asn- Lymphoblasts: Relationship of Dose and Duration of Exposure to Tumor Cell Viability. *Cancer Res.* 1982; 42:1641–1645. [PubMed: 7066887]

26. Borsi JD, Moe PJ. A comparative study on the pharmacokinetics of methotrexate in a dose range of 0.5 g to 33.6 g/m² in children with acute lymphoblastic leukemia. *Cancer*. 1987; 60:5–13. [PubMed: 3472638]
27. Rodman JH, Sunderland M, Kavanagh RL, et al. Pharmacokinetics of Continuous Infusion of Methotrexate and Teniposide in Pediatric Cancer Patients. *Cancer Res*. 1990; 50:4267–4271. [PubMed: 2194652]
28. Evans WE, Crom WR, Abromowitch M, et al. Clinical pharmacodynamics of high-dose methotrexate in acute lymphocytic leukemia. Identification of a relation between concentration and effect. *New England Journal of Medicine*. 1986; 314:471–477. [PubMed: 3456079]
29. Evans WE, Schell MJ, Pui CH. MTX clearance is more important for intermediate-risk ALL. *J Clin Oncol*. 1990; 8:1115–1116. [PubMed: 2348226]
30. Sirotinak FM, Donsbach RC, Moccio DM, et al. Biochemical and pharmacokinetic effects of leucovorin after high-dose methotrexate in a murine leukemia model. *Cancer Res*. 1976; 36:4679–4686. [PubMed: 1087182]
31. Sirotinak FM, Moccio DM, Dorick DM. Optimization of high-dose methotrexate with leucovorin rescue therapy in the L1210 leukemia and sarcoma 180 murine tumor models. *Cancer Res*. 1978; 38:345–353. [PubMed: 304375]
32. Skarby TVC, Anderson H, Heldrup J, et al. High leucovorin doses during high-dose methotrexate treatment may reduce the cure rate in childhood acute lymphoblastic leukemia. *Leukemia*. 2006; 20:1955–1962. [PubMed: 16990760]
33. Sterba J, Dusek L, Demlova R, et al. Pretreatment Plasma Folate Modulates the Pharmacodynamic Effect of High-Dose Methotrexate in Children with Acute Lymphoblastic Leukemia and Non-Hodgkin Lymphoma: "Folate Overrescue" Concept Revisited. *Clin Chem*. 2006; 52:692–700. [PubMed: 16455868]
34. Harvey RC, Mullighan CG, Wang X, et al. Identification of novel cluster groups in pediatric high-risk B-precursor acute lymphoblastic leukemia with gene expression profiling: correlation with genome-wide DNA copy number alterations, clinical characteristics, and outcome. *Blood*. Epub 2010 Aug 10.
35. Yang JJ, Cheng C, Yang W, et al. Genome-wide Interrogation of Germline Genetic Variation Associated With Treatment Response in Childhood Acute Lymphoblastic Leukemia. *Jama*. 2009; 301:393–403. [PubMed: 19176441]

Figure 1.

Box plots of (A) RBC MTX concentration, (B) RBC folate concentration, (C) RBC MTX:folate ratio, and (D) Steady state plasma MTX level (institutional MTX median) for patients in continuous remission versus those who experienced events post-intensive continuation therapy. The box represents the 1st to 3rd quartile (the middle 50% of data), the horizontal line is the median value, the bars represent the range, and the individual points are statistical outliers.

Figure 2.
Five year CCR in Hispanic, Caucasian, and African American Children

Figure 3.
Five year CCR in Patients with Steady State MTX levels ≤ 14 μ M and > 14 μ M

Table 1

Criteria for Risk Classification

DNA Index	CNS or Testicular	WBC × 10 ³ /μL	Age (years)			
			1 – 2.99	3 – 5.99	6 – 10.99	11 – 21
1.16 ¹	Negative	< 10	A ²	A	A	B ³
1.16	Negative	10–99	B	A	B	B
1.16	Negative	100	B	B	B	B
> 1.16	Negative	Any	A	A	A	A
Any	Positive	Any	B	B	B	B

¹ 1.16 or technically unsatisfactory
² A, lower risk for relapse, POG 9005
³ B, higher risk for relapse, POG 9006

Table 2

Patient Characteristics

	Frequency	Percent
Gender		
Male	598	53
Female	526	47
Race		
Caucasian	801	71
African-American	103	9
Hispanic	146	13
Other	74	7
NCI risk		
Standard risk	883	79
High risk	241	21
DNA index [/]		
1.161	761	69
> 1.161	340	31
t(1;19)	36	3

[/] 23 patients did not have DNA index results

Table 3

Outcome of Patients Completing Intensive Continuation

Outcome	Frequency (%)
No Event	838 (75)
Relapse	
Isolated Marrow	149 (13)
Isolated CNS	38 (3)
CNS + Other site	30 (3)
Extramedullary ± Marrow	56 (5)
Second Malignancy	6 (0.5)
Remission Death	7 (0.5)
Total	1124

Table 4

Comparison of patients who had events vs those in remission

	In Remission	Had Event
RBC MTX¹	353.26 (226.78, 510.75) ²	353.82 (206.96, 537.89)
RBC Folate¹	788.75 (468.32, 1072.89)	792.94 (486.82, 1051.27)
RBC MTX:Folate	0.47 (0.25, 0.85)	0.48 (0.24, 0.80)
Steady State MTX³	9.93 (8.00, 12.85)	9.45 (7.45, 12.40)

¹ pmol/ml RBC;² Median (quartiles);³ μ M

Table 5
Effect of NCI Risk Group and Gender on RBC MTX, RBC Folate, and Plasma MTX Values

	NCI Risk Group			Gender		
	Standard	High	p	Male	Female	p
RBC MTX¹	359 (225,500) ²	344 (222,537)	0.86	370 (248,528)	341 (202,497)	0.10
RBC Folate¹	766 (459,1057)	870 (570,1137)	0.09	785 (509,1067)	794 (445,1081)	0.81
RBC MTX:Folate	0.49 (0.25,0.87)	0.44 (0.24,0.76)	0.24	0.49 (0.26,0.80)	0.44 (0.24,0.89)	0.49
Steady State MTX³	9.8 (7.9,12.5)	10.0 (7.8,13.0)	0.38	9.9 (7.9, 12.5)	9.8 (7.9, 13.0)	0.83

¹ pmol/ml RBC;
² Median (quartiles);
³ μM

Table 6

Effect of Race on RBC MTX, RBC Folate, and Plasma MTX Values

	Caucasian	Hispanic	African American	p
RBC MTX¹	353(222,512) ²	348(202,497)	380(235,548)	0.63
RBC Folate¹	798(476,1071)	850(482,1229)	761(475,1033)	0.63
RBC MTX:Folate	0.49(0.25,0.86)	0.41(0.21,0.81)	0.48(0.26,0.85)	0.51
Steady State MTX³	9.9(8.1,12.9)	8.7(7.5,12.0)	10.1(7.9,12.4)	0.006

¹ pmol/ml RBC;² Median (quartiles);³ μ M