Intensification of Mercaptopurine/Methotrexate Maintenance Chemotherapy May Increase the Risk of Relapse for Some Children With Acute Lymphoblastic Leukemia

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Purpose: Thioguanine nucleotides (TGNs) mediate the cytotoxicity of mercaptopurine (MP). Methylated MP metabolites (formed by thiopurine methyltransferase [TPMT]) and methotrexate (MTX) polyglutamates can inhibit de novo purine synthesis. We explored whether dose adjustment of MP and MTX by erythrocyte (E) levels of TGN and MTX (including polyglutamates) could improve outcome in childhood acute lymphoblastic leukemia (ALL).

Patients and Methods: A total of 538 children with ALL were randomly assigned to have their oral MP/MTX maintenance therapy adjusted by white cell counts (WBC), E-TGN, and E-MTX (pharmacology group), or by WBC only (control group).

Results: After a median follow-up of 7.8 years, 79 patients had relapsed. Cox regression analysis showed an increased risk of relapse for boys (*P* = .00003), high WBC at diagnosis (*P* = .03), pharmacology arm (6.6 times increased relapse hazard for girls), high TPMT activity (*P* = .002), and high average neutrophil counts during maintenance therapy (*P* = .0009), with a significant interaction between sex and randomization group (*P* = .0007). For girls, the relapse risk was 5% in the control group and 19% in the pharmacology group (*P* = .001) because of an increased relapse hazard during the first year after cessation of therapy. TPMT activity was the most significant predictor of relapses among girls in the pharmacology arm (*P* < .0001). Overall, the TPMT activity was higher for patients who relapsed after cessation of therapy compared with those who stayed in remission (girls 19.5 *v* 17.4 U/mL, *P* = .03; boys 19.3 *v* 18.0 U/mL, *P* = .04).

Conclusion: Adding pharmacologically guided treatment intensification to dose adjustments by blood counts may not be warranted for girls, whereas new approaches to optimize maintenance therapy are needed for boys.

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TWENTY-FIVE PERCENT of children with acute lymphoblastic leukemia (ALL) are not cured by first-line therapy, and variations in the pharmacokinetics of the antimetabolites mercaptopurine (MP) and methotrexate (MTX) given orally during maintenance therapy could influence the risk of relapse.[1-3] Therapeutic drug monitoring of intravenous chemotherapy during consolidation therapy has been shown to improve the outcome in B-lineage ALL.[4] Similarly, pharmacologically based dose adjustments of oral MP/MTX maintenance therapy, which is normally tailored to the WBC, have been recommended but so far not shown to reduce relapse risk.[1,2,5]

Thioguanine nucleotides (TGN), which are incorporated into DNA, are the primary mediators of the cytotoxic effect of MP.[6] During maintenance therapy, TGN accumulate in erythrocytes (E), and E-TGN levels strongly reflect the genetically determined activity of the enzyme thiopurine methyltransferase (TPMT).[7] This enzyme methylates MP and some of its metabolites, a pathway that competes with the transformation of MP to TGN.[7] As a result of a common genetic polymorphism, the 89% of white subjects who are homozygous for high TPMT activity ($TPMT^{HH}$, wild type) will have significantly lower E-TGN levels than the 11% of heterozygous patients ($TPMT^{HL}$) and the 1 in 300 enzyme-deficient patients ($TPMT^{LL}$).[7,8] The major cytotoxic metabolites of MTX are the MTX polyglutamates, and the ability of lymphoblasts to form MTX polyglutamates is related to cure rate.[9,10] MTX and methylated MP metabolites may inhibit de novo purine synthesis.[11,12] A series of studies have indicated that E-TGN and E-MTX levels could be useful parameters for optimizing dose adjustments during maintenance therapy.[1,2,13-16]

This report presents the results of the Nordic Society of Paediatric Haematology and Oncology (NOPHO) ALL-92 study during which patients were randomly assigned to have their MP/MTX dosage adjusted by blood counts only (control group) or by a combination of blood counts and E-TGN/MTX levels (pharmacology group).

PATIENTS AND METHODS

Patients

The NOPHO ALL-92 protocol for children 1.0 to 14.9 years of age with non–B-cell childhood ALL was opened in Denmark, Finland, Iceland,

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Table 1. Characteristics

Risk Criteria	SR-ALL*	IR-ALL†	HR-ALL‡
Eligible at diagnosis§	263	264	114
Induction death	2	2	7
Relapse prior to MT	1		17
Death in CCR prior to MT	3	2	4
Bone marrow transplantation prior to MT			3
Not treated according to NOPHO protocol and risk group	12	16	19
Eligible but not randomly assigned	2	9	4
Total number randomly assigned	243	235	60
Pharmacology/control	122/121	116/119	31/29
DK/I/SF/N/S	46/4/50/50/93	54/4/51/40/86	14/0/0/15/31
Male/female	129/114	123/112	36/24
Age, years‖	4.1	4.0	3.6
Range	2.6 to 7.1	1.9 to 12.5	2.2 to 6.7
WBC‖	4.2	14.7	64
Death in CCR	1	1	1
Second cancer	3	2	1
Relapse	20	35	11
BM	15	32	11

Abbreviations: SR, standard risk; IR, intermediate risk; HR, high risk; ALL, acute lymphoblastic leukemia; BM, bone marrow; MT, maintenance therapy; CCR, complete continuous remission; DK/I/SF/N/S, Denmark, Iceland, Finland, Norway, Sweden; VHR, very high risk; NOPHO, Nordic Society of Paediatric Haematology and Oncology.

*Age, 2.0-9.9 years and WBC $< 10 \times 10^9$/L; no HR criteria.

†Age, 1.0-1.9 years or > 10.0 years and/or WBC 10-49 $\times 10^9$/L; no HR criteria.

‡WBC $\geq 50 \times 10^9$/L; T-cell, mediastinal; CNS, testicular, or lymphomatous disease, t(4;11), t(9;22), or 22q-, and M3 d14 or M2/3 d29 BM.

§1.0-14.9 years, diagnosed in 1992-1996 with non-B non-VHR ALL (and not Finish HR-ALL), and starting maintenance therapy before January 1, 1997. VHR status is assigned if age ≥ 5 years and, in addition, T-cell disease is present with one or more additional HR features, CNS leukemia, lymphomatous leukemia, or HR-ALL criteria at diagnosis, and a day 15 M3 or a day 29 M2/3 bone marrow.

‖Median age or WBC at diagnosis (75% range).

Norway, and Sweden on January 1, 1992. Criteria for classification as standard (SR), intermediate (IR), high (HR), and very high risk (VHR) are listed in Table 1. All Finnish HR patients and all VHR patients were treated by the NOPHO VHR regimen without MP/MTX maintenance therapy and were not eligible for the study.[3] The study was closed for further patient accrual on December 31, 1996, when 538 eligible patients had been randomly assigned to a regimen. A group of 641 patients were eligible at the time of diagnosis, but 38 of those patients experienced an event before the start of maintenance therapy, three patients received a bone marrow transplantation during first remission, 47 patients were not treated according to NOPHO protocols and risk groups, and 15 patients who were eligible at the start of maintenance therapy (3%) were not randomly assigned to a regimen (Table 1). One patient was lost to follow-up after 15 months of therapy and was censored at that time point. One patient moved abroad 16 months after diagnosis. However, because the family returned when the girl developed a relapse, this patient was included in the survival analyses. Nine patients for whom MP and/or MTX maintenance therapy was substituted with other drugs as a result of toxicity stayed in the study with respect to event analyses, but their pharmacological and toxicity data were included only up to the time of change in therapy. In the time-dependent analyses, the covariates calculated up to the time of change in therapy were frozen for the remainder of maintenance therapy. In addition to the 538 eligible and randomly assigned patients, 12 noneligible patients were randomly assigned and subsequently excluded.

Randomization

Patients were to be randomly assigned to a regimen within 2 weeks before the start of maintenance therapy. The median number of days from randomization to the start of maintenance therapy was 9 days. The patients were randomly assigned to have their dose of MP and MTX adjusted by either blood counts (control group) or a combination of blood counts and E-TGN $\times$ MTX (the product of E-TGN and E-MTX; pharmacology group). Patients were randomly assigned to these two groups with stratification for risk group (three divisions) and country (five divisions) within blocks of six patients. Thus, for each of these 15 subgroups defined by risk group and country, three patients were assigned to the control group and three patients to the pharmacology group for every six patients randomly assigned to a regimen.

Induction therapy consisted of prednisolone (60 mg/m^2/d on days 1 to 36, then tapered), weekly vincristine (VCR; 2.0 mg/m^2 six times), doxorubicin (40 mg/m^2 three times [non-HR] or four times [HR]), *Erwinia* asparaginase (30,000 U/m^2 10 times), and intrathecal MTX on four occasions.[3]

Consolidation therapy included high-dose MTX (HDM, 5 g/m^2 three times) for SR-ALL, whereas patients with IR- and HR-ALL received alternating series of first, cyclophosphamide (total cumulative dose, 3 g/m^2) with low-dose cytarabine and either oral MP or 6-TGN; second, HDM (IR, 5 g/m^2 and HR, 8 g/m^2; both administered four times) with either b-1 (IR only, oral MP [25 mg/m^2/d] or b-2 (HR only, high-dose cytarabine [12 g/m^2 four times]) with two 2-month-interval periods of oral weekly MTX and daily MP with two VCR/prednisolone reinductions per period; and third, 4 weeks of reinduction with dexamethasone (10 mg/m^2/d for 3 weeks, then tapered), weekly VCR (2.0 mg/m^2 four times), weekly daunorubicin (30 mg/m^2/d three times [HR] or four times [IR]), and *Erwinia* asparaginase (30,000 U/m^2 four times).[3]

Maintenance Therapy

Maintenance therapy with MP doses of 75 mg/m^2/d and MTX doses of 20 mg/m^2/wk was initiated 13 weeks (SR), 32 weeks (IR), or 63 weeks (HR) after diagnosis and continued until 2 years (IR and HR) or 2.5 years (SR) after diagnosis. During the first year of maintenance therapy, patients with SR- and IR-ALL received at 4-week intervals alternate pulses of either VCR (2.0 mg/m^2 once) and prednisolone (60 mg/m^2/d for 1 week) or HDM (5 g/m^2) until five courses of HDM had been given.[3] Every 8 weeks throughout maintenance therapy, patients with HR-ALL received reinductions of VCR (1.5 mg/m^2 once) and prednisolone (40 mg/m^2/d for 1 week) with intrathecal MTX. HDM 5- to 8-g courses during consolidation and maintenance therapy were given as 24-hour infusions with intrathecal MTX (age-adjusted doses) and leucovorin rescue from 36 hours to be continued at 6-hour intervals until plasma-MTX was less than 200 nmol/L.[3]

MP/MTX Dose Adjustments: Control Group

The doses of oral MP and MTX were targeted to a WBC of 1.5 to 3.5 × 10^9/L; reductions to 50% were recommended at a WBC less than 1.5 × 10^9/L, and therapy was interrupted at a WBC less than 1.0 × 10^9/L or a thrombocyte count less than 100 × 10^9/L. Therapy was reinitiated when blood counts were increasing and WBC was more than 1.5 × 10^9/L. Blood counts were measured at an average interval of 1 to 2 weeks. If WBC was more than 3.5 × 10^9/L, the protocol recommended upward dose adjustments of MTX and/or MP until the WBC was within the target range.

MP/MTX Dose Adjustments: Pharmacology Group

The doses of oral MP and oral MTX were targeted by WBC and thrombocyte counts as were those for the control group. In addition and unless the WBC was less than 1.5 × 10^9/L, the doses of MP and/or MTX were to be adjusted upward in steps of 20% if E-TGN × MTX was less than 1,350 (nmol/mmol hemoglobin [Hb])[2] and the treating physician regarded such dose adjustments to be tolerable. This E-TGN × MTX value represents a target level of 225 nmol/mmol Hb for E-TGN and 6.0 nmol/mmol Hb for E-MTX; these values are approximately 25% above the median levels that discriminated between patients with a good or a worse prognosis in the NOPHO ALL-88 study.[2] Because the published data on the relation of relapse risk were stronger for E-TGN than for E-MTX, dose adjustments of MP were made before adjustments of MTX until the E-TGN was more than 225 nmol/mmol Hb. However, if this value was not achieved within 8 weeks from the time of recommended adjustments, patients were to have upward dose adjustments for MP and/or MTX at the discretion of the treating physician. Because interim analyses in July 1998 (1.5 years after patient accrual had stopped) strongly indicated an increased risk of relapse for patients in the pharmacology group, pharmacologic-based dose titration was stopped and all patients still receiving maintenance therapy were thereafter adjusted by the blood counts only. At that time point, only 13 of the 24 SR-ALL patients in the pharmacology group who were still receiving therapy had more than 3 months of their therapy remaining (maximum, 8 months). Thus far, only one of those 24 patients has developed a relapse.

E-TGN/MTX and TPMT

At least once a month, unfrozen blood samples were to be sent for E-TGN/MTX analyses at the Laboratory for Pediatric Oncology (Bonkolab), H:S Rigshospitalet, Copenhagen.[17,18] If no blood samples were received for 6 weeks, a reminder was send out. If the blood sample was too small to allow analyses of both E-TGN and E-MTX, these analyses were done alternately. The time of shipment exceeded 5 days for 501 samples (5.0%), and those results were excluded from the statistical analyses because E-TGN and E-MTX are only stable in unfrozen blood for up to 5 days (K. Schmiegelow, unpublished data). If oral MTX had been taken less than 48 hours or HDM had been administered less than 1 week before blood sampling, recommendations with regard to MTX dose adjustments were based on the previous E-MTX value unless the E-MTX in question was below 6.0 nmol/mmol Hb and E-TGN × MTX was less than 1,350 (nmol/mmol Hb).[2] However, irrespective of their levels, such E-MTX values were excluded from statistical analysis of the relation between E-MTX levels and outcome. For 507 of 538 patients, the RBC TPMT activity was measured one to five times during maintenance therapy as previously described.[19] All TPMT assays were performed at least 8 weeks after the most recent blood transfusion. For 344 of these patients, erythrocyte levels of methylated MP metabolites were measured together with TPMT. Because of the low number of measurements (median, 1; 75% range, 1 to 3), these data were not included in the statistical analyses.

Statistical Methods

For patients with more than one TPMT measurement, an arithmetic mean TPMT was calculated and TPMT was considered a fixed covariate. Nonparametric methods were applied to compare the distribution of parameters between subgroups.[20] In these comparisons, patients in remission did not include those who died in remission or who developed a second malignancy. Cox proportional hazards backward regression analyses were performed, and the likelihood-ratio test was applied to test for differences in outcome.[21,22] Covariates were excluded from the models at a significance level of 0.05. Randomization group (pharmacology *v* control group), E-TGN, and E-MTX levels were tested as interacting covariates with respect to sex.[23] Where relevant, the covariates were analyzed as time-dependent continuous parameters with recalculations of the weighted means (prefix m) of these variables every time a patient treatment failed using as weight the interval between the sample in question and the next blood sample.[5] Because the background relapse intensity differed among the risk groups, the Cox analyses were stratified for risk groups assuming the same effect (ie, hazard coefficient) of possible prognostic factors across risk groups.[22]

Survival analyses were done with a basic time scale defined by the date of diagnosis with delayed entry of patients at the start of maintenance therapy, or at the time of the first E-TGN/MTX or blood count measurements after initiation of maintenance therapy if the survival analyses included such time-dependent parameters.[22] As events in the event-free survival (EFS) analyses, we included relapse, death in remission, or the diagnosis of a second malignant neoplasm, whichever occurred first. Patients who died in first remission or who developed a second cancer were censored at the time of these events in the analysis of relapse risk factors. The Kaplan-Meier method was applied for estimation of remission duration and for the generation of survival curves.[24] Subgroups were compared with the log-rank test[25] and were stratified where needed. Two-sided values of $P < .05$ were regarded as being significant. Survival analyses were performed with the SAS (SAS Institute, Cary, NC) or S-plus (Statistical Sciences, Seattle, WA) statistical software.[26]

The protocol was approved by the ethical committee of Copenhagen (no. V.200.2080/91) as well as by the local ethical committees, and participants gave informed consent according to the Helsinki Declaration.

RESULTS

The patients were followed until December 31, 2001. For patients who stayed in remission, the median length of follow-up from diagnosis was 93 months (50% range, 80 to 106 months). The EFS for 9 years (EFS_{9y}) for all children with non-B ALL and aged 1.0 to 14.9 years when diagnosed in the Nordic countries during the study period from 1992 to 2000 was 0.75 ± 0.01. Because patients who developed an event or were transplanted before the start of maintenance therapy were not eligible for the study, the EFS_{9y} for the 538 patients who entered the study was slightly better than 0.75: 0.83 ± 0.02 (SR, 0.85 ± 0.03; IR, 0.81 ± 0.03; HR, 0.79 ± 0.07).

Relapse Rate

Seventy-nine of the 538 study patients (45 in the pharmacology group) developed a relapse (93% of all relapses projected at 9 years) at a median of 38 months after diagnosis (range, 11 to 101 months), 66 of which occurred after the cessation of therapy (Table 2). Three patients died in first remission during maintenance therapy (two patients in the control group), and seven patients (three patients in the control group) developed a second cancer (one malignant brain tumor and six myelodysplasias or acute myeloid leukemias) 23 to 63 months after diagnosis.[27] Figure 1 demonstrates that for SR and IR patients, the relapse hazard peaks within the first year after cessation of therapy.

We used Cox multivariate regression analysis of all 538 patients to test the effect on relapse risk of year of diagnosis (0 for 1992 to 1993 *v* 1 for 1994 to 1996), sex, WBC at diagnosis, age at diagnosis, and randomization group. Randomization group, sex, and the interaction between these variables were significantly correlated with relapse risk (overall value of the Cox model, $P = .001$). Subsequently, we included the following time-dependent covariates in the Cox analysis: TPMT activity, the average dose of MTX and MP, the percentage of mainte-

Table 2. Distribution of Events by Sex, Risk Group, and Randomization Group

Gender	Risk Group	Stratification	CCR*	Relapse	Death in CCR	Second Cancer†
Male	Standard risk	Pharmacology group	49	11		
		Control group	58	10		1
	Intermediate risk	Pharmacology group	49	9		
		Control group	50	15		
	High risk	Pharmacology group	14	2		1
		Control group	14	4	1	
Female	Standard risk	Pharmacology group	53	7		2
		Control group	50	0	1	1
	Intermediate risk	Pharmacology group	44	12	1	1
		Control group	49	4		1
	High risk	Pharmacology group	10	4		
		Control group	9	1		

Abbreviation: CCR, complete continuous remission.
*Patients in first remission December 31, 2002.
†Second cancers include one malignant brain tumor and six myelodysplasias or acute myeloid leukemias.

nance therapy with treatment discontinuation of MP and MTX, weighted mean of the absolute neutrophil count (mANC), mE-TGN, mE-MTX, and the proportion of maintenance therapy with E-TGN, E-MTX, and mE-TGN × MTX above 225 nmol/mmol Hb, 6.0 nmol/mmol Hb, and 1,350 (nmol/mmol Hb), respectively.[2] The overall best-fit Cox model to predict risk of relapse included WBC at diagnosis, randomization group, sex (the latter two as interacting variables), TPMT activity, and mANC (Table 3). Forty-nine patients (including seven patients with a relapse) had to be excluded because they lacked TPMT activity (n = 31) or neutrophil count measurements (n = 18). Except for WBC at diagnosis, the coefficients of the significant variables in Table 3 changed less than 1% if TPMT activity was included in the Cox analysis as a dichotomous variable (≥ or < 14 U/mL).

E-TGN levels and TPMT activity were closely related. Thus, if the TPMT activity was excluded from the Cox analysis, E-TGN levels were borderline predictive of the risk of relapse ($\beta = -0.0023$, $P = .07$). Because boys did not seem to gain from pharmacological dose adjustments, and because the relapse hazard for girls randomly assigned to the pharmacology group was increased 6.6 times compared with that of the control group (9-year cumulative relapse risk, 19% ± 5% *v* 5% ± 2%; $P = .001$; Table 3; Fig 2), we performed additional analyses of drug dosage and pharmacokinetics to explore further the results.

Dose of MTX and MP

Boys received a significantly higher average dose of both MTX (median, 16.2 *v* 14.6 mg/m^2; $P = .0007$) and of MP (median, 61.3 *v* 57.2 mg/m^2; $P = .01$) than did girls. Patients in the pharmacology and control groups did not differ significantly in their average doses of MTX and MP, but if we included in the analyses only the periods during which MP was administered, boys in the pharmacology group received a higher average dose of MP compared with boys in the control arm (median, 68.5 *v* 63.8 mg/m^2; $P = .05$), whereas there was no significant difference for girls (median, 62.1 *v* 63.8 mg/m^2; $P = .85$). Conversely, girls (but not boys) in the pharmacology group were

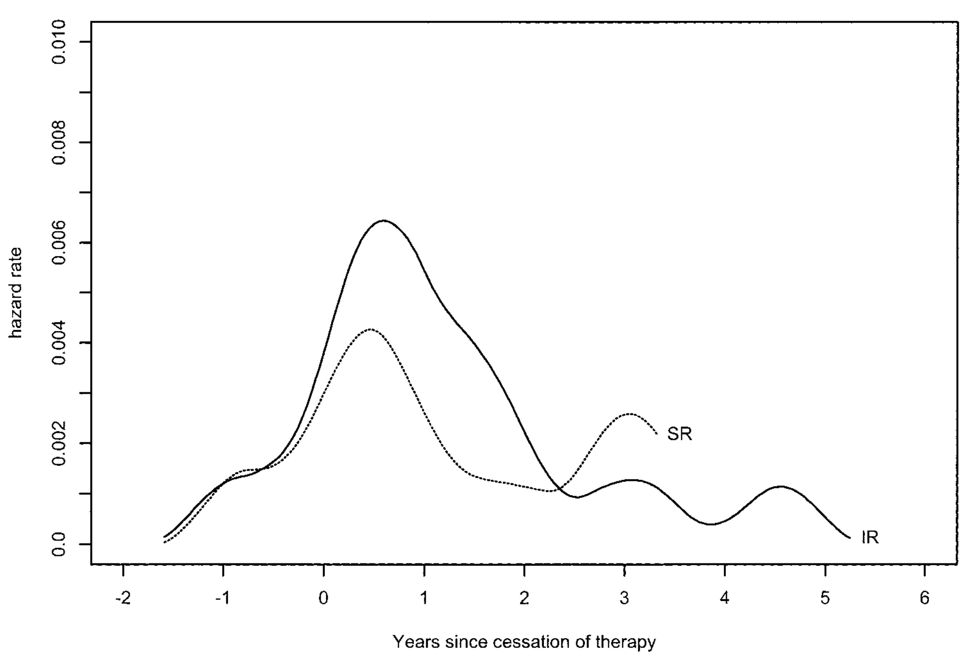

Fig 1. The hazard curves for patients with standard-risk (SR, · · ·) and intermediate-risk (IR, ——) leukemia include relapse, death, or a second cancer in first remission. Hazard curves were estimated and smoothed by kernel smoothing with bandwidths of 10 months.[22]

Table 3. Coefficients in the Cox Hazard Models: Relapses Only*

Parameter	Beta	SE, β	Relapse Hazard	*P*
Sex†	1.99	0.61	7.3	.00003
WBC at diagnosis	5.61×10^{-3}	2.26×10^{-3}	1.01	.03
Randomization group‡	1.89	0.620	6.6	.0003
Randomization group × sex,§	−2.02	0.684	0.13	.0007
mANC‖	0.609	0.175	1.83	.0009
TPMT activity	0.101	0.033	1.1	.002

Abbreviations: mANC, mean absolute neutrophil count; TPMT, thiopurine methyltransferase.

*Death in remission and second cancers were counted as censoring events.

†0 for girls, 1 for boys.

‡0 for control, 1 for pharmacology.

§The interaction between sex and randomization group, 1 for boys in pharmacology group, 0 for all other.

‖Mean absolute neutrophil count during maintenance therapy was analyzed as a time-dependent continuous variable.

Overall *P* value of the Cox model < 0.0001.

registered with significantly more cumulative MP treatment interruptions than were those in the control group (median for girls, 8% *v* 5% of the total duration of maintenance therapy; $P = .01$ compared with median for boys, 6% *v* 5%; $P = .29$). Overall, patients who relapsed did not differ in their average dose of MP or MTX from those who stayed in remission, but compared with patients who stayed in remission, the 13 patients who relapsed while receiving therapy (10 of which were boys) received lower doses of MTX (median, 12.6 *v* 15.2 mg/m^2; $P = .01$) and of MP (49. *v* 59.3 mg/m^2; $P = .06$).

TPMT Activity

Of the 507 patients for whom TPMT measurements were available, two were TPMT deficient (TPMT^{LL}, TPMT < 1.0 U/mL RBC), and 72 (14%) were classified as heterozygous (TPMT^{LH}, TPMT < 14.0 U/mL RBC, antimode of the bimodal distribution). The TPMT activity did not differ when the control group was compared with the pharmacology group (median for girls, 17.6 *v* 17.6 U/mL; $P = 1.00$ compared with median for boys, 18.3 *v* 18.2 U/mL; $P = .20$). Compared with patients with TPMT more than or equal to 14 U/mL, those with TPMT activity less than 14 U/mL received a lower average dose of MP (median, 52.4 *v* 61.5 mg/m^2; $P = .0001$), experienced more treatment interruptions (median, 9% *v* 5%; $P = .003$), had higher mE-TGN levels (median, 334 *v* 157 nmol/mmol Hb; $P = .0001$), and experienced lower mWBC levels (median, 3.1 *v* 3.4 × 10^9/L; $P = .02$). The 62 patients who relapsed after cessation of therapy and who had TPMT measurements available had significantly

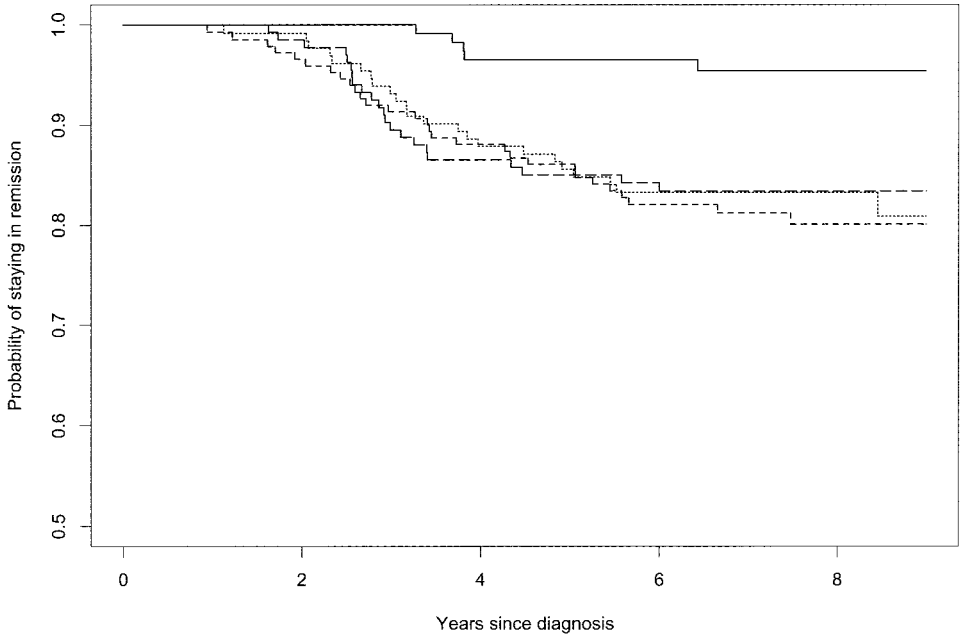

Fig 2. Kaplan-Meier curves for risk of relapse for boys and girls with respect to randomization group. Pharmacology group girls, ···; pharmacology group boys, —·—; control group girls, ——; control group boys, – – – –.

Patients at risk by year from diagnosis	0	1	2	3	4	5	6	7	8	9
Girls control group	116	116	115	114	109	109	97	71	52	23
Girls pharmacology group	134	134	132	123	116	112	97	76	47	19
Boys control group	153	152	148	139	133	130	110	84	51	28
Boys pharmacology group	135	135	131	119	115	113	98	70	48	25

higher TPMT levels than those who stayed in remission (median for girls, 19.5 *v* 17.4 U/mL; $P = .03$ compared with median for boys, 19.3 *v* 18.0 U/mL; $P = .04$). In contrast, this was not the case for those 12 patients with TPMT measurements who relapsed while receiving therapy ($P = .61$). In a multivariate analysis, the best-fit model to predict the risk of relapse after cessation of therapy among girls in the pharmacology group (17 relapses) included mANC ($\beta = 0.93$; $P = .002$) and the TPMT activity ($\beta = 0.15$; $P < .0001$). Not included in the model were WBC at diagnosis, average dose of MTX and MP, and cumulative duration of treatment interruptions of MTX and/or MP, mE-TGN, and mE-MTX.

E-TGN and E-MTX

The median number of E-TGN/MTX blood samples analyzed for patients in first remission at the end of maintenance therapy and for patients who relapsed during maintenance therapy was 16 and 12 (75% range, 7 to 30 and 4 to 22 samples), respectively (9,182 TGN/MTX measurements in total). When patients who stayed in remission were analyzed, girls and boys did not differ significantly in their mE-TGN levels (median, 169 *v* 169 nmol/mmol Hb; $P = .31$), but boys had higher mE-MTX levels (median for girls, 5.4 and median for boys, 5.8 nmol/mmol Hb; $P = .01$) and mE-TGN $\times$ MTX levels (1,087 *v* 896 nmol/mmol Hb;[2] $P = .001$). The two treatment arms did not differ in their mE-TGN levels ($P = .63$), mE-MTX levels ($P = .71$), or mE-TGN $\times$ MTX levels ($P = .55$). Patients who developed a relapse while receiving therapy had lower mE-TGN levels than those who have stayed in remission (104 *v* 169; $P = .005$), although this was not the case for those who relapsed after cessation of therapy (165 *v* 169 nmol/mmol Hb; $P = .63$). Neither mE-MTX nor mE-TGN $\times$ MTX levels differed significantly between the patients who relapsed and those who stayed in remission ($P = .13$ and $P = .76$, respectively).

Blood Counts

A total of 28,580 data sets on blood counts and/or drug doses were included in the analyses. For patients who stayed in remission, the mWBC levels did not differ significantly between boys and girls (median, 3.3×10^9/L for both sexes; $P = .59$). Compared with those in the control group, patients in the pharmacology group had lower mANC (1.9 *v* 2.0×10^9/L; $P = .04$) but did not differ significantly in mWBC (3.2 *v* 3.4×10^9/L; $P = .22$). The patients who relapsed had significantly higher mANC levels than those who stayed in remission (median, 2.2 *v* 1.9×10^9/L; $P = .0008$) but did not differ significantly in their mWBC (3.5 *v* 3.3×10^9/L; $P = .06$), average lymphocyte count levels ($P = .60$), or median monthly number of blood counts (2.9 *v* 2.5; $P = .13$). For both sexes and without adjustments for possible confounders, patients with a mANC less than 2.0×10^9/L (median of all patients) had an outcome superior to that of patients with higher mANC levels (for boys, 0.87 *v* 0.75, $P = .02$; and for girls, 0.94 *v* 0.83, $P = .01$).

DISCUSSION

Treatment intensity during maintenance therapy reflects the dose adjustment guidelines of the protocol and the physician and patient compliance.[28,29] In retrospect, the clinical effect of these factors is difficult to separate. Thus, the results of this study do not necessarily imply that dose adjustments to achieve a certain degree of systemic drug exposure during maintenance therapy will not improve cure rate. However, in contrast to the aims of the study, pharmacologically guided dose adjustments failed to improve the outcome for boys but significantly increased the relapse risk for girls. These data seem counterintuitive, but they carry important information that may expand our understanding of the way in which maintenance therapy works. Because this and previous studies indicate important sex differences in the course of childhood ALL, the results for boys and girls will be discussed separately.[23,30-32]

For boys, the NOPHO ALL-88 study and other cohorts[1,2,33] have found that E-TGN levels are related to the cure rate, although this was not the case in a recent study by Relling et al.[34] In this study, E-TGN levels were only of significance for patients who relapsed while receiving therapy. Thus, the divergent results of these studies could partly reflect the distribution of relapses while patients were receiving and not receiving therapy. In addition, E-TGN levels are surrogate markers for TPMT activity, and their prognostic significance probably reflects the effect on MP pharmacology by TPMT. Although TPMT activity is related to E-TGN, and erythrocyte and lymphoblast TPMT activities are closely related,[35] E-TGN levels will only be reflections for events in leukemic blasts, for which the end point metabolites are not cytosol TGN but rather DNA-TGN.[6] It is also likely that other MP metabolites such as methylthioinosine monophosphate (formed by TPMT) could influence the degree of DNA-TGN incorporation and cytotoxicity through inhibition of de novo purine synthesis.[12,36]

Finally, several studies have indicated that merely securing high E-TGN levels may be insufficient to reduce the risk of relapse. First, United Kingdom ALL Study Group (UKALL) and Cooperative ALL Study Group (COALL) protocols have randomly assigned patients to either MP or thioguanine (TG) as part of their maintenance therapy without significant reductions in relapse rates.[37,38] Although the patients in the COALL 05–92 study who were given TG did achieve high E-TGN levels, they did not experience significantly more leukopenia and did not achieve a significant reduction in relapse rate.[38-40] Second, patients with low TPMT activity, not least who that are TPMT deficient, can tolerate two- to 10-fold higher RBC cytosol TGN levels than can patients with a wild-type TPMT genotype and phenotype, which also indicates that cytosol TGN concentrations are not directly reflected in the DNA-TGN levels.[39-41] Thus, even if low E-TGN levels are markers for inferior treatment intensity of boys, the ideal way in which to compensate for this remains to be determined.

The cause of the significantly increased relapse rate experienced by the girls in the pharmacology group within the first year off therapy is unclear. Dose increments could have increased suppression of immune functions that play a role in the eradication of the leukemic clone. However, apart from the graft-versus-leukemia phenomenon observed after stem-cell transplantation, no clinical studies have demonstrated that the immune system plays a substantial role in the cure of childhood ALL. In addition, lymphocyte counts did not differ significantly between the control and pharmacology groups or between patients who

experienced a relapse and those who remained in remission (data not shown). Both this study and previous studies have shown that attempts to escalate the dose of MP are counteracted by longer periods of therapy withdrawal and may, in addition, preferentially increase the levels of methylated metabolites.[34,42,43] As the overall relapse risk has been reduced, the increased risk of relapse within the first year after the cessation of maintenance therapy has become obvious.[3,31,32,44,45] Thus, even though contemporary treatment intensity has reduced the risk of treatment failures, the relapse rate following cessation of therapy seems to have changed little.[46]

In this study, TPMT activity was the strongest treatment-related parameter with influence on the risk of relapse among girls in the pharmacology group. This could indicate that attempts to escalate the dose of MP lead to increased intracellular levels of methylated MP metabolites, with significant inhibition of purine de novo synthesis in the leukemic lymphoblasts for some patients with high TPMT and a delay of leukemic cells in S phase, putting these cells into a dormant state with regrowth after treatment discontinuation.[12,39] The theory of dormant leukemia with intensified maintenance therapy is indirectly supported by the Japanese Children's Cancer and Leukemia Study Group-S811 study, which demonstrated that intermittent MP/MTX therapy (possibly allowing intermittent leukemic regrowth and thus susceptibility to MP/MTX therapy) was superior to continuous MP/MTX therapy.[47] Similarly, the Dutch ALL-6 protocol gave an excellent EFS (0.81 at 8 years) for patients with low-risk ALL (WBC $< 50 \times 10^9$/L at diagnosis) that could be in part because of the use of intermittent MTX/MP maintenance therapy with 2 weeks interruption of oral MTX/MP during every 7-week cycle.[48] Although the UKALL studies in the early 1980s indicated that continuous therapy was superior to intermittent therapy, the overall outcome at that time was generally poor, and whether continuity of therapy or physician compliance with the protocol had the major effect on the improvement of outcome is not known.[49,50]

That mE-MTX was not significantly related to the overall risk of relapse (as in the NOPHO ALL-88 study) could reflect, in part, that the dose of intravenous (IV) MTX had been increased from 0.5 to 1 g/m^2 in the ALL-88 protocol to 5.0 to 8.0 g/m^2 in the ALL-92 protocol, and this could have mediated the major antileukemic effect of MTX.[2] However, measuring MTX/folate ratios may be superior to intracellular MTX levels, and further research is needed to identify the optimal way by which to monitor and MTX therapy.[51]

The relation between mANC and the risk of relapse, and the excellent outcome for girls in the control group, indicate that dose adjustments on the basis of blood counts are sufficient to optimize therapy for girls and that unnecessary treatment intensification could upset their favorable outcome. For boys, new approaches for the optimization of maintenance therapy are needed. In future trials, the value of the monitoring levels of methylated and nonmethylated MP metabolites and DNA-TGN may increase our understanding of the pharmacokinetic and pharmacodynamic background for treatment failures.

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APPENDIX

The appendix is available online at www.jco.org.

REFERENCES

1. Lilleyman JS, Lennard L: Mercaptopurine metabolism and risk of relapse in childhood lymphoblastic leukaemia. Lancet 343:1188-1190, 1994

2. Schmiegelow K, Schrøder H, Gustafsson G, et al: Risk of relapse in childhood acute lymphoblastic leukemia is related to RBC methotrexate and mercaptopurine metabolites during maintenance chemotherapy. Nordic Society for Pediatric Hematology and Oncology. J Clin Oncol 13:345-351, 1995

3. Gustafsson G, Schmiegelow K, Forestier E, et al: Improving outcome through two decades in childhood ALL in the Nordic countries: The impact of high-dose methotrexate in the reduction of CNS irradiation. Leukemia 14:2267-2275, 2000

4. Evans WE, Relling MV, Rodman JH, et al: Conventional compared with individualized chemotherapy for childhood acute lymphoblastic leukemia. N Engl J Med 338:499-505, 1998

5. Schmiegelow K, Pulczynska MK: Maintenance chemotherapy for childhood acute lymphoblastic leukemia: Should dosage be guided by white blood cell counts? Am J Pediatr Hematol Oncol 12:462-467, 1990

6. Waters TR, Swann PF: Cytotoxic mechanism of 6-thioguanine: hMutSalpha, the human mismatch binding heterodimer, binds to DNA containing S6-methylthioguanine. Biochemistry 36:2501-2506, 1997

7. Weinshilboum RM, Otterness DM, Szumlanski CL: Methylation pharmacogenetics: Catechol O-methyltransferase, thiopurine methyltransferase, and histamine N-methyltransferase. Annu Rev Pharmacol Toxicol 39:19-52, 1999

8. Lennard L, Lilleyman JS: Variable mercaptopurine metabolism and treatment outcome in childhood lymphoblastic leukemia. J Clin Oncol 7:1816-1823, 1989

9. Chabner BA, Allegra CJ, Curt GA, et al: Polyglutamation of methotrexate. Is methotrexate a prodrug? J Clin Invest 76:907-912, 1985

10. Masson E, Relling MV, Synold TW, et al: Accumulation of methotrexate polyglutamates in lymphoblasts is a determinant of antileukemic effects in vivo. A rationale for high-dose methotrexate. J Clin Invest 97:73-80, 1996

11. Bökkerink JP, Bakker MA, Hulscher TW, et al: Purine de novo synthesis as the basis of synergism of methotrexate and 6-mercaptopurine in human malignant lymphoblasts of different lineages. Biochem Pharmacol 37:2321-2327, 1988

12. Bökkerink JP, Stet EH, De Abreu RA, et al: 6-Mercaptopurine: Cytotoxicity and biochemical pharmacology in human malignant T-lymphoblasts. Biochem Pharmacol 45:1455-1463, 1993

13. Schrøder H: Methotrexate pharmacokinetics in age-fractionated erythrocytes. Cancer Chemother Pharmacol 18:203-207, 1986

14. Lennard L, Rees CA, Lilleyman JS, et al: Childhood leukaemia: A relationship between intracellular 6-mercaptopurine metabolites and neutropenia. Br J Clin Pharmacol 16:359-363, 1983

15. Lennard L, Lilleyman JS, Van Loon J, et al: Genetic variation in response to 6-mercaptopurine for childhood acute lymphoblastic leukaemia. Lancet 336:225-229, 1990

16. Schmiegelow K, Schrøder H, Schmiegelow M: Methotrexate and 6-mercaptopurine maintenance therapy for childhood acute lymphoblastic leukemia: Dose adjustments by white cell counts or by pharmacokinetic parameters? Cancer Chemother Pharmacol 34:209-215, 1994

17. Bruunshuus I, Schmiegelow K: Analysis of 6-mercaptopurine, 6-thioguanine nucleotides, and 6-thiouric acid in biological fluids by high-performance liquid chromatography. Scand J Clin Lab Invest 49:779-784, 1989

18. Kamen BA, Takach PL, Vatev R, et al: A rapid, radiochemical-ligand binding assay for methotrexate. Anal Biochem 70:54-63, 1976

19. Weinshilboum RM, Raymond FA, Pazmino PA: Human erythrocyte thiopurine methyltransferase: Radiochemical microassay and biochemical properties. Clin Chim Acta 85:323-333, 1978

20. Siegel S, Castellan NJ: Nonparametric Statistics for the Behavioral Sciences. Singapore, McGraw-Hill, 1988

21. Cox DR: Regression models and life-tables. J R Stat Soc B 34:187-220, 1972

22. Andersen PK, Borgan Ø, Gill RD, et al: Statistical Models Based on Counting Processes. New York, NY, Springer, 1993

23. Lilleyman JS, Lennard L, Rees CA, et al: Childhood lymphoblastic leukaemia: Sex difference in 6-mercaptopurine utilization. Br J Cancer 49:703-707, 1984

24. Kaplan EJ, Meier P: Non-parametric estimation from incomplete observations. J Am Stat Assoc 53:457-481, 1958

25. Mantel N: Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother 50:163-170, 1966

26. SAS Statistical Program. Cary, NC, Statistical Analysis Systems, 2001

27. Thomsen JB, Schrøder H, Kristinsson J, et al: Possible carcinogenic effect of 6-mercaptopurine on bone marrow stem cells: Relation to thiopurine metabolism. Cancer 86:1080-1086, 1999

28. Peeters M, Koren G, Jakubovicz D, et al: Physician compliance and relapse rates of acute lymphoblastic leukemia in children. Clin Pharmacol Ther 43:228-232, 1988

29. Lancaster D, Lennard L, Lilleyman JS: Profile of non-compliance in lymphoblastic leukaemia. Arch Dis Child 76:365-366, 1997

30. Lanning M, Garwicz S, Hertz H, et al: Superior treatment results in females with high-risk acute lymphoblastic leukemia in childhood. Acta Paediatr 81:66-68, 1992

31. Chessells JM, Richards SM, Bailey CC, et al: Gender and treatment outcome in childhood lymphoblastic leukaemia: Report from the MRC UKALL trials. Br J Haematol 89:364-372, 1995

32. Pui CH, Boyett JM, Relling MV, et al: Sex differences in prognosis for children with acute lymphoblastic leukemia. J Clin Oncol 17:818-824, 1999

33. Bostrom B, Erdmann G: Cellular pharmacology of 6-mercaptopurine in acute lymphoblastic leukemia. Am J Pediatr Hematol Oncol 11:80-86, 1993

34. Relling MV, Hancock ML, Boyett JM, et al: Prognostic importance of 6-mercaptopurine dose intensity in acute lymphoblastic leukemia. Blood 93:2817-2823, 2001

35. McLeod HL, Relling MV, Liu Q, et al: Polymorphic thiopurine methyltransferase in erythrocytes is indicative of activity in leukemic blasts from children with acute lymphoblastic leukemia. Blood 85:1897-1902, 1995

36. Keuzenkamp-Jansen CW, Leegwater PA, De Abreu RA, et al: Thiopurine methyltransferase: A review and a clinical pilot study. J Chromatogr Biomed Appl 678:15-22, 1996

37. Greaves L, Sinclair D: International register of current randomised protocols in childhood acute lymphoblastic leukemia. Oxford, United Kingdom, ICRF/MRC Clinical Trial Service Unit, 1996

38. Lancaster DL, Lennard L, Rowland K, et al: Thioguanine versus mercaptopurine for therapy of childhood lymphoblastic leukaemia: A comparison of haematological toxicity and drug metabolite concentrations. Br J Haematol 102:439-443, 1998

39. Erb N, Harms DO, Janka-Schaub G: Pharmacokinetics and metabolism of thiopurines in children with acute lymphoblastic leukemia receiving 6-thioguanine versus 6-mercaptopurine. Cancer Chemother Pharmacol 42: 266-272, 1998

40. Janka GE, Harms DO, Graubner U, et al: Randomized comparison of 6-mercaptopurine vs 6-thioguanine in childhood ALL: Differing cytotoxicity and interim results. Med Ped Oncol 31:267, 1998 (abstr)

41. Andersen JB, Szumlanski C, Weinshilboum RM, et al: Pharmacokinetics, dose adjustments, and 6-mercaptopurine/methotrexate drug interactions in two patients with thiopurine methyltransferase deficiency. Acta Paediatr 87:108-111, 1998

42. Schmiegelow K: Prognostic significance of methotrexate and 6-mercaptopurine dosage during maintenance chemotherapy for childhood acute lymphoblastic leukemia. Pediatr Hematol Oncol 8:301-312, 1991

43. Lennard L, Welch J, Lilleyman JS; Mercaptopurine in childhood leukaemia: The effects of dose escalation on thioguanine nucleotide metabolites. Br J Clin Pharmacol 42: 525-527, 1996

44. Miller DR, Leikin SL, Albo VC, et al: Three versus five years of maintenance therapy are equivalent in childhood acute lymphoblastic leukemia: A report from the Childrens Cancer Study Group. J Clin Oncol 7:316-325, 1989

45. Reiter A, Schrappe M, Ludwig WD, et al: Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. Blood 84:3122-3133, 1994

46. Pui CH, Dodge RK, Look AT, et al: Risk of adverse events in children completing treatment for acute lymphoblastic leukemia: St. Jude Total Therapy studies VIII, IX, and X. J Clin Oncol 9:1341-1347, 1991

47. Koizumi S, Fujimoto T, Takeda T, et al: Comparison of intermittent or continuous methotrexate plus 6-mercaptopurine in regimens for standard-risk acute lymphoblastic leukemia in childhood (JCCLSG-S811). The Japanese Children's Cancer and Leukemia Study Group. Cancer 61:1292-1300, 1988

48. Veerman AJ, Hahlen K, Kamps WA, et al: High cure rate with a moderately intensive treatment regimen in non-high-risk childhood acute-lymphoblastic leukemia. Results of protocol ALL VI from the Dutch Childhood Leukemia Study Group. J Clin Oncol 14:911-918, 1996

49. Anonymous: Improvement in treatment for children with acute lymphoblastic leukaemia. The Medical Research Council UKALL trials, 1972-84. Report to the Council by the Working Party on Leukaemia in Childhood. Lancet 1:408-411, 1986

50. Eden OB, Stiller CA, Gerrard MP: Improved survival for childhood acute lymphoblastic leukemia: Possible effect of protocol compliance. Pediatr Hematol Oncol 5:83-91, 1988

51. Mantadakis E, Smith AK, Kamen BA: Ratio of methotrexate to folate uptake by lymphoblasts in children with B-lineage acute lymphoblastic leukemia: A pilot study. J Pediatr Hematol Oncol 22:221-226, 2000