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ORIGINAL REPORT

Methotrexate-Induced Neurotoxicity and Leukoencephalopathy in Childhood Acute Lymphoblastic Leukemia

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
Methotrexate (MTX) can cause significant clinical neurotoxicity and asymptomatic leukoencephalopathy. We sought to identify clinical, pharmacokinetic, and genetic risk factors for these MTX-related toxicities during childhood acute lymphoblastic leukemia (ALL) therapy and provide data on safety of intrathecal and high-dose MTX rechallenge in patients with neurotoxicity.

Patients and Methods
Prospective brain magnetic resonance imaging was performed at four time points for 369 children with ALL treated in a contemporary study that included five courses of high-dose MTX and 13 to 25 doses of triple intrathecal therapy. Logistic regression modeling was used to evaluate clinical and pharmacokinetic factors, and a genome-wide association study (GWAS) was performed to identify germline polymorphisms for their association with neurotoxicities.

Results
Fourteen patients (3.8%) developed MTX-related clinical neurotoxicity. Of 13 patients rechallenged with intrathecal and/or high-dose MTX, 12 did not experience recurrence of neurotoxicity. Leukoencephalopathy was found in 73 (20.6%) of 355 asymptomatic patients and in all symptomatic patients and persisted in 74% of asymptomatic and 58% of symptomatic patients at the end of therapy. A high 42-hour plasma MTX to leucovorin ratio (measure of MTX exposure) was associated with increased risk of leukoencephalopathy in multivariable analysis ($P = .038$). GWAS revealed polymorphisms in genes enriched for neurodevelopmental pathways with plausible mechanistic roles in neurotoxicity.

Conclusion
MTX-related clinical neurotoxicity is transient, and most patients can receive subsequent MTX without recurrence of acute or subacute symptoms. All symptomatic patients and one in five asymptomatic patients develop leukoencephalopathy that can persist until the end of therapy. Polymorphisms in genes related to neurogenesis may contribute to susceptibility to MTX-related neurotoxicity.

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INTRODUCTION

Methotrexate (MTX) is an essential drug in the treatment of childhood acute lymphoblastic leukemia (ALL). In addition to systemic control of leukemia, it is crucial for prophylaxis and treatment of sanctuary sites, including the CNS. However, MTX can cause acute, subacute, and long-term neurotoxicities.[1-6] The mechanism of neurotoxicity is likely through disruption of CNS folate homeostasis and/or direct neuronal damage.[7-10] Subacute MTX neurotoxicity typically occurs 2 to 14 days after prolonged low-dose oral, intrathecal, or high-dose MTX and manifests with transient stroke-like symptoms, encephalopathy, seizures, and/or aphasia. Although prior reports have demonstrated that many patients can be safely rechallenged with MTX, some have recurrences of neurotoxicity.[2,11] Subsequent MTX is often omitted, potentially increasing relapse risk.

Clinical symptoms of MTX-induced neurotoxicity are often associated with leukoencephalopathy, seen as white matter hyperintensities on T2-weighted and fluid-attenuated inversion recovery magnetic resonance imaging (MRI).[1,12] Although leukoencephalopathy is low grade in

most patients, fatal diffuse necrotizing leukoencephalopathy has been reported.[13,14] Leukoencephalopathy can also develop in asymptomatic children receiving MTX, and its presence has been correlated with increasing MTX exposure.[3,15] The clinical significance of these white matter changes is unknown.[16] It is also unclear whether patients with asymptomatic leukoencephalopathy are at higher risk of developing symptoms when exposed to additional MTX. Germline polymorphisms may contribute to MTX-induced leukoencephalopathy and neurotoxicities and have included variants in *GSTP1*,[17] *MTHFR*, and *SHMT1*.[18] A comprehensive genome-wide association study (GWAS) has not been performed.

In this study, we correlate clinical symptoms of MTX-related subacute neurotoxicity with leukoencephalopathy on MRI, provide data on safety of rechallenging patients with additional MTX, and identify clinical, therapy-related, and genetic risk factors for clinical neurotoxicity and leukoencephalopathy.

PATIENTS AND METHODS

Patients and Therapy

From June 2000 to October 2007, 498 children with ALL were enrolled onto the Total Therapy XV study.[19] Of these, 408 patients enrolled at St Jude Children's Research Hospital were approached for participation in prospective MRI screening. Data from 369 patients were analyzed in this study (Data Supplement). Treatment details from the protocol specific to MTX have been included here. Two hundred seventy-eight of 369 patients received an upfront window treatment comprising high-dose MTX 1 g/m^2 (randomly assigned to 4- ν 24-hour infusions).[20] During consolidation, patients in the low-risk arm received high-dose MTX 2.5 g/m^2 infused over 24 hours for four doses, adjusted to achieve steady-state plasma concentration of 33 μmol/L, and patients in the standard/high-risk arm received 5 g/m^2 over 24 hours, adjusted to 65 μmol/L.[21] Leucovorin rescue dose for window therapy was 50 mg/m^2 at 44 hours, followed by 15 mg/m^2 every 6 hours for seven doses. During consolidation, leucovorin was administered at 5 mg/m^2 in the low-risk arm and 10 mg/m^2 in the standard/high-risk arm for five doses beginning 42 hours after initiation of MTX. Leucovorin doses were increased in patients with a 42-hour MTX level $>$ 1 μmol/L and in those with a history of delayed clearance.[21] The first intrathecal therapy was cytarabine alone, and subsequent intrathecal therapies consisted of MTX, hydrocortisone, and cytarabine in age-dependent doses. The total number of triple intrathecal therapies (ITTs) ranged from 13 to 25. No patient received prophylactic cranial irradiation. Low-dose intravenous MTX (40 mg/m^2) was administered weekly after reinduction II, with interruption for monthly pulses of dexamethasone/vincristine or cyclophosphamide/cytarabine. Total number of doses of MTX in continuation ranged from 68 to 116.

The study was approved by the institutional review board. Informed consent was obtained from parents/guardians, and assent was obtained from patients when appropriate.

Definition of MTX-Related Clinical Neurotoxicity

A neurotoxic adverse event was attributed to MTX if neurologic symptoms (eg, seizure, stroke, behavioral changes, aphasia) occurred within 2 weeks of receiving MTX (intrathecal or intravenous), and other identifiable causes were reasonably ruled out. Patients with clinical neurotoxicity were evaluated by a pediatric neurologist. Adverse events were graded according to the Common Terminology Criteria for Adverse Events (version 3.0), entered into the database in real time, and retrieved for the purpose of this study.

Pharmacokinetic Measurements

Serum MTX and plasma homocysteine concentrations were measured as previously described.[20-23] Blood samples for measuring MTX were drawn before high-dose MTX infusion and at 6, 23, and 42 hours from the start of infusion.[21] Additional MTX concentrations were measured if the 42-hour level was $\geq$ 1 μmol/L. Plasma homocysteine concentrations were measured before high-dose MTX infusion and at 23 and 42 hours in courses one and two. The concentration-time course and area under the curve over baseline (μM $\times$ hour) for homocysteine were calculated as previously described.[22]

Detection and Grading of Leukoencephalopathy

Brain MRIs were obtained at four time points during therapy: postinduction between days 33 and 46 (MRI1), postconsolidation (ie, week 1 of reinduction I; MRI2), continuation week 48 (MRI3), and continuation week 120 (MRI4). MRI4 was considered the off-therapy time point for uniformity, although boys received an additional 26 weeks of continuation chemotherapy that did not include ITT or high-dose MTX. Most patients with clinical neurotoxicity underwent additional imaging during or after the event. The protocol noncontrast MRI examinations consisted of sagittal T1, axial T1-weighted inversion recovery, axial T2, axial proton density, and axial fluid-attenuated inversion recovery pulse sequences of the brain obtained on a 1.5 Tesla MRI platform (Data Supplement). Abnormal MRIs were identified by a single neuroradiologist and graded for the extent of leukoencephalopathy by a second neuroradiologist, according to radiographic criteria of Common Terminology Criteria for Adverse Events (version 4.0). In brief, grades 1, 2, and 3 indicate involvement of $<$ one third, one third to two thirds, and $>$ two thirds of the susceptible areas of the cerebrum, respectively. The neuroradiologists were blinded to risk arm of the study.

Statistical Analyses

Logistic regression models were used to determine the association of neurotoxicity and leukoencephalopathy with patient demographics, disease features, and MTX pharmacokinetic parameters. Analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC) and R software for LINUX (version 2.15.0; http://www.r-project.org). The classification and regression tree analysis tool was used as an alternate approach to identify risk factors for leukoencephalopathy.[24]

Genotyping and GWAS

Genome-wide single-nucleotide polymorphism (SNP) genotyping was performed for 364 patients using Affymetrix 500K/6.0 array sets (Santa Clara, CA). Genotyping of 1,321 candidate SNPs was also performed for 344 patients using the Illumina GoldenGate assay (San Diego, CA) as previously described.[23,25] Race was determined by SNP genotype-based ancestry using STRUCTURE.[26] GWAS was performed for two phenotypes: leukoencephalopathy (grade 0 ν $>$ 0) and clinical neurotoxicity (presence ν absence). Multiple logistic regression models were used to test the association between leukoencephalopathy or neurotoxicity and SNP genotype, with genotype treated as an ordinal variable (AA as 0, AB as 1, and BB as 2). Pathway analysis of significant genes was performed using the g:Profiler program.[27]

RESULTS

Characteristics of Patients With Clinical Neurotoxicity Attributed to MTX

Of 369 patients, 14 (3.8%) developed MTX-related subacute neurotoxic events (Table 1). Four patients were described previously (Nos. 9, 10, 12, and 13).[1] Seven patients presented with seizures, six with stroke-like symptoms, and one with ataxia. Most episodes were brief, but ataxia persisted in Patient No. 5 for 4 weeks. All 12 patients with MRIs available at the time of the event had leukoencephalopathy (Fig 1). Overall, leukoencephalopathy was detected in all symptomatic patients at least once during the course of therapy. Screening MRI was available before the event for 10 patients. Pre-existing leukoencephalopathy was evident in seven patients, whereas three patients had normal preceding MRIs. Of 12 patients with end-therapy MRIs, leukoencephalopathy persisted in seven patients and resolved in five.

Table 1. Patient Characteristics, Details of Clinical Neurotoxic Events, and Rechallenge With MTX

Patient No.	Age (years)	Sex	CNS Status*	Therapy Arm†	MTX Before Event	Time Point in Therapy	Time From MTX to Event (days)	Neurotoxic Event	Duration of Event	Subsequent No. of High-Dose MTX Doses	Subsequent No. of ITTs	Prophylaxis	Recurrent Neurotoxicity
1	13	M	CNS 2	Standard	High-dose MTX, ITT	Consolidation course one	4	Seizure (tonic clonic)	2 minutes	3	20	No	
2	3	M	CNS 1	Low	Low-dose MTX, ITT	Continuation week 40	9	Seizure (complex partial)	24 hours	0	0	NA	
3	5	M	CNS 1	Standard	High-dose MTX, ITT	Consolidation course two	3	Seizure (tonic clonic)	5 minutes	2	13	Leucovorin after ITT	
4	15	M	CNS 1	Standard	High-dose MTX, ITT	Consolidation course two	10	Stroke-like	72 hours	0 (low dose)	7	No	
5	4	M	CNS 1	Standard	ITT	Continuation week 12	7	Ataxia	4 weeks	0	8	Leucovorin after ITT	
6	10	F	CNS 1	Standard	High-dose MTX, ITT	Consolidation course three	8	Seizure (complex partial)	24 hours	1	11	Leucovorin after ITT	
7	11	M	CNS 2	Standard	High-dose MTX, ITT	Consolidation course one	11	Seizure (tonic clonic)	20 minutes	3	13	Leucovorin after ITT	
8	2	F	CNS 2	Low	Low-dose MTX, ITT	Continuation week 36	8	Seizure (complex partial)	24 hours	0	3	No	
9	16	M	CNS 1	Standard	ITT	Continuation week 13	9	Stroke-like	24 hours	0	8	Leucovorin after ITT	
10	14	F	CNS 1	Standard	High-dose MTX, ITT	Consolidation course one	7	Stroke-like	5 hours	1 (omit 2)‡	9	Aminophylline	Headache, confusion
11	5	M	CNS 1	Standard	ITT	Continuation week 29	7	Seizure (complex partial)	7 days	0	11	No	
12	12	M	CNS 1	Standard	High-dose MTX, ITT	Consolidation course one	10	Stroke-like	48 hours	3‡	11	Aminophylline	
13	18	M	CNS 1	Standard	High-dose MTX, ITT	Consolidation course one	10	Stroke-like (and seizure)	8 hours	3	20	No	Stroke (CNS thrombus)
14	17	F	CNS 1	Standard	Low-dose MTX, ITT	Continuation week 88	11	Stroke-like	36 hours	0	1	No	

Abbreviations: CSF, cerebrospinal fluid; ITT, triple intrathecal therapy; MTX, methotrexate; NA: not applicable.
*CNS1, < 5 WBC/μL of CSF without blasts; CNS2, < 5 WBC/μL of CSF with any blasts.
†Details on risk stratification described by Pui et al.[19]
‡Second high-dose MTX and ITT given 1-2 weeks apart.

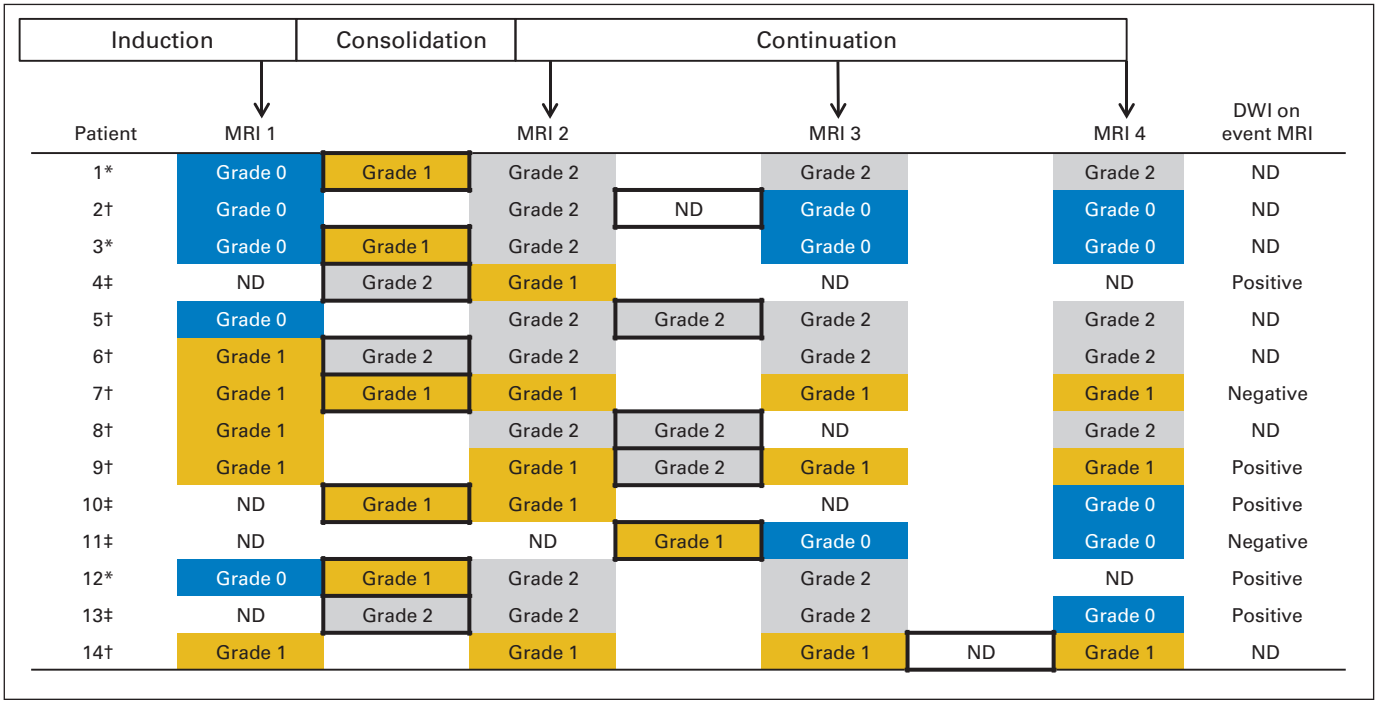

Fig 1. Grades of leukoencephalopathy in symptomatic patients at four screening time points. Boxes outlined in black indicate timing of neurotoxic event and grade of leukoencephalopathy in additional magnetic resonance imaging magnetic resonance imaging (MRI) obtained at time of the event. (*)No leukoencephalopathy on screening MRI before neurotoxic event (n = 3). (†) Leukoencephalopathy present on screening MRI before neurotoxic event (n = 7). (‡) Screening MRI not done (ND) before neurotoxic event (n = 4). DWI, diffusion weighted imaging.

MTX Rechallenge in Patients With Clinical Neurotoxicity

Thirteen patients were rechallenged with high-dose MTX or ITT. One patient (No. 2) was not rechallenged, because he required only one more dose of ITT. High-dose MTX was substituted with low-dose MTX (40 mg/m^2) for Patient No. 4. Two patients received aminophylline prophylaxis before subsequent high-dose MTX, and five patients received leucovorin rescue 24 and 36 hours after subsequent ITT. MTX-related neurotoxicity (severe headache and confusion) recurred in one patient (No. 10) when challenged with high-dose MTX. She did not receive additional high-dose MTX but received ITT with leucovorin rescue and experienced occasional headaches for the following 2 to 3 days. The other 12 patients tolerated MTX rechallenge well. Patient No. 13 developed a seizure 5.5 months after his first event but was found to have a CNS thrombus likely related to asparaginase.

Risk Factors for Clinical Neurotoxic Events

To identify risk factors, a logistic regression model with clinical and pharmacokinetic factors as explanatory variables was fitted (Table 2). Univariable analysis revealed that patients age $>$ 10 years were at higher risk for neurotoxic events than those age 1 to 10 years ($P = .003$). Patients in the standard/high-risk arm were also at higher risk for clinical neurotoxicity than those treated in the low-risk arm ($P = .016$). No risk factor retained significance in a multivariable model. The ratio of 42-hour MTX level to leucovorin dose was calculated for each consolidation course. This measure of MTX exposure relative to leucovorin did not vary significantly between patients with and without neurotoxicity.

Incidence of Leukoencephalopathy

Of 369 patients, 86 (23.3%) had evidence of leukoencephalopathy on at least one screening MRI. These included 73 (20.6%) of 355 asymptomatic patients and 13 (92.9%) of 14 patients with clinical neurotoxicity. Figure 2 shows the grades of leukoencephalopathy at various time points. No patient had radiographic grade 3 or 4 leukoencephalopathy. In asymptomatic patients, leukoencephalopathy was detected in 12.3% at MRI1, in 20.1% at MRI2, in 19.1% at MRI3, and in 15.9% at MRI4. Of 62 asymptomatic patients who developed leukoencephalopathy at any time during therapy and for whom MRI4 was available, leukoencephalopathy persisted in 46 (74.2%). In the 13 symptomatic patients with positive screening MRIs, leukoencephalopathy was detected in 50%, 100%, 72.7%, and 58.3% at the four time points, respectively. Thus, leukoencephalopathy was more prevalent in symptomatic versus asymptomatic patients at all four time points ($P < .001$). The presence of leukoencephalopathy on screening MRI1 and MRI2 indicated neurotoxic events with 50% and 100% sensitivity, respectively, but the positive predictive values were only 15.1% and 13.2% at the two time points, respectively (Data Supplement).

Natural History and Risk Factors for Leukoencephalopathy

To assess the natural history of leukoencephalopathy, we studied 74 patients with leukoencephalopathy for whom screening MRIs were available for at least three time points (Fig 3). In 30 patients (40.5%), the grade of leukoencephalopathy improved over time, including in 17 patients (23%) in whom leukoencephalopathy resolved completely. Leukoencephalopathy remained stable in 33 patients (46.6%) and worsened from grade 1 to 2 in 11 patients (14.9%). Thus, in the

Table 2. Association of Clinical and MTX Pharmacokinetic Parameters* With MTX-Related Clinical Neurotoxic Events and Leukoencephalopathy

MTX Toxicity	Summary Statistics			Univariable Logistic Regression			Multiple Logistic Regression		
	No.	Median	Range	OR	95% CI	*P*	OR	95% CI	*P*
					Clinical NT				
Age (1-10 *v* > 10 years)				0.19	0.06 to 0.57	.003	0.43	0.01 to 1.80	.249
No NT: 1-10 years	283	4.2	1.0-10.9						
NT: 1-10 years	6	4.3	2.1-10.0						
No NT: > 10 years	72	14.3	11.0-18.9						
NT: > 10 years	8	14.3	11.1-18.2						
Treatment arm (LR *v* SR/HR)				0.16	0.03 to 0.71	.016	0.42	0.06 to 2.91	.383
No NT: LR	183	NA	NA						
NT: LR	2	NA	NA						
No NT: SR/HR	172	NA	NA						
NT: SR/HR	12	NA	NA						
Total no. of triple IT therapies†				1.09	0.98 to 1.21	.127	1.08	0.93 to 1.26	.313
No NT	355	13	1.00-24.0						
NT	14	15	5.00-23.0						
Course one: homocysteine AUC > baseline (μM · hour)				1.02	0.96 to 1.08	.489	1	0.90 to 1.12	.963
No NT	347	10.5	0.00-73.2						
NT	13	15.1	0.00-22.5						
Course two: homocysteine AUC > baseline (μM · hour)				1.01	0.93 to 1.09	.886	0.95	0.82 to 1.09	.463
No NT	347	11.4	0.00-61.1						
NT	13	10.9	0.00-38.1						
Course one: ratio of 42-hour MTX to LV dose				1.07	0.88 to 1.31	.482	1.18	0.91 to 1.53	.212
No NT	317	6.2	0.47-16.2						
NT	13	6.7	1.84-11.6						
Course two: ratio of 42-hour MTX to LV dose				0.8	0.63 to 1.03	.082	0.82	0.58 to 1.15	.248
No NT	310	6.5	0.80-22.9						
NT	13	5.2	2.37-11.6						
Course three: ratio of 42-hour MTX to LV dose				0.95	0.76 to 1.19	.664	1.02	0.78 to 1.34	.892
No NT	309	6.6	1.62-18.2						
NT	11	6.7	2.38-10.8						
Course four: ratio of 42-hour MTX to LV dose				1.02	0.89 to 1.17	.766	1.05	0.91 to 1.20	.516
No NT	299	6.3	1.97-48.2						
NT	11	7.8	2.77-11.5						
					LE				
Age (1-10 *v* > 10 years)				1.59	0.34 to 1.02	.059	0.81	0.10 to 1.80	.556
No LE: 1-10 years	228	4.4	1.0-10.9						
LE: 1-10 years	61	3.5	1.2-10.8						
No LE: > 10 years	55	14.3	11.0-18.8						
LE: > 10 years	25	13.8	11.1-18.2						

(continued on following page)

Table 2. Association of Clinical and MTX Pharmacokinetic Parameters* With MTX-Related Clinical Neurotoxic Events and Leukoencephalopathy (continued)

MTX Toxicity	Summary Statistics			Univariable Logistic Regression			Multiple Logistic Regression		
	No.	Median	Range	OR	95% CI	*P*	OR	95% CI	*P*
Treatment arm (LR *v* SR/HR)				0.69	0.42 to 1.12	.133	0.83	0.41 to 1.65	.588
No LE: LR	149	NA	NA						
LE: LR	36	NA	NA						
No LE: SR/HR	134	NA	NA						
LE: SR/HR	50	NA	NA						
Total no. of triple IT therapies†				1.06	1.01 to 1.11	.023	1.07	1.00 to 1.15	.066
No LE	283	12	1.00-24.0						
LE	86	15	1.00-24.0						
Course one: homocysteine AUC > baseline (μM · hour)				1.04	1.00 to 1.07	.023	1.03	0.99 to 1.07	.145
No LE	276	10.1	0.00-44.8						
LE	84	11.7	0.00-73.2						
Course two: homocysteine AUC > baseline (μM · hour)				1.03	0.99 to 1.06	.132	0.98	0.93 to 1.04	.592
No LE	276	11.3	0.00-50.7						
LE	84	11.5	0.00-61.1						
Course one: ratio of 42-hour MTX to LV dose				1.1	1.00 to 1.21	.04	1.12‡	1.10 to 1.25	.038
No LE	256	6.1	0.47-16.2						
LE	74	6.7	1.18-15.5						
Course two: ratio of 42-hour MTX to LV dose				0.98	0.90 to 1.07	.635	0.97	0.87 to 1.08	.567
No LE	251	6.5	0.80-22.9						
LE	72	6.2	2.37-15.9						
Course three: ratio of 42-hour MTX to LV dose				1.01	0.92 to 1.11	.822	1.01	0.90 to 1.13	.889
No LE	249	6.6	1.62-17.0						
LE	71	6.7	1.86-18.2						
Course four: ratio of 42-hour MTX to LV dose				1.06	0.99 to 1.13	.118	1.05	0.98 to 1.13	.192
No LE	240	6.3	1.97-19.3						
LE	70	6.4	2.52-48.2						

NOTE. Additional clinical variables that were tested and were not significantly associated with clinical NT or LE in univariable analyses were sex, race, immunophenotype, CNS status, and leukocyte count at diagnosis.

Abbreviations: AUC, area under the curve; HR, high risk; IT, intrathecal; LE, leukoencephalopathy; LR, low risk; LV, leucovorin; MTX, methotrexate; NA, not applicable; NT, neurotoxicity (clinical); OR, odds ratio; SR, standard risk.

*Obtained during consolidation phase.

†Total No. of triple IT therapies before last screening magnetic resonance imaging.

‡OR > 1 indicates 1.12-fold higher risk of LE in those with higher ratio of plasma MTX (in μM) to LV dose (in mg/m^2 per total course).

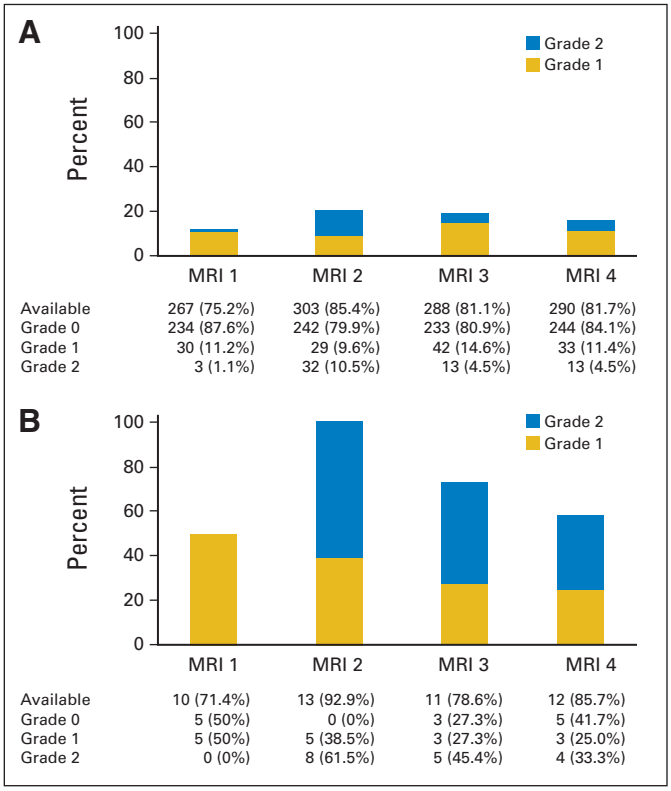

Fig 2. Number and percentage of patients with leukoencephalopathy at four screening time-points for (A) asymptomatic and (B) symptomatic patients. Incidence of leukoencephalopathy was highest after four doses of high-dose methotrexate (at time of magnetic resonance imaging [MRI] postconsolidation [MRI2]) and progressively decreased over time. At all four screening time points, incidence of leukoencephalopathy was higher in symptomatic than asymptomatic patients ($P < .001$). MRI1, MRI postinduction; MRI2, MRI postconsolidation; MRI3, MRI at continuation week 48; MRI4, MRI at continuation week 120.

majority (77%) who developed leukoencephalopathy in this subset of 74 patients, MRI abnormalities were still evident at week 120.

Table 2 and the Data Supplement show risk factors for developing leukoencephalopathy. In the univariable model, higher cumulative number of ITTs was associated with increased risk of leukoencephalopathy ($P = .023$). Clinical features were also analyzed at individual screening time points (Data Supplement). Higher MTX level at 42 hours (relative to leucovorin rescue) and higher homocysteine concentration (area under the curve over baseline) in course one were associated with increased risk of leukoencephalopathy. Of all risk factors, only the ratio of 42-hour plasma MTX concentration to leucovorin dose at the first course of high-dose MTX retained significance in a multivariable model ($P = 0{\cdot}038$).

GWAS

Genotypic analyses were adjusted for genetically determined ancestry, age, treatment arm, and CNS status. To identify SNPs related to leukoencephalopathy, genotype frequencies for patients with leukoencephalopathy (n = 85) versus those without leukoencephalopathy (n = 279) were analyzed on Affymetrix 500K/6.0 arrays. Of 347 SNPs associated with the presence of leukoencephalopathy ($P < .001$), 148 were annotated to genes. We also compared the SNP genotypes of 14 patients with clinical neurotoxicity with the genotypes of 350 asymptomatic patients. Of 206 SNPs associated with clinical neurotoxicity ($P < .001$), 103 were annotated to genes. Table 3 lists SNPs with P values $< .0001$ in both analyses. Pathway analyses of these genes per Gene Ontology biologic processes revealed over-representation of the neuron projection development pathway (GO:0031175; $P = .036$) and axon guidance pathway (GO:007411; $P = .047$). The Data Supplement provides results of association analyses of SNPs from the Illumina array (including candidate SNPs previously related to MTX disposition and toxicity).

DISCUSSION

To our knowledge, this study includes the largest cohort of patients with ALL in a contemporary therapeutic protocol who underwent serial radiologic screening for leukoencephalopathy to correlate radiographic findings with symptomatic neurotoxicity and to identify risk factors for leukoencephalopathy. Consistent with previous studies, MTX-related subacute neurotoxicity occurred in 3.8% of patients.[2,4] The incidence was as high as 19% in children receiving a regimen with suboptimal leucovorin rescue and correlated with the MTX dose to leucovorin ratio.[3,5] In our study, the ratio of 42-hour MTX level to leucovorin dose was not associated with increased risk of neurotoxic events, indicating that adequate leucovorin rescue may attenuate neurotoxic effects of MTX. Most episodes of clinical neurotoxicity were brief, and all but one patient were successfully rechallenged with high-dose MTX and/or ITT after resolution of symptoms. Aminophylline, via competitive inhibition of adenosine, is a candidate for secondary prophylaxis for MTX-related neurotoxicity, but the benefit of this modality is unclear.[1,28] The only patient with recurrent MTX-related symptoms in our cohort had received prophylaxis with aminophylline. In general, the most common modification made by physicians after the first MTX-related neurotoxic event is removal of MTX from ITT and administration of only hydrocortisone and cytarabine.[5] In our opinion, this is unnecessary, because our patients received one to 20 additional doses of ITT (some with leucovorin rescue) without recurrence of neurotoxicity.

At the time of the neurotoxic event, patients underwent diagnostic MRI, which almost always revealed at least grade 1 or 2 leukoencephalopathy, and most patients tolerated subsequent high-dose or intrathecal MTX. In addition, MRIs of 73 asymptomatic patients (20.6%) showed leukoencephalopathy. The sensitivity of MRI1 to predict clinical neurotoxicity was only 50%. Although screening MRI2 detected leukoencephalopathy in all patients who developed symptoms, it was not a good predictive tool, because eight of 14 patients developed symptoms before MRI2. Thus, leukoencephalopathy at the time of a neurotoxic event supports the diagnosis of MTX toxicity, but MRI screening is not useful to predict clinical neurotoxicity.

In prior studies, the incidence of asymptomatic leukoencephalopathy varied with type and timing of imaging, and cumulative dose of MTX, and ranged from 9% to 86% during active therapy.[15,29,30] For example, quantitative MRI segmentation techniques detected leukoencephalopathy in 86% of patients after six to seven courses of high-dose MTX (5 g/m^2).[31] However, these advanced imaging modalities are not widely available and are used primarily in research settings. Segmentation was not used in our study. The absence of a true baseline MRI is a limitation of our study. The first MRI was performed after window MTX and up to three doses of ITT. It was not feasible to

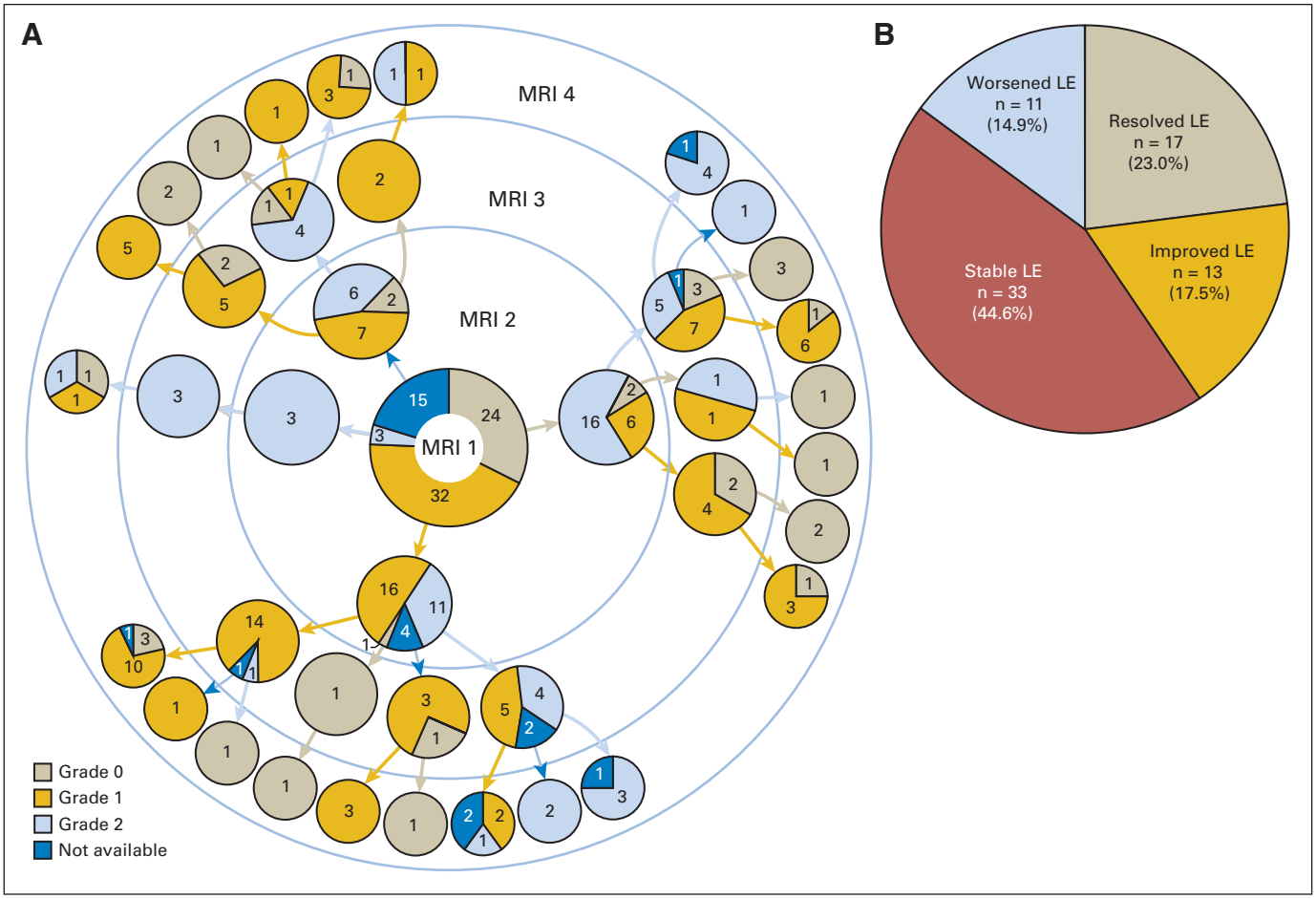

Fig 3. Natural history of leukoencephalopathy (LE) until completion of therapy. Magnetic resonance imaging (MRI) available for 74 patients with LE at minimum of three time points. (A) Detailed grading of MRIs for individual patients at four screening time points. Numbers of patients and grades of LE are indicated in pie charts. (B) Over time, MRI changes of LE resolved, improved, worsened, or were stable in 23%, 17.5%, 14.9%, and 44.6% of patients, respectively. MRI1, MRI postinduction; MRI2, MRI postconsolidation; MRI3, MRI at continuation week 48; MRI4, MRI at continuation week 120.

schedule baseline neuroimaging before initiation of therapy. However, three of 10 patients who developed neurotoxic symptoms had negative MRIs after their first dose of high-dose MTX but before their neurotoxic event, so a negative MRI cannot be assumed to presage a lack of neurotoxicity.

Leukoencephalopathy developed in 23.3% of all patients and 20.6% of asymptomatic patients. The incidence (especially grade 2) was highest after four doses of high-dose MTX and gradually decreased over time. Of those who developed leukoencephalopathy, 69% had persistent abnormal findings on MRI at the end of therapy. A longer follow-up period is required to monitor for resolution and study impact of leukoencephalopathy on long-term adverse effects, especially neurocognitive functioning. Although there was a higher risk (odds ratio, 1.12) for leukoencephalopathy associated with a higher 42-hour MTX to leucovorin ratio at consolidation course one, the clinical importance of leukoencephalopathy is unclear. Although this metric was not significantly associated with symptomatic neurotoxicity, the number of neurotoxic events was low, and the direction of association and magnitude of the odds ratio (1.18) of the MTX to leucovorin ratio was similar. Interestingly, the association of MTX/leucovorin was most evident with the first course of high-dose MTX of consolidation, suggesting that after that time, perhaps leucovorin dosing was adequate to decrease neurotoxicity or leukoencephalopathy associated with high-dose MTX. None of the patients in this study developed severe leukoencephalopathy ($\geq$ grade 3), which may be partly explained by the omission of prophylactic cranial irradiation. In addition, all patients received a minimum of five doses of leucovorin, and most doses of MTX in consolidation were targeted to achieve desired concentrations, thereby avoiding high plasma MTX concentrations.[21] Some other groups have included only three doses of leucovorin after fixed doses of high-dose MTX.[32]

We identified several SNPs that strongly influence the risk of leukoencephalopathy and/or symptomatic neurotoxicity. None of the SNPs reached genome-wide significance ($P < 5.0 \times 10^{-8}$), likely because of limited sample size and lack of patients with severe leukoencephalopathy. Of significant SNPs ($P < .0001$) that were annotated to known genes, 73% (eight of 11) were in genes important for neurogenesis. *TRIO*, *PRKG1*, *ANK1*, *COL4A2*, *NTN1*, and *ASTN2* are involved in neuronal development and migration and/or axon guidance.[33-37] *SSPN* is implicated in glial cell death[38] and *DKK2* in Wnt/β-catenin signaling, which influences neural development.[39] In the absence of a validation cohort and functional studies, findings of

Table 3. SNPs on Affymetrix Arrays Associated With Leukoencephalopathy and Clinical Neurotoxicity*

SNP ID	Chromosome	Position	Gene	MAF	Risk Allele	OR	95% CI	*P*
				Leukoencephalopathy				
rs4145201	6	17007422	—	0.33	C	0.42	0.29 to 0.61	5.14E−06
rs556269	1	164839754	*FMO9P*†	0.41	T	2.31	1.60 to 3.34	7.79E−06
rs32571	5	14227106	*TRIO*	0.48	C	0.43	0.29 to 0.63	1.73E−05
rs7590550	2	202266660	*MPP4*	0.16	G	2.65	1.69 to 4.15	2.28E−05
rs245311	5	127194498	*LOC728586*	0.29	T	2.72	1.70 to 4.35	2.88E−05
rs10842702	12	26331326	*SSPN*	0.35	C	0.44	0.30 to 0.65	3.03E−05
rs33005	5	14259537	*TRIO*	0.49	G	0.45	0.30 to 0.65	3.20E−05
rs9545873	13	81288492	—	0.42	T	2.09	1.47 to 2.97	3.75E−05
rs1904006	10	53479033	*PRKG1*	0.17	C	0.37	0.23 to 0.60	4.03E−05
rs6632675	X	13245592	—	0.18	C	0.48	0.34 to 0.68	4.08E−05
rs2065920	1	164869647	*FMO9P*†	0.36	C	0.48	0.34 to 0.69	4.46E−05
rs5762295	22	26373959	—	0.03	G	8.11	2.97 to 22.2	4.52E−05
rs1465614	2	16516774	—	0.24	C	0.42	0.28 to 0.64	4.90E−05
rs16985255	22	26371883	—	0.03	G	7.00	2.73 to 17.9	5.03E−05
rs1448686	8	137104691	—	0.41	A	0.47	0.32 to 0.68	5.66E−05
rs17584752	4	108120536	*DKK2*	0.19	C	0.42	0.28 to 0.64	5.71E−05
rs11986485	8	41659544	*ANK1*	0.29	C	0.38	0.24 to 0.61	6.55E−05
rs13267761	8	138930469	*FLJ45872*	0.27	C	0.46	0.31 to 0.67	6.83E−05
rs9466410	6	22722709	—	0.25	C	0.35	0.20 to 0.58	7.17E−05
rs6840582	4	122669925	*LOC729112*	0.28	G	2.59	1.62 to 4.15	7.27E−05
rs7320755	13	109829093	*COL4A2*	0.32	C	0.45	0.31 to 0.67	7.49E−05
rs6841032	4	122670192	*LOC729112*	0.29	G	2.59	1.61 to 4.16	7.97E−05
rs9936750	16	53729375	—	0.18	G	2.48	1.58 to 3.90	8.13E−05
rs11185944	10	91731927	*LOC119358*	0.35	G	2.06	1.44 to 2.96	8.51E−05
rs1034893	17	9013280	*NTN1*	0.17	A	0.27	0.14 to 0.52	8.92E−05
rs17133261	5	100579903	—	0.07	T	3.71	1.92 to 7.18	9.80E−05
				Clinical Neurotoxicity				
rs12379211	9	119127189	*ASTN2*	0.08	C	0.11	0.04 to 0.32	3.65E−05
rs226945	6	3662178	—	0.09	T	11.7	3.57 to 38.2	4.79E−05
rs17626001	14	42139233	—	0.07	C	0.09	0.03 to 0.28	5.17E−05
rs226962	6	3669960	*PXDC1*	0.09	A	0.07	0.02 to 0.25	5.32E−05
rs682518	6	150770134	*IYD*	0.08	G	7.66	2.81 to 20.9	6.84E−05
rs665670	6	150775949	—	0.14	T	9.17	3.03 to 27.7	8.70E−05
rs7887242	X	20931587	—	0.18	T	4.33	2.08 to 9.03	9.13E−05
rs10846690	12	123652370	—	0.13	T	9.84	3.13 to 31.0	9.28E−05
rs10886214	10	85117719	—	0.21	T	8.43	2.89 to 24.6	9.64E−05

Abbreviations: MAF, minor allele frequency; OR, odds ratio; SNP, single-nucleotide polymorphism.
*$P < .0001$.
†Pseudogene.

the GWAS remain speculative. Although consequences of these polymorphisms and the molecular mechanism of neurotoxicity are unclear, over-representation of genes involved in neurogenesis points to plausible mechanisms linking these variants with neurotoxicity. For example, viral-induced neuronal cell death can be mediated through *TRIO* signaling[40] and hypoxia-induced glial cell death by downregulation of *SSPN*.[38] SNPs in *ASTN2* are associated with autism,[41] migraine,[42] and attention-deficit hyperactivity disorder (ADHD)[43] and SNPs in *PRKG1* with ADHD[44] and Alzheimer's disease.[45] Interestingly, seven of 14 patients with clinical neurotoxicity in our cohort were also diagnosed with ADHD (four before ALL diagnosis, three post-therapy). Of these seven patients, six had inherited the risk allele (C) in rs12379211 in *ASTN2*. Because of the small number of patients and incomplete data on ADHD diagnoses in all patients, the significance of this association requires validation. Childhood ALL survivors are at risk of neurocognitive impairments, particularly attention disorders.[46] A recent investigation using a candidate SNP approach reported association of attention problems in survivors with polymorphisms in genes involved in oxidative stress and CNS integrity.[47] Additional studies will investigate whether patients who develop leukoencephalopathy during therapy are at higher risk than others for developing neurocognitive impairments.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Final approval of manuscript: All authors

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GLOSSARY TERMS

Genome-wide association study: Hypothesis-free study that evaluates the association of genetic variations throughout the entire genome with traits, using high-throughput genotyping technologies to assay single-nucleotide polymorphisms.

Magnetic resonance imaging: A procedure in which radio waves and a powerful magnet linked to a computer are used to create detailed pictures of areas inside the body. These pictures can show the difference between normal and diseased tissue.

Single-nucleotide polymorphism: Natural variations in the genomic DNA sequence present in greater than 1% of the population, with single-nucleotide polymorphisms representing DNA variations in a single nucleotide. Single-nucleotide polymorphisms are being widely used to better understand disease processes, thereby paving the way for genetic-based diagnostics and therapeutics.

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