ARTICLE

Methotrexate-related central neurotoxicity: clinical characteristics, risk factors and genome-wide association study in children treated for acute lymphoblastic leukemia

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ABSTRACT

Symptomatic methotrexate-related central neurotoxicity (MTX neurotoxicity) is a severe toxicity experienced during acute lymphoblastic leukemia (ALL) therapy with potential long-term neurologic complications. Risk factors and long-term outcomes require further study. We conducted a systematic, retrospective review of 1,251 consecutive Australian children enrolled on Berlin-Frankfurt-Münster or Children's Oncology Group-based protocols between 1998-2013. Clinical risk predictors for MTX neurotoxicity were analyzed using regression. A genome-wide association study (GWAS) was performed on 48 cases and 537 controls. The incidence of MTX neurotoxicity was 7.6% (n=95 of 1,251), at a median of 4 months from ALL diagnosis and 8 days after intravenous or intrathecal MTX. Grade 3 elevation of serum aspartate aminotransferase (P=0.005, odds ratio 2.31 [range, 1.28–4.16]) in induction/consolidation was associated with MTX neurotoxicity, after accounting for the only established risk factor, age ≥10 years. Cumulative incidence of CNS relapse was increased in children where intrathecal MTX was omitted following symptomatic MTX neurotoxicity (n=48) compared to where intrathecal MTX was continued throughout therapy (n=1,174) (P=0.047). Five-year central nervous system relapse-free survival was 89.2±4.6% when intrathecal MTX was ceased compared to 95.4±0.6% when intrathecal MTX was continued. Recurrence of MTX neurotoxicity was low (12.9%) for patients whose intrathecal MTX was continued after their first episode. The GWAS identified single-nucleotide polymorphism associated with MTX neurotoxicity near genes regulating neuronal growth, neuronal differentiation and cytoskeletal organization ($P<1x10^{-6}$). In conclusion, increased serum aspartate aminotransferase and age ≥10 years at diagnosis were independent risk factors for MTX neurotoxicity. Our data do not support cessation of intrathecal MTX after a first MTX neurotoxicity event.

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Introduction

Methotrexate-related central neurotoxicity (MTX neurotoxicity) occurs in 3-7% of children treated for childhood acute lymphoblastic leukemia (ALL)[1-3] and is characterized by seizures, stroke-like symptoms, speech disturbances and encephalopathy.[4] Although commonly encountered during clinical practice, questions remain relating to risk factors, choice of further intrathecal (IT) chemotherapy following MTX neurotoxicity and long-term neurological outcomes, which are not well defined.

Previously described risk factors for symptomatic MTX neurotoxicity include older age[1,3,5] and an interaction with cytarabine and cyclophosphamide treatment blocks.[5] Upon re-exposure to MTX in children with ALL, MTX neurotoxicity recurs in 7-24% of cases.[1,3,5] However, there is a paucity of data on central nervous system (CNS) relapse rates if CNS-directed therapy is modified following MTX neurotoxicity.[6] Genomic risk predictors may relate to neuronal development, MTX clearance or folate metabolism by-products such as homocysteine.[1,7-11]

Here we sought to analyze clinical and germline DNA factors that may predict susceptibility to MTX neurotoxicity during treatment for childhood ALL. Additionally, we assessed the incidence of neurological sequelae following an episode of MTX neurotoxicity, and, the impact that changes made to therapy following MTX neurotoxicity had on subsequent relapse risk.

Methods

We conducted a national Australian retrospective review of children diagnosed with ALL or lymphoblastic lymphoma (LBL). Consecutive patients treated on frontline Berlin-Frankfurt-Munster (BFM) or Children's Oncology Group (COG) ALL protocols between 1998 and 2013, were eligible for inclusion (see the *Online Supplementary Appendix*). The cohorts were assembled as part of the ERASE (Evaluation of Risk of ALL treatment-related Side-Effects) study.[12] Complete clinical data were collected for 1,251 children (*Online Supplementary Figure S1*). The study was approved by Hunter New England Human Research Ethics Committee (HNEHREC reference number: 12/11/21/4.01).

We defined MTX neurotoxicity as symptomatic neurotoxicity, with or without leukoencephalopathy, temporally related to intravenous (IV) or IT MTX, where MTX was deemed clinically or through record review as the most likely cause, and where other causes had been reasonably excluded. Symptomatic neurotoxicity included motor deficits, speech deficits, visual disturbances, seizures and altered level of consciousness. Toxicity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.03.[13]

Long-term neurological outcomes occurring after ALL/LBL diagnosis, focusing on epilepsy, were collected on all patients where available (n=522).

Statistical analysis was performed using IBM SPSS for Macintosh, Versions 23.0/24.0 (see the *Online Supplementary Appendix*). Overall survival (OS) was computed from date of diagnosis through to date of last contact or death from any cause. Event-free survival (EFS) was assessed as time from diagnosis to first event or last contact date in first complete remission (CR1). An event was defined as relapse, death from any cause or secondary malignancy. Leukemia-free survival (LFS) was determined from date of remission to first relapse or last contact date in CR1. CNS relapse was defined as leukemic relapse involving the CNS, either as an isolated CNS or combined CNS relapse.

Thirty-seven variables were assessed in univariate logistic regression. Factors from univariate logistic regression analysis with a *P*-value <0.0014 were considered significant, using Bonferroni correction for multiple testing. Variables associated with MTX neurotoxicity (unadjusted $P<0.10$) were further assessed in multivariable regression analysis, adjusting for gender (see the *Online Supplementary Appendix*).

Table 1. Baseline demographics.

Diagnostic information	Number (n=1,251)	% of cohort
Male	696	55.64
DIAGNOSIS		
Pre-B-ALL	1,068	85.37
B-lymphoblastic lymphoma (B-LBL)	14	1.12
T-ALL	110	8.79
T-lymphoblastic lymphoma (L-LBL)	39	3.12
Other (ALL/LBL, not specified)	20	1.6
TREATMENT PROTOCOL		
BFM-based protocols	*(n=1,033)*	
ANZCHOG Study 7 (1998-2002)[36]	239	19.10
BFM-95 (1998-2002)[37]	125	9.99
ANZCHOG Study 8 (2002-2012)[38]	608	48.60
COG A5971 (2003-2009)[39]	21	1.68
iBFM-Study 9 (2012-2016)[40]	40	3.20
COG-based protocols	*(n=218)*	
CCG1882 1991-1995)[41]	1	0.08
CCG1952 (1996-2000)[42]	16	1.28
CCG1961 (1996-2002)[43]	36	2.88
CCG1991 (2000-2005)[44,45]	54	4.32
AALL0031 (2002-2006)[46]	2	0.16
AALL0232 (2004-2011)[47]	49	3.92
AALL0331 (2005-2010)[48]	25	2.00
AALL0434 (2007-2014)[45,48]	12	0.96
AALL08P1 (2009-2011)[49]	2	0.16
AALL0932 (2010 - 2018)[45]	17	1.36
AALL1131 (2012 –[part-closure])[45]	4	0.32

B-ALL: B-cell acute lymphoblastic leukemia; T-ALL: T-cell acute lymphoblastic leukemia; B-LBL: B-cell lymphoblastic lymphoma; BFM: Berlin-Franfurt-Münster; COG: or Children's Oncology Group.

Genome-wide association study methods

Genotyping was conducted on the Illumina Infinium OncoArray-530K Beadchip (533,000 single-nucleotide polymorphisms [SNP]), using stored bone marrow or peripheral blood DNA, collected in remission[14] from children treated on BFM-based protocols.

Of the 1,021 patients treated using BFM-based therapy, 932 had an available banked DNA sample. After quality control and filtering (see the *Online Supplementary Appendix*), the resultant cohort included 707 individuals of European ancestry, as previously described.[15] Children who experienced neurotoxicity, either central or peripheral, other than MTX neurotoxicity were excluded (n=122), but those who experienced MTX neurotoxicity in addition to another type of neurotoxicity were included. The resultant genome-wide association study (GWAS) cohort of 585 patients included 48 cases and 537 controls (*Online Supplementary Figure S2*). After filtering for information score >0.4, the total number of

Table 2. **Clinical risk factors significantly associated with methotrexate neurotoxicity in regression analysis.**

Clinical Risk Factor	Univariate			Multivariate		
	P	OR	95% CI	*P*	OR	95% CI
Age at diagnosis (continuous)	<0.001	1.01	1.006-1.014			
Age≥10 years	<0.001	2.65	1.69-4.14	0.003	2.43	1.35-4.38
HR/VHR treatment[a]	0.003	2.05	1.29-3.28			
Bilirubin at diagnosis	0.026	1.03	1.003-1.05			
Peak bilirubin[b]	0.04	1.01	1.000-1.014			
Peak bilirubin > Grade 3[b]	0.03	2.6	1.10-6.13			
Peak ALTb	0.029	1.001	1.000-1.002			
Peak ALT > Grade 3[b]	0.043	1.62	1.02-2.57			
Peak AST[b]	0.018	1.001	1.000-1.002			
Peak AST > Grade 3[b]	0.004	2.38	1.33-4.26	0.005	2.31	1.28-4.16
Positive blood culture[b]	0.011	1.81	1.15-2.85			
Peak creatinine > 2 x baseline and abnormal[b]	0.005	3.27	1.44-7.45			
Insulin requirement[b]	<0.001	4.39	2.36-8.19			
Hyperglycemia[b]	0.001	2.42	1.43-4.10			

Factors that were associated with methotrexate (MTX) neurotoxicity in logistic regression analysis at a significance level $P<0.05$ (2-tailed) are listed. [a]Comparison was made between a combined high-risk cohort (high-risk "HR" and very high-risk "VHR" ALL) *versus* a combined non high-risk cohort (standard and medium risk ALL). [b]In induction/consolidation. ALT: alanine aminotransferase; AST: aspartate aminotransferase; OR: odds ratio; CI: Confidence Interval. ALT and AST elevation > grade 3 refers to levels >5x upper limit of normal (ULN). Bilirubin >grade 3 is >3x ULN based on NCI CTCAE v4.03 criteria. Significance levels were set at $P<0.0014$ and $P<0.05$ for univariate and multivariate analyses respectively.

SNP for evaluation in the GWAS for MTX neurotoxicity were 10,838,245. Imputation was conducted on 388,439 SNP (including SNP on the X chromosome) using IMPUTE2.16 GWAS was conducted correlating potential SNP with MTX neurotoxicity, using age, sex and principal components as covariates.

Results

Incidence of methotrexate neurotoxicity

The clinical cohort consisted of 1,251 children with ALL/LBL (*Online Supplementary Figure S1*) from six tertiary Australian pediatric oncology centers. There were 1,033 children treated on BFM-based protocols and 218 on COG-based protocols (Table 1). The median age at diagnosis was 59 months (range, 9-218 months) with a median duration of follow-up of 78 months (range, 3-186 months). Five-year OS, LFS and EFS were 92±0.8%, 85.6±1.1% and 83.8±1.1% respectively.

Comparative IT, IV MTX and leucovorin (folinic acid) doses according to each protocol are listed in the *Online Supplementary Appendix* and the *Online Supplementary Table S1*. IT MTX was given during induction, consolidation, CNS-directed phases, reinduction, reconsolidation and in some protocols IT MTX continued in maintenance. There were a varying number of IT MTX doses in induction. In consolidation, all protocols used IT MTX, while most used cyclophosphamide and cytarabine. Age-directed doses varied between protocols for children aged 9 years and older. For protocols that used IV MTX, doses varied considerably and could be divided into protocols that administered low (0-2.5 g/m^2 total IV MTX doses) and high (20-35 g/m^{2}total IV MTX dose) doses.

Ninety-five children (7.6% of the cohort) fulfilled the criteria for MTX neurotoxicity. Ninety-one patients experienced MTX neurotoxicity within 21 days following IV or IT MTX (*Online Supplementary Table S2*). Of four patients with MTX neurotoxicity and leukoencephalopathy >21 days following MTX, three patients had typical symptoms and leukoencephalopathy diagnosed at 22, 30 and 47 days post MTX, while one patient had seizures and leukoencephalopathy 56 days after MTX. Further information is contained in the *Online Supplementary Table S2*. All ninety-five patients were included in subsequent analyses.

The median time from ALL diagnosis to onset of MTX neurotoxicity was 4 months (range, 0-19 months, interquartile range [IQR], 2-6 months), occurring at a median of 8 days following IV or IT MTX administration (range, 0-56 days, IQR, 5-11 days).

The first episode of MTX neurotoxicity most frequently occurred after induction/consolidation in 55.8% (n=53 of 95), while 44.2% occurred during induction/consolidation. Specific timing of these events is shown in the *Online Supplementary Figure S3*. MTX neurotoxicity first events were associated with co-administration of IT MTX, cyclophosphamide and cytarabine in 53.2% patients (n=50 of 94; one patient unknown). In comparison, 23.2% (n=22 of 95) of first events were associated with concurrent IV MTX and IT MTX administration. Grade 1 neurotoxicity occurred in 4.2% of the cohort (n=4); grade 2 in 62.1% (n=59); grade 3 in 31.6% (n=30) and grade 4 in 2.1% (n=2).

Risk factors for methotrexate neurotoxicity - clinical risk factors

Clinical variables were assessed for association with MTX neurotoxicity in univariate logistic regression (Table 2 for those associated $P<0.05$, *Online Supplementary Appendix*). Variables with a *P*-value of association <0.0014 were considered significant, taking into account 37 variables as per the Bonferroni method of adjustment for multiple testing ($P<0.05/37$). Factors listed in Table 2 were analyzed further in a multivariable regression analysis. This demonstrated that age ≥10 years and peak serum AST during induction/consolidation were independent risk associations for MTX neurotoxicity (Table 2). Overall, 56.2% (n=703 of 1,251) of the cohort were available for multivariable analysis, due to availability of AST values during induction/consolidation. These two risk variables

were associated with 48.4% of MTX neurotoxicity events. There was no association between body mass index (BMI) Z scores and neurotoxicity, using BMI from diagnosis (P=0.133), end of induction (P=0.788), or end of consolidation (P=0.375).

Central nervous system disease at diagnosis

Leukemic involvement of the CNS at diagnosis was not significantly higher for children who experienced MTX neurotoxicity (P=0.414, Pearson χ^2) (*Online Supplementary Table S3*).

Dosing of methotrexate

MTX levels at 24, 48 or 54 hours following a first course of HD MTX did not correlate with incidence of MTX neurotoxicity (P=0.964, P=0.81 and P=0.866 respectively). Incidence of delayed MTX excretion is outlined in the *Online Supplementary Appendix*.

There was a significantly higher incidence of MTX neurotoxicity on protocols with a lower cumulative IV MTX dose (total dose 0-2.5 g/m^2, n=20 of 148, 13.5%) compared to protocols with a higher cumulative IV MTX dose (20-35 g/m^2, n=69 of 863, 8.0%, P=0.031, odds ratio [OR] 1.8, 95% Confidence Interval [CI]: 1.06-3.06). This difference remained when stratified for high-risk protocols, with cumulative incidence of MTX neurotoxicity of 33% (n=11 of 33) with lower dose IV MTX protocols compared to 9.7% (n=13 of 134) with higher-dose MTX protocols respectively (P<0.001).

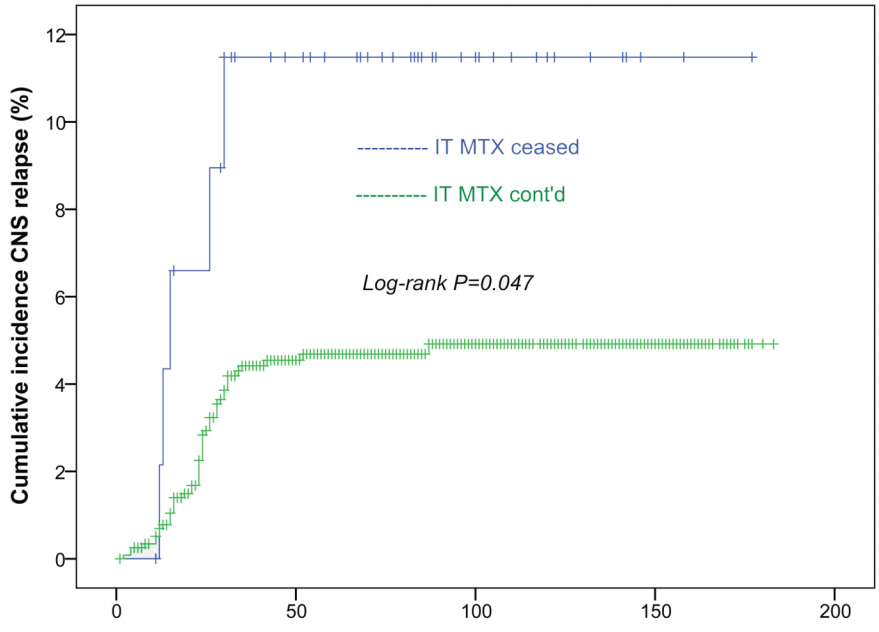
Other potential differences, such as use of vincristine during the IV MTX phases on COG-protocols, compared to BFM-based protocols which do not use vincristine during the IV MTX phases (P=0.792), use of vincristine during consolidation *versus* no vincristine (P=0.50) or higher age-adjusted IT MTX doses for children aged ≥9 years, were not significantly associated with MTX neurotoxicity (P=0.778).

Outcome following first episode of methotrexate neurotoxicity

We assessed the incidence of several clinical outcomes: including rates of CNS relapse, LFS, recurrent MTX neurotoxicity and long-term neurological outcomes in children who experienced a first episode of MTX neurotoxicity. Out of 95 patients who experienced MTX neurotoxicity, 48 (50.5%) had subsequent IT MTX permanently discontinued, 34 (35.8%) had IT MTX continued, and one patient developed MTX neurotoxicity following their last scheduled IT MTX dose. Subsequent IT management could not be determined for 12 (12.6%) cases. In patients where IT MTX was permanently discontinued, subsequent IT therapy included cytarabine alone (n=23), cytarabine and hydrocortisone (n=17), no further IT agents (n=2, in maintenance and Protocol M respectively), and in six patients the alternative IT regime was unknown.

We examined CNS relapse risk in patients who experienced MTX neurotoxicity, and assessed the impact of IT MTX continuation on CNS relapse rates. Cases were excluded if IT MTX management was unknown following neurotoxicity (n=12), where no further IT MTX doses were scheduled as per protocol (n=1), or where IT MTX was ceased due to seizures from a different etiology (n=2).

In the overall cohort where IT MTX was continued, 49 of 1.178 children experienced a CNS-based relapse (4.2%).



Number at risk										Events
Time (months)	0	25	50	75	100	125	150	175	200	
IT MTX ceased	48	43	33	24	14	8	2	1	0	5
IT MTX cont'd	1174	1011	724	551	338	207	89	9	0	49

Figure 1. Cumulative incidence of central nervous system relapse, according to intrathecal methotrexate strategy. Children who had intrathecal (IT) methotrexate (MTX) omitted permanently following symptomatic MTX neurotoxicity (n=48), had an increased risk of central nervous system (CNS) relapse compared to children who had IT MTX continued through ALL treatment (n=1,174) (P=0.047). Five-year CNS relapse-free survival was 95.4±0.6% when IT MTX was continued compared to 89.2±4.6%, when IT MTX was ceased.

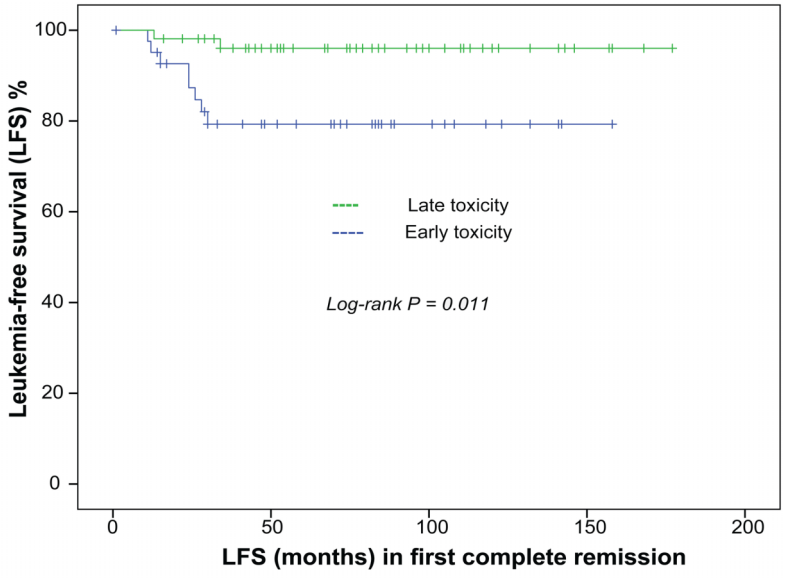
Six relapses occurred among children who had IT MTX permanently ceased following MTX neurotoxicity (n=48), and five of 48 were CNS-based relapses (10.4%). In 34 patients where IT MTX was continued following MTX neurotoxicity, there was one isolated CNS relapse (2.9%).

Cumulative incidence of CNS relapse was increased in children where IT MTX was omitted permanently following symptomatic MTX neurotoxicity (n=48) compared to

Table 3. Top single-nucleotide polymorphisms for methotrexate neurotoxicity with significance levels $P<1x10^{-6}$.

CHR	Position	SNP	Non effect allele	Effect allele	MAF	*P*	OR	OR 95% CI (lower)	OR 95% CI (upper)	Gene[a]	Location	Gene function
6	20196934	rs4712462	A	G	0.35	2.54E-07	0.26	0.16	0.45	*MBOAT1*	intron	Involved in fatty acid biomembrane synthesis. Possible role in neuronal growth and differentiation. siRNA-mediated knockdown of *MBOAT1* leads to reduced neurite growth[26]
19	14590919	rs2241357	G	A	0.2	3.60E-07	4.18	2.41	7.24	*GIPC1*	intron	Encodes for *GIPC1*, a scaffolding protein that regulates cell surface receptor dynamics in endothelial cells. Microdeletionsat this locus (19p13.12) have been reported[25]
3	195925355	rs1106479	C	T	0.16	4.08E-07	3.97	2.33	6.75	*ZDHHC19*	intron[b]	Involved in post-translational protein modification that alters trafficking, activity and localization of lipidated proteins, such as R-Ras[29]
17	747700	rs35307996	GC	G	0.2	5.70E-07	0.11	0.03	0.34	*NXN*	intron	Encoded protein acts as a redox-dependent regulator of the Wnt signaling pathway, exerting effects via dishevelled (dvl), and is involved in cell growth and differentiation[27]
19	14571966	rs74956940	C	G	0.23	6.19E-07	3.58	2.17	5.92	*PKN1*	intron	Located at 19p13.12. Encodes a protein that is involved in neuronal function and neuroprotection. siRNA-mediated knockdown of *PKN1* led to neuron apoptosis and inhibition of neurite formation[28]
9	124286453	rs62576054	G	C	0.18	7.50E-07	16.7	3.92	71.13	*HMGB1P37*	unknown	*HMGB1* family of genes contain an HMG-box domain that bends DNA, affects transcription and facilitates DNA-protein interactions[50]
13	95072136	rs9590003	G	A	0.11	9.73E-07	5.24	2.73	10.07	–	–	

This table shows the top seven single-nucleotide polymorphisms (SNP) that associate with methotrexate (MTX) central neurotoxicity at significance level $P<1x10^{-6}$, ordered by *P*-value for significance. SNP with a minor allele frequency (MAF) <1% were excluded. [a]The annotated gene was determined by cross-referencing relevant genomic databases (see the *Online Supplementary Appendix*). [b]intron (downstream variant/nc transcript variant). SNP with a minor allele frequency (MAF) <1% were excluded. Odds ratio (OR) 95% Confidence Interval (CI) (lower) and OR 95% CI (upper) refer to lower value and upper value for 95% CI for odds ratio. *P*-value from genome wide association study, where $P<5x10^{-6}$ is significant. CHR: chromosome; siRNA: small interfering RNA.



Number at risk										Events
Time (months)	0	25	50	75	100	125	150	175	200	
Early toxicity	42	33	24	17	9	4	1	0	0	8
Late toxicity	53	50	38	28	17	10	4	1	0	2

Figure 2. Timing of methotrexate neurotoxicity and risk of leukemic relapse. There is a significantly increased rate of leukemic relapse in children who experienced methotrexate (MTX) neurotoxicity early in therapy (induction/consolidation) compared to later in therapy (after consolidation, *P*=0.011). Five-year leukemia-free survival (LFS) in children who experienced early MTX neurotoxicity was 79.3±6.5% compared to 96±2.8% for children who experienced late MTX neurotoxicity.

children who had IT MTX continued through ALL treatment (n=1.174 with available LFS data) (*P*=0.047) (Figure 1). Five-year CNS relapse-free survival was 95.4±0.6% when IT MTX was continued compared to 89.2%±4.6% when IT MTX was ceased.

For children who experienced MTX neurotoxicity and had IT MTX ceased (n=48) or continued (n=34), there was no difference in CNS status (*P*=0.536) or age (age <10 *vs.* ≥10 years, *P*=0.560) (*Online Supplementary Table S4*). There was a difference in risk group status, however regression analysis did not show an impact of risk group on incidence of CNS relapse among the whole cohort (*P*=0.291, HR for high-risk group 0.72, 95% CI: 0.39-1.32). Age ≥10 years was also not associated with risk of CNS relapse (*P*=0.56, HR 0.83, 95% CI: 0.45-1.55). Distribution of MTX neurotoxicity by protocols and cases evaluable for recurrent MTX neurotoxicity are outlined in the *Online Supplementary Table S5*.

In the group where IT MTX was ceased, two of 48 had HD MTX ceased, three of 48 had HD MTX reduced, two of 48 had asparaginase ceased after neurotoxicity, and one of 48 had dexamethasone ceased in maintenance after development of osteonecrosis.

Overall, there were ten relapses in patients who experienced MTX neurotoxicity, six of whom had IT MTX ceased (and one of these patients also had HD MTX ceased). There were no other treatment modifications in these patients who experienced relapse. Specifically, in HR patients who experienced relapse (n=4), two had IT MTX ceased, two had IT MTX continued, and there were no other treatment alterations. Radiotherapy details are contained in the *Online Supplementary Appendix*.

Recurrent neurotoxicity

Six patients had recurrent neurotoxicity events, four of whom had received further IT MTX and two where ongoing IT MTX exposure could not be determined. An additional three patients who had been re-exposed to MTX did not have enough clinical information available to determine whether a subsequent neurotoxicity episode occurred. Therefore, recurrence of MTX neurotoxicity in evaluable patients occurred in 12.9% (n=4 of 31) upon IT MTX re-exposure. Three of the four patients did not receive any further IT MTX following recurrent MTX neurotoxicity. Further information regarding recurrent neurotoxicity events is shown in the *Online Supplementary Tables S5* and *S6*.

For patients who were rechallenged with IT MTX, six patients were treated according to protocols that suggest oral leucovorin with IT rechallenge, and 28 were treated on protocols without that recommendation. Of those patients evaluable for recurrent MTX neurotoxicity, there was one recurrence out of five patients who were treated on protocols that recommended oral leucovorin; and three recurrences out of 26 patients who were treated on protocols that did not recommend oral leucovorin with IT rechallenge.

Of the patients who had IT MTX ceased after a first MTX neurotoxicity event, 95.8% (n=46 of 48) did not experience further neurotoxicity episodes. One patient experienced unusual ataxic episodes during a phase where oral MTX was administered, however further clinical information was not available and in the other patient the information was unknown.

Methotrexate neurotoxicity: negative impact of occurrence during early therapy

Those who experienced MTX neurotoxicity early (during induction/consolidation, n=42) had a higher subsequent risk of leukemia relapse (all sites) compared to those

who experienced late MTX neurotoxicity after consolidation (n=53, *P*=0.011, Figure 2) (Cox regression *P*=0.036, HR 1.92 [95% CI: 1.04-3.54]). A similar number of patients had IT MTX ceased in both groups (21 of 42 in the group with early MTX toxicity; 27 of 53 who experienced late MTX toxicity). ALL risk-group did not vary significantly between those who experienced MTX neurotoxicity before or after consolidation (*P*=0.874).

Long-term neurological outcome

Long-term neurological outcome was reported at last follow-up (median 94 months, range, 3-181 months) for 41.7% (n=522 of 1,251) of patients. There was no reported mortality due to long-term neurological problems following MTX neurotoxicity. A total of 1.3% (n=7 of 522) were diagnosed with epilepsy at last follow-up. Three out of these seven children were diagnosed with epilepsy following symptomatic MTX neurotoxicity, and all remained in CR1.

Genome-wide association study results

There was no difference in the incidence of MTX neurotoxicity among the subgroup who were included and excluded from the GWAS, in relation to age, sex, timing and CTCAE grade of toxicity (*Online Supplementary Table S7*).

We found seven intronic SNP that were associated with MTX neurotoxicity at a significance level of $P<1x10^{-6}$ (Table 3), which mapped to six genes (*MBOAT-1, GIPC1, ZDHHC19, NXN, PKN1, HMGB1P37*). The rare alleles at SNP near *MBOAT-1* and *NXN* were protective (OR <1) while those at the other SNP correlated with increased risk of MTX neurotoxicity.

There were 53 additional SNP (minor allele frequency [MAF] >2%) associated with MTX neurotoxicity at a significance level of $P<5x10^{-6}$, which mapped near to seven genes (*Online Supplementary Table S8*). Further understanding of SNP function and roles was determined using contemporary online data repositories (see the *Online Supplementary Appendix*). Most SNP ($P<5x10^{-6}$) were intronic, except for rs76301301 (3' untranslated region [UTR] variant, $P=1.34x10^{-6}$) located within GIPC1 and rs7555699 located within 2 kb upstream of a 5' end of BMP8A ($P=4.54x10^{-6}$) (*Online Supplementary Table S9*).

Discussion

This is the largest cohort of children treated for ALL/LBL mapped for the incidence, risk factors, and long-term impact of MTX toxicity. Independent risk factors for symptomatic MTX neurotoxicity were age ≥10 years at diagnosis and >grade 3 elevation of serum AST during early therapy. Discontinuation of IT MTX, in an attempt to minimize further neurotoxicity after a first MTX neurotoxic event, was associated with an increased incidence of CNS relapse. Patients continuing IT MTX had only a small risk of MTX neurotoxicity recurrence (<13%). Regardless of subsequent IT management strategy, children who developed MTX neurotoxicity had an increased risk of epilepsy.

We report on independent risk factors in multivariable analysis that are associated with symptomatic methotrexate neurotoxicity: age ≥10 years at diagnosis and >grade 3 elevation of serum AST during induction/consolidation. Importantly, these factors were determined in a combined cohort of Australian children treated for ALL on either BFM or COG-based therapy. AST elevation in the current series may reflect direct hepatotoxicity from methotrexate,[17] systemic metabolic disturbances,[18] release from non-hepatic sources such as erythrocytes,[18] or a marker of longer serum exposure to MTX as has been shown for ALT elevation post HD MTX.[17]

Older age has been previously reported as a risk factor in univariate[1] and recently in multivariable analysis in a smaller cohort.[19] Potential reasons include reduced clearance of HD MTX20 or higher steady state MTX concentration following IV administration[21] in older children. Protocols that used higher IT doses (15 mg) for children ≥9 years of age were not associated with increased neurotoxicity in our study.

The observed increase in cumulative incidence of CNS relapse following permanent cessation of IT MTX therapy requires validation in contemporaneous cohorts. In study POG 9005 (1991-1995), which had the same number of cases of acute MTX neurotoxicity (n=95), 54 patients had intrathecal therapy modified without an increase in CNS relapse.[6] CNS-relapse rates in our overall cohort (n=1,251) were consistent with published rates.[22,23] Treatment of CNS relapse involves exposure to additional neurotoxic agents including CNS irradiation.[24] Taken together, these data suggest that clinicians should not cease IT MTX therapy after a first episode of MTX neurotoxicity, especially when it occurs early in therapy.

Out of the 95 patients identified with MTX central neurotoxicity, 53 fulfilled the Ponte di Legno Toxicity Working Group MTX stroke-like syndrome (SLS) definition that was published after our study commenced, i.e., symptoms within 21 days of IT/IV MTX, characteristic clinical course and/or imaging, with exclusion of other causes.4 All patients included in the GWAS experienced MTX neurotoxicity <21 days from IT/IV MTX.

We did not identify any SNP associated with MTX neurotoxicity at genome-wide levels of significance, but report seven SNP at $P<1x10^{-6}$ which require replication in larger, independent studies of MTX neurotoxicity. These seven SNP mapped near six genes, five of which have potential roles in neuronal cell growth, differentiation, development, or developmental delay phenotypes (*MBOAT-1, GIPC1, ZDHHC19, NXN, PKN1*).[25-29] A 3'UTR variant in GIPC1 was also associated with MTX neurotoxicity ($P=1.34x10^{-6}$).

A prospective study by Bhojwani *et al.*[1] reported 14 children with subacute symptomatic MTX neurotoxicity and did not identify any genome-wide significant SNP (top SNP significance level $P=3.65x10^{-6}$). We were unable to replicate their top SNP for symptomatic leukoencephalopathy. There is a critical need to validate germline risk factors for MTX neurotoxicity in a larger international study, as larger numbers will increase power of the GWAS.

There are several limitations inherent to systematic retrospective analyses. Collated clinical laboratory data were dependent on tests performed during routine clinical care, including AST values. Patients with missing data were censored and some variables were more complete than others. Clinical documentation regarding longer-term neurological function was not consistently available nor standardized, reducing the cohort for analysis.

With respect to the MTX doses, analysis was performed

on the basis of protocols that administered low dose or high dose MTX, and age-based IT MTX dosing. Where there were significant deviations to administered chemotherapy, this was usually captured in the data extraction and maintained in the database as additional phenotypic descriptors. However, it was impossible to calculate the total administered dose of IV and IT MTX per patient, therefore this could be attempted in future prospective trials to assess the relationship with MTX neurotoxicity occurrence. While the omission of IT MTX appears to be an important factor in patients who experienced a subsequent relapse, it is possible that concurrent cessation of HD MTX in one patient could have also contributed to relapse. There were no other treatment modifications identified in this cohort which could further contribute to relapse risk, however prospective capture and analysis of all treatment modifications would be important for future studies.

The main risk period in our study occurred when IT MTX was administered with cytarabine and cyclophosphamide (50 of 94 cases), which is consistent with prior publications.[2,5] Surprisingly, for children treated for high-risk ALL, there was a higher incidence of MTX neurotoxicity when a lower total dose of IV MTX was used compared to protocols using higher-dose MTX. This finding requires prospective validation and may be related to the routine use of leucovorin rescue with HD MTX.

Possible strategies for toxicity reduction include re-introducing IT MTX when cytarabine and cyclophosphamide are not co-administered, and/or additional doses of leucovorin following IT MTX. Administration of an additional leucovorin dose reduced acute neurotoxicity in a historical cohort;[30] and this could be targeted to specific risk periods such as when cyclophosphamide and cytarabine are co-administered. Caution is advised however, due to evidence that higher leucovorin doses could be associated with increased relapse risk.[31] While outside the scope of this paper, a systematic prospective study regarding secondary prophylaxis following IT MTX rechallenge would be important. Potential agents include leucovorin,[1] aminophylline[1] and the NMDA receptor antagonist dextromethorphan.[32]

In addition, drug-drug interactions that may potentiate MTX neurotoxicity or which could impact on efficacy of systemic chemotherapy such as anti-epileptic drugs should be systematically collected.[33,34] Future laboratory-based modeling using neural cells derived from pluripotent stem cells[35] could assess MTX transport, influx and efflux from the CNS including i) IT MTX pharmacokinetics and pharmacodynamics in the presence of cyclophosphamide and cytarabine and ii) MTX transport in the presence of associated SNP.

In summary, this study supports the continued use of IT MTX in patients with a first episode of MTX neurotoxicity. Continued attempts to identify and prospectively validate clinical risk factors and germline variants that indicate high risk of MTX neurotoxicity may provide an opportunity to prevent this debilitating toxicity in the future.

Disclosures

The authors declare no competing financial interests.

Contributions

MKM developed study materials, collected data, extracted patient DNA samples, wrote the manuscript, analyzed data and helped with interpretation of GWAS results; GMM and TNT wrote the study concept, supervised writing of the manuscript and assisted with interpretation of results; PMB, CG, TR, RSK collected data; MCJQ performed the GWAS; CM assisted with statistical analysis; RS, JG, DC helped with extraction of patient DNA samples; RS, DB, FA, FM, LDP assisted data collection processes; JAL assisted with data interpretation; GCT and SM provided assistance with the GWAS. All authors reviewed and approved the final version of the manuscript.

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