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

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Mateos *et al.*

Supplemental Methods, Tables and Figures

Table of Contents

1 Supplemental Methods 1
2 Comparative drug dosing 5
3 Supplemental Results 6
4 Supplemental Tables and Figures 7
5 Supplemental References 21

1 Supplemental Methods

Ethical approval

The ethical approval HNEHREC Reference Number: 12/11/21/4.01 covered approval for the clinical and genome-wide association study (GWAS). Individual consent for the clinical observational study was not required on advice from the relevant HREC. Samples that had been prospectively banked in the Sydney Children's Tumour Bank Network had individual written consent for use in future HREC-approved research.

Clinical data collection

Data were extracted from each patient's clinical, imaging and laboratory records, to capture baseline and treatment-related variables. Data were censored at 31/12/2013. Patients were excluded if less than 18 months of clinical data were available to determine occurrence of neurotoxicity (Supplemental Figure 1).

Epilepsy

Epilepsy was defined as per Fisher *et al.* (1) and cross-referenced with electroencephalogram (EEG) reports. Epilepsy was chosen as it is a defined clinical endpoint with objective diagnostic criteria supported by EEG results.

Statistical analysis

Comparison between groups, for categorical data, was conducted using Pearson chi-squared analysis; or Fisher's exact test (2-sided) where expected values were less than 5. Intergroup comparisons for continuous variables were performed using Mann-Whitney U tests or logistic regression. Comparative survival and cumulative incidence of relapse between groups were assessed using log-rank Mantel-Cox test.

Variables analysed

Variables included clinical features at presentation, treatment response, organ dysfunction at diagnosis or during treatment, and measurements of weight and body mass index at various time points. These factors were chosen based on prior literature review, and/or a hypothesized effect on drug exposure or metabolism that could contribute to MTX-induced central neurotoxicity (2-5).

Diagnostic features evaluated comprised age, cancer diagnosis, immunophenotype, ABO blood group, presence of a mediastinal mass, treatment risk-group, weight, height, body mass index (BMI), presenting white cell count, serum creatinine, liver function, and coagulation tests.

Treatment-related variables included remission status at end of induction, measurements of renal, hepatic function and coagulation parameters in induction/consolidation; and insulin-requirement during ALL therapy. Values collected for renal and liver function during induction/consolidation included peak serum creatinine, peak serum urate, lowest serum albumin, and peak values for liver function tests [bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP)] in induction/consolidation. Fibrinogen, international normalised ratio (INR) and activated partial thromboplastin time (APTT) values were collected prospectively during induction (days 0, 8,15, 22 and 29). Weight, height and BMI values were prospectively collected at diagnosis, end of induction ("Protocol 1A") and end of consolidation ("Protocol IB"). Serum methotrexate (MTX) concentration levels during the first course of HD MTX (at 24 hours, 36 hours, 42 hours, 48 hours and 54 hours) following high-dose MTX for BFM-treated patients were also assessed, in relation to MTX central neurotoxicity. Due to variability

between protocols as to when MTX levels were collected, MTX levels at 24 hours, 48 hours and 54 hours were analysed.

Alterations to chemotherapy (such as MTX dosing) and additional individual supportive care administered following neurotoxicity were extracted from patient records, as additional descriptive information where available.

Descriptive analysis of the cohort included time to neurotoxicity, days following IT or IV MTX administration, and delayed MTX excretion during any of the HD MTX courses (characterised by a MTX level higher than the protocol specified range for at least one time point).

Anthropometric values were converted to age and sex-specific Z-scores using Centers for Disease Control and Prevention (CDC) 2000 reference values within the LMSgrowth program, a recognised excel plug-in (6, 7). Z scores were then converted to pre-defined centile measurements, for example to BMI < 5^{th} centile and BMI >95^{th} centile, which is clinically defined as obesity in children (8). Categorical variables were explored for children with weight and BMI centiles < 5^{th} centile, >95^{th} centile in relation to toxicity experienced during first clinical remission (CR1), in accordance with prior reports (9).

Univariate regression analysis

Variables tested in univariate regression analysis for association with MTX central neurotoxicity included age at diagnosis, diagnostic features such as white cell count and risk group assignment, anthropometric data (height, weight at diagnosis and end of induction), liver and renal function (at diagnosis and during induction/consolidation), hyperglycemia in induction/consolidation, insulin requirement and MTX levels during high dose (HD) MTX therapy.

Multivariable regression modelling

If both the continuous and categorical version of a variable (for example, age) were significant then only one variable, as supported by significance level (*P* value and/or hazard ratio (HR)), was evaluated in multiple regression.

Backward elimination was performed using 12 variables, removing the least significant variable until all factors in the multiple regression model retained independent significance (2-

tailed $P<0.05$). The 12 included variables were: age ≥10 years, treatment risk group (high-risk [very high/high-risk] compared to standard/medium-risk groups), serum bilirubin level at diagnosis (continuous variable), peak serum aspartate aminotransferase (AST) level in induction/consolidation (continuous variable), peak serum alanine aminotransferase (ALT) level in induction/consolidation (continuous variable), peak gamma-glutamyl transferase (GGT) in induction/consolidation (categorical variable), peak serum bilirubin in induction/consolidation (categorical variable), peak creatinine in induction/consolidation > twice the creatinine value at diagnosis and abnormal (categorical variable), bacteremia in induction/consolidation, insulin requirement in induction/consolidation (categorical variable), lowest albumin in induction/consolidation (continuous variable), and weight at the end of induction (Z score).

GWAS methodology

Additional data relating to Caucasian ancestry, sample quality control, SNP filtering and the MTX neurotoxicity genomic phenotype are listed below.

After performing sample quality control on 932 DNA specimens, 57 individual patient samples were excluded. Children with non-European ancestry were excluded (n=168) to allow discovery in a homogenous ethnic population, and avoid risk of false positive associations due to population stratification. European ethnicity was defined as any of the following: Spanish IBS, Italian TSI, British GBR, White European from Utah CEU, Finnish FIN as defined by 1000 Genomes (10)).

SNPs with a missing genotype call rate <97%, minor allele frequency (MAF) <0.5% or those that deviated from Hardy-Weinberg equilibrium in European ancestry populations ($P<1.0E-04$) were excluded.

The λ(lambda) value for this MTX neurotoxicity phenotype was 1.039, where values <1.1 are acceptable (11).

2 Comparative drug dosing

Methotrexate

To better understand methotrexate (MTX) dosing on the included protocols for the ERASE cohort (n=1251), all protocols were reviewed and summary doses annotated. An outline of MTX doses is contained in Supplemental Table 1. Intravenous (IV) MTX doses were calculated based on a standard patient, CNS1 (CNS disease negative), treated on the respective protocol. BFM-based study doses were calculated as per a standard risk (SR) CNS1 patient on the protocol. COG protocol doses for standard-risk patients were calculated as for a SR-average CNS1 patient (not SR-high or LR) and a COG protocol for high-risk patients was calculated for a standard HR (not HR-high) patient.

There were a few key differences across the protocols with respect to MTX. Generally these differences related to dose of intrathecal (IT) therapy administered to older children and the use of high-dose MTX in the BFM-based protocols. High-dose MTX was administered as 5g/m^2/dose on all BFM-based protocols (total 20g/m^2) except for COG A5971, where only Arms B1 or B2 received high-dose MTX. Statistical comparisons were made based on age-adjusted IT dose pattern and use of high-dose IV versus low-dose (escalating dose IV) methotrexate.

Age-adjusted IT doses for MTX on BFM-based protocols were 8mg for 1 to < 2 years, 10 mg for 2 to < 3 years, 12 mg for ≥3 year old children. Doses for IT MTX on most COG-based protocols were 8mg for 1-1.99 years, 10mg for 2-2.99 years, 12mg for 3-8.99 years and 15 mg for ≥9years, except for CCG1882, CCG1952, CCG1961 and CCG1991 where age-adjusted doses were as per BFM-based protocols.

Most COG-based studies incorporated nil or low-dose (escalating IV) MTX dose (1-1.5g/m2/course, total dose 0-2.5g/m2 for a standard patient CNS1 on the protocol). Escalating IV MTX will be referred from hereforth as “low-dose” MTX. An exception was AALL1131, where high-dose MTX was administered to all patients.

For the comparison between high-dose and low-dose IV MTX, protocols where IV MTX doses differed in randomized arms were excluded from the analysis (AALL0232, AALL0434,

COGA5971) and AALL0331 was included in the low-dose MTX group as per doses administered to a SR patient.

Leucovorin doses per protocol are listed in Supplemental Table 1. The racemic mixture for leucovorin was administered. Dosing of leucovorin following the first episode of MTX neurotoxicity, was guided by the protocol. Five protocols (ANZCHOG Study 7, ANZCHOG Study 8, iBFM-Study 9, BFM-95, CCG1182) did not have a specific recommendation regarding leucovorin after neurotoxicity, and the rest of the protocols had a suggestion to administer leucovorin orally at hour +48, hour +60 for subsequent IT MTX doses.

3 Supplemental Results

Clinical data

High dose MTX

Where MTX neurotoxicity occurred during IV MTX (n=22), 6 patients were treated on COG and 16 on BFM treatment regimes. Of the 16 patients on BFM protocols, all received HD MTX, and of these, 5/16 had delayed MTX excretion, with creatinine elevation in 2/5 (Grade 1 (n=1), Grade 2 (n=1) with full recovery of renal function thereafter). For those with delayed MTX excretion, this occurred with the first course of MTX in 4/5 patients.

Radiotherapy

Cranial irradiation was used in frontline protocols for 9/95 patients who experienced MTX neurotoxicity. Of these 1/9 had IT MTX ceased, 6/9 had IT MTX continued, and 2/9 were unknown with respect to IT MTX management following MTX neurotoxicity. Three of these patients were HR patients. The patient who had IT MTX ceased was a HR patient who received upfront cranial RTX and did not experience disease relapse.

GWAS

Gene annotations

The annotated gene associated with a SNP was determined by cross-referencing RefSeq (12), Ensembl 74 (13) and UCSC database information (hg19, 2015 update) (14) accessed through

SNPnexus (2012 update (15), http://www.snp-nexus.org). Where there was discrepancy, a manual search was performed using NCBI dbSNP build 149 (16).

Supplemental Tables 8 and 9 outline SNPs associated with MTX neurotoxicity (P<5E-06), outside of the top 7 SNPs documented in Table 3. One SNP (rs183796502) is located close to *STIM1*, a gene that has a role in mammalian neuronal calcium stores (17) (P=3.65E-06).

4 Supplemental Tables and Figures

Supplemental Tables 1-9 and Supplemental Figures 1-3 are included below.

TREATMENT PROTOCOL	NUMBER (n=1251)	% OF COHORT	Number doses of IT MTX total (for standard risk CNS1 equivalent) [a]	IV MTX		Dose of IT MTX[c] A=8/10/12; B=8/10/12/15	VCR during consolidation	IT MTX doses induction (CNSI, standard risk on protocol)	IT MTX doses in consolidation (CNSI, standard risk on protocol)	VCR during IV MTX	CPA/AraC in consolidation	VCR in reconsolidation (2nd half of Delayed intensification or as otherwise stated)	Risk groups	Notes including standard leucovorin (LV) rescue post IV MTX assuming no delayed excretion e.g hour +42,48,54 post IV MTX; and standard LV dose following IT MTX.
			Standard risk (SR)	Type of IV MTX	Total dose[b] (g/m2)									
BFM-based protocols	*(n=1033)*													
ANZCHOG Study 7 (1998-2002) (Manuscript Ref 36)	239	19.1	Pre-maint=10; Total=13	HD	20	A	Y	2	4	N (SR), Y(HR)	Y	N	All	HD MTX: LV at +36,39,42,45,48,51 then 6 hourly until MTX <0.1microM. LV 15mg/m2/dose IV
ANZCHOG Study 8 (2002-2012) (Manuscript Ref 38)	608	48.6	11	HD	20	A	N	3	2	N (SR), Y(HR)	Y	N	All	HD MTX: LV at +42,48,54; 15mg/m2/dose IV
iBFM-Study 9 (2012-2013) (Manuscript Ref 40)	40	3.2	11	HD	20	A	N	3	2	N (SR), Y(HR)	Y	N	All	HD MTX: LV at +42,48,54; 15mg/m2/dose IV
BFM-95 (1998-2002) (Mnuscript Ref 37)	125	9.99	11	HD	20	A	N	3	2 (SR), HR total 6 in HR blocks	N (SR), Y(HR)	Y (SR/MR)	N	All	HD MTX: LV at +42,48,54; 15mg/m2/dose IV
COG A5971 (2003-2009) (Manuscript Ref 39)	21	1.68	10 (Arm A: 23)	Arm A: no IV MTX Arm B: HD	Arm B: 20	A	N	2	2 (4 if Arm A)	N	Y	N	All	*Arm A had 4 doses IT MTX in consolidation compared to 2 doses IT MTX in Arm B. Arm B (HD MTX) LV at +42,48,54; 15mg/m2/dose.
COG-based protocols	*(n=218)*													
AALL0031 (2002-2006) (Manuscript Ref 46)	2	0.16	Pre-maint=14; Total 22 (F, M)	HD	35	B	N	2	3	Y	N	Y	Ph+ALL	*IT MTX only during cycles 1-4 maintenance. Reconsolidation equates to Intensification Block 1. HD MTX: LV at +42,48,54. LV 15mg/m2/dose IV/PO
AALL0232 (2004-2011) (Manuscript Ref 47)	25	2	Different arms: Pre-maint= 14-16; Total: 24-26 (F), 28-30 (M)	randomised question: HD vs capizzi (LD)	HD: 20; LD: 1	B	Y	0-2	4	Y	Y	Y	HR ALL	* 6 cycles maint (F), 10 cycles maint (M). LV at +42,48,54 for HD MTX. LV 15mg/m2/dose IV/PO. Down syndrome (DS) patients: 5mg/m2 PO at +48,60 post IT MTX
AALL0331 (2005-2010) (Manuscript Ref 48	49	3.92	Pre-maint = 7; Total: 14 (F), 18 (M)	LD for most, HD if SR-high	LD: 1	B	Y	2	3 (2-4 for other HR arms)	Y	N (standard regime); Y(intensified regime)	N (standard regime); Y(intensified regime)	SR ALL	*7 cycles maint (F), 11 cycles maint (M), calculate for standard regime. HD MTX: LV at +42,48,54; 15mg/m2/dose IV/PO. LV at +48,60 post IT MTX for DS patients (5mg/m2 PO), except maintenance ("DS dosing")
AALL0434 (2007-2014) (Manuscript Ref 45,48)	12	0.96	Pre-maint=11; Total 22 (F), 26 (M)	LD vs HD randomised arms	LD: 1, HD: 20	B	Y	2	4	Y	Y	Y	T-ALL/T-NHL	*calculated for Low risk T-ALL patient (i.e standard, not high-risk). HD MTX: LV at +42,48,54; 15mg/m2/dose IV/PO.
AALL08P1 (2009-2011) (Manuscript Ref 49)	2	0.16	Pre-maint=11; Total 22 (F), 26 (M)	LD	1	B	Y	2	4	Y	Y	Y	HR ALL	*calculated for HR-average, 7 cycles maint (F), 11 cycles maint (M). Analysed as per pre-amendment (in LD MTX group)
AALL0932 (2010 - current) (Manuscript Ref 45)	17	1.36	Pre-maint=10; Total: 16 (F), 21 (M), doses as for AR-ALL group	LD	2.5	B	Y	2	3	Y	N	Y	SR ALL	*AALL0932: AR-ALL is standard risk, LR-ALL represents treatment de-escalation; note 6.6 maintenance cycles (F), 11 maintenance cycles (M) as determined from start of IM-1. MTX 1g/m2: LV at +42,48. Optional +54 if MTX not <0.2microM; 10mg/m2/dose IV/PO. DS dosing
AALL1131 (2012 - current) (Manuscript Ref 45)	4	0.32	Pre-maint=11; Total: 22 (F), 26 (M)	HD	20	B	Y	2	4	Y	Y	Y	VHR ALL	*for HR-ALL arm A (control arm), *7 cycles maint (F), 11 cycles maint (M). HD MTX: LV at +42,48,54; 15mg/mg2/dose. DS dosing.
CCG1882 (1991-1995) (Manuscript Ref 41	1	0.08	Pre-maint=7; Total: 14 (F), 18 (M)	0	0	A	Y	1	4	-	Y	Y	HR ALL	* calculated for standard therapy, not augmented therapy. 7 cycles maint (F), 11 cycles maint (M)
CCG1952 (1996-2000) (Manuscript Ref 42)	16	1.28	Pre-maint=11; Total 17 (F), 21 (M)	0	0	A	Y	2	3	-	N	Y	SR ALL	* 6 cycles maint (F), 10 cycles maint (M).
CCG1961 (1996-2002) (Manuscript Ref 43)	36	2.88	Pre-maint = 11; Total: 22 (F), 26 (M)	LD (intensified arm)	0 (SR), 2 (intensified arm)	A	Y (HR)	2	4	Y (HR), N/A for SR	Y	N (standard regime); Y(intensified regime)	HR ALL	*7 cycles maint (F), 11 cycles maint (M).
CCG1991 (2000-2005) (Manuscript Ref 44,45)	54	4.32	Pre-maint=10-12; Total: 16-18 (F), 20-22 (M)	LD (randomised Arm IS/ID)	0 (Arm OS/OD), LD: 2.5	A	Y	2	3	Y (Arm IS/ID)	N	Y	SR ALL	* 6 cycles maint (F), 10 cycles maint (M)

Supplemental Table 1. Doses of intrathecal (IT) and intravenous (IV) methotrexate (MTX) on included treatment protocols.

Standard risk, SR; high risk, HR; IV, intravenous; IT, intrathecal; MTX, methotrexate; F, females; M, males. HD, high dose MTX (total dose 20-35g/m2; LD, low dose MTX, total dose 0-2.5g/m2). DS = Down syndrome. DS dosing refers to standard LV oral dose 5mg/m2/dose at hour +48,60 post IT MTX. MTX doses calculated are for a standard CNS1 patient on each protocol, based on the risk-groups enrolled on to the protocol, without treatment intensification or de-intensification. [a] Total dose of IT MTX was calculated for a treatment protocol consisting of 6 maintenance ("maint") cycles (F), 10 maintenance cycles (M), where maintenance cycles are 12-weekly, unless otherwise stated above. This calculation was based on the time from commencement of interim-maintenance 1 through to end of maintenance, being total duration therapy 24 months (F), 36 months (M) from start of interim-maintenance 1. [b] The total dose for escalating doses of intravenous methotrexate on COG protocols was calculated based on maximum dose delivery, when no toxicity was experienced. [c] IT MTX age-adjusted doses: Pattern A was 8mg for 1-<2 years, 10mg for 2-<3 years, 12mg for 3 years and older. Pattern B was 8mg for 1-<2 years, 10mg for 2-<3 years, 12mg for 3-<9 years and 15mg for aged 9 years and above *Online only.*

Group	≤21 days post MTX	Typical presentation *	Characteristic MRI/ clinical course *	Number of patients
1	Y	Y	Y	53
2†	N	Y	Y	3
3‡	N	N	Y	1
4	Y	Y	N	12
5	Y	N δ	Y	13
6	Y	N ε	N	13

Supplemental Table 2. Description of timing of neurotoxicity post intravenous or intrathecal methotrexate (MTX), clinical presentation, magnetic resonance imaging (MRI) and clinical course for the cohort.

Table legend: The cohort of patients who experienced MTX central neurotoxicity has been divided into 6 groups, based on presence of diagnostic criteria. This is to allow comparison with the current Ponte di Legno Toxicity Working Group definition of methotrexate stroke-like syndrome (18), of which patients in Group 1 fulfil. Y indicates "yes" (i.e features present) and N indicates "no" (features absent). * Typical presentation and characteristic MRI/clinical course were determined as per Schmiegelow *et al* 2016 (18). Where patients did not have MRI performed, if the MRI had atypical changes or if there was lack of information regarding clinical course, these patients were deemed not to have characteristic MRI or clinical course features. † Three patients with MTX neurotoxicity > 21 days post intravenous/intrathecal MTX had typical symptoms and leukoencephalopathy (22, 30 and 47 days post MTX). These typical symptoms included - Patient 1: right-sided hemiplegia 22 days after MTX administration, in the absence of intracranial bleeding or central nervous system infection; Patient 2: reduced level of consciousness associated with confusion that occurred 30 days after MTX, with resolution after 6 days, managed as MTX neurotoxicity; Patient 3: EEG-confirmed encephalopathy and status epilepticus, onset 47 days after MTX, with resolution of symptoms after 7 days. ‡ One patient had seizures and leukoencephalopathy on MRI and was treated as MTX toxicity by the treating physician, 56 days post MTX. δ Includes seizures alone or other atypical symptoms leading to clinical concern and diagnostic imaging. ε Seizures alone. *Online only*

	CNS status at ALL diagnosis			Total patients	*P* value
Patient group	CNS 1	CNS 2	CNS 3		
No neurotoxicity	863	81	15	959	
MTX neurotoxicity	86	6	3	95	0.414

Supplemental Table 3. CNS status at ALL diagnosis for children who experienced MTX neurotoxicity.

Table legend: This table compares the CNS (central nervous system) status at ALL diagnosis between children who did and did not experience MTX neurotoxicity. There was no significant difference (P=0.414, pearson chi-squared). CNS 1, no CNS disease; CNS 2, includes <5 WCC and presence of blasts or "blood tap" as defined by the protocol; CNS 3, CNS disease. Significance level set at P<0.05, 2-tailed. *Online only.*

	IT MTX ceased (n=48)			IT MTX continued (n=34)			*P* value
Risk group	**non HR**	**HR**		**non HR**	**HR**		
	27 (56%)	21 (44%)		26 (76%)*	7 (21%)*		0.036
CNS status	**CNS 1**	**CNS 2**	**CNS 3**	**CNS 1**	**CNS 2**	**CNS 3**	
	44 (92%)	3 (6%)	1 (2%)	31 (91%)	1 (3%)	2 (6%)	0.536
Age	**< 10 years**	**≥10 years**		**< 10 years**	**≥10 years**		
	28 (58%)	20 (42%)		22 (65%)	12 (35%)		0.56

Supplemental Table 4. Baseline characteristics of patients who experienced MTX neurotoxicity. The group where IT MTX was ceased (n=48) or continued (n=34) post neurotoxicity were compared with respect to risk group, CNS status and age.

* risk group not known for 1 patient. HR, high-risk (includes high-risk and very high-risk); non HR, non high-risk (includes standard and medium risk patients). CNS 1, no CNS disease; CNS 2, includes <5 WCC and presence of blasts or "blood tap" as defined by the protocol; CNS 3, CNS disease. Significance level set at $P<0.05$, 2-tailed. *Online only.*

Protocol	Cohort	MTX Neurotox Cases	IT MTX ceased	IT MTX re-exposed	Ongoing MTX other *	Cases evaluable for recurrence	Recurrence
Study 7	239	19	10	6	3	6	0
BFM-95	125	11	3	4	4	4	1
Study 8	608	34	14	14	6	13	2**
COG A5971	21	1	1	0	0	-	-
iBFM-Study 9	40	5	1	4	0	3	0**
CCG1882	1	0	0	0	0	-	-
CCG1952	16	2	1	1	0	0	Unknown**
CCG1961	36	11	10	1	0	1	0
CCG1991	54	3	2	1	0	1	0
AALL0031	2	0	0	0	0	-	-
AALL0232	25	4	3	1	0	1	0
AALL0331	49	3	2	1	0	1	1
AALL0434	12	1	0	1	0	1	0
AALL08P1	2	0	0	0	0	-	-
AALL0932	17	1	1	0	0		
AALL1131	4	0	0	0	0	-	-
Total	**1251**	**95**	**48**	**34**	**13**	**31**	**4**

Supplemental Table 5. MTX neurotoxicity according to protocol. Number of cases per protocol of MTX neurotoxicity ("MTX Neurotox"), ongoing intrathecal (IT) MTX exposure and recurrent MTX neurotoxicity are shown for clarity regarding ongoing MTX re-exposure, and recurrence of MTX neurotoxicity. * "Ongoing MTX other" - ongoing exposure to MTX post first MTX neurotoxicity episode was unknown. ** 1 patient unknown regarding MTX neurotoxicity recurrence

Age at ALL diagnosis (months)	Gender	IT MTX strategy after first episode	Grade of first episode (CTCAE)	Second neurotoxicity episode	Subsequent IT MTX strategy
23	M	Continued	2	Leukoencephalopathy	Ceased
35	F	Unknown	3	Seizure (minimal detail)	Unknown
27	M	Unknown	3	Complex partial seizure	Unknown
172	M	Continued	3	Right hemiparesis, encephalopathy	Ceased
176	F	Continued	2	Aphasia, leukoencephalopathy	Continued
58	F	Continued	2	Leukoencephalopathy	Ceased

Supplemental Table 6. Description of recurrent neurotoxicity events. ***Online only.***

	Group excluded from GWAS (n=47)	Group included in GWAS (n=48)	*P* value for comparison
Median age at diagnosis (months)(range)	101 (16-199)	62.5 (23-196)	0.128 *
Sex (Male: Female)	22:25	26:22	0.473 †
Median timing post IV/IT MTX (days)(range)	9 (1-56) ‡	8 (0-21)	0.306 *
Median time post diagnosis (months)(range)	4.4 (0-15)	2.1 (0-19)	0.154 †□
Grade of toxicity (Grade 1&2 vs Grade 3&4) δ□	33:14	30:18	0.426 †

Supplemental Table 7. Characteristics of the methotrexate (MTX) neurotoxicity phenotype for patients included and excluded from the GWAS.

Table legend: * P value determined by Mann-Whitney U test. † *P* value determined by pearson chi-square test. ‡ One patient had MTX neurotoxicity < 21 days since last IT/IV MTX however exact number of days could not be determined therefore this analysis was for n=46 patients. δ Grading of toxicity as per CTCAE v4.03. *Online only.*

CHR	Position	SNP	Non effect allele	Effect allele	MAF	*P*	OR	OR 95 CI (lower)	OR 95 CI (upper)	Gene*	Location
1	39944180	rs6665948	A	G	0.32	4.60E-06	0.35	0.22	0.55	*MACF1*	intron
1	39944768	rs722357	C	T	0.32	4.19E-06	0.35	0.22	0.55	*MACF1*	intron
1	39945297	rs2275767	C	T	0.32	3.34E-06	0.34	0.22	0.54	*MACF1*	intron
1	39948224	rs12138051	A	G	0.32	4.00E-06	0.34	0.22	0.55	*MACF1*	intron
1	39948741	rs6691194	G	A	0.32	4.01E-06	0.34	0.22	0.55	*MACF1*	intron
1	39957301	rs7555699	G	A	0.32	4.54E-06	0.35	0.22	0.55	*BMP8A*	5'upstream
1	39958080	rs10888798	C	G	0.32	4.50E-06	0.35	0.22	0.55	*BMP8A*	intron
1	39959085	rs1809697	C	T	0.32	4.59E-06	0.35	0.22	0.55	*BMP8A*	intron
2	199177731	rs2529670	G	C	0.13	3.11E-06	3.5	2.09	5.85	*AC005235.1*	non-coding intron
2	199179415	rs2529669	G	C	0.13	2.56E-06	3.53	2.11	5.9	*AC005235.1*	non-coding intron
2	199179640	rs917312	A	G	0.13	2.50E-06	3.53	2.11	5.9	*AC005235.1*	non-coding intron
2	199181697	rs199883435	G	GTGTA	0.13	2.11E-06	3.53	2.12	5.86	*AC005235.1*	non-coding intron
2	199181699	rs201180996	G	GTA	0.13	1.97E-06	3.53	2.13	5.87	*AC005235.1*	non-coding intron
2	199181874	rs2727766	T	G	0.13	2.10E-06	3.51	2.12	5.84	*AC005235.1*	non-coding intron
2	199182389	rs2529666	C	T	0.13	2.06E-06	3.51	2.12	5.83	*AC005235.1*	non-coding intron
2	199183461	rs2529664	A	G	0.13	2.39E-06	3.45	2.09	5.7	*AC005235.1*	non-coding intron
2	199184199	rs2429092	A	T	0.13	2.25E-06	3.46	2.09	5.71	*AC005235.1*	non-coding intron
2	199184482	rs2529663	A	G	0.13	2.24E-06	3.46	2.09	5.7	*AC005235.1*	non-coding intron
2	199185059	rs2727783	T	G	0.14	2.74E-06	3.4	2.06	5.59	*AC005235.1*	non-coding intron
2	199185092	rs2529662	T	C	0.13	2.18E-06	3.46	2.1	5.71	*AC005235.1*	non-coding intron
2	199190385	rs2727767	A	G	0.13	2.15E-06	3.49	2.11	5.78	*AC005235.1*	non-coding intron
2	199192443	rs2429091	C	A	0.13	1.97E-06	3.52	2.12	5.85	*AC005235.1*	non-coding intron
2	199194805	rs2529658	G	A	0.13	2.00E-06	3.52	2.12	5.85	*AC005235.1*	non-coding intron
2	199197483	rs2529657	T	A	0.13	2.36E-06	3.48	2.1	5.76	*AC005235.1*	non-coding intron
2	199200122	rs2467044	A	G	0.13	2.37E-06	3.51	2.11	5.83	*AC005235.1*	non-coding intron
2	199200332	rs2429090	A	T	0.13	2.58E-06	3.48	2.1	5.78	*AC005235.1*	non-coding intron
2	199204986	rs10490082	A	T	0.14	4.84E-06	3.44	2.05	5.76	*AC005235.1*	non-coding intron
2	199205303	rs72916188	T	C	0.14	4.78E-06	3.45	2.06	5.78	*AC005235.1*	non-coding intron
3	195925458	rs1106480	A	C	0.15	3.54E-06	3.68	2.14	6.32	*ZDHHC19*	intron
5	154613834	rs79248818	T	C	0.02	1.53E-06	16.85	5.73	49.6	-	-
5	154614921	rs113075325	C	T	0.02	1.55E-06	16.84	5.72	49.55	-	-
10	102845241	rs17113682	G	A	0.24	3.89E-06	0.2	0.08	0.46	*TLX1NB*	-
11	3878978	rs183796502	G	A	0.33	3.65E-06	0.28	0.15	0.51	*STIM1*	-
13	95065639	rs9589996	G	T	0.11	1.03E-06	5.22	2.72	10.01	-	-
13	95067905	rs9589997	G	A	0.11	1.04E-06	5.2	2.71	9.97	-	-
13	95068457	rs9589999	G	A	0.11	1.04E-06	5.21	2.71	9.98	-	-
13	95069136	rs6650322	A	G	0.11	1.18E-06	5.13	2.68	9.81	-	-
13	95072378	rs9584226	A	C	0.11	1.09E-06	5.17	2.7	9.9	-	-
13	95075072	rs61962244	G	C	0.1	4.02E-06	5.13	2.6	10.09	-	-
13	95076104	rs7325398	T	C	0.11	1.08E-06	5.19	2.7	9.96	-	-
13	95078218	rs9590004	A	G	0.11	1.08E-06	5.2	2.71	9.98	-	-
13	95079024	rs7337957	G	A	0.1	4.20E-06	5.08	2.58	9.98	-	-
13	95079202	rs7338364	G	A	0.1	4.34E-06	5.06	2.57	9.93	-	-
14	82898979	rs1198402	G	T	0.46	4.94E-06	2.84	1.75	4.62	-	-
14	82903381	rs1953783	A	G	0.46	4.48E-06	2.86	1.76	4.64	-	-
14	82906017	rs1211307	G	A	0.46	4.51E-06	2.86	1.76	4.65	-	-
14	82910647	rs799248	T	C	0.47	3.59E-06	2.88	1.78	4.67	-	-
17	743921	rs34676006	C	T	0.2	1.41E-06	0.12	0.04	0.37	*NXN*	intron
17	758766	rs72812004	G	A	0.18	2.04E-06	0.1	0.03	0.35	*NXN*	intron
17	72671350	rs8071012	T	C	0.23	3.44E-06	4.29	2.31	7.97	*CTD-2006K23.2*	intron
19	7617733	rs202176122	T	TGATGG	0.42	3.02E-06	3.23	1.92	5.4	*PNPLA6*	intron
19	7617734	rs114424868	C	G	0.42	3.11E-06	3.22	1.92	5.4	*PNPLA6*	intron
19	7617735	rs115476330	T	A	0.42	3.11E-06	3.22	1.92	5.4	*PNPLA6*	intron
19	7617738	rs116049537	C	G	0.43	3.99E-06	3.16	1.89	5.28	*PNPLA6*	intron
19	7617740	rs116287023	G	T	0.43	3.99E-06	3.16	1.89	5.28	*PNPLA6*	intron
19	7618660	rs508740	A	G	0.45	4.21E-06	2.9	1.8	4.67	*PNPLA6*	intron
19	14568122	rs80161029	A	G	0.2	3.55E-06	3.75	2.15	6.53	*PKN1*	intron
19	14568217	rs7245494	A	G	0.21	4.68E-06	3.72	2.13	6.51	*PKN1*	intron
19	14568691	rs112483471	C	T	0.19	2.20E-06	3.87	2.22	6.76	*PKN1*	intron
19	14589068	rs76301301	T	G	0.21	1.34E-06	3.83	2.23	6.58	*GIPC1*	3'UTR

Supplemental Table 8. Single nucleotide polymorphisms (SNPs) associated with MTX central neurotoxicity (*P*<5.0E-06).

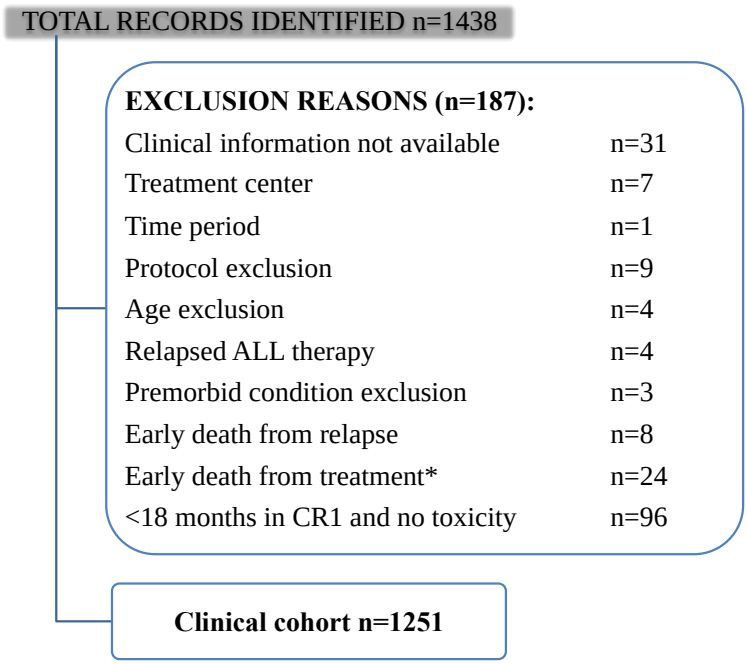
Table legend: These SNPs (n=60) are in addition to the top SNPs (*P* <1E-06) that we have described. Fifty-three of these SNPs are located near seven genes that are different to those genes associated with the top SNPs. *The annotated gene was determined by cross-referencing

RefSeq (38) , Ensembl 74 (39) and UCSC database information (hg19, 2015 update)(40) accessed through SNPnexus (2012 update (41), http://www.snp-nexus.org). SNP predicted function due to location, determined by RefSeq (38), accessed via SNPnexus (2012 update(41)). Where there was discrepancy or the gene was uncertain, a search was performed manually using NCBI dbSNP build 149 (42). "Intron", refers to intron variant within a protein-coding gene; "non-coding intron", intron variant within a non protein-coding gene, as per RefSeq nomenclature; 3'UTR, 3'untranslated region. CHR, chromosome; MAF, minor allele frequency; *P* value from genome-wide association study; OR, odds ratio; OR 95 CI (lower), lower value of 95% confidence interval for OR; OR 95 CI (upper), upper value of 95% confidence interval for OR. *Online only.*

CHR	Position	SNP	Non effect allele	Effect allele	MAF	*P*	OR	OR 95 CI (lower)	OR 95 CI (upper)	Gene*	Location
1	39957301	rs7555699	G	A	0.32	4.54E-06	0.35	0.22	0.55	*BMP8A*	5' upstream
19	14589068	rs76301301	T	G	0.21	1.34E-06	3.83	2.23	6.58	*GIPC1*	3'UTR

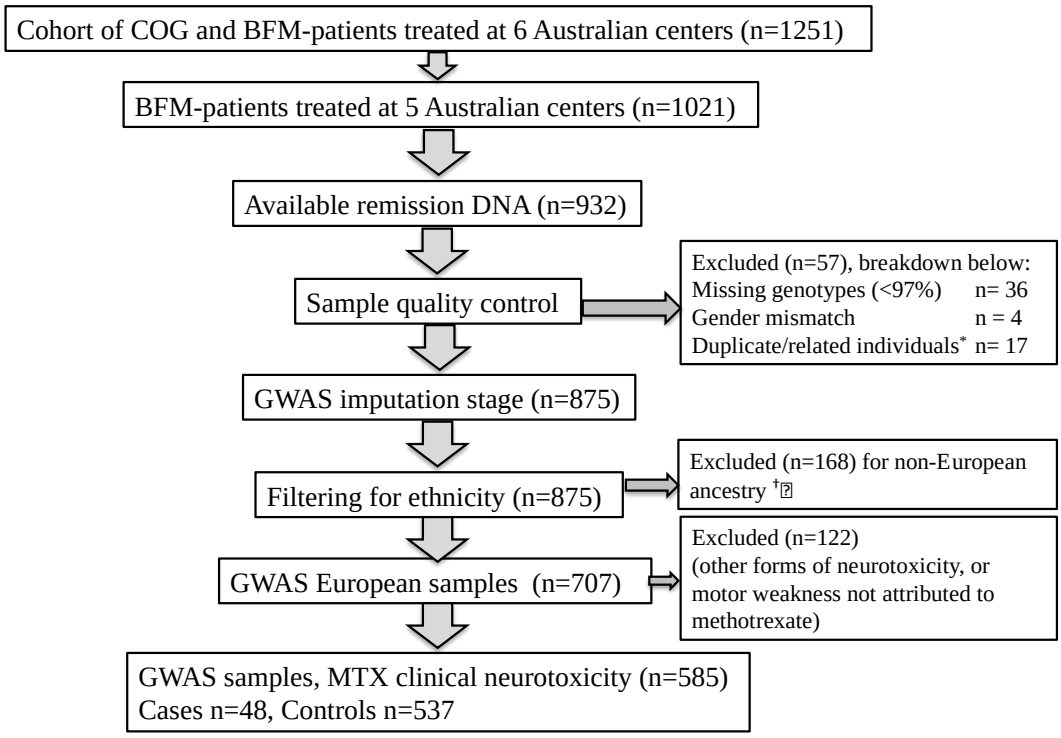
Supplemental Table 9. Single nucleotide polymorphisms (SNPs) located in regions of genes or SNPs with potential functional impact.

Table legend: *The annotated gene was determined by cross-referencing RefSeq (12) , Ensembl 74 (13) and UCSC database information (hg19, 2015 update)(14) accessed through SNPnexus (2012 update(15)). SNP predicted function due to location, determined by RefSeq (12), accessed via SNPnexus (2012 update(15)). "5'upstream" denotes SNPs that were located within 2 kb upstream of the 5' end of a transcript; "3'UTR", 3'untranslated region; CHR, chromosome; MAF, minor allele frequency; *P* values from the current genome-wide association study; OR, odds ratio; OR 95 CI (lower), lower value of 95% confidence interval; OR 95 CI (upper), upper value of 95% confidence interval



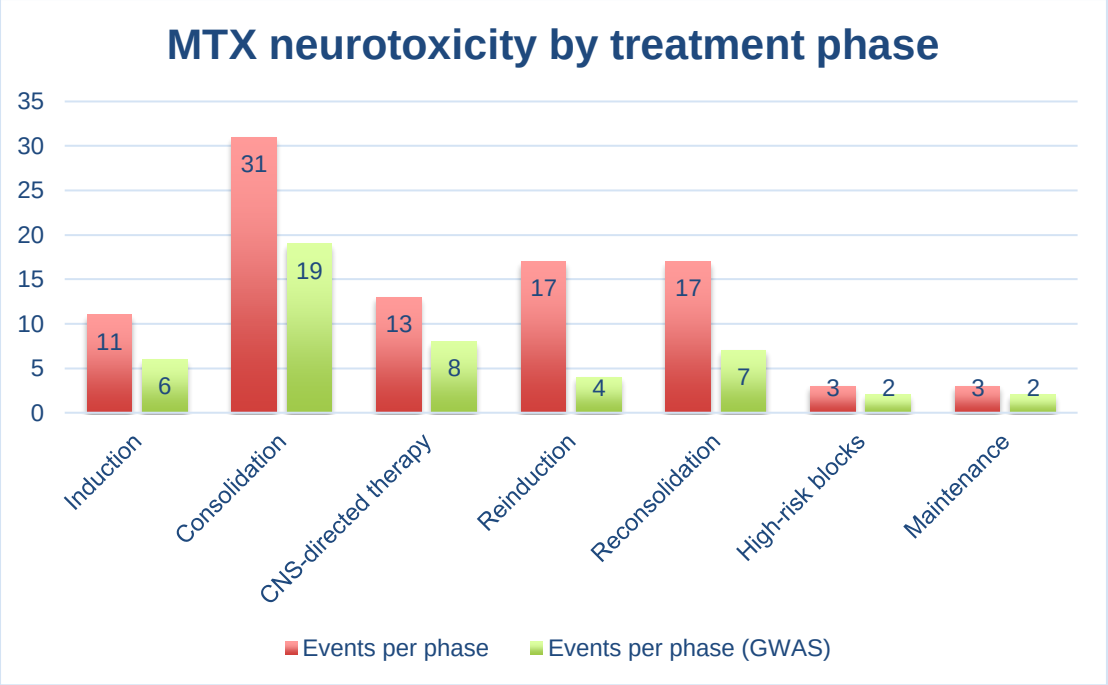
Supplemental Figure 1. Clinical cohort for symptomatic methotrexate neurotoxicity experienced in complete remission (CR1).

Figure legend: Patients without adequate clinical information to determine case or control status were excluded. *Early death from treatment: patients who experienced treatment-related mortality not related to target toxicities (VTE, neurotoxicity) were excluded. ALL, acute lymphoblastic leukemia. *Online only.*



Supplemental Figure 2. Consort diagram of 585 individuals in the GWAS discovery cohort for MTX neurotoxicity.

Figure legend: * Pi-hat threshold >0.2 † Non-European ancestry defined as per 1000 Genomes data. This figure describes the GWAS cohort, in relation to the larger clinical cohort (n=1251). Children (n=932) treated at 5 Australian centers (Sydney Children's Hospital, Children's Hospital Westmead, John Hunter Children's Hospital, Royal Children's Hospital Melbourne and Adelaide Women's and Children's Hospital) had available DNA samples for analysis. After quality control and filtering for ethnicity, there were 707 individuals of European ancestry in the GWAS analysis. Children who did not have MTX neurotoxicity but who had other types of neurotoxicity were excluded from the 'control' group. The final GWAS cohort for MTX neurotoxicity was comprised of 48 cases and 537 controls. *Online only.*



Supplemental Figure 3. MTX neurotoxicity events per treatment phase.

Figure legend: The number of symptomatic MTX neurotoxic events that occurred in each treatment phase is shown for the total cohort (n=95), with the largest number of events occurring during consolidation (n=17), reinduction (n=13) and reconsolidation (n=13). CNS-directed therapy includes “Protocol M” or “Interim Maintenance” phases. The number of events for those included in the genome-wide association study (GWAS) (“Events per phase (GWAS)”) are also depicted.

5 Supplemental References

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