The Pharmacogenomics Journal (2014), 1–7

www.nature.com/tpj

ORIGINAL ARTICLE

Genetic and metabolic determinants of methotrexate-induced mucositis in pediatric acute lymphoblastic leukemia

MAH den Hoed[1], E Lopez-Lopez[2], ML te Winkel[1], W Tissing[3], JDE de Rooij[1], A Gutierrez-Camino[2], A Garcia-Orad[2], E den Boer[4], R Pieters[1,5], SMF Pluijm[1], R de Jonge[4] and MM van den Heuvel-Eibrink[1]

Methotrexate (MTX) is an effective and toxic chemotherapeutic drug in the treatment of pediatric acute lymphoblastic leukemia (ALL). In this prospective study, we aimed to identify metabolic and genetic determinants of MTX toxicity. One hundred and thirty-four Dutch pediatric ALL patients were treated with four high infusions MTX (HD-MTX: 5 g m^{-2}) every other week according to the DCOG-ALL-10 protocol. Mucositis (National Cancer Institute grade $\geqslant 3$) was the most frequent occurring toxicity during the HD-MTX phase (20%) and occurred especially after the first MTX course. Mucositis was not associated with plasma MTX, plasma folate or plasma homocysteine levels. Patients with mucositis had higher erythrocyte folate levels at the start of protocol M than patients without mucositis (median 1.4 vs 1.2 μmol l^{-1}, $P < 0.008$), this could reflect an increased MTX uptake in mucosal cells of patients with mucositis. From 17 single-nucleotide polymorphisms in the MTX pathway, only patients with the wild-type variant of rs7317112 SNP in the *ABCC4* gene had more mucositis (AA (39%) vs AG/GG (15%), $P = 0.016$). We found no evidence that erythrocyte folate levels mediate in the association between the rs7317112 and mucositis.

The Pharmacogenomics Journal advance online publication, 4 November 2014; doi:10.1038/tpj.2014.63

INTRODUCTION

Acute lymphoblastic leukemia (ALL) represents 25% of all childhood malignancies.[1] Cure rates have reached 90% in the developed countries due to improved stratification and advanced treatment options over the past decades.[2,3] Consequently, it has gained importance to reduce toxicity of cancer treatment by identifying determinants of toxicity.

Methotrexate (MTX) is an important chemotherapeutic drug in the treatment of pediatric ALL. Side effects of MTX vary among patients and can lead to amendments of treatment with a possible impaired survival in serious cases.[4] The aim of this study is to identify metabolic and genetic determinants of MTX toxicity.

MTX enters the cell via the reduced folate carrier (RFC1/SLC19A1) or the solute carrier organic anion transporter (SLCO1B1).[5,6] In the cell, MTX is converted to MTX-polyglutamate (MTX-PG) and it inhibits dihydrofolate reductase, which depletes formation of the active form of folate; this folate depletion is cytotoxic to leukemic cells. MTX can further interfere with thymidylate synthase, 5,10-methylenetetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR) and thiopurine methyltransferase (TPMT).[7] MTX is eliminated through transporters such as multidrug resistance-associated proteins (ABCC2 and ABCC4)[8] (Supplementary Figure S1).

Several studies in ALL have suggested that variation in single-nucleotide polymorphisms (SNPs) in these aforementioned genes contribute to the inter-individual variation in MTX toxicity (Supplementary Table S1). But outcomes of previous studies were often contradictory, and they ignored the metabolic implication of SNPs. The novelty of this study is that it includes prospective monitoring of toxicity in a cohort of pediatric ALL patients, including not only genetic variation but also plasma and cellular assessment of MTX pathway metabolites (folate/homocysteine).

PATIENTS AND METHODS

Eligible for inclusion were children with newly diagnosed ALL (from November 2004 to March 2012) who were admitted to the Erasmus MC–Sophia Children's Hospital in Rotterdam or the University Medical Center Groningen (UMCG) –Beatrix Children's Hospital in Groningen. The patients were treated according to the Dutch Child Oncology Group ALL-10 protocol and were aged between 1 and 19 years. The ALL-10 protocol stratified patients into a standard-, medium- or a high-risk group. For the current study, only standard- and medium-risk patients were included, as high-risk patients received interfering concomitant drugs. Children with relevant germline aberrations, such as Down syndrome[9] and SPINK-1 mutation,[10] were excluded from this study due to their expected clinical aberrant toxicity profile (Figure 1).

The study was approved by medical ethical committee (MEC-05-358) and informed consent was obtained by parents or guardians and patients (in case they were >12 years) according to the Declaration of Helsinki.[11]

ALL-10 protocol and data collection

Patients were included before the start of protocol M, which is a 56-day treatment period and includes four courses of high-dose MTX (HD-MTX) (Supplementary Figure S2). At day 1 of protocol M, oral 6-mercaptopurine (25 mg m^{-2} daily) was started for 56 days. Patients received four courses of MTX intravenous infusions every 2 weeks at a dose of 5 g m^{-2} over 24 h starting at day 8. Each HD-MTX administration was combined with intrathecal triple therapy in a standard dose adjusted for age (8–12 mg MTX; 20–30 mg Cytosine Arabinoside; 8–12 mg Diadreson F aquosum).

[1]Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Rotterdam, The Netherlands; [2]Department of Genetics, Physical Anthropology and Animal Physiology, University of the Basque Country (UPV/EHU), Leioa, Spain; [3]Department of Oncology/Hematology, University of Groningen, University Medical Center Groningen-Beatrix Children's Hospital, Groningen, The Netherlands; [4]Department of Clinical Chemistry, Erasmus MC–University Medical Center, Rotterdam, The Netherlands and [5]Department of Pediatric Oncology/Hematology, Princess Maxima Center, Utrecht, The Netherlands. Correspondence: MM van den Heuvel-Eibrink, Department of Pediatric Oncology/Hematology, Erasmus MC–Sophia Children's Hospital, Room Na16.13, Dr Molewaterplein 60, Rotterdam 3015 GJ, The Netherlands.
E-mail: m.vandenheuvel@erasmusmc.nl
Received 6 May 2014; revised 1 September 2014; accepted 5 September 2014

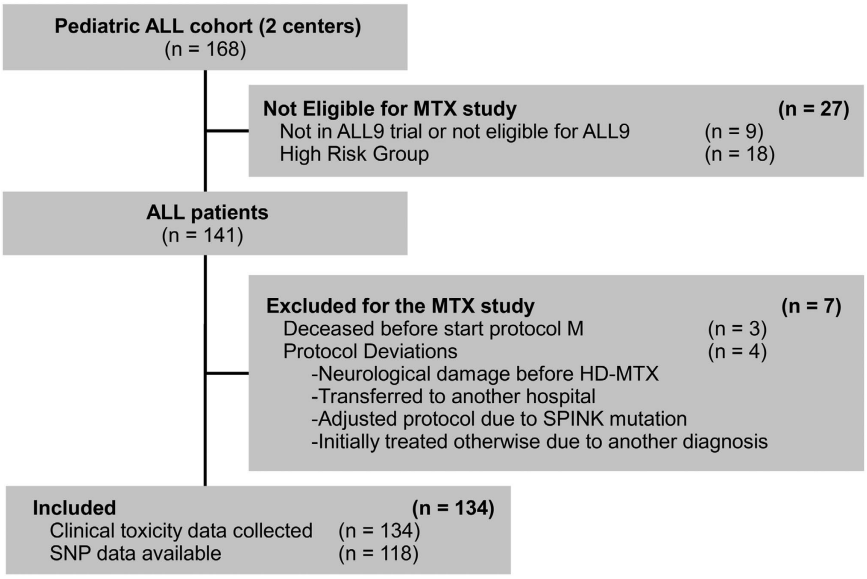

Figure 1. Flowchart of patient inclusion. ALL, acute lymphoblastic leukemia; HD-MTX, high-dose methotrexate; SNP, single-nucleotide polymorphism.

Leucovorin rescue (folinic acid: 15 mg m^{-2}) was administered every 6 h, starting at 42 h after the start of of HD-MTX administration with a minimum of three dosages. Standard supportive care guidelines included hyperhydration (2.5–3.0 l m^{-2} day^{-1}) and using sodium bicarbonate to keep the urine alkalinized (pH between 7 and 8).

Patients had a standard hospital admission of 48 h during the MTX courses. Plasma MTX levels were measured at 24 (T24) and 48 (T48) hours after starting the MTX-HD infusion. Patients were discharged from hospital as soon as MTX plasma levels at T48 were < 0.4 μmol l^{-1}. When MTX_{T48} plasma levels were > 0.4 μmol l^{-1}, hyperhydration, alkalinization and folic acid rescue was continued for a minimum of 24 h.

Toxicity assessment

A slightly modified version of The National Cancer Institute (NCI)[12] Common Terminology Criteria for Adverse Events v.3.0 score system was used to document toxicity. Toxicity was graded at five time points: just before each HD-MTX course and at the end of protocol M. The maximum experienced toxicity during and after the previous course was graded (Supplementary Figure S2, Supplementary Table S2). Relevant clinical toxicity was defined as NCI grade $\geqslant 3$, for mucosal, neurological and skin toxicity. Hospital readmissions were also recorded as a proxy for toxicity.

Metabolic determinants of toxicity

Plasma MTX was determined using the Abbot fluorescent polarization immune assay on an Abbott TDx FLx Immunology Analyzer (Abbott Diagnostics, Hoofddorp, The Netherlands). For patients in which blood samples of MTX were not exactly taken at 24 or 48 h, plasma MTX levels were extrapolated to 24 or 48 h with MwPharm (version 3.30) with the pharmacokinetic model from Rousseau and Marquet.[13]

Peripheral blood samples for measurement of MTX-pathway metabolites (plasma homocysteine and folate and erythrocyte folate) were collected from the patients in fasting state before the start of protocol M and 2 weeks after discontinuation of protocol M (Supplementary Figure S2). The EDTA tubes was kept on melting ice until centrifugation within 2 hours. Samples of MTX-polyglutamates ($MTX\text{-}PG_{1-5}$) were only collected 2 weeks after discontinuation of protocol M. All blood samples were stored at −80 °C and analyzed collectively at the end of the total study period. Erythrocyte and plasma folate were measured using electrochemiluminescence immunoassay (Modular E170, Roche, Almere, The Netherlands). Plasma homocysteine levels were analyzed using liquid chromatography–tandem mass spectrometry.[14]

Genetic determinants of toxicity

Candidate SNPs were selected based on their documented effect on enzyme activity or association with MTX toxicity by earlier published studies (Supplementary Table S1). Our selection included the following SNPs in: *MTHFR* ($rs1801133^{15-20}_{C>T}$ and $rs1801131^{18,21}_{A>C}$), MTRR ($rs1801394^{18}_{A>G}$), RFC1 ($rs1051266^{17,22}_{G>A}$), ABCC2 ($rs12826_{A>G,}$ $rs12826_{C>T,}$ and $rs3740065_{T>C}$),[23] ABCC4 ($rs1678392_{G>A}$, $rs2619312_{T>C}$, $rs7317112_{A>G}$, $rs9302061_{T>C}$, $rs9516519_{T>G}$ and $rs10219913_{T>C}$),[23] and SCLO1B1 ($rs48651564_{T>C}$).[24,25] SNPs in the gene TPMT ($rs1800462_{G>C}$, $rs1800460_{G>A}$ and $rs1142345_{A>G}$) were also selected as MTX indirectly inhibits the TPMT enzyme activity after HD-MTX infusions due to protein binding[7] and a low TPMT activity is known to cause toxicity for 6-MP[26] (Supplementary Table S3).

Genotyping

Peripheral blood was drawn at the start of protocol M, whereof genomic DNA was extracted using the Magna Pure Compact Nucleic Acid isolation kit (Roche Molecular Biochemicals, Almere, The Netherlands) in accordance with the manufacturer's instructions. Genotyping was performed using the Taqman allelic discrimination assays, PCR-restriction fragment length polymorphism or PCR sequencing. A Taqman allelic discrimination assay was performed on the Prism 7000 sequence detection system (Life Technologies, Applied Biosystems, Bleiswijk, The Netherlands) and compared with 500 healthy Dutch blood bank donors cohort.[27] PCR sequencing was performed using a BigDye terminator v1.1 Course Sequencing kit (PE Applied Biosystems, Foster City, CA, USA) on a 3130x Genetic Analyzer (Applied Biosystems). Sequence analysis was done with the CLC Workbench software (CLCbio, Aarhus, Denmark).

Statistical analysis

Clinical toxicities were included in the analyses as endpoints, these were defined as an NCI grade $>= 3$ (measured at least once during four cycles) and plasma MTX measurments. For each SNP, genotype frequency distribution was tested for Hardy–Weinberg equilibrium using the standard χ^2-test. Polymorphism groups were dichotomized into a dominant or recessive inheritance model, based on their significant association with each toxicity end point or levels of MTX or folate metabolites.[28]

Mann–Whitney *U*-test was used to examine the differences between MTX, folate and homocysteine levels and patients with and without toxicity or between the genotype categories. The χ^2-test was used to compare the frequency of toxicity between the genotype categories. Logistic regression analysis was performed and adjusted for age and

gender and, if applicable, MTX course. Finally, we tested for possible mediation of MTX levels or folate metabolites in the associations between SNPs and toxicity by following the requirements stated by Baron and Kenny.[29]

Analyses were controlled for multiple testing by repeating the analysis with measures from only the first course as an internal validation.

The significance level was set at $P = 0.05$ (two-tailed tests). Statistics were performed with SPSS Statistics Version 20.0.0.1 (SPSS, Chicago, IL, USA). Linkage disequilibrium was calculated with Haploview (version 4.2; Broad Institute, Cambridge, MA, USA),[30] using the International HapMap Project (release no. 24; http://www.hapmap.org).

RESULTS

Patients characteristics and frequency of toxicity

One hundred and thirty-four patients were included (Erasmus MC $n = 86$, UMCG $n = 48$) (Figure 1) with a median age of 5.3 years (range 1.4–18.1 years) of which 52% ($n = 70$) were male and 17 patients (13%) had T-cell ALL (Table 1).

At the start of protocol M, none of the patients showed signs of clinical toxicity (NCI $\geqslant$ grade 3). Most patients had a white blood cell count above the required trehshold of $1.5 \times 10^9\ l^{-1}$ at start of protocol M (92%, $n = 121$). However, 58% ($n = 71$) of the patients were neutropenic ($< 0.5 \times 10^9\ l^{-1}$). During protocol M, skin toxicity occurred in 7% ($n = 9$), diarrhea in 3% ($n = 2$) and neurotoxicity in 3% ($n = 2$) of the patients. Acute kidney toxicity at T48 occurred in only 1 patient (1%), and acute liver toxicity at T48 occurred in 6 patients (5%) (Figure 2).

Mucositis occurred in 20% ($n = 26$) of the patients and especially after the first course compared with the other courses (15% ($n = 18$) vs 8.1% ($n = 10$) in the other courses, $P = 0.006$). The occurrence of mucositis was not related to age, gender, immunophenotype nor neutropenia or leukopenia (Table 1).

Extra hospital admissions in between MTX courses were reported in 10 patients (8%). These were caused by severe mucositis ($n = 3$), nausea ($n = 1$), blood transfusions ($n = 2$), encephalopathy ($n = 1$), fever ($n = 2$) and unknown factors ($n = 1$). No deaths were reported during protocol M. Only mucositis was used as toxicity end point in further analyses.

Table 1. Patient characteristics of the pediatric ALL cohort compared in patients with and without mucositis during protocol M ($n = 134$)

Mucositis	*Yes* (n = *26*)	*No* (n = *104*)	P-*value*
Median age at diagnosis, range, years	5.7 (1.6–17.5)	6.4 (1.5–18.1)	0.61
Sex, n (%)			
Female	11 (42%)	50 (48%)	
Male	15 (58%)	54 (52%)	0.60
Immunophenotype, n (%)			
B-lineage	23 (89%)	88 (86%)	
T-lineage	3 (12%)	14 (14%)	0.53
Leukopenia T0, n (%)			
$< 1.5 \times 10^9\ l^{-1}$	2 (8%)	12 (11%)	
$> 1.5 \times 10^9\ l^{-1}$	24 (92%)	92 (89%)	0.57
Neutropenia T0, n (%)			
$< 0.5 \times 10^9\ l^{-1}$	20 (77%)	61 (41%)	
$> 0.5 \times 10^9\ l^{-1}$	6 (23%)	43 (59%)	0.09

Abbreviations: ALL, acute lymphoblastic leukemia; T0, measured at start of the protocol M. The analyses were repeated in patients with mucositis only during the first course, but results did not differ.

Metabolic determinants of MTX-induced toxicity

Median plasma MTX levels of all the four courses in 134 patients were 64 μmol l^{-1} at T24 ($n = 298$, range: 9–382 μmol l^{-1}) and 0.38 μmol l^{-1} at T48 ($n = 448$, range: 0.10–22 μmol l^{-1}).

There was no significant difference in median MTX plasma levels between patients with and without mucositis at T24 or T48 over all courses or per course. This was confirmed by multivariable logistic regression analyses, where we adjusted for age and gender (data not shown).

In 78 patients, the median baseline level of plasma homocysteine was 6.9 umol l^{-1} (3.3–20.2 umol l^{-1}), plasma folate level was 17.0 nmol l^{-1} (6.0–44.8 nmol l^{-1}) and erythrocyte folate level was 1.24 umol l^{-1} (0.81–3.61 umol l^{-1}).

Higher levels of baseline erythrocyte folate were found in patients with mucositis ($P = 0.012$, Figure 3). For every increase in μmol l^{-1} erythrocyte folate, the odds of developing mucositis was 1.10 (95% confidence interval (CI) 0.97–1.25). However, after removing one extreme outlier > 3 s.ds. from the mean of erythrocyte folate, a higher erythrocyte folate at baseline increased the odds of developing mucositis during protocol M (odds ratio (OR) = 1.23, 95% CI = 1.04–1.45), even after correction for age and gender (OR = 1.30, 95% CI = 1.08–1.57). Plasma folate and erythrocyte folate levels were correlated with each other ($r = 0.429$, $P < 0.001$). Plasma folate ($P = 0.907$) and plasma homocysteine ($P = 0.518$) were not associated with mucositis (Figure 3).

Compared with patients without mucositis, patients with mucositis had similar changes in erythrocyte folate, plasma folate and plasma homocysteine levels after therapy (from day 0 to 2 weeks after the end of the MTX courses). Baseline erythrocyte folate was not associated with MTX levels at T24 or T48 (at the first course or all courses) or levels of MTX-PG_{1-5} after therapy (data not shown).

Genetic determinants of MTX toxicity

All genotypes were in Hardy–Weinberg Equilibrium. χ^2-Test and univariate logistic analyses showed that only subjects with wild-type genotype rs7317112$_{A/A}$ (*ABCC4*) had more often mucositis than carriers of the G allele ($P = 0.02$; Table 2). After correction for age and gender, patients with wild-type rs7317112$_{A/A}$ genotype remained more prone to grade $\geqslant 3$ mucositis (AA, OR: 2.81, 95% CI (1.01–7.84)). All other selected SNPs were not associated to mucositis or extra hospital admissions (Table 2).

The wild-type *MTRR* rs1801394$_{A/A}$ and wild-type rs4149056$_{T/T}$ (SLCO1B1) were associated with higher T24 MTX levels, and wild-type rs7317112$_{A/A}$ (*ABCC4*) revealed higher T48 levels (Supplementary Table S4). As MTX levels were not associated with mucositis, MTX levels were not able to mediate in the association between SNPs and mucositis.

Wild-type rs4149056$_{T/T}$ (SLCO1B1) was the only genotype that was associated with higher baseline plasma folate levels (Supplementary Table S4). As rs4149056$_{T/T}$ was not associated with mucositis, rs4149056$_{T/T}$ was not able to mediate in the association between SNPs and mucositis.

Erythrocyte folate and SNP rs7317112$_{A>G}$ (*ABCC4*) are the only factors associated with mucositis. However, erythrocyte folate levels did not seem to mediate in this association between SNP and mucositis, as erythrocyte folate levels were not associated with SNP rs7317112$_{A>G}$ (*ABCC4*). In addition, erythrocyte folate and SNP rs7317112$_{A>G}$ (*ABCC4*) were also not correlated (Spearman's rho: $r = 0.002$, $P = 0.985$), neither associated (linear regression, β 0.23; 95% CI (−1.17 to 1.63)), nor interacted, as the interaction term 'rs7317112 × erythrocyte folate level' was not significant (interaction term: $P = 0.235$).

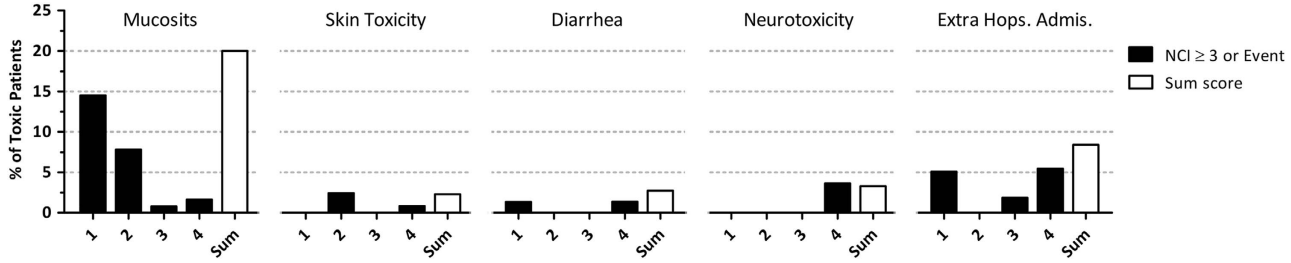

Figure 2. Prevalence of toxicity after methotrexate (MTX) courses during protocol M (5 g m^{-2} MTX). The maximum grade of toxicity after a MTX course was documented 2 weeks later, during the hospital visit for the next MTX course. The National Cancer Institute (NCI) criteria grade ⩾3 severities are depicted. '1–4' represent the consecutive MTX courses; 'Sum' represents the maximum score of toxicity during all the four courses; Extra Hops. Admis. = extra hospital admissions in between MTX courses.

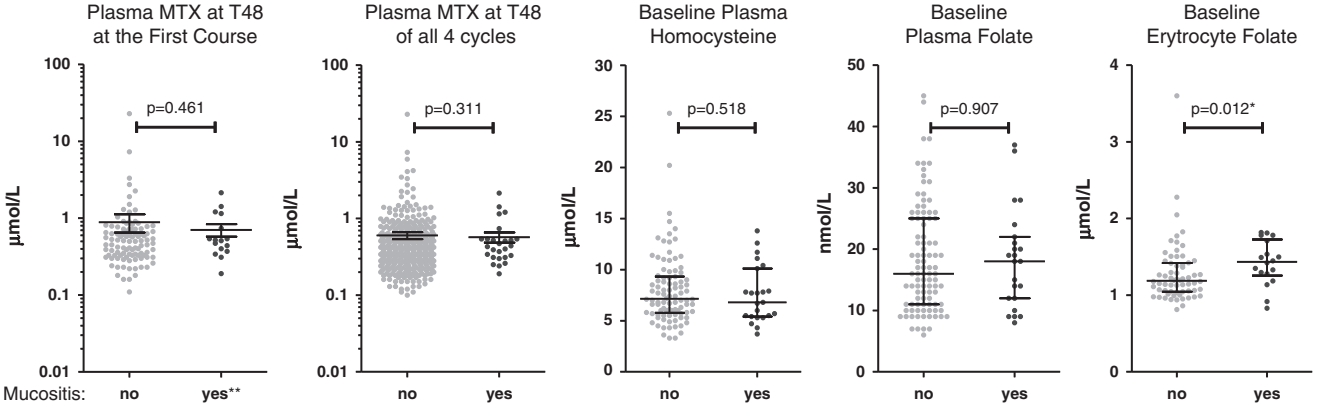

Figure 3. Comparison of plasma MTX levels, baseline folate and homocysteine levels in patients with and without mucositis during protocol M. Shown are the mean and the s.e.m. for methotrexate (MTX) levels and the median and the interquartile range for the folate metabolites. *Significant association $P < 0.05$; **Mucositis measured after only the first course.

DISCUSSION

This study evaluated the determinants of MTX-related toxicity in a prospective cohort of pediatric ALL patients. The most apparent grade ⩾3 toxicity was mucositis (20%), while other types of toxicity were observed in $<10\%$ of the patients (diarrhea, skin, neurotoxicity, kidney or liver toxicity). The occurrence of mucositis was associated with higher erythrocyte folate levels at baseline but not with baseline plasma levels of MTX, homocysteine or folate. Of the 17 selected SNPs, wild-type rs7317112$_{A/A}$ in the *ABCC4* gene was the only allelic variant that was associated with the occurrence of mucositis. As erythrocyte folate and rs7317112$_{A/A}$ were not correlated, neither associated nor interacted with each other, we can conclude that they are associated with mucositis probably through different biological pathways.

Mucositis was the most often reported toxicity, which is in line with previous studies reporting a prevalence of mucositis (NCI ⩾ grade 3) of 20–40% in pediatric ALL patients after treatment with HD-MTX 5 mg m^{-2}.[17,31–33] Mucositis was more prevalent after the first MTX course that may have been due to several factors. First, 90% of the patients started protocol M with neutropenia. It is conceivable that patients with a lower neutrophil count may have an impaired ability to be protected against oral mucosal damage, which may affect the proliferation of oral epithelial cells.[34] In our study mucositis was, however, not associated with neutropenia (continous neutrophil count or neutrophil count <0.5 umol l^{-1}). This illustrates that it is safe to start protocol M with a white blood count $>1\times10^9$ l^{-1} regardless of neutrophil count. Second, other factors contributing to mucositis after the first MTX course could be the preceding treatment with cyclophosphamide, 6-mercaptopurine and cytarabine just before protocol M.[35] These drugs are known to induce mucositis and could enhance mucositis response after the first course.[36] Finally, folinic acid is administered after the first MTX course; this increases the cellular folate levels and could therefore decrease the mucositis rate after the following MTX courses.

Mucositis was not associated with MTX plasma levels in our study. This is in line with other studies that have shown that cell injury was not related with a high MTX plasma concentration[37,38] but rather with the MTX clearance. This illustrates that plasma levels at T24 and T48 may not be the best indicator of toxicity during treatment. Alternative options such as the area under the curve of MTX clearance over a longer period of time or the measurement of the active polyglutamate form of MTX (MTX-PG)[39] may be more valuable than MTX levels. Accumulation of intracellular MTX-PGs have been shown to be associated with anti-leukemic effects and relapse in children with ALL.[39,40] However, in our cohort, we found no association between MTX-PG$_{1-5}$ measured in erythrocytes at stop protocol M and toxicity.

Mucositis is caused by intracellular depletion of folate after administering MTX that induces mucosal cell death by blocking crucial steps in the DNA synthesis.[41] It is possible that the folate status and dietary folate intake of a patient influences the occurrence of mucositis.[4] As stated above, our data showed that

Table 2. Comparison between single-nucleotide polymorphisms and mucosal toxicity

			Total		*Mucositis*				
Gene	*SNP*		n	*%*	*Yes*	*%*	*OR*	*(95% CI)*	P-*value*
ABCC2	rs12826	A/A	37	42	10	27	Reference		
		A/G-G/G	43	58	12	28	1.05	(0.39–2.80)	0.93
ABCC2	rs717620	G/G	57	68	15	26	Reference		
		G/A-A/A	23	32	7	30	1.23	(0.42–3.56)	0.45
ABCC2	rs3740065	T/T	61	80	17	28	Reference		
		T/C-C/C	19	20	5	26	0.92	(0.29–2.96)	0.57
ABCC4	rs1678392	G/G	57	70	15	26	Reference		
		G/A-A/A	21	30	7	33	1.40	(0.47–4.13)	0.37
ABCC4	rs2619312	T/T	55	63	14	25	Reference		
		T/C-C/C	25	37	8	32	1.38	(0.49–3.89)	0.36
ABCC4	rs7317112	A/A	41	53	16	39	Reference		
		A/G-G/G	39	47	6	15	0.28	(0.10–0.83)	0.016**
ABCC4	rs9302061	T/T	30	35	7	23	Reference		
		T/C-C/C	50	65	15	30	1.41	(0.50–3.98)	0.35
ABCC4	rs9516519	T/T	59	70	13	22	Reference		
		T/G-G/G	21	30	9	43	2.65	(0.92–7.67)	0.06
ABCC4	rs10219913	T/T	58	73	18	31	Reference		
		T/C-C/C	22	27	4	18	0.49	(0.15–1.67)	0.40
MTHFR	rs1801133	C/C	40	50	9	23	Reference		
		C/T-T/T	40	50	13	33	1.66	(0.61–4.48)	0.23
MTHFR	rs1801131	A/A	40	49	11	28	Reference		
		A/C-C/C	40	51	11	28	1.00	(0.37–2.67)	0.60
MTRR	rs1801394	A/A	14	16	4	29	Reference		
		A/G-G/G	66	84	18	27	0.94	(0.26–3.37)	1.00
RFC1	rs1051266	G/G	26	30	8	31	Reference		
		G/A-A/A	54	70	14	26	0.79	(0.28–2.21)	0.65
TPMT*2	rs1800462	G/G	74	92	21	28			
		G/C-C/C	6	8	1	17	NA		NA
TPMT*3B	rs1800460	G/G	74	93	21	28			
		G/A-A/A	6	7	1	17	NA		NA
TPMT*3C	rs1142345	A/A	74	93	21	28			
		A/G-G/G	6	7	1	17	NA		NA
SCLO1B1	rs4149056	T/T	58	75	18	31	Reference		
		T/C-C/C	22	25	4	18	0.49	(0.15–1.67)	0.40

Abbreviations: CI, confidence interval; NA, not analyzable because of low numbers; SNP, single-nucleotide polymorphism. rs1801133, MTHFR 677 C>T; rs1801131, 1298 A>C; rs1801394, MTRR66A>G; RFC1, SLC19A1; rs1051266, RFC1 80 G>A; rs1800462, TPMT*2 238 G>C; rs1800460, TPMT*3B 460 G>A; rs1142345, TPMT*3C 719 A>G; rs4149056, SCLO1B1 521T>C. **Significant association between mucositis after the first MTX course and the SNP.

mucositis was not associated with baseline plasma levels of homocysteine or folate, but an association was found with higher baseline levels of erythrocyte folate. This may be due to the fact that erythrocyte folate levels reflect the plasma folate levels of the previous 3 months,[42,43] whereas plasma folate levels strongly correlate with daily dietary intake. Therefore, plasma folate levels seem to reflect the biological state less precise and with more uncertainty. Individuals with higher baseline erythrocyte folate levels may have a more effective cellular uptake and retention of folate.[44,45] As MTX is structurally similar to folate and uses the same cellular metabolism and transport routes,[46,47] there could be a higher uptake of MTX by the mucosal cell. However, we did not find any association between baseline erythrocyte folate and MTX-PG_{1-5} at stop protocol M, probably due to the fact that MTX-PGs were measured 9 weeks later than the measurement of erythrocyte folate.

It remains debatable whether folate supplementation before protocol M would prevent MTX toxicity, and more clinical trials would be necessary to find the optimal folic acid dosage. However, it has been suggested that folate supplementation can counteract the anti-leukemic activity of MTX and should not be compromised by decreasing toxicity which is not life threatening.[48–52]

In this prospective study, 17 recently reported relevant candidate SNPs were included to study genetic variation. Only wild-type $rs7317112_{A/A}$ genotype in the *ABCC4* gene was identified to be associated with less occurrence of mucositis (Table 2). We also found that $rs7317112_{A/G\text{-}G/G}$ was associated with higher MTX levels at T48. Other *ABCC4* polymorphisms were previously found to be associated with a decreased clearance of MTX in a treatment protocol with 3 and 5 g m^{-2} MTX.[23] The *ABCC4* gene encodes the multi-drug resistance protein 4 (*MRP4*), a member of the ATP-binding cassette family involved in low-affinity and high-capacity efflux of molecules, such as MTX (MTX-PG1) and folate.[47] MRP4 is expressed in many tissues, such as the liver, kidney, mucosa and various blood cells.[53,54] $rs7317112_{A>G}$ is located in intron 1 of the *ABCC4* gene[55] in putative intronic enhancers and a CpG site, which could carry changes in the methylation pattern and ABCC4 expression.[56] The exact biological mechanism of $rs7317112_{A>G}$, which is associated with less mucositis and higher MTX levels, needs to be further explored.

The present study with well-documented prospectively collected data did not confirm previously found associations between SNPs and mucositis (Supplementary Table S1). Previous studies showed conflicting results regarding the association between SNPs and toxicity. Also, different toxicity end points

and various dosages of MTX hamper comparison between these studies. In addition, it is conceivable that when using very high MTX dosages in pediatric ALL (5 g m^{-2}), allelic variants become less relevant as the high dose may overrule the influence of genetic variation. The use of SNPs may therefore not be relevant in clinical practice to prevent non-life-threatening toxicity. It may be of more value to focus on treatment efficacy by personalizing MTX dosages to improve treatment.

In conclusion, mucositis occurs especially after the first MTX course, and it was the most frequently occurring toxicity in our cohort of pediatric ALL patients during the HD-MTX phase. Plasma levels of MTX, folate and homocysteine were not associated with mucositis. The only determinants of mucositis in pediatric ALL during MTX-HD treatment were a higher baseline erythrocyte folate, which may reflect the accumulation of MTX polyglutamates in mucosal cells, and the wild-type variant of SNP rs7317112 in *ABCC4*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This study was financially supported by Stichting Kinderen Kankervrij (KiKa errant, nr. 67) and an Erasmus MC translational grant (to EdB).

REFERENCES

1 Gatta G, Botta L, Rossi S, Aareleid T, Bielska-Lasota M, Clavel J *et al.* Childhood cancer survival in Europe 1999-2007: results of EUROCARE-5-a population-based study. *Lancet Oncol* 2014; **15**: 35–47.
2 Pui CH, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *J Clin Oncol* 2011; **29**: 551–565.
3 Kamps WA, van der Pal-de Bruin KM, Veerman AJ, Fiocco M, Bierings M, Pieters R. Long-term results of Dutch Childhood Oncology Group studies for children with acute lymphoblastic leukemia from 1984–2004. *Leukemia* 2010; **24**: 309–319.
4 Crews KR, Liu T, Rodriguez-Galindo C, Tan M, Meyer WH, Panetta JC *et al.* High-dose methotrexate pharmacokinetics and outcome of children and young adults with osteosarcoma. *Cancer* 2004; **100**: 1724–1733.
5 Niemi M, Pasanen MK, Neuvonen PJ. Organic anion transporting polypeptide 1B1: a genetically polymorphic transporter of major importance for hepatic drug uptake. *Pharmacol Rev* 2011; **63**: 157–181.
6 Zhao R, Diop-Bove N, Visentin M, Goldman ID. Mechanisms of membrane transport of folates into cells and across epithelia. *Annu Rev Nutr* 2011; **31**: 177–201.
7 Wennerstrand P, Martensson LG, Soderhall S, Zimdahl A, Appell ML. Methotrexate binds to recombinant thiopurine S-methyltransferase and inhibits enzyme activity after high-dose infusions in childhood leukaemia. *Eur J Clin Pharmacol* 2013; **69**: 1641–1649.
8 Krajinovic M, Moghrabi A. Pharmacogenetics of methotrexate. *Pharmacogenomics* 2004; **5**: 819–834.
9 Ravindranath Y. Down syndrome and leukemia: new insights into the epidemiology, pathogenesis, and treatment. *Pediatr Blood Cancer* 2005; **44**: 1–7.
10 Witt H, Luck W, Hennies HC, Classen M, Kage A, Lass U *et al.* Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. *Nat Genet* 2000; **25**: 213–216.
11 Assembly WMAG. *The Declaration of Helsinki — Sixth Revision*. Helsinki, Finland, 2008.
12 Trotti A, Colevas AD, Setser A, Rusch V, Jaques D, Budach V *et al.* CTCAE v3.0: development of a comprehensive grading system for the adverse effects of cancer treatment. *Semin Radiat Oncol* 2003; **13**: 176–181.
13 Rousseau A, Marquet P. Application of pharmacokinetic modelling to the routine therapeutic drug monitoring of anticancer drugs. *Fundam Clin Pharmacol* 2002; **16**: 253–262.
14 Ducros V, Belva-Besnet H, Casetta B, Favier A. A robust liquid chromatography tandem mass spectrometry method for total plasma homocysteine determination in clinical practice. *Clin Chem Lab Med* 2006; **44**: 987–990.
15 Urano W, Taniguchi A, Yamanaka H, Tanaka E, Nakajima H, Matsuda Y *et al.* Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. *Pharmacogenetics* 2002; **12**: 183–190.
16 van Ede AE, Laan RF, Blom HJ, Huizinga TW, Haagsma CJ, Giesendorf BA *et al.* The C677T mutation in the methylenetetrahydrofolate reductase gene: a genetic risk factor for methotrexate-related elevation of liver enzymes in rheumatoid arthritis patients. *Arthritis Rheum* 2001; **44**: 2525–2530.
17 Shimasaki N, Mori T, Samejima H, Sato R, Shimada H, Yahagi N *et al.* Effects of methylenetetrahydrofolate reductase and reduced folate carrier 1 polymorphisms on high-dose methotrexate-induced toxicities in children with acute lymphoblastic leukemia or lymphoma. *J Pediatr Hematol Oncol* 2006; **28**: 64–68.
18 Huang L, Tissing WJ, de Jonge R, van Zelst BD, Pieters R. Polymorphisms in folate-related genes: association with side effects of high-dose methotrexate in childhood acute lymphoblastic leukemia. *Leukemia* 2008; **22**: 1798–1800.
19 Aplenc R, Thompson J, Han P, La M, Zhao H, Lange B *et al.* Methylenetetrahydrofolate reductase polymorphisms and therapy response in pediatric acute lymphoblastic leukemia. *Cancer Res* 2005; **65**: 2482–2487.
20 Seidemann K, Book M, Zimmermann M, Meyer U, Welte K, Stanulla M *et al.* MTHFR 677 (C-->T) polymorphism is not relevant for prognosis or therapy-associated toxicity in pediatric NHL: results from 484 patients of multicenter trial NHL-BFM 95. *Ann Hematol* 2006; **85**: 291–300.
21 de Jonge R, Hooijberg JH, van Zelst BD, Jansen G, van Zantwijk CH, Kaspers GJ *et al.* Effect of polymorphisms in folate-related genes on in vitro methotrexate sensitivity in pediatric acute lymphoblastic leukemia. *Blood* 2005; **106**: 717–720.
22 Jansen G, Mauritz RM, Assaraf YG, Sprecher H, Drori S, Kathmann I *et al.* Regulation of carrier-mediated transport of folates and antifolates in methotrexate-sensitive and-resistant leukemia cells. *Adv Enzyme Regul* 1997; **37**: 59–76.
23 Lopez-Lopez E, Ballesteros J, Pinan MA, Sanchez de Toledo J, Garcia de Andoin N, Garcia-Miguel P *et al.* Polymorphisms in the methotrexate transport pathway: a new tool for MTX plasma level prediction in pediatric acute lymphoblastic leukemia. *Pharmacogenet Genomics* 2013; **23**: 53–61.
24 Ramsey LB, Bruun GH, Yang W, Trevino LR, Vattathil S, Scheet P *et al.* Rare versus common variants in pharmacogenetics: SLCO1B1 variation and methotrexate disposition. *Genome Res* 2012; **22**: 1–8.
25 Radtke S, Zolk O, Renner B, Paulides M, Zimmermann M, Moricke A *et al.* Germline genetic variations in methotrexate candidate genes are associated with pharmacokinetics, toxicity, and outcome in childhood acute lymphoblastic leukemia. *Blood* 2013; **121**: 5145–5153.
26 Krynetski E, Evans WE. Drug methylation in cancer therapy: lessons from the TPMT polymorphism. *Oncogene* 2003; **22**: 7403–7413.
27 Griffioen PH, de Jonge R, van Zelst BD, Montserrate Brouns R, Lindemans J. Detection and allele-frequencies of the 833T>C, 844ins68 and a novel mutation in the cystathionine beta-synthase gene. *Clin Chim Acta* 2005; **354**: 191–194.
28 Sole X, Guino E, Valls J, Iniesta R, Moreno V. SNPStats: a web tool for the analysis of association studies. *Bioinformatics* 2006; **22**: 1928–1929.
29 Baron RM, Kenny DA. The moderator-mediator variable distinction in social psychological research: conceptual, strategic, and statistical considerations. *J Pers Soc Psychol* 1986; **51**: 1173–1182.
30 Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**: 263–265.
31 Buitenkamp TD, Mathot RA, de Haas V, Pieters R, Zwaan CM. Methotrexate-induced side effects are not due to differences in pharmacokinetics in children with Down syndrome and acute lymphoblastic leukemia. *Haematologica* 2010; **95**: 1106–1113.
32 Liu SG, Li ZG, Cui L, Gao C, Li WJ, Zhao XX. Effects of methylenetetrahydrofolate reductase gene polymorphisms on toxicities during consolidation therapy in pediatric acute lymphoblastic leukemia in a Chinese population. *Leuk Lymphoma* 2011; **52**: 1030–1040.
33 Faganel Kotnik B, Grabnar I, Bohanec Grabar P, Dolzan V, Jazbec J. Association of genetic polymorphism in the folate metabolic pathway with methotrexate pharmacokinetics and toxicity in childhood acute lymphoblastic leukaemia and malignant lymphoma. *Eur J Clin Pharmacol* 2011; **67**: 993–1006.
34 Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest* 2000; **80**: 617–653.
35 van Kooten Niekerk PB, Schmiegelow K, Schroeder H. Influence of methylene tetrahydrofolate reductase polymorphisms and coadministration of antimetabolites on toxicity after high dose methotrexate. *Eur J Haematol* 2008; **81**: 391–398.
36 Niscola P, Romani C, Cupelli L, Scaramucci L, Tendas A, Dentamaro T *et al.* Mucositis in patients with hematologic malignancies: an overview. *Haematologica* 2007; **92**: 222–231.
37 Cheng KK. Association of plasma methotrexate, neutropenia, hepatic dysfunction, nausea/vomiting and oral mucositis in children with cancer. *Eur J Cancer Care (Engl)* 2008; **17**: 306–311.

38 Maiguma T, Hayashi Y, Ueshima S, Kaji H, Egawa T, Chayama K *et al.* Relationship between oral mucositis and high-dose methotrexate therapy in pediatric acute lymphoblastic leukemia. *Int J Clin Pharmacol Ther* 2008; **46**: 584–590.
39 Masson E, Relling MV, Synold TW, Liu Q, Schuetz JD, Sandlund JT *et al.* Accumulation of methotrexate polyglutamates in lymphoblasts is a determinant of antileukemic effects in vivo. A rationale for high-dose methotrexate. *J Clin Invest* 1996; **97**: 73–80.
40 Mikkelsen TS, Sparreboom A, Cheng C, Zhou Y, Boyett JM, Raimondi SC *et al.* Shortening infusion time for high-dose methotrexate alters antileukemic effects: a randomized prospective clinical trial. *J Clin Oncol* 2011; **29**: 1771–1778.
41 Kremer JM. Toward a better understanding of methotrexate. *Arthritis Rheum* 2004; **50**: 1370–1382.
42 Ganji V, Kafai MR. Trends in serum folate, RBC folate, and circulating total homocysteine concentrations in the United States: analysis of data from National Health and Nutrition Examination Surveys, 1988-1994, 1999-2000, and 2001-2002. *J Nutr* 2006; **136**: 153–158.
43 Bailey LB. *Folate in Health and Disease*, 2nd edn. Taylor & Francis, 2010.
44 de Rotte MC, de Jong PH, Pluijm SM, Calasan MB, Barendregt PJ, van Zeben D *et al.* Association of low baseline levels of erythrocyte folate with treatment nonresponse at three months in rheumatoid arthritis patients receiving methotrexate. *Arthritis Rheum* 2013; **65**: 2803–2813.
45 Kim YI, Fawaz K, Knox T, Lee YM, Norton R, Arora S *et al.* Colonic mucosal concentrations of folate correlate well with blood measurements of folate status in persons with colorectal polyps. *Am J Clin Nutr* 1998; **68**: 866–872.
46 Goodsell DS. The molecular perspective: methotrexate. *Oncologist* 1999; **4**: 340–341.
47 Assaraf YG. The role of multidrug resistance efflux transporters in antifolate resistance and folate homeostasis. *Drug Resist Updat* 2006; **9**: 227–246.
48 Pinedo HM, Zaharko DS, Bull JM, Chabner BA. The reversal of methotrexate cytotoxicity to mouse bone marrow cells by leucovorin and nucleosides. *Cancer Res* 1976; **36**: 4418–4424.
49 Rask C, Albertioni F, Schroder H, Peterson C. Oral mucositis in children with acute lymphoblastic leukemia after high-dose methotrexate treatment without delayed elimination of methotrexate: relation to pharmacokinetic parameters of methotrexate. *Pediatr Hematol Oncol* 1996; **13**: 359–367.
50 Khanna D, Park GS, Paulus HE, Simpson KM, Elashoff D, Cohen SB *et al.* Reduction of the efficacy of methotrexate by the use of folic acid: post hoc analysis from two randomized controlled studies. *Arthritis Rheum* 2005; **52**: 3030–3038.
51 Sterba J, Dusek L, Demlova R, Valik D. Pretreatment plasma folate modulates the pharmacodynamic effect of high-dose methotrexate in children with acute lymphoblastic leukemia and non-Hodgkin lymphoma: 'folate overrescue' concept revisited. *Clin Chem* 2006; **52**: 692–700.
52 Cohen IJ, Wolff JE. How long can folinic acid rescue be delayed after high-dose methotrexate without toxicity? *Pediatr Blood Cancer* 2014; **61**: 7–10.
53 Russel FG, Koenderink JB, Masereeuw R. Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules. *Trends Pharmacol Sci* 2008; **29**: 200–207.
54 Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S *et al.* BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. *Genome Biol* 2009; **10**: R130.
55 McLaren W, Pritchard B, Rios D, Chen Y, Flicek P, Cunningham F. Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. *Bioinformatics* 2010; **26**: 2069–2070.
56 Samuelsson J, Alonso S, Ruiz-Larroya T, Cheung TH, Wong YF, Perucho M. Frequent somatic demethylation of RAPGEF1/C3G intronic sequences in gastrointestinal and gynecological cancer. *Int J Oncol* 2011; **38**: 1575–1577.

Supplementary Information accompanies the paper on the The Pharmacogenomics Journal website (http://www.nature.com/tpj)