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

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Abstract

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. 134 Dutch pediatric ALL patients were treated with four high infusions MTX(HD-MTX: 5 g/m^2) every other week according to the DCOG-ALL10 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. Higher erythrocyte folate levels measured at start of protocol M(median 1.2 µmol/L vs. 1.4 µmol/L, p<0.008), which could reflect an increased MTX uptake in mucosal cells, were associated with more mucositis. From 17 single nucleotide polymorphisms(SNPs) in the MTX pathway, only patients with the wild-type variant of rs7317112 SNP in *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.

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 last 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 solute carrier organic anion transporter (SLCO1B1)[5, 6]. In the cell, MTX is converted to MTX-polyglutamate (MTX-PG) and it inhibits dihydrofolate reductase (DHFR) which depletes formation of the active form of folate; this folate depletion is cytotoxic to leukemic cells. MTX can further interfere with thymidylate synthase (TS), 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] (supplemental figure 1).

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 (supplemental table 1). 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 1-19 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], 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 older than 12 years) according to the Declaration of Helsinki[11].

ALL10 Protocol and data collection

Patients were included before start of Protocol M, which is a 56-day treatment period including four courses of High Dose-MTX (HD-MTX) (supplemental figure 2). At day 1 of protocol M, oral 6-Mercaptopurine (25 mg/m^2 daily) was started for 56 days. Patients received 4 courses of MTX intravenous (IV) infusions every 2 weeks at a dose of 5 g/m^2 over 24 hrs 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). Leucovorin rescue (folinic acid: 15 mg/m^2) was administered every 6 hours, starting at 42 hours after start of the of HD-MTX administration with a minimum of three dosages. Standard supportive care guidelines included hyperhydration (2.5–3.0 L/m^2/day) and using sodium bicarbonate to keep the urine alkalinized (pH between 7 and 8).

Patients had a standard hospital admission of 48 hours 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 below 0.4 µmol/L. When MTX_{T48} plasma levels were higher than 0.4 µmol/L, hyperhydration, alkalinization and folic acid rescue was continued for a minimum of 24 hours.

Toxicity assessment

A slightly modified version of The National Cancer Institute (NCI)[12] Common Terminology Criteria for Adverse Events v.3.0 (CTCAE) 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 (supplemental figure 2, supplemental table 2). Relevant clinical toxicity was defined as NCI grade ≥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 hours, plasma MTX levels were extrapolated to 24 or 48 hours with MwPharm (version 3.30) with the pharmacokinetic model from Rousseau[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 two weeks after discontinuation of protocol M (supplemental figure 2). The EDTA tubes was kept on melting ice until centrifugation within two hours. Samples of MTX-polyglutamates ($MTX\text{-}PG_{1\text{-}5}$) were only collected two 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, 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 (supplemental table 1). Our selection included the following SNPs in; *MTHFR* (rs1801133$_{C>T}$[15-20] and rs1801131$_{A>C}$[18, 21]), MTRR (rs1801394$_{A>G}$[18]), RFC1 (rs1051266$_{G>A}$[17, 22]), 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](supplemental table 3).

Genotyping

Peripheral blood drawn at start of protocol M whence genomic DNA was extracted using the Magna Pure Compact Nucleic Acid isolation kit (Roche Molecular Biochemicals, Almere, Netherlands) in accordance with the manufacturer's instructions. Genotyping was performed using Taqman allelic discrimination assays, PCR-RFLP or PCR sequencing. A Taqman allelic discrimination assay was performed on the Prism 7000 sequence detection system (Life Technologies, Applied Biosystems, Bleiswijk, 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 CLC Workbench software (CLCbio, Aarhus, Denmark).

Statistical Analysis

Clinical toxicity was defined as an NCI grade ≥ 3, and plasma MTX measurements were included in the analysis as endpoints. For each SNP, genotype frequency distribution was tested for Hardy–Weinberg equilibrium (HWE) 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 endpoint 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. Lastly, 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 et al.[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 Inc., Chicago, IL, USA). Linkage disequilibrium was calculated with Haploview (version 4.2; Broad Institute, Cambridge, MA, United States)[30], using International HapMap Project (release #24; http://www.hapmap.org).

Results

Patients characteristics and frequency of toxicity

134 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 ≥ grade 3). At the start of protocol M, most patients had white blood cell count above the required threshold of 1.5×10^9/L (92%, n=121). However, 58% (n=71) patients were neutropenic ($<0.5 \times 10^9$/L). 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 to 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 endpoint in further analyses.

Metabolic determinants of MTX-induced toxicity

Median plasma MTX levels of all the four courses in 134 patients were 64 µmol/L at T24 (n=298, range: 9-382 µmol/L) and 0.38 µmol/L at T48 (n=448, range: [0.10-22 µmol/L]).

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, were we adjusted for age and gender (data not shown).

In 78 patients, the median baseline level of plasma homocysteine was 6.9 umol/L [3.3-20.2 umol/L], and plasma folate level was 17.0 nmol/L [6.0-44.8 nmol/L] and erythrocyte folate level was 1.24 umol/L [0.81-3.61 umol/L].

Higher levels of baseline erythrocyte folate were found in patient with mucositis (p=0.012, figure 3). For every increase in µmol/L erythrocyte folate the odds of developing mucositis was 1.10 (95%CI 0.97-1.25). However after removing one extreme outlier more than 3 standard deviations from the mean of erythrocyte folate, a higher erythrocyte folate at baseline increased the odds of developing mucositis during protocol M (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 to mucositis (figure 3).

Compared to patients without mucositis, patients with mucositis had similar changes in erythrocyte folate, plasma folate and plasma homocysteine levels after therapy (from day 0 to two 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 (HWE). χ^2-test and univariate logistic analyses showed that only subjects with wildtype genotype rs$7317112_{A/A}$ (*ABCC4*) had more often mucositis than carriers of the G allele (p=0.02)(table 2). After correction for age and

gender, patient with wild-type rs7317112$_{A/A}$ genotype remained more prone to grade ≥ 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 wildtype *MTRR* rs1801394$_{A/A}$ and wildtype rs4149056T/T (SLCO1B1) were associated with higher T24 MTX levels and wildtype rs7317112$_{A/A}$ (*ABCC4*) revealed higher T48 levels (supplemental table 4). As MTX levels were not associated with mucositis, MTX levels are not able to mediate in the association between SNPs and mucositis.

Wildtype rs4149056$_{T/T}$ (SLCO1B1) was the only genotype that was associated with higher baseline plasma folate levels (supplemental table 4). As rs4149056$_{T/T}$ was not associated with mucositis, rs4149056$_{T/T}$ is 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*) are also not correlated (*spearman's rho: r= 0.002, p=0.985*), neither associated (*linear regression,β 0.23 ; 95%CI (-1.17 – 1.63)*), nor do they interact, as the interaction term "*rs7317112*erythrocyte folate level*" was not significant (interaction term*:* p=0.235).

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 less than 10% of the patients (diarrhea, skin, neurotoxicity, kidney, nor 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 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 do they interact 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 which 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 (neutrophil count or neutrophil count <0.5 umol/L). This illustrates that it is safe to start protocol M with a WBC > 1 x 10^9/L regardless of neutrophil count. Secondly, 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]. Lastly, folinic acid is administered after the first MTX course, this will increases 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, which 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 show that 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]. Since 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-PG's 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 had been suggested that folate supplementation can counteract the anti-leukemic activity of MTX and effectively should not be compromised by decreasing toxicity that is not life threatening[48-52].

In this prospective study, 17 recently reported relevant candidate SNPs were included to study genetic variation. Only wildtype 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 like 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 (supplemental table 1). Previous studies show conflicting results regarding the association between SNPs and toxicity. Also, different toxicity endpoints 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 gr/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-treating 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 reflects the accumulation of MTX polyglutamates in mucosal cells, and the wild-type variant of SNP rs7317112 in *ABCC4.*

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Figure Legends

Figure 1: Flowchart of patient inclusion

Abbreviations: ALL=acute lymphoblastic leukemia; HR=high risk; NEL=not eligible; SNP=single nucleotide polymorphism; n= number.

Figure 2: Prevalence of Toxicity after MTX courses during Protocol M (5 gr/m2 MTX)

The maximum grade of toxicity after a MTX course was documented 2 weeks later, during the hospital visit for the next MTX course. NCI criteria grade ≥ 3 severities are depicted. Abbreviations: "1-4" = represent the consecutive MTX courses; "Sum" = represents the maximum score of toxicity during all the four courses; Hops. Admis. = extra hospital admissions in-between MTX courses.

Figure 3: Comparison of baseline folate and homocysteine levels in patients with and without mucositis during protocol M

Shown are the mean and the SEM for MTX levels and the median and the interquartile range for the folate metabolites. Abbreviations: * = significant association $p<0.05$; ** = mucositis measured after only the first course.

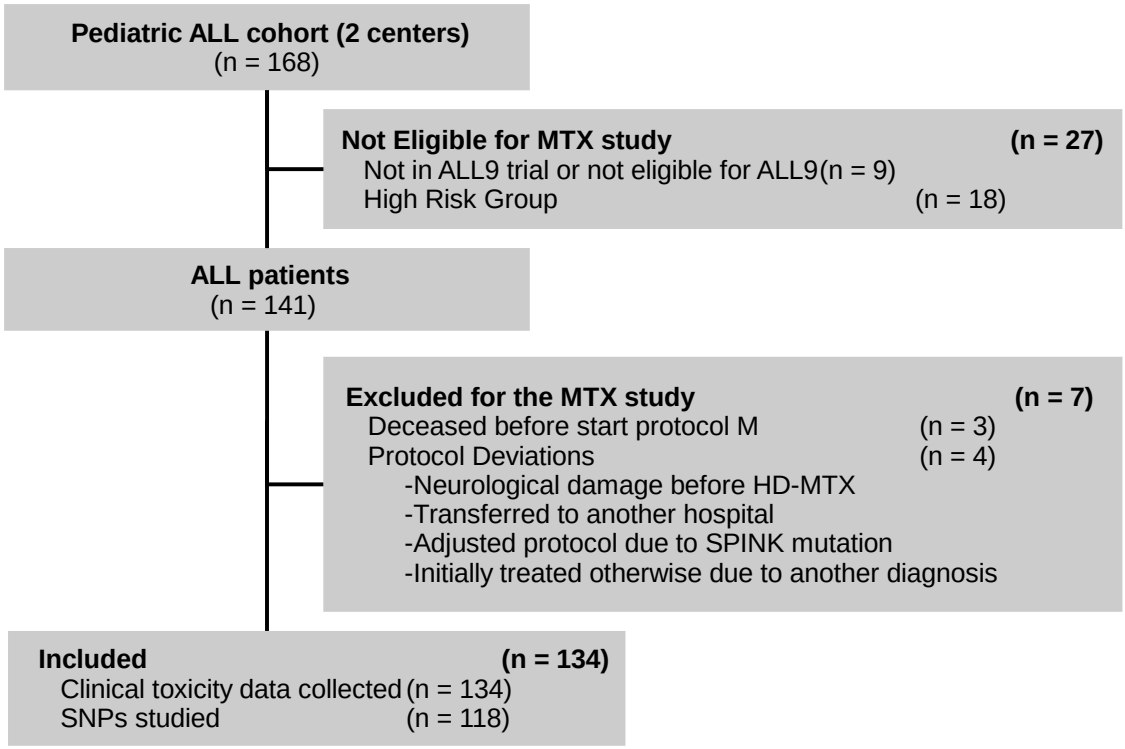


Figure 1: Flowchart of patient inclusion

Abbreviations: ALL=acute lymphoblastic leukemia; HR=high risk; NEL=not eligible; SNP=single nucleotide polymorphism; n= number.

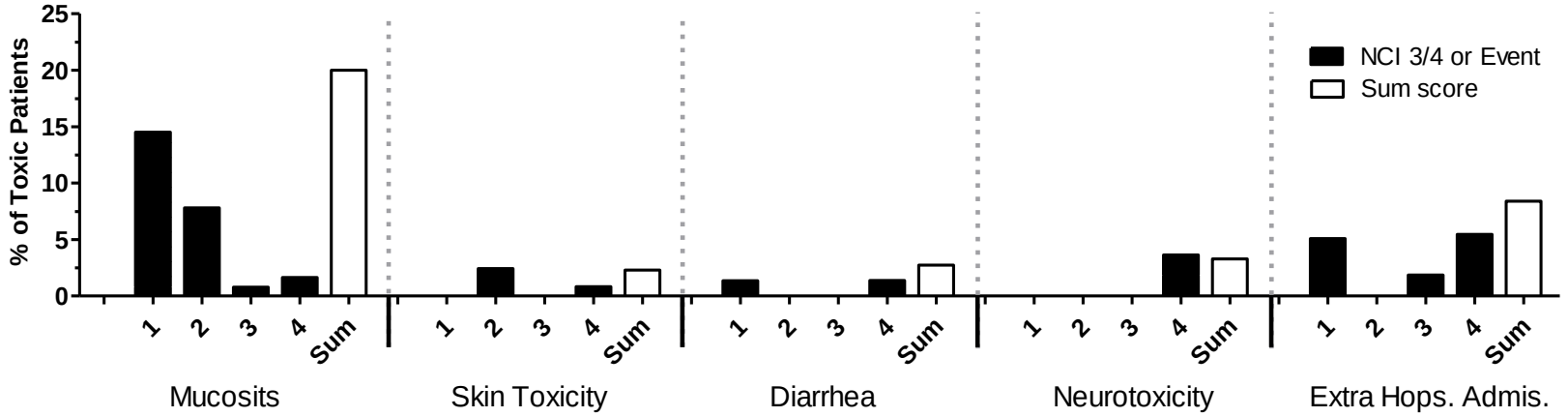


Figure 2: Prevalence of Toxicity per MTX course during Protocol M (5 gr/m2 MTX)

The maximum grade of toxicity after a MTX course was documented 2 weeks later, during the hospital visit for the next MTX course. NCI criteria grade ≥ 3 severities are depicted. Abbreviations: "1-4" = represent the consecutive MTX courses; "Sum" = represents the maximum score of toxicity during all the four courses; Hops. Admis. = extra hospital admissions in-between MTX courses.

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	2 (8%)	12 (11%)	
$> 1.5 \times 10^9$/L	24 (92%)	92 (89%)	0.57
Neutropenia T0, n (%)			
$< 0.5 \times 10^9$/L	20 (77%)	61 (41%)	
$> 0.5 \times 10^9$/L	6 (23%)	43 (59%)	0.09

The analyses were repeated in patients with mucositis only during the first course, but results did not differ. Abbreviations: ALL = Acute Lymphoblastic Leukemia; n = number; T0 = measured at start protocol M; L = liter.

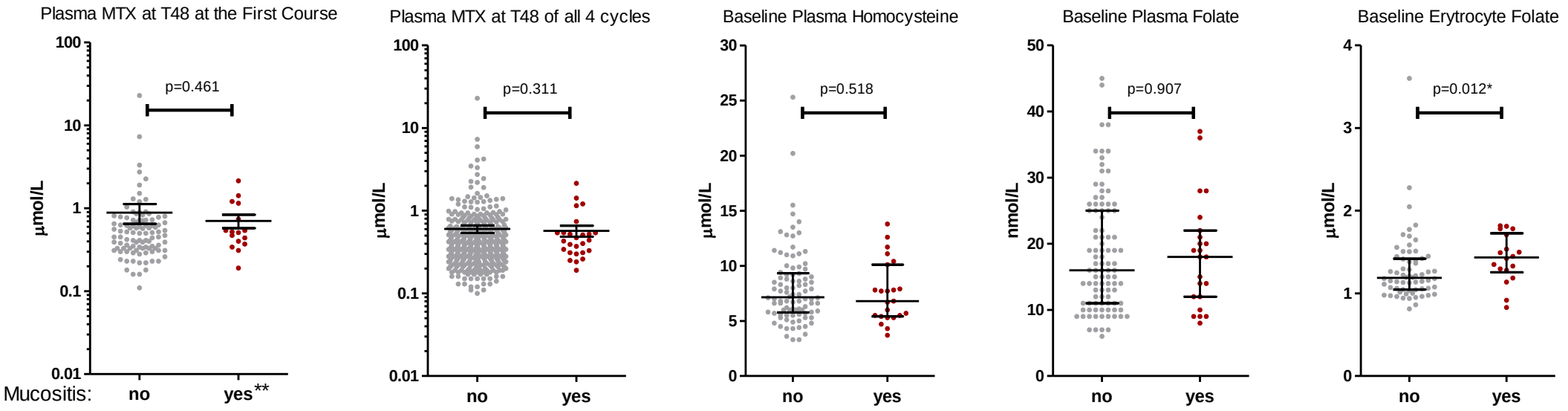


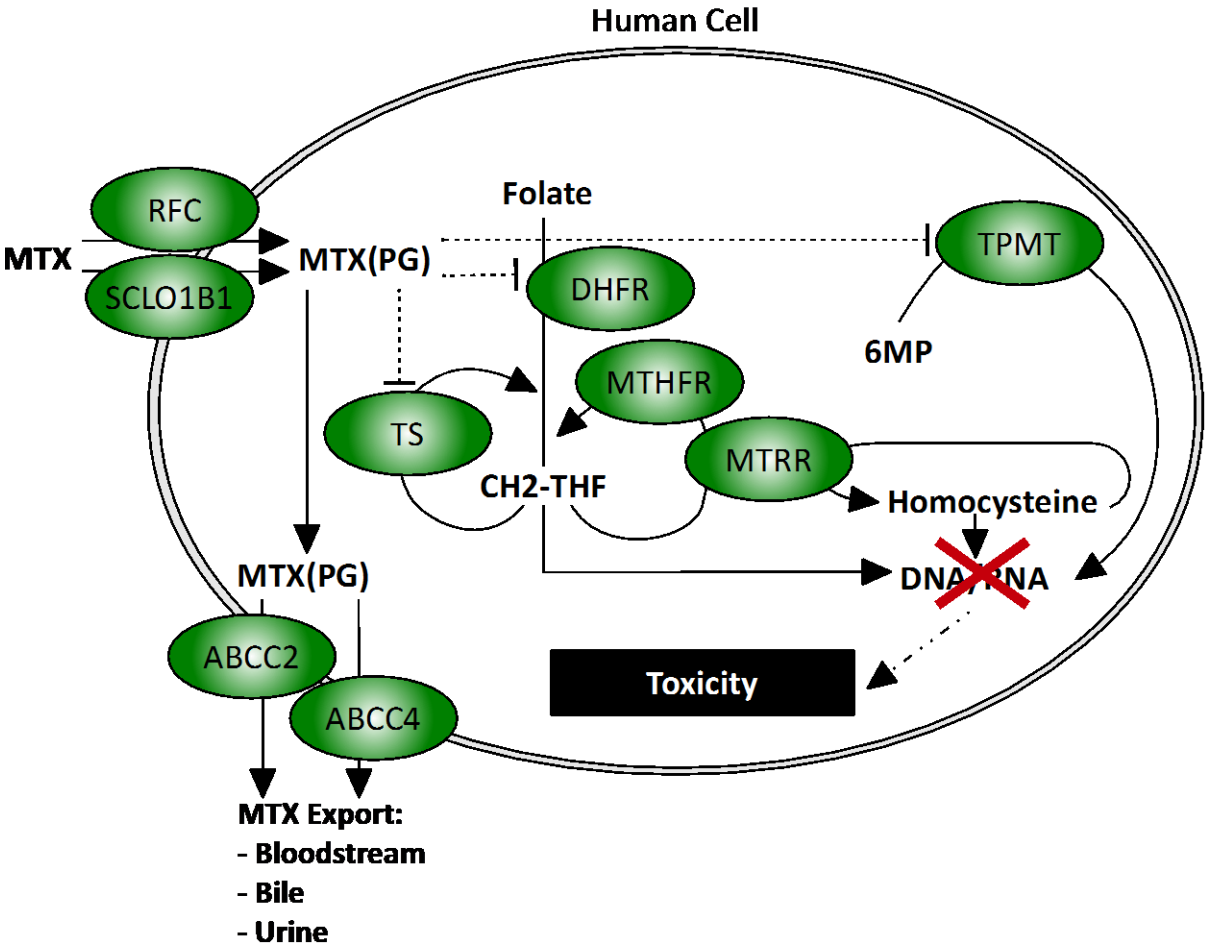
Figure 3: Comparison of baseline folate and homocysteine levels in patients with and without mucositis during protocol M

Shown are the mean and the SEM for MTX levels and the median and the interquartile range for the folate metabolites. Abbreviations: * = significant association $p<0.05$; ** = mucositis measured after only the first course.

Table 2: Comparison between single nucleotide polymorphisms and mucosal toxicity

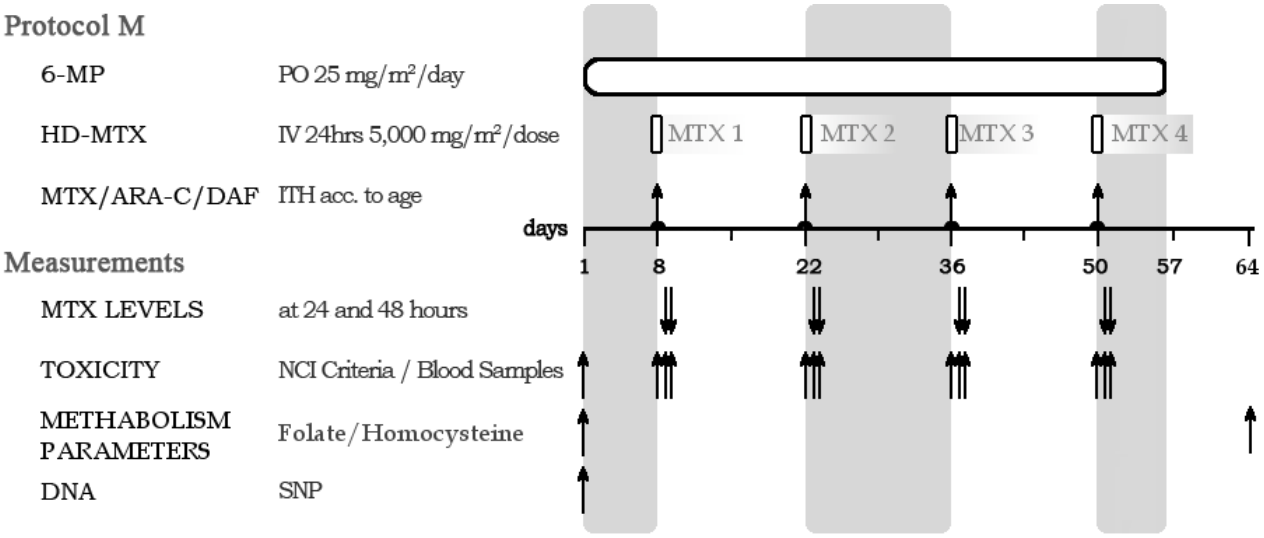
					Mucositis				
Gene	**SNP**		**n**	**(%)**	**yes**	**(%)**	**OR**	**(95%-CI)**	**p-value**
ABCC2	rs12826	A/A	37	42%	10	(27%)	Reference		
		A/G - G/G	43	58%	12	(28%)	1.05	(0.39-2.80)	0.93
ABCC2	rs717620	G/G	57	68%	15	(26%)	Reference		
		G/A - A/A	23	32%	7	(30%)	1.23	(0.42-3.56)	0.45
ABCC2	rs3740065	T/T	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: ALL= Acute Lymphoblastic Leukemia; n=number; ** = significant association between mucositis after the first MTX course and the SNP; NA = not analyzable because of low numbers; 95%CI = 95% confidence interval; rs1801133 = MTHFR 677 C>T; rs1801131 = 1298 A>C; rs1801394 = MTRR 66A>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.



Supplemental figure 1: Genes in the folate metabolism pathway and MTX transporters

Abbreviations: 6-MP, 6-mercaptopurine; MTX, methotrexate; RFC, reduced folate carrier; MTX-PG, methotrexate-polyglutamate; TS, thymidylate synthase ; CH2-THF, 5,10-Methylenetetrahydrofolate (5,10-CH2-THF); MTHFR, methylenetetrahydrofolate reductase; MTRR, methionine synthase reductase; ABCC, ATP-binding cassette, sub-family C. MTX is eliminated primarily by renal excretion, and ~ 10% of each dose is excreted unchanged in the bile.[1]



Supplemental figure 2: Overview of protocol M, of the ALL10 protocol.

6-mercaptopurine (6-MP) was given every day since start protocol M. High Dosage Methotrexate (HD-MTX) was given intravenously (IV) in four courses at day 8, 22, 36 and 50 of protocol M (MTX1, MTX2, MTX3, MTX4), as also intrathecal medication (ITH). MTX plasma levels were measured at 24 and 48 hours after infusion. Clinical Toxicity was determined at baseline and also at the start of each of the four MTX courses. DNA material was collected at start and samples for metabolism parameters were withdrawn from patients at start and two weeks after the last MTX course. Metabolism parameters included levels of erythrocyte folate, plasma folate and plasma homocysteine.

Supplemental table 1: Global overview of studies in pediatric ALL on associations between candidate polymorphisms and MTX toxicity, MTX levels and/or folate metabolites.

		Overall Toxicity*		Mucositis		High MTX serum levels/ Poor clearance		Erythrocyte Folate		Serum Folate / Homocysteine	
		Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
ABCC2	rs12826		2		2		2				
ABCC2	rs717620		2, 3	(+) [2]	3	(+) [4]	2, 3				
ABCC2	rs3740065	3	2		2, 3	(+) [2]	3				
ABCC4	rs1678392		2		2		2				
ABCC4	rs2619312		2		2		2				
ABCC4	rs7317112	(+) [2]		(-)	2		2				
ABCC4	rs9302061		2		2	(+) [2]					
ABCC4	rs9516519		2		2		2				
ABCC4	rs10219913		2		2		2				
MTHFR	rs1801133	(+) [5-13] (-) [14-18]	19-28	(+) [7, 10, 12, 24]	8, 19, 21, 25, 29	(+) [11, 12, 17, 27]	8, 13, 20, 28, 30				
MTHFR	rs1801131	(+) [9, 12, 14, 17, 24] (-) [16, 28]	5, 7, 8, 10, 13, 19, 25, 26		7, 8, 10, 12, 19, 25	(+) [17]	8, 13, 20, 28, 30				
MTRR	rs1801394		12	(+) [28]	12	(+) [31]					
RFC1	rs1051266	(+) [12, 32]	20-23, 26-28, 31, 33	(+) [25, 28]	12, 21, 31, 33	(+) [32, 34]	20, 27, 30, 33				
TPMT	rs1800462	(+) [35, 36]									
TPMT	rs1800460	(+) [6, 35, 36]	16, 26								
TPMT	rs1142345	(+) [6, 22, 35-37]	16, 26								
SCLO1B1	rs4149056		38	(+) [39]	38	(+) [2, 31, 38-41]	13, 30				

The numbers refer to the references. *overall toxicity includes the following toxicities: mucosal/gastro-intestinal, skin, neutropenia, anemia, thrombocytopenia, nausea, diarrhea and neurological toxicity; +=variant is associated with more toxicity; - = variant is associated with less toxicity; No = no association of variant with toxicity; * = association that was found in the current study.

Other available literature that was not mentioned in this table: [42-44]=not available online ; [45]=letter to the editor ; [46-51]=Meta analyses MTHFR gene ; [52]=meta analyses RFC gene

Supplemental table 2: Slightly modified NCI criteria used for the grading toxicity

		Subclinical toxicity		Threshold for clinical relevant toxicity		
Adverse event	grade 0	grade 1	grade 2	grade 3	grade 4	grade 5
ASAT/ALAT T48	Normal	> N - 2.5 x N	2.5 x N - 5 x N	5 x N - 20 x N	> 20 x N	Death
Creatinine T48	Normal	< 1.5 x N	1.5 x N - 3.0 x N	3.1 x N - 6.0 x N	> 6.0 x N	Death
Changes in the skin	Normal	Erythema	Dry desquamation, vasculitis, pruritis	Moist desquamation, ulceration	Exfoliative dermatitis, necrosis	Death
Mucositis/ stomatitis of the oral cavity (clinical exam)	Normal	Erythema of the mucosa	Patchy ulcerations	Confluent ulcerations, bleeding with minor trauma	Tissue necrosis, significant spontaneous bleeding	Death
Mucositis/ (functional/ symptomatic)	Normal	Minimal symptoms, normal diet	Symptomatic, but can eat and swallow modified diet	Symptomatic and unable to adequately aliment or hydrate orally	Symptoms associated with life-threatening consequences	Death
Diarrhoea*	Normal	Increase of <4 stools per day over baseline	Increase of 4-6 stools per day over baseline, iv fluids indicated <24hrs, not interfering with ADL	Increase of >=7 stools per day over baseline or incontinence, interfering with ADL	Life threatening consequences	Death
Central neurotoxicity	Normal	Mild somnolence, or agitation; drowsiness	Somnolence <50% of the time, moderate disorientation	Somnolence >50% of the time, severe disorientation, hallucinations	Coma, seizures	Death
Peripheral neurotoxicity	Normal	Paraesthesia's mild subjective weakness	Severe paraesthesia's and/or mild weakness	Unbearable paraesthesia's, deficits in motor function	Paralysis	Death

This table represents the slightly modified NCI criteria used for the ALL10 protocol. *Diarrhea was not questioned in UMCG. Abbreviations: NCI, National cancer institute.

Supplemental table 3: Overview of all the SNPs in this study

Gene	dbSNP ID	Variation	Position	location	Chromosome position		all groups n%		HWE	PCR Primer
ABCC2	Rs12826	A>G	74858	Downstream	10;101612320	wild type	50	42%	0.69	F: GGC ATT TGC ATT TCC ACT
						heterozygous	52	44%		R: CCT GGA GAA TTT GTA AAT CAC A
						mutant	16	14%		
ABCC2	Rs717620	G>A	5116	5"UTR	10;101542578	wild type	80	68%	0.52	F: AGG GCT TTT TAG TCA CAT GTC
						heterozygous	36	31%		R: AGA CCA ATT GCA CAT CTA ACA
						mutant	2	2%		
ABCC2	Rs3740065	T>C	68231	Intron 29	10;101605693	wild type	94	80%	0.17	F: CCC CCA GGT GAG CTC TA
						heterozygous	21	18%		R: CAG CGG CAA AAC TGC TA
						mutant	3	3%		
ABCC4	Rs1678392	G>A	8811856	Intron 26	13;95722180	wild type	81	70%	1	F: AGC GAT TTT CCT GCT TCA
						heterozygous	31	27%		R: TCC AGG TAC CCA CAT GTA AGT
						mutant	3	3%		
ABCC4	Rs2619312	T>C	8812715	Intron 26	13;95723039	wild type	74	63%	1	F: TGT GGG AAT TTA AGA TGA GAT TT
						heterozygous	39	33%		R: TTG GGG GTC TGA TTT CTG
						mutant	5	4%		
ABCC4	Rs7317112	A>G	9013199	Intron 1	13;95923523	wild type	62	53%	0.37	F: GCC AGC GTG TGA CCT T
						heterozygous	44	37%		R: GGG GAC AGA GCC AGA CT
						mutant	12	10%		
ABCC4	rs9302061	T>C	9056380	Upstream	13:95966704	wild type	41	35%	0.57	F: CGT GGT GCT AGA TTA CAT CAA
						heterozygous	60	51%		R: CCA GGA TCC CAA GAA ATT AG
						mutant	17	14%		
ABCC4	Rs9516519	T>G	8762133	3”utr	13;95672457	wild type	83	70%	0.53	F: CCT GGG ACC TTT TGT ACT TTA T
						heterozygous	33	28%		R: TGT GGT TTG TTG GAC TGA AC
						mutant	2	2%		
ABCC4	Rs10219913	T>C	8790611	Intron 28	13:95700935	wild type	86	73%	0.3	F: GCC CCT AAA TAA GAG CAA CTC
						heterozygous	28	24%		R: GGG AAC AAC CTT TAA CAA GAA C
						mutant	4	3%		
MTHFR	Rs1801133	C>T	677	Exon 4	1:11778965	wild type	59	50%	0.06	F: TGA AGG AGA AGG TGT CTG CGG GA
						heterozygous	42	36%		R: AGG ACG BTB CGG TGA GAG TG
						mutant	17	14%		
MTHFR	Rs1801131	A>C	1298	Upstream	1:11854476	wild type	58	49%	0.83	F: GGG AGG AGC TGA CCA GTG CAG
						heterozygous	49	42%		R: GGG GTC AGG CCA GGG GCA G
						mutant	11	9%		
MTRR	Rs1801394	A>G	66	Upstream	5:7870973	wild type	19	16%	0.19	F: CAG TTT CAC TGT TAC ATG CCT TG
						heterozygous	66	56%		R: CAA TTT TTG AGA CCA TTT AGT CT
						mutant	33	28%		
SLC19A1 (RFC1)	Rs1051266	G>A	80	Exon4	21:46957794	wild type	35	30%	0.36	F: TCC AGG CAC AGT GTC ACC TTC
						heterozygous	54	46%		R: TGC TCC CGC GTG AAG TTC T

Supplemental table 3: Overview of all the SNPs in this study

Gene	dbSNP ID	Variation	Position	location	Chromosome position		all groups n%		HWE	PCR Primer
						mutant	29	25%		
TPMT*2	Rs1800462	G>C	238	Exon 5	6:18143955	wild type	109	92%	0.18	*F: TGT AAA ACG ACG GCC AGT*
						heterozygous	8	7%		*R: GTA TGA TTT TAT GCA GGT TTG*
						mutant	1	1%		
TPMT*3B	Rs1800460	G>A	460	Exon 7	6:18139228	wild type	110	93%	0.15	*F: AGGCAGCTAGGGAAAAAGAAAGGTG*
						heterozygous	7	6%		*R: CAAGCCTTATAGCCTTACACCCAGG*
						mutant	1	1%		
TPMT*3C	Rs1142345	A>G	719	Exon 10	6:18130918	wild type	110	93%	1	*F: GAG ACA GAG TTT CAC CAT CTT GG*
						heterozygous	8	7%		*R: CAG GCT TTA GCA TAA TTT TCA AT TCC TC*
						mutant	0	0%		
SCLO1B1	rs4149056	T>C	521	Intron 4	12:21331549	wild type	88	75%	0.69	Assay ID: C__30633906_10
						heterozygous	29	25%		
						mutant	1	1%		

Abbreviations: F=forward strand; R=reverse strand.

Supplemental table 4: Comparison between single nucleotide polymorphisms and Toxicity, MTX levels and Folate metabolites

			Extra Hosp.					MTX levels T24					MTX levels T48				
			n	(%)	yes	(%)	*p*	n	Median	(range)		*p*	n	Median	(range)		*p*
ABCC2	rs12826	A/A	37	42%	5	(10%)		139	65.59	(36.26-	143.50)		220	0.44	(0.12-	2.14)	
		A/G - G/G	43	58%	5	(8%)	0.66	145	63.29	(24.43-	190.53)	0.94	169	0.39	(0.20-	22.89)	0.94
ABCC2	rs717620	G/G	57	68%	9	(12%)		85	64.90	(24.43-	190.53)		122	0.44	(0.12-	22.89)	
		G/A - A/A	23	32%	1	(3%)	NA	199	63.70	(32.70-	146.40)	0.51	267	0.35	(0.20-	1.42)	0.05
ABCC2	rs3740065	T/T	61	80%	7	(8%)		71	65.59	(24.43-	190.53)		**75**	**0.42**	**(0.20-**	**22.89)**	
		T/C - C/C	19	20%	3	(13%)	0.42	213	58.06	(36.26-	142.26)	0.26	**314**	**0.34**	**(0.12-**	**0.68)**	**0.00**
ABCC4	rs1678392	G/G	57	70%	6	(8%)		74	65.59	(24.43-	146.40)		113	0.41	(0.12-	1.33)	
		G/A - A/A	21	30%	4	(13%)	0.47	206	63.54	(41.34-	190.53)	0.94	269	0.40	(0.13-	2.14)	0.62
ABCC4	rs2619312	T/T	55	63%	6	(8%)		103	68.79	(32.70-	146.40)		143	0.41	(0.12-	1.33)	
		T/C - C/C	25	37%	4	(10%)	1.00	181	63.20	(24.43-	190.53)	0.58	246	0.39	(0.13-	22.89)	0.96
ABCC4	rs7317112	A/A	41	53%	2	(3%)		**133**	**60.01**	**(32.70-**	**146.40)**		**188**	**0.32**	**(0.12-**	**1.42)**	
		A/G - G/G	39	47%	8	(15%)	NA	**151**	**74.00**	**(24.43-**	**190.53)**	**0.04**	**201**	**0.40**	**(0.21-**	**22.89)**	**0.00***
ABCC4	rs9302061	T/T	30	35%	3	(8%)		192	61.62	(32.70-	190.53)		245	0.42	(0.20-	22.89)	
		T/C - C/C	50	65%	7	(9%)	1.00	92	65.26	(24.43-	146.40)	0.54	144	0.38	(0.12-	2.14)	0.34
ABCC4	rs9516519	T/T	59	70%	5	(6%)		78	66.57	(24.43-	146.40)		118	0.40	(0.12-	22.89)	
		T/G - G/G	21	30%	5	(15%)	0.13	206	62.00	(32.70-	190.53)	0.33	271	0.40	(0.13-	2.14)	0.96
ABCC4	rs10219913	T/T	58	73%	7	(8%)		**60**	**63.20**	**(24.43-**	**190.53)**		100	0.39	(0.13-	2.14)	
		T/C - C/C	22	27%	3	(10%)	0.72	**224**	**75.12**	**(49.93-**	**146.40)**	**0.02**	289	0.42	(0.12-	22.89)	0.46
MTHFR	rs1801133	C/C	40	50%	8	(14%)		125	63.54	(32.70-	142.26)		190	0.40	(0.13-	1.33)	
		C/T - T/T	40	50%	2	(4%)	NA	159	67.54	(24.43-	190.53)	0.23	199	0.41	(0.12-	22.89)	0.74
MTHFR	rs1801131	A/A	40	49%	4	(7%)		159	73.50	(24.43-	146.40)		199	0.41	(0.12-	22.89)	
		A/C - C/C	40	51%	6	(11%)	0.74	125	61.32	(32.70-	190.53)	0.05	190	0.40	(0.13-	2.14)	0.57
MTRR	rs1801394	A/A	14	16%	2	(12%)		**229**	**81.56**	**(59.00-**	**139.35)**		328	0.42	(0.12-	1.42)	
		A/G - G/G	66	84%	8	(8%)	NA	**55**	**60.39**	**(24.43-**	**190.53)**	**0.00***	61	0.40	(0.13-	22.89)	0.71
RFC1	rs1051266	G/G	26	30%	4	(12%)		202	64.77	(40.11-	190.53)		275	0.44	(0.12-	22.89)	
		G/A - A/A	54	70%	6	(8%)	0.49	82	64.87	(24.43-	143.50)	0.65	114	0.39	(0.13-	2.14)	0.49
TPMT	rs1800462	G/G	74	92%	9	(9%)		17	64.87	(24.43-	190.53)		29	0.40	(0.12-	22.89)	
		G/C - C/C	6	8%	1	(11%)	NA	267	66.81	(40.11-	96.45)	NA	360	0.40	(0.30-	0.67)	NA
TPMT	rs1800460	G/G	74	93%	9	(9%)		16	64.90	(24.43-	190.53)		25	0.41	(0.12-	22.89)	
		G/A - A/A	6	7%	1	(13%)	NA	268	61.01	(40.11-	96.45)	NA	364	0.40	(0.30-	0.67)	NA
TPMT	rs1142345	A/A	74	93%	9	(9%)		17	64.87	(24.43-	190.53)		26	0.39	(0.12-	22.89)	
		A/G - G/G	6	7%	1	(13%)	NA	267	66.81	(40.11-	96.45)	NA	363	0.42	(0.35-	0.67)	NA
SCLO1B1	rs4149056	T/T	58	75%	6	(7%)		**82**	**59.77**	**(24.43-**	**190.53)**		104	0.42	(0.12-	22.89)	
		T/C - C/C	22	25%	4	(14%)	0.28	**202**	**74.20**	**(47.42-**	**146.40)**	**0.02***	285	0.37	(0.13-	1.15)	0.14

Supplemental table 4. Comparison between single nucleotide polymorphisms and Toxicity, MTX levels and Folate metabolites

			Erythrocyte Folate Levels T0				Plasma Folate Levels T0				Plasma Homocysteine Levels T0			
			n	Median	(range)	*p*	n	Median	(range)	*p*	n	Median	(range)	*p*
ABCC2	rs12826	A/A	43	1.30	(0.81- 3.61)		68	15.98	(6.01- 44.80)		68	7.60	(3.30- 25.30)	
		A/G - G/G	39	1.14	(0.83- 2.28)	0.32	50	16.99	(6.55- 37.92)	0.75	50	7.00	(3.60- 15.50)	0.46
ABCC2	rs717620	G/G	25	1.29	(0.95- 1.83)		38	13.86	(7.45- 44.80)		38	6.80	(3.30- 25.30)	
		G/A - A/A	57	1.19	(0.81- 3.61)	0.12	80	17.54	(6.01- 44.13)	0.26	80	7.40	(3.30- 20.20)	0.87
ABCC2	rs3740065	T/T	19	1.16	(0.83- 2.28)		24	18.24	(8.05- 37.50)		24	6.55	(3.60- 15.50)	
		T/C - C/C	63	1.27	(0.81- 3.61)	0.10	94	15.22	(6.01- 44.80)	0.22	94	7.65	(3.30- 25.30)	0.37
ABCC4	rs1678392	G/G	22	1.35	(0.83- 2.28)		34	18.60	(7.38- 44.13)		34	7.80	(3.60- 14.70)	
		G/A - A/A	58	1.19	(0.81- 3.61)	0.15	81	16.15	(6.01- 44.80)	0.78	81	6.90	(3.30- 25.30)	0.41
ABCC4	rs2619312	T/T	**31**	**1.38**	**(0.83- 3.61)**		44	19.32	(7.38- 44.13)		44	7.70	(3.60- 14.70)	
		T/C - C/C	**51**	**1.14**	**(0.81- 2.05)**	**0.01**	74	15.72	(6.01- 44.80)	0.28	74	7.00	(3.30- 25.30)	0.88
ABCC4	rs7317112	A/A	39	1.21	(0.81- 3.61)		56	16.48	(6.01- 37.50)		56	7.20	(3.30- 14.70)	
		A/G - G/G	43	1.23	(0.83- 2.28)	0.86	62	16.81	(7.35- 44.80)	0.68	62	7.10	(3.60- 25.30)	0.48
ABCC4	rs9302061	T/T	56	1.22	(0.81- 2.28)		77	15.92	(6.01- 44.80)		77	7.10	(3.30- 25.30)	
		T/C - C/C	26	1.22	(0.94- 3.61)	0.71	41	17.30	(8.77- 35.98)	0.36	41	7.70	(3.30- 15.50)	0.67
ABCC4	rs9516519	T/T	23	1.29	(0.83- 2.28)		35	16.22	(7.38- 44.13)		35	7.70	(3.60- 14.70)	
		T/G - G/G	59	1.19	(0.81- 3.61)	0.37	83	16.51	(6.01- 44.80)	0.43	83	7.00	(3.30- 25.30)	0.43
ABCC4	rs10219913	T/T	19	1.18	(0.94- 2.05)		32	16.95	(6.01- 37.92)		32	8.15	(3.80- 25.30)	
		T/C - C/C	63	1.26	(0.81- 3.61)	0.39	86	16.33	(6.55- 44.80)	0.55	86	7.00	(3.30- 15.50)	0.25
MTHFR	rs1801133	C/C	38	1.28	(0.83- 2.23)		59	15.92	(7.38- 44.80)		59	7.10	(3.30- 25.30)	
		C/T - T/T	44	1.20	(0.81- 3.61)	0.30	59	16.59	(6.01- 34.02)	0.89	59	7.45	(3.30- 20.20)	0.82
MTHFR	rs1801131	A/A	45	1.21	(0.83- 3.61)		60	17.46	(6.01- 44.80)		60	7.15	(3.30- 25.30)	
		A/C - C/C	37	1.26	(0.81- 1.83)	0.90	58	14.66	(6.55- 37.50)	0.23	58	7.20	(3.30- 15.50)	0.65
MTRR	rs1801394	A/A	66	1.24	(0.81- 3.61)		99	17.30	(6.01- 44.80)		99	7.00	(3.30- 25.30)	
		A/G - G/G	16	1.19	(0.83- 2.28)	0.58	19	14.58	(7.35- 37.50)	0.33	19	8.30	(3.60- 20.20)	0.72
RFC1	rs1051266	G/G	58	1.27	(0.83- 3.61)		83	16.22	(6.01- 44.13)		83	7.05	(3.30- 25.30)	
		G/A - A/A	24	1.16	(0.81- 2.23)	0.16	35	17.61	(7.35- 44.80)	0.60	35	7.55	(4.40- 20.20)	0.62
TPMT	rs1800462	G/G	4	1.12	(0.99- 1.29)		9	19.19	(9.59- 34.02)		9	8.00	(5.00- 14.70)	
		G/C - C/C	78	1.24	(0.81- 3.61)	0.33	109	16.44	(6.01- 44.80)	0.45	109	7.10	(3.30- 25.30)	0.33
TPMT	rs1800460	G/G	4	1.12	(0.99- 1.29)		8	20.38	(9.59- 34.02)		8	8.25	(5.00- 14.70)	
		G/A - A/A	78	1.24	(0.81- 3.61)	0.33	110	16.48	(6.01- 44.80)	0.51	110	7.05	(3.30- 25.30)	0.20
TPMT	rs1142345	A/A	4	1.12	(0.99- 1.29)		8	17.71	(9.59- 25.96)		8	8.25	(5.60- 14.70)	
		A/G - G/G	78	1.24	(0.81- 3.61)	0.33	110	16.48	(6.01- 44.80)	0.81	110	7.05	(3.30- 25.30)	0.19
SCLO1B1	rs4149056	T/T	22	1.32	(0.95- 2.28)		**30**	**20.01**	**(6.01- 44.80)**		30	6.80	(3.60- 25.30)	
		T/C - C/C	60	1.21	(0.81- 3.61)	0.25	**88**	**15.72**	**(6.55- 44.13)**	**0.03**	88	7.70	(3.30- 20.20)	0.24

Abbreviations: ALL = Acute Lymphoblastic Leukemia; n=number; * = significant association in the first MTX course as well; T0 = at baseline

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