

research article

Association between *SLC19A1* gene polymorphism and high dose methotrexate toxicity in childhood acute lymphoblastic leukaemia and non Hodgkin malignant lymphoma: introducing a haplotype based approach

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Background. We investigated the clinical relevance of *SLC19A1* genetic variability for high dose methotrexate (HD-MTX) related toxicities in children and adolescents with acute lymphoblastic leukaemia (ALL) and non Hodgkin malignant lymphoma (NHML).

Patients and methods. Eighty-eight children and adolescents with ALL/NHML were investigated for the influence of *SLC19A1* single nucleotide polymorphisms (SNPs) and haplotypes on HD-MTX induced toxicities.

Results. Patients with rs2838958 TT genotype had higher probability for mucositis development as compared to carriers of at least one rs2838958 C allele (OR 0.226 (0.071–0.725), $p < 0.009$). Haplotype TGTCCG (H4) statistically significantly reduced the risk for the occurrence of adverse events during treatment with HD-MTX (OR 0.143 (0.023–0.852), $p = 0.030$).

Conclusions. *SLC19A1* SNP and haplotype analysis could provide additional information in a personalized HD-MTX therapy for children with ALL/NHML in order to achieve better treatment outcome. However further studies are needed to validate the results.

Key words: acute lymphoblastic leukaemia; genetic polymorphism; haplotype; methotrexate

Introduction

Inter-individual variability in treatment responses and treatment related toxicities is an important issue in clinical practice. It can lead to therapeutic failures or adverse drug events (ADE). Identification and characterization of genetic polymorphisms

and haplotypes involved in drug metabolism, transport and mechanism of action would provide important information about individual inherited differences in drug metabolism and treatment response in order to optimize treatment outcome.¹

The solute carrier 19A1 (*SLC19A1*), trivially referred as reduced folate carrier (RFC), located in a

40719 bp region at chromosome 21q22.3, is coding for solute folate carrier (*RFC1*), a major transporter of folates and antifolates into the cell. The antifolate methotrexate (MTX) is a cytotoxic drug which is used in current treatment regimens for childhood acute lymphoblastic leukaemia (ALL)², and non-Hodgkin malignant lymphoma (NHML).^{3,4} The most frequently studied *SLC19A1* polymorphism is rs1051266 in the second exon of the *SLC19A1*, which results in amino acid substitution of arginine for histidine (H27R)⁵ in transmembrane domain 1 (TMD1), a region implicated in substrate binding and/or translocation^{6,7}, is expected to alter SLC transport properties. Other polymorphisms have been described to date, but the functional consequences of other single nucleotide polymorphisms (SNPs) are not known yet.⁸

Several studies so far investigated the association of rs1051266 with high dose MTX (HD-MTX) treatment outcome and treatment related toxicities in ALL patients.⁹⁻¹³ Some of them reported a protective role of rs1051266 AA genotype^{14,15}, while in others either an increased risk of MTX-induced toxicities and fatal outcomes in rs1051266 A carriers^{9,10}, or even no association between *SLC19A1* genotype and MTX-induced toxicities was report-

ed.^{11,13,16,17} Recently, SNPs in microRNAs (miRNAs) that are involved in regulation of *SLC19A1* were investigated. SNP in miR-595 which might affect *SLC19A1* regulation and could affect MTX levels in patients with paediatric B-cell ALL was reported.¹⁸

To date all studies but one¹⁹ evaluated the role of individual *SLC19A1* SNPs in the MTX toxicities and outcomes of ALL treatment. The discovery of HapMap (Humane gene database) made it possible to analyse many SNPs at a time. Between the former and the latter a tagSNP approach has been introduced recently. A tagSNP is a representative SNP in a region of the genome with high linkage disequilibrium. Using the tagSNPs reduces number of SNPs needed to be genotyped to capture the common variations across the genome.²⁰ Relatively small numbers of tagSNPs will therefore mark each of the common haplotypes within the gene region.²¹

In the present study we investigated a possible association between *SLC19A1* haplotypes, defined by common functional variants and tagSNPs, and MTX related toxicities in 88 children and adolescents with ALL/NHML. The aim was to provide additional insight into improved, cost-effective approach for a personalized HD-MTX therapy for children with ALL/NHML based on haplotype analysis in order to achieve better treatment outcome.

TABLE 1. Demographic and clinical characteristics of children with ALL/NHML and MTX treatment toxicity

Characteristic	ALL patients (N = 88)
Gender (%)	
Male	41 (46.6)
Female	47 (53.4)
Median age at diagnosis, years (range)	4.58 (0.3–16.6)
Median body surface area, m ² (range)	0.73 (0.4–1.9)
Leukaemia subtype (%):	
B-cell	72 (81.8)
T-cell	13 (14.7)
Undetermined	3 (3.4)
Median No. leukocytes at diagnosis, 10 ⁹ cells/L (range)	9.35 (1–1650)
Median No. thrombocytes at diagnosis, 10 ⁹ cells/L (range)	72 (3–553)
Median haemoglobin conc., g/l (range)	84 (43–148)
Median % of blasts at diagnosis (range)	23.5 (0–97)
Median MTX dose, mg (range)	3300 (540–9200)
MTX response	Present, N (%)
Relapse	8 (9.2)**
Exitus	4 (4.6)**
All MTX-induced ADEs*	48 (54.5)
Leukopenia	33 (37.5)
Thrombocytopenia	16 (18.2)
Mucositis	15 (17.0)
Neurotoxicity	7 (8.0)**

*data was collected after the first cycle of MTX, **data is missing for one patient

Patients and methods

Study design

The study group included 88 children and adolescents (41 male, 47 female) with ALL/NHML diagnosed and treated in the period from 1990 to 2008 at the Department of oncology and haematology, University Children's Hospital in Ljubljana, Slovenia. All the patients were Central European Caucasians and below the age of 18 years at the time of diagnosis. Clinical and demographic data were obtained from the medical records and a questionnaire by a single abstractor blinded to genotypes and are presented in Table 1. Each patient enrolled in the study received a high MTX dose (77.3% of patients received 5 g/m² and 22.7% received 2 g/m² of MTX, according to treatment protocols) on 4 separate occasions and concomitant leucovorin rescue therapy to reduce MTX toxicity. Overall, 352 courses of HD-MTX applications were analysed.

All patients and/or their parents or legal guardians gave their written informed consent to participate in the study. The study was approved by the Republic of Slovenia National Medical Ethics

Committee and was carried out according to the Helsinki Declaration.

Outcome measurement

Adverse drug events (ADE) were recorded retrospectively from patients' files throughout the course of the HD-MTX treatment. ADEs were classified as (1) leukopenia, defined as white blood cells count less than 5×10^9 per litre, (2) thrombocytopenia, defined as thrombocyte count less than 140×10^9 per litre, (3) mucositis, determined and graded according to the WHO oral toxicity scale classification²² or based on physician's descriptions in medical records, need for analgesia and/or total parenteral nutrition and (4) neurotoxicity, determined and graded according to National Cancer Institute Common Toxicity Criteria Version 1 and included patients with seizures (regardless of grade) and patients with grade 3 or 4.²³ The data on relapse and exitus were also recorded. All the reported ADEs were used for the evaluation of MTX toxicity.

SLC19A1 SNP selection

A total of seven SNPs (rs2838951, rs2838956, rs2838958, rs1051266, rs1131596, rs2838451 and rs17004785) in *SLC19A1* spanning 40 kbp across the chromosomal 21q22.3 region were selected. SNP selection was based on known function from the literature and/or National Center for Biotechnology Information SNP database (NCBI) and block-tagging ability. Among all, two SNPs were a functional variant (rs1051266 and rs1131596), while five (rs2838951, rs2838956, rs2838958, rs2838451, rs17004785) were tagging SNPs, selected based on International HapMap Project CEPH data (phase II, March 2008) with the following criteria: $r^2 > 0.8$, and the frequency of minor allele greater than 5%. Tagging SNP selection was done using HaploView software version 4.1 (Supplementary Table 1).²⁴

SLC19A1 genotyping

In total 5 ml of peripheral blood was collected into tubes with sodium citrate and stored for short term at -20°C until DNA isolation. Genomic DNA was isolated from peripheral blood leukocytes using a Qiagen FlexiGene kit (Qiagen GmbH, Germany). Genotyping was performed using a fluorescence-based competitive allele-specific (KASPar) assay.²⁵ Real time PCR was performed on the ABI 7900HT in a 4 μL reaction mix containing 0.055 μL of

KASPar allele specific PCR assay, 2 μL of KASPar PCR Master Mix (2x concentration, containing KTaQ polymerase and ROX), 2.2 mM MgCl_2 and 10 ng of DNA.

Statistical analysis

SNP based analyses

Binary logistic regression analysis with the addition of independent variables, such as sex, age at time of diagnosis and body surface area (BSA) was done using SPSS for Windows version 16.0.1 software (Statistical Package for the Social Sciences, Chicago, IL). The risk for toxicity of MTX treatment was expressed as odds ratio (OR) with 95% confidence intervals (CI). The level of significance was set to 0.05. Deviation from Hardy-Weinberg equilibrium (HWE) was calculated for each SNP using Chi-square test. The level of deviation was set to 0.05. Power calculations were done using G*Power software version 3.1.0. Our study had an 80% statistical power to detect medium effect sizes (0.331) with an alpha level of 0.05. The correction for false-positive findings was done using a method for detecting false-positive report probability (FPRP) described by Wacholder *et al.*²⁶

The cut-off for noteworthy results was set as 0.5. The expected ORs were based on the previous reports of an association of SNPs in *SLC19A1* with MTX-toxicity.^{9,10,11,14,15} Prior probability was set to high ($\pi = 0.1$) due to well documented association of *SLC19A1* and MTX transport.²⁷

Haplotype based analysis

SLC19A1 haploblocks and linkage disequilibrium (LD) between the selected SNPs were visualized using HaploView software version 4.1.²⁴ *SLC19A1* haplotype construction was performed using Tagger pairwise method.

Results

Patient characteristics

Of the 88 patients recruited, 48 (54.5%) patients suffered from MTX-induced adverse events. Specific MTX-induced ADEs after the first cycle of MTX and their frequencies are presented in Table 1.

Association between individual SNPs and MTX toxicity

The characteristics and the minor allele frequencies (MAF) of the investigated SNPs in *SLC19A1* are

TABLE 2. The influence of rs2838958 genotype on mucositis development - univariate logistic regression analysis

Degree of mucositis	rs2838958 genotype		OR (95% CI)	P
	TT (%)	TC + CC (%)		
0	18 (66.7)	53 (89.8)	reference	
1	4 (14.8)	4 (6.8)	0.340 (0.077–1.500)	0.154
2	4 (14.8)	2 (3.4)	0.170 (0.029–1.007)	0.051
4	1 (3.7)	0 (0.0)	- *	
1–4	9 (33.3)	6 (10.2)	0.226 (0.071–0.725)	0.009
Mean value ± STD	0.59	0.14		0.007

* Calculation was not possible

presented in Supplementary Table 1. In one patient genotyping failed for all SNPs, while genotyping for rs2838951 and rs17004785 failed in two patients. The observed frequencies of the studied SNPs in ALL/NHML patients did not deviate from HWE (data not shown).

With regard to specific ADEs, univariate logistic regression analysis revealed only the association of rs2838958 with the occurrence of mucositis development ($p = 0.009$). We observed that patients with rs2838958 TT genotype had higher probability for mucositis development as compared to carriers of at least one polymorphic rs2838958 C allele (OR 0.226 (0.071–0.725), $p < 0.009$) (Table 2).

We then investigated the possible association between SLC19A1 polymorphisms and MTX-induced ADEs with the multivariate analysis using an additive model (*per allele*), but a statistically significant relationship could be established neither for overall nor for specific ADEs (Table 3). A margin-

ally significant impact on the risk of mucositis was observed for rs2838951 (OR 2.160 (0.909–5.129), $p = 0.081$).

We also performed haplotype analysis to ascertain whether the combination of polymorphisms that define a specific haplotype impacts the occurrence of ADEs. As shown in Figure 1 the investigated polymorphisms were lying in the same haplotype.

The frequencies of SLC19A1 haplotypes in patients with ALL and NHML that were treated with HD-MTX are shown in Table 4. The Haplotype TGTTCCC (H2), which has wild type alleles present on all loci, was selected as the reference haplotype for the analysis. By frequency, it is the second most common haplotype in the patient test group with a frequency of 33.4%. The haplotype CACCCCG (H1) had polymorphic alleles present on the first four and on the last locus, with wild type alleles present on the fifth and sixth loci. The

TABLE 3. The impact of SLC19A1 genotypes on the occurrence of any and specific MTX-induced adverse events using multivariate analysis – an additive model

SNP ID	Any adverse events		Leukopenia		Thrombocytopenia		Mucositis		Neurotoxicity	
	OR (CI 95%)	P								
rs1131596	0.719 (0.354–1.458)	0.360	0.609 (0.310–1.197)	0.150	1.170 (0.532–2.576)	0.696	1.292 (0.525–3.170)	0.579	0.542 (0.146–2.016)	0.361
rs1051266	1.392 (0.686–2.824)	0.360	1.643 (0.836–3.230)	0.150	0.855 (0.388–1.881)	0.696	0.775 (0.316–1.906)	0.579	0.542 (0.146–2.016)	0.361
rs2838958	1.221 (0.599–2.490)	0.583	1.684 (0.849–3.339)	0.136	0.556 (0.240–1.291)	0.172	0.476 (0.182–1.243)	0.130	0.453 (0.122–1.677)	0.236
rs2838956	1.411 (0.686–2.899)	0.349	1.742 (0.878–3.458)	0.113	0.689 (0.303–1.566)	0.374	0.705 (0.283–1.759)	0.454	0.596 (0.163–2.182)	0.434
rs17004785	0.849 (0.280–2.572)	0.772	1.393 (0.476–4.079)	0.545	0.854 (0.218–3.349)	0.821	0.672 (0.148–3.055)	0.606	1.226 (0.255–5.899)	0.800
rs12483377	1.475 (0.504–4.318)	0.478	1.825 (0.672–4.956)	0.238	1.688 (0.540–5.280)	0.368	0.930 (0.249–3.473)	0.914	1.061 (0.232–4.861)	0.939
rs2838951	1.261 (0.666–2.412)	0.483	0.740 (0.392–1.394)	0.351	1.652 (0.786–3.473)	0.185	2.160 (0.909–5.129)	0.081	1.693 (0.577–4.967)	0.337

The estimates for the odds ratio (OR) were adjusted to the following variables: age, sex, and treatment regimen.

haplotype TGTTGTC (H3) had wild type alleles present on the first four and on the last locus, with polymorphic alleles present on the fifth and sixth. The haplotype TGTTCGG (H4) had wild type alleles present at all loci, except for the last one, while haplotype CATTCCC (H5) had polymorphic alleles present on the first, second, fifth and sixth locus, with wild type alleles on the remaining three.

H1 was the most frequent haplotype in the patient test group with a frequency of 39.6%. Together, H1, H2, and H3 haplotypes accounted for 82.4% of all haplotypes. In patients with ALL and ML, the haplotypes H4 and H5 were more rarely represented, with the frequency of a single haplotype between 1 to 5%. Therefore haplotype H5 and the other very rare haplotypes have been grouped together as 'rare' for further statistical analysis.

We examined whether there is a link between the *SLC19A1* haplotypes and ADEs in patients treated with HD-MTX. We included the age and sex of the patient, and the treatment regimen as co-variables in the model. We found a statistically significant association of haplotype H4 in the *SLC19A1* gene and the treatment regimen on the occurrence of any possible MTX-induced ADEs. Patients who were treated with ALL-BFM 95 were at an increased risk for the occurrence of any MTX-induced ADEs (OR 2.050 (1.065–3.943), $p = 0.031$), while those with haplotype H4 had a reduced risk for the occurrence of any possible ADE after treatment with HD-MTX (OR 0.143 (0.023–0.852), $p = 0.030$) (Table 5). The impact of the *SLC19A1* haplotypes on specific MTX-induced ADEs was not observed, probably due to the small number of individual toxic events (Table 5).

Discussion

In the present study we have used haplotype based approach to investigate the association of genetic variability in *SLC19A1* gene with the high-dose MTX-induced ADEs in paediatric ALL and NHML patients. *SLC19A1* is one of the main influx transporters for folates and antifolates, however the reports on the impact on genetic variability on the MTX treatment outcome are conflicting. Protective role of rs1051266 AA genotype^{14,15}, as well as increased risk for MTX-induced ADEs¹⁰ or no association of rs1051266 was reported.^{11,13,16}

Among the so far published studies on the role of *SLC19A1* in the treatment of ALL with MTX all studies but one¹⁹ have only investigated the most

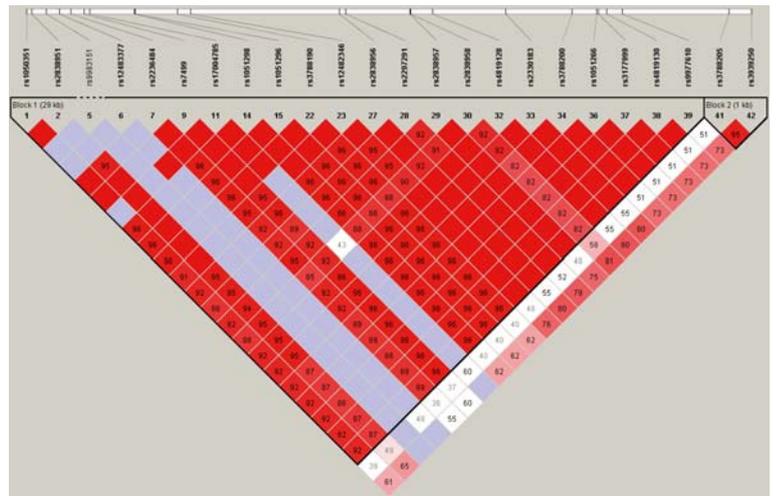


FIGURE 1. Graphic presentation of haploblocks and selected SNPs in *SLC19A1*.

TABLE 4. *SLC19A1* haplotype frequencies in patients with ALL and NHML

Haplotype*	Frequency	Standard error
H1 = CACCCCG	0.396	0.037
H2 = TGTTCCC	0.334	0.036
H3 = TGTTGTC	0.094	0.022
H4 = TGTTCGG	0.053	0.017
H5 = CATTCCC	0.017	0.010
Other	0.106	

* SNP rs1131596, rs1051266, rs2838958, rs2838956, rs17004785, rs12483377, rs2838951 from 5' to 3', respectively

common functional SNP rs1051266 (*SLC19A1* G80A), which may alter the transport characteristics of *SLC19A1*.²⁸ By using a haplotype based approach we were able to investigate the impact of genetic variability of SNPs that are in linkage disequilibrium within the broader region within of the *SLC19A1* on the occurrence of MTX-induced ADEs and disease outcome and effectiveness of treatment with HD-MTX in children and adolescents with ALL/NHML.

Among the investigated *SLC19A1* polymorphisms univariate logistic regression analysis showed that the rs2838958 TT genotype increases the risk of developing mucositis. This association remained borderline significant in the multivariate analysis using the additive model. The other *SLC19A1* polymorphisms were not associated with the occurrence of MTX-induced ADEs.

In the haplotype based analysis we found that H4 haplotype (TGTTCGG), which, excluding the last locus (rs2838951), has wild type alleles present,

TABLE 5. The impact of gender, age, treatment regimen and SLC19A1 haplotypes on the occurrence of any and specific (thrombocytopenia, mucositis, and neurotoxicity) MTX-induced adverse events

	All		Thrombocytopenia		Mucositis		Neurotoxicity	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Gender	2.385 (0.908–6.259)	0.078	1.615 (5.134–5.076)	0.413	1.523 (0.423–5.472)	0.520	0.531 (0.093–3.024)	0.475
Age	1.028 (0.931–1.134)	0.588	9.526 (8.422–1.078)	0.440	1.054 (0.935–1.190)	0.396	1.088 (0.931–1.272)	0.289
Treatment regimen	2.050 (1.065–3.943)	0.031*	9.839 (4.620–2.095)	0.962	1.676 (0.689–4.075)	0.255	1.282 (0.397–4.139)	0.678
H1 = CACCCCG	1.000 (0.472–2.117)	0.943	6.615 (2.769–1.579)	0.352	0.419 (0.150–1.167)	0.096	0.309 (0.070–1.364)	0.120
H3 = TGTTGTC	1.110 (0.335–3.618)	0.844	9.220 (2.254–3.750)	0.891	0.593 (0.122–2.877)	0.515	0.951 (0.175–5.150)	0.934
H4 = TGTTCCC	0.143 (0.023–0.852)	0.030*	3.617 (4.568–2.541)	0.379	0.005 (0.0–NC)	0.985	0.014 (0.0–NC)	0.992
Rare haplotypes	2.002 (0.580–6.775)	0.275	9.113 (2.607–3.171)	0.871	2.002 (0.580–6.775)	0.275	2.002 (0.580–6.775)	0.275

*p < 0.05; H2 was the reference haplotype

statistically significantly reduces the risk for the occurrence of ADEs during treatment with HD-MTX. Since the frequency is low in our sample it is difficult to ascertain its clinical relevance given the number of patients and the small number of individual toxic events.

Haplotype based approaches have been rarely used when investigating HD-MTX induced adverse events in ALL and NHML patients. One study investigated the influence of *MTHFR* SNPs and haplotypes on treatment response²⁹ and HD-MTX toxicity^{30,31}, while the other studies focused on DNA repair and cell cycle processes on risk for leukaemia development using haplotype based approach.^{32–36}

Only study of Lopez-Lopez focused on MTX transporters. This study examined 384 SNPs in 12 transporter genes, including *SLC19A1*, and their relationship to MTX plasma levels and MTX-related toxicities.¹⁹ They found no association between *SLC19A1* haplotypes and MTX plasma levels. However they confound the results of Treviño and colleagues on association between *SLCO1B1* rs11045879 polymorphism and toxicity and observed for the first time a significant association with MTX plasma levels and rs9516519 in *ABCC4*, rs3740065 and haplotype GCGGG in *ABCC2*.¹⁹

However, haplotype based approach has been used in analysing the influence of the polymorphisms in the broader region of *SLC19A1* on the occurrence of low dose MTX-induced ADEs in rheumatoid arthritis (RA) patients in order to facilitate the design of personalized low dose MTX

treatment.^{37,38} Bohanec Grabar *et al.* reported that individual SNP and haplotype analysis suggest that rs1051266 could be a functional variant altering MTX toxicity; rs1051266 and rs1131956 were significantly associated with protection against discontinuation of treatment owing to MTX toxicity. Rs2838956 was significantly associated with protection against skin ADEs, while two common *SLC19A1* haplotypes carrying rs1051266 and rs1131956 minor alleles had a protective effect towards MTX induced ADEs. Significant association was also found for rs1051266 and rs1131956 with infection.³⁷ Lima *et al.* reported that *SLC19A1* and genotypes and haplotypes may help to identify RA patients with increased risk of MTX-related gastrointestinal toxicity since protective effect of carriers of wild type allele of rs1051266 and rs2838956 regarding MTX induced gastrointestinal toxicity as well as association between GGAG haplotype for *SLC19A1* rs7499, rs1051266, rs2838956 and rs3788200 with MTX gastrointestinal toxicity was established.³⁸

The results of the haplotype based analysis indicate, that other polymorphisms that are in linkage disequilibrium with rs1051266 and rs1131956, may impact the functioning of *SLC19A1* via, for example, alterations in *SLC19A1* splicing.^{39,40} As well, other genes coding for proteins that are involved in the transport and metabolism of MTX can also impact pharmacokinetics and the occurrence of MTX-induced AE, such as *SLCO1B1* SNPs that are associated with lower clearance of MTX and *SLCO1A2* as well.⁴¹ Furthermore, SNP in miR-595

which might affect *SLC19A1* regulation and could affect MTX levels in patients with paediatric B-cell ALL was recently reported.¹⁸

The results of our study show that *SLC19A1* genotyping could be one of the factors that could contribute to safer and more effective treatment with HD-MTX, especially for identifying patient groups at risk of mucositis development in Slovenian patients. As the occurrence of ADEs and the treatment outcome could be affected by other genetic factors involved in the transport and metabolism of MTX as well as by concomitant supportive therapy and the current pathophysiological condition of the patient the results of our study should be confirmed in a larger number of patients.

The main limitation of our study, which is probably also one of the reasons for the inconsistent results among studies published so far, is the limited sample size. Since ALL is a rare disease in children, the number of patients that could be as comparable as possible in the entire treatment process is limited. We therefore also included five patients with NHML in the study, who were treated according to the same protocol as patients with ALL.

The great advantage of our study is that all subjects come from a small and very homogeneous population⁴², that the patient group is clinically well defined and very homogeneous given the type and course of treatment, centralized treatment of patients with an established treatment and supportive regimen. The group was treated by the same medical team in a single centre according to standard criteria.

For genotyping, we used a method that would be suitable for genetic testing in clinical practice, as it is easy to perform, fast, affordable, and enables simultaneous analysis of multiple samples and different polymorphisms if they are run under the same conditions, and also require small amounts of DNA for analysis.

Employing a haplotype based approach we tried to examine the impact of genetic variability in the entire region of the studied gene and also included the possible functional polymorphisms that are in linkage disequilibrium with the selected SNPs. With this approach an even stronger influence of genetic variability of *SLC19A1* with MTX-induced ADEs could be shown as opposed to taking into account only individual SNPs.

The observations of our study provide additional information to the ongoing discussion about the most suitable biological markers to be evaluated during treatment with HD-MTX in order to achieve more efficient, safer and more rational

treatment of children and adolescents with ALL. Additional prospective pharmacogenetic studies on higher sample sizes are needed to further evaluate a possible impact of genetic variability of the MTX transporters that have an impact on pharmacokinetics of MTX and enzymes involved in MTX metabolism that could help us to identify patients that are threatened by serious ADEs during HD-MTX treatment.

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