Germline genetic variations in methotrexate candidate genes are associated with pharmacokinetics, toxicity, and outcome in childhood acute lymphoblastic leukemia

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Susanne Radtke,1 Oliver Zolk,2 Bertold Renner,2 Marios Paulides,1 Martin Zimmermann,3 Anja Möricke,4 Martin Stanulla,4 Martin Schrappe,4 and Thorsten Langer1

1Department for Pediatric Oncology and Immunology, Late Effects Surveillance System Center, University Hospital for Children and Adolescents, Erlangen, Germany; 2Institute of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen-Nuremberg, Erlangen, Germany; 3Department of Pediatric Hematology and Oncology, Hannover Medical School, Hannover, Germany; and 4Department of Pediatrics, University Hospital Schleswig-Holstein, Campus Kiel, Kiel, Germany

Key Points

- Polymorphisms in the MTX pathway genes substantially influence the kinetics and response to high-dose MTX therapy in childhood ALL.
- Germline variants in SLCO1B1, thymidylate synthase, and methylenetetrahydrofolate reductase are significantly associated with kinetics, toxicity, and outcome.

Introduction

The outcome for pediatric cancer patients has improved dramatically over the past 6 decades, with 5-year survival rates currently at 75% vs 20% in the 1950s.1,2 Accordingly, the cure rates of acute lymphoblastic leukemia (ALL), which is the most common childhood cancer, are higher than 80% due to optimized chemotherapy protocols.3,4 Most protocols consist of methotrexate (MTX) as a key chemotherapeutic agent with high therapeutic efficiency. Nevertheless, MTX causes severe dose-limiting adverse events during MTX courses (R2 < 0.015) with event-free survival in the ALL-BFM 2000 study population. Genetic variations substantially influence the kinetics and response to high-dose MTX therapy in childhood ALL. (Blood. 2013;121(26):5145-5153)

The pharmacogenetics of methotrexate (MTX) was investigated in a large cohort of pediatric patients with acute lymphoblastic leukemia (ALL). Four hundred ninety-nine children with ALL from the ALL-BFM (Berlin–Frankfurt–Münster) 2000 trial who received 1996 courses of MTX at 5 g/m2 were genotyped for 8 single nucleotide polymorphisms in 5 candidate genes of the MTX/folate pathway. Patients’ MTX pharmacokinetics, MTX toxicities, and outcomes were correlated with the genotypes. The interindividual variability in MTX kinetics had a substantial genetic component between 68% and 75%. The SLCO1B1 rs4149056 variant was significantly associated with MTX kinetics. In a multiple regression model, MTX area under the concentration time curve (AUC)0-48h increased by 26% (P < .001) per SLCO1B1 rs4149056 C allele. MTX AUC0-48h was a significant predictor of overall toxic adverse events during MTX courses (R2 = 0.043; P < .001), whereas the thymidylate synthase rs34743033 tandem repeat polymorphism was predictive of stomatitis (R2 = 0.018; P = .009), a frequent side effect of high-dose MTX. Multiple Cox regression analyses revealed an association of minimal residual disease (hazard ratio 7.3; P < .001) and methylenetetrahydrofolate reductase rs1801131 (hazard ratio 3.1; P = .015) with event-free survival in the ALL-BFM 2000 study population. Genetic variations substantially influence the kinetics and response to high-dose MTX therapy in childhood ALL. (Blood. 2013;121(26):5145-5153)
 due to small study cohorts, heterogeneous study populations, and/or treatment protocols or replication failure limit its significance.13–16

The aim of the present study was to confirm positive associations in a large homogeneous subpopulation from the multicenter ALL-BFM (Berlin–Frankfurt–Münster) 2000 trial. The primary endpoint was the effect of genetic variants in candidate genes on MTX pharmacokinetics. Because altered MTX pharmacokinetics may affect adverse drug reactions and treatment efficacy, overall toxicity, stomatitis (which is a frequent adverse event specifically of high-dose MTX), and event-free survival (EFS, time from diagnosis to relapse, secondary neoplasm, or death from any cause) were included as secondary endpoints in an explorative analysis. Based on the strength of evidence of previously published association studies, the following candidate genetic variants were selected for our analysis: rs4149056, rs2306283, and rs11045879 in SLCO1B1; rs717620 in ABCC2; rs1051266 in SLC19A1; rs1801131 and rs1801133 in MTHFR; and rs34743033 in TYMS.

All patients of our study cohort were similarly treated with MTX regarding the ALL-BFM 2000 protocol for patients within the standard risk (SR), medium risk, and slow early responder (SER) minimal residual disease (MRD) class, thereby excluding interacting effects of protocol inconsistency on MTX pharmacokinetics and response.

### Methods

#### Study population

Between August 1, 1999, and November 30, 2005, 2300 patients with ALL (aged ≥1 to <18 years) were enrolled in the ALL-BFM 2000 trial (ClinicalTrials.gov: NCT003430118) in Germany. The institutional review board of the Hannover Medical School approved the study and informed consent was obtained from patients and/or their legal guardians in accordance with the Declaration of Helsinki. Diagnostics and treatment arm assignments were performed according to the ALL-BFM 2000 protocol.17 Treatment arm assignment was based on MRD analysis after induction (day 33) and consolidation therapy (day 78) and the presence of clinical high-risk (HR) criteria (Table 1). MRD stratification required at least 2 polymerase chain reaction (PCR)-MRD targets with a sensitivity of at least $10^{-4}$. MRD was analyzed by real-time quantitative–PCR reaction analysis of “leukemia-specific” junctional regions of rearranged immunoglobulin genes and T-cell receptor genes, which can be considered as “DNA fingerprints” of the leukemic cells. Molecular marker identification was patient specific and failed in some patients. If MRD evaluation was not available, patients were assigned to the intermediate treatment arm or, based on clinical parameters, to the HR group. A detailed description is provided in the supplemental data.

### Treatment and clinical data

Patients were treated according to the ALL-BFM 2000 protocol. During the extracompartment phase (protocol M), patients received 4 courses of MTX every 2 weeks at a dose of 5000 mg/m² body surface area in each course. MTX was given as a 500-mg/m² loading infusion over 0.5 hour followed by a 4500-mg/m² intravenous infusion for another 23.5 hours. MTX administration, leukovorin rescue, intravenous hydration, urinary alkalization therapy, and monitoring were standardized according to the ALL-BFM 2000 study protocol (supplemental Table 1). The MTX dose was reduced in children with Down syndrome (trisomy 21) because of their suspected poor tolerance to antineoplastic drugs (n = 5). In cases (n = 19) when children experienced severe MTX toxicity with the standard dose, dose adjustments were made in subsequent courses.

MTX toxicities during each MTX course were reported by applying a 5-step scoring scheme (0 = none to 4 = highest grade of toxicity) based on the National Cancer Institute (NCI) toxicity criteria (supplemental Table 2).

### Selection of polymorphisms for genotyping

Genetic variants were selected based on the strength of evidence of published association studies. For instance, there is a huge body of work on the nonsynonymous variants rs1801131 and rs1801133 in the MTHFR gene, which were associated with a lower probability of EFS or hepatic toxicity.18–22 rs1051266 in SLC19A1, resulting in a less efficient transportor, has been associated with more adverse events and worse overall prognosis.23–26 A polymorphic tandem repeat in the 5′UTR of the TYMS enhancer region (rs34743033) was associated with a significantly greater chance of response to treatment in 205 ALL patients treated with MTX.27 A genome-wide association study implicated the intronic SNP rs11045879 in the SLC19A1 transporter gene, which was in linkage disequilibrium (LD) with the functional SLCO1B1 SNP rs4149056, with MTX clearance and gastrointestinal toxicity in 640 ALL patients.28 The nonsynonymous SNP rs2306283 in SLCO1B1 was also recognized as a predictor of MTX clearance in 434 children with ALL.29 Our own results suggest an association of the nonsynonymous SNP rs717620 in ABCC2 with MTX kinetics,30 which demanded replication in a large cohort.

### Sample size calculation

The sample size depends on the primary endpoint (MTX kinetics, eg, MTX clearance), the method of calculating the test statistic (multiple linear regression analysis), and the proposed effect size of the predictive variables ($R^2$, ie, the amount of variance in the dependent variable that can be explained by the predictive variables). The following predictive variables were suggested: SLCO1B1 (rs4149056, rs2306283, and rs1045879), SLC19A1 (rs1051266), ABCC2 (rs717620), MTHFR (rs1801131 and rs1801133), and TYMS (rs34743033) genotypes, age, sex, and MRD class. For the a priori analysis, we used an estimate of the effect size from previous research. According to Trevisío et al15 the 2 most powerful predictors of MTX clearance were the treatment regimen ($R^2 = 17.9\%$) and the SLCO1B1 genotypes.
Because the treatment regimen was similar for all patients in our ALL-BFM 2000 study population (and thus was not considered as a predictive variable), we defined $R^2 = 9.3\%$ as the anticipated effect size of our study. With these preconditions the minimum required sample size at a nominal type I error rate of 0.01 and a power level of 90% was 312. We exceeded the required sample size by inclusion of N = 415 patients.

During analysis of the data we recognized that the white blood cell (WBC) count significantly differed between our subcohort and the total ALL-BFM 2000 cohort. To avoid any bias, we considered WBC count as an additional predictive variable. Reestimation of the statistical power with WBC count as an additional predictive variable revealed that the multiple regression analysis provided a power of 98% for detecting the prespecified effect of $R^2 = 9.3\%$ of the predictive variables on MTX clearance (at a nominal type I error rate of 0.01) in our study population (N = 415).

Genotyping

DNA was extracted from remission BM samples by using the QIAamp DNA Blood Midi Kit (Qiagen GmbH, Hilden, Germany) and stored at −20°C until use. Genotyping for rs4149056, rs11045879, and rs2306283 in \textit{SLCO1B1}; rs717620 in \textit{ABCC2}; rs1801131 and rs1801133 in \textit{MTHFR} was carried out using the TaqMan Pre-Developed Assay Reagents for Allelic Discrimination (assay ID: C\_\_30633906\_\_10, C\_\_31106904\_\_10, C\_\_1901697\_\_20, C\_\_2814642\_\_10, C\_\_850486\_\_20, C\_\_1202883\_\_20; Applied Biosystems, Foster City, CA). SLC19A1 rs1051266 was genotyped with the following primers and probes: (forward) 5\'GCCTGACCCCGAGCT-3\', (reverse) 5\'CATGAAGCCGTAGAAGCAAAGGTA-3\', (probe 1) 5\'VIC-ACACGAGGTGCCGCC-3\', (probe 2) 5\'FAM-ACGAGGCGCCGCC-3\'. Amplification was performed in a final volume of 6 μL containing 5 ng DNA, 4.5 pmol of each primer, 1.0 pmol of each probe, 3 μL of 2× Type-It Fast Genotyping Master Mix (contains buffer, passive reference dye ROX, deoxynucleotides, and Taq DNA polymerase; Qiagen, Hilden, Germany), and 1.2 μL of Q-Solution (Qiagen, Hilden, Germany) by use of the ABI Prism Sequence Detector 7900 (Applied Biosystems). Cycle parameters were as follows: initial denaturation at 95°C for 5 minutes and then 45 cycles at 95°C for 15 seconds and at 60°C for 30 seconds. After PCR, fluorescence yield for the 2 dyes was measured. SDS 2.1 software (Applied Biosystems) was used to plot and automatically call genotypes on the basis of a 2-parameter plot with fluorescence intensities of FAM and VIC. The 28 bp tandem repeat polymorphism in \textit{TYMS} (rs34743033) was genotyped according to the method of Hori et al\cite{29} using the sense primer 5\'GTGCTCTCCTGCCGTTCCCC-3' and the reverse primer 5\'GCTCCGAGCCGCGCACAGCGATGGCGCGGG-3'. PCR amplicons were analyzed using 3% agarose gel electrophoresis. The expected PCR product sizes were 214 bp (2 repeats), 242 bp (3 repeats), and 270 bp (4 repeats).

Each SNP was tested for conformance of genotype frequencies to those expected under Hardy-Weinberg equilibrium with a $x^2$ goodness-of-fit test. LD was calculated with Haploview (version 4.2; Broad Institute, Cambridge, MA)\cite{30}.

Pharmacokinetics

The MTX area under the concentration time curve (AUC$[0-t]$), peak MTX plasma concentration at the end of infusion (C$[24h]$), and MTX clearance were estimated based on at least 4 MTX plasma concentrations per course up to 48 hours from the start of infusion. Individual pharmacokinetic data were analyzed using the software package Phoenix WinNonlin/NLME (version 6.2.1, Pharsight Corp, St. Louis, MO). Plasma concentration data were fit to a 2-compartment infusion model with first-order elimination. Both dosing intervals (0–0.5 hours and 0.5–24 hours) with individual dosing rates were included in the model. Initial estimates (for apparent volume of distribution and rate constants between compartments) were derived from previous publications and set to $V = 10$ L, $k_e = 0.5$ h$^{-1}$, $k_{12} = 0.07$ h$^{-1}$, and $k_{21} = 0.12$ h$^{-1}$\cite{31}. Clearance was calculated from primary estimates, and AUC

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**Figure 1.** Consolidated Standards of Reporting Trials (CONSORT) diagram of entry criteria and study cohort characteristics for the study population.
values were obtained from model-predicted concentration time courses. In rare cases when patients did not receive the standard MTX dose of 5000 mg/m², for the purpose of comparison, AUC and C₄₈ were dose normalized. We assumed that MTX kinetics are approximately linear over the dose range, that is, the plasma concentration that results from the dose is proportional to that dose and the rate of elimination of the drug is proportional to the concentration. Individual parameters were modeled for each of the 4 high-dose methotrexate courses, and corresponding results were transferred to statistical analysis.

Genetic component r₂GC

The repeated drug application methodology can be used to estimate heritability without using a twin study design. 31,32 This method relies on data obtained from studies in which a group of individuals receives an identical dose of a drug on 2 or more occasions. Also, it allows for the calculation of variance (ie, SD² [standard deviation]) in a pharmacokinetic parameter within an individual and between individuals. The genetic component (r₂GC) as a measure of heritability of MTX kinetics was calculated according to the following equation: r₂GC = (SDₜ² − SDₕ²)/SDₜ², where SDₜ² is the SD between patients (influenced mainly by variation in environmental factors, genetic factors, and measurement errors) and SDₕ² is the SD within patients who received MTX at 4 cycles (influenced only by environmental factors and measurement errors).

Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics 20 software (SPSS, Chicago, IL). Linear multiple regression models for the effect on pharmacokinetic parameters were calculated, with genotypes, age, sex, WBC, and MRD class as independent variables. All polymorphisms were included except SLCO1B1 rs11045879, which was in LD with SLCO1B1 rs4149056 and thus was excluded from the regression models for reasons of collinearity. Investigation of secondary endpoints was exploratory, that is, P values were not corrected for multiple endpoints. The association between SNPs and sum toxicity scores was calculated using multiple linear regression analysis, with age, sex, MRD class, WBC, AUC₀-₄₈, and genotypes as independent variables. The overall toxicity score for each patient was calculated by adding up the grading of all adverse events that occurred in 17 toxicity categories during 4 courses of MTX. Similarly, the stomatitis score was calculated by adding up the NCI stomatitis grading of the 4 MTX courses. Associations between SNPs and EFS were determined by multiple analysis using the Cox proportional hazards model with age, DNA index, TEL/AML1, MRD class, AUC₀-₄₈, and effect of the SNPs. Fisher exact test and χ² test were used to compare frequency distributions, and log-rank test was used to compare survival data between 2 patient populations. Bivariate correlations were tested with Pearson correlation test. Statistical significance was accepted for values of P < .05. Nominal P values are shown throughout.

Results

Patient characteristics and genotyping

Patient characteristics are summarized in Figure 1. Study endpoints, such as survival rates (Table 2) and MTX toxicity grading (not shown), did not significantly differ between ALL-BFM 2000 patients with genotype data included in our study and those without genotype data. Notably, our study cohort consisted of significantly more male patients (P = .025) and more patients with WBC count ≥ 50 000 at diagnosis (P < .001). Thus, our subcohort was not fully reflective of the overall ALL-BFM 2000 cohort.

Genetic component of MTX pharmacokinetics

Substantial interpatient variability was observed in C₄₈ (coefficient of variation [CV] = 45.5%), AUC₀-₄₈ (CV = 44.5%), and MTX clearance (CV = 49.4%). Variation in MTX pharmacokinetics is substantially influenced by heredity as revealed by r₂GC. The estimated genetic component contributing to variation in MTX C₄₈, AUC₀-₄₈, and clearance was 0.75, 0.62, and 0.68, respectively, suggesting that genetic factors contribute to MTX pharmacokinetics.

Association of SNPs in candidate genes with MTX pharmacokinetics

MTX clearance decreased with increasing age (Figure 2A; P = 4.7 × 10⁻³). Moreover, an influence of sex on MTX kinetics was observed previously. 16 Therefore, age and sex were included as
Figure 2. Influence of age and genetic polymorphisms on pharmacokinetic parameters. (A) Correlation between age and the average AUC_{0-48h} (upper panel) and between age and the average MTX clearance (lower panel) of each patient. (B) Association of the SLCO1B1 rs4149056 SNP with pharmacokinetic parameters. Genotype-dependent change in the area under the concentration time curve (AUC_{0-48h}, the peak MTX plasma concentration at the end of infusion (C_{24h}), and the MTX clearance. Error bars show 5% to 95% percentiles. Shown are P values obtained from the multiple regression models with age, sex, MRD class, WBC, and genotypes as independent variables. Jonckheere trend test assuming an a priori ordering consistent with an additive allelic effect: P < .0001 (AUC_{0-48h} and C_{24h}), P < .001 (clearance).

Table 3. Identification of predictive variables for MTX C_{24h}, MTX AUC_{0-48h}, and MTX clearance by regression analysis

<table>
<thead>
<tr>
<th>Included variables</th>
<th>MTX AUC_{0-48h}</th>
<th>MTX C_{24h}</th>
<th>MTX clearance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B (95% CI)</td>
<td>P</td>
<td>R^2 (%)</td>
</tr>
<tr>
<td>SLCO1B1 rs4149056</td>
<td>146.9 (94.4, 199.4)</td>
<td>6.8 x 10^{-8}</td>
<td>5.4</td>
</tr>
<tr>
<td>SLCO1B1 rs2306283</td>
<td>56.7 (-98.3, -15.1)</td>
<td>7.7 x 10^{-3}</td>
<td>1.6</td>
</tr>
<tr>
<td>Age</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Age, sex, WBC count, MRD class, and SLCO1B1 rs4149056, SLCO1B1 rs2306283, SLC19A1 rs1051266, ABC2 rs717620, MTHFR rs1801131, MTHFR rs1801133, and TYMS rs34743033 genotypes were considered as independent variables in the regression analyses. Because SLCO1B1 rs11045879 was in LD with SLCO1B1 rs4149056, SLCO1B1 rs11045879 was not included in the regression models for reasons of collinearity. This table shows significant predictor variables included in the stepwise regression models. Age was a significant predictor variable of MTX clearance only.

Association of SNPs in candidate genes with MTX toxicity

Overall toxicity was evaluated by applying a sum score that integrates both frequency and severity of adverse events during protocol M. Regression analysis with sex, age, MRD class, WBC, MTX AUC_{0-48h}, and genotypes as independent variables revealed that MTX AUC_{0-48h} was the most significant predictor of high overall toxicity scores (regression coefficient B = 0.013; 95% confidence interval [CI], 0.007, 0.019; R^2 = 0.043; P = 2.9 x 10^{-5}).

Stomatitis, a common side effect of chemotherapy in children with ALL, is closely attributed to the use of (high-dose) MTX and therefore has been considered to be relatively specific for MTX. Surprisingly, MTX AUC_{0-48h} was not related to the stomatitis sum toxicity score. However, the TYMS rs34743033 tandem repeat polymorphism was the only significant predictor of stomatitis in a multiple regression model considering sex, age, MRD class, WBC, MTX AUC_{0-48h}, and genotypes as independent variables (B = -0.48; 95% CI, -0.84, -0.12; R^2 = 0.018; P = .009). The stomatitis toxicity score decreased with increasing number of TYMS independent variables into the regression analyses. To exclude any bias, we also considered WBC and MRD as predictive variables. When genotypes were allowed to compete in the regression model (adjusted for sex, age, WBC, and MRD class), SLCO1B1 rs4149056 and rs2306283 remained significant predictors of AUC_{0-48h} (Table 3). Consistent with the mathematical relationship between the pharmacokinetic parameters (AUC and the steady-state concentration at the end of infusion are inversely proportional to the clearance), the SLCO1B1 genotypes were also significantly associated with MTX C_{24h} and MTX clearance (Table 3). In these multiple regression models, SLCO1B1 genotypes explained 6.8%, 7.0%, and 3.4% of the interindividual variability in MTX C_{24h}, AUC_{0-48h}, and clearance, respectively. Both genotypes at the SLCO1B1 locus were more powerful predictors of MTX kinetics than age. Supplemental Figure 1 shows MTX AUC_{0-48h} levels stratified according to the SLCO1B1 rs2306283/rs4149056 diplotype. Per dysfunctional rs4149056 C allele, MTX AUC_{0-48h} increased by 26%, MTX C_{24h} increased by 24%, and MTX clearance decreased by 18%. A significant association was also found between SLCO1B1 rs11045879, which is in strong LD with SLCO1B1 rs4149056, and the pharmacokinetic parameters. Pharmacokinetic parameters significantly changed in a gene dose-dependent manner (Figure 2B), suggesting a per allele effect consistent with a codominant model of association.

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*3 alleles. Figure 3 shows the incidence of stomatitis stratified according to the NCI grade and \textit{TYMS} rs34743033 genotypes.

**Association of SNPs in candidate genes with EFS**

In a multiple Cox regression analysis, which included age, WBC, DNA index, TEL/AML1, AUC\(_{0-48h}\), MRD class, and genotypes as independent variables, MRD class and \textit{MTHFR} rs1801131 SNP were significantly associated with EFS (Figure 4). The adjusted hazard ratios were 7.3 (95% CI = 2.5 - 21.3; \(P = 3.2 \times 10^{-4}\)) for MRD-SER vs MRD SR patients and 3.1 (95% CI = 1.2 - 7.7; \(P = .015\)) for homozygous carriers of the \textit{MTHFR} rs1801131 C allele vs patients with the AA reference genotype.

**Discussion**

By investigating key genes involved in the MTX pathway, our study aimed to detect genetic polymorphisms associated with MTX pharmacokinetics, toxicity, and outcome. The strongest association was observed between the \textit{SLCO1B1} rs4149056 SNP and MTX pharmacokinetic parameters such as \(C_{24h}\), \(AUC_{0-48h}\), and clearance.

The \textit{SLCO1B1} carrier is mainly expressed in the liver where it is located on the sinusoidal membrane of human hepatocytes.\(^{33,34}\) The carrier acts as an influx transporter for endogenous compounds (eg, bile acids and thyroid hormones)\(^{35}\) and for many xenobiotics and drugs.\(^{7}\) Several findings support the notion that \textit{SLCO} is important for hepatic uptake and thus for the pharmacokinetics of MTX. First, independent studies have shown that \textit{SLCO1B1} can transport MTX in vitro.\(^{28,35}\) Second, in transgenic mice with functional and specific expression of human \textit{SLCO1B1} in the liver, the AUC for intravenous MTX was 1.5-fold decreased compared with wild-type mice, demonstrating a marked and possibly rate-limiting role for human \textit{SLCO1B1} in MTX elimination in vivo.\(^{36}\) Third, using a genome-wide association study approach, the tagging SNP rs11045879 in \textit{SLCO1B1}, which is in LD with the rs4149056 loss-of-function SNP, was associated with reduced MTX clearance in patients with ALL.\(^{13}\) The latter study demonstrated the clinical significance of \textit{SLCO1B1} for MTX clearance for the first time and highlighted the role of \textit{SLCO1B1}'s polymorphic expression for the interindividual variability in MTX pharmacokinetics.

A subsequent study, however, failed to show a universally statistically significant association between \textit{SLCO1B1} rs11045879, which is in LD with \textit{SLCO1B1} rs4149056, and MTX plasma concentrations in ALL patients (corrected \(P = .08\)).\(^{14}\) Our study, which was performed in a cohort that provides sufficient statistical power, clearly replicates the association found by Treвиño et al.\(^{15}\) A study by Ramsey et al, which was published during preparation of our manuscript, confirmed the association of \textit{SLCO1B1} rs4149056 with MTX clearance in a genome-wide association study.\(^{37}\)

In contrast to the loss-of-function phenotype of the \textit{SLCO1B1} rs4149056 variant,\(^{7}\) the rs2306283 variant, another common coding SNP in \textit{SLCO1B1}, increased the transport activity of OATP1B1 for MTX in vitro.\(^{28}\) In accordance with enhanced cellular uptake of MTX in vitro, presence of the mutant rs2306283 A allele was related to increased MTX clearance and decreased MTX AUC\(_{0-48h}\) in our patients. The \textit{SLCO1B1} rs2306283 G allele, thus, in part counter-balances the effects of the mutant rs4149056 C allele on MTX kinetics.

Because altered MTX pharmacokinetics may affect treatment efficacy and adverse drug reactions, survival and MTX toxicity were investigated as secondary endpoints in an explorative analysis. We used an overall toxicity sum score that integrated both frequency and severity of 17 toxicity items reported during protocol M. Among the parameters and genotypes tested in the regression model, MTX AUC\(_{0-48h}\) was a significant predictor of adverse events. The rather weak association of MTX AUC\(_{0-48h}\) with adverse events—the regression model explained only 4.3% of variance—might be due, in part, to leucovorin rescue and unclear attribution of some types of adverse events to MTX.

In fact, causality assessment of adverse drug reactions is a general problem that also holds true for suspected toxicity of MTX in our study. In the ALL BFM-2000 study causality assessment scales were not applied. Thus, reported adverse events are most likely due to MTX but may also originate from cytostatic comedication, such as 6-mercaptopurine. Moreover, early symptomatic treatments for pain, diarrhea, nausea, and vomiting can cause adverse events or modify MTX toxicity.

Stomatitis is an adverse event that has been attributed specifically to high-dose MTX, as applied in protocol M. Surprisingly, in the multiple regression analysis, stomatitis was not associated with MTX AUC\(_{0-48h}\) but with the \textit{TYMS} tandem repeat polymorphism. Stomatitis was significantly less frequent and/or less severe in patients carrying at least 1 \textit{TYMS} triple repeat (\textit{TYMS} *3) allele compared with homozygous *2 carriers. It is known that an increased number of tandem repeats increases \textit{TYMS} mRNA and protein expression and that overexpression of \textit{TYMS} might be a mechanism by which individuals with at least 1 *3 allele develop resistance, that is, tolerate high-dose MTX with fewer side effects. A previous study reported that homozygosity for the \textit{TYMS} triple repeat was associated with poorer outcome of ALL patients treated with high-dose MTX; however, this was not confirmed in our study.

Conter et al showed that among patients presenting high MRD levels at day 33 (\(>10E-3\)), those with no MRD detectable at day 78 had a favorable outcome, whereas those with MRD still present at day 78 (MRD-SER) had a marked increase in the risk of relapse.\(^{17}\) Our data confirmed the previous results by the demonstration that MRD-SER patients had significantly worse EFS than MRD-SR or MRD-MR patients. After adjustment for age, WBC, TEL/AML1, DNA index, MRD class, and AUC\(_{0-48h}\), our association analysis of individual polymorphisms showed a significant association of the \textit{MTHFR} rs1801131 SNP with EFS. Variants in folate pathway genes have been found to be associated with relapse-free
survival and EFS of pediatric ALL patients in previous studies.\textsuperscript{22,38,39}
Some studies failed to replicate significant associations. Similarly, our study could not confirm associations of folate pathway gene polymorphisms with EFS, except for the association with the \textit{MTHFR} rs1801131 variant, suggesting that the overall contribution to outcome is small and cannot be explained by this variant alone. Whether variants in a specific gene affect EFS rates may depend on the specific treatment protocol. Remission rates, relapse, or secondary leukemia depend on the respective protocol applied in a study. Thus, the association between \textit{MTHFR} rs1801131 and outcome may be specific for the ALL-BFM 2000 protocol and must not apply to other protocols for treatment of pediatric ALL.

Our results may help to guide and optimize MTX treatment of individual patients based on genetic markers.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Association of the \textit{MTHFR} rs1801131 genotype (A) and MRD status (B) with EFS. HR, hazard ratio.}
\end{figure}

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\section*{Authorship}
Contribution: S.R. conceived and designed the study, collected and assembled data, did data analysis and interpretation, wrote the manuscript, and gave final approval of the manuscript; O.Z. conceived and designed the study, collected and assembled data, did data analysis
and interpretation, wrote the manuscript, gave final approval of the manuscript, and provided administrative support; B.R. did data analysis and interpretation, wrote the manuscript, and gave final approval of the manuscript; M.P. conceived and designed the study, wrote the manuscript, and gave final approval of the manuscript; M.Z. collected and assembled data, did data analysis and interpretation, wrote the manuscript, gave final approval of the manuscript, and provided study material or patients; A.M. collected and assembled data, did data analysis and interpretation, wrote the manuscript, gave final approval of the manuscript, and provided study material or patients; M. Stanulla collected and assembled data, did data analysis and interpretation, wrote the manuscript, gave final approval of the manuscript, and provided study material or patients; T.L. conceived and designed the study, collected and assembled data, did data analysis and interpretation, wrote the manuscript, gave final approval of the manuscript, and provided financial and administrative support.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: T. Langer, University Hospital for Children and Adolescents, Loschgestrasse 15, 91054 Erlangen, Germany; e-mail: thorsten.langer@uk-erlangen.de.

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