Relationship Between Polymorphisms in Methotrexate Pathway Genes and Outcome of Methotrexate Treatment in a Cohort of 119 Patients with Juvenile Idiopathic Arthritis

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ABSTRACT. ***Objective.*** To identify clinical and pharmacogenetic determinants of efficacy and toxicity of methotrexate (MTX) in juvenile idiopathic arthritis (JIA) over time.

Methods. A cohort of 119 consecutive patients with JIA treated with MTX was reviewed. The Juvenile Arthritis Disease Activity Score including 71 joints was used to measure disease activity. Nonresponders were patients who did not reach a minimum of 30% improvement after 6 months of treatment or were switched to biologic drugs in the first 6 months because of inefficacy. All adverse events (AE) were noted. Genotyping of single-nucleotide polymorphisms (SNP) in the genes coding for MTX transporters, folate pathway, and adenosine pathway was performed using real-time PCR methods. Univariate and multivariable penalized logistic and Cox regression were used to analyze data.

Results. Thirty patients (25.8%) were defined as nonresponders and 55 (47.2%) were switched to biologics during the followup. Sixty-five patients (54.5%) reported AE in a total of 405 patient-years, and 10 patients (8.4%) discontinued MTX because of AE. *AMPD1* rs17602729 and *MTHFD1* rs2236225 were associated with gastrointestinal AE while the latter together with *MTRR* rs1801394 also demonstrated associations with developing hepatoxicity. *MTHFR* rs1801131, *ABCG2* rs2231137, wild-type of *MTR* rs1805087, and wild-type of *ABCC2* rs2273697 were identified as potential markers for discontinuing MTX treatment because of AE. *MTHFR* rs1801133, *MTRR* rs1801394, and *ABCC2* rs2273697 were associated with switching to biologics.

Conclusion. SNP in different MTX metabolic pathways influence treatment with MTX. Genetic variability is a better marker for toxicity than efficacy. (First Release June 1 2017; J Rheumatol 2017;44:1216–23; doi:10.3899/jrheum.160950)

Key Indexing Terms:
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Juvenile idiopathic arthritis (JIA) is one of the most common chronic diseases in childhood, with a prevalence of 1 in 1000 children. Methotrexate (MTX) in a dose of 10–15 mg/m^2/week is the most important disease-modifying antirheumatic drug (DMARD) used in JIA and is recommended as an initial DMARD therapy[1]. Around 65%–70% of children reach and sustain remission with MTX. In the remaining patients, the delay in optimal treatment can lead to functional disability, and therefore it would be of great clinical importance to identify MTX-refractory patients earlier[2].

While MTX has a long history as a drug, its precise mode of action is still not completely understood. MTX enters the cell by the solute carrier 19A1 and is pumped out of the cell

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by ATP-binding cassette (ABC) transporters. Inside the cell, activated MTX polyglutamates inhibit several enzymes in the folate pathway, thereby disrupting the purine and pyrimidine synthesis. It is generally assumed that MTX in the dose used for rheumatic diseases does not considerably inhibit cell division[3,4]. MTX also inhibits the enzymes in the adenosine pathway, resulting in the release of endogenous adenosine, which is thought to be responsible for the site-specific anti-inflammatory effects of MTX[3].

There is growing evidence that single-nucleotide polymorphisms (SNP) within the MTX pathway genes are significant contributors to interindividual differences in response to MTX and adverse events (AE) in adults[3,5,6,7], as well as patients with JIA[8–17]. However, several candidate SNP that emerged from other fields of medicine were not evaluated in JIA[18,19]. To our knowledge, no study included SNP in all known MTX metabolic pathways and assessed toxicity and efficacy over time in JIA. Therefore, the aim of our study was to identify clinical and pharmacogenetic factors to predict efficacy and toxicity of MTX in JIA over time.

MATERIALS AND METHODS

Study design and patient selection. The design of our study was a single-center, descriptive observational cohort study. The data were collected retrospectively with a longitudinal followup. The inclusion criteria were a definite diagnosis of JIA according to the International League of Associations for Rheumatology criteria[20] and past or current treatment with MTX. In total, 126 consecutive patients with JIA treated with MTX at the University Children's Hospital Ljubljana from September 2011 to October 2014 were eligible to participate in the study. The study was approved by the National Ethics Committee for Research in Medicine and was carried out according to the Helsinki Declaration (approval number 39/11/09). Patients with all JIA subtypes were recruited at their regular visits. Seven patients were excluded from the study because they refused to participate (n = 2), received MTX for isolated uveitis (n = 4), or had missing data (n = 1). All patients were Central European white and their parents or guardians gave their written informed consent prior to enrollment.

Data were retrospectively collected from the medical records for 78/119 patients (65%) who were already taking MTX at the start of the study or had been taking MTX in the past. Forty-one patients (34%) started taking MTX during the course of our study and their data were collected prospectively. Patients were followed up at regular intervals by the standardized protocol used at our department until the study was finished. Patients were evaluated at least 4 times: upon MTX introduction, 3 months and 6 months after treatment was introduced, and at the last followup visit. All patients were also evaluated in case of a flare. Starting oral dose of MTX was 10 mg/m^2 and increased to 15 mg/m^2 in the first 3 months if required.

Concomitant treatment with nonsteroidal antiinflammatory drugs, bridging therapy with oral corticosteroids, and intraarticular corticosteroid injections were allowed. All patients were receiving folic acid supplementation. Biologic therapy because of refractory disease was introduced by a standard protocol used in our department.

Definition of response. Response to treatment was measured using the Juvenile Arthritis Disease Activity Score including 71 joints (JADAS-71)[21]. The response was measured as a percentage of improvement in the JADAS-71 score at 3 and 6 months after treatment introduction and at the last followup visit. Nonresponders were patients who did not achieve the minimum improvement of 30% in the score 6 months from baseline and those who required therapy escalation to biologic drugs because of inefficacy in the first 6 months.

Inactive disease, remission while taking or not taking therapy, and flares were noted. Inactive disease was defined as JADAS score of a maximum of 1[22], remission while taking therapy as continuously inactive disease for 6 months, and remission while not taking therapy as continuously inactive disease for 12 months without any antiarthritis drug[23]. Flare was defined as worsening of the disease that required treatment intervention after inactive disease had already been achieved.

Recording of AE. All AE that were reported by the patients throughout the course of MTX treatment were recorded. AE were grouped by organ systems: (1) gastrointestinal AE (GAE): nausea, vomiting, abdominal pain; (2) hepatotoxicity: serum transaminases above the upper range of normal; (3) bone marrow toxicity; (4) dermatological complaints; (5) central nervous system toxicity; (6) renal toxicity; (7) infections; and (8) allergic reactions. The AE were divided into categories of mild, moderate, and severe in accordance with the Common Terminology Criteria for AE[24]. All AE that were the reason for the patient to discontinue the treatment were noted separately.

Genotyping. In total, 5 ml of peripheral blood was collected into tubes with sodium citrate and stored for DNA isolation. Genomic DNA was isolated from peripheral blood leukocytes using commercial kits (Flexigene, Qiagen). SNP were analyzed with real-time PCR–based methods using TaqMan (Applied Biosystems) and KASPar assays (KBiosciences). The following polymorphisms were determined in folate pathway: *MTHFD1* rs2236225, *MTHFR* rs1801133, *MTHFR* rs1801131, *MTR* rs1805087, and *MTRR* rs1801394; adenosine pathway: *ATIC* rs2372536, *AMPD1* rs17602729, and *ITPA* rs1127354; and in the genes for MTX transporters: *ABCC2* rs2804402, *ABCG2* rs 2231142, *ABCC2* rs2273697, *ABCB1* rs1045642, *ABCG2* rs2231137, *SLC19A1* rs1051266, *ABCC2* rs717620, *SLCO1B1* rs11045879, *SLCO1B1* rs4149056, and *SLCO1B1* rs2306283. Polymorphic *TYMS* region was analyzed by multiplying promoter region as previously described[25]. PCR products were analyzed on 3% agarose gel, and visualized using ethidium bromide staining.

Statistical analysis. Patient characteristics of responders and nonresponders were compared using the 2-tailed Fisher's exact test for categorical data, and the 2 sample Student t test was used for numerical data. Log-rank test was used to compare responders and nonresponders in time-to-event endpoints (time to inactive disease, remission, AE, escalation to biological treatment). Univariate logistic regression models were used to estimate the association between JADAS 30% nonresponse after 6 months and each of the polymorphisms. Results were reported as OR with 95% CI and p values. A significance level of $\alpha = 0.05$ was used in the univariate analyses. We also estimated a multivariable logistic regression model with the Least Absolute Shrinkage and Selection Operator (LASSO) penalty, which included all the polymorphisms; the use of this model is appropriate when the number of covariates is large compared with the number of events, and it performs some type of variable selection: the polymorphisms whose penalized OR were not shrunk to 1 were considered as associated to MTX response. The dominant genetic model was used. Wild-type was the reference category.

A similar approach was used for time-to-event endpoints: univariable and multivariable cause-specific Cox models with LASSO penalization were used to estimate the association between the polymorphisms and a specific event; the univariable results were summarized with HR, 95% CI, and p values, the multivariable with HR. All statistical analyses were carried out using the R language[26].

RESULTS

Patient characteristics. A total of 119 patients were included in the analysis, 91 girls (76.5%) and 28 boys (23.5%). Patient characteristics are presented in Table 1. Mean time of disease duration before MTX was started was 13 months in the whole cohort and 6.9 months in the subgroup of patients with polyarticular disease (rheumatoid factor–positive and -negative). Mean starting dose of MTX was 10.2 mg/m^2 and

Table 1. Patient group characteristics and characteristics of responders and nonresponders. Values are mean (SD) unless otherwise specified.

Characteristics	Patient Group	Nonresponders	Responders	p
Patients, n (%)*	119	30 (25.8)	86 (74.1)	†
Female, n (%)	91 (76.5)	23 (76.7)	66 (76.7)	1
Age at baseline, yrs	6.9 (4.1)	6.3 (4.4)	7.1 (3.9)	0.303
Disease duration until start of MTX therapy, mos	13 (23)	7.2 (10)	15.6 (26)	0.076
ESR at baseline, mm/h	36 (28)	35 (27.5)	36 (28.7)	0.958
No. active joints at baseline	9 (8.6)	9.5 (10.6)	9 (7.9)	0.770
PGA at baseline	5.3 (1.9)	5.3 (1.9)	5.3 (1.8)	0.607
PGE at baseline	7.1 (2.1)	7.4 (1.8)	7 (2.2)	0.333
JADAS-71 at baseline	23.4 (11.2)	23.6 (12.7)	23.3 (10.6)	
JIA subtype, n (%)				0.181
Systemic	10 (8.4)	1 (3.3)	9 (10.5)	
Persistent oligoarticular	24 (20.2)	8 (26.7)	16 (18.6)	
Extended oligoarticular	24 (20.2)	8 (26.7)	15 (17.4)	
Polyarticular RF-negative	41 (34.5)	9 (30)	32 (37.2)	
Polyarticular RF-positive	5 (4.2)	0 (0)	5 (5.8)	
Psoriatic	11 (9.2)	2 (6.7)	8 (9.3)	
Enthesitis-related	2 (1.7)	2 (6.7)	0 (0)	
Undetermined	2 (1.7)	0 (0)	2 (2.3)	
Inactive disease, n (%)	82 (70.1)	9 (30.0)	73 (84.9)	0.0013
Median time to inactive disease, mos	5.9	14.5	5	NR
Concomitant oral steroids upon reaching inactive disease, n (%)**	29 (35.4)	3 (33.3)	26 (35.6)	NR
Flare after reaching inactive disease, n (%)**	59 (72.0)	7 (77.8)	52 (60.5)	NR
Median time to flare after reaching inactive disease, mos	9.1	3.8	9.8	NR
MTX dose to reach inactive disease, mg/m^2/week	11.5 (2.1)	11.6 (2.0)	11.5 (2.1)	NR
Remission on therapy, n (%)	54 (46.6)	3 (10.0)	51 (59.3)	0.151
Median time to remission on therapy, mos	21.9	28.8	14.9	NR
Remission without therapy, n (%)	20 (17.2)	1 (3.3)	19 (22.1)	0.684
Median time to remission without therapy, mos	76.5	NR	76.5	NR
Remission without therapy at last followup, n (%)	16 (13.7)	1 (3.3)	15 (17.4)	NR
No. flares	1.45 (1.5)	1.3 (1.1)	1.5 (1.5)	NR
Medication				
Starting dose of MTX, mg/m$^{2/}$week	10.2 (1.4)	10.3 (1.2)	10.2 (1.4)	0.792
Switched to higher dose of MTX, n (%)***	77 (66.3)	25 (83.3)	53 (60.4)	< 0.0001
Subcutaneous MTX, n (%)	42 (35.3)	9 (30.0)	33 (38.3)	0.345
Switched to biologic therapy, n (%)	55 (47.2)	26 (86.7)	29 (33.7)	< 0.0001
Median time to biologic therapy, mos	44.0	5.9	65.3	NR
Switched to biologic therapy in the first yr of treatment, n (%)	31 (26.5)	22 (73.3)	9 (10.5)	NR

* Three patients were excluded from the efficacy study. ** Analyses performed only in the patients who reached inactive disease. *** Higher dose is > 12.5 mg/m^2/week. † p value comparing responders and nonresponders. ESR: erythrocyte sedimentation rate; PGA: physician's global assessment; PGE: parent's general evaluation of well-being; JADAS-71: Juvenile Arthritis Disease Activity Score including 71 joints; JIA: juvenile idiopathic arthritis; RF: rheumatoid factor; MTX: methotrexate; NR: not reported.

mean dose at 6 months was 12.0 mg/m^2. Twenty patients (16.8%) discontinued MTX in less than 6 months because of inefficacy or toxicity. At 6 months, 48.0% of the patients still taking MTX were receiving a higher dose. Forty-two patients (35%) were switched to subcutaneous MTX to achieve higher efficacy. The mean followup of our patients from the beginning of the disease was 45 months.

Treatment efficacy. Out of 116 patients evaluated for efficacy, 30 (25.8%) were defined as MTX nonresponders at 6 months of therapy. The following patients were excluded from efficacy assessment: 1 because of missing data, 1 because of MTX withdrawal before reaching 6 months of treatment following severe AE, and 1 because of changed indication for treatment. The groups of MTX responders and nonresponders did not differ statistically in any of the clinical variables evaluated at baseline (Table 1). Disease duration prior to treatment was not associated to efficacy of MTX ($p > 0.05$).

Analysis of AE. AE data are presented in Table 2. Sixty-five patients (54.5%) reported AE in a total of 405 patient-years. Most patients with AE (83%) developed them in the first year of treatment. The most common AE were gastrointestinal, which occurred in 39 patients (32.8%), followed by hepatotoxicity in 28 patients (23.5%). In addition, 25 patients (21.0%) had moderate hepatotoxicity and were included in the further analyses. AE were considered mild in 50 (42%) and moderate in 43 patients (36.1%). A total of 36 patients (30.3%) reported infectious illness during the study period;

Table 2. Adverse events in patient population.

Adverse Events	n (%)
Overall adverse events	65 (54.5)
Estimated cumulative incidence after 6 mos/12 mos*	40.0%/51.2%
Severe AE	14 (11.8)
Discontinued MTX because of AE	10 (8.4)
Discontinued MTX because of AE while receiving MTX monotherapy	7 (5.9)
Estimated cumulative incidence after 6 mos/12 mos*	3.1%/5.8%
Gastrointestinal AE	39 (32.8)
Estimated cumulative incidence after 6 mos/12 mos*	21.5%/31.9%
Hepatotoxicity	28 (23.5)
Moderate hepatotoxicity	25 (21.0)
Estimated cumulative incidence after 6 mos/12 mos*	13.5%/16.3%
Infections	36 (30.3)
Severe infections	13 (10.2)
Dermatological AE	11 (9.2)
Hematological AE	1 (0.8)
Renal AE	0 (0)
Central nervous system AE	1 (0.8)

* Estimated proportion of patients who experienced the event in the first 6 months/12 months of therapy. Estimates were obtained using Kaplan-Meier method. AE: adverse events; MTX: methotrexate.

however, only 13 patients (10.9%) reporting severe infection were included in further analyses. One patient had a severe AE of persistent leukopenia. Apart from these, no other severe AE were noted.

Of the 10 patients (8.4%) who discontinued MTX because of AE, 4 had nausea/vomiting, 4 had persistently elevated liver transaminases, 1 had persistent leukopenia, and 1 discontinued MTX because of worsening of an existing dermatological condition. Three patients developed AE after biologic therapy was introduced and were not included in further analyses. We estimated that 3.1% of patients discontinued MTX because of AE in the first 6 months and 5.8% in 1 year.

Pharmacogenetic determinants of MTX treatment efficacy. All of the investigated SNP except *TS**2/*3 were in Hardy-Weinberg equilibrium. The minor allele frequencies and genotype frequencies are shown in Supplementary Table 1 (available with the online version of this article). The number of successful analyses per each SNP and numbers of polymorphic alleles are shown in Supplementary Table 2 (available with the online version of this article). Significant results are shown in Table 3 and Table 4, and all results are shown in Supplementary Table 3 (available with the online version of this article). JADAS 30% nonresponse after 6 months was associated with *ABCC2* rs2804402 genotype ($p = 0.048$) in univariate analysis, but the penalized multivariable logistic regression analysis did not confirm the association. However, this SNP, together with 5 others, was statistically significantly associated with time to reach inactive disease in multivariable penalized Cox regression [folate pathway: *MTR* rs1805087 (HR 1.58), *MTHFR* rs1801131 (HR 1.19); adenosine pathway: *ITPA* rs1127354 (HR 1.36), *ATIC* rs2372536 (HR 1.14); MTX transporters: *ABCC2* rs2273697 (HR 1.55 for wild-type), and *ABCC2* rs2804402 (HR 1.07)]. No associations were found between the investigated SNP and time to flare or reaching remission while taking and not taking therapy (Supplementary Table 3, available with the online version of this article).

Pharmacogenetic determinants of MTX AE. Statistically significant results are shown in Table 5 and Table 6, and all results are shown in Supplementary Table 4 (available with the online version of this article). These were associated to time to develop GAE in the univariate analyses: *AMPD1* rs17602729 ($p = 0.011$) from adenosine pathway, *MTHFD1* rs2236225 ($p = 0.015$) from folate pathway, and MTX trans-

Table 3. Pharmacogenetic determinants of MTX treatment efficacy. Results of all performed analyses are shown in Supplementary Table 3 (available with the online version of this article).

SNP	Nonresponse to MTX			Time to Inactive Disease		
	Univariate Analysis		Penalized Cox Regression OR,	Univariate Analysis		Penalized Cox Regression, HR
	Associated Allele, OR, 95% CI	p		Associated Allele, HR, 95% CI	p	
Folate pathway						
MTHFR rs1801133	T, 0.64, 0.27–1.49	0.299	1	T, 1, 0.64–1.56	0.999	1
MTHFR rs1801131	C, 0.66, 0.28–1.52	0.325	1	C, 1.26, 0.8–1.97	0.317	**1.19**
MTR rs1805087	G, 0.96, 0.38–2.28	0.921	1	G, 1.64, 1.02–2.63	**0.042**	**1.58**
MTRR rs1801394	G, 1.22, 0.37–5.08	0.752	1	G, 0.81, 0.43–1.51	0.509	1
Adenosine pathway						
ATIC rs2372536	G, 0.99, 0.43–2.33	0.984	1	G 1.18, 0.76–1.85	0.457	**1.14**
ITPA rs1127354	A, 1.57, 0.42–5.17	0.480	1	A, 1.6, 0.82–3.11	0.170	**1.36**
MTX transporters						
ABCC2 rs2804402	**G, 0.37, 0.14–0.99**	**0.048**	1	G, 1.14, 0.65–2.01	0.640	**1.07**
ABCC2 rs2273697	A, 1.06, 0.45–2.44	0.894	1	G wt, 0.62, 0.39–0.99	**0.043**	**1.55**

Wt was associated with tested variable, and in all other analyses, polymorphic allele was associated with tested variable. Significant data are in bold face. MTX: methotrexate; SNP: single-nucleotide polymorphism; wt: wild-type.

Table 4. Pharmacogenetic determinants of escalation to biologic drugs because of inefficacy or AE. Results of all performed analyses are shown in Supplementary Table 3 (available with the online version of this article).

SNP	Univariate Analysis		Penalized Cox Regression
	Associated Allele, HR, 95% CI	p	HR
Folate pathway			
MTHFR rs1801133	T, 1.78, 1–3.15	**0.050**	**1.41**
MTHFR rs1801131	C, 0.58, 0.34–0.98	**0.043**	1
MTR rs1805087	G, 0.88, 0.47–1.65	0.693	1
MTRR rs1801394	G, 1.64, 0.65–4.13	0.296	**1.17**
Adenosine pathway			
ATIC rs2372536	G, 0.85, 0.5–1.46	0.562	1
ITPA rs1127354	A, 0.92, 0.37–2.33	0.866	1
MTX transporters			
ABCC2 rs2804402	G, 1.07, 0.55–2.08	0.84	1
ABCC2 rs2273697	A, 1.59, 0.94–2.68	0.086	**1.05**

Significant data are in bold face. AE: adverse events; SNP: single-nucleotide polymorphism; MTX: methotrexate.

porter *SLCO1B1* rs11045879 (p = 0.045). The association of *AMPD1* rs17602729 and *MTHFD1* rs2236225 remained significant in the multivariable model (HR 1.61 and 1.55, respectively).

In the univariate analysis, no SNP were significantly associated with time to moderately elevated liver transaminase; nevertheless 11 SNP were statistically significantly associated in the multivariate analyses, including *MTRR* rs1801394 with the strongest association (HR 2.76), *MTHFD1* rs2236225 (HR 1.64 for wild-type), *MTHFR* rs1801133 (HR 1.12), *MTR* rs1805087 (HR 1.25 for wild-type) *TYMS* rs34743033 (HR 1.01), *ATIC* rs2372536 (HR 1.24), *ABCB1* rs1045642 (HR 1.28), *ABCG2* rs2231137 (HR 1.54 for wild-type), *SLC19A1* rs1051266 (HR 1.10), *ABCC2* rs717620 (HR 1.29), and *ABCC2* rs2804402 (HR 1.05).

All patients who discontinued MTX because of toxicity had at least 1 polymorphic allele of *MTHFR* rs1801131 in comparison with 52% of the patients who tolerated the drug throughout the followup period. None of the patients who discontinued MTX carried any polymorphic alleles of *MTR* rs1805087, *ABCG2* rs2231137, and *ABCC2* rs2273697 in comparison with 32%, 71%, and 41% of patients who did not (Table 6).

In the final part of our analyses, we investigated the association between the SNP and the time to escalation to biologic therapy, either because of inefficacy or because of AE. In the univariate analyses, *MTHFR* rs1801133 (p = 0.050) and *MTHFR* rs1801131 (p = 0.043) were significantly associated and in the multivariate analysis, the association of *MTHFR* rs1801133 (HR 1.41) also persisted with *MTRR* rs1801394 (HR 1.17) from folate pathway and transporter *ABCC2* rs2273697 (HR 1.05) being associated with escalation to biologic therapy (Table 4).

Table 5. Pharmacogenetic determinants of MTX toxicity. Results of all performed analyses are shown in Supplementary Table 4 (available with the online version of this article).

SNP	Time to First GI AE			Time to First Liver-associated AE		
	Univariate		Penalized Cox Regression	Univariate		Penalized Cox Regression
	Associated Allele, HR, 95% CI	p	HR	Associated Allele, HR, 95% CI	p	HR
Folate pathway						
RFC1 rs1051266	C, 0.95, 0.48–1.87	0.877	1	C, 2.09, 0.71–6.15	0.180	**1.09**
MTHFR rs1801133	T, 1.06, 0.56–2.02	0.861	1	T, 1.74, 0.71–4.23	0.224	**1.12**
MTHFR rs1801131	C, 1.06, 0.56–2.02	0.849	1	C, 1.11, 0.50–2.47	0.801	1
MTR rs1805087	G, 1.26, 0.63–2.50	0.515	1	A wt, 0.75, 0.28–2.03	0.572	**1.25**
MTRR rs1801394	G, 3.16, 0.76 –13.18	0.115	1	G, 4.29, 0.58 –31.85	0.154	**2.76**
MTHFD1 rs2236225	**G wt, 0.44, 0.22–0.85**	**0.015**	**1.55**	G wt, 0.49, 0.21–1.16	0.105	**1.64**
TYMS rs34743033	3R, 1.52, 0.67–3.46	0.317	1	*3, 0.95, 0.35–2.61	0.925	**1.01**
Adenosine pathway						
ATIC rs2372536	G, 1.36, 0.70–2.67	0.365	1	G, 1.42, 0.61–3.33	0.414	**1.24**
AMPD1 rs17602729	**A, 2.35, 1.22–4.53**	**0.011**	**1.61**	A, 0.78, 0.29–2.11	0.630	1
MTX transporters						
ABCG2 rs2231142	T, 0.64, 0.28–1.45	0.283	1	T, 1.22, 0.5–2.96	0.656	1
ABCG2 rs2231137	T, 0.67, 0.16–2.76	0.575	1	C wt, 0.51, 0.07–3.80	0.509	**1.54**
ABCC2 rs717620	T, 1.14, 0.6–2.15	0.694	1	C wt, 0.76, 0.33–1.74	0.515	**1.29**
ABCC2 rs2804402	G, 1.06, 0.46–2.42	0.895	1	G, 1.58, 0.53–4.69	0.409	**1.05**
ABCC2 rs2273697	A, 0.99, 0.52–1.90	0.981	1	A, 1.03, 0.46–2.30	0.940	1
ABCB1 rs1045642	G, 1.87, 0.86–4.08	0.115	1	G, 1.53, 0.57–4.10	0.400	**1.28**
SLCO1B1 rs11045879	**C, 1.93, 1.01–3.66**	**0.045**	1	C, 1.34, 0.59–3.02	0.483	1

* 3 means 3R, triple repeat of 28 base pairs. Wt was associated with tested variable, and in all other analyses, polymorphic allele was associated with tested variable. Significant data are in bold face. MTX: methotrexate; GI: gastrointestinal; AE: adverse events; SNP: single-nucleotide polymorphism; wt: wild-type.

Table 6. Pharmacogenetic determinants of MTX discontinuation because of AE. Percentage of patients carrying at least 1 polymorphic allele is shown. Because of the small number of patients (n = 7), statistical analyses were unreliable. Results of all performed analyses are shown in Supplementary Table 4 (available with the online version of this article).

SNP, Allele	Patients Who Did Not Discontinue MTX Because of AE, n = 110	Patients Who Discontinued MTX Because of AE, n = 7
Folate pathway		
RFC1 rs1051266, C	72	43
MTHFR rs1801133, T	61	29
MTHFR rs1801131, C	**53**	**100**
MTR rs1805087, G	**32**	**0**
MTRR rs1801394, G	86	100
MTHFD1 rs2236225, A	68	71
TYMS rs34743033, 3R	77	57
Adenosine pathway		
ATIC rs2372536, G	59	71
AMPD1 rs17602729, A	25	14
MTX transporters		
ABCG2 rs2231142, T	23	14
ABCG2 rs2231137, T	**71**	**0**
ABCC2 rs717620, T	41	29
ABCC2 rs2804402, G	82	86
ABCC2 rs2273697, A	**41**	**0**
ABCB1 rs1045642, G	72	71
SLCO1B1 rs11045879, C	37	57

Significant data are in bold face**.** MTX: methotrexate; AE: adverse events; SNP: single-nucleotide polymorphism.

DISCUSSION

The ultimate goal of treating JIA is achieving and sustaining disease remission. MTX is an important drug for the treatment of JIA, but early predictors are needed to identify patients who will not respond to MTX or will develop AE[2]. In our present study, none of the clinical variables was associated with response to treatment; however, specific genetic markers were identified that may be associated to efficacy and toxicity of MTX in JIA. Our study was based on a well-described patient cohort representative of the whole JIA population followed in the tertiary care pediatric rheumatology center, and was one of the most comprehensive to evaluate common functional SNP in all MTX pathways. Moreover, the longitudinal followup approach allowed us to collect data on AE for a total of 405 patient-years, contributing to the reliability of our findings.

MTX treatment efficacy results of our study are consistent with previous studies demonstrating that almost half of the patients required escalation of therapy to biologics[27]. These represent the most important target group for a therapeutic intervention that could be based on pharmacogenetic data. Different definitions of response to treatment make the comparisons of published studies difficult. Therefore, the JADAS scoring system, which could become the cornerstone for response evaluation because of its completeness and practical application, was used in our study[21].

AE are the reason for MTX withdrawal in around 20% of adult patients with rheumatoid arthritis (RA). In children, AE are less pronounced and in our cohort, 10 patients (8.4%) discontinued MTX because of AE, a comparable number to other studies[8,10]. Possibly children use less concomitant drugs and are more compliant to folic acid supplementation, which reduces hepatic and GAE[28]. GAE lower the quality of life and lead to reduced compliance[29], and these occurred in almost one-third of our patients, which is comparable to previous studies[10,30].

Several SNP were evaluated for the first time in JIA in our study. In particular, ABC transporters were evaluated in only a few studies, but have consistently shown statistically significant associations with response. In a study of 285 children with JIA, 2 SNP in ABC transporters were predictors of first-year response to MTX[9]. These results were not replicated in our cohort; however, according to our clinical experience it seems more appropriate to evaluate MTX effects after 6 months of therapy. In our study, *ABCC2* rs2804402, which that has not been analyzed before in JIA, was statistically significantly associated with nonresponse in univariate analysis. *ABCC2* rs2273697 was statistically significantly associated with time to inactive disease as well as being involved in switching to biologics. This SNP was evaluated for the first time regarding MTX treatment, emphasizing the need for further pharmacogenetic studies to analyze the involvement of transporters. Nevertheless, a gene within the same subfamily, *ABCC7,* showed association with response to MTX in a genome-wide association study on 759 patients with JIA[17].

In the previous pharmacogenetic studies in patients with JIA, AE were not assessed and not subdivided according to the organ system or severity[8,11,12,31,32], or only a limited number of SNP were analyzed[13,14,33]. Identifying patients with AE that make them discontinue MTX would be of major clinical importance. Because of a small number of patients experiencing this event in our study (n = 7), statistical analyses were unreliable. Interestingly, their genetic background was different, because all these patients carried polymorphic alleles of *MTHFR* rs1801131 and only wild-type alleles of *MTR* rs1805087, *ABCG2* rs2231137, and *ABCC2* rs2273697. MTHFR is a key enzyme in the folate homeostasis. SNP in its gene are widely researched and believed to contribute to reduced enzymatic activity. The role of *MTHFR* rs1801131 is not consistent; in a study on RA, it was associated with toxicity[34], but some studies found different or no association[5,10,13,32]. To our knowledge, *ABCC2* rs2273697 and rs2804402 have not yet been evaluated in relation to MTX treatment. We hypothesize that the efflux rate of MTX polyglutamates might be decreased in the presence of SNP in ABC transporter genes, resulting in intracellular accumulation of active metabolites[18].

Gastrointestinal- and liver-associated AE were 2 main reasons to discontinue MTX and both were associated with specific genetic markers. The carriers of *AMPD1* rs17602729 and of wild-type *MTHFD1* rs2236225 had almost 2× the higher hazard rates for developing GAE compared with mutated patients, which has not been shown in previous studies that focused on assessment of efficacy rather than AE[8,12,35,36]. In a prediction model of GAE of MTX in JIA, the involvement of *AMPD1* rs17602729 was not significant[11]. Its functional role is not clear, although it leads to severely truncated peptide[3]. Similarly, *MTHFD1* rs223622 is also believed to be a functional SNP affecting enzyme thermostability and shifting the balance of folate pools[3,4], and in our present study the wild-type also had a 1.64 times higher hazard rate for hepatotoxicity compared with mutated patients. Altogether, 11 SNP were significantly associated with hepatotoxicity and some of them were also associated with treatment discontinuation because of AE, emphasizing that more attention should be paid to AE in pharmacogenetic studies in children with JIA. Carriers of *MTRR* rs1801394 had around a 2.5× higher hazard rate both to develop hepatotoxicity and to discontinue MTX because of AE. Our results are consistent with research in JIA, where *MTRR* rs1801394 was included in the prediction model for MTX-associated GAE[11]. Its involvement regarding AE has been shown in previous studies on RA[5,34]. Increased risk of toxicity can be explained by low homocysteine remethylation resulting from lower enzymatic activity[3].

To find pharmacogenetic tests that would be most useful in clinical practice, we have focused on patients who required escalation to biologics, combining inefficacy and AE. To our knowledge, this approach has not been used before. Significant associations were found with *MTHFR* rs1801133, *MTRR* rs1801394, and *ABCC2* rs2273697. *MTHFR* rs1801133 has shown associations with AE in studies on children as well as in several metaanalyses in patients with RA[13,33,37,38]. Additionally, it has also been associated with nonresponse to MTX[34,39]. Similarly, *MTRR* rs1801394 was included in both a prediction model for nonresponse in JIA[9] as well as exhibited associations with AE in our study and previous ones[5,34]. Therefore, it seems that the combined approach used in our study could integrate the different influences of these SNP.

Some limitations of our study should be considered. The size of our cohort was relatively small (n = 119) and did not include an independent validation cohort. Patients were followed by the standard protocol used in our department; however, some heterogeneity of our cohort must be taken into account because some patients were reviewed retrospectively. This could also influence the reporting of AE, especially those that are not an objective laboratory result.

Our results suggest that SNP in different MTX metabolic pathways influence treatment with MTX and that genetic variability is a better marker for toxicity than efficacy. *AMPD1* rs17602729 and *MTHFD1* rs223622 were associated with GAE while the latter together with *MTRR* rs1801394 also demonstrated the strongest association with developing hepatoxicity. *MTHFR* rs1801131, *MTR* rs1805087, *ABCG2* rs2231137, and *ABCC2* rs2273697 were identified as potential markers for discontinuing MTX treatment because of AE. *MTHFR* rs1801133, *MTRR* rs1801394, and *ABCC2* rs2273697 were associated with switching to biologics because of inefficacy or toxicity, indicating patients who could benefit most from pharmacogenetic testing in clinical environment. Construction of a prediction model based on both clinical and pharmacogenetic determinants is needed.

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ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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