ABCB1 and *ABCC3* Gene Polymorphisms Are Associated with First-year Response to Methotrexate in Juvenile Idiopathic Arthritis

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ABSTRACT. ***Objective.*** Although methotrexate (MTX) is the most widely prescribed drug in juvenile idiopathic arthritis (JIA), 30% of patients fail to respond to it. To individualize treatment strategies, the genetic determinants of response to MTX should be identified.

Methods. A cohort of 287 patients with JIA treated with MTX was studied longitudinally over the first year of treatment. MTX response was defined as the American College of Rheumatology pediatric 70 criteria (ACRped70). We genotyped 21 single-nucleotide polymorphisms in 13 genes related to MTX polyglutamylation and to cellular MTX uptake and efflux. Potential associations between ACRped70 and genotypes were analyzed in a multivariate model and corrected for these 3 covariates: disease duration prior to MTX treatment, physician's global assessment of disease activity at baseline, and MTX dose at all study visits.

Results. MTX response was more often achieved by patients variant for the adenosine triphosphate-binding cassette transporter B1 (*ABCB1*) gene polymorphism rs1045642 (OR 3.80, 95% CI 1.70–8.47, p = 0.001) and patients variant for the *ABCC3* gene polymorphism rs4793665 (OR 3.10, 95% CI 1.49–6.41, p = 0.002) than by patients with other genotypes. Patients variant for the solute carrier 19A1 (*SLC19A1*) gene polymorphism rs1051266 were less likely to respond to MTX (OR 0.25, 95% CI 0.09–0.72, p = 0.011).

Conclusion. *ABCB1* rs1045642, *ABCC3* rs4793665, and *SLC19A1* rs1051266 polymorphisms were associated with response to MTX in 287 patients with JIA studied longitudinally. Upon validation of our results in other JIA cohorts, these genetic determinants may help to individualize treatment strategies by predicting clinical response to MTX. (First Release Aug 1 2012; J Rheumatol 2012;39:2032–40; doi:10.3899/jrheum.111593)

Key Indexing Terms:
METHOTREXATE JUVENILE IDIOPATHIC ARTHRITIS GENE POLYMORPHISMS

Juvenile idiopathic arthritis (JIA) is the most frequent rheumatic disease in infants, affecting 1 in 1000 children, and is an important cause of disability[1]. Methotrexate (MTX) is the most widely used disease-modifying antirheumatic drug (DMARD) in JIA[2]. Although patients can go into prolonged remission, 30% of the patients treated with MTX do not respond to the drug[2]. The delay in identifying the optimal treatment at an early stage of the disease can lead to joint damage. Therefore, there is a need to identify determinants of response to MTX that can be used to individualize treatment strategies.

In weekly low-dose MTX treatment, MTX polyglutamates accumulate intracellularly and thus inhibit several key enzymes in the folate metabolism and *de novo* purine synthesis (Figure 1)[3,4]. MTX polyglutamates correlate with MTX efficacy in adult rheumatoid arthritis (RA)[5,6,7,8]. Nonresponders accumulate fewer MTX polyglutamates in red blood cells compared to responders in an early phase of treatment[6]. Single-nucleotide polymorphisms (SNP) in genes involved in MTX transport and polyglutamylation affect intracellular MTX accumulation[9]. MTX enters mammalian cells mainly through the solute carrier 19A1/reduced folate carrier (*SLC19A1*/*RFC*) and is additionally transported into the cell through the solute carrier 46A1/proton-coupled folate transporter (*SLC46A1*/*PCFT*) and the folate receptors (*FOLR*) 1 and 2[4]. Members of the adenosine triphosphate (ATP) binding cassette (*ABC*) transporters,

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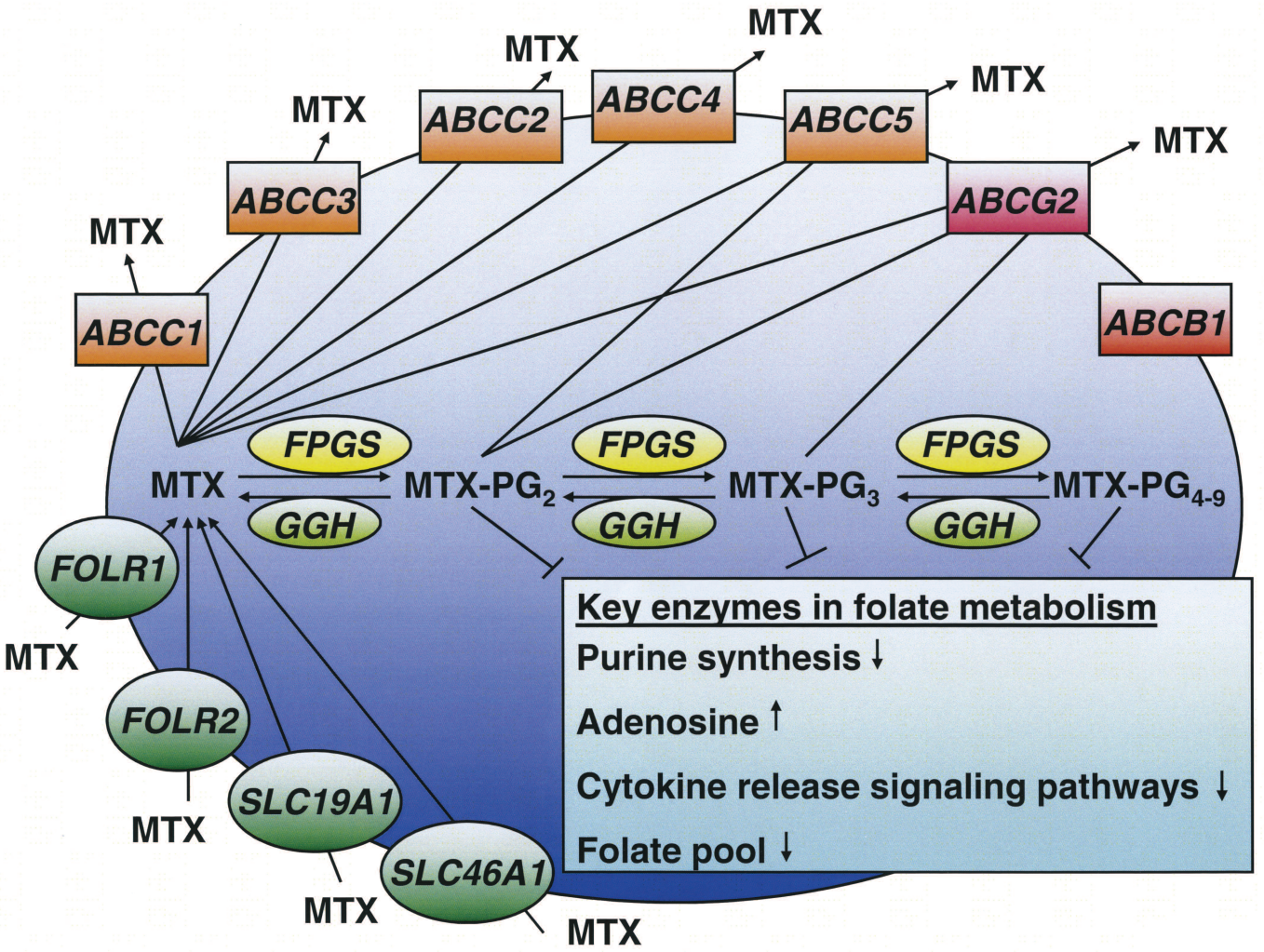

Figure 1. Cellular MTX transport routes for MTX influx and efflux in relation to polyglutamylation and mechanisms for arthritis suppression. MTX polyglutamates (MTX-PG) can inhibit several key enzymes in folate metabolism and may cause a decreased *de novo* purine biosynthesis, increased adenosine release, direct or indirect effects on cytokine release signaling pathways, and folate depletion, all of which may lead to suppression of arthritis. *ABCB1*, *ABCC1*, *ABCC2*, *ABCC3*, *ABCC4*, *ABCC5*, *ABCG2:* adenosine triphosphate-binding cassette transporter subfamily B/C/G member 1/2/3/4/5; *FPGS*: folylpolyglutamate synthetase; *FOLR1/2*: folate receptor 1/2; *GGH:* gamma-glutamyl hydrolase; *SLC46A1/19A1*: solute carrier 46A1/19A1.

including *ABCB1*/P-glycoprotein (P-gp), multidrug resistance proteins (*MRP/ABCC*), and breast cancer resistance protein (*BCRP/ABCG2*), function as ATP-dependent MTX efflux transporters[4]. Cellular retention of MTX is mediated by the dynamic interplay between formation of MTX polyglutamates through folylpolyglutamate synthetase (*FPGS*) and MTX polyglutamate breakdown through gamma-glutamyl hydrolase (*GGH*)[3].

In contrast to RA[10], studies in JIA examining associations of SNP in genes involved in MTX transport (uptake/efflux) and polyglutamylation are scarce[11,12,13,14,15,16,17]. Moreover, they report inconsistent findings and the majority has a cross-sectional design. Therefore, the aim of our study was to perform a comprehensive analysis of SNP in genes involved in cellular MTX transport and polyglutamylation in relation to MTX response in a longitudinal JIA cohort. We hypothesize that SNP in genes involved in MTX transport and polyglutamylation affect response to MTX in JIA.

MATERIALS AND METHODS

Patients and study design. We used a cohort study performed at the University Medical Center Utrecht (UMCU), Wilhelmina Children's Hospital, The Netherlands. The cohort included 295 patients who started MTX therapy between 1990 and 2010. Patients with a confirmed JIA diagnosis according to the International League of Associations for Rheumatology criteria were included[18]. Patients were excluded if full clinical data or blood for DNA analysis were not available. All patients gave informed consent. The study was approved by the Medical Ethics Committee of the UMCU and was in compliance with the Declaration of Helsinki. Patients had been systematically followed at 0, 3, 6, and 12 months after initiation of MTX therapy using a standardized report form on disease activity. Information was collected from the patients' medical files at every visit until 1 year after the start of MTX therapy. The data were disease activity, MTX usage and route of administration, MTX dose, reasons for ending MTX treatment, concomitant therapy, and laboratory measurements.

Definition of response. The international validated core set criteria for the assessment of patients with JIA was used to define disease activity: (1) physician global assessment of disease activity on a 10-cm visual analog scale (PGA); (2) parent/patient assessment of overall well-being using the Childhood Health Assessment Questionnaire; (3) functional ability, measured using the Childhood Health Assessment Questionnaire on a 0–3 scale; (4) number of joints with active arthritis, defined by the presence of swelling and/or limitation of movement accompanied by pain and/or tenderness; (5) number of joints with limited range of motion, defined as a loss of at least 5 degrees in any articular movement from the normal amplitude; (6) erythrocyte sedimentation rate (mm/first hour). MTX response was defined by the American College of Rheumatology 70 pediatric criteria (ACRped70)[19]: Patients with > 70% improvement in at least 3 of the 6 criteria, without > 30% worsening in 1 of the remaining variables, were defined as good clinical responders. Use of anti-tumor necrosis factor-α (TNF-α) was a criterion for nonresponse.

SNP selection. SNP in genes involved in MTX transport and polyglutamylation were selected based on the following criteria: minor allele frequency (MAF) > 0.10 in the Hapmap and National Center for Biotechnology Information (NCBI) database[20,21] or a proven functionality in relation to MTX, JIA, RA, or folate metabolism[22,23,24,25,26,27,28,29,30]. If no information was known for a particular gene, we selected tagging SNP by Hapmap database and Haploview (version 4.2, 29 April 2008)[20]. We chose an MAF > 0.10 instead of the commonly chosen > 0.05. Because our sample size was relatively small, we expected that SNP with an MAF < 0.10 would not have sufficient data distribution for statistical analysis. Preferably, 2 SNP were selected per gene, which were located in different haplotype blocks. The following 21 SNP in 13 genes were chosen: *ABCB1* rs1128503, rs2032582, rs1045642; *ABCC1* rs35592, rs3784862; *ABCC2* rs4148396, rs717620; *ABCC3* rs4793665, rs3785911; *ABCC4* rs868853, rs2274407; *ABCC5* rs2139560; *ABCG2* rs13120400, rs2231142; *FPGS* rs4451422; *FOLR1* rs11235462; *FOLR2* rs514933; *GGH* rs10106587, rs3758149; *SLC46A1* rs2239907; and *SLC19A1* rs1051266. Subsequently, we calculated the gene coverage[31] to assess the percentage of genetic variation that was covered by the investigated SNP of all the genetic variation possible within each gene.

We standardized our SNP nomenclature based on the probes labeled with fluorescent dyes VIC and FAM, for which the Taqman assays were designed for allele detection. The major allele was analyzed as wild-type allele and the minor allele as variant allele.

A haplotype is a combination of alleles at adjacent locations on the chromosome that are transmitted together. We included haplotype analysis in our study to test whether the effect of the haplotypes on MTX response was larger than that of the corresponding SNP alone. Lewontin's D prime (D') and correlation coefficient (R^2) were calculated by Haploview to assess linkage disequilibrium of SNP within each gene. SNP that were in linkage disequilibrium (D' ≠ 0) with a correlation coefficient < 0.80 were selected for haplotype reconstruction by the phase method[32].

Genotyping. Genomic DNA was isolated from 0.2 ml EDTA whole blood with a Total Nucleic Acid Extraction kit on a MagNA Pure LC (Roche Molecular Biochemicals, Almere, Netherlands). Genotyping was performed using Taqman allelic discrimination assays on the Prism 7000 sequence detection system (Life Technologies, Applied Biosystems, Bleiswijk, Netherlands). Each assay consisted of 2 allele-specific minor groove binding probes, labeled with VIC and FAM. The primer and probe sequences were ordered from stock by Applied Biosystems and otherwise by their Assay-by-Design service (*ABCB1* rs1128503, rs2032582, rs1045642, and *SLC19A1* rs1051266). Samples in which the Taqman did not perform an automatic calling were rejected. Of these samples, duplicate samples were genotyped. When the Taqman could not perform an analysis the second time, the result was included as missing in the database. For every new genotyping test in our laboratory, 50 random blood samples were analyzed. From these results a wild-type, heterozygous, and homozygous variant control sample was chosen. In each run with patient samples, control samples for each genotype were included. A run was rejected when the results for the control samples changed from the original results. For 5% of the patients, duplicate samples were run for each SNP on random patients. All allele frequencies were compared with Hapmap and NCBI databases[20,21] and if discrepancies existed, samples were sequenced to confirm genotypes. Therefore, we designed primers for these SNP. The quality-control samples were sequenced with the obtained primers. Deviation from Hardy-Weinberg equilibrium (HWE) was tested.

Statistical analysis. Before analysis we plotted the percentage responders within each genotype group, and the inheritance of all SNP followed the recessive mode of inheritance. We therefore chose a recessive inheritance model to increase the statistical power. Consequently, genotypes and haplotypes were divided accordingly: genotypes into wild-type/heterozygous = 0 and homozygous variants = 1; haplotypes into heterozygous and all other homozygous haplotypes = 0; and homozygous for the specific haplotype = 1. For example, for the *ABCB1* haplotype GCA, the patients with the genotypes rs1128503 GG, rs2032582 CC, and rs1045642 AA = 1, and for all other patients = 0. Statistical analyses were done with SPSS PASW 17.02 for Windows (SPSS Inc., Chicago, IL, USA) unless stated otherwise. P values < 0.05 were considered significant.

SNP or haplotypes with sufficient distribution of data for statistical analysis (at least 1 responder and 1 nonresponder for each genotype on every visit) were further analyzed for associations with MTX response. The associations between genotype, or haplotype and response, were analyzed with a generalized linear mixed model to account for the correlations between the repeated measurements and to obtain an overall OR and CI over the whole treatment period[33]. Generalized linear mixed models were fitted using SAS v. 9.2 (SAS Institute Inc., Cary, NC, USA). A random intercept logistical model was used. This model considers random variation within individuals and random variation between individuals. We used empirical (sandwich) estimators to make analysis robust against misspecification of the covariance structure and to adjust for small-sample bias. The estimation is based on integral approximation by adaptive quadrature.

Univariate relations between genotype or haplotype and ACRped70 with a significance of p < 0.2 were further investigated in a multivariate analysis. This analysis combined potential univariate associations (p < 0.2) with clinical covariates, namely disease duration prior to start of MTX treatment, PGA at baseline, and MTX dose, which were previously reported to be significantly associated with MTX response in JIA[12].

To test whether our results had multiple testing problems, we tested the significant SNP from the multivariate analysis also in relation with ACRped50 as criterion for MTX response. We also used an alternative outcome (responders as patients with an ACRped70 at 2 or more consecutive visits) to obtain an ordinary logistic regression analysis to test our significant results. Finally, we used a Bonferroni correction to assess our significant results.

RESULTS

Patient characteristics. Blood for DNA isolation was available for 295 patients. Five patients were excluded because longitudinal clinical data could not be retrieved and 3 patients were excluded because they received biologicals (anakinra) at start of MTX. That left 287 patients for further analyses. Baseline characteristics are shown in Table 1. Of the 287 patients, 29 (10.1%) were ACRped70 responders after 3 months, 83 (28.9%) after 6 months, and 132 (46.0%) after 12 months of MTX therapy. After 3 months, 1 patient received anti-TNF-α therapy; after 6 months, 3 patients; and after 12 months, 17 patients, because of insufficient response to MTX. Those patients were considered nonresponders on those visits. Patients taking sulfasalazine were

Table 1. Characteristics of patients with juvenile idiopathic arthritis (JIA) at the time of starting methotrexate (MTX) treatment.

Characteristics	n = 287
Polyarticular JIA, n (%)	107 (37.3)
Systemic-onset JIA, n (%)	47 (16.4)
Oligoarticular persistent JIA, n (%)	63 (22.0)
Oligoarticular extended JIA, n (%)	48 (16.7)
Enthesitis-related JIA, n (%)	11 (3.8)
Psoriatic JIA, n (%)	11 (3.8)
Male sex, n (%)	104 (36.2)
Age, yrs, median (range)	9.0 (1.4–18.8)
Disease duration at MTX start, yrs, median (range)	1.4 (0.0–15.6)
PGA, median (range)	3.4 (0.0–10.0)
Joints with limited motion, median (range)	2 (0–26)
Joints with active arthritis, median (range)	3 (0–30)
C-HAQ disability, mean (SD)*	1.1 (0.7)
C-HAQ well-being, cm, mean (SD)*	4.3 (2.7)
ESR, mm/h, median (range)	24 (1–140)
RF seropositivity, n (%)**	23 (8.0)
MTX dose at start (mg/m^2/wk), median (range)	9.6 (2.8–25.0)
NSAID, n (%)	250 (87.1)
Sulfasalazine, n (%)	8 (2.8)
Oral steroids, n (%)	43 (15.0)
Intraarticular steroids, n (%)	41 (14.3)

* C-HAQ was assessed for 280 patients, included after 1994, when the C-HAQ was introduced in our clinic. ** RF was assessed for 234 patients. PGA: physician global assessment of disease activity; C-HAQ: Child Health Assessment Questionnaire; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; NSAID: nonsteroidal antiinflammatory drugs.

not considered nonresponders. Despite the heterogeneity of the study population, we did observe equal MTX response rates among different JIA subtypes.

SNP analysis. Only the *ABCC2* rs717620 SNP deviated from HWE (p = 0.038). However, this SNP had a low number of homozygous variants (5 patients). This could have contributed to the HWE p value < 0.05. We decided to keep this SNP in the analysis. Failure for genotyping was between 0 and 6% per SNP. Allele frequencies for *ABCC3* rs4793665, *ABCC3* rs3785911, *ABCC4* rs868853, and *ABCC4* rs2274407 were not confirmed in the Hapmap/NCBI database and therefore a sequencing analysis was performed. For all 4 SNP investigated, the sequencing analysis confirmed the expected SNP. There were < 5% discrepancies between duplicate runs.

Of the 21 genotyped SNP, statistical analyses, for the univariate association between genotype and MTX response in JIA, could be performed on 17 SNP (Table 2). For the other 4 SNP investigated, there was insufficient distribution of data for statistical analysis (not at least 1 responder and 1 nonresponder for each genotype on every visit). A p value < 0.2 for ACRped70 after univariate analysis was observed for the following 6 SNP: *ABCB1* rs1045642 (p = 0.002), *ABCC1* rs35592 (p = 0.045), *ABCC3* rs4793665 (p = 0.005), *ABCG2* rs13120400 (p = 0.036), *FPGS* rs4451422 (p = 0.087), and *SLC19A1* rs1051266 (p = 0.054). These SNP were entered together in a multivariate model and were corrected for the clinical covariates disease duration prior to the start of MTX treatment, PGA at baseline, and dose of MTX (Figure 2). Three of these 6 investigated SNP remained significant (p < 0.05) in this multivariate analysis. *ABCB1* rs1045642 showed a 3.80 higher OR (95% CI 1.70–8.47, p = 0.001), and *ABCC3* rs4793665 a 3.10 higher OR (95% CI 1.49–6.41, p = 0.002) to achieve an ACRped70 response in the first year after start of MTX therapy, whereas *SLC19A1* rs1051266 showed a 0.25 lower OR (95% CI 0.09–0.72, p = 0.011) to achieve the ACRped70 response.

To address the issue of subtype heterogeneity, we investigated whether the effect sizes of the significant SNP remained the same in the oligoarticular and polyarticular JIA subtypes only. We found similar effects sizes as those reported for MTX response in the entire JIA cohort, namely *ABCB1* rs1045642, OR 4.07 (95% CI 1.40–11.90, p = 0.010), *ABCC3* rs4793665, OR 2.78 (95% CI 1.07–7.19, p = 0.036), and *SLC19A1* rs1051266, OR 0.09 (95% CI 0.01–0.65, p = 0.017). There were no differences in the frequency of ACRped70 responses in patients taking oral MTX and patients receiving parenteral MTX. We also checked the prevalence of SNP between routes of administration. The MAF for the patients taking oral MTX at baseline (n = 270) were comparable with the MAF for the patients receiving parenteral MTX at baseline (n = 17).

Table 3 shows the reconstructed haplotypes. None of the haplotypes remained significant after multivariate analysis.

DISCUSSION

In our longitudinal study, we identified 2 SNP that were potentially associated with a positive MTX response and 1 SNP associated with a negative MTX response in patients with JIA. The presence of *ABCB1* rs1045642 or *ABCC3* rs4793665 variant genotypes increased the likelihood of becoming an MTX responder 2–3-fold. For *SLC19A1* rs1051266, the likelihood decreased 2–3-fold. For children who failed to respond to MTX, the delay in finding the appropriate treatment may be crucial for their disease outcome, with the risk of joint damage and potentially permanent disability[34]. Therefore, identifying determinants of MTX response would be a major development in JIA therapy.

The SNP in the *ABCC1*, *ABCC2*, *ABCC5*, *ABCG2*, *FPGS*, *FOLR1*, *FOLR2*, *GGH*, and *SLC46A1* genes were not associated with response to MTX in our study. In a recent study[13], a total of 14 genes in the MTX pathway in relation to MTX response were investigated in a cross-sectional JIA cohort and replication cohort. Similarly to our study, the authors did not find a significant association for SNP in the genes *FPGS* and *GGH* with response to MTX. Another recent cross-sectional study in 92 Japanese patients with JIA also showed no evidence for a relation between SNP in *FPGS* and *GGH* and response to MTX[14].

To our knowledge, our longitudinal study is the first to

Table 2. Genes and 21 single-nucleotide polymorphisms (SNP) within cellular methotrexate (MTX) transport routes and polyglutamylation in relation to response (ACRped70) over the first year of MTX therapy in 287 patients with juvenile idiopathic arthritis. Analyses were performed according to a recessive inheritance model.

SNP	GC (%)	HWE p	WT/het/var	MAF	Genotype Frequency (WT/het/var)	OR (95% CI) Univariate	p	Study
ABCB1 rs1128503	7.5	0.560	GG/GA/AA	0.40	0.38/0.46/0.16	1.85 (0.64–5.35)	0.254	22
ABCB1 rs2032582	7.5	0.682	CC/CA/CT/TA/AA	0.38/0.02	0.36/0.47/0.01/0.02/0.13	1.86 (0.63–5.52)	0.263	22
ABCB1 rs1045642	7.5	0.060	GG/GA/AA	0.48	0.29/0.45/0.26	3.72 (1.62–8.55)	0.002	23
ABCC1 rs35592	2.8	0.175	TT/TC/CC	0.23	0.61/0.32/0.07	4.93 (1.04–23.26)	0.045	24
ABCC1 rs3784862	2.8	0.260	AA/AG/GG	0.28	0.53/0.38/0.09	1.49 (0.49–4.50)	0.482	24
ABCC2 rs4148396	6.3	0.329	CC/CT/TT	0.36	0.39/0.49/0.12	1.02 (0.34–3.03)	0.973	24
ABCC2 rs717620	6.3	0.038*	CC/CT/TT	0.19	0.63/0.35/0.02	2.87 (0.14–58.82)	0.493	24
ABCC3 rs4793665	3.8	0.347	CC/CT/TT	0.57	0.17/0.52/0.31	2.99 (1.39–6.41)	0.005	25
ABCC3 rs3785911	3.8	0.298	AA/AC/CC	0.32	0.48/0.41/0.11	1.09 (0.35–3.40)	0.879	†
ABCC4 rs868853	0.5	0.638	TT/TC/CC	0.07	0.86/0.14/0.00	**		26
ABCC4 rs2274407	0.5	0.243	CC/CA/AA	0.06	0.87/0.13/0.00	**		26
ABCC5 rs2139560	17.5	0.092	GG/GA/AA	0.40	0.34/0.53/0.13	0.51 (0.18–1.46)	0.208	‡
ABCG2 rs13120400	9.1	0.622	TT/TC/CC	0.27	0.53/0.39/0.08	0.17 (0.03–0.89)	0.036	24
ABCG2 rs2231142	9.1	0.351	GG/GT/TT	0.11	0.80/0.18/0.02	**		27
FPGS rs4451422	16.2	0.568	AA/AC/CC	0.40	0.37/0.46/0.17	2.14 (0.90–5.13)	0.087	‡
FOLR1 rs11235462	5.6	0.925	TT/TA/AA	0.16	0.71/0.27/0.02	**		†
FOLR2 rs514933	7.1	0.514	TT/TC/CC	0.35	0.44/0.43/0.13	0.59 (0.20–1.73)	0.338	28
GGH rs10106587	14.9	0.992	AA/AC/CC	0.29	0.50/0.42/0.08	2.28 (0.54–9.62)	0.260	‡
GGH rs3758149	14.9	0.921	GG/GA/AA	0.30	0.49/0.42/0.09	0.69 (0.19–2.47)	0.563	24
SLC46A1 rs2239907	48.4	0.643	CC/CT/TT	0.44	0.32/0.48/0.20	1.05 (0.42–2.63)	0.914	29
SLC19A1 rs1051266	57.3	0.839	CC/CT/TT	0.37	0.39/0.47/0.14	0.34 (0.11–1.02)	0.054	30

* *ABCC2* rs717620 had a low number of homozygous variants (5 patients). As this could have contributed to the HWE p value < 0.05, we kept this SNP in the analysis. ** Insufficient distribution of data for statistical analysis (not at least 1 responder and 1 nonresponder for each genotype on every visit). † No tagging SNP were available and an SNP with minor allele frequency > 0.10 was chosen. ‡ Tagging SNP were selected by Hapmap database and Haploview. ACRped70: American College of Rheumatology 70% pediatric criteria; GC: gene coverage; HWE: Hardy-Weinberg equilibrium; WT: wild type; het: heterozygous; var: variant; *ABCB1*/*ABCC1*/*ABCC2*/*ABCC3*/*ABCC4*/*ABCC5*/*ABCG2:* adenosine triphosphate-binding cassette transporter subfamily B/C/G member 1/2/3/4/; *FPGS:* folylpolyglutamate synthetase; *FOLR1*/*FOLR2:* folate receptor 1/2; *GGH:* gamma glutamyl hydrolase; *SLC 46A1*/*SLC19A:* solute carrier 46A1/19A1; rs: reference SNP number.

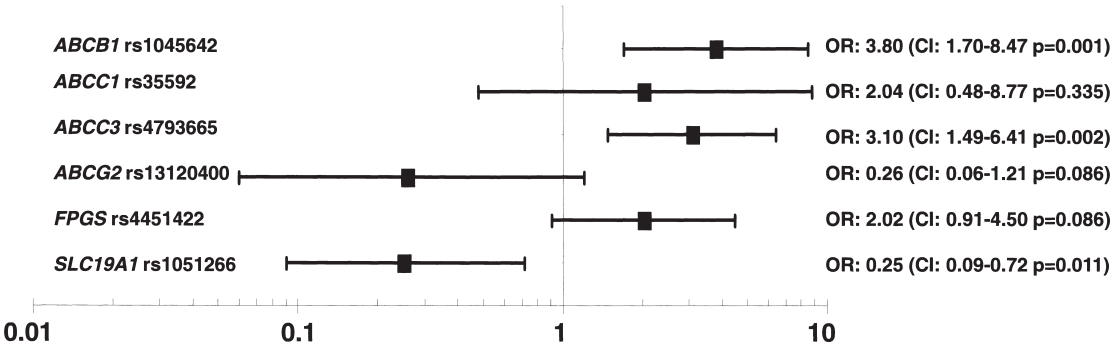

Figure 2. Multivariate analysis of relation between *ABCB1* rs1045642, *ABCC1* rs35592, *ABCC3* rs4793665, *ABCG2* rs13120400, *FPGS* rs4451422, and *SLC19A1* rs1051266, and American College of Rheumatology 70% pediatric criteria with OR, 95% CI, and p values. Covariates in multivariate analysis: disease duration prior to start of MTX treatment, physician's global assessment of disease activity at baseline, and MTX dose. *ABCB1*, *ABCC1, ABCC3*, *ABCG2*: adenosine triphosphate-binding cassette transporter subfamily B/C/G member 1/2/3; *FPGS*: folylpolyglutamate synthetase; *SLC46A1*/*19A1*: solute carrier 46A1/19A1.

evaluate *ABCB1* and *ABCC3* gene polymorphisms with response to MTX in patients with JIA. Previous studies in adult patients with RA reported a positive association[35,36], a negative association[37], and no statistically significant association[38,39,40] between *ABCB1* polymorphisms and response to MTX. *ABCB1* belongs to the efflux transporters of the *ABC* superfamily, subfamily B, and was formerly referred to as multidrug resistance 1 gene. The product of the *ABCB1* gene is P-gp[4]. Although the *ABCB1* rs1045642 polymorphism is synonymous (i.e., not leading to amino acid

Table 3. Haplotypes of SNP in genes within cellular MTX transport routes and polyglutamylation in relation to response (ACRped70) over the first year of MTX therapy in JIA. Haplotype analysis was performed according to a recessive inheritance model and therefore only homozygous haplotypes were analyzed.

Gene	rs Numbers	Haplotypes	Frequency	OR (95% CI) Univariate	p Univariate
ABCB1	rs1128503/rs32032582/rs1045642	GCA	0.10	*	
ABCB1	rs1128503/rs32032582/rs1045642	AAA	0.37	2.44 (0.80–7.46)	0.117
ABCB1	rs1128503/rs32032582/rs1045642	GCG	0.46	0.37 (0.15–0.89)	0.026
ABCC2	rs4148396/rs717620	TC	0.17	1.23 (0.24–6.29)	0.806
ABCC2	rs4148396/rs717620	TT	0.19	2.87 (0.14–58.82)	0.493
ABCC2	rs13120400/rs2231142	CC	0.63	1.08 (0.51–2.30)	0.837
ABCG2	rs13120400/rs2231142	TT	0.11	*	
ABCG2	rs13120400/rs2231142	CG	0.27	0.17 (0.03–0.89)	0.036
ABCG2	rs13120400/rs2231142	TG	0.62	2.10 (1.00–4.39)	0.049
GGH	rs10106587/rs3758149	AA	0.29	1.11 (0.31–3.91)	0.875
GGH	rs10106587/rs3758149	CG	0.30	1.29 (0.36–4.57)	0.692
GGH	rs10106587/rs3758149	AG	0.41	0.74 (0.29–1.92)	0.541
Gene	rs Numbers	Haplotypes	Frequency	OR (95% CI) Multivariate	p Multivariate
ABCB1	rs1128503/rs32032582/rs1045642	AAA	0.37	3.01 (0.72–5.65)	0.184
ABCB1	rs1128503/rs32032582/rs1045642	GCG	0.46	0.48 (0.21–1.10)	0.081
ABCG2	rs13120400/rs2231142	CG	0.27	0.26 (0.05–1.42)	0.120
ABCG2	rs13120400/rs2231142	TG	0.62	1.69 (0.83–3.43)	0.149

* Insufficient distribution of data for statistical analysis (not at least 1 responder and 1 nonresponder for each haplotype on every visit). MTX: methotrexate; SNP: single-nucleotide polymorphism; ACRped70: American College of Rheumatology 70% pediatric criteria; *ABCB1/ABCC2/ABCG2:* adenosine triphosphate-binding cassette transporter subfamily B/C/G member 1/2; *GGH:* gamma glutamyl hydrolase.

exchange), it is associated with altered P-gp expression and reduced P-gp function[41]. Early *in vitro* experiments in cell lines with high levels of MTX resistance suggested that P-gp could transport MTX[42,43]. From this perspective, the *ABCB1* rs1045642 polymorphism may result in impaired cellular efflux of MTX in heterozygous and homozygous variants, with concomitant increased intracellular MTX levels and increased MTX efficacy. However, recent research showed that MTX is unlikely to be a substrate of P-gp[44,45]. P-gp is expressed as a cell membrane-associated protein in natural killer cells, CD4 and CD8 lymphocytes, and bone marrow progenitor cells[46] and plays a role in the transport of some inflammatory mediators, in particular bioactive lipids[47]. This could explain why *ABCB1* gene polymorphisms have been associated with increased response to MTX in adult RA[35,36] and in JIA in our study; if the *ABCB1* rs1045642 polymorphism is associated with a diminished extrusion of inflammatory mediators, it could facilitate a better therapeutic effect of MTX. Collectively, changes in the physiological function of P-gp could provide an alternative explanation for the association between the *ABCB1* rs1045642 polymorphism and MTX response.

ABCC3 is involved in the efflux of MTX[4,48]. The rs4793665 SNP is located in the 5'-promoter region of the *ABCC3* gene and was associated with significantly lower *ABCC3* transcript levels and a trend toward lower protein expression in human liver, and it could affect the binding of nuclear proteins to the *ABCC3* promoter[49]. Less expression of *ABCC3* transporter could have a positive effect on the cellular retention of MTX, leading to higher intracellular levels (Figure 1). This could explain our finding that the rs4793665 SNP was associated with response to MTX. However, others have shown that this polymorphism determined neither the expression of the *ABCC3* gene nor the response to MTX therapy in acute leukemia[50]. Nevertheless, the treatment dosage is much lower in the JIA context, and thus these studies are not comparable. We expect that SNP in efflux transporters have a greater influence on low-dose MTX therapy.

The membrane transporter *SLC19A1* is involved in the influx of MTX. Previously, we associated *SLC19A1* rs1051266 with an increased risk of pediatric acute lymphoblastic leukemia and elucidated the effects of this carrier on MTX metabolism[30]. SNP in *SLC19A1* have been associated with response to MTX in RA[8] but not in JIA[13]. The association between *SLC19A1* rs1051266 ($p = 0.011$) and MTX response was not significant after Bonferroni adjustments (significant p value = 0.05/17 SNP tested = 0.003); hence this finding should be judged with some skepticism. Therefore, the *SLC19A1* rs1051266 needs to be replicated in larger JIA cohort studies.

Haplotype analysis revealed no associations between haplotypes and MTX response in JIA. Therefore, our results suggest that testing of the 3 *ABCB1* SNP has no additional

value, and that determination of the rs1045642 SNP alone may suffice.

Some limitations of our study should be considered. Because of the large number of SNP tested, the observed positive associations may be spurious. However, when we analyzed all SNP in relation to ACRped50, similar results were obtained. Multivariate analysis yielded OR of 3.18 (95% CI 1.41–7.19, p = 0.006), 3.47 (95% CI 1.66–7.25, p = 0.001), and 0.34 (95% CI 0.12–0.95, p = 0.040) to be an ACRped50 responder for *ABCB1* rs1045642, *ABCC3* rs4793665, and *SLC19A1* rs1051266, respectively. In addition, we alternatively defined MTX responders as patients with an ACRped70 at 2 or more consecutive visits. Ordinary logistic regression analysis on this alternative outcome measure for MTX response yielded results comparable to those of the repeated measures analysis using generalized linear mixed modeling: *ABCB1* rs1045642, OR 2.46 (95% CI 1.39–4.34, p = 0.002), *ABCC3* rs3785911, OR 1.86 (95% CI 1.07–3.22, p = 0.003), and the *SLC19A1* rs1051266, OR 0.38 (95% CI 0.14–1.01, p = 0.053). Further, if Bonferroni adjustments for multiple comparisons were applied (significant p value = 0.05/17 SNP tested = 0.003), *ABCB1* rs1045642 (p = 0.001) and the *ABCC3* rs4793665 (p = 0.002) SNP remained significant with MTX response.

Our findings can only be interpreted as associations, because the selected SNP may be in linkage disequilibrium with the true causal variant. For the other genes investigated in our study, gene coverage (Table 2) was not high enough (0.5%–57.3%) to conclude that there is no association between these genes and response to MTX, because not all the genetic variation within these genes was covered with our analysis. We are aware of the relatively small sample size (n = 287) of our cohort. This may have caused overestimation of OR[51]. Therefore, this study should be replicated in a cohort with a larger sample size. Finally, our study lacks an independent validation cohort and so our results should be replicated. For that, multicenter studies with large patient numbers are needed, which for rare diseases such as JIA can be difficult. Therefore, an international collaboration is warranted to pool clinical data for analysis of gene associations and to validate the observed associations.

Unlike other studies that examined the associations of SNP within genes in the MTX metabolic pathway with MTX response in JIA[11,12,13,15], we analyzed our data longitudinally. A study in patients with RA revealed that multiple measurements per patient with the same number of patients reduces the between-subject variability and will increase power[52]. In addition, we showed earlier that response to MTX in JIA can fluctuate over time and thus should be analyzed in a longitudinal way[53]. For this reason we did not apply a multifactor dimensionality reduction (MDR) analysis on our data. Recently, other authors[15,54] have introduced MDR into the field of predicting MTX response in arthritis. This is an elegant method to reveal interactions between covariates on an outcome in a cohort. However, for MDR analysis, our longitudinal MTX response data have to be transformed into 1 binary variable, missing cases have to be removed, and continuous data have to be stratified. This would mean a loss of most of the benefits of longitudinal analysis[52,53]. Instead, we chose to analyze our data with a generalized linear mixed model to make use of the longitudinal character of our data. Nonetheless, MDR identified identical SNP significantly associated with MTX response compared to the general linear mixed model.

Our longitudinal study is the first, to our knowledge, to associate *ABCB1* and *ABCC3* gene polymorphisms with response to MTX in patients with JIA. *ABCB1* rs1045642, *ABCC3* rs4793665, and *SLC19A1* rs1051266 are possibly associated with improved MTX response according to ACRped70 criteria. These polymorphisms may be used to optimize the treatment of patients with JIA.

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