677TT Genotype Is Associated with Elevated Risk of Methotrexate (MTX) Toxicity in Juvenile Idiopathic Arthritis: Treatment Outcome, Erythrocyte Concentrations of MTX and Folates, and MTHFR Polymorphisms

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ABSTRACT. ***Objective.*** To investigate whether methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphisms and erythrocyte concentration of methotrexate (EMTX) could serve as predictors of methotrexate (MTX) efficacy and toxicity in patients with juvenile idiopathic arthritis (JIA).

Methods. Genetic analyses and EMTX and folate assessment were performed in 69 patients with JIA aged 2.5–19.6 years (30 male) treated with MTX using a dose-escalation protocol and classified as full responders (disease inactivity; n = 51) or nonresponders (< 30% improvement in pediatric American College of Rheumatology-30 criteria while receiving ≥ 15 mg/m^2/week parenteral MTX for at least 3 months; n = 18).

Results. Nonresponders were treated with the higher median MTX dose (17.2 vs 12.6 mg/m^2/week; $p < 0.0001$) and accumulated more EMTX (217 vs 106 nmol/l; $p < 0.02$) and erythrocyte folates (763 vs 592 nmol/l; $p = 0.052$) than responders. Analysis of MTHFR allele and genotype frequencies in relation to response failed to detect association. The frequency of any adverse effect was 29.4% in responders and 33.3% in nonresponders ($p = 0.77$). The frequency of 677T allele was elevated in patients with adverse effects (52.4% vs 20.9%; OR 3.88, 95% CI 1.8–8.6, $p < 0.002$). The probability of any adverse effect was significantly higher in patients with 677TT compared to the 677CC genotype (OR 55.5, 95% CI 2.9–1080, $p < 0.001$).

Conclusion. MTHFR genotyping may have a predictive value for the risk of MTX-associated toxicity in patients with JIA. Despite the lack of therapeutic effect, nonresponders accumulated adequate concentrations of EMTX. (J Rheumatol First Release July 1 2010; doi:10.3899/jrheum.091427)

Key Indexing Terms:
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Over the last 2 decades low-dose weekly methotrexate (MTX) has been commonly used in the treatment of juvenile idiopathic arthritis (JIA)[1]. Although its efficacy and safety in children and adolescents with JIA have been documented in multiple clinical trials[2,3], interpatient variability of efficacy and variety of side effects remain a clinical concern. About 10% of children fail to improve while receiving MTX[4] and 10% to 76% exhibit some common side effects[5]. Dose and route of administration need to be tailored individually in order to achieve early and sustained therapeutic effect[4,6].

Plasma concentration of the parent drug is not useful for MTX therapeutic monitoring due to its short plasma half-life. It has been postulated that long-acting polyglutamylated intracellular MTX metabolites (EMTX) mediate most of the antiinflammatory effects of MTX. EMTX may reflect its pharmacokinetic variability as well as bioavailability[7,8].

A growing body of evidence is now available to support an important contribution of various genetic polymorphisms in MTX xenobiotic metabolic pathways to interpatient variability in therapeutic response as well as toxicity. Methylene tetrahydrofolate reductase (MTHFR) is associated with regeneration of reduced folates. It mediates synthesis of 5-methyltetrahydrofolate, the carbon donor required for methionine synthesis. Two relatively common single-nucleotide polymorphisms (SNP), C677T and A1298C, have been studied in the MTHFR gene[9,10,11,12,13,14,15,16,17,18]. Several studies in adults with rheumatoid arthritis (RA) treated with MTX investigated associations between C677T and A1298C polymorphisms and clinical variables of disease outcome and/or toxicity, with inconsistent results[9,11,12,13,14,16,17,18,19]. Studies evaluating a possible role of EMTX for therapeutic monitoring have been inconclusive[7,8,20].

We aimed to combine assessment of EMTX and MTHFR polymorphisms in JIA patients with clearly defined response status in order to evaluate their possible predictive value for the efficacy and toxicity of MTX treatment.

MATERIALS AND METHODS

Patients and study protocol. Patients were recruited prospectively from the pediatric rheumatology outpatient clinic population of the Department of Paediatrics and Adolescent Medicine, 1st Medical School, Charles University in Prague, between 2005 and 2008. The study was approved by the local research ethics committee and informed consent was obtained from the patients and/or their legal guardians.

To be eligible patients must have had a definitive diagnosis of JIA according to the International League of Associations for Rheumatology criteria[21,22,23] and documented disease activity with MTX therapy for at least 3 months prior to recruitment. Only patients within extreme ends of the response spectrum (full responders and nonresponders) were enrolled.

For the purpose of this study, responders were defined as having achieved disease inactivity on medication with MTX monotherapy according to Wallace, *et al*[24]. Criteria for inactive disease included no active arthritis; no fever, rash, serositis, splenomegaly, or generalized lymphadenopathy attributable to JIA; no active uveitis; normal erythrocyte sedimentation rate (ESR) or CRP; and physician's global assessment of disease activity indicating clinical disease quiescence[24].

Using the American College of Rheumatology (ACR) Pediatric 30 (ACR Pedi30) definition of improvement[24,25,26,27], therapeutic efficacy was assessed monthly during the dose escalation and at 3-monthly intervals while patients received a stable dose. In nonresponders, at least 3 of any 6 JIA core set variables did not improve by a minimum of 30% and no more than 1 of the remaining variables improved by more than 30%[24]. Core set outcome variables included number of joints with active arthritis, joints with limited range of motion, physician's global assessment of disease activity [10 cm visual analog scale (VAS)], parent's global assessment of the child's overall well-being (10 cm VAS), disability index of the Childhood Health Assessment Questionnaire (CHAQ), and ESR[28,29,30]. To qualify as a nonresponder, the patient must have been treated with a minimum of 15 mg/m^2/week MTX subcutaneously for at least 3 months[4].

Before entering the study, patients were treated with MTX using a standard dose-escalation department protocol. Over the first 3–6 months, initial weekly MTX dose of 7.5–10 mg/m^2 orally was titrated according to efficacy and toxicity evaluations up to the weekly dose of around 15 mg/m^2 (maximum 20–25 mg). Patients with persistent disease activity taking oral MTX and requiring more than 10 mg/m^2 were switched to subcutaneous administration[6]. MTX injections were chosen as an initial treatment modality in polyarthritis patients with high disease activity and in small children (usually below 4 years of age). In addition to MTX, most patients received once-weekly folic acid (5–10 mg/week, 24–48 hours after MTX), and were allowed to take one nonsteroidal antiinflammatory drug, usually ibuprofen.

Toxicity was prospectively monitored at each visit by routine questioning and laboratory tests (full blood count, liver function tests). MTX toxic effects of any grade were defined as those related to the gastrointestinal tract (stomatitis, nausea, vomiting, abdominal pain/discomfort), liver function (alanine and/or aspartate aminotransferase equal to or above twice the upper normal limit), bone marrow suppression (any cytopenia), and other (alopecia, behavioral changes, headache, nodulosis).

MTX concentration in erythrocytes. In patients who had been treated a minimum of 3 months and received a stable MTX dose for at least 8 weeks, venous blood samples were drawn just before weekly administration of MTX. Samples were collected into standard EDTA-coated tubes and processed within 1 hour. Erythrocytes were separated, washed in ice-cold 0.9% NaCl, and hemolyzed as described[31]. Hematocrit of the suspension of washed erythrocytes was measured with a hematology analyzer (Celltac E; Nihon Kohden, Tokyo, Japan) using the calculation: EMTX = (MTX concentration in the hemolysate)/hematocrit. Plasma samples and erythrocyte suspensions were stored no longer than 1 month at –20°C before analysis. Plasma MTX and EMTX were determined by high-performance liquid chromatography methods using fluorimetric detection after post-column derivatization in a photochemical reactor as described[32,33]. During sample preparation, all MTX polyglutamates were hydrolyzed to MTX. Thus, EMTX concentration represented the sum of all polyglutamates in the erythrocyte.

Folate concentration in erythrocytes. Erythrocyte concentration of folates was measured on the Elecsys analyzer using an automated electrochemiluminescence immunoassay (ECLIA), including RBC Folate Hemolyzing Reagent and Elecsys Folate II kit (Roche, Prague, Czech Republic).

Genotype analysis. Genomic DNA was extracted from white blood cells. The A1298C and C677T polymorphisms of the MTHFR gene were analyzed by polymerase chain reaction restriction fragment length polymorphism method as described[34,35]. The rheumatologists evaluating the efficacy and safety of MTX (PD, DN, JH) were blinded to the results of genotyping and EMTX and folate analysis.

Statistical analysis. Differences between responders and nonresponders were assessed using Mann-Whitney U test or chi-square test. Allele and genotype frequencies were compared by 2-sided Fisher exact test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated for the chance of response and the risk of overall adverse effects of MTX therapy. Univariate and multivariate logistic regression models were used to analyze the influence of 677C>T and 1298A>C polymorphisms on the frequencies of response and adverse effects. In these models, the numbers of 677T and 1298C alleles (0, 1, or 2) for each patient served as independent variables. A p value < 0.05 was considered statistically significant. Calculations were performed using Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA).

RESULTS

Stratification of patients according to treatment response. A total of 69 Caucasian children were enrolled. Based on the treatment efficacy assessment, 51 patients (74%) were classified as full responders and 18 (26%) as nonresponders. Their demographic and disease characteristics were similar (Table 1). Only 4 patients with persistent oligoarthritis had a history of chronic uveitis (all responders).

In nonresponders, disease activity persisted despite treatment with subcutaneous MTX at a 37% higher dose than in responders ($p < 0.0001$), who received the drug orally (n =

Table 1. Patient and treatment characteristics.

Characteristic	Response to MTX Therapy		p
	Responders	Nonresponders	
No.	51	18	—
ILAR subtype*, n	26, 15, 7, 1, 1, 1	13, 1, 2, 2, 0, 0	
Male/female	20/31	10/8	0.28
Age, yrs**	8.2 (2.8–19.6)	9.8 (2.5–17.1)	0.54
Body surface area, m^2**	0.96 (0.57–1.93)	1.06 (0.57–1.79)	0.55
Disease duration†, yrs**	2.2 (0.5–17.0)	1.9 (0.7–4.2)	0.31
MTX therapy duration†, yrs**	1.4 (0.3–11.5)	1.3 (0.5–4.1)	0.93
ESR††, mm/h**	37 (3–116)	28 (2–140)	0.034
Active joints††, n**	6 (2–40)	6 (2–59)	0.87
Joints with limited motion††, n**	6 (2–40)	7 (2–59)	0.63
Route of MTX administration† (oral/subcutaneous)	24/27	0/18	< 0.0001
Weekly MTX dose†, mg/m^2**	12.6 (7.4–20.0)	17.2 (11.8–24.2)	< 0.0001
Folic acid supplementation, n (%)	44 (82.3)	17 (94.4)	0.67
Weekly dose of folic acid, mg**	10 (5–10)	10 (10–10)	0.12

† At the time of sampling. †† Before initiation of MTX. * Polyarticular, oligoarticular persistent, oligoarticular extended, systemic, enthesopathic, psoriatic. ** Median (range).

24) or subcutaneously (n = 27). The majority of patients from both groups took folic acid and its weekly dose did not differ between the groups (Table 1). Only 12 patients (all nonresponders) received other medication at the time of the study (corticosteroids n = 11, sulfasalazine n = 1, etanercept n = 1, cyclosporine n = 1).

MTX toxicity. Mild to moderate toxicity was noted in a total of 21 patients (30.4%). Gastrointestinal complaints (mucosal, nausea, vomiting, abdominal pain) were recorded in 16, hepatopathy in 3, and alopecia in 2 patients. Other adverse effects (bone marrow suppression, behavioral changes, nodulosis) were not seen. The frequency of overall adverse effects was 29.4% in responders (15/51) and 33.3% in nonresponders (6/18) (p = 0.77).

EMTX and folate concentration in erythrocytes. Measurements of EMTX/erythrocyte folate were available for 51/40 responders and 13/14 nonresponders. The median EMTX concentrations in nonresponders [217 (interquartile range, IQR, 91.4–354) nmol/l] were 2-fold higher than those in responders [106 (IQR 65.3–168) nmol/l] (p < 0.02; Figure 1A). Nonresponders tended to have higher concentrations of erythrocyte folates compared to responders [763 (IQR 583–935) nmol/l vs 592 (487–751) nmol/l, respectively; p = 0.052; Figure 1B].

Association of MTX efficacy/toxicity and MTHFR polymorphisms. Results of the MTHFR SNP analysis were available in all 18 nonresponders and in 46/51 (90.2%) responders. Allele and genotype frequencies are summarized in Table 2.

Full clinical response was achieved in 4/8 (50%) carriers of the 677TT compared to 19/25 (76%) carriers of 677CT and to 23/31 (74.2%) carriers of 677CC genotype. Patients with 677TT genotype had 2.9-times lower probability of response in comparison to patients with the reference genotype 677CC. However, this difference was not significant (OR 0.35, 95% CI 0.07–1.73, p = 0.22). The frequency of T allele among MTX responders was 29.3% and among nonresponders 38.9% (OR 0.65, 95% CI 0.29–1.46, p = 0.30; Table 2). Full response was achieved in the following numbers of polymorphic allele carriers: 4/6 (66.7%) with 1298CC, 22/29 (75.9%) with 1298AC, and 20/29 (70%) with 1298AA genotypes. The probability of response did not differ among different genotypes (Table 2). The frequency of C allele among MTX responders and nonresponders was 30.4% and 30.6%, respectively (OR 0.99, 95% CI 0.43–2.30, p = 0.99; Table 2). In univariate and multivariate regression analyses, the presence of neither 677T nor 1298C allele was associated with an altered response rate (data not shown).

Adverse effects (any type) occurred in all 8 carriers of the homozygous 677TT genotype (100%), in 6/25 (24%) carriers of 677CT, and in 7/31 (22.6%) carriers of 677CC. All 677TT homozygotes had combined genotype 677TT/1298AA. The probability of any adverse effect was significantly and markedly elevated in patients with 677TT genotype, compared to the reference genotype 677CC (OR 55.5, 95% CI 2.9–1080, p < 0.001). Frequency of T allele was significantly higher in patients with adverse effects than in those without (52.4% vs 22.1%, respectively; OR 3.88, 95% CI 1.8–8.6, p < 0.002; Table 2). Using this unadjusted OR, the false-positive report probability of detecting a conservative OR of 2 was 13%, 62%, and 94%, respectively, for the high (0.1), moderate (0.01), and low (0.001) prior probabilities of true association. In contrast to the MTHFR C677T genotype, association between the A1298C genotype and adverse effects was much weaker. Adverse effects were detected in 2/6 (33.3%) of 1298CC, in 6/29 (20.7%) of 1298AC, and in 13/29 (44.8%) of 1298AA genotype carri-

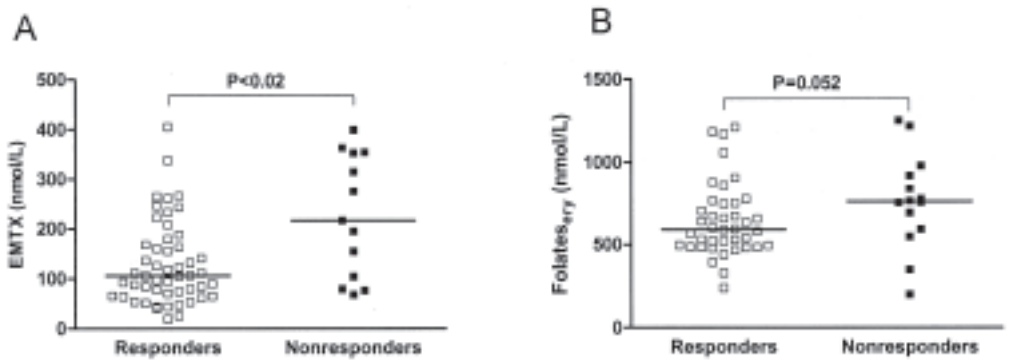

Figure 1. EMTX (A) and erythrocyte folates (B) in patients with JIA according to the therapy response. Horizontal lines are medians.

Table 2. The MTHFR polymorphisms and genotypes in relation to patients' responses to MTX and occurrence of side effects.

MTHFR Polymorphism	All Patients, n (%)	Response to MTX Therapy		OR (95% CI)	Overall Side Effects,		OR (95% CI)
		Responders, n (%)	Nonresponders, n (%)		Yes, n (%)	No, n (%)	
677C>T alleles							
T	41 (32.0)	27 (29.3)	14 (38.9)	0.65 (0.29–1.46)	22 (52.4)	19 (22.1)	3.88* (1.8–8.6)
C	87 (68.0)	65 (70.7)	22 (61.1)	—	20 (47.6)	67 (77.9)	—
1298A>C alleles							
C	39 (30.4)	28 (30.4)	11 (30.6)	0.99 (0.43–2.30)	9 (21.4)	30 (34.9)	0.51 (0.22–1.20)
A	89 (69.6)	64 (69.6)	25 (69.4)	—	33 (78.6)	56 (65.1)	—
677C>T genotype							
TT	8 (12.5)	4 (8.7)	4 (22.2)	0.35 (0.07–1.73)	8 (38.1)	0 (0)	55.5** (2.9–1080)
CT	25 (39.1)	19 (41.3)	6 (33.3)	1.10 (0.32–3.73)	6 (28.6)	19 (44.2)	1.08 (0.31–3.76)
CC	31 (48.4)	23 (50.0)	8 (44.4)	—	7 (33.3)	24 (55.8)	—
1298A>C genotype							
CC	6 (9.4)	4 (8.7)	2 (11.1)	0.90 (0.14–5.85)	2 (9.5)	4 (9.3)	0.62 (0.097–3.9)
AC	29 (45.3)	22 (47.8)	7 (38.9)	1.41 (0.44–4.51)	6 (28.6)	23 (53.5)	0.32 (0.10–1.02)
AA	29 (45.3)	20 (43.5)	9 (50.0)	—	13 (61.9)	16 (37.2)	—

Fisher exact test: * $p < 0.002$, ** $p < 0.001$ compared with CC (or C) as reference genotype (allele).

ers. The probability of any adverse effect tended to be lower in homozygotes or heterozygotes for 1298C allele as compared to homozygous carriers of 1298A allele, but the differences did not reach statistical significance. The frequency of C allele among patients with and without adverse effects was 21.4% and 34.9%, respectively (OR 0.51, 95% CI 0.22–1.20, $p = 0.15$; Table 2). In both univariate (OR 1.46, 95% CI 1.15–1.74, $p < 0.005$) and multivariate (OR 1.46, 95% CI 1.10–1.96, $p < 0.01$) regression analyses, only MTHFR 677T allele was confirmed as a factor strongly associated with an increased frequency of overall adverse effects.

DISCUSSION

We investigated relationships between the 2 common SNP of the MTHFR gene, C677T and A1298C, and the efficacy and safety of MTX in children and adolescents with JIA. The ACR Pedi30 nonresponders and responders with inactive disease were selected among the MTX-treated patients. MTHFR SNP were detected and MTX and folate polyglutamates in erythrocytes were assayed after the patients had been treated at sufficient length with a stable, sufficiently high dose of MTX. To our knowledge, this is the first study in JIA children simultaneously evaluating the contribution of pharmacogenetic and metabolic markers to MTX efficacy and toxicity.

Significantly increased risk of overall MTX side effects was found in carriers of the 677T allele. Further, all homozygotes for this variant, which is associated with a decreased activity of MTHFR, experienced adverse effects, as compared to 22.6% of homozygotes for the wild-type 677C allele. This represents a 55-fold elevated risk for adverse effects in homozygotes for the mutated allele. Observed adverse effects included gastrointestinal complaints, elevation of aminotransferases, and alopecia. In addition, compared to the carriers of 677TT genotype, patients with the reference genotype 677CC had a 3-fold higher chance to achieve the full clinical response to MTX.

However, this difference was not statistically significant. Separate examination of the A1298C polymorphism in relation to the effects of MTX showed no association.

Evaluating possible false positivity of this finding due to the limited number of patients we applied the concept of false-positive report probability (FPRP), as suggested by Wacholder, *et al*[36] and introduced to the field of MTX genetic association studies by Lee and colleagues[37]. The high to moderate probability level set-up appears justified: in a metaanalysis of 8 studies in adults an increased risk of MTX toxicity was found in carriers of the MTHFR C677T SNP (OR 1.71, 95% CI 1.32–2.21)[38]. Our estimates of the FPRP are similar to those retrospectively determined by Dervieux[39] for the study of Wessels, *et al*[14], and lower compared to the studies of Dervieux, *et al*[10]. Therefore, our finding of association of the C677T SNP and adverse effects of MTX in patients with JIA is noteworthy, and in accord with conclusions of recent studies of different SNP in adults with RA treated with MTX[10,14,39].

One previous study in MTX-treated children with JIA investigated the influence of MTHFR SNP on treatment outcome and toxicity[40]. In that retrospective study, the probability of improvement was higher in carriers of the 1298C allele, whereas no association was found for the C677T SNP polymorphism. In agreement with our findings, heterozygotes for the 677T genotype exhibited adverse effects more frequently than homozygotes for the wild-type allele. Pharmacogenetic studies in adult patients with RA have provided inconsistent conclusions. This may be explained in part by different study design and outcome measures, coadministered drugs, patient ethnicity, etc. According to recent reviews[38,41], clinical data in adults support possible association between the C677T variant and increased MTX toxicity.

During treatment, pharmacologically active MTX polyglutamates with up to 5 glutamic acid residues accumulate in cells[42]. Polyglutamylation enhances intracellular retention of the drug and facilitates its affinity for several folate-dependent enzymes[43]. These metabolites have a longer half-life than MTX itself, enabling once weekly dosing. The steady-state EMTX level could be an indicator of longterm MTX exposure. It is influenced by several factors, including bioavailability of MTX, elimination kinetics, and patient compliance. Under well controlled conditions of prospective clinical studies, the steady-state concentration of EMTX correlated strongly with the area under the concentration-time curve (AUC) of plasma MTX, reflecting its bioavailability, as shown in patients with RA[7] and psoriasis[44]. Nevertheless, weak to moderate correlation of EMTX with MTX dose was found[45,46]. Circulating erythrocytes lack folylpolyglutamate synthetase and MTX polyglutamates are mainly formed in bone marrow progenitor cells. Therefore, EMTX may reflect MTX polyglutamate concentrations in immunocompetent cells, e.g., lymphocytes, and work as a bioindicator of the effect[47].

Most studies with adult RA patients suggest that higher EMTX concentration is associated with better response[7,8,10,11,48]. Hornung, *et al*[7] monitored clinical effect during the followup period (52 weeks) using preliminary ACR core criteria (no response, 20%, 50% improvement). Patients with RA classified as responders had a significantly higher mean steady-state EMTX, but significantly higher dose at the same time. Since the correlation between the dose and EMTX was found, the MTX dose might have affected EMTX concentration in nonresponders who were underdosed[7]. In adults with RA, 2- to 3-fold higher concentrations of EMTX were found in responders and partial responders as compared to nonresponders[8]. However, MTX was administered orally and mean doses used in all 3 groups were similar and low (≤ 11.2 mg/week). Nonresponders might have achieved response if treated parenterally with a higher MTX dose[8]. Dervieux, *et al* used a dose-escalation protocol in adults with RA receiving oral MTX, and found approximately 20% lower accumulation of EMTX in patients with the lower than median improvement in the Disease Activity Score-28 as compared to the better responders[10]. Moreover, 3 nonresponders had 33% lower EMTX concentrations compared to responders. In another study with adult patients, the probability of a good response was 20% to 30% lower in patients with EMTX level below 60 nmol/l[10,11,48].

Kristensen, *et al*[20] investigated the relationship between EMTX and clinical and laboratory measures in children with JIA. The study design did not allow a cross-sectional analysis of the relation between EMTX and disease measures, and patients served as their own controls. Within the 3-month interval, spontaneous fluctuation in disease activity did not reflect intraindividual changes in EMTX[20]. In our study, the dose-escalation protocol allowed switching from oral to subcutaneous administration, which resulted in an exclusive use of the subcutaneous route in nonresponders, who received 37% higher MTX dose than responders. Nonresponders also presented higher EMTX levels, suggesting that exposure to MTX was maximized and that the lack of response was associated with pharmacodynamic factors rather than pharmacokinetics. Our assay for EMTX did not allow us to separately quantify concentrations of individual polyglutamates. Therefore we were not able to evaluate the proportion of longer-chain polyglutamates that might better reflect efficacy of MTX, as shown in adult patients[10]. Moreover, route of administration contributed to the pattern of polyglutamation of MTX in children with JIA in a recent study[49]. A higher proportion of long-chain (3 to 5) polyglutamates was observed in patients treated subcutaneously, and, conversely, a higher proportion of short-chain (1 + 2) derivatives was found in patients treated orally[49].

Concentration of erythrocyte folates is one of the important factors in MTX therapeutic response. In our study, patients classified as nonresponders tended to have higher

concentrations of erythrocyte folates compared to full responders. Importantly, results of large cross-sectional studies in adults also established that increased erythrocyte folates were associated with high disease activity[10,50].

Evaluation of relationships between MTHFR gene SNP (C677T and A1298C) and MTX efficacy and safety in children with JIA under simultaneous control for both antifolate and folate status revealed that carriers of the 677T allele have a 3.9-fold increased risk for adverse effects of MTX. This elevation may be attributed to a 55-fold augmented risk in patients carrying 677TT, which almost exclusively combines with 1298AA genotype. Nonresponders to the dose-escalation protocol had EMTX concentrations twice as high as those of responders and a similar rate of adverse effects. We found no significant relationship between EMTX and treatment efficacy. Analysis of MTHFR allele and genotype frequencies in relation to response failed to detect any significant association. The significance of our results is limited by small patient numbers, thus estimation of MTHFR 677/1298 haplotype distributions and statistical evaluation of their influence could not be done. This area definitely deserves further investigation.

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