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The impact of C677T and A1298C MTHFR polymorphisms on methotrexate therapeutic response in East Bohemian region rheumatoid arthritis patients

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Abstract Some single-nucleotide polymorphisms (SNPs) might be predictive of methotrexate (MTX) therapeutic outcome in rheumatoid arthritis (RA). The aim of this study was to determine whether SNPs in the methylenetetrahydrofolate reductase (MTHFR) gene are predictive of MTX response. Comparison was made using EULAR response criteria and according to the change of DAS28 (ΔDAS28) after a 6-month MTX treatment in RA patient cohort. The two SNPs C677T (rs1801133) and A1298C (rs1801131) have been genotyped. A total of 120 patients were enrolled in the study, and all of them fulfilled the American College of Rheumatology 1987 RA criteria and are currently or previously taking MTX oral treatment, either as a monotherapy ($n = 65$) or in a combination with other disease-modifying antirheumatic drugs ($n = 55$). Genotyping was performed using qPCR allelic discrimination. We did not found any association of C677T and A1298C genotypes with MTX treatment inefficacy in dominant model (OR 1.23, 95 % CI 0.57–2.65, $P = 0.697$; and OR 0.98, 95 % CI 0.47–2.14, $P = 1.0$, respectively), or in recessive and codominant models. However, when ΔDAS28 after a 6-month therapy was used as a measure of treatment efficacy, the 677CT and 1298AC genotypes were found to be significantly associated with less favorable response to MTX ($P = 0.025$ and $P = 0.043$, respectively). In addition, even lower ΔDAS28 was determined for double-mutated 677CT–1298AC heterozygotes. It means that a synergistic effect of 677CT and 1298AC genotypes was observed. Nevertheless, the DAS28 baseline was lower here comparing to other genotypes. Unexpectedly, quite the opposite trend—i.e., better response to MTX—was found in genotypes 677CC–1298CC and 677TT–1298AA. It is an intriguing finding, because these double-mutated homozygotes are known for their low MTHFR-specific activity. Global significance was $P = 0.013$, $\eta^2 = 0.160$—i.e., large-size effect. Thus, our data show greater ability of 677CC–1298CC and 677TT–1298AA genotypes to respond to MTX treatment.

Keywords Rheumatoid arthritis · Methotrexate · Methylenetetrahydrofolate reductase · Polymorphism

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Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disorder being the most common from chronic inflammatory joint

disease. It has an overall prevalence of 0.5–1 % in European Caucasian populations, with a female/male ratio of 3:1 [1]. *Methotrexate (MTX) is a part of the first treatment strategy in patients with active RA* [2, 3] and is a highly effective agent both as monotherapy and in combination with glucocorticoids, other conventional synthetic disease-modifying antirheumatic drugs (DMARDs) and biologic DMARDs. MTX thus continues to serve as an anchor drug in RA [4]. As monotherapy with or without glucocorticoids, it is effective in DMARD-naïve patients and leads to low disease activity states or 70 % improvement rates according to the criteria of the American College of Rheumatology (ACR; which correspond to nearly a state of low disease activity) [5] in about 25–50 % of patients with early RA within 6–12 months [6–12]. The maximum effect of MTX is attained only after 4–6 months of treatment [8–11, 13]. In this respect, the optimal dose (25–30 mg per a week with folate supplementation), or somewhat less in the case of dose-limiting side effects [14], should be maintained for at least 8 weeks as an important aspect on the way to ultimate treatment success [15]. Moreover, 15–30 % of the patients develop severe adverse drug reaction (ADR) to MTX therapy [9, 13, 16, 17].

According to the selection of patients at low risk of ADR to MTX, we would be able to adjust and increase MTX dose to intensify therapy. On the other hand, patients supposed to be resistant to MTX therapy or presumed at high risk of ADR based on genotyping could be switched to other DMARDs.

In last decade, numerous studies have reported significant associations between single-nucleotide polymorphisms (SNPs) in gene-encoding enzymes related to the pharmacokinetics and pharmacodynamics of MTX, and its treatment efficiency and ADR. MTX therapeutic effect is achieved by inhibiting *enzymes of the folate and adenosine pathways.* Therefore, MTX response is influenced by 5, 10-methylenetetrahydrofolate reductase (MTHFR) enzyme activity. Although the MTHFR is not directly inhibited by MTX or by its polyglutamated forms (MTXPG). MTHFR catalyses the conversion of 5, 10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, the major circulating form of folate, and a carbon donor for the vitamin B_{12} dependentremethylation of homocysteine to methionine [18]. In addition to adenosine and folate pathways, MTX is considered to be involved also in the de novo nucleotide synthesis and methionine pathways. Moreover, these pathways can be inhibited by MTX and/or MTXPG [18].

Two SNPs of the *MTHFR* have been mainly studied C677T (rs1801133) and A1298C (rs1801131). However, conflicting data were seen for the SNPs [19–22], and the recent meta-analysis did not find any association of the SNPs with MXT treatment outcome [23].

The aim of the study was to determine whether SNPs in the *MTHFR* are predictive of MTX response. Comparison of EULAR responders/non-responders was made using dominant, recessive, co-dominant models and according to the change of DAS28 (ΔDAS28) after a 6-month MTX treatment in RA patient cohort of the East Bohemian population. The two SNPs 677C > T (rs1801133) and 1298A > C (rs1801131) of the *MTHFR* have been genotyped by qPCR allelic discrimination.

Methods

Patient characteristics, study design

Monocentric, regional, retrospective and prospective, cross-sectional study has been performed. There were 186 patients enrolled in study and genotyped, all of whom fulfilled the American College of Rheumatology (ACR) 1987 RA criteria [23], and who currently or previously taking MTX oral treatment, either as a monotherapy or in a combination with other DMARDs. In retrospective part of the study, there were patients studied with beginning of MTX treatment in history, from 2002 toward ($n = 162$). In the prospective part, there were patients with current beginning of MTX treatment enrolled during recruiting period ($n = 24$). Enrolled patients were treated for RA with peroral MTX at the Second Department of Internal Medicine University Hospital, Hradec Kralove. Seventy patients (blood donors) were enrolled into control group (CG) for verification of genotypic distribution in our cohort. Demographic data have not been determined. All probands (including control group) gave their written informed consent before being enrolled. All patients were adult's of Caucasian origin and living in East Bohemian (central European) region of the Czech Republic. The study was approved by the Ethics Committee of the University Hospital, Hradec Kralove, Czech Republic, and was conducted in accordance with the Declaration of Helsinki principles. Clinical data were available from 120 patients: mean age 58.5 years, SD ± 12.6, age of 29–85 years and from 32 male (26.7 %; Table 1). Sixty-five patients were treated by MTX monotherapy or MTX together with glucocorticoids. Fifty-five patients were treated by MTX in combination with conventional synthetic or biologic DMARDs. The concomitant medications were sulfasalazine ($n = 18$), leflunomide ($n = 10$), hydrochloroquin ($n = 18$), cyclosporine ($n = 8$), biologics ($n = 7$) and glucocorticoids ($n = 86$). Mean dose of MTX treatment in MTX monotherapy group was 11.7 ± 2.9 mg per week, and in patients treated with MTX together with other DMARDs, the dose was 11.0 ± 2.7 mg per week. Folate supplementation was provided in all patients, and the dose of folic acid was 20 mg taking 1 day after MTX.

Treatment response was evaluated using DAS28 score and based on EULAR response criteria at the beginning

Table 1 Characteristics in enrolled rheumatoid arthritis subjects and comparison of responders with non-responders after 6-month methotrexate treatment according to EULAR response criteria

	Responders ($n = 80$)	Non-responders ($n = 40$)	P value (responders vs. non-responders)
Age (years), mean ± SD	58.8 ± 13.2	57.8 ± 11.4	0.684
Female gender, n (%)	62 (77.5 %)	26 (65.0 %)	0.189
DAS28, start of MTX, mean ± SD	3.06 ± 1.47	4.89 ± 2.65	<0.001
DAS28, after 6-month MTX, mean ± SD	1.65 ± 1.19	3.96 ± 1.44	<0.001
C677T			
CC	36 (45)	16 (40)	0.722
CT	36 (45)	21 (52.5)	
TT	8 (10)	3 (7.5)	
A1298C			
AA	38 (47.5)	19 (47.5)	0.233
AC	33 (41.3)	20 (50)	
CC	9 (11.2)	1 (2.5)	

Comparison of genotypes in patients with MTX discontinuation for adverse event and rheumatoid arthritis group

MTX discontinuation, adverse events

	C677T			A1298C		
Genotypes	CC	CT	TT	AA	AC	CC
MTX discontinuation for adverse event, n (%), $n = 16$	10 (62.5)	6 (37.5)	0	5 (31.2)	9 (56.2)	2 (12.5)
RA total group, n (%), $n = 120$	52 (43.3)	57 (47.5)	11 (9.2)	57 (47.5)	53 (44.2)	10 (8.3)
P value, comparison of genotypes	$P = 0.220$			$P = 0.342$		

DAS28 disease activity score in 28 joints, *MTX* methotrexate, *C* C allele, *T* T allele, *A* A allele, *RA* rheumatoid arthritis, *AE* adverse event

of MTX treatment, prospectively at entry into the study or retrospectively from patient's file (in case of patients with history of MTX treatment) and after 6 month of therapy. Measurement of 28 joint count of tender, swollen joint and erythrocyte sedimentation rate (ESR) with calculation of the DAS28 was taken for each patient [24, 25]. Disease activity was defined: DAS28 <2.8, remission; =2.8 and ≤3.2, low disease activity; DAS28 = 3.2 ≤ 5.1, moderate active disease; DAS28 ≥5.1, severe disease. In this study, we dichotomized patients into non-responder versus moderate/good responder groups. The European League Against Rheumatism (EULAR) response criteria based on the DAS28 were used. EULAR criteria define good responders as patients with a mean DAS28 >2.6 and <3.2 and with reduction in DAS28 >1.2 during the treatment [26]. In second part of study, MTX response was determined by ΔDAS28. ΔDAS28 is characterized by change in DAS28 between DAS28 at beginning of MTX and DAS28 after sixth month of MTX treatment. ΔDAS28 was measured in all patients.

The association of genotype with efficacy of MTX was evaluated after 6 months by comparing the genotype distribution in patients with good and moderate clinical response (responders) versus non-responders.

For evaluation of toxicity, all reported adverse events (AEs) during first 6 months of MTX treatment were used. AEs were reported in patients' files. Each AE was described by its duration, frequency, severity, an assessment of its cause and its relationship to the study medication. In general, the dose of MTX was lowered temporarily in case of mild AEs. In case of a severe AE, MTX was discontinued. MTX treatment was discontinued owing to toxicity $n = 16$ (dyspepsia $n = 6$, hepatopathia $n = 2$, diarrhea $n = 2$, infection $n = 4$, leukopenia $n = 1$, alopecia $n = 1$). One patient discontinued MTX owing to inefficacy and include into non-responders group.

At the entry into study, demographic data were collected such as age, sex, smoking, duration of disease and joint symptoms. In addition, laboratory parameters such as C-reactive protein (CRP; mg/l), anti-cyclic citrulinated peptide antibodies (ACPA), rheumatoid factors (RF), ESR (mm/h), blood count, bilirubin plasma levels, liver enzymes (ALT, AST, ALP) plasma activities and creatinine clearances (calculated according to the Cockcroft–Gault formula), the occurrence of dyspepsia and infections were measured at the entry into study (start of MTX treatment) or retrospectively from patient´s file (patients with history of MTX treatment) and after 6 months of observation. The turbidimetry (using commercial kit COBAS from Roche analyzed using Modular analysator) was provided for the evaluation of CRP (normal range 0–5 mg/l). For determination of ACPA, the ELISA analysis was done by using commercially available kit purchased from Immunoscan (Euro-Diagnostica, Sweden). RF

Table 2 Association of single-nucleotide polymorphisms (C677T and A1298C) SNPs with peroral methotrexate treatment outcomes using EULAR response criteria

Polymorphism	Responders $n = 80$, n (%)	Non-responders $n = 40$, n (%)	OR (95 % CI) P value
Total group, $n = 120$			
C677T			
CC versus TT			1.18 (0.278–5.061) $P = 1.000$
CC	36 (45)	16 (40)	1.23 (0.57–2.65) $P = 0.697$
CT + TT	44 (55)	24 (60)	
TT	8 (10)	3 (7.5)	1.34 (0.193–13.96) $P = 1.000$
TC + CC	72 (90)	37 (92.5)	
A1298C			
AA versus CC			4.50 (0.530–38.18) $P = 0.260$
AA	(47.5)	19 (47.5)	1.00 (0.47–2.14) $P = 1.000$
AC + CC	42 (52.5)	21 (52.5)	
CC	9 (11.2)	1 (2.5)	1.41 (0.51–4.55) $P = 0.432$
CA + AA	71 (88.7)	39 (97.5)	
MTX monotherapy, $n = 65$			
C677T	47	18	
CC versus TT			1.43 (0.139–14.69) $P = 1.000$
CC	21 (44.7)	6 (33.3)	1.62 (0.52–5.03) $P = 0.410$
CT + TT	26 (55.3)	12 (66.7)	
TT	5 (10.6)	1 (5.6)	1.33 (0.58–2.35) $P = 0.672$
TC + CC	42 (89.4)	17 (94.4)	
A1298C	47	18	
AA versus CC			$P = 0.317$
AA	23 (48.9)	7 (38.9)	1.51 (0.50–4.56) $P = 0.471$
AC + CC	24 (51.1)	11 (61.1)	
CC	6 (12.8)	0	$P = 0.562$
CA + AA	41 (87.2)	18 (100)	

Comparison corresponded to a codominant model (CC vs. TT) or (AA vs. CC), dominant model [CC vs. (CT + TT)] or [AA vs. (AC + CC)], recessive model [TT vs. (TC + CC)] or [CC vs. (CA + AA)]

C C allele, *T* T allele, *A* A allele, *MTX* methotrexate, *OR* odds ratio

level was detected using the ELISA kit Rheumatoid Factor IgG, IgA, IgM (Orgentec, Germany).

Analyses were corrected for confounders, including age, sex, baseline DAS28, MTX dose, presence of the *MTHFR* 677TT genotype and the use of other DMARDs.

Clinical predictors of RA activity

Potential clinical predictors of disease activity were chosen based on the literature reports [27, 28]. Clinical predictors included age, sex, cigarette smoking status (non-smoker, current smoker), RF status, ACPA status and another/prior DMARDs use.

Genotyping

Patients were genotyped using standard genotyping assays. Blood samples were collected in EDTA vacutainer tubes. Genomic DNA was extracted from 200 µl aliquots using QIAamp DNA Blood Mini Kit (Qiagen, Netherlands). Genotyping was performed by qPCR allelic discrimination using commercial TaqMan assays (Life Technologies/Thermo Fisher, USA) with FAM/VIC labeled allele-specific probes, specifically assay C_850486_20 for *MTHFR* A1298C (rs1801131) and C_1202883_20 for *MTHFR* C677T (rs1801133). The reactions contained 50–100 ng of DNA in 1× TaqMan genotyping master mix (Life

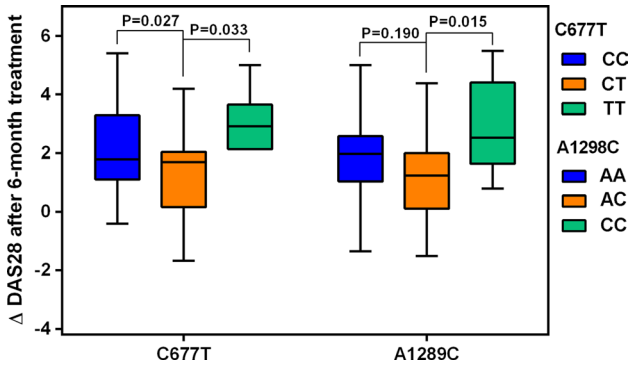

Fig. 1 Comparison of C677T and A1298C polymorphisms according to ΔDAS28 after 6-month methotrexate treatment

Technologies/Thermo Fisher, USA) with 1× assay in total volume of 20 µl. Thermal cycling was as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 92 °C for 15 s and 60 °C for 90 s. Real-time PCR and data analysis were performed using RotorGene 6000 system (Corbett Life Science/Qiagen, USA). Genotypes were determined in 100 % samples.

Statistical analysis

Logistic regression analysis was used for the dichotomous outcome measures in non-responders versus moderate/good responders according to the EULAR criteria. Results are expressed as the odds ratio (OR) with 95 % confidence interval (95 % CI). Differences between responders and non-responders were evaluated using the Mann–Whitney *U* test or the Chi-square test. The effect of the genetic variants on DAS28 change (ΔDAS28) was assessed via one-way ANOVA with wild-type homozygosity, heterozygosity and variant homozygosity as separate factor levels.

Furthermore, technology GML/generalized linear model technique, BOOTSTRAP type was used in the analysis. Statistical differences of clinical and laboratory parameters among haplotypes were analyzed by independent *t* test or ANOVA test. Statistical significance was considered at $P < 0.05$. All statistical analyses were performed using the SPSS statistical package version 16 (SPSS Inc., Chicago, IL, USA).

Results

Distribution of C677T and A1298C SNPs in RA patients

In the first examination, we observed no statistically significant ($P = 0.722$) difference in genotype distribution between RA patients and controls for the C677T SNP. T homozygotes of this polymorphism were not at an increased risk of RA (OR 1.370 95 % CI 0.343–5.474, $P = 0.656$).

In addition, we found no significantly ($P = 0.233$) different distribution of genotypes between RA patients and controls for the A1298C SNP in the *MTHFR*. C homozygotes of this polymorphism were not at an increased risk of RA (OR 4.944 95 % CI 0.604–40.476 $P = 0.104$).

When the pooled cohort was stratified according to genotype, 11 (9.2 %) 677TT homozygotes, 57 (47.5 %) 677CT heterozygotes, 10 (8.3 %) 1298CC homozygotes and 53 (44.2 %) 1298AC heterozygotes were found, respectively (Table 1). The allele frequencies were 32.9 % for T allele of C677T SNP (95 % CI 0.57–2.65) and 30.4 % for C allele of A1298C SNP (95 % CI 0.47–2.14), respectively. The distributions of genotypes in patients are shown in Table 1. We observed the development of AE $n = 38$ (dyspepsia 15, infection 11, nodulosis due to MTX discontinuation 4, alopecia 2, hematology 2, allergy, 1 pulmonary 1, others 2). Incidence of AE did not differ between genotypes in SNPs (for C677T $P = 0.29$ and A1298C $P = 0.45$).

Groups divided by genotyping were not statistically different by age, gender, DAS28 at start, MTX discontinuation and MTX dose at start (Table 3). SNPs were in Hardy–Weinberg equilibrium.

Association of C677T and A1298C SNPs with the MTX treatment response according to EULAR criteria

When treatment efficacy was determined according to the EULAR response criteria, 80 of 120 patients (66.7 %) have been classified as responders and 40 (33.3 %) have been considered as non-responders (Table 1). We revealed no evidence to support association of the *MTHFR* SNPs with efficacy of the treatment with low-dose MTX in a cohort of Czech RA patients based on DAS28 treatment response stratification using a dominant, recessive and codominant models (Tables 1, 2).

Association of C677T SNP with the MTX treatment response according to ΔDAS28

Next, we analyzed efficacy of MTX treatment using ΔDAS28 (expressed by ΔDAS28 after a 6-month treatment) as a quantitative parameter of the MTX treatment efficacy. When ΔDAS28 was used as a measure of MTX treatment efficacy, *MTHFR* 677CT genotype was significantly associates with less favorable response to MTX ($P = 0.025$, $\eta^2 = 0.112$—medium effect size; Fig. 1). This association was not detected in case of group with combination of MTX treatment with other DMARDs (conventional synthetic or biologic; $P = 0.886$), but only in MTX monotherapy group. Post hoc tests showed significantly low efficacy of MTX treatment in carriers of

Table 3 Characteristics of rheumatoid arthritis patients treated with peroral methotrexate, comparison of different genotypes in C677T and A1298C polymorphisms

n	Total group	MTX monotherapy			*P* value comparison different genotypes of MTX monotherapy group	MTX +other DMARDs			P_r value MTX monotherapy group vs. MTX + other DMARDs group
	120	Genotypes				Genotypes			
		CC	CT	TT		CC	CT	TT	
		27	32	6		25	25	5	
Age (years), mean ± SD	58.5 ± 12.6	60.1 ± 12.7	60.3 ± 11.7	53.0 ± 17.3	0.107	61.7 ± 11.7	53.1 ± 12.3	55.0 ± 13.0	0.678
Female, *n* (%)	88 (73.3)	21 (77.8)	18 (56.3)	3 (50.0)	0.469	21 (84.0)	21 (84.0)	4 (80.0)	0.058
Age at beginning of RA (years), mean ± SD	45.6 ± 14.4	48.9 ± 14.1	46.9 ± 13.8	45.3 ± 19.9	0.285	46.6 ± 13.7	39.8 ± 14.2	43.0 ± 15.6	0.258
RF positivity, MTX start, *n* (%)	69 (60.5)	16 (60.0)	19 (60.0)	3 (50.0)	0.891	14 (65.2)	14 (60)	3 (60.0)	0.669
ACPA positivity baseline, *n* (%)	82 (70.8)	17 (94.4)	15 (68.2)	2 (40.0)	0.018	13 (81.3)	12 (66.7)	1 (33.3)	0.404
ACPA positivity at 6th month, *n* (%)	34 (45.3)	16 (57.1)	9 (32.1)	3 (50)	0.011	12 (44.4)	13 (48.1)	2 (7.4)	0.364
MTX dose at start (mg), mean ± SD	11.34 ± 2.84	11.67 ± 3.02	11.67 ± 2.73	11.67 ± 3.76	0.844	11.10 ± 2.98	11.00 ± 2.80	10.00 ± 0.00	0.161
MTX dose at 6th month (mg), mean ± SD	12.38 ± 3.67	12.89 ± 3.58	12.58 ± 3.62	12.08 ± 3.32	0.467	12.20 ± 3.70	12.32 ± 4.24	10.00 ± 1.77	0.259
ESR (mm/h), mean ± SD. ($n = 113$)	33.7 ± 22.5	36.12 ± 23.0	27.4 ± 20.6	55.3 ± 9.2	0.079	35.4 ± 21.4	31.5 ± 26.8	34.8 ± 14.7	0.286
ESR (mm/h), mean ± SD. ($n = 115$)	20.19 ± 17.32	17.11 ± 10.27	19.94 ± 22.28	36.33 ± 19.83	0.073	23.17 ± 17.00	17.41 ± 15.54	17.60 ± 10.46	0.385
DAS 28, MTX start, mean ± SD	4.45 ± 1.41	4.76 ± 1.25	3.85 ± 1.38	5.44 ± 1.34	0.10	4.49 ± 1.55	4.54 ± 1.39	4.65 ± 1.06	0.718
DAS 28, at 6th month MTX, mean ± SD	3.09 ± 1.36	2.72 ± 1.08	2.69 ± 1.20	2.83 ± 1.05	0.823	3.37 ± 1.43	3.63 ± 1.61	3.73 ± 1.28	0.010
Smoking status, yes, *n* (%)	14 (11.8)	3 (15.4)	4 (15.6)	4 (16.7)	0.706	2 (12.0)	1 (4.0)	0	0.999
n	Total group	MTX monotherapy			P_{gen} value comparison different genotypes of MTX monotherapy group	MTX +other DMARDs			P_{ter} value MTX monotherapy group vs. MTX + other DMARDs group
		Genotypes				Genotypes			
		AA	AC	CC		AA	AC	CC	
	120	30	29	6		27	24	4	
Age (years), mean ± SD	58.5 ± 12.6	58.1 ± 12.8	60.2 ± 13.2	63.8 ± 8.7	0.813	60.0 ± 11.8	55.0 ± 12.6	50.8 ± 16.4	0.087
Female, *n* (%)	88 (73.3)	17 (56.7)	19 (65.5)	6 (100)	0.681	22 (81.5)	21 (87.5)	3 (75.0)	1.000
Age at beginning of RA (years), mean ± SD	45.6 ± 14.4	43.9 ± 13.8	50.6 ± 15.5	51.3 ± 3.1	0.034	44.2 ± 14.5	43.4 ± 14.1	35.5 ± 14.0	0.507
RF positivity, MTX start, *n* (%)	69 (60.5)	18 (64.3)	12 (44.4)	6 (100.0)	0.011	18 (69.2)	13 (56.5)	2 (50.0)	0.572
ACPA positivity baseline, *n* (%)	82 (70.8)	16 (80.0)	14 (66.7)	4 (100.0)	0.192	10 (62.5)	15 (75.0)	1 (100.0)	0.504
ACPA positivity at 6th month, n (%)	34 (45.3)	14	11	3	0.327	10	14	3	0.101
MTX dose at start (mg), mean ± SD	11.34 ± 2.84	11.58 ± 3.25	11.76 ± 2.66	11.67 ± 2,58	0.249	10.19 ± 1.82	11.56 ± 3.02	12.50 ± 5.00	0.720

Table 3 continued

n	Total group	MTX monotherapy			P_{gen} value comparison different genotypes of MTX monotherapy group	MTX +other DMARDs			P_{ter} value MTX monotherapy group vs. MTX + other DMARDs group
		Genotypes				Genotypes			
		AA	AC	CC		AA	AC	CC	
	120	30	29	6		27	24	4	
MTX dose at 6th month (mg), mean ± SD	12.38 ± 3.67	12.83 ± 3.76	12.59 ± 3.50	12.00 ± 2.74	0.845	11.48 ± 4.34	12.52 ± 3.09	13.13 ± 4.73	0.919
ESR (mm/h), mean ± SD. ($n = 113$)	33.7 ± 22.5	34.4 ± 19.9	30.4 ± 22.2	46.3 ± 31.4	0.403	33.9 ± 20.3	32.4 ± 28.2	38.0 ± 14.7	0.691
ESR (mm/h), mean ± SD. ($n = 115$)	20.19 ± 17.32	22.33 ± 20.36	18.48 ± 17.93	18.67 ± 11.50	0.664	19.08 ± 15.52	19.77 ± 16.17	30.67 ± 17.90	0.436
DAS 28, MTX start, mean ± SD	4.45 ± 1.41	4.49 ± 1.27	4.04 ± 1.43	5.40 ± 1.71	0.294	4.41 ± 1.22	4.61 ± 1.68	4.82 ± 1.06	0.934
DAS 28, at 6th month MTX, mean ± SD	3.09 ± 1.36	2.71 ± 1.17	2.78 ± 1.15	2.45 ± 0.84	0.215	3.40 ± 1.52	3.81 ± 1.50	2.60 ± 0.69	0.058
Smoking status, yes, *n* (%)	14 (11.8)	6 (20.0)	2 (6.9)	2 (40.0)	0.125	2 (7.4)	1 (4.2)	1 (25.0)	0.450

MTX methotrexate, *DMARDs* disease-modifying antirheumatic drugs, *RA* rheumatoid arthritis *RF* rheumatoid factors, *ACPA* anti-cyclic citrulinated peptide antibodies, *ESR* erythrocyte sedimentation rate, *VAS* visual analog scale, *DAS 28* disease activity score in 28 joints

677CT genotype versus either wild-type homozygous CC or mutant homozygous TT carriers (Fig. 1). Difference of mean ΔDAS28 in CT versus CC genotypes was 0.885, 95 % CI (0.105, 1.667); $P = 0.027$. In case CT versus TT, the difference was ΔDAS28 1.448, 95 % CI (0.119, 2.776); $P = 0.033$. No significant result between CC and TT homozygotes ($P = 0.407$) was detected (Fig. 1). Demographic characteristics and clinical parameters of patients are presented in Table 3.

Association of A1298C SNP with the MTX treatment response according to ΔDAS28

Regarding A1298C SNP, the similar significant difference between 1298AC and 1298AA genotypes was found only in MTX monotherapy group ($P = 0.043$, $\eta^2 = 0.097$—medium effect size). No effect was found in case of the cohort of patient treated with MTX in combination with other DMARDs (synthetic or biologic; $P = 0.272$; *data not shown*). Post hoc tests showed significantly low response of MTX associated with 1298AC genotype in comparison with the 1298CC genotype carriers. The difference of mean ΔDAS28 in 1298AC versus 1298CC carriers was 1.691, 95 % CI (0.340, 3.041); $P = 0.015$. In case 1298AC versus 1298AA genotype carriers, the difference of DAS28 was 0.520 95 % CI (0.264, 1.304); $P = 0.190$. No significant results were found between 1298CC and 1298AA homozygous carriers ($P = 0.087$; Fig. 1). Demographic characteristics and clinical parameters of patients are shown in Table 3.

Correlation of ΔDAS28 with MTX dose

Next, we further analyzed the mean ΔDAS28 in homozygotes. We found that the higher dose of MTX lead to better response in DAS28, i.e., 1.19 DAS28/10 mg MTX ($P = 0.02$). Therefore, the correlation with MTX dose was performed in next analyses. The comparison was expressed by using the variable mean change of DAS28 per 10 mg MTX/week.

In case of polymorphism C677T, mean response on MTX treatment (expressed by decrease in DAS28 after a 6-month treatment) in CC homozygotes was found 1.59 DAS/10 mg MTX (median of MTX dose); 95 % CI (0.12,3.06); $P = 0.034$, in CT heterozygotes 0.70 DAS28/10 mg; 95 % CI (−0.82,2.22); $P = 0.36$ and in homozygotes TT 1.83 DAS28/10 mg; 95 % CI (−1.70,5.37); $P = 0.31$, respectively.

Regarding A1298C polymorphism, in AA homozygotes, DAS28 changed by 1.92 DAS28/10 mg; 95 % CI (0.43,3.41); $P = 0.012$, in AC heterozygotes by 0.43 DAS28/10 mg; 95 % CI (−1.08,1.94); $P = 0.57$ and in CC homozygotes by 1.33 DAS28/10 mg; 95 % CI

(−1.59,4.24); $P = 0.37$, respectively. Correction of MTX dose showed significant decrease in DAS28 in 677CC and 1298AA wild-type homozygotes after 6-month MTX treatment in comparison with minor homozygotes and heterozygotes.

Combination of C677T and A1298C SNPs

Further, we investigated whether C677T and A1298C SNPs may have a synergistic effect on response to MTX treatment determined using ΔDAS28. In our patient cohort were not found the 677TT–1298CC homozygotes with four mutant alleles and either 677TT–1298AC or 677CT–1298CC heterozygotes with three mutant alleles. Remaining combinations of C677T and A1298C SNPs stratified patients into six subgroups (Table 4). The heterozygotes with one mutant allele (group 2: 677CC–1298AC and 677CT–1298AA genotypes) were found to be significantly associated with less favorable response to MTX (see Fig. 2). Even lower ΔDAS28 was determined for group 3—heterozygotes 677CT–1298AC with two mutant alleles (see Fig. 2)—i.e., a synergistic effect of 677CT and 1298AC genotypes was found, which should be, however, considered with caution as the DAS28 baseline was lower here comparing to other genotypes. Surprisingly, just the opposite trend (i.e., heightened mean ΔDAS28—better response to MTX) was found in group 4—homozygotes with two mutant alleles of either 677CC–1298CC or 677TT–1298AA type (see Fig. 2). It is an intriguing finding, because these double-mutated homozygotes are known for their low MTHFR-specific activity [29–31]. Global significance was $P = 0.013$, $\eta^2 = 0.160$—i.e., large-size effect. Analysis of haplotype distribution between pairs of loci demonstrated the presence of significant linkage disequilibrium = 0.0355 (i.e., 35.5 % of maximum theoretically achievable value) between *MTHFR* A1298 and C677T polymorphisms ($P = 0.001$).

Discussion

Our data showed no association between clinical aspects (gender, diagnosis age and smoking status) and distribution of C677T and A1298C genotypes. Nevertheless, earlier studies indicated that clinical variables could play certain roles. Male gender is associated with better response to MTX therapy [32]. On the other hand, smokers are the worst responders to MTX, presenting a higher disease activity and severity [33]. ACPA and ANAs auto-antibodies found in RA are strongly correlated with erosive disease, worse functional status and higher disease activity associated with non-response [34, 35]. Combination of non-current smoking, ACPA and ANAs positivity, higher HAQ, NSAIDs utilization, per oral administration route and the 677TT *MTHFR* genotype can be possible predictive factor of non-response to MTX [36]. Low folate intake affects individuals carrying the 677TT genotype. Lower plasma folate levels are at risk of MTX non-response, ADR and elevated plasma homocysteine levels [37]. The folic acid supplementation correlates with ethnicity [38]. Moreover, folate status is affected by local diets.

There was found clinical significance of 677CT (but not 1298AC) heterozygotes, in many clinical studies [39–42]. Rather conflicting results were yielded consider association of *MTHFR* SNPs with response to MTX in RA. Many studies found no association of a genotype with overall MTX-induced toxicity, whereas other studies found associations with GI toxicity [19, 23, 43]. Homozygous 677CC genotype patients have a better outcome (lower DAS28). The same was observed for homozygous 1298AA patients (considering EULAR response using DAS28), but also C-allele carriers with an improvement in the therapy were reported. Recently, large meta-analyses summarized studies reporting the association of the *MTHFR* SNPs in RA patients treated with MTX response using EULAR criteria in responders and non-responders [19, 23, 43–46]. Recent meta-analysis provided sufficient data (with over 1,400 patients for the C677T analysis and over 660 patients for the A1298C analysis) for studying association of both SNPs with toxicity [44]. However, there were not sufficient data to perform a meta-analysis of MTX efficacy. Another recent meta-analysis suggested that the C677T and A1298C *MTHFR* SNPs are not reliable predictors of response to MTX treatment in RA patients [23]. This analysis included data from 1,375/1,140 patients for the C677T/A1298C SNP efficacy analysis and from 2,043/1,239 patients for the toxicity analysis. Very recent meta-analysis included twelve studies comprising a total of 2,288 RA patients [45]. Their results suggest that the C677T and A1298C SNPs are associated with MTX toxicity in RA patients [45]. Both *MTHFR* SNPs were found associated with MTX treatment response in multivariate analysis [47].

Overall, above-mentioned studies largely differ in many aspects. Not all earlier studies discriminated between the heterozygous and homozygous genotypes [44]. There are differences in study designs and settings (retrospective/prospective, inpatient/outpatient), environmental variability, definition of MTX efficacy and toxicity, used genetic models, therapeutic regimens, MTX dose etc. In the current study, we demonstrate that the A1298C and C677T SNPs showed predictive values only in the case of the low-dose MTX monotherapy group. Our dosing (7.5–15 mg) is comparable to that which was used in recent meta-analysis [23] that enrolled patients with beginning of treatment from 2002 toward. In 2003, common dose of MTX was 10 mg per week. From that time on, effectiveness of higher dosing

Table 4 Characteristics of rheumatoid arthritis patients treated with peroral methotrexate, comparison of different genotypes in combination C677T and 1298AC polymorphisms

	Genotypes						
	1298AC–677CT $n = 16$	1298AA–677CT $n = 16$	1298AC–677CC $n = 13$	1298AA–677CC $n = 8$	1298AA–677TT $n = 6$	1298CC–677CC $n = 6$	*P* value* comparison different genotypes
Age (years), mean ± SD	57.9 ± 13.4	68.2 ± 9.4	63.1 ± 12.9	52.6 ± 13.0	53.0 ± 17.3	63.6 ± 8.66	0.152
Female, *n* (%)	9 (56.2)	9 (56.2)	10 (76.9)	5 (62.5)	3 (50)	6 (100)	0.176
Age at beginning of RA (years), mean ± SD	48.6 ± 15.0	45.2 ± 12.7	53.1 ± 16.4	40.1 ± 11.8	45.3 ± 19.9	51.3 ± 3.1	0.313
ACPA positivity baseline, *n* (%)	7 (42.9)	14 (90)	11 (88.9)	8 (100)	2 (40)	6 (100)	0.016
ACPA positivity at 6th month, *n* (%)	2 (12.5)	13 (85.7)	13 (100)	8 (100)	5 (75)	2 (40)	0.036
MTX dose at start (mg), mean ± SD	12.1 ± 2.7	11.2 ± 2.7	11.3 ± 2.6	12.2 ± 4.1	11.7 ± 3.8	11.7 ± 2,6	0.82
ESR (mm/h), baseline, mean ± SD.	46.3 ± 31.4	28.3 ± 20.8	35.0 ± 23.3	29.4 ± 11.9	55.3 ± 9.2	31.4 ± 2.9	0.235
DAS28, MTX start, mean ± SD	3.6 ± 1.5	4.1 ± 1.2	4.6 ± 1.2	4.6 ± 1.0	5.4 ± 1.3	5.3 ± 1.7	0.062
DAS28, at 6th month MTX, mean ± SD	2.4 ± 0.8	2.7 ± 1.2	2.7 ± 1.2	2.7 ± 1.2	2.8 ± 1.0	2.7 ± 1.1	0.983
ΔDAS28	0.9 ± 1.5	1.4 ± 1.2	1.7 ± 1.4	1.9 ± 1.7	2.6 ± 1.8	2.9 ± 1.7	0.021
MTHFR-specific activity (nmol formaldehyde/mg protein per h) by Chango et al. [30]	11.8	16.7	19.7	33.0	7.3	17.0	

Comprarison of ΔDAS28 in combination C677T and 1298AC polymorphisms with MTHFR-specific activity by Chango et al. [30]

MTX methotrexate, *RA* rheumatoid arthritis, *RF* rheumatoid factors, *ACPA* anti-cyclic citrulinated peptide antibodies, *ESR* erythrocyte sedimentation rate, *DAS28* disease activity score in 28 joints, *ΔDAS28*, change of DAS28, *MTHFR* methylenetetrahydrofolate reductase

* *P* value—comparison of characteristics of the patients of four groups in Fig. 2

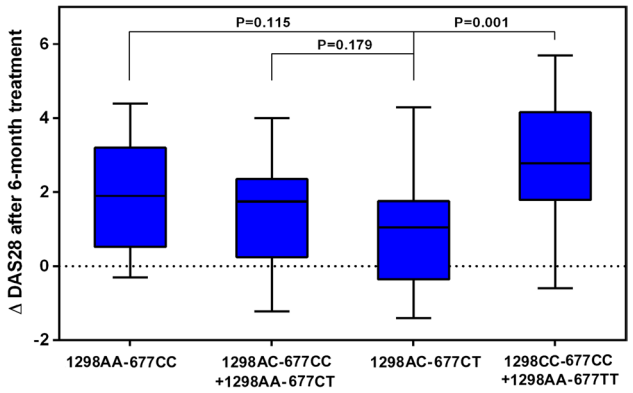

Fig. 2 Comparison of combination C677T and A1298C polymorphisms according to ΔDAS28 after 6-month methotrexate treatment

has been demonstrated [4, 48]. Recently recommended dose of MTX (20–30 mg) should be maintained for at least 8 weeks [3, 15, 24].

In addition, it seems to be critical, whether MTX is used as monotherapy or in combination with other DMARDs. In our "MTX and other DMARDs" group of patients, no remarkable differences in ΔDAS28 values were observed. Thus, the usage of other DMARDs effectively obscures impacts of different genotypes on the outcome of the MTX treatment. Other DMARDs might effect on antibodies production. On the other hand, DAS28 at sixth month of MTX treatment was higher in the "MTX + other DMARDs" group than in the MTX monotherapy group (see Table 3). Suboptimal response to MTX monotherapy may due to the addition of other DMARDs after third month. It should be possible reasons of higher DAS28 in this group at sixth month.

Many previous studies and meta-analyses used a dominant model (assuming dominant effects of the minor alleles) as well as recessive and codominant models. We examined the effects of minor alleles (CC versus CT + TT for the C677T SNP and AA vs. AC + CC for the A1298C SNP) in the dominant model. The OR of EULAR responders versus non-responders showed no significant association with C677T and A1298C SNPs.

The potential issue in studying pharmacogenetics is the impact of multiple SNPs on the efficacy or toxicity of MTX. While a single SNP may not have significance alone, the combination of several SNPs for given protein may lead to significant changes in function that either increase or decrease toxicity or efficacy or both. Interestingly, a synergistic interaction of the double heterozygotes 677CT–1298AC of the *MTHFR* in hyperhomocysteinemia has been described [49]. This combined heterozygosity was observed in 28 % of the neural tube defect patient compared with 20 % among controls, resulting in an odds ratio 2.04. The data suggested that combined heterozygosity for this common mutations accounts for a proportion of folate-related neural tube defects, which is not explained by homozygosity for the C677T mutation [31]. Choe et al. [50] investigated relationships of C677T and A1298C SNPs with MTX-related toxicities in Korean patients with RA taking low-dose MTX. The proportion of patients with the 677C–1298A haplotype who experienced toxicity was greater than the proportion of those with 677C/1298C haplotype ($P = 0.032$, OR 2.085). In our study, relatively large group of 677CT and 1298AC heterozygotes showed statistical significance in dependence of low response to MTX treatment on these polymorphisms. This fact was reinforced by a very low response to MTX treatment (determined with ΔDAS28) in the double heterozygotes 677CT–1298AC (Figs. 1, 2). Similar results have never been reported before and indicate association of C677T and A1298C with MTX treatment response.

The C677T and A1298C mutations result in decreased specific activity of the MTHFR enzyme, which is even more pronounced in combined heterozygotes and the most striking in double-mutated homozygotes [29–31]. Our ΔDAS28 values showed appropriate tendency (i.e., correlation with specific activities of MTHFR) in genotypes wt/677CC–1298AA, 677CC–1298AC, 677CT–1298AA and 677CT–1298AC (compare our Table 4 with Table 1 in Frosst et al. [29]; Table 2 in van der Put et al. [31]; Table 2 in Chango et al. [30]). Nevertheless, double-mutated homozygotes (i.e., genotypes 677CC–1298CC and 677TT–1298AA) were associated here with favorable response to MTX treatment (see Table 4), although exactly opposite outcome was expected on the base of low specific activities of mutant MTHFR enzymes [29–31].

Study limitations

This is a single-center retrospective study. Patients in this study did not receive recently recommended dose of MTX. The low dose of MTX could lead to low frequency of AEs and to relatively low cumulative rate of discontinuation. The 677TT–1298CC homozygotes with four mutant alleles and either 677TT–1298AC or 677CT–1298CC heterozygotes with three mutant alleles were not found in this study. Similarly, the 677CT–1298CC and 677TT–1298CC genotypes were not observed, for example, in 119 neonatal cord fetal tissue samples [51]. Apparently increased numbers of mutant *MTHFR* alleles lead to decreased viability and possible selection disadvantage among fetuses [51]. Therefore, just wild-type homozygotes (677CC–1298AA), heterozygotes with one mutant allele (677CC–1298AC, 677CT–1298AA), heterozygotes with two mutant alleles (677CT–1298AC) and homozygotes with two mutant alleles (677CC–1298CC and 677TT–1298AA) were studied here. The significance of synergism of C677T and A1298C SNPs

in the *MTHFR* gene on MTX treatment response in RA patients needs to be confirmed in future larger studies.

Conclusions

In this study, we did not find any association of C677T and A1298C variants on MTX treatment inefficacy in dominant, recessive, codominant models according to EULAR criteria. However, when reduction in DAS28 (ΔDAS28) was used as a measure of MTX treatment efficacy, the 677CT and 1298AC heterozygosity had statistically significant influence on reduction in response to MTX monotherapy. Moreover, homozygous double-mutant genotypes 677CC–1298CC and 677TT–1298AA showed increased ability to respond to MTX treatment, despite of a remarkably low specific activity of the affected MTHFR enzyme (repeatedly reported in literature). In conclusion, the results of this study suggest that SNPs C677T and A1298C in the *MTHFR* gene are predictive of low-dose peroral MTX efficacy using ΔDAS28 after a 6-month MTX treatment in RA adult patient cohort of the East Bohemian population. According to the study results, it is necessary to focus on combination of these SNPs in MTX pharmacogenetics. Contradictory impacts of *MTHFR* polymorphisms on MTHFR-specific activity and response to MTX treatment in the case of double-mutant homozygotes need further clarification.

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Conflict of interest None of the authors have financial interests that could create a potential competing interest or the appearance of a conflict of interest with regard to the work.

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