Influence of *MTHFR* C677T polymorphism on methotrexate monotherapy discontinuation in rheumatoid arthritis patients: results from the GAPAID European project

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Abstract

Objective

Methotrexate (MTX) is the most widely prescribed drug for rheumatoid arthritis (RA) patients, but 45% of them discontinue therapy within two years, either due to inefficacy or toxicity. Several authors have reported contradictory results related to C677T polymorphism in the MTHFR gene and response to MTX in RA. The purpose of this study was to further explore this genotype-response association in a European RA population.

Methods

This retrospective longitudinal study included a total of 269 RA patients from Italy and Hungary, of whom 73.2% had available data on MTX treatment (197 patients). C677T polymorphism (rs1801133) was genotyped by quantitative PCR using TaqMan assays. Genotype association analysis and Kaplan-Meier method were used for statistical comparisons between patients continuing and patients who abandoned MTX treatment.

Results

A total of 85 out of the 197 RA patients (43%) abandoned MTX treatment by the time of analysis. No significant genotype-MTX discontinuation association was found for the overall population, either at the end of the study (p=0.375), or during the follow-up (p=0.324). When the analysis was restricted to the 68 patients on MTX monotherapy, a borderline association (OR 3.15, 95% CI 0.93-10.67, p=0.057) was noted with the recessive genetic model. In agreement with that, a Kaplan-Meier analysis showed a significantly shorter time-to-discontinuation of MTX monotherapy for homozygous carriers of the T-allele (p=0.042).

Conclusions

These results demonstrate that the C677T polymorphism in the MTHFR gene is involved in MTX monotherapy discontinuation in a multicentre European patient cohort, confirming previous results.

Key words

methotrexate, rheumatoid arthritis, pharmacogenetics, single nucleotide polymorphism, methylenetetrahydrofolate reductase

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Introduction

Methotrexate (MTX) is an anti-metabolite drug recommended by the European League Against Rheumatism (EULAR) as the first-line therapy for rheumatoid arthritis (RA) (1, 2). MTX displays a favourable risk/benefit ratio, a good safety profile, as well as a low cost, and many clinical trials have shown its ability to decrease disease activity and slow down the structural damage in RA (3, 4). Nevertheless, only 55% of patients remain on this therapy beyond two years, either due to lack of efficacy, or to adverse effects (5, 6). It has been demonstrated that an early selection of the most effective treatment for RA patients is mandatory to improve their long-term outcomes, such as joint damage, disability or mortality (6-8). Therefore, validated biomarkers that aid in the identification of RA patients that will benefit from a MTX therapy or that will help optimise its dose are urgently needed (6, 9).

Several pharmacogenetic studies have demonstrated associations between genetic modifications in sequences coding for enzymes involved in the metabolism of folic acid and discontinuation of MTX therapy (10), C677T polymorphism (rs1801133) in the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene being the most widely studied (1, 10-12). MTHFR is a central regulatory enzyme in the folic acid pathway, since it catalyses the irreversible conversion of 5,10- methylenetetrahydrofolate, required for purine and thymidine synthesis, to 5-methylhydrofolate, which acts as a carbon donor for the re-methylation of homocysteine to methionine (1, 4, 12). The C677T polymorphism causes an Alanine to Valine replacement at codon 222 of the MTHFR protein, which results in a decreased enzyme activity, elevation of plasma homocystein levels and an altered distribution of folate (10, 12, 13).

Although the relationship between patient´s response to MTX and C677T polymorphism has extensively been studied, conclusive data do not exist yet (1, 4, 6, 11, 12, 14, 15). Two meta-analyses conducted by Owen *et al*. (1) and Lee *et al*. (16), concluded that there was no convincing evidence supporting this particular association in independent RA patient cohorts. Several groups, in contrast, have found significant associations between these two variables (11, 17, 18), and even two meta-analyses carried out by Spyridopoulou *et al*. (19) and Fisher *et al*. (20), demonstrated a significant association of C677T polymorphism with MTX toxicity. Previous studies carried out by our group with a cohort of 468 Spanish RA patients also demonstrated that C677T polymorphism in the *MTHFR* gene is associated with MTX toxicity (21).

The GAPAID (Genes And Proteins for AutoImmunity Diagnostics) consortium was created within the European Union's Seventh Framework Programme for Research and Technological Development (FP7), with the aim of validating gene and protein biomarkers for autoimmune diseases, such as RA. Given the importance of MTX in the treatment of RA, our previous results, and the controversy that still exists in the field, the present work was conducted to study the association between C677T polymorphism and response to MTX in an independent patient cohort with the aim of shedding more light on this important issue.

Material and methods

Study population

A cohort of 269 RA patients fulfilling the American College of Rheumatology (ACR) classification criteria for RA (22) was consecutively recruited between August 2012 and October 2013 at the Departmet of Clinical and Experimental Medicine of the University of Pisa (Italy) and the Department of Rheumatology and Immunology of the University of Pécs (Hungary). Clinical data of these patients were retrospectively evaluated. A total of 133 RA patients from Italy and 136 from Hungary were included. All these patients comprised the RA patient cohort of the GAPAID (Genes And Proteins for AutoImmunity Diagnostics, ref. 314971) project funded by the European Union's Seventh Framework Programme for Research and Technological Development (FP7). Within this cohort, 197 RA patients had MTX treatment data available, which consisted of MTX starting date,

stopping date (if ever), and whether it was administered as monotherapy or in combination with any other treatments. Patients were deemed to have failed MTX treatment if they discontinued treatment either because of an adverse effect or because of inefficacy (non responders), as previously described by Plant *et al*. (23). Patients that did not discontinue MTX treatment were classified as responders.

Genotyping assays

A blood sample was obtained from each patient. DNA was purified by NucleoSpin® 96 Blood Core Kit (Macherey-Nagel, Düren, Germany). Quantity (ng/µl) and quality (260/280 and 260/230 absorbances) values were obtained for each DNA sample with Qubit® 2.0 Fluorometer (Life Technologies, California, USA) using dsDNA HS Assay Kit (Life Technologies) and a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Wilmington, USA), respectively, before the genotyping process.

The genotyping of C677T polymorphism (rs1801133) in *MTHFR* gene was carried out by quantitative polymerase chain reaction (PCR) in a *7500 RT-PCR* System instrument using TaqMan® probes (Assay ID: C_1202883_20; Life Technologies) and following the manufacturer´s instructions. Specific alleles for each patient were assigned using TaqMan® Genotyping Software v.1.3 (Life Technologies).

Statistical analyses

Two different statistical approaches were used within this work. Firstly, the possible association between C677T genotype and MTX discontinuation at the end of the follow-up was assessed by different genetic association tests (allelic, genotypic, additive, recessive and dominant), after having tested for its deviation from Hardy-Weinberg equilibrium (HWE). The magnitude of association was expressed as an odds ratio (OR) with 95% confidence interval (CI). Secondly, a Kaplan-Meier analysis was performed in order to study whether significant differences exist between C677T genotypes and the time until MTX discontinuation. We defined the time of MTX treatment as the difference in years between the first day of treatment with MTX and the date of MTX discontinuation. Patients that did not abandon MTX treatment were censored at final follow-up. We tested all genotypes with a recessive model (TT *vs*. CT + CC). The Log Rank test was used to evaluate the null hypothesis of no differences between the survival curves for each genotype level. For both statistical approaches, in addition to the overall population, patients on MTX monotherapy and patients on combined therapy were separately studied.

Golden Helix SVS software suite 7 (Golden Helix, Bozeman, MT, USA) was used to test the genotype-MTX discontinuation association and deviation from HWE, and the *Survival* Package (v. 2.36–14) in R for the Kaplan-Meier analysis. SPSS version 18.0 (SPSS, Chicago, IL, USA) software was used for additional statistical associations between demographic and clinical variables with MTX discontinuation or sample origin, as well as to test for the possible genetic differentiation between analised populations, using Student's *t*-test or χ^2 test, depending on the variable being studied. The threshold for statistical significance was set at p-value = 0.05.

Ethics and consent

This study was approved by the Ethics Committee of the University Hospital of Pisa (ref. no: 45066/2012) and the Hungarian Scientific and Research Ethics Board (ref. no: 24973-1/2012 EKU). The procedures followed were in accordance with the Helsinki Declaration of 1975. All patients gave writen informed consent.

Table I. Clinical characteristics of the RA patients included in the study and comparison between Italian and Hungarian patients.

Variable	Total (n=197)	Italy (n=69)	Hungary (n=128)	*p*-value
Age (at inclusion) (years)	59.3 (12.5)	60.8 (13.3)	58.5 (12.1)	0.213
Sex, Female (%)	79.2	72.5	82.8	0.088
Disease duration (years)	14.53 (7.97)	14.7 (9.1)	14.4 (7.3)	0.794
Age of onset (years)	44.84 (14.15)	46.3 (15.7)	44.1 (13.2)	0.291
RF positivity (%)	61.7	64.9	60.3	0.554
Anti-CCP positivity (%)	67.9	69.6	66.9	0.706

RF: rheumatoid factor; anti-CCP: anti-cyclic citrullinated peptide antibodies.
Values are given as percentage or mean (standard deviation).

Results

The study cohort included 197 RA patients (69 Italian and 128 Hungarian), with a mean age at disease onset of 44.84±14.15 (mean ± standard deviation, SD) years, and an average time of evolution from disease onset of 14.53±7.97 years. No significant differences in demographic and clinical variables were found between Italian and Hungarian RA patients (Table I). As shown in Table II, no significant associations were found between clinical and demographic variables and MTX discontinuation when the whole population was analysed. A total of 85 patients (43.15%) discontinued MTX therapy after 3.8±3.9 years (non responders), whereas 112 (56.85%) continued on MTX treatment by the time of recruitment (8.24±4.27 years treatment by then) (responders).

Among all the RA patients included in the study, only 68 were treated with MTX monotherapy, 48 of which did not discontinue treatment by the time of recruitment (responders), while 20 dropped out (non responders). No significant associations were found between demographic and clinical variables and MTX monotherapy discontinuation (Table III), except for the age of disease onset, where we observed that a younger age was associated with an increased MTX monotherapy discontinuation (MTX responders: 51.2±14.0 years; MTX non responders: 39.7±16.1 years; p=0.006).

MTFHR polymorphism genotyping analysis gave rise to a 100% call-rate and genotype frequencies were in HWE (p>0.05) in the overall population. The minor allele (T) frequency was 0.39,

Table II. Comparison of the clinical and demographic characteristics between MTX responders and non-responders.

Variable	Total (n=197)	MTX responders (n=112)	MTX non responders (n=85)	*p*-value
Age (at inclusion) (years)	59.3 (12.5)	59.2 (12.33)	59.4 (12.9)	0.909
Sex, Female (%)	79.2	81.3	76.5	0.413
Disease duration (years)	14.53 (7.97)	13.68 (6.59)	15.63 (9.41)	0.090
Age of onset (years)	44.84 (14.15)	45.64 (13,82)	43.78 (14.59)	0.360
RF positivity (%)	61.7	60.2	63.8	0.623
Anti-CCP positivity (%)	67.9	69.4	65.9	0.604

MTX: methotrexate; RF: rheumatoid factor; anti-CCP: anti-cyclic citrullinated peptide antibodies. Values are given as percentage or mean (standard deviation).

Table III. Clinical and demographic characteristics of the RA patients on MTX monotherapy included in the study.

Variable	Total (n=68)	MTX responders (n=48)	MTX non responders (n=20)	*p*-value
Age (at inclusion) (years)	61.5 (13.2)	63.5 (12.2)	56.7 (14.5)	0.079
Sex, Female (%)	72.1	77.1	60.0	0.129
Disease duration (years)	13.91 (8.11)	12.60 (5.87)	17.00 (11.43)	0.176
Age of onset (years)	47.81 (15.43)	51.19 (13.97)	39.70 (16.09)	0.006*
RF positivity (%)	60.34	62.5	55.5	0.414
Anti-CCP positivity (%)	61.8	66.7	50	0.155

MTX: methotrexate; RF: rheumatoid factor; anti-CCP: anti-cyclic citrullinated peptide antibodies. Values are given as percentage or mean (standard deviation). *indicates *p*-value <0.05.

which is in agreement with other studies published before. No association was observed between C677T genotype and the geographical origin of the sample (Italy or Hungary) (p=0.222).

In a first statistical approach, the association between C677T genotype and the fact of discontinuing MTX treatment was studied (Table IV). No association was found in the overall population (p=0.375), whereas when studying patients treated with MTX monotherapy alone, an almost significant association was detected in the recessive model (p=0.057). No associations were found with the allelic, genotypic, additive, or dominant genetic models (data not shown).

In a second statistical approach, the time to MTX discontinuation was compared between C-allele carriers and TT homozygous individuals. A Kaplan-Meier analysis showed no relationship between the C677T genotype under a recessive model and MTX treatment discontinuation when studying all the RA patients (p=0.324, Fig. 1A), nor when analysing only those patients with a combined therapy (p=0.955, Fig. 1B). In contrast, a significant difference between genotypes was found in the time to MTX treatment discontinuation when including only patients on MTX monotherapy. In fact, patients carrying the C-allele showed a higher probability of MTX monotherapy continuation compared with patients with a TT genotype (p=0.042, Fig. 1C).

Discussion

In the present work, we have studied the association of C677T polymorphism (rs1801133) in the *MTHFR* gene with the discontinuation of MTX treatment in a European RA population. We have observed a borderline significant association of the TT genotype with MTX monotherapy discontinuation, as well as a significant difference in the time to MTX monotherapy withdrawal between RA patients with TT and CT/CC genotypes.

One of the main strengths of this work is that patients from two different coun-

Table IV. Frequency distributions, odds ratios (OR) and univariate association *p*-values for discontinuation of MTX therapy by genotype for SNP rs1801133 on MTHFR gene.

	MTHFR, C667T (rs1801133) Genotype frequencies, n (%)			Frequency of risk genotype-carriers*, n (%), and ORs			
Total cohort (n=197)	CC	CT	TT	TT*	CC or CT	OR (95% CI)	*p*-value
Responders	42 (37.5)	53 (47.3)	17 (15.2)	17 (15.2)	95 (84.8)	1.40 (0.67 – 2.93)	0.375
Non responders	33 (38.8)	35 (41.2)	17(20.0)	17 (20.0)	68 (80)		
Total	75 (38.1)	88 (44.7)	34 (17.2)	34 (17.2)	163 (82.8)		
MTX combined therapy (n= 129)	CC	CT	TT	TT*	CC or CT	OR (95% CI)	*p*-value
Responders	29 (45.3)	25 (39.1)	10 (15.6)	10 (15.6)	54 (84.4)	0.982 (0.38-2.55)	0.969
Non responders	28 (43.1)	27 (41.5)	10 (15.4)	10 (15.4)	55 (84.6)		
Total	57 (44.2)	52 (40.3)	20 (15.5)	20 (15.5)	109 (84.5)		
MTX monotherapy (n= 68)	CC	CT	TT	TT*	CC or CT	OR (95% CI)	*p*-value
Responders	13 (27.1)	28 (58.3)	7 (14.6)	7 (14.6)	41 (85.4)	3.15 (0.93-10.67)	0.057
Non responders	5 (25.0)	8 (40.0)	7 (35.0)	7 (35.0)	13 (65.0)		
Total	18 (26.5)	36 (52.9)	14 (20.6)	14 (20.6)	54 (79.4)		

MTX: methotrexate; OR: odd ratio; CI: confidence interval.

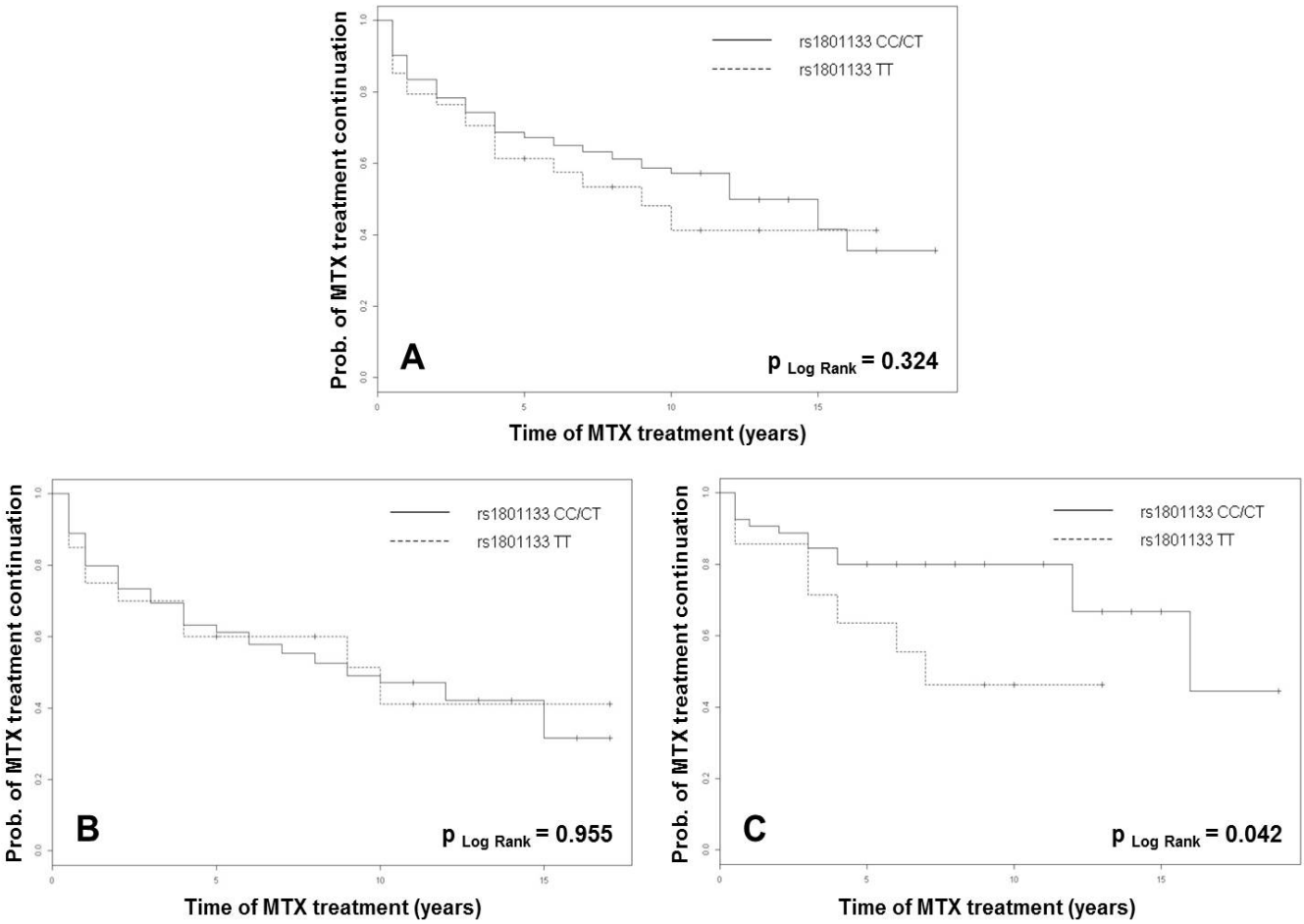

Fig. 1. Kaplan-Meier analysis of time to MTX treatment discontinuation of RA patients by the C677T polymorphism in MTHFR under a recessive genetic model. **A.** Analysis including all recruited RA patients (TT n=34; CT/CT n=163); **B.** Analysis with those patients on a combined therapy (TT n=20; CT/CT n=109); **C.** Analysis with those patients on MTX monotherapy (TT n=14; CT/CT n=54).

tries have been included. The lack of significant differences among countries for the analysed features allowed the combination of the two patient populations, obtaining results of more general clinical interest. In addition, Kaplan-Meier analysis allowed us to include patients that started therapy at different times and with different disease duration (14.5 years on average). It is one of the best options to be used to measure the fraction of subjects having an outcome after a certain period of time, and it also allowed us comparing curves for two different groups of patients by means of the Log Rank test, allowing for those observations that are censored. Many studies, including our previous work with a Spanish population, have found significant associations of the C677T polymorphism with either MTX toxicity (11, 17, 18, 21, 24-27) or efficacy (28, 29), whereas some others have also described a lack of association (30-34). Several important differences in terms of clinical study design exist among previous studies on this subject.

Firstly, MTX can be prescribed as monotherapy or in combination with other agents, including biologics, and over the last two decades drug combinations based on MTX have been used increasingly to treat patients with RA (10, 35). It has been suggested that the combination of different treatments might add variability to the results (1). Most studies that did not detect associations were carried out with patients in combined therapies (30-34). Our findings further support the restricted inclusion of patients on monotherapy in order to reduce the variability and obtain conclusive results. In fact, among the 197 patients enrolled, only 68 have been prescribed with MTX monotherapy, and interestingly, significant genotype-MTX response associations have only been found in this patient subgroup.

A second important issue that needs to be considered as causative of such controversial results is the inconsistency in patient outcome criteria and measurements. Different populations have been studied using varying definitions of drug toxicity and efficacy, which might lead to selection bias (1, 4, 36). Our study has defined MTX responders as patients who did not discontinue treatment, and non responders as those who discontinued MTX therapy, regardless of whether it was due to inefficacy or toxicity, as previously reported by Plant *et al*. (23). Considering the elevated cost of alternative treatments, it is highly unlikely that patients interrupt MTX treatment for reasons different from lack of efficacy or adverse effects. Addition-

ally, we consider that from a clinical point of view, discontinuation of MTX treatment is a more relevant and objective endpoint than other specific definitions of toxicity or inefficacy, since the clinician would need to prescribe another drug in any case. Furthermore, these outcome criteria would facilitate the comparison of results between different studies.

Thirdly, the discrimination between heterozygote and homozygote genotypes was also different among published studies, since several did not find individuals with two copies of the variant allele (TT) (12). The meta-analysis by Owen *et al*., for example, was performed using a dominant model (CC *vs* CT, TT) in order to allow the inclusion of more studies (1). However, *in vitro* studies of MTHFR enzyme activity showed that heterozygotes (CT) have 60% *in vitro* enzyme activity, and the homozygous variant TT genotype has only 30% of the homozygous wild-type (CC) enzyme activity (10), suggesting that it might be important to analyse homozygous TT patients separately in order to find more obvious associations. In accordance with this, we only found statistically significant associations under the assumption of a recessive model, this is, when patients genotyped as TT were compared with the remaining. We obtained very similar results in our previous study with a Spanish cohort of RA patients (21), and some other authors also reported similar findings (11, 28, 37, 38).

In addition to the genetic association analyses, demographic and clinical features have also been analysed within this study and no significant association of any of them with MTX discontinuation has been identified with the overall population. However, when the analysis was restricted to patients on MTX monotherapy, a significant association was observed between the age at RA disease onset and MTX discontinuation, a younger age of onset being related to a higher probability of MTX treatment interruption. Interestingly, we obtained a very similar result in our previous study with the Spanish population, where MTX intolerant patients were on average 4 years younger at disease onset (21). Tutuncu *et al*. (39) also reported that toxicities related to MTX were seen more frequently in a group of patients with a younger-onset RA compared to the elder-onset RA group, but they hypothesised that it could be attributable simply to the fact that the patients with a younger disease onset were on higher dose of MTX. We lack the information on MTX dosage to try to evaluate this hypothesis, but it would be interesting to further explore this association in the future.

Despite the clinical relevance of these results, further prospective studies would provide additional evidence of the specific impact of *MTHFR* gene variants on MTX discontinuation. However, Yazici has recently stated some advantages of retrospective studies over randomised controlled trials, since they do not require pre-selection of patients and the time of evolution from the first MTX dosage application can be longer (40). It would also be interesting to explore this genotype-outcome association including a significant number of RA patients from different ethnic groups, since our study was restricted to Caucasians. Moreover, the independent analysis of RA patients with a TT genotype and on MTX monotherapy reduced the sample size of the analysis, which limited its statistical power. Finally, since more *loci* are increasingly being found involved in MTX pharmacogenetics (6), it would be useful to explore their effect in our RA population in order to obtain a combination of genes that better selects the patients that would benefit from a MTX treatment.

In summary, our results confirm an increased probability of MTX monotherapy discontinuation for RA patients carrying the homozygous 677T variant allele in the *MTHFR* gene.

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