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Genetic polymorphisms in key methotrexate pathway genes are associated with response to treatment in rheumatoid arthritis patients

S A Owen[1], **S L Hider**[2], **P. Martin**[1], **I N Bruce**[1], **A Barton**[1], and **W Thomson**[1]

[1]Arthritis Research UK Epidemiology Unit, Manchester Academic Health Sciences Centre, The University of Manchester, Stopford Building, Manchester, UK.

[2]Arthritis Research UK, National Primary Care Centre, Keele University, Staffordshire, United Kingdom

Abstract

We investigated the effect of Single Nucleotide Polymorphisms (SNPs) spanning 10 methotrexate (MTX) pathway genes, namely *AMPD1*, *ATIC*, *DHFR*, *FPGS*, *GGH*, *ITPA*, *MTHFD1*, *SHMT1*, *SLC19A1* (RFC) and *TYMS* on the outcome of MTX treatment in a UK rheumatoid arthritis (RA) patient cohort. Tagging SNPs were selected and genotyping performed in 309 patients with predefined outcomes to MTX treatment. Of the 129 SNPs tested, 11 associations were detected with efficacy (p-trend ≤ 0.05) including four SNPs in the *ATIC* gene (rs12995526, rs3821353, rs7563206 and rs16853834), 6 SNPs in the *SLC19A1* gene region (rs11702425, rs2838956, rs7499, rs2274808, rs9977268, rs7279445) and a single SNP within the *GGH* gene (rs12681874). Five SNPs were significantly associated with adverse events; three in the *DHFR* gene (rs12517451, rs10072026, and rs1643657) and two of borderline significance in the *FPGS* gene. The results suggest that genetic variations in several key MTX pathway genes may influence response to MTX in RA patients. Further studies will be required to validate these findings and if confirmed these results could contribute towards a better understanding of and ability to predict MTX response in RA.

Keywords

Rheumatoid Arthritis; Methotrexate; polymorphism; pharmacogenetics; ATIC; RFC

*Correspondence to Address: [1]Arthritis Research UK Epidemiology Unit, Manchester Academic Health Sciences Centre, The University of Manchester, Stopford Building, Manchester, M13 9PT, UK Tel: 0044-161-275-1638 Fax: 0044-161-275-5043 Wendy.Thomson@manchester.ac.uk.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic disabling disease, requiring long-term treatment. Drug therapy is a key component of the treatment pathway and disease modifying anti rheumatic drugs (DMARDs) provide the mainstay of therapy, with mounting evidence suggesting that earlier treatment with DMARDS offers benefits in the longer term [1, 2]. There are several DMARDs available, but in clinical practice methotrexate (MTX) is increasingly recognised as the anchor drug for the treatment of RA [3-6]. This is because of substantial clinical experience, established efficacy, superior continuation rates, affordability and the fact that treatment with MTX not only reduces disease activity in the short term, but can also delay or stabilise the development of bone erosions in some patients over the longer term [7, 8]. Nevertheless, there is still significant variability in patient responses to treatment, with an estimated one third of patients failing to respond to MTX either due to lack of efficacy or adverse events (AE) [9-11]. As a result of this inter-patient variability in response and the fact that no predictive tests are available, routine blood and liver function testing are required in clinical practice that can be costly and inconvenient for patients [12]. For these reasons MTX represents an interesting target for pharmacogenetic testing, to identify response predictors that could maximise response and minimise toxicity.

Treatment response is a complex multi-factorial trait with various contributing factors, including individual patient factors (age, sex, ethnicity, co-morbidities), disease specific factors (disease duration, severity, activity) and genetic factors [1, 13-15] A complex interplay of several genes encoding proteins involved in drug uptake and disposal, absorption, retention, distribution, metabolism and interaction with cellular targets can influence drug actions and thus these genes represent logical targets for pharmaocogenetic testing. The actual mechanism of action of low-dose MTX, used in the treatment of RA, is still not fully understood, but it is thought that the anti-inflammatory effects, mediated by adenosine release, are more important than the anti-proliferative effects [16-18]. In order to enter the cell, MTX is internalised by the reduced folate carrier (SLC19A1/RFC), with impaired transport correlated with MTX resistance [19, 20]. Once internalised, MTX requires intracellular polyglutamation, controlled by the polyglutumation-deconjugation cycle which is instigated by the enzymes γ- folypolyglutamate synthetase (FPGS) and glutamyl hydrolase (GGH), respectively [21]. It is these active MTX-polyglutamates (MTX-Pg) that determine MTX functional status and play an important role in directly suppressing various enzymes, such as dihydrofolate reductase (DHFR), thymidylate synthase (TYMS), 5-aminoimidazole 4-carboximide ribonucleotide (AICAR) transformylase (ATIC) and having an indirect effect on methylenetetrahydrofolate reductase (MTHFR).

The association of polymorphisms in these genes with MTX response have been described by various groups, although many of these studies have tested isolated polymorphisms within a few genes relevant to MTX metabolism. Our aim was to examine several important MTX pathway genes to determine if single nucleotide polymorphism (SNP) markers, selected to comprehensively cover the genes, were associated with MTX treatment response outcomes in a well-characterised group of patients with established RA.

PATIENTS AND METHODS

Study subjects and outcomes

Details of the patients included in this study and the methods by which they were recruited are outlined elsewhere [22]. In brief, all subjects were considered eligible for inclusion if they had taken MTX monotherapy for at least 3 months for RA, aged over 18 years, of white Caucasian ethnic origin and classified as having RA according to the ACR 1987 criteria [23]. The patient cohort was recruited retrospectively from two hospitals: The University Hospital of North Staffordshire (UHNS) and Central Manchester NHS Foundation Trust (CMFT). Patients were identified either via an electronic database (UHNS) or case note review (CMFT) (Table 1). Eligible patients had to fulfill one of three defined outcomes to MTX: (i) good responder (physician statement of good response plus a stable dose of MTX for at least 6 months, with an ESR <20 and/or normal CRP); (ii) inefficacy failure (physician statement of inefficacy plus failure to reduce ESR and/or CRP by at least 20% with MTX therapy for at least 3 months at a minimum dose of 15mg/wk) or (iii) AE failure (AE had to be persistent or serious and lead to treatment cessation: verified by medical record review. Furthermore, the AE had to resolve on treatment cessation and, in the case of GI AE, recur after MTX re-challenge). Individuals that did not meet one of these defined outcomes were not included in the study. Ethical approval for the study was obtained from North Staffordshire LREC (Ref 03/20) and Central Manchester LREC (Ref 03/CM/315) and subjects written consent was obtained according to the Declaration of Helsinki.

Selection of single-nucleotide polymorphisms (SNPs) and genotyping

Ten candidate genes were selected for study on the basis of putative involvement in the MTX metabolic pathway and previous evidence from the literature. They included genes involved in MTX cellular influx (*SLC19A/RFC*), polyglutamation (*GGH, FPGS*), folate pathway (*DHFR, SHMT1, MTHFD1*), purine synthesis (*ATIC),* pyrimidine synthesis (*TYMS*), adenosine pathway (*AMPD1, ATIC*) and *ITPA* (Supplementary Figure 1).

For each gene SNPs were selected based on a pair wise tagging SNP approach, supplemented with other commonly investigated SNPs from the literature. Marker coverage of each gene was extended to include the 10-kb upstream and downstream flanking region. Tag SNPs for each gene were selected from the CEPH/CEU Hapmap dataset (release 22) (http://www.hapmap.org) and this downloaded SNP data was then filtered through the *Haploview* software (http://www.broad.mit.edu/haploview/ [24] and pair-wise tagging SNPs (r^2 cut off > 0.8 and MAF >5%) were selected for genotyping**.** In addition to the tag SNPs identified, we also included additional SNPs in each gene in case of SNP failure, 7 duplicate SNPs for quality control purposes and one 28 base pair variable number tandem repeat (VNTR) located in the 5′UTR of the *TYMS* gene.

Genotyping

SNP genotyping was performed using the Sequenom iPLEX® MASS ARRAY platform according to the manufacturer's instructions (Sequenom San Diego, CA http://www.sequenom.com). Genotyping for the VNTR was performed in a 5 μl reaction volume, using primer sequences as described by Zhang *et al* [25]. Amplicons were electrophoresed

through a 3% agarose gel and visualised with ethidium bromide staining. Quality control (QC) procedures before analysis were used such that 80% sample and polymorphism genotyping success rate was required and any samples and polymorphisms failing to meet this threshold were removed from further analysis.

Statistical analysis

Each polymorphism was tested for association with MTX efficacy and AE. Genotype and allele frequencies were compared between the groups and analysed as a nested case-control study with the responders as the referent category, using STATA version 9.2 software (StataCorp, Texas, USA) and the $\chi 2$ test for trend implemented in PLINK [26]. Two different analyses were conducted; 1) 'responders vs inefficacy failures' and 2) 'responders vs AE failures'. The threshold for significance was defined at trend $p \leq 0.05$ and associations were expressed as trend p values, allelic odds ratios (ORs) and their 95% confidence intervals (CIs). Where variants were significantly associated with a trend $p \leq 0.05$, tests of inheritance of the minor allele under dominant, recessive and multiplicative models were also conducted. Deviation from the Hardy–Weinberg equilibrium (HWE) in the responders group was tested using a chi-squared test with a threshold of $p \leq 0.05$.

Haplotype analysis and association was performed using Haploview software and conditional logistic regression was used to determine whether independent genetic effects existed [24].

RESULTS

Patient characteristics

The clinical and demographic features of the subjects included in the analysis have been described previously and are also presented in table 1 [22]. Subjects with established RA treated with MTX monotherapy were included in the study (median age at MTX onset=54.2 years); folic acid supplementation was taken by all patients and NSAIDs were allowed. Analysis of response to MTX was based on 309 patients comprising a maximum 147 good responders (48 %), 101 inefficacy failures (33 %) and 61 adverse event failures (19 %). The observed AEs included; gastrointestinal (n=24), abnormal liver function (n=20), and other AE's (n=17) (Other AE's comprising: haematological (n=7), skin rashes (n=6), renal (n=1), headaches and pneumonitis (n=3)).

Genetic association

145 SNPs were selected for testing within 10 genes. 7 duplicate SNPs were shown to be concordant; these SNPs were removed from analysis along with a further 9 SNPs, which failed QC, based on a genotyping success of <80% and deviation from HWE ($p<0.05$). This left 129 SNPs for further analysis. Of the 129 SNPs analysed, eleven SNPs in 3 gene regions (*ATIC, GGH, SLC19A1/RFC*) were found to be significantly associated (trend $p \leq 0.05$) with MTX efficacy and five SNPs in two genes (*DHFR* and *FPGS)* with AE (table 2 and 3). Overall no significant associations were detected with the genes*, SHMT1, MTHFD1, AMPD1, ITPA* and *TYMS* and MTX related efficacy and toxicity (supplementary table 1)

Association with MTX efficacy

Four of the SNPs associated with MTX inefficacy (trend p≤ 0.05) lie within the *ATIC* gene: Two SNPs in high LD using genotype data from our cohort (rs12995526 & rs7563206, r^2 = 0.98) and a single SNP (rs16853834) were associated with a poor response to MTX (OR 1.65, 95%CI 1.13-2.42, OR 1.60, 95%CI 1.10-2.33 and OR 1.70 95%CI 1.02-2.82 respectively) and one SNP (rs3821353) with a good response to MTX (OR 0.51, 95%CI 0.31-0.84) (Table 2). Six SNPs within the *SLC19A1*/*RFC* gene region were associated with a poor response to MTX: two SNPs in high LD (rs2274808 & rs9977268, r^2=0.86), rs2838956 & rs7499 (r^2=0.74) and rs11702425 and one with borderline significance, rs7279445 (Table 2). Finally one SNP within the *GGH* gene (rs12681874) was associated with a good response to MTX (OR 0.54 95% CI 0.30-1.00).

In the *ATIC* and *SLC19A1 (RFC)* genes, as some SNPs were in high LD, we used conditional logistic regression to determine if the associations were independent of each other. The significantly associated markers were conditioned against the effect of the most significant marker in the gene but there was no evidence of independence demonstrated in this sample (data not shown). Similarly, haplotype analysis did not reveal haplotypic effects (data not shown).

Association with MTX toxicity

With regard to MTX related toxicity, two SNPs (rs10072026 and rs1643657) within the *DHFR* gene region were associated with a reduced risk of MTX related AE (OR 0.43 95%CI 0.19-0.99 & OR 0.60 95%CI 0.39-0.99) and another (rs12517451) showed evidence of association with an increased risk of AE (OR 1.68, 95%CI 1.03-2.75). In addition two SNPs in high LD (r^2=0.96) within the *FPGS* gene (rs1054774 & rs44511422) showed borderline evidence of association with an increased risk of AE (Table 3). Both SNPs in the *FPGS* gene (rs1054774 & rs4451422) were highly significant under a recessive model of inheritance (OR 3.03, 95% CI 1.14-7.99 and OR 3.60, 95% CI 1.39-9.33 respectively) (Table 3).

Replication of previous pharmacogenetic results

Twelve of the 145 SNPs were included in order to validate previously reported associations from the literature (Table 4). Of these, one SNP in the *DHFR* gene (rs1650697) for which a proxy SNP (rs12517451) (r^2 =1.0) was genotyped provided evidence of association with AEs (OR= 1.68, 95%CI 1.03-2.75) and two SNPs in the *FPGS* gene (rs1544105 & rs10106) for which proxies were genotyped showed borderline evidence of association (trend p = 0.06) with an increased risk of AE, with a further increased risk with carriage of two copies of the minor allele under a recessive model (Table 4). No significant associations were revealed with any of the other previously reported SNPs (Table 4).

DISCUSSION

The ability to individually tailor MTX treatment to meet individual patient's needs remains an important goal and would be valuable if applied in clinical practice. Our study has identified 16 SNPs, some novel and others replicating previous findings, in 5 key MTX metabolic pathway genes which show evidence for association with MTX efficacy or AE's

in this cohort of RA patients. No significant associations or replications of previous associations of SNPs within the genes, *SHMT1, MTHFD1, AMPD1, ITPA,* and *TYMS* with MTX efficacy or AE's were found.

There have been a number of studies conducted to determine MTX response in RA patients, with the majority adopting a candidate gene approach, genotyping isolated SNPs within the gene and testing for association. Our study also focused on candidate genes in the MTX metabolic pathway, partly because this approach has proven successful for a number of other common treatments. One of the best examples is the identification of polymorphisms in the vitamin K (*VKORC1*) and cytochrome p450 (*CYP2C9*) genes which influence response to warfarin where findings have since been validated in independent studies [27-30]. Our study had the additional strength that, rather than testing single SNPs in specific genes, we systematically screened selected MTX pathway genes ensuring gene coverage following quality control measures exceeded 85% when compared to the HapMap data. In this way, we can confidently exclude association with a number of genes in our cohort for effect sizes >1.5. This was a retrospective study and patients were recruited to the study based on phenotypes defined using the data available in the patients notes. We set out to define phenotypes for both inefficacy and AE's in order to minimise variation and maximise the power to detect significant genetic effects. Furthermore, all of the patients were recruited within a well-defined geographical area and comprised an ethnically homogenous patient population.

Despite these strengths, our study has several limitations: Firstly, incomplete knowledge of the MTX metabolic pathway means that we may have failed to screen some important genes and although we have found several SNPs to be associated with either efficacy or AE in this study; it is likely that combinations of risk SNPs will be more predictive of response to MTX than individual SNP effects, as shown in previous studies [31-33]. It would be interesting to look at combinations of the polymorphisms reported here in an independent patient cohort. Secondly, the sample size was modest (n=309); although this represents one of the largest cohorts studied to date in the context of MTX pharmacogenetics in RA, the power to detect effect sizes less than 1.5 was consequently limited. Thirdly, retrospective data collection can introduce biases: this can be due to limitations of missing information on important clinical variables that are thought to be predictive of response to MTX, for example, disease specific factors, co-morbidities or folate. Thus, apart from folic acid, which all patients received at a dose of at least 5mg/week, we did not adjust for clinical factors in the analysis. Phenotypes were pre-defined using the data available in the medical notes and a broad term was used to define good responders and inefficacy failures that took into account physician statement, inflammatory markers (CRP and ESR), dose of MTX and time on treatment. Whilst these criteria were selected to identify those with well-validated response measures, many researchers now advocate using the DAS score, because it is used clinically to base treatment decisions. Unfortunately, the DAS information was not available for many patients in the current cohort. This remains an area of ongoing debate for pharmacogenetic studies, with a need to standardise outcome measures and should be a consideration for future prospective studies, including other important patient reported outcomes, measures of disability and psychological outcome measures. Finally ensuring good gene coverage inevitably results in multiple testing and a consequent increased likelihood of false positive

findings. Given that 129 SNPs have been tested for both response and inefficacy, a Bonferroni corrected p-value threshold of $p < 2 \times 10^{-4}$ could be used as the threshold for claims of significance. At this threshold, none of the SNPs would have remained statistically associated with response. We present the uncorrected p-values because, due to the limited sample size, the fact that corrective procedures may unnecessarily reduce power and a proportion of our positive findings do support those from previous studies suggesting that the effects seen may be true. For example, we report borderline association with a SNP (rs1054774) within the *FPGS* gene with MTX AE. Interestingly a SNP in high LD with our associated SNP, namely rs1544105, has been associated with response in a previous study [34, 35]. Also, a SNP mapping to the *DHFR* gene (rs1650697) and perfectly correlated (r^2= 1) with rs12517451, which we report to be associated with AE, specifically liver AE (data not shown), was associated with the occurrence of hepatitis in RA patients in an independent study [36]. Finally, a SNP mapping to the *ATIC* gene and tested in this RA cohort for the first time has been reported to be associated with MTX in a JIA cohort as described later. In terms of MTX efficacy, several studies have investigated the role of the *SLC19A1* (RFC) gene, focusing on the *RFC* 80A/C non-synonymous SNP (rs1051266), which results in a substitution of arginine to histidine at codon 27 in the first transmembrane domain of the RFC protein [34, 36-43]. Our results did not replicate previous findings of association with this particular SNP, in keeping with the results from some studies [34, 36, 39, 42]. We did however find six other SNPs both in the *RFC* gene and a neighbouring gene *COL18A1* which associated with MTX efficacy. As the *RFC* gene is highly polymorphic in humans and shows strong LD across the gene, some of these variants were correlated with other less commonly reported polymorphisms in the RA literature [42] . This may well suggest that the overall contribution of polymorphisms on phenotype cannot be explained by the most commonly reported SNP (rs1051266) alone, but that other variants within the gene may also be important influencing response to MTX.

Several previous RA studies have reported a SNP (rs2372536) located in exon 5 of the *ATIC* gene, leading to a threonine to serine substitution, to be associated with both response and AE to MTX [31, 32, 36, 44]. Our finding of no association with this SNP and MTX response is in keeping with those of Takatori *et al* and Sharma *et al* [35, 39]. Differences seen with the present study and others could be explained by inter study variability; small sample sizes; comparing patients with different stages of disease (early onset [45] vs established RA [44], different outcomes to measure efficacy and AE and different treatment regimes However, we have found several other SNPs in the *ATIC* gene to be associated with response (efficacy) to MTX in this study and one of these (rs12995526) has also been found to be associated with MTX response in two independent patient cohorts with juvenile idiopathic arthritis (JIA) with a similar magnitude of effect [46]. Increasing evidence suggests that the anti-inflammatory role mediated by adenosine is key to MTXs mode of action and furthermore previous work reported from our group has demonstrated the importance of adenosine receptors and response to MTX [22]. The fact that SNPs in the same gene are showing evidence for association with MTX response in different cohorts suggest that this gene may play a role in determining efficacy. It is possible that multiple variants within the gene contribute or there may be a single causal variant but different LD patterns in different populations may mean that different SNPs show evidence for association.

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In summary, results from this study replicate some previous findings reported in the literature and at the same time we report associations between several MTX pathway genes, namely *GGH, ATIC, DHFR* and *SLC19A1 (RFC)* and either efficacy or AE in MTX-treated RA patients. In particular there is growing evidence to support the role of the *ATIC* gene in the response to MTX treatment. Many of the SNPs reported here reside in non-coding regions, therefore impact on functional properties of the gene are unknown and thus no specific biological mechanisms can be proposed. Further investigation in a larger, prospectively collected cohort with well-defined outcome and clinical measures is required to confidently confirm the association of these SNPs with MTX treatment response. Once confirmed, fine mapping will be needed to determine the actual causal variant. One of the most important applications will be in providing biological insight into the mechanisms by which some patients respond to MTX whilst others do not.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Patients demographics and baseline characteristics

	Responders	IE failure	AE failure
Number patients (%)	147 (48)	101 (33)	61 (19)
Age at diagnosis in years median (range)	50.1 (41.2-58.8)	46.4 (35.9-53.5)	44.9 (39-56.8)
Gender: female (%)	103 (70):	77 (76.2):	50 (82)
Age at MTX start in years median (range)	57.4 (49.6-64.6)	52.8 (46.3-59.3)	52.2 (46.5-60.7)
RF +ve status * (%)	99 (67.4)	76 (75.3)	45 (73)
Erosions § (%)	115 (78)	93 (92)	52 (87)
Copies SE * (%) 0	27 (19)	15 (15)	13 (22)
1	66 (47)	40 (41)	24 (41)
2	48 (34)	43 (44)	22 (37)
No previous DMARDs median (range)	2 (0-3)	2 (1-3)	2 (2-4)
MTX first DMARD (%)	40 (27.2)	21(21.8)	7 (11.5)

SE= Shared epitope, RF= rheumatoid factor, DMARD= disease modifying anti-rheumatic drug, MTX= methotrexate,, AE= adverse event, IE= inefficacy,

* n= 297 with information,

§ n=301 with information

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Table 2

SNPs significantly associated with MTX efficacy (p trend ≤ 0.05)

				MAF (%)		Genotype frequencies (%)							
Gene	**SNP**	**Base Pairs**	**SNP position**	**R**	**IE**	**Responders**			**Inefficacy failures**			**Trend p**	***Allelic OR (95%CI)**
		(1/2)				**1_1**	**1_2**	**2_2**	**1_1**	**1_2**	**2_2**	**R vs IE**	
ATIC	rs7563206	C/T	Intron	42.4	54.3	45 (32.4)	70 (50.4)	24 (17.3)	20 (21.3)	46 (48.9)	28 (29.8)	0.01	1.60 (1.10-2.33)
	rs3821353	G/T	Intron	24.4	14.4	83 (59.7)	44 (31.7)	12 (8.6)	67 (71.3)	27 (28.7)	0 (0.0)	0.009	0.51 (0.31-0.84)
	rs12995526	C/T	Intron	42.4	54.9	45 (34.1)	62 (47.0)	25 (18.9)	20 (22.0)	42 (46.2)	29 (31.9)	0.01	1.65 (1.13-2.42)
	rs16853834	C/T	Exonic 5′UTR	13.9	21.5	96 (73.8)	32 (24.6)	2 (1.5)	54 (62.8)	27 (31.4)	5 (5.8)	0.04	1.70 (1.02-2.82)
GGH	rs12681874	C/T	Intron	16.9	10.0	104 (78.2)	25 (18.8)	4 (3.0)	77 (86.5)	9 (10.1)	3 (3.4)	0.04	0.54 (0.30-1.00)
SLC19A1	rs11702425[#]	T/C	Exon	26.9	40.7	75 (53.2)	56 (39.7)	10 (7.1)	34 (35.1)	47 (48.5)	16 (16.5)	0.001	1.86 (1.26-2.74)
	rs2838956	A/G	Intron	38.3	47.4	52 (36.9)	70 (49.6)	19 (13.5)	24 (24.7)	54 (55.7)	19 (19.6)	0.04	1.45 (1.00-2.10)
	rs7499	G/A	Exonic 5′ UTR	35.4	45.3	59 (41.8)	64 (45.4)	18 (12.8)	27 (28.1)	51 (53.1)	18 (18.8)	0.02	1.50 (1.03-2.19)
	rs2274808[#]	C/T	Intron	21.8	33.0	88 (62.9)	43 (30.7)	9 (6.4)	45 (46.4)	40 (41.2)	12 (12.4)	0.009	1.76 (1.17-2.67)
	rs9977268[#]	C/T	Intron	18.9	28.1	95 (67.9)	37 (26.4)	8 (5.7)	52 (54.2)	34 (35.4)	10 (10.4)	0.02	1.67 (1.08-2.58)
	rs7279445[#]	C/T	Intron	45.0	54.2	43 (30.5)	69 (48.9)	29 (20.6)	18 (18.8)	52 (54.2)	26 (27.1)	0.05	1.44 (0.99-2.08)

[*] based on carriage of the minor (rare) allele;

[#] = In An overlapping gene *COL18A1* MAF= minor allele frequency, R= Responder, IE= Inefficacy failure to MTX, CI= Confidence Interval 1_1= major allele homozygote 1_2= heterozygote 2_2= minor allele homozygote

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Table 3

SNPs significantly associated with MTX related AE (p trend ≤ 0.05)

				MAF (%)		Genotype frequencies (%)							
Gene	**SNP**	**Base pairs**	**SNP Position**	**R**	**AE**	**Responders**			**Adverse events**			**Trend p**	***Allelic OR (95% CI)**
		(1/2)				**1_1**	**1_2**	**2_2**	**1_1**	**1_2**	**2_2**	**R vs AE**	
DHFR	rs12517451	C/T	Intron	20.0	29.7	91 (65.0)	42 (30.0)	7 (5.0)	32 (54.2)	19 (32.2)	8 (13.6)	0.04	1.68 (1.03-2.75)
	rs1643657	A/G	Intron	31.0	21.2	66 (48.2)	57 (41.6)	14 (10.2)	36 (61.0)	21 (35.6)	2 (3.4)	0.04	0.60 (0.39-0.99)
	rs10072026	T/C	Exonic 3′UTR	12.7	5.9	107 (75.9)	32 (22.7)	2 (1.4)	53 (89.8)	5 (8.5)	1 (1.7)	0.04	0.43 (0.19-0.99)
FPGS	rs1054774	A/C	5′ gene	39.6	49.0	48 (35.8)	67 (50.0)	19 (14.2)	16 (30.8)	21 (40.4)	15 (28.8)	0.06	1.52 (0.98-2.34)
	rs4451422	T/A	Exonic 5′ UTR	40.0	49.0	46 (34.3)	69 (51.5)	19 (14.2)	15 (28.8)	23 (44.2)	14 (26.9)	0.06	1.49 (0.97-2.30)

*based on carriage of the minor (rare) allele, MAF= minor allele frequency, R= Responders AE= Adverse events to MTX, CI= Confidence Interval 1_1= major allele homozygote 1_2= heterozygote 2_2= minor allele homozygote

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Table 4

Results for SNPs previously found to show evidence of association with MTX response

Gene (ref)	SNP from literature	Reported assoc	Position	Proxy SNP typed	MAF			R vs AE		R vs IE	
					R	AE	IE	Trend p	*Allelic OR (95% CI)	Trend p	*Allelic OR (95% CI)
ITPA [44, 45, 47]	rs1127354	Efficacy	94 C/A		9.6	9.3	8.8	0.93	0.97 0.47-2.02	0.76	0.90 0.48-1.71
ATIC [31-33, 35, 39, 45, 47, 48]	rs2372536	AE/Efficacy	347 C/G		32.8	34.6	32	0.74	1.08 0.67-1.74	0.86	0.94 0.63-1.39
DHFR [36]	**rs1650697**	**AE (Hepatitis)**	**−473 G/A**	**rs12517451**	**20.0**	**29.7**	**23.7**	**0.04**	**1.68 1.03-2.75**	0.35	1.24 0.80-1.93
GGH [49, 50]	rs11545078	Efficacy	452 C/T		10.7	11.9	8.6	0.74	1.12 0.57- 2.20	0.49	0.80 0.42-1.50
AMPD1 [35, 45, 47]	rs17602729	Efficacy	34 C/T		13.2	19.6	12.1	0.13	1.61 0.88-2.94	0.72	0.91 0.51-1.61
MTHFD1 [47, 51]	rs17850560	Efficacy	1958 G/A	rs2236225	46.8	50.8	49.0	0.44	1.18 0.75-1.81	0.64	1.09 0.76-1.56
SLC19A1 [31, 33, 34, 36-40, 42, 43, 52]	rs1051266	AE /Efficacy	80 G/A		41.4	42.0	47.8	0.91	1.02 0.66-1.59	0.17	1.29 0.89-1.89
FPGS [34, 35, 49]	**rs1544105**	**Efficacy**	**G/A**	**rs1054774**	**39.2**	**49.0**	**37.6**	**0.06**	**1.52 0.98-2.34**	0.48	088 0.60-1.29
	rs10106		**1994 A/G**	**rs4451422**	**39.9**	**49.0**	**39.9**	**0.06**	**1.49 0.97-2.30**	0.66	0.99 0.68-1.47
TYMS [31-33, 41, 43, 53-55]	28bp VNTR	AE/Efficacy	N/A		§45.0	46.5	47.9	0.80	1.06 0.69-1.64	0.53	1.12 0.78-1.62
	6bp deletion		N/A		33.5	29.3	29.0	0.43	0.83 0.51-1.32	0.31	0.81 0.54-1.28
SHMT1 [32, 43, 48, 51]	rs1979277	AE/Efficacy	1420 C/T		31.9	29.7	35.6	0.67	0.89 0.56-1.43	0.41	1.17 0.80-1.73

*based on carriage of the minor (rare) allele**; emboldened SNP significant or approaching statistical significance**

§= 2R2R genotype MAF= minor allele frequency, AE= Adverse event failure IE=Inefficacy failure, OR= odds ratio, CI= Confidence Interval, VNTR= Variable number tandem repeat