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RESEARCH ARTICLE

Wang et al: MTX genetics, MTXPGs and RA outcomes

MTX pathway gene variants, erythrocyte methotrexate polyglutamates, and treatment outcomes in rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) exhibits significant inter-patient variability in response to and toxicity from methotrexate (MTX). The clinical utility of erythrocyte methotrexate polyglutamates (MTXPGs) and MTX-pathway pharmacogenetics remains uncertain. This study investigates the relationships between MTX-pathway gene polymorphisms, erythrocyte MTXPG levels, and MTX treatment outcomes in RA. In a single-center, cross-sectional cohort study conducted in southern Fujian from 2017 to 2020, we analyzed 140 Han Chinese RA patients who had been receiving stable low-dose oral MTX (7.5–15 mg/week) for at least three months. Genotyping was performed using MassARRAY, and MTXPG levels 1–6 were quantified in red blood cells via LC-MS/MS. Data on treatment efficacy (measured by ACR20 and clinical scales) and MTX-related adverse drug reactions (ADRs) were collected, with associations analyzed through univariate and multivariable models. MTXPG levels 1–3 were detectable in all patients, while longer-chain MTXPGs were infrequent. The *SLCO1B1* 521T>C polymorphism was independently associated with lower levels of MTXPG1 (B=−1.119), MTXPG2 (B=−0.924), and total MTXPG (B=−0.849), all with P-values ≤0.045. However, MTXPG levels did not correlate with MTX efficacy or ADRs. The *GGH* 401C>T polymorphism was associated with a reduced ACR20 response (OR=0.421, p=0.021) and higher visual analog scale (VAS) and patient global assessment (PGA) scores. Additionally, the variants *SLCO1B1* 521T>C and *ABCB1* 3435C>T were linked to higher scores in the Patient Health Global Assessment (PHGA) and Health Assessment Questionnaire (HAQ). In this low-dose MTX cohort, erythrocyte MTXPGs did not predict clinical outcomes. However, variants in *SLCO1B1*, *GGH*, and *ABCB1* emerged as exploratory candidate markers for MTX response, warranting validation in larger prospective cohorts.

Keywords: Rheumatoid arthritis, methotrexate, genetic polymorphisms, methotrexate polyglutamate.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease, which can damage joints and multiple extra-articular organs [1]. The global age-standardised prevalence and incidence rate of RA increased in varying figures. If not treated in time, symptoms of RA will seriously affect the patient's ability to work and to perform daily activities [2]. Owing to the efficacy and safety profile, affordability, and flexible administration of methotrexate (MTX), several guidelines have recommended MTX as a first-line treatment for RA [3-5]. However, MTX has a considerable inter-patient variability, with 30-50% of patients not achieving remission, and up to 30% suffering adverse reactions (ADRs) that necessitate discontinuation because of toxicity [6, 7]. Multiple studies have indicated that individual differences in drug response may be attributed to intracellular and plasma drug concentrations [8, 9]. Unfortunately, it is difficult to accurately monitor serum MTX concentrations in RA patients because of its rapid decline in plasma [10]. Direct monitoring of MTX concentrations has proven unreliable in predicting clinical outcomes, and the search for more robust biomarkers of MTX treatment response remains ongoing.

In the treatment of RA, a small dose of MTX (7.5-20.0 mg) is often used once a week. When administered subcutaneously or orally, MTX is transported into cells and undergoes polyglutamation to form methotrexate polyglutamates (MTXPGs). γ-glutamyl hydrolase deconjugates MTXPGs in a competing reaction, resulting in an array of chain lengths (MTXPG1-6) [11]. MTXPGs are the active intracellular metabolite of MTX and the average half-life of the MTXPGs is about 1-4 weeks, during which they continuously exert anti-rheumatic effects [12]. MTXPGs accumulate in red blood cells (RBCs) and are easy to detect. Several methods can be employed to determine MTXPG levels in RBCs, among which the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method described recently was capable of directly measuring individual concentrations of MTXPG1-6 [13]. Therefore, studies have reported that MTXPGs are related to drug efficacy and are potential biomarkers for RA treatment response [14, 15]. Nevertheless, potentially because of sample size limitations, ancestry heterogeneity, and other confounding factors, there is ongoing discussion among different studies on whether MTXPG levels could predict the efficacy and safety of MTX in RA patients [14, 16].

As shown in Figure 1, there are several key metabolic enzymes involved in the transport and metabolism of MTX. MTX is transported into the cell via Reduced Folate Carrier 1(RFC1) and Solute Carrier Organic Anion Transporter 1B1(SLCO1B1). Intracellular MTX is catalyzed by Folylpolyglutamate Synthase (FPGS) to produce MTXPGs, which are then hydrolyzed by Gamma-Glutamyl Hydrolase (GGH) back to the parent compound MTX. When MTXPGs are converted back to MTX by GGH, the drug is rapidly transported out of the cell by the ATP-Binding Cassette (ABC) family pump. MTXPGs inhibit Dihydrofolate Reductase (DHFR), thereby preventing the reduction of dihydrofolate to tetrahydrofolate, a reaction that is catalyzed by Methylenetetrahydrofolate Reductase (MTHFR) during one-carbon-unit transfer. Several studies report that genetic polymorphisms in MTX transport and metabolism genes influence treatment response in RA patients, although others have reported paradoxical findings [17-24]. Regarding the impact of genetic polymorphisms in methotrexate pathway genes (GPMTX) on intracellular MTXPG levels, few studies are available and the findings are inconsistent [25-28]. Taking RFC1 80G>A as an example, Ando et al. reported that RFC1 80G>A was significantly associated with the detectability of MTXPG5, whereas an Indian study found no association between this variant and MTXPG levels. Consequently, the relationship between GPMTX and MTXPG levels remains unclear [27, 28]. Hence, we have selected 9 single nucleotide polymorphisms (SNPs) of 8 key genes in the transport and metabolism of MTX, with well-studied but controversial, and we measured MTXPG levels in RBCs that utilized the more specific and sensitive LC-MS/MS method to clarify their respective influences on MTX response and to delineate the genotype–metabolite relationship in RA patients.

MATERIALS AND METHODS

Patients

The study enrolled 140 patients with RA on stable oral MTX pulse therapy at a tertiary hospital from October 2017 to July 2020. These patients were enrolled in our study according to the following inclusion and exclusion criteria. Inclusion criteria: All patients were from southern Fujian and were of Han nationality; had been diagnosed with RA according to the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) [29]; had

received stable oral MTX pulse therapy (7.5-15.0 mg once weekly for ≥3 months, with the concentration of MTX in RBCs reached stability); and had maintained stable concomitant medications for ≥ 4 weeks before enrolment. Hepatic function had to be Child-Pugh class A or B with transaminases ≤ 2 × the upper limit of normal; renal function required an eGFR ≥ 60 mL/min/1.73 m². Exclusion criteria: patients receiving concomitant drugs known to alter MTX pharmacokinetics; those with significant cardiac, hepatic, pulmonary, or renal disease; or individuals unable or unwilling to provide informed consent.

In our study, the sample size was calculated according to the rule of "10 events per variable" [30, 31]. Treatment adherence was an inclusion criterion, and a 10% dropout rate was assumed in the sample-size calculation. Forward calculation estimated that this study would require a sample size of 67 to 156 cases. In addition, in the back-calculation validation, 23 to 78 cases were required in the linear regression and generalized linear models, so our study has a favorable statistical power.

Data collection

Demographic and medication information was collected by online electronic systems. A standard data-collection form was used to record each patient's name, sex, age, ethnicity, height, weight, time from last dose to sampling, treatment regimen, and past medication history. Efficacy was evaluated using the American College of Rheumatology 20(ACR20) diagnostic criteria for RA patients, which divided patients into effective and ineffective groups. Some widely used efficacy evaluation scales including tender joint count 28 (TJC28), swollen joint count 28 (SJC28), visual analogue scale (VAS), patients' global assessment (PGA), physicians' global assessment (PHGA), health assessment questionnaire (HAQ) were also adopted as complementary methods to evaluate. MTX-related ADRs were assessed based on the causality criteria of the National Adverse Drug Reaction Monitoring Centre. Patients whose ADRs were rated as "definite", "very likely", or "possible" were assigned to the ADR group, whereas those rated as "possibly unrelated", " to be evaluated", or "unable to evaluate" constituted the non-ADR group. In this cross-sectional study, the ACR20 response was evaluated as a composite outcome measure based on changes from baseline. The assessment period spanned from the initiation of MTX therapy in patients to the date of MTXPG measurement. All participants were required to have

been maintained on a stable dose of MTX for at least three months prior to the ACR20 assessment, ensuring that MTXPG levels had reached a steady state. Concurrent with the ACR20 assessment, baseline patient characteristics, concomitant medications, and the occurrence of ADRs were collected. ADRs were captured through medical record review, in-person interviews, and questionnaires. In the assessment of ADRs, hepatotoxicity was defined as an elevation in aspartate aminotransferase or alanine aminotransferase levels exceeding the upper limit of the laboratory's normal reference range. A patient was considered to have experienced hepatotoxicity during the period (from the initiation of MTX administration to the date of MTXPG measurement), if they met the hepatotoxicity criteria at least once within that period.

Genetic analysis

The genomic DNA of each subject was extracted from 4 mL of peripheral venous blood that had been stored in an EDTA-coated tube at -80°C. The MassARRAY method that mainly applies the principle of time-of-flight mass spectrometry to directly perform SNP typing was used to detect and analyze the distribution of nine loci of *GGH 401C>T*, *ABCC2 24 C>T*, *MTHFR 677C>T*, *ABCB1 3435C>T*, *ABCC4(rs9516519)*, *MTHFR1298A>C*, *RFC1 80G>A*, *FPGS G>A* and *SLCO1B1 521T>C* in RA patients from southern Fujian. According to the protocol provided by the company, a special DNA extraction kit was utilized to extract from EDTA blood samples. DNA quality was checked by electrophoresis of 5 μL aliquots on 1% agarose gels in 1× TAE buffer at 120-180 V for 15 min. A single, sharp band indicated high-molecular-weight, non-degraded DNA of sufficient concentration for subsequent Polymerase Chain Reaction (Figure S1). MALDI-TOF System (Sequenom, The USA) was employed for SNP genotype detection to obtain genotype data.

MTXPG levels monitoring

For sample pre-treatment, we used milli-Q water for lysis of red blood cells in EDTA anticoagulated whole blood, followed by protein precipitation with perchloric acid [32]. The complete sample pretreatment procedure was as follows: 200 μL of thawed whole blood was mixed with 400 μL of ultrapure water for red blood cell lysis, followed by the addition of 400 μL of a 4% perchloric acid aqueous solution. The mixture was vortexed and centrifuged at 10,000 rpm for 5 minutes. The supernatant

was loaded onto an Oasis MAX solid-phase extraction cartridge, which had been preconditioned sequentially with 1 mL of methanol and 1 mL of water. After sample loading, the cartridge was washed with 1 mL of 5% ammonia and then 1 mL of 100% methanol. The analyte was eluted using 0.8 mL of a methanol-water (6:4, v/v) solution containing 2% formic acid. The eluate was dried under a gentle stream of nitrogen (or: in a water bath) at 40°C. The residue was reconstituted in 100 µL of 0.1% NH_4OH, centrifuged at 10,000 rpm for 5 minutes, and the supernatant was transferred to an autosampler vial for LC-MS analysis.

As reported by Hawwa et al., haematocrit introduced <5% error into MTXPG concentration measurements. Therefore, the influence of haematocrit and RBC count was deemed negligible [33]. These authors also demonstrated that MTXPGn remained stable at -80 °C and 25°C for two months, confirming adequate stability for our analytical procedure. Hence, no in-house stability testing was performed [33]. Steady-state concentrations under stable dosing of MTXPG1-6 in RBCs were quantified by LC-MS/MS. The performance parameters of the MTXPG analytical method were fully validated according to the FDA (Bioanalytical Method Validation Guidance for Industry) and EMEA (Guideline on bioanalytical method validation) guidelines (Table S1). For example, we conducted the following analyses: (1) determination of detection limits and quantification limits of the method; (2) measurement of recovery and precision (Table S2); (3) correlation coefficients and linear ranges of the standard curves of MTXPG (Table S3). The liquid chromatography-mass spectrometry (LC/MS) system were AB SCIEX 4000+ liquid chromatography-tandem mass spectrometer (AB SCIEX, Concord, Ontario, Canada) with an electrospray ionization source. MTXPG1-6 standards were purchased from Schircks Laboratories (Jona, Switzerland). Lot numbers of the MTXPG1-6 standards were as follows: MTXPG1(16.411 lot 014), MTXPG2(16.412 lot105), MTXPG3(16.413 lot 17), MTXPG4(16.414 lot 12), MTXPG5(16.415 lot 15), MTXPG6 (16.416 lot 31). The purity of the standard met the requirements. Ammonium bicarbonate, LC-MS-grade methanol, and formic acid were obtained from TEDIA (Fairfield, USA), while perchloric acid and acetic acid were purchased from Sinopharm Chemistry Reagent Co. Ltd (Shanghai, China). Chromatography was performed on 10 µL aliquots after partial-loop injection, using a Beckman C8 column (4.6×250 mm, 5 µm) (Beckman, CA, USA) maintained at 35°C. The mobile phase

consisted of (A) 10 mM ammonium bicarbonate buffer adjusted to pH 10 with 25% ammonia solution and (B) methanol at a flow rate of 0.5 mL/min. The elution program was performed as follows: 0-0.5 min isocratic hold 5% B, 0.5-6.0 min, linear gradient 5-60% B; 6.0-6.5 min, isocratic 60% B; 6.5-7.5 min, linear gradient 60-100% B; 7.5-9.0 min, isocratic 100% B; 9.0-10.0 min, linear gradient 100-5% B ; and 10.0-15.0 min, isocratic 5% B. The electrospray ionisation source was operated in positive-ion mode, and quantification was carried out by multiple-reaction monitoring. The monitoring ion pairs of MTXPG1-6 were as follows: MTXPG1: 455.2/308.2; MTXPG2: 584.3/308.2; MTXPG3: 713.2/308.2; MTXPG4: 842.5/308.2; MTXPG5: 486.6/308.2; MTXPG6: 550.9/308.2. In calculating MTXPG levels, concentrations below the limit of detection (LOD) were treated as zero, and concentrations ≥ LOD and < limit of quantification (LOQ) were included at their measured values.

Statistical analysis

All data were analyzed using SPSS 22.0 software (IBM Corporation, Armonk, NY, USA). First, Hardy-Weinberg equilibrium was tested to verify that the distribution of gene polymorphisms was consistent with a genetically balanced population. Then, univariate analysis was adopted to analyze continuous and categorical variables. Numerical data were presented as mean ± standard deviation (SD) or median (interquartile range) and were tested for normality using the Kolmogorov-Smirnov test. If the data followed a normal distribution, t-test or analysis of variance was used for group comparison. If the data did not follow a normal distribution, Spearman's rank correlation analysis was used for correlation analysis, and the Mann-Whitney U test or Kruskal-Wallis test was employed for group comparisons. Chi-square test or Fisher's exact test were applied to the comparison of categorical data. Finally, variables ($P < 0.20$) in the univariate analysis were incorporated into multiple linear regression, binary logistic regression, or generalized linear models to analyze the relationship of GPMTX and MTXPG levels and their impact on RA treatment response. A two-sided P-value < 0.05 was set as the level of significance in our study.

Ethical statement

This study was registered in the Chinese Clinical Trial Registry and approved by the local internal Ethics Review Board (Ethics No. 41||2018-05-20). The study was

conducted according to the guidelines of the Declaration of Helsinki, and all patients signed an informed consent form before the study.

RESULTS

Basic patient information

140 participants including 36 males and 104 females were collected from RA patients in the southern Fujian region, with a median age of 50 years, a median height of 160 centimeters, weight of 55 kilograms. With respect to demographic characteristics, there was no difference between the effective and ineffective groups (Table 1).

Gene distribution

In this study, the success rate for sample genotyping was 100%, with no duplicate genotyped samples identified. Apart from the fact that the *ABCC4* variant (rs9516519) was not detected and that *ABCC2 24C>T* deviated from Hardy-Weinberg equilibrium ($X^2 = 8.451$, $P < 0.05$), all remaining polymorphisms were in equilibrium. Hence, we excluded *ABCC4* (rs9516519) and *ABCC2 24C>T* from the subsequent study. Upon comparing the results of this study with the PharmaGKB and NCBI SNP databases, it was discovered that the variant frequencies of *GGH 401T*, *MTHFR 677T*, *ABCB1 3435T*, *MTHFR 1298C*, *RFC1 80A*, *FPGS A*, and *SLCO1B1 521C* in the south Fujian population were similar to those found in the East Asian population.

MTXPG levels

MTXPG1, MTXPG2, and MTXPG3 were detected in all patients (concentration range: MTXPG1 0.430-52.700 nmol/L; MTXPG2 0.980-49.700nmol/L MTXPG3 0.482-36.500nmol/L); MTXPG4 was detected in 19 patients (12 results were above the LOD but below the LOQ, while the remaining 7 results were within the concentration range: 3.56-7.370 nmol/L); MTXPG5 was detected in 10 patients (9 results were above the LOD but below the LOQ, only 1 results above the LOQ with the concentration range 5.740 nmol/L); MTXPG6 was detected in 3 patients (all results were above the LOD but below the LOQ); the concentration range of MTXPGs (total MTXPG) were 1.762-152.273 nmol/L, of whom 135 patients had MTXPG levels lower than 60 nmol/L (Figure 2).

Correlation of GPMTX with MTXPG levels

We analyzed the effects of seven SNPs in MTX-pathway genes on intracellular MTXPG levels. Univariate and multivariate analyses were performed with genotype and clinical parameters as independent variables, and MTXPG1, MTXPG2, MTXPG3 and MTXPGs as dependent variables. As shown in Figure 3, genotype of SLCO1B1 521T>C was significantly correlated with MTXPG1 (regression coefficient (B) = -1.119, 95% confidence interval: -2.301- -0.206, P = 0.016), MTXPG2 (B = -0.924, 95% CI: -1.65- -0.197, P = 0.013), and MTXPGs (B = -0.849, 95% CI: -1.681- -0.018, P = 0.045) in generalized linear models. The diagnostic plot of standardized residuals against predicted values showed that the standardized residuals were randomly distributed around the zero line, with no obvious trend or heteroscedastic pattern, indicating that the assumption of homogeneity of variance was largely satisfied. The influence diagnostic plot revealed that all Cook's distances were well below the threshold of 1.0 for strong influential points, suggesting that the model diagnostic results were stable and reliable (Figure S2-S7).

Correlation of MTXPG levels with efficacy and ADR of MTX in RA patients

A total of 35 patients (25%) experienced ADRs in this study, most frequently nausea/vomiting (n = 12), abdominal distension (n = 5), dizziness (n = 4) and hepatotoxicity (n = 4). The Mann-Whitney U test was used for group comparisons, and Spearman's rank correlation was employed to assess the association of MTXPG1-3 and total MTXPG levels with MTX efficacy as measured by efficacy evaluation scales. We found that RBC levels of MTXPG1 (P = 0.015), MTXPG2 (P = 0.025), and total MTXPG (P = 0.048) were significantly correlated with the HAQ score (Figure 4).

Correlation of GPMTX with efficacy and ADR of MTX in RA patients

We applied univariate and multivariate analyses to examine the effects of seven SNPs in MTX-pathway genes, demographic characteristics and concomitant medication on the efficacy and ADRs of MTX. In univariate analyses (Figure 4), the genotypes of *GGH 401C>T* had an impact on efficacy(Fisher's exact test: P=0.045), VAS (P=0.010) scores and PGA scores (P=0.043), *ABCB1 3435C>T* were associated with HAQ score (P=0.045), *SLCO1B1 521T>C* had an impact on PHGA scores (P=0.026).

No significant associations were observed for the remaining genotypes with either efficacy or ADR groups.

Multivariate analyses of GPMTX and MTXPG levels with efficacy of MTX in RA patients

In multivariate analyses, a logistic regression model (Figure 5a, events: 60/140) showed that *GGH 401C>T* contributed to the difference in efficacy (odds ratio (OR) = 0.421, 95% CI: 0.202-0.879, P=0.021). MTXPG2 level was not significantly correlated with efficacy of MTX (OR =1.006, 95%CI: 0.948-1.067, P = 0.846). A multiple linear regression model (Figure 5b) showed that *GGH 401C>T* had an impact on VAS scores (B = 0.763, 95% CI: 0.094-1.431, P = 0.026) and PGA scores (B = 0.721, 95% CI: 0.023-1.419, P = 0.043), *SLCO1B1 521T>C* had an impact on PHGA scores (B = 1.083, 95% CI: 0.258-1.909, P = 0.011), and *ABCB1 3435C>T* had an impact on PHGA scores (B = 0.715, 95% CI: 0.015-1.414, P = 0.045) and HAQ score (B = 0.378, 95% CI: 0.004-0.752, P = 0.048). MTXPG2 level was also not found to correlate with VAS scores (B = 0.005, 95% CI: -0.048-0.057, P = 0.862), PGA scores (B = 0.016, 95% CI: -0.039-0.070, P = 0.571), and PHGA scores (B = 0.029, 95% CI: -0.026-0.083, P = 0.297). In the multiple linear regression model, the significant associations of RBC levels of MTXPG1 (B = 0.023, 95% CI: -0.032-0.078, P = 0.412), MTXPG2 (B = -0.004, 95% CI: -0.105-0.098, P = 0.945), and total MTXPGs (B = -0.009, 95% CI: -0.051-0.033, P = 0.672) with the HAQ score disappeared. Since neither MTXPG levels nor seven SNPs demonstrated a statistically significant association with MTX-related ADRs in the univariate analyses (P > 0.2), a multivariate model for ADRs was not constructed.

DISCUSSION

In previous studies, there was a great deal of discussion about whether GPMTX and MTXPG levels could be more stable biomarkers to predict the efficacy and safety of MTX in RA patients. Moreover, whether GPMTX had an effect on MTXPG levels remained to be investigated. We conducted this prospective study to comprehensively explore these controversial relationships. The following findings were obtained in this low-dose, single-centre, Han-Chinese RA cohort: (1) The genotype of *SLCO1B1 521T>C* may be associated with MTXPG levels in RBCs. (2) Under these constrained dose-exposure conditions, MTXPG levels may not predict the efficacy and safety of

MTX in RA patients. (3) *GGH 401C>T*, *SLCO1B1 521T>C* and *ABCB1 3435C>T* genotypes may be exploratory candidate markers forecasting the efficacy of MTX.

Currently, few scholars have explored the relationship between GPMTX and MTXPG levels in RA patients. Thus, the correlation remains ambiguous. Apart from the SLCO1B1 521T>C genotype, no statistically significant associations were observed between other SNPs and MTXPG levels in this study. A study from Japan found that *SLCO1B1 521T>C*, *RFC1 80G>A*, *ABCB1 3435C>T*, and *MTHFR 1298A>C* genotypes were not related to MTXPG levels in 55 Japanese RA patients receiving MTX monotherapy [16]. An Indian study did not find correlation between the genotypes of *ABCB1 3435C>T*, *FPGS G>A*, *GGH 401C>T*, RFC1 80G>A and intracellular MTXPG levels in 117 RA patients [27]. Studies from East Asian populations and ethnic groups indicated that most SNPs involved in MTX transport and metabolism showed no significant association with MTXPG levels. According to the PharmaGKB and NCBI SNP databases, the mutation frequencies of the genotypes investigated in this study were similar to those in East Asian populations, which may serve as a reference. However, using a generalized linear model, we found that *SLCO1B1 521T>C* significantly influenced MTXPG1, MTXPG2, and total MTXPG levels, which contrasts with previous negative reports. This discrepancy may be attributed to two factors. First, the study population consisted exclusively of Han Chinese individuals, with high ethnic homogeneity, potentially amplifying genetic effects specific to the Han ethnicity. Second, LC-MS/MS was employed to directly quantify individual MTXPG1-6 subtypes within erythrocytes, offering higher sensitivity and specificity, thereby enabling the untangling of differential regulation of various MTXPG chain lengths by *SLCO1B1 521T>C*. It must be noted that this study employed a cross-sectional, small-sample design. Although sample processing and quality control strictly adhered to the guidelines, the findings require further validation through large-sample, prospective cohort studies.

Concurrently, we found that there was no correlation between the concentrations of MTXPG1, MTXPG2, MTXPG3, and MTXPGs and therapeutic efficacy or ADRs in RA patients of the southern Fujian area. Addressing the efficacy aspect, several studies have reported the same results as our study. While there are some scholars who have reported opposite findings . The MIRACLE trial and a meta-analysis both confirmed that elevated erythrocyte MTXPG concentrations were associated with

decreased disease activity in rheumatoid arthritis [34, 35]. The MIRACLE trial was a 48-week randomized, open-label, parallel-group study involving 300 MTX-naïve patients who initiated treatment with oral MTX at 10 mg/week or 1 mg/day, escalating to the maximum tolerated dose by week 12 [34]. The meta-analysis encompassed various immune-mediated inflammatory diseases and different study designs, with no restriction on the route of MTX administration [35]. The detection methods of MTXPG levels and regional populations in these studies were also different. We employed a cross-sectional design, enrolling only Chinese patients who had been on a stable oral MTX dose of 7.5-15.0 mg/week for at least three months. Because of the low dose, single route of administration, and population homogeneity, no association between MTXPG levels and treatment response was detected in this study.

With respect to ADRs, the majority of studies were consistent with our study. There were two possible reasons why correlations were not detected by researchers. On one hand, RA patients taking low-dose MTX have good long-term tolerability [36]. On the other hand, concomitant medication was an important influencing factor. Patients with RA often require combination therapy to control disease activity. Excessively strict restrictions would substantially reduce enrollment feasibility and fail to reflect real-world efficacy profiles. At enrollment, all concomitant medications were required to have been stably used for ≥4 weeks, thereby indirectly controlling fluctuations through medication stability. Moreover, we reanalyzed the influence of these concomitant medications on toxicity and efficacy. Except for NSAIDs, no significant correlation was found between other concomitant medications and efficacy and ADRs of MTX. Because we also did not find any correlation between GPMTX and MTXPG levels with the ADRs of MTX, the use of NSAIDs did not have an impact on the conclusions of our study. However, it has been reported in the literature that folate supplementation could prevent MTX-related ADRs, an effect that could influence the correlation between MTXPG levels and ADRs [37]. Thus, to clarify this relationship, it is necessary to conduct a prospective study on RA patients who are initially treated with MTX monotherapy.

Univariate and multiple analyses indicated a correlation between *GGH 401C>T*, *SLCO1B1 521T>C*, *ABCB1 3435C>T* and efficacy-related indicators. Especially for the *GGH 401* genotype, patients with *GGH 401CC* genotype showed an efficacy rate

of only 46% relative to those with the *GGH 401CT* genotype. Previous studies have reported that SNPs of GGH gene may affect the GGH activity. The polymorphism of 401C >T in the GGH promoter region can increase the expression of GGH, and *GGH 401C>T* can increase the activity of GGH [38, 39]. GGH, encoded by the GGH gene, is an enzyme involved in the deglutamination of MTXPG, promoting the elimination of MTXPG from the cells. In this study, we found that the CC genotype might increase GGH activity or expression compared with the CT genotype. This increase could enhance MTXPG elimination and thereby reduce MTX efficacy. SLCO1B1 that is expressed at the basolateral membrane of hepatocytes transports MTX into cells. A double-blind and controlled study found that, compared with the wild-type group of *SLCO1B1 521T>C*, the area under the concentration-time curve of MTX increased 4.2-fold and the peripheral clearance rate decreased significantly in variant group [40]. Zhang et al. also reported that compared with the wild-type group, the plasma concentration of MTX increased significantly (P = 0.001) [41]. Therefore, compared with the mutant type group, the wild-type group (*SLCO1B1 521 TT*) may decrease plasma concentration of MTX, which is associated with reduced MTX efficacy. MTX is transported out of the cell by ABCB1. A meta-analysis showed a significant association between *ABCB1 3435C>T* and MTX efficacy under the recessive model (CC vs CT+TT; OR = 1.35; 95% CI: 1.01-1.82; P = 0.047) [18]. We also found that compared with patients with the CT genotype, the CC genotype was associated with MTX efficacy. Three genes were critical genes impacting the metabolism and elimination of MTX. In terms of the design of our study, we incorporated demographic characteristics, which permitted us to take more factors into account in our analyses. This may also explain why the current results differ from those previously reported. Therefore, our study indicates that *GGH 401C>T*, *SLCO1B1 521T>C* and *ABCB1 3435 C>T* genotypes might serve as exploratory candidate markers of the efficacy of MTX in RA patients.

There are some limitations in our study. Firstly, Although RBC sampling is practical, RBCs are surrogates and do not fully reflect intracellular MTXPG levels in synovial or lymphoid cells, which are more pharmacologically relevant. MTXPG4, MTXPG5, and MTXPG6 were only detected in a subset of patients, and we were unable to explore the correlation between their concentrations and gene polymorphisms, efficacy and ADRs of MTX therapy in RA patients. In addition, only the single

measured result of MTXPG levels was detected and applied, and repeated measurements could not be conducted during the patients' follow-up period. It was not possible to explore the variation in MTXPG levels with patient duration of administration. Thirdly, due to cost and sample size limitations, confounding factors such as baseline disease activity, lifestyle habits, adherence (lack of objective evaluation), and any potential biomarkers or genetic factors were not taken into account in our study. That could bias our interpretation of certain results. Fourth, we conducted this study in Han Chinese patients. As such, it is unclear whether these findings can be generalized to other ethnic groups.

CONCLUSION

In summary, we employed MassARRAY and LC-MS/MS methods to comprehensively explore the relationship of GPMTX, MTXPG levels and effect of these in the treatment of RA. In this low-dose, single-centre, Han-Chinese RA cohort, we detected no correlation between RBC MTXPG levels and GPMTX, or the efficacy and safety of MTX. This null finding may reflect the narrow dose-exposure window, and should not be generalised to higher-dose populations. *GGH 401C>T*, *SLCO1B1 521T>C* and *ABCB1 3435C>T* genotypes may serve as exploratory candidate markers of MTX efficacy in this specific cohort. Genotype of *SLCO1B1 521T>C* may be associated with MTXPG levels in RBCs. These findings provided an experimental basis for the rational individualization of MTX medication in RA patients. However, the correlation between MTXPG levels and MTX-related ADRs remains to be explored because of the favourable tolerability of low-dose MTX and the influence of concomitant medication. Furthermore, this study is a small-sample, single-center exploratory analysis. The current findings are only preliminary clues and may serve as candidate biomarkers for priority testing in future large-scale, prospective studies, which still require independent validation.

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TABLES AND FIGURES WITH LEGENDS

Table 1. Baseline demographic characteristics of patients with rheumatoid arthritis

Characteristics	All patients (*n*=140)	Effective (*n*=60)	Ineffective (*n*=80)	*p* value
Age, years, mean ± SD	50.0±12.0	48.8±12.3	50.2±11.3	0.504
Height, cm, median (IQR)	160(155.0-165.0)	160(155.0-166.5)	158(155.0-163.8)	0.246
Weight, kg, median (IQR)	55(49.3-63.4)	55.25(50.0-63.8)	55(48.6-63.4)	0.956
Gender, male (%)	36(25.7)	15(25.0)	21(26.3)	0.867
Time*, hour, median (IQR)	4.5(3.0-6.8)	4.75(3.0-7.0)	4.5(2.0-6.0)	0.610
Dose+, mg/week, median (IQR)	10(10-10)	10(10-10)	10(10-10)	0.506
Course#, years, median (IQR)	1(0.3-4.9)	1(0.3-3.1)	1(0.3-5.0)	0.445
Duration§, month, median (IQR)	5.5(3.0-13.8)	6(3.0-14.8)	4(3.0-12.0)	0.116

*Time refers to the interval between the last dose and sampling after achieving steady-state concentrations, which occurs under stable dosing following a minimum of three months.+Dose, the dosage of the drug; #Course, the course of the disease; §Duration, the duration of the drug. **Abbreviations**: SD: Standard deviation; IQR: Inter quartile range; RA: Rheumatoid arthritis.

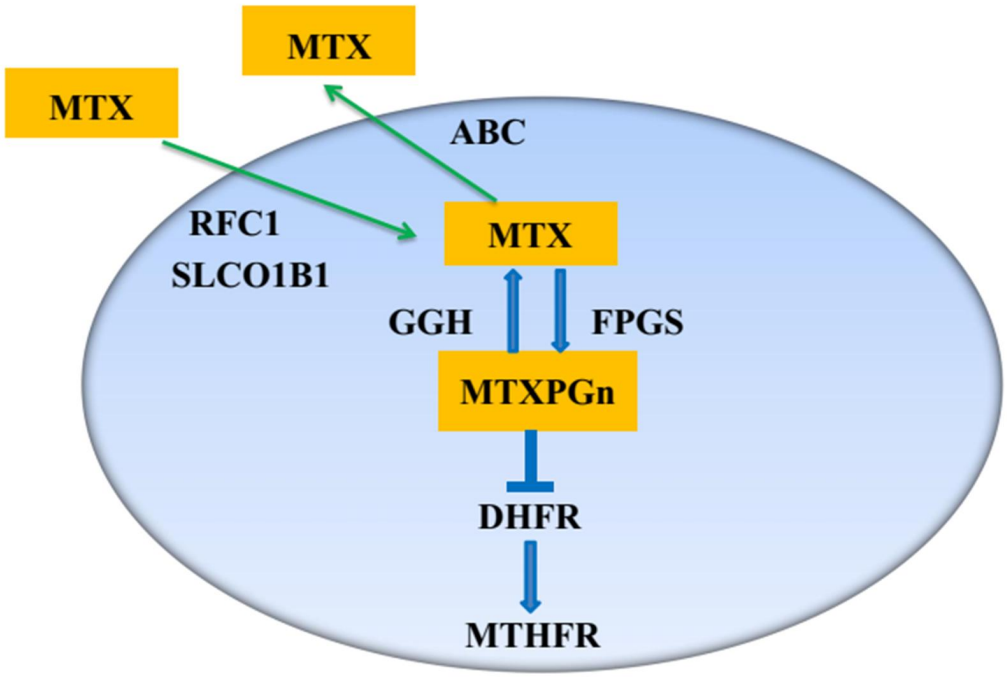

Figure 1. Schematic overview of MTX transport, intracellular MTXPGn formation, and key steps in the MTX folate pathway. MTX enters cells via RFC1 and SLCO1B1 and is converted by FPGS into MTXPGn. MTXPGn can be deglutamated by GGH back to MTX, which may be exported by ABC transporters. MTXPGn inhibit DHFR, thereby perturbing downstream folate/one-carbon metabolism involving MTHFR. Polyglutamate chain length is indicated by n. **Abbreviations**: MTX: Methotrexate; MTXPGn: Methotrexate polyglutamates; RFC1: Reduced folate carrier 1; SLCO1B1: Solute carrier organic anion transporter 1B1; FPGS: Folylpolyglutamate synthase; GGH: Gamma-glutamyl hydrolase; ABC: ATP-binding cassette; DHFR: Dihydrofolate reductase; MTHFR: Methylenetetrahydrofolate reductase.

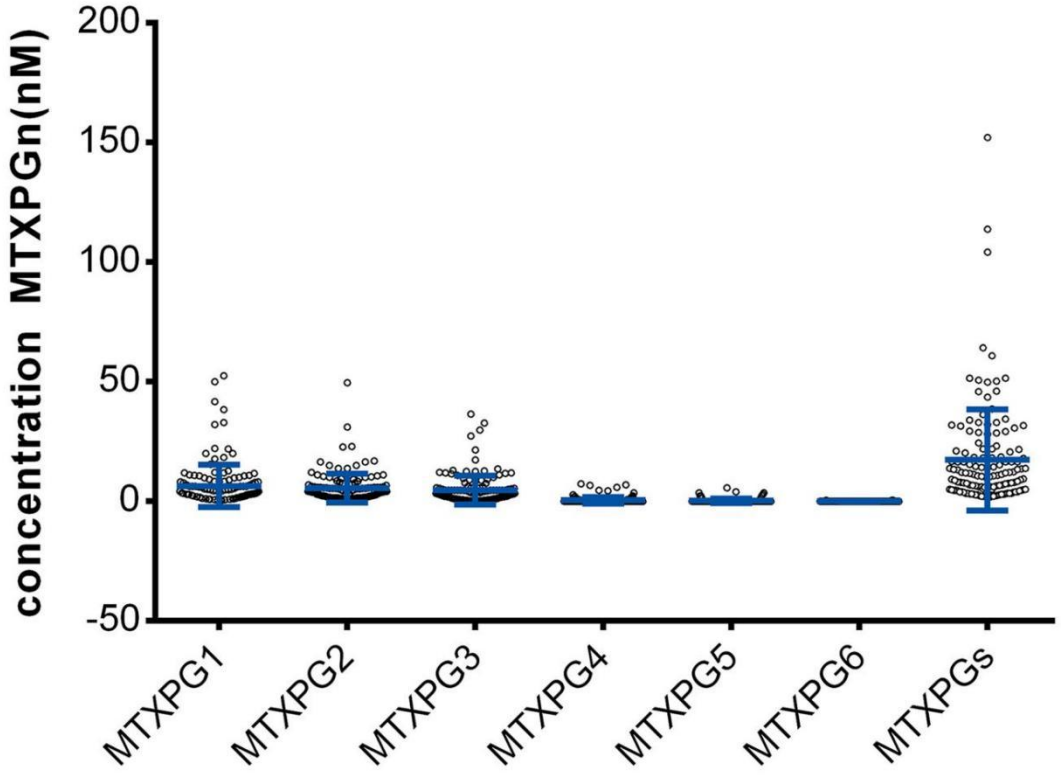

Figure 2. Distribution of erythrocyte MTXPG concentrations. MTXPG1–3 were detected in all patients (ranges: MTXPG1 0.430–52.700 nM; MTXPG2 0.980–49.700 nM; MTXPG3 0.482–36.500 nM). MTXPG4 was detected in 19 patients (12 results ≥LOD and <LOQ; 7 results 3.56–7.370 nM). MTXPG5 was detected in 10 patients (9 results ≥LOD and <LOQ; 1 result 5.740 nM). MTXPG6 was detected in 3 patients (all results ≥LOD and <LOQ). Total MTXPGs ranged from 1.762–152.273 nM, and 135 patients had total MTXPGs <60 nM. Dots represent individual patients; blue bars indicate mean ± SD. **Abbreviations**: MTXPG: Methotrexate polyglutamate; MTXPGs: Total methotrexate polyglutamates; LOD: Limit of detection; LOQ: Limit of quantification; SD: Standard deviation.

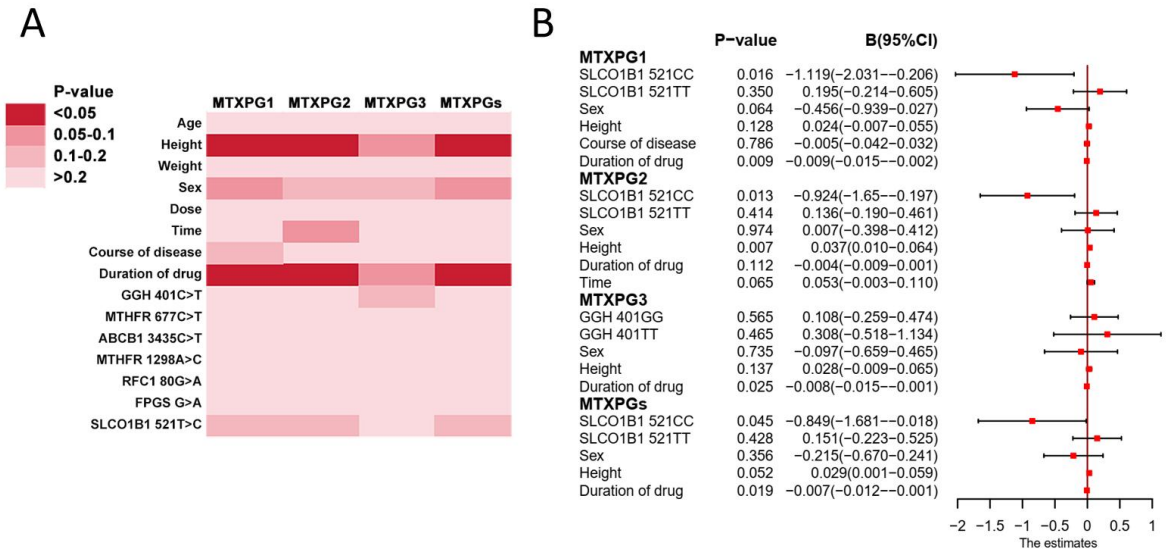

Figure 3. Associations of MTX-pathway SNPs and clinical covariates with erythrocyte MTXPG concentrations. (A) Heatmap summarizing univariate analyses between demographic/treatment variables and seven MTX-pathway SNPs versus MTXPG1, MTXPG2, MTXPG3 and total MTXPGs; colour intensity denotes the P-value category (scale shown). **(B)** Forest plot of covariate-adjusted effects from outcome-specific generalized linear models; points indicate regression coefficients (B) and whiskers the 95% CI. Consistent with the multivariable models, *SLCO1B1* 521T>C was significantly associated with MTXPG1, MTXPG2 and total MTXPGs. Genotypes were modelled as categorical variables, and B estimates represent differences relative to the reference genotype. **Abbreviations**: MTXPG: Methotrexate polyglutamate; MTXPGs: Total methotrexate polyglutamates; SNP: Single nucleotide polymorphism; B: Regression coefficient; CI: Confidence interval; GGH: Gamma-glutamyl hydrolase; MTHFR: Methylenetetrahydrofolate reductase; ABCB1: ATP-binding cassette subfamily B member 1; RFC1: Reduced folate carrier 1; FPGS: Folylpolyglutamate synthase; SLCO1B1: Solute carrier organic anion transporter 1B1.

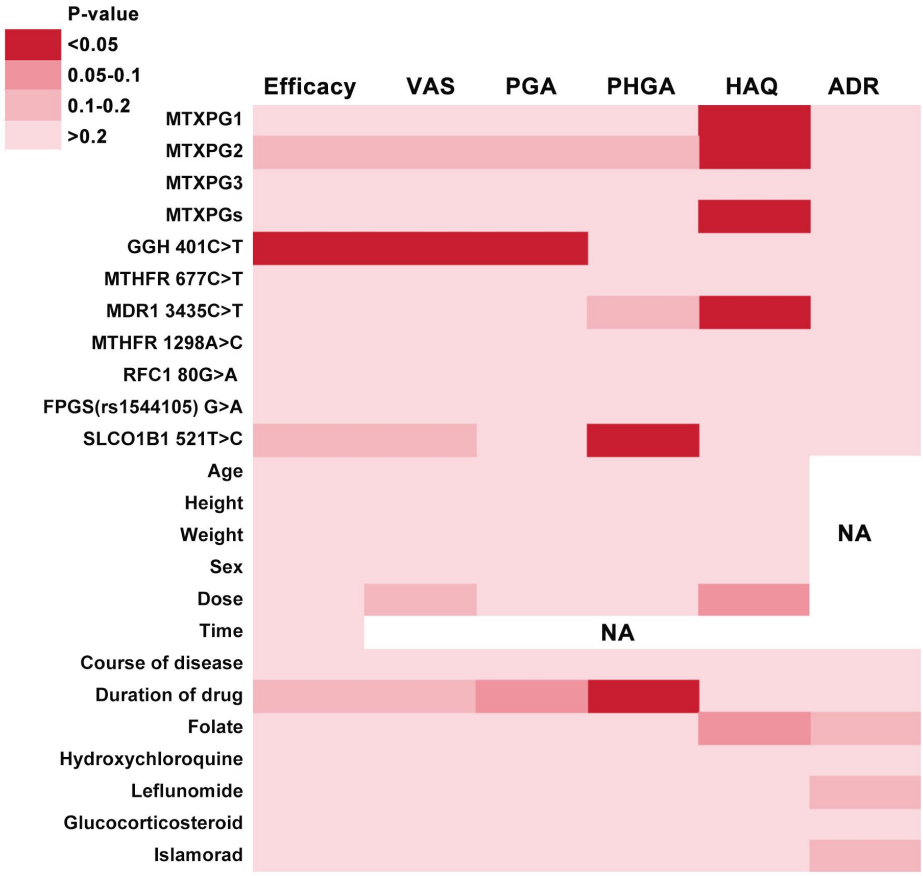

Figure 4. Heatmap summary of univariate associations between erythrocyte MTXPG measures, MTX-pathway SNPs, and clinical covariates with MTX treatment outcomes in RA. Columns show efficacy (ACR20 responder status), VAS, PGA, PHGA, HAQ, and MTX-related ADRs; rows include MTXPG1–3 and total MTXPGs, seven MTX-pathway SNPs, demographic/treatment variables, and concomitant medications. Cell shading denotes *p* value categories (key shown); NA indicates outcomes not assessed for the corresponding predictor. In univariate testing, MTXPG1, MTXPG2 and total MTXPGs were significantly associated with HAQ, *GGH* 401C>T with efficacy as well as VAS and PGA, *ABCB1* (MDR1) 3435C>T with HAQ, and *SLCO1B1* 521T>C with PHGA; no significant associations were observed for ADRs. **Abbreviations**: RA: Rheumatoid arthritis; MTX: Methotrexate; MTXPG: Methotrexate polyglutamate; MTXPGs: Total methotrexate polyglutamates; ACR20: American College of Rheumatology 20% response; VAS: Visual analogue scale; PGA: Patient's global assessment; PHGA: Physician's global assessment; HAQ: Health Assessment Questionnaire; ADR: Adverse drug reaction; SNP: Single nucleotide polymorphism; NA: Not applicable; GGH: Gamma-glutamyl hydrolase;

MTHFR: Methylenetetrahydrofolate reductase; ABCB1/MDR1: ATP-binding cassette subfamily B member 1 (multidrug resistance 1); RFC1: Reduced folate carrier 1; FPGS: Folylpolyglutamate synthase; SLCO1B1: Solute carrier organic anion transporter 1B1.

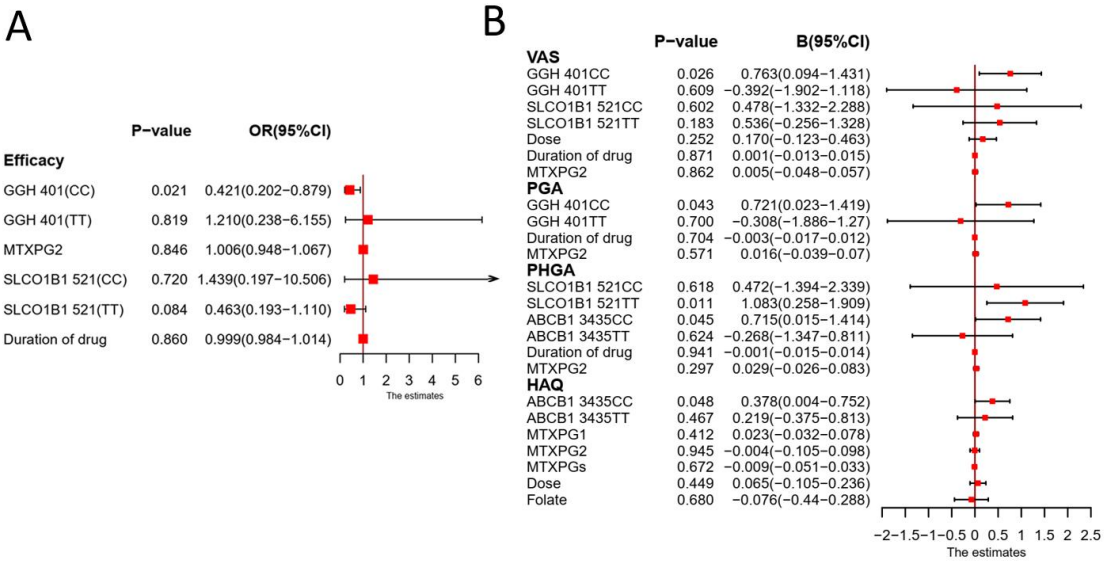

Figure 5. Multivariable associations of MTX-pathway genotypes and erythrocyte MTXPG concentrations with MTX efficacy in RA. (A) Logistic regression model for ACR20 response (events: 60/140), including *GGH* 401C>T, *SLCO1B1* 521T>C, MTXPG2 and duration of drug; results are shown as OR with 95% CI. **(B)** Multiple linear regression models for efficacy scales, shown as B with 95% CI: VAS (*GGH* 401C>T, *SLCO1B1* 521T>C, dose, duration of drug, MTXPG2), PGA (*GGH* 401C>T, duration of drug, MTXPG2), PHGA (*SLCO1B1* 521T>C, *ABCB1* 3435C>T, duration of drug, MTXPG2), and HAQ (*ABCB1* 3435C>T, MTXPG1, MTXPG2, MTXPGs, dose, folate). Genotypes are displayed as homozygous groups (CC or TT) relative to the heterozygous reference (CT). Squares indicate point estimates and horizontal lines the 95% CI; vertical lines denote the null effect (OR=1 in A; B=0 in B).
Abbreviations: RA: Rheumatoid arthritis; MTX: Methotrexate; MTXPG: Methotrexate polyglutamate; MTXPGs: Total methotrexate polyglutamates; ACR20: American College of Rheumatology 20% response; OR: Odds ratio; B: Regression coefficient; CI: Confidence interval; VAS: Visual analogue scale; PGA: Patient's

global assessment; PHGA: Physician's global assessment; HAQ: Health Assessment Questionnaire; GGH: Gamma-glutamyl hydrolase; SLCO1B1: Solute carrier organic anion transporter 1B1; ABCB1: ATP-binding cassette subfamily B member 1.

SUPPLEMENTAL DATA

Supplemental data are available at the following link:

https://www.bjbms.org/ojs/index.php/bjbms/article/view/13544/4105