Pharmacogenetic aspects of methotrexate in a cohort of Colombian patients with rheumatoid arthritis

JOHN LONDONO[1], EUGENIA-LUCIA SALDARRIAGA[1], JUAN C. RUEDA[1,2], RODRIGO GIRALDO-BUSTOS[1], JOSE-IGNACIO ANGARITA[1], LUISA RESTREPO[1], JESUS BALLESTEROS-MUÑOZ[1], CAMILO GONZÁLEZ[1], MARIA J. OSPINA[1], SOFIA ARIAS-CORREAL[1], VIVIANA REYES-MARTINEZ[1], SANTIAGO BERNALMACIAS[1], CATALINA VILLOTA-ERASO[1], PEDRO SANTOS-MORENO[3], NANCY MARTINEZ-RODRIGUEZ[4] and ANA M. SANTOS[1]

[1]Grupo Espondiloartropatías, Rheumatology Department, Universidad de La Sabana; [2]Biosciences Programme, Faculty of Medicine and Engineering, Universidad de La Sabana, Chía, Cundinamarca 250001; [3]BIOMAB, Centro de Atencion Integral en Artritis Reumatoide, Bogotá 10231, Colombia; [4]Unidad de Investigación Epidemiológica en Endocrinología y Nutrición (UIEEN), Hospital Infantil de México Federico Gomez, Ciudad de Mexico 06720, Mexico

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Abstract. Methotrexate (MTX) is the most commonly used disease-modifying antirheumatic drug for the treatment of rheumatoid arthritis (RA). However, over time, ~40% of patients may experience therapeutic failure or drug toxicity. The genetic variability of the enzymes involved in the MTX metabolic pathway seem to serve an important role in the eventual therapeutic failure or drug toxicity. Depending on the enzymes affected, the toxicity or the therapeutic response may change. The present study reports some of the polymorphisms identified in enzymes in the MTX metabolic pathway that are present in a group of Colombian patients with RA, and assesses the associations of these polymorphisms with toxicity or therapeutic response to the medication. A total of 400 patients with RA were evaluated, of which 76% were women. the average age was 60.7±13.9 years and the duration of the disease was 13.2±10.9 years. The disease activity scoring method, DAS28-CRP, was used to evaluate the therapeutic response. Toxicity was determined based on reports of adverse events during the evaluation of the patients. The single nucleotide polymorphisms (SNPs) assessed using reverse transcription-PCR in the present study were MTHFR C677T, A1298C, ATIC C347G, RFC-1-G80A, FPGS-AG and DHFR-CT. The SNPs of MTHFR C677T (P=0.05) and A1298C (P=0.048) were significantly associated with the efficacy of MTX, and DHFR-CT (P=0.01) and ATIC C347 (P=0.005) were significantly associated with documented toxicity. Haematological, hepatic or renal toxicity was not associated with any of the SNPs. The results obtained in Colombian patients with RA receiving MTX are similar to those reported in other populations; however, the SNPs associated with a lack of response previously reported in the literature were not observed in our data. The SNPs identified in the present study may be used as biomarkers to predict response to MTX in terms of efficacy and toxicity in Colombian patients with RA.

Correspondence to: Professor Ana M. Santos, Grupo Espondiloartropatías, Rheumatology Department, Universidad de La Sabana, Campus del Puente del Común, Km. 7, Autopista Norte de Bogotá, Chía, Cundinamarca 250001, Colombia
E-mail: ana.santos@unisabana.edu.co

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory joint disease. RA affects ~1% of the population worldwide and it is at least three times more common in women compared with men (1). In Colombia, the prevalence of RA was recently estimated to be 1.49% (2). Early diagnosis and effective treatment with disease modifying anti-rheumatic drugs can prevent joint destruction, reduce functional limitation, improve the quality of life, and reduce morbidity and mortality associated with the natural progression of the disease (3-6).

Methotrexate (MTX) has been the first-choice drug for RA treatment since the 1980s (7). The therapeutic effectiveness and safety of MTX has been reported in numerous studies in various populations, and its continuous use has been shown to reduce disease activity and prevent bone destruction (8-10). However, only 45% of patients receiving MTX as a monotherapy achieve significant results in the medium term; and 15-30% of patients with RA stop the treatment after 3 years of use due to gastrointestinal, cutaneous, haematological and hepatic toxicity (11,12).

To explain the variability in the clinical response to MTX, several factors have been studied, including patient-dependent factors (such as, sex, age at RA onset and disease duration),

RA-related factors (such as immunological mechanisms conditioning inflammatory response duration and severity) and the presence of autoantibodies [such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP)]. In addition, genetic variability of the enzymes involved in the metabolic pathway of MTX has been studied, and this variability may be a factor associated with the therapeutic efficacy and/or safety of MTX (13-18).

MTX is a folic acid analogue with immunomodulatory and anti-inflammatory properties. MTX enters the cell through reduced folate carrier 1, which is encoded by the SLC19A1 gene, and exits the cell via members of the ATP-binding cassette transporter family, also known as multidrug resistance-associated proteins (14). Inside the cell, MTX is converted to methotrexate polyglutamate (MTXPG), which inhibits dihydrofolate reductase (DHFR) (19) and indirectly inhibits the methylenetetrahydrofolate reductase (MTHFR), which is involved in novo purine synthesis. MTXPGs intervene in pyrimidine synthesis through inhibition of thymidylate synthetase enzyme, which converts deoxyuridylate into deoxythymidylate. MTXPGs also inhibits the AICAR transformylase enzyme, which is encoded by the ATIC gene; this leads to the intracellular accumulation of adenosine nucleotides and 5-amino-4-imidazole carboxamide, thereby increasing extracellular adenosine, a potent anti-inflammatory agent when dephosphorylated (20). MTXPGs have been shown to inhibit the proliferation of lymphocytes, monocytes, macrophages and the production of proinflammatory cytokines (such as TNFα, IL-2, IL-4, IL-6, IL8 and IL-10) (21,22).

The aim of the present study was to establish the association between SNPs in the metabolic pathway of MTX and its therapeutic efficacy and safety concerning Colombian patients with RA.

Patients and methods

Patients. The present study involved 400 patients with RA, diagnosed according to the ACR/EULAR 2010 classification criteria (23), who attended the outpatient clinic at Hospital Militar Central and Centro de Atención Integral en Artritis Reumatoide (BIOMAB) in Bogota. O the recruited cohort, 304 (76%) patients were female and 96 (24%) were male. The mean ± standard deviation age was 60.7±13.9 years (Range 20-72.5 years), and the mean duration of the disease was 13.2±10.9 years. For every patient, disease-related information and medical history were collected through a structured, pre-validated form (24). Disease activity was quantified using the DAS28-CRP scoring system (25). Adverse events (AEs) were determined by directly interviewing the patients; information obtained was verified with medical records. Venous blood samples were taken for measuring the levels of inflammation-related biomarkers in the serum using ultrasensitive c-reactive protein (CRP) and erythrocyte sedimentation rate tests. The presence of RF and anti-CCP were determined in all patients, and other laboratory evaluations, such as blood count, transaminases, alkaline phosphatase and creatinine, were included. The clinical and demographic characteristics of the patients are presented in Table I.

MTX efficacy was defined as <3.2 DAS28-CRP, hepatic toxicity as >3 times the normal transaminase value; haematological toxicity as a leucocyte count <4,000 mm^3, haemoglobin (Hb) levels <9.5 gr/dl and a platelet count <150,000 mm^3; and renal toxicity as creatinine levels >1.5 mg/dl. The study was performed in accordance with the ethical standards of the Hospital Militar Central and Centro de Atención Integral en Artritis Reumatoide (BIOMAB) and in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (26). Informed consent was obtained from all individual participants included in the study.

Table I. Clinicopathological characteristics of the recruited cohort.

Characteristics	Patients, n=400
Age, mean ± SD	60.7±13.9
Age at RA initiation, mean ± SD, years	47.6±15.1
RA duration, mean ± SD, years	13.2±10.9
Sex	
Female (%)	304 (76.0)
Male (%)	96 (24.0)
Medium-low socioeconomic level (%)	314 (78.4)
Cardiovascular comorbidities (%)	180 (45.0)
Osteoporosis (%)	81 (20.3)
Positive RF (%)	361 (90.2)
Positive anti-CCP (%)	326 (81.6)
usCRP ≥5.5 mg/l (%)	176 (44.1)
DAS-28-ESR, mean ± SD	3.89±1.46
DAS-28-CRP, mean ± SD	3.67±1.40
Synthetic DMARDs	
MTX (%)	346 (86.8)
Leflunomide (%)	145 (36.2)
Antimalarial (%)	78 (19.5)
Sulfasalazine (%)	46 (11.5)
Tofacitinib (%)	8 (2.1)
Most frequently combined synthetic DMARDs	
MTX + Leflunomide (%)	68 (17.1)
MTX + Antimalarial drugs (%)	49 (12.2)
MTX + Sulfasalazine (%)	22 (5.6)
Biological DMARDs plus MTX (%)	110 (27.5)

SD, standard deviation; RA, rheumatoid arthritis; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide; usCRP, ultrasensitive C-reactive protein; DAS-28, Disease activity score-28; ESR, erythrocyte sedimentation rate; DMARD, disease modifying anti-rheumatic drugs; MTX, methotrexate.

DNA extraction. Blood samples were taken from all patients who signed the informed consent form. The samples were placed in EDTA tubes and a Wizard Genomic DNA Purification kit (cat. no. A1120; Promega Corporation) was used for extracting DNA, according to the manufacturer's protocol. A spectrophotometer (NanoDrop 2000®; Thermo Fisher Scientific, Inc.) was used for adjusting the

concentration to 20 μg/μl and electrophoresis was used to assess the integrity of the DNA. The samples were stored at -80˚C until required.

Identifying SNPs. The *rs* (SNP reference) of each SNP studied was identified; *rs*1801133 for MTHFR C677T, *rs*1801131 for MTHFR A1298C, *rs*2371536 for ATIC C347G, *rs*1051266 for RFC-1-G80A, rs1544105 for folylpolyglutamate synthetase (FPGS)-AG and *rs*10072026 for DHFR C829T. dbSNP reference numbers were obtained from ncbi.nlm.nih.gov/snp/ (Table SI). Primers were obtained from Roche Diagnostics and used according to the manufacturer's protocol. SNPs were genotyped using TaqMan assays on a LightCycler 480 Fast Real-time PCR system (Roche Diagnostics). Information related to the sequence of the primers and thermocycling conditions are show in Tables SI and SII.

Statistical analysis. Central tendency and dispersion measurements (mean ± standard deviation) were used to quantitatively express continuous variables, and tables and percentages were used to express categorical variables. A χ^2 test was used to compare categorical variables (with Fisher's correction when necessary). For continuous variables, comparisons were made using a Student's t-test or Mann-Whitney U test, dependent on the distribution of the data. To confirm the existence of an independent association between the MTX SNPs and the toxicity or efficacy of the medication, logistical regression analysis was performed using SPSS version 25 (IBM Corp.). The associations identified were quantified as odds ratios (ORs) and 95% confidence intervals (95% CIs). P≤0.05 was considered to indicate a statistically significant difference.

Results

Associations between polymorphisms and MTX efficacy. The patients were classified for MTHFR C677T, A1298T, DHFR-CT and FPGS-AG SNPs according to ancestral and mutated genotypes, to identify the SNP which resulted in the most efficacious response to MTX. Significant results were identified for MTHFR C677T (OR, 1.62; 95% CI, 1.02-2.68; P=0.05) and A1298T SNPs (OR, 1.74; 95% CI, 1.01-3.02; P=0.048; Table II).

Associations between polymorphisms and lack of MTX efficacy. When comparing SNP genotypes associated with a lack of efficacy of MTX for ATIC, ATIC C347G and RFC-1-G80A enzymes, no significant associations were identified in the population studied. Additionally, it was found that all patients were heterozygous for RFC (Table III).

Associations between polymorphisms and MTX toxicity. Significant differences were identified for DHFR-CT (OR, 1.93; 95% CI, 1.13-3.30; P=0.0095) and ATIC C347G (OR, 2.0; 95% CI, 1.2-3.36; P=0.005) when evaluating relevant polymorphisms associated with drug toxicity in the cohort studied (Table IV).

Patients with and without MTX treatment were compared to assess the associations between haematological, hepatic and renal toxicity. MTX-related leukopenia was documented, however there were no significant differences in any of the other laboratory parameters assessed (Table V).

Discussion

In the present study, an association between the therapeutic response to MTX and MTHFR SNPs (C677T: *rs*1801133 and A1298C: *rs*1801131, respectively) was identified in the population studied. There were no associations found for DHFR-CT (*rs*10072026) and FPGS-AG (rs1544105), in agreement with a previous study (19). To the best of our knowledge, the present study is the first to simultaneously investigate the relationship of SNPs of the primary enzymes involved in the metabolic process of MTX with MTX response and toxicity in Colombian patients with RA. Similar results have been described by Urano *et al* (27) in a group of 106 Chinese patients with RA, where subjects with a MTHFR 677C-1298C haplotype required a lower dose of MTX to control the inflammatory symptoms, which in-turn resulted in fewer AEs. Similarly, Xiao *et al* (28) identified MTHFR SNPs in 110 Japanese patients and found that MTHFR C677T (*rs*1801133) was associated with an improved clinical response, whereas SNPs *rs*1801133 and *rs*2274976 were associated with frequent development of primarily gastrointestinal AEs, including anorexia, diarrhoea, dyspepsia, gastrointestinal haemorrhage, hepatitis, nausea or vomiting and pancreatitis.

The aforementioned results are relevant for the information already reported in healthy Colombian populations from the cities of Bogota, Medellin, Barranquilla and Cali, in which comparisons of the ancestral homozygous genotype C/C with mutant heterozygotic C/T and MTHFR T/T demonstrated significant differences. These mutant genotypes may result in an increased risk of toxicity and AEs in individuals that receive MTX for AR treatment (29).

However, there are contradictory reports when comparing with the results from cohorts from other countries. In a Mexican population, 70 patients with RA exhibited greater hepatic toxicity associated with the MTHFR A1298C mutant allele (*rs*180113) (30). The same association has been observed in a Dutch population of 205 patients with RA in whom MTX treatment was initiated, with a follow-up of 6-months. Among these 205 patients, patients with a mentioned polymorphism more frequently presented with pneumonitis, and skin, gastrointestinal or hepatic toxicity (31).

These findings seem to be consistent with outcomes observed in patients with other rheumatic diseases treated with MTX. In a Slovenian population study performed with a cohort of 119 patients with idiopathic juvenile arthritis (with 6 months follow-up), 55% of the patients had side effects and required a change of pharmacological therapy. These patients had an increased frequency of MTHFR SNP *rs*1801131, which was associated with hepatic and gastrointestinal toxicity, and in MTHFR *rs*1801133, which was associated with early discontinuation of MTX treatment (32).

The present study analysed the relationship of SNPs from the enzymes in the MTX metabolic pathway that have been associated with a lack of therapeutic response to the drug previously; these were: ATIC, ATIC C347G and RFC-1-G80A. However, no significant association was identified for any SNPs of these enzymes with response to MTX. These results are in contrast to a study by Dervieux *et al* (33) in which a group of 108 North American patients with RA were followed-up for 3 months after the initiation MTX therapy. The RFC-1-G80A

Table II. Association between genetic polymorphisms and methotrexate efficacy.

Polymorphism	n	Activity (%)	Remission (%)	Odds ratio (95% confidence interval)	P-value
MTHFR C677T	344	189	155	1.62 (1.0-2.68)	0.05[a]
CC	81	37 (20)	44 (28)		
TT	263	152 (80)	111 (72)		
MTHFR A1298T	381	219	162	1.74 (1.01-3.02)	0.04[a]
AA	312	172 (79)	140 (86)		
TT	69	47 (21)	22 (14)		
DHFR-CT	394	225	165	1.03 (0-68-1.55)	0.48
CC	233	131 (58.2)	95 (57.6)		
TT	161	91 (40.5)	68 (41.2)		
FPGS-TC	400	225	165	1.92 (0.69-1.41)	0.4
AA	137	74 (32.9)	57 (34.5)		
GG	263	151 (67.7)	108 (65.5)		
ATIC	386	222	184	0.81 (0.36-1.78)	0.99
CC	35	22	13		
TT	351	200	151		
ATIC C347G	385	222 (57.6)	163 (42.3)	0.82 (0.51-1.30)	0.4
CC	146	88 (60.3)	58 (39.7)		
GG	239	134 (56)	105 (44)		
RFC1 A80G	388	223 (57.4)	165 (42.6)	-	-
AA	-	-	-		
GG	388	223 (57.4)	165 (42.6)		

[a]P≤0.05. MTHFR, methylenetetrahydrofolate reductase; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase.

Table III. Association between genetic polymorphisms and lack of methotrexate efficacy.

Polymorphism	n	Activity (%)	Remission (%)	Odds ratio (95% confidence interval)	P-value
ATIC	386	222	164	1.27 (0.62-2.61)	0.5
CC		22 (10)	13 (8)		
TT		200 (90)	151 (92)		
ATIC C347G	385	222	163	1.18 (0.78-1.80)	0.41
CC		88 (40)	58 (36)		
GG		134 (60)	105 (64)	-	-
RFC1-GA	388	223	165		
GG		0	0		
AA		223	165		

ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; RFC, reduced folate carrier.

SNP was analysed and found to be associated with more prominent inflammatory activity, such as increased joint count and increased visual analogue scale pain rating. Similar results were reported for the ATIC C347G SNP (*rs*237253) in the same study.

The present study reported similar results when comparing the relationship of SNPs associated with MTX toxicity with other populations regarding DHFR-CT (*rs*10072026) and ATIC C347G (*rs*2371536) SNPs. A study of 211 central European patients with RA determined the primary polymorphisms from the enzymes involved in the metabolic pathway of DTX, identifying an association for ATIC C347G with a higher risk of toxicity (34). Similar results were reported by Salazar *et al* (35) in 124 Spanish patients with RA treated with MTX monotherapy; it was identified that the ATIC polymorphism was associated with increased AEs, including hepatotoxicity, gastrointestinal disorders or alopecia. A DHFR polymorphism was also evaluated and was found to

Table IV. Association between genetic polymorphisms and methotrexate toxicity.

Polymorphism	n	No toxicity	Toxicity	Odds ratio (95% confidence interval)	P-value
MTHFR C677T	398	317	81	1.23 (0.58-2.58)	0.584
CC	92	70	22		
TT	306	247	59		
MTHFR A1298T	389	312	80	2.36 (0.51-10.88)	0.268
AA	320	250	70		
TT	69	59	10		
DHFR-CT	392	313	81	1.7 (1.00-2,87)	0.045[a]
CC	231	176	55		
TT	161	136	25		
FPGS-TC	398	317	81	1.54 (0.46-5.20)	0.479
AA	136	113	23		
GG	262	204	58		
ATIC	394	314	80	1.36 (0.52-3,58)	0.526
CC	35	31	4		
TT	359	283	76		
ATIC C347G	394	314	80	2.16 (1.06-4.40)	0.034[a]
CC	35	31	4		
GG	359	283	76		
RFC1 A80G	396	315	81	-	-
AA		-	-		
GG	396	315	81		

[a]P<0.05. MTHFR, methyl tetrahydrofolate reductase; DHFR, dihydrofolate reductase; ATIC, 5-aminoimidazol-4-carboxamida ribonucleotide formyltransferase.

Table V. Results of laboratory tests in patients treated with and without MTX.

Laboratory test	MTX	Without MTX	P-value
AST, U/l	24.2±10.04	23.11±15.1	0.685
ALT, U7l	27.50±17.7	19.43±9.0	0.813
Creatinine mg/dl	0.80±0.22	0.84±0.32	0.562
Leukocytes, cells/mm^3	6,105.8±2,196.3	7,459.47±5,131	0.056
Haemoglobin, g/dl	14.18±1.62	14.13±2	0.914
Platelet count, mcl	287,664.47±87,326.7	264,000±56,675.9	0.192

AST, aspartate transaminase; ALT, alanine aminotransferase; MTX, methotrexate.

be associated with improved treatment response without increased toxicity.

Owen *et al* (36) studied polymorphisms regarding MTX management in 309 UK patients, and the results showed that the DHFR mutation (*rs*12517451) was associated with toxicity; however, rs10072026 and rs1643657 were associated with a reduced risk of MTX-related AEs.

Cardiovascular (CV) comorbidities have been observed in 45% of the Colombian population. MTHFR polymorphisms have also been implicated in the onset of CV disease in a healthy population; specifically, patients with the MTHFR C677T polymorphism may have an increased risk of cardiovascular disease. Furthermore, individuals who are homozygotic for the MTHFR C677T polymorphism exhibit increased levels of homocysteine, and high concentrations of this amino acid represent an additional risk of CV and cerebrovascular disease (37). Since CV diseases are the most common cause of morbidity and mortality in patients with RA, it has been proposed that MTHFR polymorphisms represent an additional and non-traditional risk of patients experiencing CV events. Another prospective, observational study involving a Spanish population of 612 patients (mean age, 53 years), who were followed up for 14 years, reported an association between CV events and endothelial dysfunction with a MTHFR A1298C genotype, but not with the C677T genotype (38).

The present study has some limitation. First, there was no follow up of patients to evaluate the evolution of DAS28 over time as well as its toxicity. However, the relatively large sample size decreases biases, which strengthen the results of the present study. Due to the design of the study from the beginning, a COX regression analysis after the univariate analysis was not performed.

In conclusion, the Colombian population shares similarities regarding SNPs associated with MTX efficacy and toxicity; however, the polymorphisms associated with inefficacy according to the literature were not observed in the present study. The variability of the results suggests that the discrepancies between populations may underlie the different outcomes, suggesting these SNPs should be assessed in additional countries. However, the SNPs identified may be used to establish biomarkers for predicting the MTX response in terms of efficacy and toxicity in Colombian patients with RA.

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Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

All authors were involved in drafting the article or revising it critically for important intellectual content. JL, RGB, LR, JBM, PSM and AMS conceived and designed the study. JL, ELS, JCR, RGB, JIA, LR, JBM, CG, SAC and AMS acquired the data. JL, ELS, JCR, RGB, LR, MJO, VRM, SB, CVE, NMR and AMS analysed and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Universidad de La Sabana (approval no. Acta 1470/12-12-2017) and the Hospital Militar Central (approval no. Acta Nº 005/20-3-2017). The project and the committee-approved consent was explained to all the patients included in the study, whom provided signed consent. Confidentiality information was maintained and the principles of the declaration of Helsinki Declaration and Colombian Ministry of Health resolution 8430/1993 were followed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. van der Woude D and van der Helm-van Mil AHM: Update on the epidemiology, risk factors, and disease outcomes of rheumatoid arthritis. Best Pract Res Clin Rheumatol 32: 174-187, 2018.
2. Londoño J, Peláez Ballestas I, Cuervo F, Angarita A, Giraldo R, Rueda JC, Ballesterosa JG, Baquero R, Forero E, Cardiel M, *et al*: Prevalence of rheumatic disease in Colombia according to the Colombian Rheumatology Association (COPCORD) strategy. Prevalence study of rheumatic disease in Colombian population older than 18 years. Rev Colombiana de Reumatol 25: 245-256, 2018.
3. Anderson J, Caplan L, Yazdany J, Robbins ML, Neogi T, Michaud K, Saag KG, O'Dell JR and Kazi S: Rheumatoid arthritis disease activity measures: American College of Rheumatology recommendations for use in clinical practice. Arthritis Care Res (Hoboken) 64: 640-647, 2012.
4. Smolen JS, Landewé R, Breedveld FC, Buch M, Burmester G, Dougados M, Emery P, Gaujoux-Viala C, Gossec L, Nam J, *et al*: EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2013 update. Ann Rheum Dis 73: 492-509, 2014.
5. Singh JA, Furst DE, Bharat A, Curtis JR, Kavanaugh AF, Kremer JM, Moreland LW, O'Dell J, Winthrop KL, Beukelman T, *et al*: 2012 update of the 2008 American College of Rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis. Arthritis Care Res (Hoboken) 64: 625-639, 2012.
6. O'Dell JR, Leff R, Paulsen G, Haire C, Mallek J, Eckhoff PJ, Fernandez A, Blakely K, Wees S, Stoner J, *et al*: Treatment of rheumatoid arthritis with methotrexate and hydroxychloroquine, methotrexate and sulfasalazine, or a combination of the three medications: Results of a two-year, randomized, double-blind, placebo-controlled trial. Arthritis Rheum 46: 1164-1170, 2002.
7. Aletaha D and Smolen JS: Diagnosis and management of rheumatoid Arthritis: A review. JAMA 320: 1360-1372, 2018.
8. Emery P, Breedveld FC, Hall S, Durez P, Chang DJ, Robertson D, Singh A, Pedersen RD, Koenig AS and Freundlich B: Comparison of methotrexate monotherapy with a combination of methotrexate and etanercept in active, early, moderate to severe rheumatoid arthritis (COMET): A randomised, double-blind, parallel treatment trial. Lancet 372: 375-382, 2008.
9. Treon SP and Chabner BA: Concepts in use of high-dose methotrexate therapy. Clin Chem 42: 1322-1329, 1996.
10. Verhoeven AC, Boers M and Tugwell P: Combination therapy in rheumatoid arthritis: Updated systematic review. Br J Rheumatol 37: 612-619, 1998.
11. van Ede AE, Laan RF, Blom HJ, De Abreu RA and van de Putte LB: Methotrexate in rheumatoid arthritis: An update with focus on mechanisms involved in toxicity. Semin Arthritis Rheum 27: 277-292, 1998.
12. Grim J, Chládek J and Martínková J: Pharmacokinetics and pharmacodynamics of methotrexate in non-neoplastic diseases. Clin Pharmacokinet 42: 139-151, 2003.
13. Edno L, Bressolle F, Gomeni R, Bologna C, Sany J and Combe B: Total and free methotrexate pharmacokinetics in rheumatoid arthritis patients. Ther Drug Monit 18: 128-134, 1996.
14. Cronstein BN: The mechanism of action of methotrexate. Rheum Dis Clin North Am 23: 739-755, 1997.
15. Blits M, Jansen G, Assaraf YG, van de Wiel MA, Lems WF, Nurmohamed MT, van Schaardenburg D, Voskuyl AE, Wolbink GJ, Vosslamber S and Verweij CL: Methotrexate normalizes up-regulated folate pathway genes in rheumatoid arthritis. Arthritis Rheum 65: 2791-2802, 2013.
16. Umićević Mirkov M and Coenen MJ: Pharmacogenetics of disease-modifying antirheumatic drugs in rheumatoid arthritis: Towards personalized medicine. Pharmacogenomics 14: 425-444, 2013.

17. Zhu H, Deng FY, Mo XB, Qiu YH and Lei SF: Pharmacogenetics and pharmacogenomics for rheumatoid arthritis responsiveness to methotrexate treatment: The 2013 update. Pharmacogenomics 15: 551-566, 2014.
18. Romão VC, Lima A, Bernardes M, Canhão H and Fonseca JE: Three decades of low-dose methotrexate in rheumatoid arthritis: Can we predict toxicity? Immunol Res 60: 289-310, 2014.
19. Boughrara W, Benzaoui A, Aberkane M, Moghtit FZ, Dorgham S, Lardjam-Hetraf AS, Ouhaibi-Djellouli H, Teixeira EP and Boudjema A: No correlation between MTHFR c.677 C>T, MTHFR c.1298 A>C, and ABCB1 c.3435 C>T polymorphisms and methotrexate therapeutic outcome of rheumatoid arthritis in West Algerian population. Inflamm Res 66: 505-513, 2017.
20. Ranganathan P: An update on methotrexate pharmacogenetics in rheumatoid arthritis. Pharmacogenomics 9: 439-451, 2008.
21. Lee YH and Bae SC: Association of the ATIC 347 C/G polymorphism with responsiveness to and toxicity of methotrexate in rheumatoid arthritis: A meta-analysis. Rheumatol Int 36: 1591-1599, 2016.
22. Restrepo LF, Giraldo R, Londoño J, Pinzón C, Cortes A, Ballesteros G and Santos MA: Farmacogenética del metotrexato en artritis reumatoide. Revisión sistemática harmacogenetics of methotrexate in rheumatoid arthritis: A systematic review. Revista Colombiana de Reumatología 23: 102-114, 2016.
23. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO III, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, *et al*: 2010 rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. Ann Rheum Dis 69: 1580-1588, 2010.
24. Santos AM, Saldarriaga EL, Giraldo-Bustos R, Ballesteros-Muñoz JG, Rueda JC, Cuervo FM, Angarita JI, Vásquez AY, Arias-Correal S, González CA, *et al*: Dickkopf 1 protein circulating levels as a possible biomarker of functional disability and chronic damage in patients with rheumatoid arthritis. Clin Rheumatol 37: 795-801, 2017.
25. Muñoz JGB, Giraldo RB, Santos AM, Bello-Gualteros JM, Rueda JC, Saldarriaga EL, Angarita JI, Arias-Correal S, Vasquez AY and Londono J: Correlation between rapid-3, DAS28, CDAI and SDAI as a measure of disease activity in a cohort of Colombian patients with rheumatoid arthritis. Clin Rheumatol 36: 1143-1148, 2017.
26. World Medical Association: World Medical Association Declaration of Helsinki: Ethical principles for medical research involving human subjects. JAMA 310: 2191-2194, 2013.
27. Urano W, Taniguchi A, Yamanaka H, Tanaka E, Nakajima H, Matsuda Y, Akama H, Kitamura Y and Kamatani N: Polymorphisms in the methylenetetrahydrofolate reductase gene were associated with both the efficacy and the toxicity of methotrexate used for the treatment of rheumatoid arthritis, as evidenced by single locus and haplotype analyses. Pharmacogenetics 12: 183-190, 2002.
28. Xiao H, Xu J, Zhou X, Stankovich J, Pan F, Zhang Z, Xu S, Lian L and Ding C: Associations between the genetic polymorphisms of MTHFR and outcomes of methotrexate treatment in rheumatoid arthritis. Clin Exp Rheumatol 28: 728-733, 2010.
29. Romero-Sánchez C, Gómez-Gutierrez A, Gómez PE, Casas-Gomez MC and Briceño I: C677T (RS1801133) MTHFR gene polymorphism frequency in a colombian population. Colomb Med (Cali) 46: 75-79, 2015.
30. Mena JP, Salazar-Páramo M, González-López L, Gámez-Nava JI, Sandoval-Ramirez L, Sánchez JD, Figuera LE, Muñoz-Valle FJ, Vazquez del Mercado M and Dávalos IP: Polymorphisms C677T and A1298C in the MTHFR gene in Mexican patients with rheumatoid arthritis treated with methotrexate: Implication with elevation of transaminases. Pharmacogenomics J 11: 287-291, 2011.
31. Wessels JA, de Vries-Bouwstra JK, Heijmans BT, Slagboom PE, Goekoop-Ruiterman YP, Allaart CF, Kerstens PJ, van Zeben D, Breedveld FC, Dijkmans BA, *et al*: Efficacy and toxicity of methotrexate in early rheumatoid arthritis are associated with single-nucleotide polymorphisms in genes coding for folate pathway enzymes. Arthritis Rheum 54: 1087-1095, 2006.
32. Zajc Avramovič M, Dolžan V, Toplak N, Accetto M, Lusa L and Avčin T: Relationship between polymorphisms in methotrexate pathway genes and outcome of methotrexate treatment in a cohort of 119 patients with Juvenile idiopathic arthritis. J Rheumatol 44: 1216-1223, 2017.
33. Dervieux T, Furst D, Lein DO, Capps R, Smith K, Walsh M and Kremer J: Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum 50: 2766-2774, 2004.
34. Grabar PB, Rojko S, Logar D and Dolzan V: Genetic determinants of methotrexate treatment in rheumatoid arthritis patients: A study of polymorphisms in the adenosine pathway. Ann Rheum Dis 69: 931-932, 2010.
35. Salazar J, Moya P, Altés A, Díaz-Torné C, Casademont J, Cerdà-Gabaroi D, Corominas H and Baiget M: Polymorphisms in genes involved in the mechanism of action of methotrexate: Are they associated with outcome in rheumatoid arthritis patients? Pharmacogenomics 15: 1079-1090, 2014.
36. Owen SA, Lunt M, Bowes J, Hider SL, Bruce IN, Thomson W and Barton A: MTHFR gene polymorphisms and outcome of methotrexate treatment in patients with rheumatoid arthritis: Analysis of key polymorphisms and meta-analysis of C677T and A1298C polymorphisms. Pharmacogenomics J 13: 137-147, 2013.
37. Fan AZ, Yesupriya A, Chang MH, House M, Fang J, Ned R, Hayes D, Dowling NF and Mokdad AH: Gene polymorphisms in association with emerging cardiovascular risk markers in adult women. BMC Med Genet 11: 6, 2010.
38. Palomino-Morales R, Gonzalez-Juanatey C, Vazquez-Rodriguez TR, Rodriguez L, Miranda-Filloy JA, Fernandez-Gutierrez B, Llorca J, Martin J and Gonzalez-Gay MA: A1298C polymorphism in the MTHFR gene predisposes to cardiovascular risk in rheumatoid arthritis. Arthritis Res Ther 12: R71, 2010.