**MTRR A66G, RFC1 G80A, and MTHFR C677T and A1298C Polymorphisms and Disease Activity in Mexicans with Rheumatoid Arthritis Treated with Methotrexate**

Mirna Gisel González-Mercado,1–3 Fernando Rivas,4 M. Patricia Gallegos-Arreola,1 M. Cristina Morán-Moguel,1,3 Mario Salazar-Páramo,5,6 Laura González-López,5,6 J. Iván Gámez-Nava,5,6 J. Francisco Muñoz-Valle,5 Ricardo Medina-Coss y León,1 Anahí González-Mercado,1,3 Mario A. Aceves,5,6 Nory O. Dávalos,3 Agustín Macías-Chumacera,6 and Ingrid P. Dávalos1,3

**Aim:** To investigate the relationships of polymorphisms in genes whose protein products are related in the metabolic pathway of folic acid, particularly MTRR A66G, RFC1 G80A, and MTHFR C677T and A1298C, and disease activity in Mexican patients with rheumatoid arthritis (RA) treated with methotrexate (MTX).

**Materials and Methods:** Sixty-eight patients with RA were included in the study who were being treated with MTX, either with or without other drugs. In addition to general data, disease activity was measured by the disease activity score 28 (DAS28). Single nucleotide polymorphisms (SNPs) genotyping was performed by allelic discrimination using real-time polymerase chain reaction.

**Results:** Differences in genotype (homozygotic or heterozygotic for each allele), allele distributions, and phenotype were not statistically different between the RA group and control populations. We did not find any association between the studied polymorphisms and disease activity nor with the intragroup variables (e.g., clinical activity, body mass index, and single- or combined-drug treatment) or between genetic markers; we also did not find any association within the RA group or between the RA group and control populations.

**Conclusion:** Additional studies of more polymorphisms related to this or other metabolic pathways are required to determine the influence of genetics on disease activity in RA.

**Keywords:** MTRR, RFC1, MTHFR, rheumatoid arthritis, DAS28, methotrexate

**Introduction**

RHEUMATOID ARTHRITIS (RA) is a chronic and progressive inflammatory disease that is characterized by cell proliferation and inflammation of the joint synovial membranes (McInnes and Schett, 2011). RA is an autoimmune disease and, thus, its etiology is multifactorial with genetic and environmental components, including diet (Oliver and Silman, 2009). Disease-modifying anti-rheumatic drugs (DMARDs) are used to decrease inflammation and pain, prevent joint damage, and preserve patient functional capacity. The DMARD methotrexate (MTX) is an antagonist of the essential nutrient folic acid, and it is the most commonly used drug to treat RA, either alone or in combination with other drugs (Calabrese et al., 2001). Although the mechanism of action of MTX in patients with autoimmune diseases is not well understood, it seems to have both antiproliferative and anti-inflammatory effects (Cutolo et al., 2001). Differences in one or more etiologic factors may predispose patients to RA onset or varying degrees of disease...
severity (Oliver and Silman, 2009; McInnes and Schett, 2011). Consequently, RA onset, prognosis, and response to MTX could be affected by environmental factors or genetic variations in folic acid metabolism (Inoue and Yuasa, 2014).

Previous studies have investigated associations between RA clinical activity and treatment response and the deoxyribonucleic acid (DNA) variants of genes associated with folic acid metabolism such as MTRR, RFC1, and MTHFR (Berkus et al., 2004; Hughes et al., 2006; Wessels et al., 2006; Rubini et al., 2008; Inanir et al., 2013; Salazar et al., 2014; Saad et al., 2015a, 2015b, 2016; Muralidharan et al., 2016; Remuzgo-Martinez et al., 2016). MTRR gene is located on chromosome 5p15.3 and encodes for the enzyme methionine synthase reductase, involved in the reductive regeneration of cob(I)alamin (vitamin B12) cofactor required for the maintenance of methionine synthase in a functional state (Jacques, 2003). MTRR A66G polymorphism (rs1801394) has been identified with a global minor allele frequency (MAF) G = 36% (https://www.ncbi.nlm.nih.gov/snp).

In the Caucasian population, GG genotype has been associated with an increase in plasma homocysteine (Hcy) levels, having a greater effect than the AG genotype (Gaughan et al., 2001).

RFC1 gene is located on chromosome 21q22.3 and encodes for the reduced folate transporter, which plays an important role in folate metabolism and also works as a transporter of the MTX into the cell (Matherly et al., 2007). RFC1 G80A polymorphism (rs1051266) has an overall MAF of A = 49% (https://www.ncbi.nlm.nih.gov/snp) and this genetic variant might cause an alteration in the folate transporter, affecting the availability of folate (Dervieux et al., 2004).

MTHFR gene encodes the methylene-tetrahydrofolate reductase enzyme and is located on chromosome 1p36.3. This enzyme catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for Hcy remethylation to methionine (Goyette et al., 1998). MTHFR C677T polymorphism (rs1801133) has a global MAF of T = 24% (https://www.ncbi.nlm.nih.gov/snp), which causes a thermolabile variant of the protein, altering the enzymatic function (Van Der Put et al., 1998).

In homozygous individuals, this variant is correlated with a decrease in enzyme activity (35%), elevated Hcy, low levels of folic acid, and reduced formation of 5-methyltetrahydrofolate, a predominant form of folate (Froist et al., 1995). Otherwise, a global MAF of C = 25% (https://www.ncbi.nlm.nih.gov/snp) has been reported in the MTHFR A1298C polymorphism (rs1801131) and causes loss of 40% enzyme activity in individuals homozygous for the mutant allele and only in combination with the C677T polymorphism causes hyperhomocysteinemia (Van Der Put et al., 1998).

In this study, we investigated potential associations between RA severity and the single nucleotide polymorphisms (SNPs) MTRR A66G, RFC1 G80A and MTHFR C677T and A1298C.

Materials and Methods

Study population

We analyzed data for 68 Mexican Mestizo patients (67 women and 1 man) with RA diagnosed according to the American College of Rheumatology criteria (ACR, 1987) (Arnett et al., 1988). Patients were seen in the rheumatology service clinic at Regional Hospital 110, Instituto Mexicano del Seguro Social (IMSS) or at the Hospital Civil de Guadalajara “Fray Antonio Alcalde.” and they signed a written informed before the sampling of peripheral blood when they accepted their voluntary participation in the study. All of the patients had been previously diagnosed with RA for at least 1 year and had been receiving MTX for at least 3 months. DAS criteria were used to evaluate their RA activity (low activity score, <3.2; moderate, 3.2–5.1; and high, >5.1) (Prevo et al., 1995; Fransen et al., 2003; Makinen et al., 2005).

Control population

Each polymorphism had a different control population, for MTRR A66G n = 50 (Shi et al., 2003), RFC1 G80A n = 121 (Rodarte, unpublished data), MTHFR C677T n = 82 (González-Mercado et al., 2014), and MTHFR A1298C n = 94 (González-Mercado et al., 2014).

DNA analysis

We extracted DNA from blood samples in accordance with the methods of Miller and Gustinich (Miller et al., 1989; Gustinich et al., 1991). Polymorphisms MTRR A66G, RFC1 G80A and MTHFR C677T, and A1298C were typed in an allelic discrimination assay by using TaqMan 5′ exonuclease probes (Applied Biosystems, Foster City, CA) with the ABI 7300 real-time PCR system (Applied Biosystems).

Statistical analysis

Genotype and allele frequencies were obtained by direct counting. Data analysis included comparisons of allele frequencies and genotypes between the RA patient group and control normal Mexican population distributions (Shi et al., 2003; González-Mercado et al., 2014; Rodarte, unpublished data). All control genotypes were in agreement with Hardy–Weinberg equilibrium (HWE). We performed distribution comparisons by the exact, chi square, and likelihood ratio tests by using SPSS statistical package, v. 22 (IBM®). Quantitative variables were subjected to mean comparisons by Student’s t-test or one-way analysis of variance.

Results

The general characteristics of the study participants were as follows: The mean age was 53.7 years with a SD ± 10.7 (range 72–76). Most previous studies, including some studies in Mexican populations, have estimated that the female-to-male ratio of patients with RA is ~ 3:1 (Rodríguez-Acosta et al., 2001; Spindler et al., 2002). In our study, we identified 67 women and only 1 man who had been diagnosed with RA.

The disease activity score 28 (DAS28) values found in the patients in this study had a mean of 4.7 ± 1.4 SD (range 2.3–7.8). According to the DAS28 (Fransen et al., 2003), disease activity levels were as follows: low, 14 patients; moderate, 28 patients; and high, 27 patients. Forty-one patients were receiving MTX monotherapy, 26 patients were receiving MTX plus an additional DMARD, and 2 patients were receiving MTX plus two additional DMARDs. DMARDs other than MTX were leflunomide (n = 15), sulfasalazine (n = 8), chloroquine (n = 4), and penicillamine (n = 3). Since MTX monotherapy is widely recommended in most RA patients as the first line of treatment (Rodríguez-Valverde et al., 2004; Van...
monotherapy versus combined therapy. There were no significant differences with body mass index (BMI), and group with respect to clinical activity (Fig. 1). There were also no significant differences within the RA patient Polymorphism Group MTRR A66G, increased activity in the genotypes 2/2 of the polymorphisms studied and there was a trend toward (Table 1). DAS28 values were compared with the genotypes No statistically significant differences between the groups with RA and various Mexican healthy populations, we found four polymorphisms included in this study between patients with RA and control populations were not statistically significant. RA, rheumatoid arthritis; SNPs, single nucleotide polymorphisms.

### Polymorphism analysis

Polymorphism distributions for the RA patient group and for the control populations are presented in Table 1. The genetic frequencies of MTRR A66G, RFC1 G80A, and MTHFR C677T and A1298C SNPs have been analyzed in previous studies of several Mexican populations; the genotypes were in agreement with HWE (Shi et al., 2003; González-Mercado et al., 2014; Rodarte, unpublished data).

When comparing the allele and genotype frequencies of the four polymorphisms included in this study between patients with RA and various Mexican healthy populations, we found no statistically significant differences between the groups (Table 1). DAS28 values were compared with the genotypes of the polymorphisms studied and there was a trend toward increased activity in the genotypes 2/2 of the polymorphisms MTRR A66G, RFC1 G80A, and MTHFR A1298C; however, there were no significant differences within the RA patient group with respect to clinical activity (Fig. 1). There were also no significant differences with body mass index (BMI), and monotherapy versus combined therapy.

### Discussion

#### General characteristics

Although disease onset can occur at any age (Rodríguez-Acosta et al., 2001; Firestein, 2005), the Diagnóstico y Tratamiento de Artritis Reumatoide del Adulto, (Diagnosis and Treatment of Adult RA) reports that the mean age at diagnosis is 40±10 SD years in Mexico (Barrera-Cruz et al., 2010). In this series, the mean age at disease onset was 44.7±12.0 SD years, which is similar to that reported in other populations.

In our patients, the mean BMI was 26.9±4.8 SD (range 15–41), which is above the threshold for overweight. Other studies have reported similar findings with respect to increased BMI in Mexican patients with RA (Puente-Torres et al., 2009). However, the proportion of overweight patients that we observed is comparable to that of the general Mexican population (INEGI, 2016).

#### Polymorphism analysis

Studies of the genotype and allele frequencies of the polymorphisms that we analyzed have revealed that they vary widely worldwide (Dávalos et al., 2001; Boughrara et al., 2015; Saad et al., 2015a; Li et al., 2016; Remuzgo-Martínez et al., 2016). Several studies have investigated the associations of these polymorphisms with autoimmune diseases, including systemic lupus erythematosus (Summers et al., 2016; Shi et al., 2003; Saad et al., 2015a; Li et al., 2016; Remuzgo-Martínez et al., 2016).

### Table 1. Genotypes and Allele Frequencies for MTRR A66G, RFC1 G80A, and MTHFR C677T and A1298C Single Nucleotide Polymorphisms in Rheumatoid Arthritis Patients and Control Mexican Populations

| Polymorphism     | Group                        | Genotype, counts (%) | Allele, counts (%) |
|------------------|------------------------------|----------------------|--------------------|
|                  |                              | 1/1 | 1/2 | 2/2 | p | 1 | 2 | p |
| MTRR A66G        | RA                           | 38 (56) | 25 (37) | 5 (7) | 0.67 | 101 (74) | 35 (26) | 0.40 |
|                  | Control                      | 32 (64) | 15 (30) | 3 (6) | 0.18 | 79 (79) | 21 (21) | 0.40 |
| RFC1 G80A        | RA                           | 19 (28) | 37 (54) | 12 (18) | 0.99 | 75 (55) | 61 (45) | 0.97 |
|                  | Control                      | 34 (28) | 65 (54) | 22 (18) | 0.75 | 133 (55) | 109 (45) | 0.52 |
| MTHFR C677T      | RA                           | 23 (34) | 32 (47) | 13 (19) | 0.75 | 78 (57) | 58 (43) | 0.52 |
|                  | Control                      | 23 (28) | 42 (51) | 17 (21) | 0.75 | 88 (53) | 76 (47) | 0.52 |
| MTHFR A1298C     | RA                           | 43 (63) | 21 (31) | 4 (6) | 0.70 | 107 (79) | 29 (21) | 0.38 |
|                  | Control                      | 54 (57) | 32 (34) | 8 (9) | 0.70 | 140 (74) | 48 (26) | 0.38 |

*Allele 1: A in MTRR 66, G in RFC-1 80, C in MTHFR 677, and A in MTHFR 1298. Allele 2: G in MTRR 66, A in RFC-1 80, T in MTHFR 677, and C in MTHFR 1298. Differences in genotype, phenotype (homozygous or heterozygous for each allele), and allele distributions between the RA group and control populations were not statistically significant.*

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**FIG. 1.** Comparison between DAS28 mean values and the genotypes of each polymorphism in patients with RA treated with MTX. A slight upward trend in disease activity was observed in genotypes 2/2 of polymorphisms (MTRR A66G \( p = 0.73 \), RFC1 G80A \( p = 0.55 \), MTHFR A1298C \( p = 0.48 \)) with the exception of MTHFR C677T \( p = 0.58 \); however, it was not statistically significant. DAS28, disease activity score 28; RA, rheumatoid arthritis.
| Disease and MTX response | Population | n | Polymorphism | Allele frequency | Association | References |
|--------------------------|------------|---|--------------|-----------------|-------------|------------|
| RA and MTX response      | Spain      | 61 RA patients responders/16 RA patients no responders | MTHFR C677T | RA responders C 0.68/ T 0.32, RA no responders C 0.75/T 0.25 | N.A. | Salazar et al. (2014) |
| RA and MTX response      | Netherlands | 205 RA patients | MTRR A66G | Not available | N.A. | Wessels et al. (2006) |
| RA and MTX response      | India      | 327 RA patients/322 controls | RFC1 G80A | RA patients G 0.57/A 0.43/ Controls G 0.57/A 0.43 | N.A. | but polymorphism confers protection for RA response | Muralidharan et al. (2016) |
| RA and MTX toxicity      | Mexico     | 57 RA patients without toxicity/13 RA patients with toxicity | MTHFR C677T | RA without toxicity C 0.55/T 0.45, RA with toxicity C 0.58/T 0.42 | N.A. | Mena et al. (2011) |
| RA and MTX toxicity      | Tunisia    | 141 RA MTX tolerant/MTX intolerant | MTRR A66G | MTX tolerant A 0.51/G 0.49, MTX intolerant A 0.52/G 0.48 | N.A. | Chaabane et al. (2016) |
2008), multiple sclerosis (Naghibalhossaini et al., 2015), and thyroiditis (Arakawa et al., 2012). Previous studies of RA patients have focused on disease evolution (Brambila-Tapia et al., 2012; Remuzgo-Martínez et al., 2016) and severity or the therapeutic response to MTX. Associations of these polymorphisms with MTX treatment response seem to be consistently significant, but studies of their association with disease onset and clinical severity have yielded conflicting results (Table 2).

The role of polymorphisms MTRR A66G, RFC1 G80A, and MTHFR C677T and A1298C in RA and other autoimmune diseases remains unclear.

Our failure to find an association between RA and these SNPs could be due to the small sample size included in this study or to the weak contribution of these genes to RA onset and evolution, either in Mexico or worldwide.

Table 2. (Continued)

| Disease                  | Population | n     | Polymorphism | Allele frequency | Association                                      | References        |
|--------------------------|------------|-------|--------------|------------------|-------------------------------------------------|------------------|
| RA and serum MTX levels  | Japan      | 100 RA patients | RFC1 G80A | G 0.37            | N.A.                                            | Fukino et al. (2007) |
|                          |            |       |              | A 0.63            |                                                 |                  |
|                          |            |       | MTHFR C677T  | C 0.57            |                                                 |                  |
|                          |            |       |              | T 0.43            |                                                 |                  |
|                          |            |       | MTHFR A1298C | A 0.80            |                                                 |                  |
|                          |            |       |              | C 0.20            |                                                 |                  |
| RA and MTX               | Japan      | 170 RA patients | RFC1 G80A | G 0.48            | G allele may be associated with lower intracellular MTX uptake and poor efficacy | Hayashi et al. (2013) |
|                          |            |       |              | A 0.52            |                                                 |                  |
| RA and DAS28             | Japan      | 55 RA patients | MTRR A66G | A 0.75            | N.A.                                            | Kato et al. (2012) |
|                          |            |       |              | G 0.25            |                                                 |                  |
|                          |            |       | RFC1 G80A    | G 0.47            |                                                 |                  |
|                          |            |       |              | A 0.53            |                                                 |                  |
|                          |            |       | MTHFR C677T  | C 0.6             |                                                 |                  |
|                          |            |       |              | T 0.4             |                                                 |                  |
|                          |            |       | MTHFR A1298C | A 0.86            | AA genotype had lower mean DAS28 than 1298AC/CC genotypes |                  |
|                          |            |       |              | C 0.14            |                                                 |                  |

DAS28, disease activity score 28; MTX, methotrexate; N.A., no association.

By the European League Against Rheumatism. Although we found no statistical differences between polymorphisms and disease activity, there was a trend in three of the polymorphisms studied (MTRR 66GG, RFC1 80AA, and MTHFR 1298CC) and we concluded that perhaps a larger sample size might have yielded data with statistical significance.

This study has some limitations to be discussed: Because this study was exploratory, there was no previous information related with our main objective of investigating potential associations between RA severity and the SNPs MTRR A66G, RFC1 G80A, and MTHFR C677T and A1298C in the Mexican population from Western Mexico. In the results of the comparisons (described in Table 1) of allele and genotype frequencies observed in RA cases versus controls, we were not able to exclude the probability of an insufficient statistical power to identify differences between these two groups (type II error). Nevertheless, we consider that this study might help future investigations based in our data on the computation of the sample size required to perform these comparisons.

Otherwise, we have observed that although the n of controls increases, the allelic frequencies of the polymorphisms are maintained, since a minimum of 100 alleles is required. Our results represent relevant information demonstrating that these polymorphisms might not be related with disease severity in RA. These findings support the importance of seeking other genetic factors that might predispose to the observed phenotype differences in the severity of this disease among these patients.
In conclusion, in this study, we did not find any significant associations between RA or RA characteristics such as activity disease and polymorphisms MTRR A66G, RFC1 G80A, and MTHFR C677T and A1298C. Additional studies that include greater numbers of patients and more polymorphisms related to this or other metabolic pathways are required to determine the influence of genetics on disease activity in RA in Mexican populations, and thus provide greater knowledge about individualized pharmacological therapies for a better response to treatment.

Author Disclosure Statement

No competing financial interests exist.

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