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

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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 dam-

age, 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 etiological factors may predispose patients to RA onset or varying degrees of disease

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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* (Berkun *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-Martínez *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 (Frosst *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

services 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) (Prevoo *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 Gustincich (Miller *et al.*, 1989; Gustincich *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

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 (%) ^a		
		1/1	1/2	2/2	p	1	2	p
<i>MTRR</i> A66G	RA	38 (56)	25 (37)	5 (7)	0.67	101 (74)	35 (26)	0.40
	Control (Shi <i>et al.</i> , 2003) (n=50)	32 (64)	15 (30)	3 (6)		79 (79)	21 (21)	
<i>RFC1</i> G80A	RA	19 (28)	37 (54)	12 (18)	0.99	75 (55)	61 (45)	0.97
	Control (Rodarte, unpublished results) (n=121)	34 (28)	65 (54)	22 (18)		133 (55)	109 (45)	
<i>MTHFR</i> C677T	RA	23 (34)	32 (47)	13 (19)	0.75	78 (57)	58 (43)	0.52
	Control (González-Mercado <i>et al.</i> , 2014) (n=82)	23 (28)	42 (51)	17 (21)		88 (53)	76 (47)	
<i>MTHFR</i> A1298C	RA	43 (63)	21 (31)	4 (6)	0.70	107 (79)	29 (21)	0.38
	Control (González-Mercado <i>et al.</i> , 2014) (n=94)	54 (57)	32 (34)	8 (9)		140 (74)	48 (26)	

^aAllele 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.

RA, rheumatoid arthritis; SNPs, single nucleotide polymorphisms.

der Heijde *et al.*, 2005; GUIPCAR, 2011), we compared the DAS28 between patients undergoing mono- and combined therapy. The average DAS28 was 4.64 for monotherapy and 4.76 for combined therapy ($p=0.74$).

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 (Puentes-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.*,

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.

TABLE 2. ASSOCIATIONS BETWEEN POLYMORPHISMS AND RHEUMATOID ARTHRITIS DISEASE COURSE AND CLINICAL RESPONSE TO METHOTREXATE TREATMENT IN PREVIOUS STUDIES

Disease	Population	n	Polymorphism		References
			Allele frequency	Association	
RA and MTX response	Spain	61 RA patients responders 16 RA patients no responders	<i>MTHFR</i> C677T	N.A.	Salazar <i>et al.</i> (2014)
			RA responders		
			C 0.68		
			T 0.32		
			RA no responders		
			C 0.75		
			T 0.25		
			<i>MTHFR</i> A1298C		
			RA responders		
			A 0.67		
			C 0.33		
			RA no responders		
			A 0.56		
			T 0.46		
RA and MTX response	Netherlands	205 RA patients	<i>MTRR</i> A66G	N.A.	Wessels <i>et al.</i> (2006)
RA and MTX response	India	327 RA patients 322 controls	Not available	N.A. but polymorphism confers protection for RA response	Muralidharan <i>et al.</i> (2016)
			<i>RFC1</i> G80A		
			RA patients		
			G 0.57		
			A 0.43		
			Controls		
			G 0.57		
			A 0.43		
RA and MTX toxicity	Mexico	57 RA patients without toxicity 13 RA patients with toxicity	<i>MTHFR</i> C677T	N.A.	Mena <i>et al.</i> (2011)
			RA without toxicity		
			C 0.55		
			T 0.45		
			RA with toxicity		
			C 0.58		
			T 0.42		
			<i>MTHFR</i> A1298C		
			RA without toxicity		
			A 0.79		
			C 0.21		
			RA with toxicity		
			A 0.58		
			C 0.42		
RA and MTX toxicity	Tunisia	141 RA MTX tolerant/MTX intolerant	<i>MTRR</i> A66G	N.A.	Chaabane <i>et al.</i> (2016)
			MTX tolerant		
			A 0.51		
			G 0.49		
			MTX intolerant		
			A 0.52		
			G 0.48		
			<i>MTHFR</i> C677T		
			MTX tolerant		
			C 0.75		
			T 0.25		
			MTX intolerant		
			C 0.65		
			T 0.35		
			<i>MTHFR</i> A1298C		
			MTX tolerant		
			A 0.70		
			C 0.30		
			MTX intolerant		
			A 0.74		
			C 0.26		

(continued)

TABLE 2. (CONTINUED)

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

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

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.

Polymorphisms and disease activity

The patients of this study showed the three levels of disease activity, with the most frequent being moderate activity. Although there are clinical or laboratory measures used in clinical practice, a good individual performance in the assessment of disease activity has not been demonstrated, so it has been decided to use DAS28 for a better classification of the disease (Prevoo *et al.*, 1995; Fransen *et al.*, 2003), which is used as the basis for the evaluation of the response to treatment established

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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