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Original article

EFFECT OF REDUCED FOLATE CARRIER GENE POLYMORPHISM (G80A) ON THE METHOTREXATE LEVEL IN PATIENTS WITH RHEUMATOID ARTHRITIS AND ITS RELATION TO THE DISEASE ACTIVITY

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ABSTRACT

Methotrexate (MTX) is the primary conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) used to treat rheumatoid arthritis (RA). Interindividual variability in its efficacy and toxicity has been partially attributed to genetic polymorphisms affecting folate metabolism and transport. The reduced folate carrier-1 (RFC1), encoded by the SLC19A1 gene, is a key transporter responsible for MTX cellular uptake. The RFC1 G80A (rs1051266) polymorphism may alter transporter function, influencing MTX plasma levels and therapeutic outcomes. This study attempted to evaluate the impact of RFC1 G80A polymorphism on MTX plasma concentration and its association with the activity of RA disease. This cross-sectional study was carried out at the Rheumatology Center in Duhok, Iraq, from September 2024 to June 2025. Ninety RA patients receiving oral MTX therapy were recruited. MTX levels were quantified by ELISA, and genotyping of the RFC1 G80A polymorphism was performed by PCR-RFLP. Statistical analyses were conducted to evaluate genotype associations with MTX levels, DAS-28 activity score, and laboratory parameters. The study cohort (mean age: 50.1 ± 11.9 years) was predominantly female (91.1%). Genotype frequencies were GG (53.3%), GA (35.6%), and AA (11.1%). Although MTX levels were numerically highest in AA individuals (330.1 ± 70.5 ng/mL), the difference was not significant in statistical terms (p=0.82). However, AA carriers had significantly higher DAS-28 scores (4.3 ± 1.5) than GG carriers (3.8 ± 1.2; p=0.009), suggesting poorer disease control. No substantial variation has been noticed in inflammatory or hematological parameters between genotype groups. The RFC1 G80A polymorphism may influence MTX pharmacokinetics and RA disease activity. The AA genotype appears to be associated with non-significantly higher MTX levels and worse disease control. Pharmacogenetic profiling could help optimize MTX therapy in RA.

KEYWORDS: Reduced folate carrier, RFC1, G80A polymorphism, Rheumatoid arthritis, Methotrexate, Pharmacogenomics.

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease mostly attacking the synovial joint and causes gradual joint degradation, disability, and systemic complications if left untreated. Its global prevalence ranges between 0.5% and 1% worldwide, with women being more likely to have it and individuals living in urban and northern climates (Bullock *et al.,* 2019). Clinically, RA is characterized by joint swelling, morning stiffness, fatigue, and weight loss; additionally, extra-articular symptoms may include interstitial lung disease, vascular inflammation, and subcutaneous nodules (Das & Padhan, 2017).

Methotrexate (MTX), an antagonist of folic acid, serves as the basis of RA management and is classified as a conventional synthetic disease-modifying anti-rheumatic drug (csDMARD). It is typically used as first-line monotherapy or in conjunction with other DMARDs and biological agents (Chiusolo *et al*., 2012).

The pathophysiology of RA involves complex relationships between genetic susceptibility and environmental triggers. T lymphocytes, B cells, macrophages, and pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-1) are central to disease initiation and progression (Brown *et al.,* 2016). Despite extensive research, etiology remains multifactorial, with genetic factors such as HLA-DR4 and non-HLA loci contributing significantly to disease susceptibility and heterogeneity in treatment response (Szostak *et al.*, 2020).

The efficacy of MTX is due to its anti-inflammatory and anti-proliferative characteristics, which result from its inhibitory effect of enzymes in the folate pathway, including dihydrofolate reductase and thymidylate synthase (Nomair *et al.,* 2024). Once inside the cell, MTX is polyglutamated and retained in the

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cytoplasm, prolonging its therapeutic action (Dervieux *et al*., 2004).

The cellular transport of MTX is mainly accomplished by the reduced folate carrier-1 (RFC1), encoded by the SLC19A1 gene located on chromosome 21q22.3. The function of RFC1 is essential for folate and antifolate drug uptake, especially in proliferating tissues (Lv *et al*., 2019). A common single-nucleotide polymorphism (SNP) in exon 2 of the RFC1 gene, G80A (rs1051266), results in an amino acid change (arginine to histidine), which may modify transporter efficiency and alter intracellular drug accumulation (Kung *et al*., 2014).

Several studies have evaluated the clinical relevance of RFC1 G80A polymorphism in various conditions, including cancer and RA, with inconsistent results. Some findings suggest that the A allele may lead to decreased MTX uptake and accumulation, thereby reducing efficacy and possibly requiring dose adjustments or combination therapy (Ranganathan & McLeod, 2006; Owen *et al*., 2013). Conversely, others report that AA genotype carriers may experience increased MTX levels, potentially due to impaired drug efflux or altered retention mechanisms (Chango *et al*., 2000).

Pharmacogenomics—the study of how genetic variation influences drug response—has emerged as a promising approach to personalize RA treatment. Identifying predictive markers, such as RFC1 G80A, could enable clinicians to tailor MTX dosing, minimize toxicity, and improve therapeutic outcomes (Stamp et al., 2011). The importance of personalized therapy is underscored by the wide interindividual variability observed in MTX pharmacokinetics and clinical response, which are influenced not only by genetics but also by age, sex, BMI, renal function, concomitant medications, and disease activity (Wessels *et al*., 2007).

In the Iraqi population, especially in the Kurdistan Region, pharmacogenomic data on RFC1 polymorphisms and their correlation with MTX efficacy in RA patients are lacking. This knowledge gap hinders the development of targeted strategies for optimizing RA therapy. Therefore, this study was conducted to evaluate the relationship between RFC1 G80A polymorphism and MTX plasma levels in a cohort of RA patients attending a tertiary rheumatology center in Duhok, Iraq. Additionally, the association between genotype, disease activity (as measured by DAS-28), and inflammatory biomarkers (ESR, CRP) was assessed.

The findings of this study may contribute to the growing body of evidence supporting the role of genetic testing in RA treatment and offer insights into the applicability of pharmacogenetic testing in routine clinical practice in Middle Eastern populations.

2. MATERIALS AND METHODS

Study Design and Ethical Approval:

The Rheumatology Center in Duhok, Kurdistan Region, Iraq, was the site of this cross-sectional analytical study, which was conducted from September 2024 to June 2025. Ethical approval was obtained from the General Directorate of Health Scientific and Ethical Committee (Approval No. 31072024-6-29). Participants were all provided with informed permission before joining, and the study took place in accordance with the Declaration of Helsinki.

Study Population:

A total of 90 adult patients diagnosed with rheumatoid arthritis (RA) according to the categorization criteria established by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) in 2010 (Aletaha *et al*., 2010), were consecutively recruited. The criteria for involvement were: (1) a history of RA for a minimum of six months, (2) ongoing oral MTX therapy for at least 1 month, and (3) stable MTX dosing regimen during the last 4 weeks. Patients were excluded if they had (1) irregular or non-adherent MTX intake, (2) hepatic or renal dysfunction, or (3) concurrent use of cytotoxic drugs or biological agents. Demographic and clinical information including sex, age, a body mass index (BMI), and duration of the disease, age at diagnosis, duration and dosage of MTX therapy, and comorbid conditions were obtained using a pretested organized questionnaire. Weight and height were measured using standardized equipment, BMI is expressed as weight in kilograms divided by height in meters squared (kg/m²). Disease activity was assessed using the Disease Activity Score in 28 joints (DAS-28), was collected from patients' datasheets. This score incorporates tender and swollen joint counts, erythrocyte sedimentation rate (ESR), and the patient's global health assessment on a visual analog scale (VAS) (Prevoo *et al*., 1995).

Laboratory Investigations:

Venous blood samples were collected under sterile conditions. Complete blood count (CBC), liver function tests [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], renal function tests (serum creatinine and blood urea), and inflammatory markers such as C-reactive protein (CRP) and ESR were measured using standard automated methods in the hospital laboratory. Immunological markers, including rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (Anti-CCP), were also recorded for each participant. Methotrexate plasma concentrations were determined using a competitive enzyme-linked immunosorbent assay (ELISA) method. A commercial MTX ELISA kit (BT LAB, China) was employed according to the manufacturer's instructions and performed at Zeriland Medical Laboratory/Duhok.

Genomic DNA Extraction:

For genotyping, carried out at Zeriland Medical Lab in Duhok/Iraq, whole blood was obtained in ethylenediaminetetraacetic acid (EDTA) tubes and stored at −20°C until analysis. Genomic DNA was extracted using a modified salt-out procedure as mentioned by Miller *et al*. (1988), which provides high-yield and high-purity DNA suitable for downstream molecular applications. The concentration and the purity of the isolated DNA were evaluated using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA) at 260 and 280 nm wavelengths. Samples with A260/A280 ratios between 1.8 and 2.0 were considered acceptable for PCR analysis (Fleige & Pfaffl, 2006).

Genotyping of RFC1 G80A Polymorphism:

The RFC1 G80A polymorphism (rs1051266) was analyzed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, as described previously by Owen *et al*. (2013). PCR was conducted in a 25 µL reaction volume that includes 12.5 µL of Taq DNA polymerase master mix (Addbio, South Korea); 2 µL forward primer (5′-AGT GTC ACC TTC GTC CCC TC-3′); 2 µL reverse primer (5′-CTC CGT GAA GTT CTT-3′); and 2 µL of DNA were used in the 25 µL

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reaction volume,.5 µL of distilled water (nuclease-free water) was also added. Primers were obtained from Humanizing Genomics (Macrogen, South Korea)

The PCR procedure included five minutes of initial denaturation at 95°C; thirty-five cycles of denaturation at 95°C for 15 seconds, annealing at 62°C for 30 seconds, and extension for 30 seconds; and a final seven-minute extension at 72°C. Following the manufacturer's instructions, 0.2 µL of the restriction enzyme HaeII (Haemophilus aegyptius II, New England Biolabs, USA) was used to treat 12.5 µL of the PCR products (230 bp) with .

enzymatic digestion. The samples were then incubated at 37 °C for three hours. The resulting fragments were separated using 3% agarose gel electrophoresis and stained with DNA Safe dye (AddBio, South Korea).

The genotypes were analyzed as follows: GG homozygotes indicate two fragments measuring 126 bp and 104 bp (complete digestion); GA heterozygotes display three bands (230 bp, 126 bp, and 104 bp), while AA homozygotes present one undigested band of 230 bp (Stamp *et al*., 2011), as displayed in Figure 1

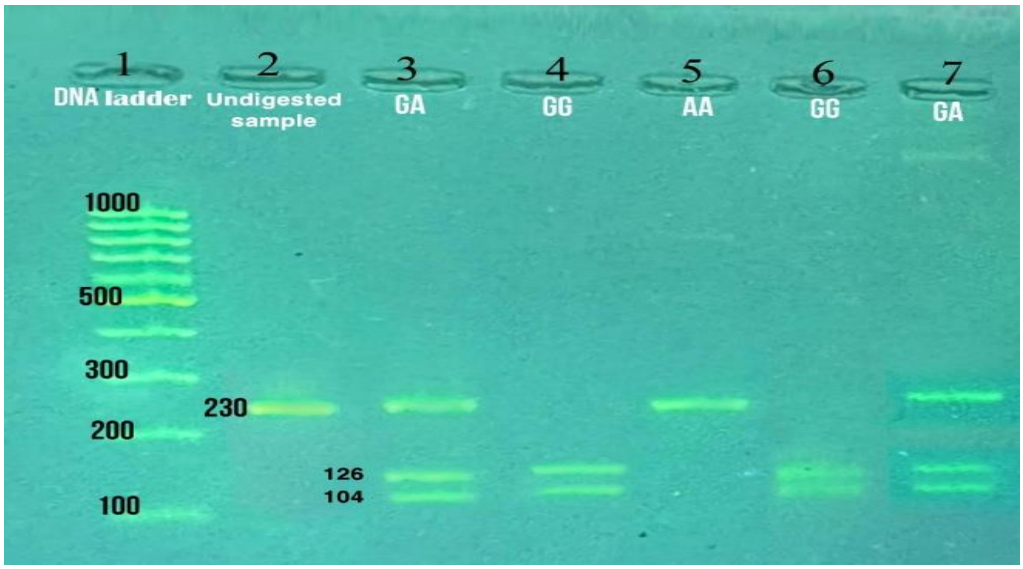

Figure 1: RFLP-PCR genotyping of rs1051266 (80G>A) utilizing particular primers and enzyme digestion. Lane 1: ladder 100 bp; Lane 2: amplified material without digestion used as a control (230bp); Lanes 3 and 7: heterozygous (GA) variant that retains the 230 bp band in addition to forming 126 and 104 bp. Lanes 4 and 6 are homozygous (GG) variants that exhibit full digestion of 230bp, resulting in two bands of 126 and 104 bp. Lane 5 homozygous (AA) variant with 230bp band persistence, indicating no enzyme digestion.

Statistical Analysis:

Statistical analysis was conducted using IBM SPSS version 26.0 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation (SD). The Shapiro–Wilk test was employed to assess the normality of distribution for continuous variables. For normally distributed variables, comparisons between two groups were made using the independent t-test, while comparisons among three or more groups were analyzed using one-way ANOVA. Categorical variables were expressed as frequencies and percentages and analyzed using the Chi-square test or Fisher's exact test where appropriate. A p-value of less than 0.05 was considered statistically significant. Hardy-Weinberg equilibrium (HWE) was tested to validate the genetic distribution. Linear regression models were applied to examine the associations between RFC1 genotypes and MTX levels.

3. RESULTS

Patient Demographics and Clinical Characteristics:

A total of 90 patients diagnosed with rheumatoid arthritis (RA) and receiving MTX therapy were included in this study. Females comprised the majority (91.1%, n = 82), with a mean age of 50.1 ± 11.9 years. The mean body mass index (BMI) was 29.6 ± 6.0 kg/m², significantly higher in females compared to males (29.9 ± 6.1 vs. 26.4 ± 3.4, p = 0.04), consistent with the female predominance and obesity trend in RA cohorts.

The mean age at RA diagnosis was 35.2 ± 12.9 years. Participants had been diagnosed for an average of 14.3 ± 10.4 years and had been taking MTX for a mean of 9.6 ± 7.8 years. The average MTX dose was 14.25 ± 3.5 mg per week. Common comorbidities included hypertension (42.2%), diabetes mellitus type II (22.2%), and hypothyroidism (16.7%). Prednisolone (62.2%) and folic acid (87.8%) were frequently prescribed as adjuncts to MTX therapy (Table 1).

Table 1: Demographic and Clinical Characteristics of RA Patients Stratified by Sex

Variables	Total (N=90)	Male (N=8)	Female (N=82)	*p*-value
Age (years)	50.1 ± 11.9	48.4 ± 10.6	50.3 ± 12.1	0.65*
BMI	29.6 ± 6.0	26.4 ± 3.4	29.9 ± 6.1	0.04*

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Age at Diagnosis (years)	35.2 ± 12.9	39.6 ± 9.9	34.8 ± 13.1	0.28*
Disease Duration (years)	14.3 ± 10.4	9.6 ± 8.9	14.8 ± 10.4	0.16*
MTX Duration (years)	9.6 ± 7.8	7.1 ± 7.2	9.9 ± 7.8	0.31*
MTX Dose (mg/week)	14.25 ± 3.5	13.5 ± 3.25	14.25 ± 3.5	0.55*
Hypertension	42.2% (38/90)	25% (2/8)	43.9% (36/82)	0.32†
Diabetes Mellitus	22.2% (20/90)	0% (0/8)	24.4% (20/82)	0.12†
Prednisolone Use	62.2% (56/90)	50% (4/8)	63.4% (52/82)	0.49†

p-values from an independent t-test (male vs. female).
†p-values from Chi-square/Fisher's exact test.
* = statistically significant ($p < 0.05$).

Distribution of RFC1 G80A Genotypes and Methotrexate Plasma Concentrations Across Genotypes:

Genotyping for the RFC1 G80A polymorphism (rs1051266) showed a distribution of: GG: 53.3% (n = 48); GA: 35.6% (n = 32); AA: 11.1% (n = 10).

The observed genotype frequencies were in Hardy–Weinberg equilibrium ($p > 0.05$) and similar to other population studies on RA pharmacogenetics.

The mean MTX plasma concentration was highest in patients with the AA genotype (330.1 ± 70.5 ng/mL), followed by GA (325.1 ± 65.0 ng/mL) and GG (318.0 ± 62.4 ng/mL). Although these values demonstrated a rising trend with the presence of the A allele, the differences were not statistically significant ($p = 0.82$) (Table 2).

This finding suggests a potential gene-dose effect, as previously reported, in which the A allele may reduce the efficacy of the RFC1 transporter, leading to reduced MTX efflux, higher intracellular retention, and greater serum accumulation (Table 2).

Table 2: MTX Plasma Levels and Disease Duration Across RFC1 G80A Genotypes

Genotype	Frequency	MTX Level (ng/mL)	Disease Duration (years)	p-value
GG	53.3% (48)	318.0 ± 62.4	13.8 ± 10.1	Ref.
GA	35.6% (32)	325.1 ± 65.0	15.2 ± 11.3	0.68*
AA	11.1% (10)	330.1 ± 70.5	14.5 ± 9.8	0.79*

P-values from linear regression (adjusted for age/sex).

Disease Activity Score (DAS-28) and Genotype Association:

There was a significant association between RFC1 G80A genotypes and RA disease activity, as measured by DAS-28. Patients with the AA genotype exhibited significantly higher DAS-28 scores compared to those with the GG genotype ($p = 0.009$), indicating greater disease activity and suboptimal MTX response. This supports prior reports that polymorphisms in folate pathway genes can influence MTX therapeutic response (Ranganathan & McLeod, 2006; Wessels *et al.*, 2007). The DAS-28 scores are presented in Table 3.

Table 3: DAS-28 scores Stratified by RFC1 Genotypes

Genotype	Frequency	DAS-28 (Mean ± SD)	p-value
GG	53.3% (48)	3.8 ± 1.2	Ref.
GA	35.6% (32)	4.1 ± 1.4	0.15*
AA	11.1% (10)	4.3 ± 1.5	0.009*

DAS-28: Disease Activity Score; *28*

Hematological and Inflammatory Markers by Genotype:

Inflammatory biomarkers (CRP and ESR) and hematologic indices (hemoglobin, white blood cells [WBC], platelets) were analyzed across genotypes. CRP levels: highest in AA genotype (24.5 ± 35.2 mg/L), followed by GA (20.8 ± 31.4) and GG (16.2 ± 25.1), though not statistically significant ($p = 0.57$). ESR followed a similar trend: AA (35.0 ± 22.9 mm/hr) > GA (32.1 ± 24.7) > GG (28.3 ± 20.5), $p = 0.49$.

Hematologic parameters, including hemoglobin, WBC, and platelet counts, showed no significant difference between the categories ($p > 0.05$ for all), suggesting that RFC1 G80A polymorphism does not contribute significantly to hematologic toxicity (Table 4).

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Table 4: Laboratory Parameters by RFC1 Genotype

Parameter	GG (N=48)	GA (N=32)	AA (N=10)	*p*-value
Hemoglobin (g/dL)	12.7 ± 1.2	12.3 ± 1.4	12.1 ± 1.6	0.21*
WBC (×10³/μL)	8.5 ± 2.8	9.4 ± 3.5	9.8 ± 3.2	0.18*
Platelets (×10³/μL)	268.1 ± 75.3	281.4 ± 82.1	287.0 ± 89.4	0.64*
ESR (mm/hr)	28.3 ± 20.5	32.1 ± 24.7	35.0 ± 22.9	0.49*
CRP (mg/L)	16.2 ± 25.1	20.8 ± 31.4	24.5 ± 35.2	0.57*
MTX Concentration (ng/mL)	318.0 ± 62.4	325.1 ± 65.0	330.1 ± 70.5	0.82*

p-values from ANOVA (comparing GG, GA, AA genotypes).

4. DISCUSSION

Methotrexate continues to be the cornerstone of RA management due to its proven efficacy, tolerability, and affordability. However, interindividual variability in treatment response and adverse effects poses a significant challenge in optimizing therapeutic outcomes. This variability has been partially attributed to pharmacogenetic factors, particularly polymorphisms in genes involved in transporting MTX and metabolism. One such key gene is the RFC1 gene, also known as SLC19A1, which encodes the primary transporter responsible for MTX uptake into cells. In this context, the present study evaluated the impact of RFC1 G80A (rs1051266) polymorphism on serum levels of MTX and disease activity in Iraqi RA patients from the Kurdistan Region.

In our cohort, the AA genotype was associated with the highest mean MTX serum levels (330.1 ± 70.5 ng/mL), followed by GA (325.1 ± 65.0) and GG (318.0 ± 62.4). Although this trend was not statistically significant, it suggests a potential gene-dose effect, where the presence of the A allele may lead to decreased efficiency of RFC1-mediated MTX cellular uptake, resulting in its accumulation in the plasma. This aligns with earlier studies reporting that the A allele may alter RFC1 protein structure, reducing intracellular drug uptake and increasing extracellular retention (Chango *et al*., 2000; Wessels *et al*., 2007).

Dervieux *et al* (2004) demonstrated that reduced MTX uptake associated with RFC1 polymorphisms may impair the formation of active MTX polyglutamates, which are crucial for inhibiting folate-dependent enzymes involved in DNA synthesis. Similarly, Chango *et al*. (2000) described that the 80A variant leads to conformational changes in the RFC1 protein, thus reducing MTX transport efficiency. Although our findings support this biological plausibility, the lack of statistical significance may be due to the small number of AA genotype carriers (n = 10), which reduced the power to detect inter-group differences.

Importantly, our study found a strong relationship between RFC1 G80A genotype and RA disease activity**,** as measured by DAS-28 scores. Patients with the AA genotype had significantly higher DAS-28 scores (4.3 ± 1.5) than those with GG genotype (3.8 ± 1.2, p = 0.009**)**, indicating higher activity of the disease and a poorer MTX therapy response. This finding matches data from Wessels *et al*. (2007) and Owen *et al*. (2013), who demonstrated that the A allele is correlated with weakened MTX effectiveness and higher disease severity.

A potential mechanism may involve altered intracellular MTX levels and reduced polyglutamate formation, leading to insufficient inhibition of pro-inflammatory pathways such as TNF-α and IL-6. The higher levels of CRP and ESR observed in AA genotype carriers in our study, although not statistically significant, also support this hypothesis and point toward a more active disease state.

Our findings are broadly consistent with those from Asian and Middle Eastern populations. A study by Lv *et al*. (2019) on a Chinese RA cohort stated that the RFC1 80AA genotype was correlated with poorer response to MTX and higher inflammatory activity, mirroring our results. Likewise, Kung *et al*. (2014) in their meta-analysis revealed that RFC1 80G>A polymorphism significantly influences MTX efficiency, especially in non-Caucasian populations. However, studies from European populations have shown mixed results, possibly due to ethnic differences in allele frequency, linkage disequilibrium with other variants, or gene–environment interactions (Huizinga & Wessels, 2008).

While not statistically significant, CRP and ESR levels were higher in AA genotype carriers, suggesting a biologically plausible link between RFC1 polymorphism and persistent inflammation. Stamp *et al*. (2011) previously demonstrated that MTX pharmacodynamics, including its anti-inflammatory properties**,** may be attenuated Within people that have the A allele, resulting in higher systemic inflammation. Our findings add to this body of evidence and support the use of genotype-based personalization of MTX therapy to improve outcomes.

The clinical implications of these findings are substantial. RA patients with the RFC1 AA genotype possibly risked of inadequate response to standard MTX therapy. Identification of this genotype could justify early escalation to combination DMARDs or biologic agents, thereby avoiding prolonged disease activity and joint damage. Furthermore, pharmacogenetic profiling could assist in dose adjustment, particularly in populations with high frequencies of the A allele.

Given the growing emphasis on personalized medicine, incorporating pharmacogenomics into RA treatment algorithms

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could help optimize MTX use, reduce toxicity, and improve long-term outcomes. However, this approach must be validated through large-scale, prospective studies before routine clinical implementation.

Limitations:

This research possesses multiple limitations. The cross-sectional method prevents any causal relationship between the genetic makeup of RFC1 and MTX response. The lowered samples of males and the lowered sample size of the AA subgroup restricted the ability of statistical analysis to identify variations in some parameter values. Third, while MTX plasma concentration was measured, MTX polyglutamates, the pharmacologically active intracellular forms, were not assessed. Finally, the study did not evaluate gene–gene interactions, which are increasingly recognized as critical in pharmacogenomics.

CONCLUSION

This study suggests that the RFC1 G80A polymorphism may influence MTX pharmacokinetics and its therapeutic effectiveness in RA patients. Although genotype differences in MTX levels were not statistically significant, individuals with the AA genotype tended to have higher MTX levels, worse disease activity, and greater inflammation, indicating a reduced response to treatment. The A allele may hinder MTX uptake into cells, limiting its effectiveness. These findings highlight the potential of RFC1 G80A genotyping as a predictive biomarker to guide personalized RA therapy. The study calls for larger, multiethnic research to validate these results and assess the clinical utility of incorporating pharmacogenetic testing into routine care.

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Ethical Approval:

Approval for the study was obtained by the Scientific and Ethical Committee of the General Directorate of Health (Approval No. 31072024-6-29). Informed consent was obtained from all participants prior to their involvement.

Author Contributions:

R.G.A.: Collected data and performed all investigations, including ELISA and molecular analyses.
A.A.E.: Designed the study, supervised the investigations, analyzed the data, and wrote the manuscript.

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