ISSN: 0067-2904

MTHFR Gene Polymorphisms in Iraqi Kurdish Rheumatoid Arthritis Patients: Relation to Methotrexate Response and Toxicity

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Received: 20/2/2022 Accepted: 12/6/2022 Published: 30/12/2022

Abstract.

Methotrexate (MTX) is still one of the gold standard treatments for rheumatoid arthritis (RA). It shows diverse outcomes in blood level and clinical response, this was demonstrated by its relation to the genetic polymorphism in the pharmacogenetic study. This study aimed to investigate the role of methylenetetrahydrofolate reductase (MTHFR) gene polymorphisms in relation to MTX efficacy and toxicity in Iraqi Kurdish RA patients. Sixty-four RA patients were involved in this study with an average age of 47.78 ±14.08 and female to male ratio of (8.1). Diagnosis and disease activity were confirmed. Blood analyses, including those of laboratory markers of disease activity, were done. The 28 joint disease activity score (DAS28-CRP) was calculated. MTHFR gene polymorphisms were analyzed by real-time polymerase chain reaction. The most frequent genotypes which were identified in RA patients were the CT genotype of the C677T single nucleotide polymorphism (SNP) (51.6%) and the AC genotype of the A1298C SNP (48.4%). Patients with non-response to treatment had high frequencies of genotypes CT and TT (58.0% and 12.0%) of the C677T SNP respectively, as compared to those in the responder group; 28.6% and 0.0%); T-allele was associated with drug non-responding OR=4.17, P value=.0.009, meanwhile; genotypes AC and CC of the A1298C SNP were seen in (54.0% and 16.0%) in non-responder group. Patients with active RA had increased frequencies of CT and TT genotypes of the C677T SNP (60.0% and16.0%) respectively as compared to those who were in remission (26.6% and 0.0%); T-allele was associated with high disease activity; OR = 5.11. No association was found between C677T SNP and A1298C SNP, and MTX level status (P> 0.05). However, the variant alleles (T and C) were associated with the MTX toxic level (OR: 2.05, 95% CI [0.97 – 4.32]) and (OR: 1.99, 95% CI [0.96 – 4.18]) respectively. This study suggests that genetic polymorphisms of MTHFR SNP (C677T and A1298C) are associated with MTX efficacy but not toxicity in RA patients. This may assist the physicians in personalizing RA treatment in Iraqi patients.

Keywords: RA, MTHFR polymorphism, MTX efficacy and toxicity, ACCP.

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تعدد الأشكال الجينية لمثيلين رباعي هيدروفولات المختزلة في مرضى التهاب المفاصل الرثوي في كردستان العراق: العلاقة بالميثوتريكسيت استجابته وسميته

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الخلاصة

لا يزال الميثوتريكسيت احد العلاجات القياسية الذهبية لالتهاب المفاصل الرثوي .اظهر(MTX) نتائج متنوعة فى المستوى بالدم والاستجابة السريرية له، وهذه كانت مبينة خلال علاقته بتعدد الأشكال الجينية في الدراسة الوراثية الدوائية . الدراسة الحالية هدفت إلى التحري عن دورتعدد الأشكال الجينية لميثيلين رباعي هيدروفولات المختزلة فيما يتعلق بفعالية الميثوتريكسيت وسميته في مرضى التهاب المفاصل الرثوي في كردستان العراق. شارك في هذه الدراسة 64 مريضًا من مرضى التهاب المفاصل الرثوي بمتوسط عمر 47.78 ± 14.08 ونسبة الإناث إلى الذكور (1:8). ان اجراء الفحص السريري للتأكد من تشخيص ونشاط المرض قد تم من قبل أخصائي أمراض الروماتيزم . وقد تم فحص تحاليل الدم للعلامات البيوكيميائية والبيولوجية لنشاط المرض.وكذلك تم حساب درجة نشاط المرض لـ 28 مفصل (DAS28–CRP). اما تحليل تعدد الأشكال الجينية لميثيلين رباعي هيدروفولات فقد تم عن طريق تفاعل البلمرة المتسلسل في الوقت الحقيقي. كانت الأنماط الجينية الأكثر شيوعًا التي تم اكتشافها في مرضى الالتهاب المفاصل الرثوي هي٪ 51.6 لـ CT (C677T SNP) و ٪ 48.4لـ AC (A1298C SNP) . وكان لدى المرضى الذين لم يستجيبوا للعلاج ترددات عالية من الانماط CT و TT (58.0٪ و 12.0٪) على التوالي ، مقارنة مع أولئك المستجيبين للعلاج (28.6٪ و 0.0٪) على التوالي ؛ الاليل T ارتبط بعدم الاستجابية للدواء = (OR 4.17، (P=.0.009) .في نفس الوقت؛ الأنماط الجينية AC و CC لوحظت في (54.0٪ و 16.0٪) في المجموعة الغير المستجيبة للعلاج. المرضى الذين يعانون من التهاب المفاصل الرثوي النشط لديهم زيادة في ترددات الانماط الجينية لـ TT,CT (C677T) (60 % و16%) على التوالي مقارنة مع أولئك الذين مرضهم غير نشط (26.6 ٪ و 0.0 ٪). الاليل T ارتبط بالنشاط المرضي العالي (OR=5.11) .لم يتم العثور على ارتباط بين C677T وA1298C) وحالة مستوى الميثوتريكسيت في الدم (p>0.05). ومع ذلك ، فإن الأليلات المتغيرة (T وC) كانت مرتبطة بالمستوى السمي للميثوتريكسيت [4.32 – 0.97] CI 95٪ (OR: 2.05) و[4.18 – 0.96] CI 95٪ (OR: 1.99) على التوالي .هذه الدراسة اقترحت أن تعدد الأشكال الجينية لميثيلين رباعي هيدروفولات C677T و A1298C مرتبطة بفعالية الميثوتريكسيت ولكن ليس مع سميته في مرضى الالتهاب المفاصل الرثوي وهذا , قد يساعد الاطباء في تخصيص علاج التهاب المفاصل الرثوي في المرضى العراقيين.

Introduction

Rheumatoid arthritis (RA) is one of the autoimmune diseases that is characterized by chronic inflammation of the joints which is followed by the destruction of joint cartilage and bony erosion and ends with joint deformities and disabilities [1]. To date, several options were available for the treatment of patients with RA, including the following medications: nonsteroidal anti-inflammatory drugs, corticosteroids, disease-modifying antirheumatic drugs (DMARDs), and biological agents. The most commonly used DMARD world wildly for RA treatment is methotrexate (MTX) [2-5].

MTX is a folate antagonist that is a well-established treatment for inflammatory and autoimmune conditions. Recently, MTX is the first-line DMARDs in the RA therapy, because

of their useful activities as anti-inflammatory and immunomodulatory [6]. The MTX action's mechanisms are not clear, but are displayed to include: raising the release of endogenous adenosine and T cells apoptosis, cellular adhesion molecules changed expression, changes in the production of cytokine, formation of bone, and responses of humoral [7]. MTX is an antimetabolite that inhibits the conversion of dihydro folic (FH_2) into tetrahydro folic acid (FH_4), - the cofactor that plays an important role in the synthesis of amino acids and nucleic acids and amino acids- due to binding to dihydrofolate reductase (DHFR) in the folate pathway [8].

Methylene tetrahydrofolate reductase (MTHFR) is an enzyme that has a key role in the modify proteins, synthesis of DNA, repair, and methylation by helping the body process folate. The MTHFR gene polymorphisms localized on chromosome 1 (1p36.3), were the object of plenty works. The two most studied missense mutations were C677T substitution (rs1801133) which is the first one and A1298C substitution (rs1801131) which is the second one, and both were described as having an association with the MTX outcomes regarding toxicity and/or efficiency in patients with RA [9-10].

The C677T mutation occurs by converting alanine for a valine through substituting C by T in exon 4 of the SNP rs1801133 in position 222 of the protein. The A1298C mutation formed through converting glutamine to alanine by altering A with C in exon 7 of the SNP rs1801131 in position 429 of the protein [11].

Furthermore, clinical response to MTX treatment in RA shows personal variations in side effects and efficacy, and 33% of patients were failed to get remission; due to low efficacy or toxicity has a great impact on discontinuation of MTX in the treatment of RA patients as reported by several studies [3,12]. Moreover, many factors possibly influence therapeutic outcomes and disease course in patients treated by MTX, these factors include environmental, physiological, pathological, and genetic ones [13-14].

Several previous studies, tried to clarify the clinical response to MTX and the genetic variations in patients with RA, therefor great interest was raised by pharmacogenomics studies to explain genetic polymorphisms in MTHFR [15-16].

Moreover, polymorphisms in genes of enzyme metabolizing MTX bring about an individual variation of MTX efficacy and toxicity. The single SNPs of MTHFR were associated with MTX outcomes in RA. Nevertheless, the results of these studies are controversial and inconclusive [17-18].

However, only little is known about the MTHFR polymorphism in the Iraqi population, especially among those with rheumatoid arthritis. Thus, it is important to study frequencies of genotypes and alleles of MTHFR SNPs C677T and A1298C in rheumatoid arthritis and to determine their association with the efficacy and toxicity of MTX as this polymorphism might provide a clue about the clinical application of MTX in RA patients. Recently, similar to our approach, others were tried to seek the genetic role in Iraqi RA patients [19-20].

Material and Methods

Subjects

Our Case-control study involved 64 healthy subjects with a mean age of 46.98±15.1 years and Sixty-four RA patients with a mean age of 47.78 ±14.08 years and female to male ratio (8.1). Patients were diagnosed with RA according to the criteria of ACR / EULAR 2010

(American college of rheumatology / European League Against Rheumatism.) [21]. Subjects were recruited in Duhok Center for Rheumatic Disease and Medical Rehabilitation (DCRDMR) as case and control was the staff of Azadi teaching hospital. All patients were treated mainly with MTX with an average weekly dose of 13.71±0.73 mg/week it has been taken orally duration of 7.97±0.77 years. Disease activity score-28 with CRP (DAS28-CRP) was calculated and MTX adverse effects were recorded. Eventually, response to treatment has been evaluated using (DAS28-based EULAR response criteria) [24], accordingly, RA patients were classified into the responder group (their DAS28-CRP ≤2.6 and they were in remission) and the non-responders' group (their DAS28-CRP>2.6).

Metabolite Analysis

Whole blood was collected in Ethylene Diamine Tetra Acetic Acid (EDTA) tubes for MTHFR gene polymorphisms study. Another volume of blood was collected in a gel tube, for laboratory investigations involving MTX blood level, Anti cyclic citrullinated peptide (ACCP), C-reactive protein (CRP), and Homocysteine (Hcys) [22-23]. Human Methotrexate ELISA KIT (Bioassay Technology Laboratory, China) was used for the determination of MTX serum level (Bio-Tek reader, USA). ACCP, CRP, and Hcys were analyzed using (Cobas 6000, Japan). Immune assay and chemistry based on CLIA (chemiluminescent immune assay) and turbidometry method using the special kit (Roche Diagnostics GmbH, Germany).

Genetic Analysis

DNA was extracted from leukocytes via an automated machine called SaMag-12 devise designed for this purpose (Sacace biotechnologies Sri, Italy) using a SaMag blood DNA Extraction kit from the same source. The A1298C and C677T (MTHFR genetic polymorphisms) were analyzed by real-time PCR detection - Rotor-Gene Q. (Qiagen Hilden, Germany) with software (Rotor-Gene Q Series Software) using MTHFR Real-Time PCR kits (SNP biotechnology, Turkey).

Statistical Analysis

Statistical analysis was performed using the IBM SPSS Version 25. Hardy-Weinberg equilibrium was used for the estimation of alleles' frequencies. The Fisher's exact test or Pearson Chi-square test were used for comparing frequencies of genotypes and alleles, and MTX level status. One Sample T-test and independent T-test were used for Means comparison. Two-sided P-value (<0.05) was considered statistically significant. The association was done using the Odd Ratio (OR) and the 95% confidence interval (95%CI) were calculated manually.

Results and Discussion

The study is considered to be one of the primitive prospective works which was done as a pharmacogenetic study on polymorphisms of the MTHFR genes and their correlation with methotrexate response (or efficacy) and toxicity in the Iraqi Kurdish population. The relation between genetic mutability and medication outcomes shown by pharmacogenetic studies, polymorphisms of the MTHFR genes was one of the most important ones that can impact the outcomes of MTX treatment by impacting the activity of the enzyme and MTX metabolism [25-26].

General characteristics of the study population.

The Demographics and Clinico-Biochemical variables of the studied population enrolled in the study was illustrated in **Table 1**. All 64 patients who were enrolled in the study had

typical clinical and biological features of rheumatoid arthritis. The biochemical parameters investigated then the results show a statistically significant difference between both groups ($p<0.05$).

Table 1: Comparison of disease-related characteristics between RA patients and healthy subjects

Characteristics	RA patients (N=64)	Healthy subjects (N=64)	P-value
1-Demographic			
Age (years)	47.78±14.08	46.98±15.1	0.67
Male N (%)	7(10.9)	8(12.5)	0.78
Female N (%)	57(89.1)	56(87.5)	/
F/M ratio	8.1	7.0	/
2-Clinical			
DAS28-CRP	4.38±1.42	/	/
RA duration (years)	11.19±7.55	/	/
3-Biochemical			
ACCP (U/ml).	106.46±95.64	5.2±2.3	0.00
CRP (mg/l.)	25.06±20.06	3.9±1.7	0.00
Hcys (umol/l)	17.30±8.52	7.2±3.4	0.00

Distribution of genotype and allele frequency of MTHFR polymorphisms in patients with RA and healthy subjects.

To illustrate the relation of MTHFR polymorphisms with RA susceptibility. The comparison of frequencies of C677T and A1298C genotypes and alleles distribution between RA patients and healthy subjects were analyzed and shown in Table 2.

Our study results revealed that the difference between frequencies of MTHFR genotypes distributed between RA patients (as a case group) and healthy subjects (as a control group) was not significant ($p> 0.05$), but the difference regarding alleles of both SNPs was significant ($p<0.05$). Although, homozygous recessive genotypes TT of the C677T and CC of the A198C were associated with RA susceptibility in the studied population (OR:4.44 and 2.88) respectively, indicating that these polymorphisms were associated with RA susceptibility in the Iraqi Kurdish population which was in conflict to the results of Gonzalez-Mercado et al. [27].

Table 2: Distribution of disease-related genetic characteristics between RA patients and healthy subjects (N=64)

Genetic MTHFR polymorphism	RA patients N (%)	Healthy subjects N (%)	OR, 95%CI	P-value
C677T SNP Genotypes				**0.066**
CC	25(39.1)	37(57.8)	/	/
CT	33(51.6)	25(39.1)	1.95, [0.94-4.04]	0.069
TT	6(9.4)	2(3.1)	4.44, [0.83-23.8]	0.063
Alleles				**0.027**
C	83(64.84)	99(77.3)	/	/
T	45(35.16)	29(22.7)	1.85, [1.07-3.21]	/
A1298CSNP Genotypes				**0.10**
AA	25(39.1)	36(56.3)	/	/
AC	31(48.4)	24(37.5)	1.86, [0.89-3.89]	0.098
CC	8(12.5)	4(6.2)	2.88, [0.78-10.61]	0.102
Alleles				**0.042**
A	81(63.28)	96(75)	/	/
C	47(36.72)	32(25)	1.74, [1.02-2.98]	/

The relation between MTHFR polymorphisms and MTX outcomes (MTX efficacy)

The distribution of MTHFR polymorphisms genotypes and alleles between responders to MTX and non-responder groups were shown in **Table 3**. The percentage of RA patients who responded to MTX treatment was 21.88%. The most considerable findings and clues from the present study were the presence of a significant association between MTHFR polymorphism genotypes and alleles of both SNP C677T and A1298C, and MTX efficacy ($p < 0.05$). Weak relation between the efficacy of MTX and, two MTHFR gene polymorphism were displayed by some other studies [28-29]. In contrast to our results, Shuang et al. didn't find any association between genotypes and alleles of MTHFR SNPs (C677T and A1298C) and MTX efficacy [30].

Regarding C677T, this study revealed that genotypes and alleles appear at higher frequencies of homozygous dominant genotype CC and dominant allele C in the responder group 71.4% and 85.7%. respectively ($p < 0.05$). The dominant allele C of the C677T was significantly associated with MTX efficacy (OR:4.17, 95% CI [1.35-12.92] which seemed to have an association with better drug response. However, all homozygous recessive genotype TT were present in the non-responder group ($p < 0.05$), when compared to the data which was collected from other available studies, a direct relation to RA and response to MTX had appeared. Dervieux et al., revealed that a lack of response to MTX was associated with TT genotype of the C677T polymorphism [31].

Concerning A1298C, our study showed that higher frequencies of homozygous dominant genotype AA and dominant allele A (71.4% and 85.7%) respectively were emerged in the MTX responders' group. The association of dominant AA genotype with MTX response was strongly significant $p < 0.05$. The dominant allele A was associated with MTX responding (OR:4.53, $p<0.05$). Similarly, regarding A-allele, Berkani et al. showed a significant association between A-allele and response to MTX, while regarding AA genotype they did not show any significant association with the MTX response [32]. One of the studies done in Indian subjects by Ghodke Puranik et al.; explained that RA patients with one dominant A-allele at least (AA or AC of A1298C genotypes) could benefit from MTX treatment when compared with those who have the homozygous recessive genotype CC [29]. On the other hand, Shakari et al. showed that homozygous recessive genotype CC and heterozygous genotype AC has a significant association with not responding to MTX drug ($p < 0.05$), and RA patient who were carrying recessive allele C has significant association with not responding to MTX ($p < 0.05$) [18]. In contrast to our result, Grabar et al. did not detect any significant association between MTX response and MTHFR A1298C genotypes [33].

Table 3: Distribution of disease-related genetics characteristics in relation to response to treatment in RA patients

MTHFR polymorphism	Responder (N=14) N (%)	Non-responder (N=50) N (%)	OR: [95%CI]	P-value
C677T SNP Genotypes				**0.016**
CC	10(71.4)	15(30.0)	/	/
CT	4(28.6)	29(58.0)	0.21 [0.06-0.77]	0.014
TT	0(0.0)	6(12.0)	0.05 [0.00-4.62]	0.081
Alleles				**0.009**
C	24(85.7)	59(59.0)	4.17 [1.35-12.92]	/
T	4(14.3)	41(41.0)	0.24 [0.08-0.74]	/
SNP A1298C Genotypes				**0.014**
AA	10(71.4)	15(30.0)	/ /	/
AC	4(28.6)	27(54.0)	0.22 [0.06-0.83]	0.02
CC	0(0.0)	8(16.0)	0.04 [0.0-3.41]	0.043
Alleles				**0.005**
A	24(85.7)	57(57.0)	4.53 [1.46-14.01]	/
C	4(14.3)	43(43.0)	0.22 [0.07-0.68]	/

Association between MTHFR and MTX level status

The distribution of MTHFR polymorphisms genotypes and alleles between MTX toxic and non-toxic blood level groups were shown in Table 4.

Focusing on the relation between MTX toxicity and two MTHFR gene polymorphism, our study revealed that RA patients who took MTX drug attained MTX toxic blood levels at a higher percentage 34 (53.13%). Even though, no significant difference was observed between MTX blood level status and the two MTHFR SNPs (C677T and A1298C) genotypes and alleles. Similarly, same observation was seen in other studies regarding A1298C [25-26].

In terms of C677T SNP, our results showed that the heterozygous genotype CT and the homozygous recessive genotype TT in addition to recessive allele T have a higher frequency (55.9%,14.7%, and 42.6%) respectively in those with MTX toxic level. Also, they are associated with MTX toxic blood levels (OR: 2.04, 7.5, and 2.04), but to extent not reached a significant level ($p > 0.05$). In a similar manner, some studies described the association of C677T SNP homozygous recessive genotype TT and heterozygous genotype CT, and MTX toxicity [34-35]. while some other studies revealed the association of the recessive allele T with MTX toxicity [35-37]. In contrast, Qiu, et al., and Jing Huang et al. showed that there was no significant association observed between MTX toxicity and the C677T alleles frequency in the East Asian populations. [38-39].

In respect to A1298C, our results revealed the association of heterozygous genotypes AC and the homozygous recessive genotype CC and recessive allele C with the MTX toxicity (OR:2.08, 4.5, and 2.0) but to an extent not attained significant level ($P>0.05$). However, the risk of MTX toxicity was raised in RA patients with homozygous recessive genotype CC and heterozygous AC [40]. Similarly, Choe et al. noted that a higher frequency of RA patients who carried homozygous recessive genotype CC could present with drug toxicity when compared to those who had homozygous dominant AA genotype [41].

Table 4: Distribution of genetic characteristics in relation to MTX blood level status in RA patient

MTHFR polymorphism	Toxic (N=34) N (%)	Non-Toxic (N=30) N (%)	OR (95%CI)	P-value
C677T SNP Genotypes				**0.12**
CC	10(29.4)	15(50.0)	/	/
CT	19(55.9)	14(46.7)	2.04[0.71-5.89]	0.185
TT	5(14.7)	1(3.3)	7.5[0.76-74.16]	0.056
Alleles				**0.059**
C	39(57.4)	44(73.3)	0.49[0.23-1.03]	/
T	29(42.6)	16(26.7)	2.04[0.97-4.32]	/
SNP A1298C Genotypes				**0.16**
AA	10(29.4)	15(50.0)	/	/
AC	18(52.9)	13(43.3)	2.08[0.71-6.07]	0.179
CC	6(17.6)	2(6.7)	4.5[0.75-26.93]	0.085
Alleles				**0.064**
A	38(55.9)	43(71.7)	0.50[0.24-1.05]	/
C	30(44.1)	17(28.3)	2.0 [0.95-4.18]	/

Relation between MTX and biochemical characteristics.

The RA disease-related biochemical characteristics level groups were distributed among RA patients with MTX level status (toxic and non-toxic) groups as illustrated in **Table 5**.

The positive level of ACCP, CRP, and Hcys were strongly associated with MTX toxicity (OR:18.29, 37.71, and 15.0) respectively. The difference between ACCP groups and MTX level status was statistically significant, similarly, same difference was noted between groups of ACCP, CRP, and of Hcys, and MTX level status (P <0.05). Similar to our study, Chaabane et al., observed the significant association between MTX toxicity and elevated level of Hcys in RA patients p <0.05 [42]. Lima et al., showed that positive ACPA could use as potential predictive factor for not-responding to MTX drug [16].

Table 5: Distribution of Biochemical markers in relation to MTX level Status groups in RA patients

Biochemical characteristic	Toxic N (%)	Non-Toxic N (%)	OR [95%CI]	P-Value
ACCP				0.00
Negative (≤17.0 IU/ml)	2(5.9)	16(53.3)	0.05 [0.01-0.27]	/
Positive (>17.1 IU/ml)	32(94.1)	14(46.7)	18.29 [3.7-90.44]	/
CRP				0.00
Negative (≤ 6.0 mg/L)	1(2.9)	16(53.3)	0.03 [0.00-0.22]	/
Positive (>6.0 mg/L)	33(97.1)	14(46.7)	37.71 [4.55-312.58]	/
Hcys				0.00
Normal level (≤15.0 umol/l)	4(11.8)	20(66.7)	0.07[0.02-0.24]	/
Increased level (>15.0 umol/l)	30(88.2)	10(33.3)	15.0[4.13-54.50]	/

Relation between DAS28-CRP and MTX level status.

Distribution of DAS28-CRP grade among RA patients with MTX level status (toxic and non-toxic) groups was demonstrated in **Table 6**. with increasing MTX toxicity the disease activity worsens when crossed over moderate to reach high grade. However, high DAS28-CRP grade had strong association with MTX toxic level significantly (p<0.05, OR:41.17).

Furthermore, the majority of RA patients with remission grades had MTX with a non-toxic level. Similarly, Wang et al., indicated that disease activity might be one of the factors that influence MTX toxicity independently, he also demonstrated that patients with good MTX efficacy had lower DAS28 grade [35].

Table 6: Distribution of MTX level status among DAS28-CRP groups of RA patients

DAS28 MTX Level	High N (%) 25(39.0)	Moderate N (%) 24(37.5)	Low N (%) 1(1.6)	Remission N (%) 14(21.9)	OR and [95%CI]	P-value
Toxic	**19(55.5)**	14(41.2)	0(0.0)	1(2.9)	**41.17, [4.42-383.42]**	0.00
Non-Toxic	6(20.0)	10(33.3)	1(3.3)	13(43.3)	/	/

Conclusion

Our study revealed a significant association between genotypes and alleles of the two MTHFR gene polymorphisms (C677T SNP and A1298C SNP) and the response to MTX treatment و but didn't detect any significant difference between both MTHFR polymorphisms genotypes and alleles, and MTX toxicity statistically.

Ethical Clearance

This study was approved by Scientific Committee at the University of Duhok, the Ministry of Higher Education and Scientific Research, and the Ministry of Health in Kurdistan Regional Government in Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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