

Biogenetic Markers for Predicting Response to Immunotherapy in Rheumatoid Arthritis

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ABSTRACT

Background: TNF- α plays a critical role in the pathogenesis of RA. Gene polymorphisms occurring in this pro-inflammatory cytokine or their receptors may influence responses to biological therapy. **Objectives:** This study aimed to evaluate the impact of -238G/A(rs361525), -308G/A(rs1800629), -376G/A(rs1800750), +489G/A(rs80267059) SNPs in TNF- α and +587T/G(rs1061622), +884A/G(rs5746032) SNPs in TNFR2 genes on responsiveness to TNF inhibitors as well as their effect on serum levels of TNF- α and TNFR2. **Subjects and methods:** Sixty patients with RA treated with anti-TNF therapy (30 responders and 30 non-responders) were allocated to this study. SNPs in the TNF- α and TNFR2 genes were studied by three different techniques: PCR-sequencing, PCR-RFLP, and q-PCR-TaqMan assay. TNF- α and TNFR2 serum levels were determined using the ELISA technique. **Results:** TNF- α -308 (GA), +489 (GA), and TNFR2 +587 (TG) genotypes were found to be more associated with non-responsiveness to TNF than homozygous genotypes (OR: 1.3, 2.5, and 2.0, respectively). On other hand, TNF- α -238 and -376 (GA) genotypes, were found to be more associated with TNFi responsiveness than homozygous genotypes (OR: 0.172 and 0.22, respectively). However, none of them reached a significant level. Furthermore, the studied SNPs were found to be unrelated to serum levels of TNF- α and TNFR2. **Conclusion:** According to our findings, the TNF- α -238G/A, -308G/A, -376G/A, +489G/A, and TNFR2 +587T/G, +884A/G SNPs were not significantly associated with the responsiveness of RA patients to biological therapy and had no effect on the serum levels of TNF- α and TNFR.

Keywords: rheumatoid arthritis, single nucleotide polymorphisms pharmacogenetics, TNF- α , TNFR2.

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I. INTRODUCTION

Rheumatoid arthritis (RA), a chronic autoimmune illness characterized by synovial inflammation and progressive joint destruction, affects up to 1% of the world's population [1]. Although the pathogenic mechanism of RA is unknown, it is established that tumor necrosis factor (TNF- α) plays an important role in the inflammatory process of this prevalent autoimmune-mediated condition [2]. TNF- α is a possible biomarker for RA susceptibility, progression, and treatment, according to growing data [3]. When it is released, it binds to TNF-receptor I (TNFR1, p55) or tumor TNF-receptor II (TNFR2, p75) and stimulates a cascade of pro-inflammatory cytokines, resulting in persistent inflammation and joint destruction [4]. Several TNF inhibitors (TNFi) are being utilized as clinical therapy for RA patients, including infliximab (IFX), etanercept (ETN), and adalimumab (ADA). These anti-TNF therapies have a high affinity for TNF, preventing it from engaging to its receptor and thus inactivating it [5]. TNF inhibitors are the most effective RA treatment because they minimize inflammation and joint degeneration in many treated patients. However, 30-40% of patients do not respond well or develop resistance to [6]. These differences indicate that RA patients may have different genetic regulatory systems, resulting in distinct cytokine expression patterns [7]. The reasons for this lack of responsiveness merit investigation.

In this regard, pharmacogenetics may improve response prediction prior to the start of anti-TNF therapy. Several research have been undertaken to investigate the association between TNF- α and TNFR2 polymorphisms and responsiveness to anti-TNF therapy, but the findings are controversial [8]–[10]. Three earlier investigations in Iraq investigated the relationship between genetic variants in the TNF- α promoter region and RA susceptibility [11]–[13]. However, these investigations examined only one or two SNPs. More recently, examined the link between three SNPs in the TNF- α promoter region and the tendency for ETN resistance [11].

Because stratification analysis revealed that risk alleles differed by ethnicity [14], beside relevant studies are still limited in Iraq. As a result, we sought to evaluate the relationship of six SNP markers in the TNF- α gene (-238G/A;rs361525, -308G/A;rs1800629, -376G/A;rs1800750, and +489G/A;rs80267059) and the TNFR2 gene (+587T/G;rs1061622 and +884A/G;rs5746032) with the responsiveness of patients with RA to anti-TNF therapy, and to study the effects of these SNPs on the serum levels of TNF- α and TNFR2 in Iraqi population.

II. METHODS

A. Study Design and Subjects

The case-control study included 60 anti-TNF treated RA patients, including 30 non-responders aged 35–67 years, 3 males, and 27 females (as cases), and 30 responders with a mean age 27–70 years, 9 males, and 21 females (as controls), who visited the biologic center at Basra Teaching Hospital between November 2021 and March 2022. The disease activity score in 28 joints-erythrocyte sedimentation measure was used to assess patients' clinical responses (DAS28-ESR). The accepted clinical definition of responsiveness is the improvement of clinical signs and symptoms in the induction phase, as determined 12–16 weeks after initiation of treatment, respectively, with IFX, ADA, and ETN. Patients must have achieved remission (defined as a DAS28 score of 2.6) by the end point to be classed as a good responder. A high degree of disease activity (DAS28 score of >5.1) by end point was considered as non-response [15]–[17]. Table I shows the clinical and demographic features of the patients.

B. Inclusion Criteria

Patients with established RA had to be over the age of eighteen and have no history of missed doses. A diagnosis of disease was made by a rheumatologist based on the revised 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for RA [18].

C. Exclusion Criteria

Participants with concurrent immune-mediated diseases, endocrine abnormalities, severe liver or kidney disease, or pregnant women were excluded from participating in the study. Participants were also ineligible if they had corticosteroid medication within the previous three months. Patients who had been taking TNFi for less than four months, more than one year, or who had switched to another TNFi in the previous three months were not eligible for this study.

D. Ethical Considerations

The current case-control study was approved by the Ethics Research Committee of the Basra Health Institute (No: 448/2021; Date: 03/11/2021). To participate in this study, all subjects provided written informed consent.

E. Blood Sampling

Five milliliters of venous blood were drawn from each participant and placed in a sterile K2-EDTA tube and gel tube. A blood sample in a K2-EDTA tube was used for DNA extraction and genotyping. Blood in a gel tube was used to separate serum for measuring TNF- α and TNFRII circulating levels. All samples were kept at -20°C until they were used.

F. DNA Extraction

The Wizard Genomic DNA Extraction Kit (Promega, USA) was used to isolate genomic DNA from peripheral blood leukocytes according to the manufacturer's recommendation. All isolated DNA samples were examined for quality and quantity using agarose gel electrophoresis

and a Thermo Scientific NanoDrop 1000 spectrophotometer (Thermo Scientific, USA).

G. Polymerase Chain Reaction (PCR) protocol

Target sequences of genomic DNA were amplified using specific primers listed in (Table I) supplied by Alpha DNA Biotechnology Company (Canada). The PCR reaction mixture contained 12.5 μ L GoTaq® (Green Master Mix) (2x); 1 μ L for each primer (10 pmol/L); 8.5 μ L of nuclease-free water; and 2 μ L of template DNA in a final volume of 25 μ L. The PCR amplifications were performed with a thermocycler (Applied Biosystem, US) following these conditions: initial denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 30 sec, a 45-second annealing step at 52°C for primer A and 57°C for primer B and C, and extension at 72°C for 1 min. Final extension time of 8 minutes at 72°C. Agarose gel electrophoresis was used to visualize the amplified products.

H. Genotyping

We detected different SNPs by three different techniques:

1) DNA Sequencing

TNF- α Promoter (-238 G/A, and -308 G/A, -376 G/A) SNPs were amplified using following primers: forward primer 5'-AACCAGCATTATGAGTCTC-3' and reverse primer 5'-AACAACTGCCTTTATATGTC-3' [19] that give product of 677bp in size, and TNFRII exon 6 (+587 T/G) SNP was amplified using following primers: forward primer 5'-ACTCTCCTATCCTGCCTGCT-3' and reverse primer 5'-TTCTGGAGTTGGCTGCGTGT-3' [20] that give product of 242bp in size, then genotyped by sequencing. TNF- α gene amplification and identification were carried out using the previously mentioned PCR protocol. Then after, 20 μ L of amplicon were sent for sequencing by the Sanger method using a DNA analyzer (illumine, MacroGen firm, South Korea) The Bio Edit application was used to examine and align the results [21] and Nucleotide BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=Blas tSearc).

I. PCR-RFLP

TNF- α intron 1 (+489G/A) SNP was amplified using the following primers: forward primer 5'-GGAGAGAAGCAACTACAGAC-3' and reverse primer 5'-CACACTTAGTGAGCACCTTC-3' [22], [23] that give product of 551bp in size. After that, Allele-specific restriction enzymes (Tail, Cat No. ER1142, Thermo Scientific™) digested the amplified products at 65°C for 3 hours according to the manufacturer's instructions. Digestion mixture of the reaction was performed in a final volume of 31 μ L which included 10 μ L of PCR reaction mixture, 18 μ L nuclease-free water, 2 μ L 10X Buffer R, and 1 μ L Tail (Maell) (10U/ μ L). After a 2% agarose gel electrophoresis at 80V for 80 minutes, the fragments were examined under ultraviolet light. The sizes of the obtained fragments for genotype GG were 111, 159, and 281 bp; for genotype AA: 159 and 392 bp; and for genotype GA: 111, 159, 281, and 392 bp.

J. C-qPCR-TaqMan assay

The exon 6 (+884 A/G) SNP of TNFRII gene was genotyped using the TaqMan dual probe allelic

discrimination assay (probe assay: C_30915118_10) on a Step One 7500 fast real-time PCR system (Applied Biosystems, USA) according to the manufacturer's instructions (Thermo Scientific™, USA). The reaction mixture consisted of two solutions: 2X TaqMan master mix (Cat No.4371353) and 20X assay working stock (probes with VIC and FAM fluorophore attachments). The probes were designed to match the +884A allele or +884G allele. Isolated DNA samples, in addition to the reaction mixture and controls, were added into a 0.1ul PCR tube. In this study, thermal cycling conditions were as follows: polymerase activation at 95°C for 10 minutes, denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute for 40 cycles. The plate was examined for the presence of VIC and FAM fluorophores at 551 and 517 nm, respectively. VIC or FAM fluorescence alone determined whether the sample had allele A or allele G, whereas both VIC and FAM fluorescence determined whether the sample was heterozygous for either allele.

K. 2.6 Cytokine Assay

In the current study, serum TNF- α (Cat No. SL1761Hu) and TNFR2 (Cat No. SL3063Hu) levels were quantified using the SUNLOG ELISA kit according to the manufacturer's instructions. The optical density of each sample and standards were read by a fully automated ELISA analyzer (Elisys Uno-HUMAN) at 450 nm. A standard curve was used to calculate the protein concentrations.

L. Statistical Analysis

The Fisher's Exact test, chi-square test, Mann-Whitney U test, Person Chi-square, t-test, and ANOVA test were performed using SPSS v.26 software. Because the goal was to detect a class effect, no adjustment for anti-TNF therapy type was made. Age and gender were not included as covariates because no age or gender effects were identified across all data sets. The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to see if the data had a normal distribution.

III. RESULTS

Table I shows the demographic and clinical characteristics of the participant groups. Except for morning stiffness and DAS 28, which were higher in non-responders (53.76 \pm 58.190), (5.7783 \pm 0.40214) than in responder patients (15.73 \pm 22.806), (2.1970 \pm 0.37705) with a highly significant difference (p=0.0001), other variables did not differ significantly between the responder and non-responder RA groups.

Study showed that none of the TNF- α -238, -308, -376 and +489 (G/A); TNFR2+587T/G polymorphisms have mutant homozygous genotype. Study also showed that all subjects (100%) carried homozygous AA genotype in case of +884 A/G SNP, so no statistics was computed for this SNP. The sequence chromatogram results were shown in Fig. 1. The GG genotype of the -308G/A SNP was detected in (83.3%) of non-responders and (86.7%) of responders (OR=0.769, 95% CI: 0.185-3.195), whereas heterozygous GA genotype was detected in (16.7%) of non-responders

and (13.3%) of responders (OR=1.3, 95%CI:0.313-5.404). Similarly, non-responders had a higher frequency of the A allele (8.3% vs. 6.7%; OR=1.273; 95%CI:0.325-4.991), and responders had a higher frequency of the G allele (93.3% vs. 91.7%; OR=0.786; 95%CI:0.2-3.007). In spite of these differences, the P-value did not reach a significant level (Table II).

TABLE I: DISPLAY DETAILED DEMOGRAPHIC AND CLINICAL INFORMATION ABOUT THE SUBJECTS WHO HAVE BEEN ENROLLED

Demographic characteristics at enrolment	Responder n=30	Non-responder n=30	P-value
Age** years	48.27 \pm 12.457	50.70 \pm 9.447	0.371
M	9 (30.0%)	3(10.0%)	0.152
F	21(70.0%)	27/ (90.0%)	
Yes	5 (16.7%)	5 (16.7%)	1.000
No	25(83.3%)	25(83.3%)	
Urban	21 (70%)	21 (70%)	1.000
Rural	9 (30%)	9 (30%)	
Disease duration**years	9.87 \pm 4.974	12.13 \pm 7.868	0.328
Yes	10(33.3%)	15(50%)	0.190
No	20(66.7%)	15(50%)	
Morning stiffness**(minutes)	15.73 \pm 22.806	53.67 \pm 58.190	0.0001
Yes	21(70%)	23(77%)	0.559
No	9(30%)	7(33%)	
BMI**(kg/m2)	29.0557 \pm 3.49616	29.7090 \pm 6.19367	0.900
DAS 28**	2.1970 \pm 0.37705	5.7783 \pm 0.40214	0.0001

Values are *number and percentage or **mean \pm SD. Abbreviations: n: number of cases; SD: standard deviation; M: male; F: female; RA: rheumatoid arthritis; BMI: Body Mass Index; DAS 28: disease activity score; bold text: significant p-value.

TABLE II: SHOWS THE FREQUENCY OF TNF-A (-308G/A) GENOTYPES AND ALLELES IN RESPONDER AND NON-RESPONDER PATIENTS, AS WELL AS THEIR CORRELATION WITH TNF-A CIRCULATING LEVELS

SNP	Molecular study			Cytokine assay	
Genotype Allele	Non-responder group vs. Responder group			TNF- α serum level Median (pg/ml)	
-308G>A (rs1800629)	Non-Responder (n=30) N (%)	Responder (n=30) N (%)	OR (95% CI) P-value	Non-responder (n=30) N (%)	Responder (n=30) N (%)
GG	25(83.3)	26(86.7)	0.769 (0.185–3.195) 1.000 1.300	20.0	20.0
GA	5(16.7)	4(13.3)	(0.313–5.404) 1.000	22.3	22.15
AA	-	-	0.786 (0.200–3.077) 1.000 1.273	-	-
G	55(91.7)	56(93.3)	(0.325–4.991) 1.000		
A	5(8.3)	4(6.7)			
P-value				0.656	0.446

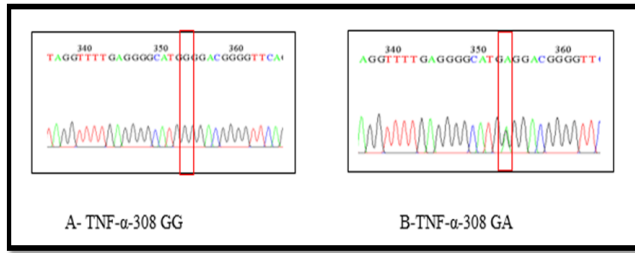


Fig. 1. Sequence chromatogram results of TNF- α (-308G/A) SNP showing two genotypes.

The TNF- α -238 genotyping showed that the GG genotype was higher in non-responders (96.7%) as compared to responders (83.3%) (OR=5.813, 95%CI:0.635–52.631), while GA genotype was higher in responders (16.7%) as compared to non-responders (3.3%) (OR=0.172, 95%CI:0.019–1.576). The frequency of the G allele was also elevated in non-responders (98.3% vs. 91.7%; OR=5.363; 95%CI:0.607–47.619). The A allele frequency was higher in responders (8.3% vs. 1.6%; OR=0.186; 95%CI:0.021–1.646). In spite of these differences, the P-value did not reach a significant level (Table III).

TABLE III: SHOWS THE FREQUENCY OF TNF-A (-238G/A) GENOTYPES AND ALLELES IN RESPONDER AND NON-RESPONDER PATIENTS, AS WELL AS THEIR RELATIONSHIP TO TNF-CIRCULATING LEVELS

SNP	Molecular study			Cytokine assay	
Genotype	Non-responder group vs. Responder group			TNF- α serum level Median (pg/ml)	
Allele	Non-responder (n=30) N (%)	Responder (n=30) N (%)	OR (95%CI) P-value	Non-responder (n=30) N (%)	Responder (n=30) N (%)
-238G>A (rs361525)					
GG	29(96.7)	25(83.3)	5.813 (0.635–52.631)	20.2	21.6
GA	1(3.3)	5(16.7)	0.172 (0.019–1.576)	16.0	15.7
AA	-	-	0.195	-	-
G	59(98.3)	55(91.7)	5.363 (0.607–47.619)		
A	1(1.6)	5(8.3)	0.207 (0.021–1.646)		
			0.207		
	P-value			0.453	0.266

The TNF- α -376 GG genotype was found to be higher in non-responders (96.7%) than in responders (86.7%) (OR=4.464, 95% CI: 0.468–41.667), while the GA genotype was found to be higher in responders (13.3%) than in non-responders (3.3%) (OR=0.224, 95% CI: 0.024–2.136). Similarly, the G allele was also higher in non-responders compared to responders (98.3% vs. 93.3%; OR=4.219; 95%CI:0.457–38.462), whereas A allele frequency was higher in responders compared to non-responders (6.7% vs. 1.7%; OR=0.237; 95%CI: 0.026–2.188). In spite of these

differences, the P-value did not reach a significant level (Table IV).

TABLE IV: SHOWS THE FREQUENCY OF TNF-A (-376G/A) GENOTYPES AND ALLELES IN RESPONDER AND NON-RESPONDER PATIENTS, AS WELL AS THEIR RELATIONSHIP TO TNF-A CIRCULATING LEVELS

SNP	Molecular study			Cytokine assay	
Genotype	Non-responder group vs. Responder group			TNF- α serum level Median (pg/ml)	
Allele	Non-responder (n=30) N (%)	Responder (n=30) N (%)	OR (95%CI) P-value	Non-responder (n=30) N (%)	Responder (n=30) N (%)
-376G>A (rs1800750)					
GG	29(96.7)	26(86.7)	4.464 (0.468–41.667)	20.2	21.75
GA	1(3.3)	4(13.3)	0.353 (0.024–2.136)	16.0	17.5
AA	-	-	0.224	-	-
G	59(98.3)	56(93.3)	4.219 (0.457–38.462)		
A	1(1.7)	4(6.7)	0.364 (0.026–2.188)		
			0.237		
	P-value			0.453	0.127

Regarding TNF- α +489 SNP, the responders mostly carried GG genotype (83.3%) in comparison to (66.7%) in non-responders (OR:0.4; 95%CI:0.118–1.361), whereas GA genotype was more frequent in non-responders compared to responders (33.3% vs. 16.7%; OR:2.5; 95%CI:0.735–8.502). The frequency of the G allele was higher in responders (91.7% vs. 83.3%; OR = 0.455; 95% CL: 0.154–1.420). The frequency of the A allele was higher in non-responders (16.7% vs. 8.3%; OR=2.2; 95%CI:0.704–6.877). In spite of these differences, the P-value did not reach a significant level (Table V; Fig. 2).

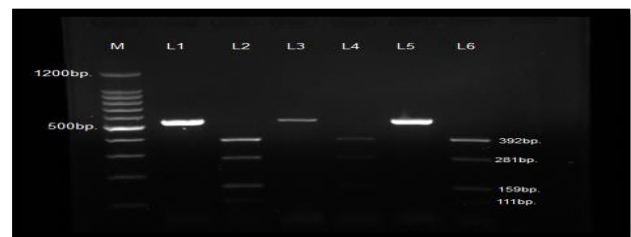


Fig. 2. A photomicrograph of the TNF- α + 489G/A polymorphism reveals a heterozygote GA genotype in Lanes 2, 4, and 6, with the G allele appearing at 111, 159, and 281 bp and the A allele appearing at 159, 392 bp. Lanes 1, 3, and 5 showed PCR products at 551bp. M showed a DNA ladder of 100bp.

The TT genotype of the TNFR II +587 SNP was present in 100% of responders and 93.3% of non-responders (OR=0.483, 95%CI:0.370–0.630), whereas TG genotype was present in 0.0% of responders and 6.7% of non-responders (OR=2.07, 95%CI:1.587–2.703).

TABLE V: SHOWS THE FREQUENCY OF TNF-A (+489G/A) AND TNFR II (+587T/G) GENOTYPES AND ALLELES IN RESPONDER AND NON-RESPONDER PATIENTS, AS WELL AS THEIR RELATIONSHIP TO TNF-A AND TNFR II CIRCULATING LEVELS, RESPECTIVELY

SNP			Molecular study			Cytokine assay			SNP			Molecular study			Cytokine assay		
Genotype			Non-responder group vs. Responder group			TNFR- α serum level Median (pg/ml)			Genotype			Non-responder group vs. Responder group			TNFR II serum level Median (pg/ml)		
Allele									Allele								
+489G>A(rs80267959)			Non-responder (n=30) N (%)	Responder (n=30) N (%)	OR (95%CI) P-value	Non-responder (n=30) N (%)	Responder (n=30) N (%)		+587 T>G(rs1061622)			Non-responder (n=30) N (%)	Responder (n=30) N (%)	OR (95%CI) P-value	Non-responder (n=30) N (%)	Responder (n=30) N (%)	
GG			20(66.7)	25(83.3)	0.400 (0.118–1.361)	22.5	21.6		TT			28(93.3)	30(100)	0.483 (0.370–0.630)	16.1	15.1	
GA			10(33.3)	5(16.7)	0.136 2.500 (0.735–8.502)	17.1	15.7		TG			2(6.7)	0(0)	0.492 2.070 (1.587–2.703)	15.15		
AA			-	-	0.136	-	-		GG			-	-	0.492			
G			50(83.3)	55(91.7)	0.455 (0.145–1.420)				T			58(96.7)	60(100)	0.967 (0.923–1.013)			
A			10(16.7)	5(8.3)	0.168 2.200 (0.704–6.877)				G			2(3.3)	0(0)	0.496 1.034 (0.987–1.084)			
					0.168									0.496			
			P-value			0.441 0.254						P-value			0.803		

The frequency of the T allele was present in 100% of responders and in 96.7% of non-responders (OR=0.967; 95%CI: 0.923–1.013). The frequency of the G allele was present in 0.0% of responders and in 3.3% of non-responders (OR = 1.034; 95% CI: 0.987–1.084) (Table V). Furthermore, there was no significant relationship between the median of serum TNF- α and TNFR II levels and the homozygous and heterozygous genotypes of different polymorphisms in corresponding genes (Tables II–V).

IV. DISCUSSION

TNF inhibitors have advanced the treatment of RA patients, improving their quality of life by reducing joint inflammation and damage. It has been demonstrated that TNFi inhibits the production of other pro-inflammatory cytokines in RA synovial fluid, such as IL-1 and GM-CSF [24]. Unfortunately, growing research indicates that therapy response varies amongst patients [6]. As a result, considering the wide range of TNFi currently available and their high costs, identifying pre-therapeutic predictors of response is becoming a top goal.

In this regard, pharmacogenetics may improve response prediction prior to the start of anti-TNF therapy. SNPs in the TNF- α promoter region, as well as intronic SNPs, may play a functional role by affecting gene transcription or expression levels [25], [26]. A TNFR II genetic polymorphism may cause conformational changes that affect the intracellular transmission of signals generated when TNF- α binds to TNFR II [27].

Several research have been conducted to investigate the impact of SNPs in the TNF- α and TNFR II genes as a predictor of treatment response; however, the results are conflicting. The disparities were explained by the fact that the subjects were from various races and areas [28]–[31]. A

conflicting result may also be due to these association analyses based on independent SNPs; Analyzing each SNP independently ignores inherent gene interaction in the body, resulting in statistically lost micro-effect genes [25].

To the best of our knowledge, relevant research in Iraq are still limited; consequently, the aim of our study was to evaluate the role of TNF- α (-308, -238, -376, and +489 (G/A) and TNFR II (T+587G and A+884G) polymorphisms in influencing the outcome of anti-TNF therapy and to study the effects of these SNPs on the serum levels of TNF- α and TNFR II in Iraqi population.

Our results regarding TNF- α -308 SNP genotyping revealed that GG genotype was present in 86.7% responders and 83.3% non-responders, while GA genotype was present in 13.3% responders and 16.7% non-responders. The odd ratio shows that the non-responding of heterozygous patients (GA) to TNFi is higher by 1.3 than that of homozygous patients (GG), which suggests it may act as a possible predisposing factor for poor response or non-responsiveness (95%CI:0.313–5.404). However, the differences were not statistically significant, so the -308 polymorphism was not associated with response to TNFi. Our findings are consistent with those of [32], who observed no relation between the TNF-308G>A SNP and treatment response. Our findings are consistent with the findings of [9] meta-analyses, which found no significant difference in the percentage of A allele carriers between groups that responded to treatment and those that did not. In a cross-sectional investigation of 80 Iraqi RA patients treated with ETN, [33] discovered no significant difference in the availability of GA and AA genotypes of the -308G/A polymorphism between responsive and non-responsive groups. Our findings, on the other hand, contradict three prior studies that found a link between TNF-308 GG

genotype and improved response to IFX, ETN, and ADA in RA patients [34]–[36].

Our study reported that GG genotype frequency of -238 polymorphism in non-responders was higher compared with the responders (96.7% vs. 83.3%), while the frequency of GA genotype in responders was higher compared with the non-responders (16.7% vs. 3.3%). The odd ratio of heterozygous genotype (GA) suggests that it may act as a possible protective factor against non-responsiveness (OR=0.172, 95%CI: 0.019–1.576). However, the differences did not reach a significant level, so the -238 polymorphism was not associated with response to TNFi. Our finding was consistent with a study completed by [37] on 280 RA patients who had been treated with different TNFi that showed no statistical differences in -238G/A genotypes and allele frequencies between patient groups. Likewise, a cohort study of 107 consecutive patients with JIA conducted by [38] found that the -238 G/A SNP was not associated with response to anti-TNF treatment. Nevertheless, our result was contradictory to the [39] study, which showed that the -238 GG genotypes have a better disease course. On the contrary, [40] study reports a significant association between TNF- α -238 GG genotype and G allele with a negative response to TNF inhibitors.

The investigation of the +489G/A polymorphism revealed a higher frequency of GG genotype in responders compared to non-responders (83.3% vs. 66.7%), whereas GA genotype was frequent in non-responders (33.3% vs. 16.7%). The odd ratio shows that the non-responding of heterozygous patients (GA) to TNFi is higher by 2.5 than that of homozygous patients (GG), which suggests it may act as a possible predisposing factor for poor response or non-responsiveness (95%CI:0.735–8.502). However, the differences were not significant, so the +489 polymorphism was not associated with TNFi response. Our results agreed with those of [41] who reported a lack of association between the +489G>A SNP and therapeutic efficacy in 58 RA patients receiving infliximab.

In the case of the TNFR2 +587 T/G SNP, the TG genotype was found in only 2/30 of the non-responders compared to 0/30 of responders RA group (6.7% vs. 0%). The risk of non-response to anti-TNF drugs was higher in people carrying the TG genotype than in people carrying the TT genotype (OR:2.0; 95%CI:1.587–2.703). However, the differences did not reach a significant level, so the TNFR2 +587 polymorphism was not associated with response to TNFi, which is in line with [42]–[46] found that patients with the TG+GG genotype of rs1061622 had poorer responses to anti-TNF biological drugs at 3 months and 12 months than patients with the TT genotype.

The discrepancy between prior studies and the current study could be attributed to variances in ethnic origin, where stratification analysis revealed that different ethnicities would have different risk alleles [14], or to the number of studied persons. There was no significant association between the serum levels of TNF- α and TNFR2 and

homozygous and heterozygous genotypes of different polymorphisms in corresponding genes. [47] showed no significant difference ($p>0.05$) in TNF- α protein levels in RA patients between -308 GG and AA or GA genotypes. Similarly, we also concurred with [12] and [48] findings, who demonstrated no relationship between the -308, -238 G/A polymorphism and serum TNF- α levels in Iraqi and Iranian RA patients, respectively. Our findings, on the other hand, contradicted the findings of [49], who discovered that the -308 GA/AA genotype was related with elevated TNF- α levels. In contrast, [36] discovered a substantial link between the -308 GG genotype and higher circulating TNF- α levels in Chilean RA patients treated with ADA.

LIMITATION

The sample size of RA patients is relatively small, which may not be representative of the entire population. This may be related to the minimal number of patients who meet the inclusion criteria.

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