Arch Rheumatol 2022;37(4):613-625
doi: 10.46497/ArchRheumatol.2022.9272

ORIGINAL ARTICLE

Association of tumor necrosis factor-alpha promoter region gene polymorphism at positions -308G/A, -857C/T, and -863C/A with etanercept response in Iraqi rheumatoid arthritis patients

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ABSTRACT

Objectives: This study aims to evaluate the association between polymorphisms in the promoter region of the tumor necrosis factor-alpha (TNF-α) gene at locations -308G/A, -857C/T, and -863C/A with the tendency of being non-responder to etanercept.

Patients and methods: Between October 2020 and August 2021, a total of 80 patients (10 males, 70 females; mean age: 50 years; range, 30 to 72 years) with rheumatoid arthritis (RA) receiving etanercept for at least six months were included. The patients were divided into two groups responders and non-responders, based on their response after six months of continuous treatment. Following polymerase chain reaction amplification of the extracted deoxyribonucleic acid, sequencing by Sanger method was performed to identify the polymorphism at the TNF-α promoter region.

Results: In the responder group, the GG genotype of (-308G/A) and the AA genotype of (-863C/A) were both significantly present. The CC genotype of (-863C/A) was significantly present in the non-responders group. The CC of (-863C/A) SNP was the only genotype that appeared to increase the likelihood of being resistant to etanercept. The GG genotype of (-308G/A) was negatively correlated with the likelihood of being a non-responder. The (-857CC) and (-863CC) genotypes were significantly more prevalent in the non-responders group.

Conclusion: The presence of the (-863CC) genotype, alone or in combination with (-857CC), is linked to an increased likelihood of becoming a non-responder to etanercept. The GG genotype of -308G/A and the AA genotype of -863C/A significantly increase the likelihood of becoming responder to etanercept.

Keywords: Etanercept, genetic polymorphism, response, rheumatoid arthritis, tumor necrosis factor-alpha.

Biological agents or biological response modifiers are a highly effective class of therapies for rheumatoid arthritis (RA) that have been in use for nearly a decade.[1] These medications are intended to target the inflammatory pathways in RA patients that contribute to joints damage.[1] Biological agents are protein molecules that have been genetically modified.[2] Most fundamental class of biological agents are blocker of tumor necrosis factor-alpha (TNF-α) such as etanercept (ETN), golimumab, infliximab, adalimumab, and certolizumab.[2]

Etanercept (Enbrel, Pfizer, NY, USA) is one of the most commonly used biological disease-modifying antirheumatic drugs (DMARDs) approved in 1998.[3] It is a humanized fusion protein consisting of the human TNF-α p75 receptor extracellular ligand-binding portion

Received: October 18, 2021 ***Accepted:*** November 30, 2021 ***Published online:*** March 03, 2022

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Citation:
Mohammed S, Zalzala M, Gorial F. Association of tumor necrosis factor-alpha promoter region gene polymorphism at positions -308G/A, -857C/T, and -863C/A with etanercept response in Iraqi rheumatoid arthritis Patients. Arch Rheumatol 2022;37(4):613-625.

connected to the Fc portion of the human immunoglobulin G1.[4] It is well established that ETN is considerably safe and highly effective in reducing adverse clinical manifestations of RA.[5] However, ETN is more expensive than other conventional antirheumatics, and it adds a significant pressure on medication budgets, whether at the individual level or the national health plan levels.[6] Additionally, it is unexplained why certain patients with RA do not respond satisfactorily to anti-TNF medication, including ETN.[7] About 30 to 40% of ETN users do not respond adequately to TNF-α inhibitors.[8]

Genetic polymorphism is one of the most important influences on the response, as the TNF-α gene promoter region is highly polymorphic.[9] Compared to other variables that may influence or modify the ETN response, genetic determinants would remain consistent throughout a patient's lifetime.[10] Accordingly, polymorphism testing helps to distinguish patients with a high response from inadequate or poor ones. In addition, early recognition of patients who would not respond to anti-TNF medications would allow for a fast switch to alternative medicines, giving the patient a better chance of rapidly reaching treatment goals.[11]

Numerous studies published over the last several years have revealed conflicting findings regarding a possible association between anti-TNF-α response and polymorphisms in the TNF-α gene at different positions such as -308, -238, -857, and +489[12-14] or other associated genes such as TNF-α receptors (TNFR1 and TNFR2).[15] In addition, several meta-analyses have been performed to inspect the relationship among different single nucleotide polymorphisms (SNPs) and anti-TNF-α responsiveness.[16] However, the outcomes of these studies vary from one population to another due to racial and ethnic variance in pharmacogenetics, which occurs due to dissimilarity in the allele frequencies in different populations. Thus polymorphism analysis in various populations is essential to determine the genetic variables that may influence the response to ETN.[17]

To the best of our knowledge, earlier investigations did not examine the effect of the -863C/A polymorphism in RA patients' responsiveness to ETN. To date, no previous study performed in Iraq to examine the effect of any SNPs in the TNF-α promoter region on the tendency to be a non-responder to ETN. In the present study, we aimed to determine whether the presence of SNPs in the promoter region of the TNF-α gene at positions -308G/A(rs1800629), -857C/T (rs1799724) and -863C/A (rs1800630) in a sample of Iraqi RA patients could influence the patients' response to ETN.

PATIENTS AND METHODS

This single-center, cross-sectional study was conducted at Baghdad Teaching Hospital, Department of Rheumatology, between October 12th, 2020 and August 8th, 2021. The present study is a part of a large study which was conducted, with an eligible convenient sample of eighty Iraqi RA patients with established RA according to the revised 2010 American College of Rheumatology (ACR)/European League against Rheumatism (EULAR) Classification Criteria for RA.[18] Our center serves a wide variety of communities in Iraq, including rural, urban, and inner-city areas from several governorates. All patients were diagnosed and treated under specialist supervision. Initially, 97 patients with active RA using ETN alone as a single treatment who met the inclusion criteria were screened. However, only 86 patients accepted to participate in this study and only 80 patients (10 males, 70 females; mean age: 50 years; range, 30 to 72 years) completed the requirements of the study. Inclusion criteria were as follows: having confirmed RA according to revised 2010 ACR/EULAR RA classification criteria; having high disease activity according to disease activity score based on 28 joints (DAS28) and erythrocyte sedimentation rate (ESR); i.e., the DAS28-ESR should be more than 5.1 at baseline; and requiring ETN treatment consistently for at least six months, with no history of missed doses. Exclusion criteria were as follows: using ETN for less than six months or more than one year; having concomitant diseases other connective tissue diseases; using additional DMARDs with ETN; and missing data.

Clinical evaluation and patient groups

As shown in Figure 1, the patients were classified into two groups according to EULAR response criteria,[19] which are based on the

clinical response as determined by the DAS28[20] following at least six continuous months of ETN treatment.

When the DAS28 value after six months was reduced from a high value of ≥5.1 at baseline to a value less than 5.1 and with a change in DAS28 of greater than 0.6, the patient was designated an ETN responder. After six months of ETN treatment, the patient was classified as a non-responder, if the DAS28 value did not fall below 5.1 and if the change in the DAS28 was less than 0.6.

The patients were distributed according to their responses into two groups. The first group (Group A, n=41) consisted of RA patients who responded clinically to ETN. The second group (Group B, n=39) consisted of RA patients who failed to respond to ETN.

Data collection

Data on demographic characteristics (age, weight, disease duration, recent lab data such as ESR, white blood cell (WBC) count, tender and swollen joints, patients and Visual Analog Scale (VAS) scores were collected via patient interviews using a patient information chart explicitly designed for this study.

Sample collection and preparation

Each patient's forearm vein was punctured to obtain 5 mm of venous blood. Two milliliters of blood were converted into an ethylenediaminetetraacetic acid (EDTA) tube for deoxyribonucleic acid (DNA) extraction.

DNA extraction

The Promega ReliaPrepTM Blood gDNA Miniprep System for Genomic DNA (Promega Corp., WI, USA) provides a practical approach for purifying DNA from blood samples. Polymerase chain reaction (PCR) was used for enzymatic amplification with the Master Taq polymerase enzyme and a hybrid thermal cycler.

Primer

The TNF-α gene DNA sequences were taken from the NCBI GenBank database. Primer Premier 3 software was used to generate PCR primers (Table 1), with a melting temperature

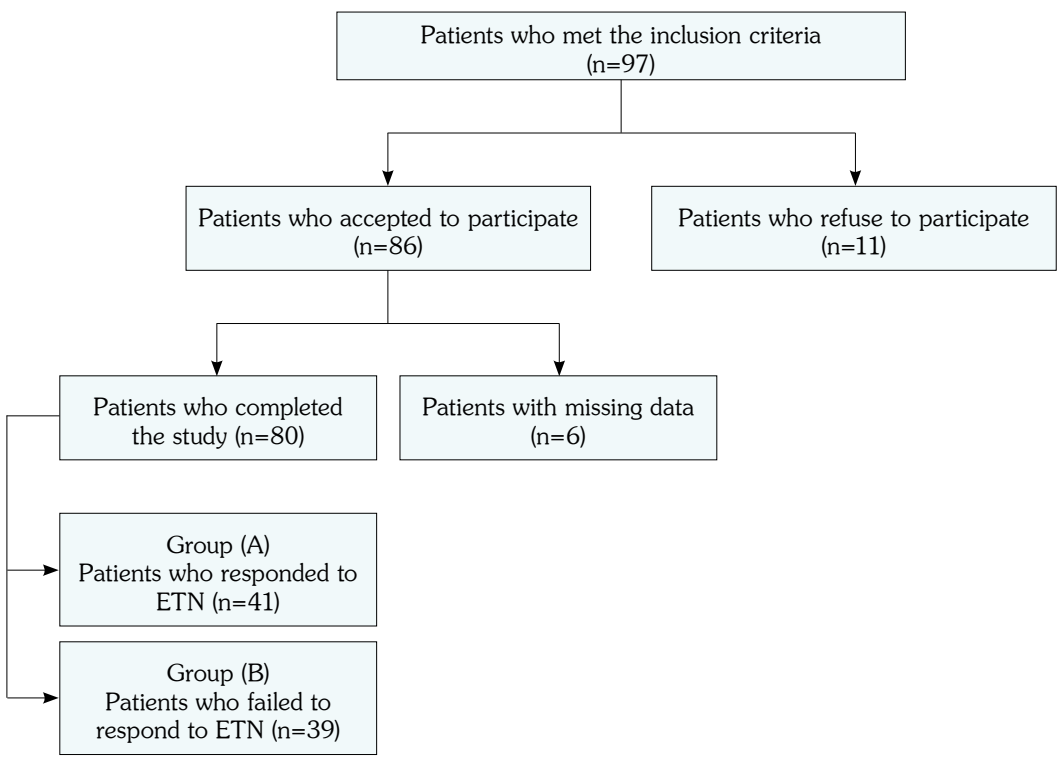

Figure 1. Study flowchart.

Table 1. The sequences of the primers, annealing temperature, product size (bp)

Primer name	Sequence	Annealing Temp. (°C)	Product size (bp)
TNF-α 1-F	5`-TGTAAAACGACGGCCAGTCTCAGAGAGCTTCAGGGATA-3`	60	966
TNF-α 1-R	5`-CAGGAAACAGCTATGACCGGGACACACAAGCATCAA-3`		

TNF-α 1-F: The forward primer; TNF-α 1-R: The reverse primer.

of (58 to 62°C), a primer length of (18 to 23) nucleotides, and a PCR amplicon length of (800 to 1000) base pairs.

Primer optimization and PCR amplifications

To determine the optimal annealing temperature for primers, we amplified the DNA template using the identical primer pair (Forward) (Reverse) at annealing temperatures of 55, 58, 60, 63, and 65°C. The best annealing temperature for the primer was 60°C as seen in Figure 2. The PCR amplifications were performed with 20 µL volumes containing 10 µL GoTaq® (Green Master Mix) (2×); 1 µL for each primer (10 pmoL); 6 µL nuclease-free water, then, 2 µL of template DNA. The following temperature program was used for PCR cycling with PCR Express (Thermal Cycler; Bio-Rad Laboratories Inc., Hercules, CA, USA): The DNA denatured at high temperature of 94°C for 4 min initially followed by 30 cycles of denaturation at 94°C for 30 sec; annealing occurs at 60°C for 30 sec; and extension at 72°C for 30 sec. A final extension incubation period of 7 min at 72°C was included, followed by a 10 min incubation at 4°C to stop the reactions.

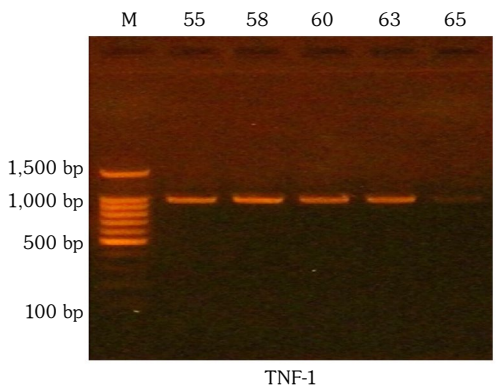

Figure 2. Primer optimization at ennealing temperatures of 55, 58, 60, 63 and 65°C.
TNF: Tumor necrosis factor.

Sequencing of PCR products

The PCR product was sequenced by Sanger method of sequencing using DNA analyzer (ABI3730XL) (Macrogen Corp., Seoul, South Korea). The results were obtained by electronic mail and analyzed with the use Geneious Prime software version 2021.1.1 (Biomatters Ltd., Auckland, New Zealand; www.geneious.com).

Statistical analysis

Statistical analysis was performed using the IBM SPSS for Windows version 26.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism (GraphPad Software, CA, USA). Continuous variables were expressed in mean ± standard error of the mean (SEM) of the values. Allele and genotypes were presented in number and frequency. The Shapiro-Wilk test was used to test the normality of the results. The unpaired t-test was used for normally distributed data to determine a significant difference in demographic characteristics and parameters between the responders and non-responders. One-way analysis of variance (ANOVA) was used to analyze the difference between the means of more than two groups. Then, a post-hoc analysis was used whenever a significant difference between three sample means was revealed by the ANOVA. The chi-square test or Fisher exact test was used to test group differences of proportions. The phi correlation coefficient (phi) was used to measure the correlation between each genotype and the likelihood of being a non-responder. A p value of <0.05 was considered statistically significant.

Table 2. Demographic data and clinical characteristic parameters of the study

	Responders group (n=41)			Non-responders group (n=39)			
Category	n	%	Mean±SD	n	%	Mean±SD	*p*
Age (year)			49.5±10.5			51.2±12.0	0.49[a]
Sex							
Male	6	14.6		4	10.3		0.55[c]
Female	35	85.4		35	89.7		0.55[b]
Weight (kg)			80.6±14.5			78.0±12.8	0.40[a]
Disease duration (year)			10.1±6.8			8.3±3.7	0.15[a]
Baseline WBC			11.1± 2.1			11.7±1.8	0.21[a]
WBC after six months			7.3±1.7			9.0±2.2	0.0003*[a]
Baseline ESR (mm/h)			49.9±20.1			67.3±19.8	0.0002*[a]
ESR after six months (mm/h)			21.1±13.9			56.4±21.1	<0.001*[a]
TNF-α (pg/mL)			78.6±34.1			113.4±54.5	0.0010*[a]
Baseline DAS28			5.6±0.3			6.1±0.4	0.06*[a]
DAS28 after six months			3.3±0.8			5.7±0.5	<0.001*[a]

SD: Standard deviation; WBC: White blood cell counts; ESR: Erythrocyte sedimentation rate; TNF-α: Tumor necrosis factor-alpha; DAS28: Disease activity score in 28 joints; a: Independent 2 sample t-test; b: Chi-square test; C: Fisher exact test; *: Significant difference between the groups.

RESULTS

Demographic, disease and baseline clinical characteristics variables of the study groups

Demographic data of the study groups are presented in Table 2.

Prevalence of genotypes and alleles for all patients

Table 3 highlights the high proportions of GG genotypes in the -308G/A. Notably, in the -857C/T and -863C/A, the CC genotype was the most prevalent.

Table 3. Genotypes and alleles frequencies of -308G/A, -857C/T, -863C/A in RA patients (n=80)

Genotypes	n	%	n	%	n	%
-308G/A						
Genetic variant	AA		GA		GG	
No. (%)	6	7.5	13	16.25	61	76.25
Allele	G		A			
No. (%)	74	93.75	19	23.75		
-857C/T						
Genetic variant	CC		CT			
No. (%)	63	78.75	17	21.25		
Allele	C		T			
No. (%)	80	100	17	21.25		
-863C/A						
Genetic variant	AA		CA		CC	
No. (%)	9	11.25	13	16.25	58	72.5
Allele	C		A			
No. (%)	71	88.75	22	27.5		

Table 4. Difference in genotype and alleles frequencies of -308G/A, -857C/T, -863C/A between responders and non-responders

	Responders' group (n=41)		Non-responders' group (n=39)		
Genotypes	n	%	n	%	*p*
-308G/A					
AA	1	2.4	5	12.8	0.10
GA	4	9.8	9	23.1	0.13
GG	36	87.8	25	64.1	0.012*
G	40	53.4	34	46.6	0.1
A	5	12.2	14	35.8	0.012
-857C/T					
CC	33	80.5	30	76.9	0.69
CT	8	19.5	9	23.1	0.69
C	41	100	39	100	1
T	8	19.5	9	23	0.69
-863C/A					
AA.	8	19.5	1	2.6	0.01*
CA	8	19.5	5	12.8	0.18
CC	25	61	33	84.6	0.01*
C	33	80.4	38	97.4	0.02*
A	16	39	6	25	0.001*

A Chi-square test or Fisher exact test was used to identify the statistical difference between the groups. *: Significant difference between the groups.

Additionally, the results of this study indicated that there was a significant incidence the GG genotype of -308G/A (p=0.01) and the AA genotype of -863C/A (p=0.01) in respondents' group, whereas CC genotypes of -863C/A were significantly present in non-respondents' group (p=0.01) (Table 4).

Regarding the difference in alleles frequencies between the responders and non-responders, the results show a significant difference in C and A alleles of -863C/A SNP (Table 4).

Correlations between genotypes and the likelihood of being non-responder

The phi coefficient analysis was used to investigate the correlation between each genotype and the tendency of being non-responder to ETN. Table 5 shows that the only genotype that appeared to raise the likelihood of being resistant to ETN was the CC of (-863C/A) SNP. On the other hand, The GG genotype of -308G/A was negatively correlated with the tendency for being a non-responder.

Correlations between the genotypes and difference in DAS28 over six months

As shown in Table 6, most of the differences between various genotypes regarding the

Table 5. Correlation between each genotype and the likelihood of being a non-responder

Genotypes	Phi-coefficient	*p*
-308G/A		
AA	0.171	0.13
GA	0.172	0.12
GG	-0.245	0.02*
-857C/T		
CC	-0.013	0.91
CT	0.013	0.91
-863C/A		
AA	-0.217	0.05
CA	-0.148	0.18
CC	0.311	0.005*

Phi-correlation coefficient was used to find the correlation between each genotype and the likelihood of being a non-responder. *: Significant difference between the groups.

Table 6. The change in DAS28 over six months between different genotypes in -308G/A, -857C/T, -863C/A TNF-α

Genotypes	Mean±SD	Mean±SD	Mean±SD	*p*
-308G/A				
Genetic variant	AA	GA	GG	
ΔDAS28	0.92±1	1.37±1.19	1.012±1.24	0.45[a]
-857C/T				
Genetic variant	CC	CT		
ΔDAS28	1.31±0.15	1.14±0.3		0.61[b]
-863C/A				
Genetic variant	AA	CA	CC	
ΔDAS28	2.26±0.77	1.29±1.16	1.13±1.20	0.03*[a]

SD: Standard deviation; ΔDAS28: The change in disease activity score of 28 joints over six months; a: One-way ANOVA used to find the statistical difference; b: Unpaired t-test used to find the statistical difference; *: Significant difference between the groups.

change in DAS28 after six months of ETN treatment were not statistically significant. The only significant change was in the (-863C/A) SNP's genotypes. Post-hoc analysis revealed a significant difference (p=0.023) between the AA and CC genotypes.

Relationship between the presence of multiple genotypes and tendency of being non-responder

To determine if the existence of multiple genotypes in the same patient could result in non-response to ETN, the SNPs with the highest TNF-α level were selected. Then, the frequency of occurrence of these SNPs was determined to see whether there was a difference between responders and non-responders. As shown in Table 7, there was no statistically significant difference in the distribution of these genotypes between the two groups, except for the (-857CC) and (-863CC) genotypes, which were considerably more prevalent (up to 70%) in the non-responders group.

Table 7. The distribution of multiple genotypes between the responders and non-responders' groups

	Response group No. (n=41)		Nonresponse group No. (n=39)		
	n	%	n	%	*p*
(-308AA) (-857CC) (-863CC)	0	0	3	7.6	0.11
(-857CC) (-863CC)	15	36.5	27	69.2	0.004*
(-308AA) (-857CC)	0	0	4	10.25	0.05

A chi-square test or Fisher exact test was used to identify the statistical difference between the groups; *: Significant difference between the groups.

DISCUSSION

Pharmacogenetics is a major breakthrough in the research for genetic markers indicating the biological response to TNF-α inhibitors. Numerous polymorphisms have been examined in relation to TNF-α inhibitors responsiveness.[21] The current study examined the association between three SNPs in the TNF-α promoter region and the proclivity for resistance to ETN, a commonly used TNF-α inhibitor in the treatment of RA patients. Regarding the demographic characteristics, the results of the current study were comparable to those of another Iraqi study that assessed beliefs regarding medications among a sample of Iraqi patients with RA.[22] Concerning the genetic polymorphism testing, the present study results in a sample of 80 RA patients treated with ETN showed three SNPs in the promoter region of the TNF-α gene (-308G/A, -857C/T, and -863C/A).

Three previous studies[23-25] conducted in Iraq explored the relation between a genetic

polymorphism in the promoter section of TNF-α and RA in a sample of Iraqi patients. However, none of them examined the association between these SNPs and resistant to ETN. Additionally, these investigations examined only one or two SNPs, in contrast to the current study. The existence of these polymorphic sites in the current and earlier researches indicates that TNF-α site in Iraqi patients is highly polymorphic and may alter ETN responsiveness.

Globally, many studies have examined the effect of a combination of SNPs in the TNF-α gene on the response to anti-TNF-α.[16] The SNPs -857C/T, -308G/A, -238G/A, and +489G/A in the TNF-α gene and their association to therapeutic efficacy were evaluated in 58 RA patients taking infliximab.[26] Similarly, an association study of five TNF-α related SNPs (-308G/A, -238G/A, and -857C/T) was conducted in 280 RA patients treated with different TNF-α inhibitors.[27] All of these studies demonstrated that TNF-α was worthy to investigate to determine the effect of genetic variation on biological drug response. Regarding the prevalence of -308G/A genotypes, the current study confirmed that the GG genotype of -308G/A was present in more than three-quarters of the participants, followed by heterozygote GA and the lowest one, homozygote AA. In addition, the G allele was present in more than 90% of patients, while the A allele was only present in 25% of patients. These findings are consistent with the Alanzy et al.[24] study, which covered Iraqi RA patients in Babylon province and revealed that GG and GA genotypes were present in 60% and 40% of RA patients, respectively, and the proportion of G to A allele was 80/20. Regarding the AA genotype, the results of Alanzy et al.[24] showed that none of the patients had AA genotype, unlike the present study results, which found six patients with AA genotype. Nevertheless, the findings of the present study differ from those of Ahmed et al.,[25] who identified a significant prevalence (58%) of AA genotype in the Iraqi RA patients in Najaf province, whereas GG was observed in (19%) only and the proportion of G to A was 30/70. The difference between prior studies and the current study could be related to the fact that the present study included patients from several governorates in Iraq, which may reflect some ethnic diversity, as opposed to earlier studies, which only examined patients from one governorate.

Various studies have examined the effect of the -308G/A polymorphism on TNF-α blocker responsiveness and the effect of these variants on increased susceptibility to and severity of RA.[28-31] The present study outcomes indicated no significant variance in the availability of GA and AA genotypes of the -308G/A polymorphism between responsive and non-responsive groups; however, the GG genotype was significantly present in the responsive group. This result is consistent with many studies that show RA patients with a -308GG genotype respond better to ETN than patients with other genotypes.[28-30]

Although the current study did not discover any association between the AA genotype of -308G/A and the proclivity for being non-responder to ETN in Iraqi patients, this result contradicts with earlier findings. Previous studies showed a correlation between the AA genotype and the proclivity for non-response in Hungarian,[32] Sweden,[33] Spain,[34] and Switzerland[35] patients in which those with -308 A/A homozygotes respond less favorably to ETN than patients with other genotypes in these four populations. The discrepancy between this study and other studies can be explained by the fact that the -308A allele is linked to other genes implicated in the severity of RA or resistance to ETN.[36] However, this linkage disequilibrium with other genes may not be present in our sample RA patients in addition to the current study's low availability of A allele, or ethnic discrepancy may have contributed to the study's failure to prove the influence of the AA genotype. Concerning the prevalence of -857C/T genotypes, the result showed that the CC genotype was the most prevalent, occurring in more than three-quarters of participants. Furthermore, the C allele was detected in all patients, whereas the T allele was only discovered in 18.75% of patients.

There was no prior study performed in Iraq and investigated -857C/T on RA or even other individuals to compare it to; however, the results were comparable to those of another study with a Caucasian patient with RA[37] and Iranian patients

with cystic fibrosis[38] which investigated the -857C/T in RA and cystic fibrosis respectively. However, the present finding contrasts with a Chinese study that found a much greater incidence of the -857T allele in patients with RA.[39]

Moreover, the current study demonstrated that both genotypes CC and CT were similarly distributed between responsive and non-responsive patients, with no statistically significant difference between the two groups and no correlation with ETN response. This result is in contrast to a study by Kang et al.,[40] which found that patients with the T allele of the TNF-α -857C/T SNP responded better to ETN therapy than homozygotes for the C allele, suggesting that the T allele might become a valuable genetic marker for response prediction in Korean RA patients. Additionally, the current results contradicted two other studies conducted in Poland[41] and China,[39] which discovered a relationship between -857C/T and anti-TNF therapy response and that the TT genotype was associated with a more excellent response to anti-TNF-α therapy. Also, in Chinese Han patients with ankylosing spondylitis, SNPs at -857CC genotypes can predict a favorable response to TNF-α blockers.[42] Additionally, a meta-analysis of diverse ethnic groups discovered an association between Caucasians' -857C/A C allele and their responsiveness to TNF-inhibitors, but not in Asians.[43] Alternatively, numerous studies demonstrate that TNF-857C enhances the response to ETN, while there is inconsistent and non-significant evidence for this connection.[44] This discrepancy in outcomes of the current study with previous studies can be related to the low prevalence of the T allele in Iraqi RA patients who participated in the present study.

In case of -863C/A genotypes, the present study findings indicated that the CC genotype was the most prevalent in more than half of the participants, followed by the CA genotype, and the AA genotype was the least prevalent. In terms of allele distribution, the C allele was the most prevalent, occurring in more than 87% of patients, whereas the A allele occurred in only 30% of patients.

This study is the first to investigate the genotyping of -863C/A in Iraqi RA patients or even other patients. However, the findings were comparable to those of a study that examined the risk of RA in the Chinese Han population with RA.[45] In addition, the results are comparable to a study that examined the association between Behçet's disease and TNF-α gene polymorphisms in a sample of Moroccan patients.[46] Moreover, the results are comparable to a study that examined the relationship between the -863C/A polymorphism and type 2 diabetes in a Tunisian diabetic patient population.[47]

The most remarkable result of the current study was a statistically significant positive link between the CC genotype and a tendency to be non-responder to ETN and a statistically significant link of AA genotypes with a tendency to be a responder. Likewise, the current investigation confirmed that the C allele was significantly more prevalent in the non-responsive group, while the A allele was significantly observed in the responsive group.

It is interesting to note that our study is the first to examined the relationship between the TNF-α -863C/A polymorphism and response to ETN in RA patients. However, in two studies involving RA patients from northwest China's Han population[48] and Thai RA patients,[49] TNF-α -863C/A was strongly associated with RA susceptibility. Nevertheless, this association was not confirmed in another Pakistani study,[50] or the results showed a weak association in the North Indian population study.[51]

The present study findings can be explained by the fact that carriers of the rare -863AA genotype had lower serum TNF-α levels and a significant change in DAS28 than carriers of the CC genotype. This finding is consistent with the findings of a prospective study done by Boehm et al.[52] that measured TNF-α levels in patients genotyped for TNF-α -863C/A. The DAS28 score is the most often used outcome measure in investigating therapy response in RA.[53] The current investigation could not confirm a significant association between a change in DAS28 after six months of continuous ETN treatment and specific genotype, except for the AA genotype of (-863C/A), which is associated with the most remarkable change in DAS28. However, numerous prior investigations demonstrate that some genotypes are associated

with a considerable effect on DAS28 variation. A study examined the effect of the (-308G/A) polymorphism on ETN treatment in RA discovered that the GG genotype was linked with a more notable change in DAS28.[54] The same effect of GG genotypes on DAS28 change was also confirmed with infliximab.[55] Another study discovered that the change in DAS28 was significantly lower in patients with C homozygous for the -857C/T variant, whereas the T allele caused a significant alteration in DAS28, compared to carriers of the C allele.[41]

Furthermore Kang et al.[40] found a non-significant change in ACR20 or ACR70 linked with -1031T/C and -863C/A polymorphisms in a sample of Korean RA patients. The discrepancy between the results of the current study and that of previous studies can be explained, as subjective scores, particularly DAS28, are vulnerable to heterogeneity depending on the reporting clinician. In addition, it is self-evident that joint tenderness measurement and patient perceptions of disease activity are two areas prone to confounding, as there is no objective metric to validate clinical judgment.[53]

In addition to the differences in research design, clinical outcomes assessed (DAS28-ESR *vs.* DAS28-CRP), anti-TNF medicine utilized, concomitant disease-modifying antirheumatic therapies (DMARDs) in some studies, sex ratios, and sample size. To ascertain whether the presence of numerous genotypes in a single patient can result in a tendency to be non-responder to ETN, the SNPs with the highest TNF-α level were chosen. Following that, the frequency of occurrence of these SNPs together was assessed to determine whether there was a difference between the responders' and non-responders' groups. Except for the existence of both (-857CC) and (-863CC) genotypes together, the current investigation found no significant difference in the distribution of these genotypes between the two groups. This finding can be explained by the fact that both genotypes are associated with an increased risk of being non-responder to ETN or high TNF-α level according to several previous studies[40,43,52] and, thus, the presence of both genotypes in the same patient increases the likelihood of being non-responder.

Compared to previous studies, the results revealed controversial outcomes. According to the study done by Udalova et al.,[56] the -857C and -863A alleles, which is associated with a high TNF-α producer, were related with a low anti-TNF-α response, whereas the -857T and -863C haplotype, which is linked with a low TNF-α producer, was associated with a high anti-TNF-α response. Another study that examined gene polymorphisms of -238G/A, -308G/A and -857C/T found that the -238G, -308G, or -857C haplotype in a homozygous form was significantly accompanying with a lesser response as measured by (ACR50) with adalimumab.[57]

This study has several limitations. First, this study has a relatively small sample size, which may be attributed to the restricted number of patients who used ETN monotherapy and all other inclusion criteria were met. Second, as the current study is limited to a single-center, caution may be required before generalizing the findings to all Iraqi RA patients. However, our center serve patients from different governorates in Iraq. Finally, the authors were unable to conduct a more powerful prospective cohort study by selecting only patients with specific genotypes and following them for six months, owing to the small number of patients who received only ETN, insufficient financial resources, and the lengthy time frame required to recruit a sufficient number of patients and conduct this type of study.

In conclusion, the presence of the -863CC genotype, alone or in combination with -857CC, is linked to an increased likelihood of becoming a non-responder to ETN, implying that it is a valuable marker in Iraqi RA patients for predicting the tendency to be a non-responder. The GG genotype of -308G/A and the AA genotype of -863C/A, on the other hand, significantly increase the likelihood of becoming a responder to ETN. These results suggest the need for investigating RA patients for the availability of the -863CC genotype with -857CC before administration of ETN.

Ethics Committee Approval: The study protocol was approved by the Scientific and Ethical Committee in College of Pharmacy, University of Baghdad and Rheumatology Medical Department at Baghdad Teaching

Hospital (date: 03.10.2020, no: RECACPUB-3102020B). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patient Consent for Publication: A written informed consent was obtained from each patient.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: The researcher who perform the practical work in hospital: S.M.; The 1st supervisor for the PhD. research, writing guide, statistics: M.Z.; The second supervisor who responsible for patients selection,writing guide: F.G.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding: The authors received no financial support for the research and/or authorship of this article.

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