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The Impact of rs767455 and rs1061622 Polymorphisms on Treatment Outcomes in Iraqi Ankylosing Spondylitis Patients Taking Etanercept

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ABSTRACT

Background: Ankylosing spondylitis (AS) is inflammation of the sacroiliac joints and spine, associated with clinical symptoms such as pain and stiffness in the vertebral column, after which, in a considerable number of individuals, new bone growth occurs.

Objective: The current research study attempted to find out whether the presence of SNPs in TNF receptor [*TNFRSF1A* (rs767455), *TNFRSF1B* (rs1061622)] encoding genes could influence patients' outcomes to etanercept in a specimen of Iraqi AS patients.

Patients and methods: Sixty patients with established AS receiving only etanercept were selected to be enrolled in this research with a mean age of 40.75 ± 8.67 years, 51 patients of them were males and only 9 patients were females. Patients were classed as "responders" if just obtained a BASDAI 50 clinical response and as "non-responders" if they can't achieve a BASDAI 50 clinical elaboration after at least 6 months treatment. After PCR products amplification of purified blood DNA, TNF receptor (TNFRSF1A and TNFRSF1B) genes SNPs were established by Sanger sequencing.

Results: The analysis of this study expressed that there was a significant incidence of TT genotype of rs1061622 (P = 0.022) in responder group, whereas the TG genotype of the same SNP was considerably present in the group that did not respond (P = 0.002). Finally, a non-significant difference existed in alleles and genotypes frequency between responder and non-responder groups of rs767455 SNP in *TNFRSF1A* gene.

Conclusions: The wild TT genotype of rs1061622 predicts etanercept responsiveness in ankylosing spondylitis patients. The TG genotype of the same SNP increases the probability of non-responding.

Keywords: Ankylosing spondylitis, Genetic polymorphism, *TNFRSF1A*, *TNFRSF1B*, Etanercept.

INTRODUCTION

Ankylosing spondylitis (AS) is characterized by inflammation of the sacroiliac joints (SIJ) and spine, with associated clinical symptoms including pain and stiffness in the spine, after which, in a considerable number of individuals, new bone growth occurs [(1)]. Ankylosing spondylitis is an autoimmune illness that arises as a result of complicated interplay between genetic and environmental variables [(2)]. Although HLA-B27 remains the strongest association in almost all groups, a considerable multi-genic hereditary component is [(3)]. In response to an infection or tissue injury, the immune system strongly activates a signaling cascade that includes the pro-inflammatory molecule tumor necrosis factor-alpha (TNF-α). Patients with AS, in particular, respond well to TNF-α antagonist therapy [(4)].

Etanercept (ETN) has been examined in a wide variety of rheumatologic disorders [(5)]. ETN is the only soluble TNF receptor that the FDA has licensed for therapeutic use, and it is typically administered in weekly doses of 50 mg subcutaneously (or 25 mg twice weekly), either by self-injection or by a a caregiver [(6)]. TNF-α is a strong immunological peacemaker that is crucial to the pathophysiology of ankylosing spondylitis. TNF-α accomplishes its function via attaching to the receptors on the cell surface (TNFRI, encrypt through *TNFRSF1A* and TNFRII, encrypt through *TNFRSF1B*) [(7)]. A variant of a genetic sequence might create adaptive abnormalities in the TNF receptors, resulting in alterations produced by TNF-α via aberrant signaling [(8)].

Twenty percent to forty percent of patients, unfortunately, failed to respond well to tumor necrosis factor inhibitors (TNFi) biological therapies. Recent studies have been examined the significance of genetic markers in anti-TNF pharmacological therapies in ankylosing spondylitis patients [(9, 10)], but Iraq lacked these

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investigations. Identification of pharmacogenetic markers enabling treatment, only those patients who will respond without the danger of unwanted effects will be treated. This would considerably improve the efficacy of treatment and reducing expenses [(11)].

According to our knowledge, no prior research has been conducted in Iraq to explore the influence of SNPs within the *TNFRSF1A* and *TNFRSF1B* genes upon the propensity to respond to ETN in AS patients. The current research study, therefore, attempted to find out whether the presence of SNPs in TNF receptor [*TNFRSF1A* (rs767455), *TNFRSF1B* (rs1061622)] encoding genes could influence the patients' outcomes to ETN in a specimen of Iraqi AS patients.

SUBJECTS AND METHODS

Inclusion criteria: Patients who were diagnosed AS according to modified New York criteria [(12)]; on etanercept for at least 6 months and without a prior missed dosage history were included.

Exclusion criteria: Patients with coexisting other connective tissue diseases, any chronic infectious diseases, cancer, hepatic or renal dysfunction, endocrine gland insufficiency, hematological and cardiac conditions, multiple sclerosis ,using etanercept for less than 6 months, using synthetic disease modifying anti-rheumatic drugs (sDMARDs) in addition to etanercept, and patient with inadequate data.

Data collection

The demographic information and adverse effects of ETN were acquired by direct interviews with patients using a patient records' sheet prepared specifically for this study.

Clinical evaluation:

To analyze disease signs, symptoms, medical history, and laboratory data for all patients who participated in the study, direct interviews were conducted. Assessment of the patient's disease activity by calculating Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Bath Ankylosing Spondylitis Functional Index (BASFI) was done.

DNA extraction:

For DNA extraction, two ml of the obtained venous blood specimen were transformed to EDTA tube. Promega ReliaPrep^{TM} Organizing Method for Genomic DNA (Promega Corporation, USA) offers a feasible method for DNA purification from samples of blood. Loci portions of TNFRSF1A and TNFRSF1B genes were amplified by conventional type of PCR.

Primers:

Premier 3 software was utilized for the generation PCR primers. Primers for rs767455 forward (F): 5′-TGT AAA ACG ACG GCC AGT GTG TCG GAC GCT TAT CTA TAT C-3′ and reverse (R): 5′-CAG GAA ACA GCT ATG ACC TCT CTC CCT TTC AAA CTT CTC-3′; and for rs1061622 forward (F): 5′-TGT AAA ACG ACG GCC AGT GCC CTA AGC TAGG AAA GTT ATG-3′, reverse (R) 5′-CAG GAA ACA GCT ATG ACG CAG ACA GAA GGA GTG AATG-3′.

The products of PCR were sent for Sanger sequencing using automated DNA sequencer (ABI3730XL, Macrogen Corporation South Korea). The results were received by email then analyzed using Geneious prime software.

Ethical approval:

The Ethical and Scientific Committee in the College of Pharmacy at University of Baghdad, Iraq agreed to give their permission to the cross-sectional study that was conducted with approval number: RECACPUB-3102020D. The current study was conducted at the Rheumatology Unit, Baghdad Teaching Hospital of Medical City, Baghdad, Iraq from January to December 2021. Prior to data collection, signed consent was obtained from each participant.

Out of 76 participants met the study's participation criteria in the study, only 60 were involved in the study, where 12 patients rejected the involvement and 4 were eliminated for missing data. The patients were determined to have AS in accordance with the modified New York Criteria [(12)] with a mean age of 40.75 ± 8.67 years, fifty-one patients of them were males and only nine patients were females. As a 50% improvement in disease activity score; Bath ankylosing spondylitis disease activity index 50 (BASDAI 50) was defined as a response to therapy [(13)]. Patients were categorized as responders (R) and as non-responders (NR), as demonstrated in **figure (1)**.

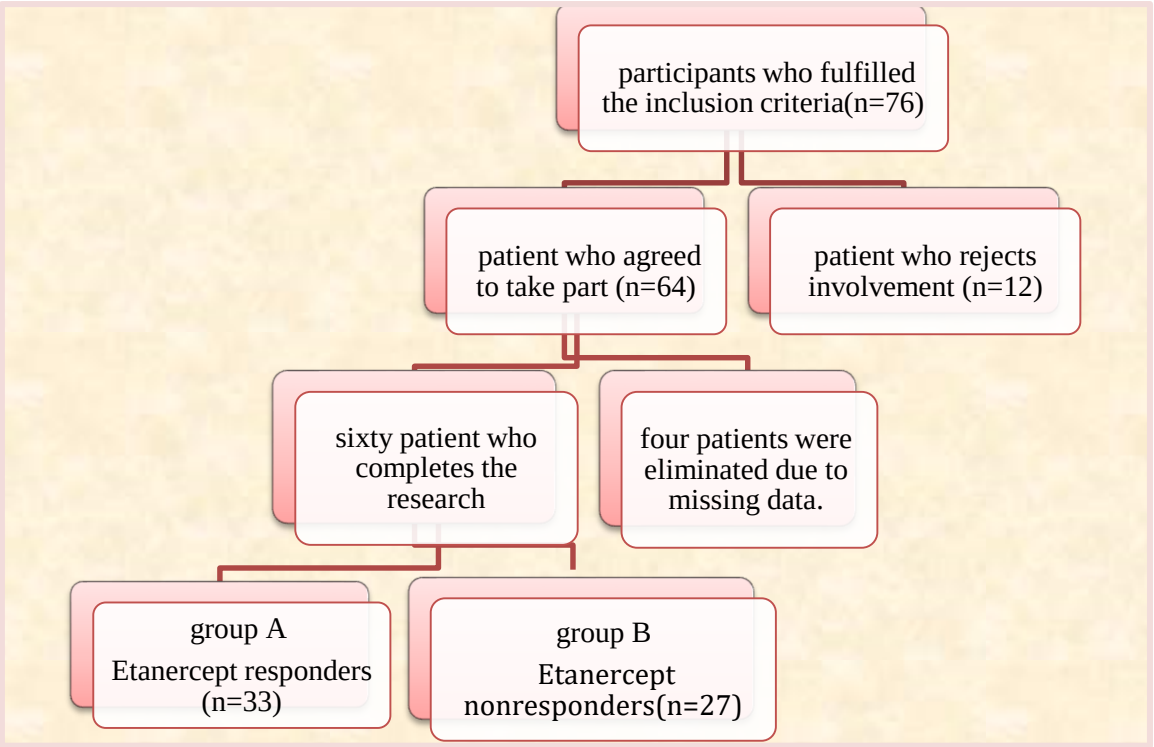

Figure (1): Schematic representation of the research design.

Statistical analysis

IBM SPSS Statistics (Version 26, IBM Corp., 2019) was used for the statistical analysis of the collected data. Variables with continuous values were stated as mean ± standard deviation (SD). Discrete variables were presented using their number and percentage. Allele and genotype percentages and frequencies were reported by direct count. P ≤ 0.05 was considered as significant. Chi-square test and Fischer exact test were used for data arrangement and analysis. To determine the link between each genotype and the likelihood of being a responder, the phi coefficient was applied.

RESULTS

AS demographics and disease characteristics:

Demographic data and clinical characteristics variables of the research participants are expressed in table (1). The mean of age was 40 ± 7.9 and 41.5 ± 9.7 years for responder, non-responder to etanercept groups, respectively (*P*=0.559). The study results revealed that there was a significant difference (*P*=0.0008) in gender distribution, in which the percentage of males in responder group was 97%, whereas it was 70.4% in non-responder group, whilst the percentage of females in responder and non-responder group was 3% and 29.6%, respectively.

Genotyping:

The products of DNA extraction then fractionated on1.5% agarose gel and stained with ethidium bromide and visualized using gel imaging system, as shown in **figures (2) and (3)**.

Prevalence of *TNFRSF1A*/*TNFRSF1B* gene polymorphism in all AS patients:

As presented in **table (2)**, the AG heterozygote genotype of rs767455 was more frequent (43%) than homozygote wild AA genotype and homozygote mutant GG genotype. The prevalence of wild homozygote TT genotypes of rs1061622 was high, whereas the mutant homozygote genotypes of this SNPs were present in only 8.3% of patients. Furthermore, in respect to the distinction in genotypes frequency between the two study groups, the analysis of this study expressed that there was a substantial prevalence of TT genotype of rs1061622 (*P* = 0.022) in responder group, whereas the TG genotype of the same SNP was significantly present in non-responder group (*P* = 0.002), as revealed in **table (3)**. Finally, there was a nonsignificant differenced in alleles and genotypes frequency between responder and non-responder groups of rs767455 SNP in *TNFRSF1A* gene.

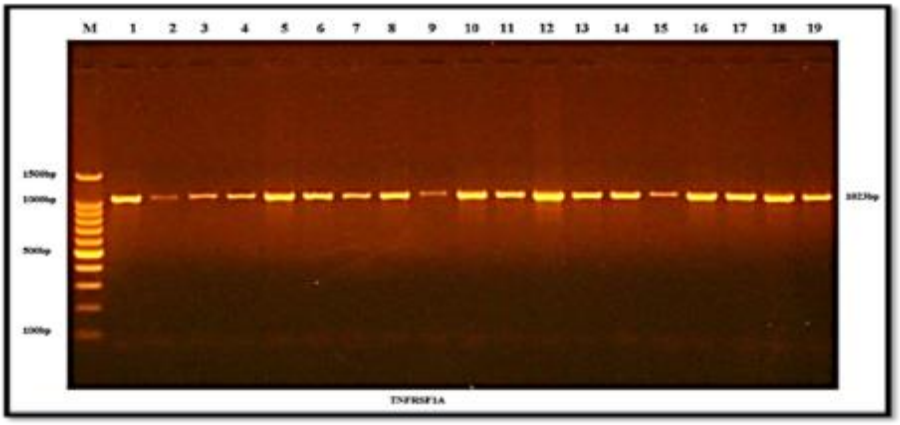

Figure (2): Electrophoresis of a 1023 bp fragment of human *TNFRSF1A* gene as electrophoresed for 1 hour on ethidium bromide-stained agarose (1.5%) gel at 70 volt/cm^2 and 1× TBE buffer. Lane M: 100-1500 bp ladder marker. Lanes 1-19 resemble 1023 bp PCR products

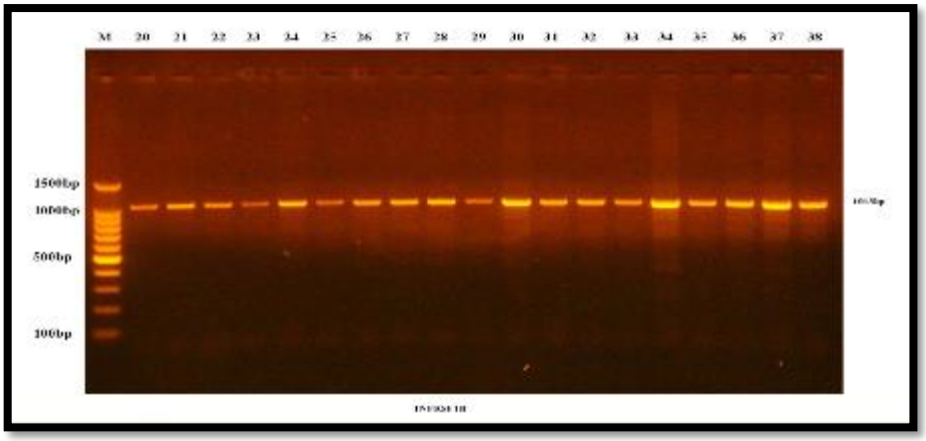

Figure (3): Electrophoresis of a 1013 bp fragment of human *TNFRSF1A* gene as electrophoresed for 1 hour on ethidium bromide-stained agarose (1.5%) gel at 70 volt/cm^2 and 1× TBE buffer. Lane M: 100-1500 bp ladder marker. Lanes 1-19 resemble 1023 bp PCR products

Table (1): Demographic data of the responder and non-responder study groups

Variable	Category	Responder (n=33)	Non-responder (n=27)	*P*-value
Age (years), mean ± SD	-	40.0±7.90	41.0±9.70	0.559
BMI kg/m^2, mean ± SD	-	28.4±5.60	29.7±5.30	0.340
Use NSAIDs	Yes	2.0 (6.10)	6.0 (22.2)	0.124
	No	31 (96.9)	21 (77.8)	
Family Hx of AS, n (%)	Yes	7.0 (21.2)	8.0 (29.6)	0.454
	No	27 (78.8)	19 (70.4)	
Gender, n (%)	Male	32 (97.0)	19 (70.4)	0.0008*
	Female	1.0 (3.03)	8.0 (29.6)	
Smoking status, n (%)	Non-smoker	10 (30.3)	16 (59.3)	0.077
	Active smoker	18 (54.5)	9.0 (33.3)	
	Ex-smoker	5.0 (15.2)	2.0 (7.4.0)	
Disease duration, n (%)	< 5 years	17 (51.5)	15 (55.6)	0.755
	≥ 5 years	16 (48.5)	12 (44.4)	
Presence of, n (%)	Osteoporosis	9.0 (27.3)	5.0 (19.2)	0.471
	Uveitis	3.0 (9.10)	3.0 (11.5)	0.999
	IBD	0.0 (0.00)	2.0 (7.70)	0.190

*Significant difference between two groups, BMI=Body mass index, Hx=History, IBD=Inflammatory bowel disease

Table (2): Genotypes and alleles frequency of rs767455A/G and rs1061622T/G in ankylosing spondylitis patients (n=60(

SNPs					
rs767455A/G			**rs1061622T/G**		
Genotype	**Number**	**Percentage**	**Genotype**	**Number**	**Percentage**
AA	24	40.0	TT	32	53.3
AG	26	43.3	TG	23	38.3
GG	10	16.7	GG	5.0	8.40
Allele	**Frequency**		**Allele**	**Frequency**	
A	74	61.7	T	87	72.5
G	46	38.3	G	33	27.5

Table (3): Distribution of rs767455A/G and rs1061622T/G polymorphism and individual alleles in the study groups

		Responder n=33		**Non-responder, n=27**		
SNPs	**Genotype**	**Number**	**Percentage**	**Number**	**Percentage**	***P*-value**
rs767455A/G	**AA**	12	36.4	12	44.5	0.520
	AG	15	45.5	11	40.7	0.710
	GG	6.0	18.2	4.0	14.8	1.000
Allele	A	39	59.0	35	64.8	0.520
	G	27	41.0	19	35.2	
rs1061622T/G	**TT**	22	66.7	10	37.0	0.022*
	TG	70.	21.2	16	59.3	0.002**
	GG	4.0	12.1	1.0	3.70	0.360
Allele	T	51	77.3	36	66.7	0.190
	G	15	22.7	18	33.3	0.220

*****Significant difference between two groups; ** highly significant difference between two groups; rs: reference SNP; wild genotypes are indicated in bold text

Association between genotypes and probability of being responder:

Phi correlation coefficient analysis showed that there was a strong association between each genotype and the predisposition to be responder to ETN. **Table (4)** displayed that the homozygote wild **TT** genotype of rs1061622 seemed to enhance the probability of being responder to ETN in AS patients. Nevertheless, the heterozygote TG genotype of rs1061622 showed a negative and significant correlation for responsiveness to ETN, whilst the other genotypes showed either a positive or negative relationship but did not reach the statistically significant level.

Table (4): Relationship among genotypes and the probability of responding

SNPs	**Genotype**	**Phi-coefficient**	***P*-value**
rs767455A/G	**AA**	-0.082	0.362
	AG	0.047	0.714
	GG	0.045	0.728
rs1061622T/G	**TT**	0.295	0.022*
	TG	-0.389	0.003**
	GG	0.152	0.241

Adverse events and associated SNPs of ETN therapy:

To investigate whether SNPs in the *TNFRSF1A* and *TNFRSF1B* alter the severity of deleterious effects from ETN use, we looked at the relationship between genotype and the occurrence of these adverse events. Infection, injection site reaction, headache, and rash. For each variant, these two SNPs were contrasted between patients that did not experience any adverse effects and cases that did. At rs767455 and rs1061622, two polymorphism genotypes, namely A/A and T/G, were statistically significant contributors to the prevalence of infection after ETN therapy (P = 0.027 and 0.005, respectively) as shown in **table (5)**. Whilst, the present result showed a non-significant difference between genotypes with increased risk of developing injection site reaction, headache, and rash adverse effect.

Table (5): Distribution of studied patients according to occurrence of infection, as a side effect and encountered alleles

		Infection				
		Yes, n=17		**No, n=43**		
SNPs	**Genotype**	**Number**	**Percentage**	**Number**	**Percentage**	***P*-value**
	AA	11	64.7	13	30.2	
rs767455 A/G	AG	4.0	23.5	22	51.2	0.027
	GG	2.0	11.8	8.0	18.6	
	TT	4.0	23.5	28	65.1	
rs1061622 T/G	TG	12	70.6	11	25.6	0.005*
	GG	1.0	5.90	4.0	9.30	
		Injection site reaction				
		Yes, n=21		**No, n=39**		
SNPs	**Genotype**	**Number**	**Percentage**	**Number**	**Percentage**	***P*-value**
	AA	8.0	38.1	16	41.0	
rs767455 A/G	AG	9.0	42.9	17	43.6	0.932
	GG	4.0	19.1	6.0	15.4	
	TT	10	47.6	22	56.4	
rs1061622 T/G	TG	9.0	42.9	14	35.9	0.808
	GG	2.0	9.5	3.0	7.7	
		Headache				
		Yes, n=11		**No, n=49**		
SNPs	**Genotype**	**Number**	**Percentage**	**Number**	**Percentage**	***P*-value**
	AA	7.0	63.3	17	34.7	
rs767455 A/G	AG	4.0	36.4	22	44.9	0.110
	GG	0.0	0.0	10	20.4	
	TT	4.0	36.4	28	57.1	
rs1061622 T/G	TG	5.0	45.5	18	36.7	0.290
	GG	2.0	18.2	3.0	6.1	
		Rash				
		Yes, n=12		**No, n=48**		
SNPs	**Genotype**	**Number**	**Percentage**	**Number**	**Percentage**	***P*-value**
	AA	5.0	41.7	19	39.6	
rs767455 A/G	AG	5.0	41.7	21	43.8	0.990
	GG	2.0	16.6	8.0	16.7	
	TT	5.0	41.7	27	56.3	
rs1061622 T/G	TG	6.0	50.0	17	35.4	0.633
	GG	1.0	8.30	4.0	8.3	

DISCUSSION

Pharmacogenomics allows clinicians to use genetic information to prevent diseases, improve diagnostic tests, and choose medicines with the best probability of success and the fewest side effects [14]. Recently, many genetic studies have been performed in Iraq [15, 16]. The first biological DMARDs that are considered second-line therapy are TNFi [17].

In the current research, the mean age in responder and non-responder groups showed non-significance difference (P = 0.559). The study results revealed that there was a significant difference (P = 0.0008) in gender distribution, in which the percentage of males in responder group was 97%, whereas it was 70.4% in non-responder group. On the other hand, the percentages of females in responder and non-responder groups were 3% and 29.6%, respectively. These results are consistent with various previous studies [18-20].This pharmacogenetic observational study is the first study in Iraq that analyzed the association of SNPs in TNFRI gene (rs767455A/G) and in TNFRII (rs1061622T/G) with outcomes of treatment in Iraqi patients with AS taking ETN.

Concerning the frequency of the prevalence of rs767455 in all AS patients, this current research shown that the AG heterozygote of rs767455 was more frequent (43%) than homozygote wild AA genotype and homozygote mutant GG genotype. This result agrees with **Zhao *et al.*** [21] how found that heterozygote variant was found in 91 patients. Moreover, in the present research, the A allele was found in over 60% of patients, but G allele was just present in about 38% of patients' population and this result is consistent with previous research done by **Chen *et al.*** [22].

There has been no previous research conducted in Iraq and closely examined G36A on AS or any other disease to match it along with.

Considering the distinction in rs767455 genotypes frequency between the two groups, the analysis of this study showed no statistically significant difference in the allele and genotype frequency of this SNP. Similar findings are reported by **Zhao *et al.*** [21].

Regarding the frequency of the prevalence of genotypes in rs1061622 in all AS patients, the current data showed that there was a high prevalence of wild homozygote TT genotype, occurring in more than half the patients (53.3%). Besides, the T allele was found in more than 70% of participants, but G allele was just present in about 27% of participants. This result was in agreement with a study done by **Xing-Rong *et al.*** [23]. Similarly, a Polish study performed by **Swierkot *et al.*** [24] reported a high frequency of TT genotype (60%) compared to TG and GG genotypes in rheumatoid arthritis patients.

Considering the difference in genotype frequencies between the two groups, the analysis of this study revealed that the responder group significantly had the TT genotype of rs1061622 (P = 0.022), whereas the non-responder group significantly had the TG genotype of the same SNP (P = 0.002), suggesting differences in soluble TNFR p75 expression and function.

The outcome seems to be in agreement with result of previous study done by **Schiotis *et al.*** [25] and **Xing-Rong *et al.*** [23].

However, the current findings disagree with Iraqi study done by **Hadi *et al.*** [26] on psoriatic patients, which revealed heterozygote genotypes of *TNFRSF1B* SNP can predict a positive prognosis responsiveness to TNF-α inhibitors [26].

Considering investigating whether SNPs in the genes which encodes *TNFRSF1A*/*TNFRSF1B* alter the severity of deleterious effects from ETN use, this study looked at the relationship between genotype and the occurrence of infection, injection site reaction, headache, and rash (adverse events associated with ETN use). These two SNPs were examined between patients with and without adverse effects for each genotype. ETN can cause side effects despite being well-tolerated. Pharmacogenetics applications may reduce adverse effects and improve safety. Thus, identifying these side effects may enhance drug tailoring and reduce unnecessary toxicity in TNF-α inhibitor patients [27].

It is usually regarded that the most typically reported adverse reactions with ETN are injection site responses, skin rash, runny nose, and infections [28]. In line with the outcomes of this study, the most common adverse effects were injection site reaction (35%) followed by infection (28.3%), then headache and rash with incidence percent about 38%. However, the result of this study revealed, at rs767455 and rs1061622, two polymorphism genotypes, namely A/A and T/G, were significantly associated with the occurrence of infection after ETN (P = 0.027; P = 0.005, respectively). Nevertheless, TNF's had essential role in immunological protection over pathogenic organisms, it is physiologically probable that reducing TNF-α would increase infection prevalence [29].

This was the first study to investigate the relationship between genetic variation in TNFR (I & II) encoded genes and the most common side effects of ETN in patients with AS, therefore there was no prior research against which to compare the results.

There are significant limits to the generalizability of our study's findings which ought to be taken into account. First of all, the limited sample size is a drawback of this study. Specifically, despite the fact that ETN is a highly effective treatment for AS, its expense and stringent inclusion criteria are frequently the primary reasons why published observations in AS patients, as well as our own study, typically have a small sample size. Second, as the present research was conducted at one institute. In the future, it is anticipated that a multi-center study will be conducted in order to involve more and more diverse

patients. Third, our study did not analyze numerous other genetic variants, such as those on IL-10 and IL-6.

CONCLUSION

The homozygote wild TT genotype of rs1061622 predicts ETN responsiveness in Iraqi ankylosing spondylitis patients. The TG genotype of the same SNP substantially increases the probability of non-responding. These findings suggest that AS patients should be tested for rs1061622 TG genotype before receiving ETN.

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Author contribution: Authors contributed equally to the study.

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