ORİJİNAL ARAŞTIRMA ORIGINAL RESEARCH

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# Investigation of Relation Between *MDR1* Gene and Ankylosing Spondylitis: Case Control Research

## *MDR1* Geni ile Ankilozan Spondilit Arasındaki İlişkinin İncelenmesi: Olgu Kontrol Araştırması

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**ABSTRACT Objective:** In this study, the relationship between the *MDR1* gene and ankylosing spondylitis (AS) was investigated. Genetic and environmental factors are known to play an important role in the pathogenesis of AS. It is obvious that different patients have different reactions to prescribed drugs and drug dosages during standard treatment. One of these factors is the P-glycoprotein encoded by the *MDR1* gene. The most widely studied alleles of this gene are rs1045642-NM_000927.4:c.3435T>C, p. Ile1145Ile (C3435T), rs2032582-NM_000927.4:c.2677T>G/A, p. Ser893Ala/Thr (G2677T/A) and silent rs1128503-NM_000927.4:c.1236T>C, p. Gly412Gly (C1236T). **Material and Methods:** The study group was formed from 34 patients with biological therapy, 32 patients with other treatments (66 AS patients in total) and 32 healthy individuals. DNA isolation was performed using a High Pure PCR Template Preparation Kit. RNA isolation was performed with TRIzol manual isolation. For quantitative real-time polymerase chain reaction, a Roche LightCycler FastStart DNA Master device HybProbe was used. **Results:** There was no statistically significant difference between groups regarding the investigated polymorphisms. In the biological therapy [tumor necrosis factor alpha (TNF-α) inhibitor] group, a significant decrease was found in *MDR1* gene expression (p<0.001). **Conclusion:** In conclusion, we could say that there is no relation between single-nucleotide polymorphism and treatment choice and response for AS patients. Decreased MDR1 expression can be explained by the possible downregulatory effect of TNF-α inhibitors.

**Keywords:** Ankylosing spondylitis; *MDR1*; genetic polymorphisms; gene expression; anti-TNF

**ÖZET Amaç:** Bu çalışmada *MDR1* geni ile Ankilozan spondilit araştırıldı. Genetik ve çevresel faktörlerin ankilozan spondilit (AS) patogenezinde önemli rol oynadığı bilinmektedir. Standart tedavi sırasında farklı hastaların, reçeteli ilaçlara ve ilaç dozajlarına karşı farklı tepkileri olduğu açıktır. Bu faktörlerden biri, *MDR1* geni tarafından kodlanan P-glikoproteindir. Bu genin en çok çalışılan allelleri rs1045642-NM_0009 27.4:c.3435T>C, p. Ile1145Ile (C3435T), rs2032582- NM_ 000927. 4:c.2677T>G/A, p. Ser893Ala/Thr (G2677T/A) ve sessiz rs1128503-NM_000927.4:c.1236T>C, p. Gly412Gly (C1236T)'dir. **Gereç ve Yöntemler:** Otuz dört biyolojik tedavi, 32 diğer tedavileri alan toplam 66 AS hastası ve 32 sağlıklı herhangi bir ilaç kullanmayan bireyden oluşan grup karşılaştırıldı. DNA izolasyonu High Pure PCR Template Preparation Kiti kullanılarak gerçekleştirildi. RNA izolasyonu trizolle yapıldı. Kantitatif gerçek zamanlı polimeraz zincir reaksiyonu, Roche LightCycler FastStart DNA Master HybProbe cihazında gerçekleştirildi. **Bulgular:** Çalışmamızın sonucunda, araştırılan polimorfizmlerle ilgili gruplar arasında istatistiksel olarak anlamlı bir fark yoktu. Biyolojik tedavileri [tümör nekrozis faktör alfa (TNF-α)] kullanan AS grubunun, *MDR1* gen ekspresyonu arasında anlamlı bir farklılık bulundu (p<0,001). **Sonuç:** Sonuç olarak AS hastalarında tek nükleotid polimorfizmi ile tedavi seçimi ve yanıt arasında bir ilişki olmadığını söyleyebiliriz. Azalan MDR1 ekspresyonu, TNF-α inhibitörlerinin olası baskılayıcı regülatör etkisi ile açıklanabilir.

**Anahtar Kelimeler:** Ankilozan spondilit; *MDR1*; genetik polimorfizm; gen ekspresyon; anti-TNF

Spondyloarthropathy (SpA) is a group of chronic inflammatory diseases that affect the vertebral column, peripheral joints and other organs.[1] Ankylosing spondylitis (AS), the most common form of SpA, generally onsets at the 3rd decade of life and is seen 2.4 times more often in males than in fe-

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males.[2] Because of this imbalance, there was some suspicion about the relation between this disease and the X chromosome, which were denied afterwards.[3] The exact etiology and pathogenesis of spondyloarthritis, including AS, is still unknown.[4] However, it is known that there is an excellent role of genetic and environmental factors in the pathogenesis of these disease.[5]

Treatment of AS includes physical and medical therapy. Current recommendations on treatment were published by Assessment of SpondyloArthritis International Society in 2016 and by American College of Rheumatology in 2019.[6,7] Medical therapy can be divided into biologic [tumor necorsis factor alpha (TNF-α) inhibitors] and non-biologic therapy (nonsteroidal anti-inflammatory drugs, analgesics, glucocorticoids, disease-modifying anti-rheumatic drugs).

It is evident that different patients have different reactions to prescribed drugs and drug dosages during standard treatment. While some patients demonstrate a better response to treatment and minimal side effects, some experience severe side effects such as toxic reaction with lethal outcome without any benefit of treatment at all. The drug response varies based on pharmacokinetic and pharmacodynamic mechanisms. Genetic variations of enzymes that regulate drug metabolism, transporters, receptors or cofactors have an effect on these mechanisms.[9] It is known that the differences in drug influence or elimination between individuals are based on polymorphisms in specific genes. The prediction of drug response and selection of proper medicine and dosage are essential components of "precision medicine."[10]

Multidrug resistance is the development of resistance to multiple and different drugs. One of the causes of inefficient intracellular drug concentrations is the disruption of membrane permeability, and p-glycoprotein (P-gp) has been shown to be responsible for this. Human P-gp removes from the cell (efflux) toxic substances entering the cell and a number of chemicals, such as chemotherapeutics, by using adenosine triphosphate (ATP) energy.[11]

The *MDR1* gene is located on chromosome 7 and codes a P-gp. P-gp is a member of the "ATP-Binding Cassette (ABC) transporter" protein family and is responsible for functioning as an ATP-dependent pump that transports drugs out of the cell.[12,13]

Different single-nucleotide polymorphisms (SNPs) were determined in the *MDR1* gene, and the effect of this phenomenon on P-gp expression was also observed.[14]

Among *MDR1* gene SNPs, silent rs1045642, NM_000927.4:c.3435T>C, p. The Ile1145Ile (known as C3435T) variant located on the 26th exon draws more attention and is responsible for the decreased expression and activity of P-gp.[15] Other most emphasized SNPs regularly observed on the *MDR1* gene in the literature are rs2032582, NM_000927.4: c.2677T>G/A, p. Ser893Ala/Thr (known as G2677T/A) and silent rs1128503, NM_000927. 4:c.1236T>C, p. Gly412Gly (known as C1236T).[16]

Although C3435T is silent, it is reported to be associated with reduced P-gp function when found along with other common non-synonymous SNPs in any haplotype, and G2677T/A variants result in missense mutations that alter the intracellular side of P-gp.[17,18]

In our study, we investigated the profile of *MDR1* mRNA expression and distribution of C3435T, C1236T, and G2677A/T SNPs in AS patients, which were divided into 2 groups (biologic therapy receiving and non-biologic therapy receiving groups) and healthy controls.

## MATERIAL AND METHODS

The research included 66 (38 male and 28 female) patients diagnosed with AS using the Modified New York Classification Criteria who received inpatient and outpatient care at Erciyes University, Faculty of Medicine, Department of Physiotherapy and Rehabilitation and at the Department of Medical Genetics and 32 healthy volunteers (Group 3). Patients diagnosed with AS were divided into 2 study groups, which included 34 patients (Group 1) receiving biological treatment and 32 (Group 2) patients receiving non-biological therapy. The study was approved by the Ethics Committee of Erciyes University (date: August 20, 2013, no: 2013/521) and is in full compliance with all relevant codes of experimentation

and legislation. Signed consent was obtained from all participants. The study was conducted in accordance with the Principles of the Declaration of Helsinki.

### DNA ISOLATION AND GENOTYPING FOR *MDR1* C3435T, C1236T, G2677A/T SNPS

Two mL of venous blood samples with ethylene diamine tetra acetic acid (EDTA) were obtained from patients and the control group volunteers and used for the extraction of genomic DNA samples according to the manufacturers' instructions by using an extraction kit (MagNA Pure LC DNA Isolation Kit, Roche Diagnostics, GmbH, Mannheim, Germany) on an automated DNA extraction device (MagNA Pure LC 2.0, Roche Diagnostics Ltd., Rotkreuz, Switzerland). *MDR1* C3435T (rs1045642), C1236T (rs1128503), and G2677A/T (rs2032582) were investigated by quantitative real-time polymerase chain reaction (Q-RT-PCR). Genotyping was carried out by analysis of the melting temperature with the LightCycler 480 system (Roche, Germany). Cycling conditions were as follows: 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 10 seconds, 72°C for 10 seconds and melting curve 95°C for 30 seconds, 40°C for 2 minutes, 70°C continuous, a final cooling phase consisting of one cycle of 30 seconds at 40°C.

### RNA ISOLATION, REVERSE TRANSCRIPTION-PCR AND QUANTITATIVE REAL-TIME PCR

Nine mL of venous blood samples with EDTA were obtained from patients, and the control group was used to study gene expression. Total RNA extractions were performed using TRIzol reagent (Roche, Germany) from venous blood samples based on the manufacturers' instructions. RNA was stored at -80°C until use. First-strand cDNA synthesis was performed using a cDNA synthesis High Fidelity cDNA synthesis kit (Roche, Germany) according to the provided protocol. cDNA was amplified for 10 minutes at 29°C and 5 minutes at 85°C. Real-Time Ready Catalogue Assay LOT 900015809 (Roche, Germany), 1 µl of each primer (20 pmol/µl), LightCycler 480 Probes Master Mix (Roche, Germany) and 5 µl of cDNA were added to each well of the PCR plate. Q-RT-PCR reactions were performed using a LightCycler 480 (Roche, Germany) device for *MDR1* gene expression. The beta-actin (β-actin) gene was used as a housekeeping gene. The cycling conditions were as follows: 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 30 seconds, 72°C for 1 minute and a final cooling phase consisting of one cycle of 30 seconds at 40°C. Target gene copy numbers were normalized using the beta-actin (β-actin) gene. The delta-delta CT method was applied for relative quantification.

### STATISTICAL ANALYSIS

In the evaluation of normality of data, histogram, Q-Q graphics and the Shapiro-Wilk test were used. For comparison of data between groups chi-square test, independent t-test and Kruskal-Wallis (in multiple comparisons Dunn Bonferroni test) used.

The descriptive statistics of the data are displayed as count and percentages [n (%)] and the normally distributed variable displayed as the mean±standard deviation. Data analysis was performed using GraphPad Prism V7.0 (GraphPad Software, CA, USA) software $p<0.05$ was considered statistically significant.

Allele and genotype frequencies comparison between all groups and gnomAD database performed for revealing the responsible SNP, and comparison of total subjects in this study and gnomAD database was performed to see if the results are biased because of difference in population.

## RESULTS

There were no significant differences in age ($p>0.5$) and gender distribution ($p>0.5$) between groups (Table 1).

**TABLE1:** Age and gender distribution comparison between groups.

| Gender | Group 1 | Group 2 | Control | p value |
|---|---|---|---|---|
| Male (n) | 18 | 20 | 18 | >0.5 |
| Female (n) | 14 | 14 | 14 | |
| Age | | | | |
| Mean±SD | 40.94±9.44 | 33.90±8.71 | 29.75±4.70 | >0.5 |

SD: Standard deviation.

## POLYMORPHISM RESULTS

The results of all allele frequencies and genotype frequencies for Groups 1 and 2, the control group and the gnomAD database are shown in Table 2, Table 3. No differences were observed in the population included in this study in either allele frequencies or genotype frequencies (Table 2, Table 3). However, a small number of patients with the homozygote c.1236T=(wild-type) genotype were observed in Group 2 (Table 3).

The performed statistical analysis (chi-square test) between all subjects in this study and the gnomAD database showed slight differences; for the c.2677T>G/A SNP GG and TG genotypes and for the c.3435T>C SNP genotypes, although it is accepted that c.3435T=is wild-type, CC and CT genoty pes are seen more frequently in the gnomAD database (Table 3).

Fourteen different genotype combinations (C1236T- G2677T/A-C3435T) were observed. In AS

**TABLE 2:** Allele frequencies for Group 1, 2, control group and gnomAD database.

| | | allele count; allele frequency | | | | gnomAD | |
|---|---|---|---|---|---|---|---|
| Variant | ACMG Classification | Group 1 (n=34) | Group 2 (n=32) | Control (n=32) | Total (n=98) | (n; allele frequency) | p value |
| c.1236T>C | Benign | 30; 0.441 | 40; 0.625 | 31; 0.484 | 101; 0.515 | 154059; 0.5452 | 0.135a |
| c.1236= | Wild-type | 38; 0.559 | 24; 0.375 | 33; 0.516 | 95; 0.485 | 128523; 0.4548 | 0.401[b] |
| c.2677T>G | Benign | 30; 0.441 | 36; 0.562 | 31; 0.484 | 97; 0.495 | 155027; 0.5498 | 0.443[a] |
| c.2677T>A | VUS | 2; 0.029 | 1; 0.016 | 2; 0.032 | 5; 0.025 | 10221; 0.0363 | 0.154[b] |
| c.2677= | Wild-type | 36; 0.53 | 27; 0.422 | 31; 0.484 | 94; 0.48 | 116722; 0. 4139 | |
| c.3435T>C | Benign | 29; 0.426 | 32; 0.5 | 27; 0.453 | 88; 0.449 | 144371; 0.5105 | 0.266[a] |
| c.3435= | Wild-type | 39; 0.574 | 32; 0.5 | 37; 0.547 | 108; 0.551 | 138353; 0.4895 | 0.085[b] |

[a]Chi-square test between Group 1, Group 2, Control group and gnomAD database.
[b]Chi-square test between Total subjects in this study and gnomAD database.

**TABLE 3:** Genotype frequencies for Group 1, 2, control group and gnomAD database.

| Genotype | Group 1, n=34 (% within group) | Group 2, n=32 (% within group) | Control, n=32 (% within group) | Total, n=98 (% within group) | gnomAD (% within group) | p value |
|---|---|---|---|---|---|---|
| c.1236T>C | | | | | | |
| CC | 7 (20.60) | 10 (31.30) | 7 (21.90) | 24 (24.49) | 43876 (31.05) | 0.117[a] |
| CT | 16 (47.10) | 20 (62.50) | 19 (59.40) | 55 (56.12) | 66307 (46.93) | 0.180[b] |
| TT | 11 (32.40) | 2* (6.30) | 6 (18.80) | 19 (19.39) | 31108 (22.02) | |
| c.2677T>G/A | | | | | | |
| GG | 5 (14.70) | 7 (21.90) | 6 (18.80) | 18 (18.37) | 45274 (32.11) | 0,0977[a] |
| AA* | 0 | 0 | 0 | 0 | 327 (0.23) | 0.0177[b] |
| TA | 2 (5.90) | 1 (3.10) | 2 (6.25) | 5 (5.10) | 9567 (6.79) | |
| TG | 18 (52.90) | 21 (65.60) | 17 (53.10) | 56 (57.14) | 64479 (45.73) | |
| TT | 9 (26.50) | 3 (9.40) | 7 (21.90) | 19 (19.39) | 21338 (15.13) | |
| c.3435T>C | | | | | | |
| CC | 5 (14.70) | 5 (15.60) | 7 (21.90) | 17 (17.34) | 38978 (27.58) | 0.136[a] |
| CT | 19 (55.90) | 22 (68.80) | 15 (46.90) | 56 (57.14) | 66327 (46.94) | 0.052[b] |
| TT | 10 (29.40) | 5 (15.60) | 10 (31.30) | 25 (25.52) | 36013 (25.48) | |

[a]Chi-square test between Group 1, Group 2, Control group and gnomAD database.
[b]Chi-square test between Total subjects in this study and gnomAD database.
*Excluded from statistical analyse because of 0 value

patients and the control group, the most commonly observed genotype combinations were found to be CT-GT-CT, TT-TT-TT, and CC-GG-CT (Table 4). No significant differences were detected between groups (p=0.269), except for the TT-TT-TT combination in Group 2, which was observed in only one patient (p<0.05).

### *MDR1* GENE MRNA EXPRESSION RESULTS

*MDR1* gene mRNA expression between males (0.26±0.14) and females (0.28±0.18) was not statistically significant (p>0.05) (Figure 1).

A significant difference between groups in *MDR1* gene expression (p<0.001) was observed (Figure 2). Group 1 (0.16±0.09) *MDR1* mRNA expression was lower than Group 2 (0.27±0.12) (p=0.0022) and the control group (0.34±0.09) (p<0.001). Although the Group 2 expression level was lower than that in the control group, the differences were not statistically significant (p=0.0514) (Table 5).

## DISCUSSION

The exact cause of AS and another SpA is not yet known. However, it is known that genetic and environmental factors play an essential role in pathogenesis. A twin study conducted in the UK suggested that the role of genetic factors in the occurrence of the disease is 97%.[5] Another study found that the disease was diagnosed in both monozygotic twins five times more than in dizygotic twins.[19] These findings suggest that in the development of the disease, genetic factors came ahead of environmental factors.

In a study conducted between 200 drug-resistant and 115 drug-sensitive epilepsy patients by Siddiqui et al., more drug-resistant patients carried the CC genotype at position 3435. However, no significant difference between the 2 groups was determined while researching the CT genotype. Drug-resistant epilepsy patients were also compared with the healthy control group (n=200); drug-resistant patients were found to carry significantly more CC genotypes and fewer TT genotypes. Based on the statement that the C3435T polymorphism does not lead to amino acid changes, Siddiqui et al. proposed that this polymorphism has a linkage with the potential amino acid

**TABLE 4:** Observed genotype combinations and their frequencies.

| No | Genotype combinations (C1236T- G2677T/A-C3435T) | Group 1 | Group 2 | Control | Total |
|---|---|---|---|---|---|
| 1 | CT-GT-CT | 15 | 16 | 12 | 43 |
| 2 | TT-TT-TT | 9£ | 1¥ | 6£,¥ | 15 |
| 3 | CC-GG-CT | 2 | 4 | 3 | 9 |
| 4 | CC-GG-CC | 3 | 3 | 1 | 7 |
| 5 | CT-GT-TT | 1 | 3 | 2 | 6 |
| 6 | CC-GA-CC | 2 | 1 | 1 | 4 |
| 7 | TT-GT-CT | 2 | 0 | 0 | 2 |
| 8 | CT-TT-TT | 0 | 1 | 1 | 2 |
| 9 | CC-GT-CC | 0 | 1 | 1 | 2 |
| 10 | CT-GG-CC | 0 | 0 | 2 | 2 |
| 11 | CT-GT-CC | 0 | 0 | 2 | 2 |
| 12 | CC-GT-CT | 0 | 1 | 0 | 1 |
| 13 | TT-TT-CT | 0 | 1 | 0 | 1 |
| 14 | CC-GA-TT | 0 | 0 | 1 | 1 |

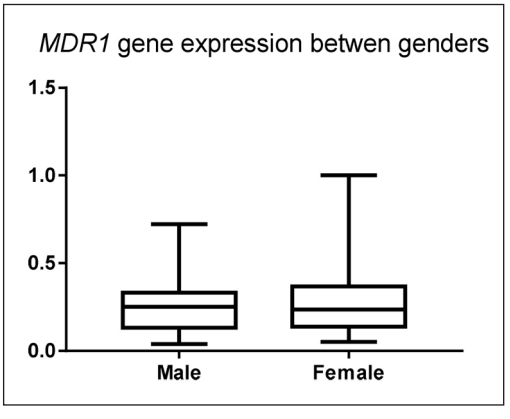


**FIGURE 1:** *MDR1* gene mRNA expression comparison between genders.

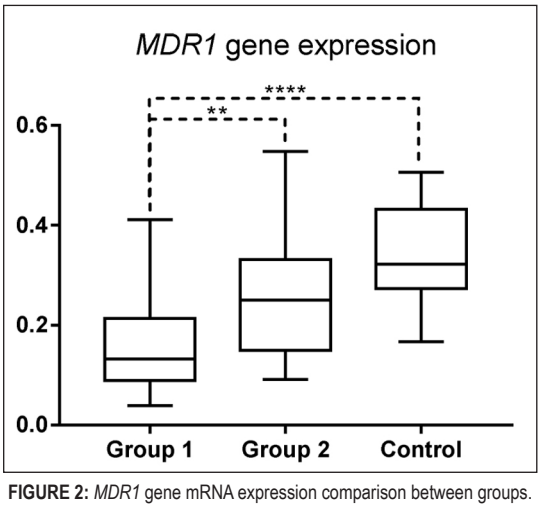


**FIGURE 2:** *MDR1* gene mRNA expression comparison between groups.

**TABLE 5:** Multiple comparisons test result of *MDR1* gene mRNA expression comparison between genders.

| Dunn's multiple comparisons test | Mean rank diff, | Significant? | Summary | Adjusted p value |
|---|---|---|---|---|
| Group 1 vs. Group 2 | -22.88 | Yes | ** | 0.0022 |
| Group 1 vs. Control | -39 | Yes | **** | <0.0001 |
| Group 2 vs. Control | -16.12 | No | ns | 0.0514 |
| Test details | Mean rank 1 | Mean rank 2 | Mean rank diff | n1 |
| Group 1 vs. Group 2 | 25.35 | 48.23 | -22.88 | 30 |
| Group 1 vs. Control | 25.35 | 64.35 | -39 | 30 |
| Group 2 vs. Control | 48.23 | 64.35 | -16.12 | 31 |

changing polymorphisms in *MDR1*.[20] In our study, no differences were observed in allele frequencies (Table 2).

Kimchi-Sarfaty et al. emphasized that each of C3435T, G2677T and C1236T SNPs changed the substrate specificity. However, the haplotype carrying all three SNPs reached a significant level in this effect. These data support the idea that the primary cause of the altered function of P-gp is not SNP variations but, rather, the haplotype formed by these polymorphisms.[16] In our study, no differences were observed in genotype frequencies (Table 3).

The slight differences in genotype frequencies between the population in this study and the gnomAD database observed in this study can be explained by the different origins of the study populations.

As a result of the linkage analysis between C3435T, G2677T/A and C1236T, a strong disequilibrium was observed between these SNPs in different populations. For example, Tang et al. reported a connection between these polymorphisms in the Chinese, Malay and Indian populations.[21] Similarly, Kim et al. reported a strong link between the C3435T, G2677T/A and C1236T SNPs found in European Americans and African Americans.[22] Tanabe et al. reported an association of the 3435T allele with the 2677A or 2677T allele in 94% of samples obtained from 65 Japanese individuals.[18] Similarly, in the Turkish population, significant linkage disequilibrium between these 3 SNPs was identified.[23] No significant differences were detected between groups in genotype combinations in our study.

There is a need to mention that in Group 2, c.1236T=(wild-type) SNP and TT-TT-TT genotype combinations of all SNPs investigated in this study are seen less. As it may sound paradoxical, maybe, after all, there is some relation between non-wild-type (c.1236T=) variants and patients with better responses to non-biological treatment. Different molecular mechanisms and relationships can be investigated to confirm this theory in the case of obtaining the same results from studies performed with more patients.

Thörn et al. investigated *MDR1* mRNA levels in the gastrointestinal tract. They observed no difference in mRNA levels between genders.[24] In our study, there was also no statistically significant relationship between *MDR1* gene expression and sex.

A significant difference between groups in *MDR1* gene expression was observed. While looking into the graph describing expressions, we see that the highest expression is in the control group (Figure 2). It is decreased in Group 2, non-biological treatment receiving patients and much more decreased in Group 1, biological treatment receiving patients. This result can be explained by the observation of Poller et al. that TNF-α increased the protein expression of P-gp in the human hCMEC/D3 cell line.[25] Therefore, treatment of Group 1 patients with TNF-α inhibitors may cause reduced *MDR1* gene expression. If it is true, TNF-α inhibitors can be used as drug dose regulator agents in the treatment of resistant diseases.

The statistically insignificant difference between Group 2 and the control groups is expected, as there was no expectation that the use of drugs or disease influences *MDR1* gene expression.

## CONCLUSION

This is the first study in which the effects and frequencies of 3 *MDR1* gene SNPs and the expression of the *MDR1* gene were described in an AS patient population. Our study showed no significant difference in the frequencies of C3435T, C1236T, and G2677T/A SNPs between the healthy population, and AS diagnosed patients who were receiving biological and non-biological therapy.

*MDR1* mRNA expression in the TNF-α inhibitor-treated group decreased significantly compared with that in the other groups. The obtained effect of TNF-α inhibitors on *MDR1* gene expression can be the subject of further investigations as dose regulatory agents for other drugs in treating of resistant diseases.

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### *Conflict of Interest*

*No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.*

### *Authorship Contributions*

***Idea/Concept:*** *Fatma Kurt Çolak;* ***Design:*** *Fatma Kurt Çolak, Keziban Korkmaz Bayram;* ***Control/Supervision:*** *Çetin Saatçı, Mehmet Kirnap;* ***Data Collection and/or Processing:*** *Fatma Kurt Çolak, Keziban Korkmaz Bayram,* ***Analysis and/or Interpretation:*** *Fatma Kurt Çolak, Arslan Bayram;* ***Literature Review:*** *Fatma Kurt Çolak;* ***Writing the Article:*** *Fatma Kurt Çolak, Arslan Bayram;* ***Critical Review:*** *Munis Dündar, Çetin Saatçi, Mehmet Kirnap;* ***References and Fundings:*** *Munis Dündar, Çetin Saatçi;* ***Materials:*** *Mehmet Kirnap, Fatma Kurt Çolak.*

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