

# Association of the genetic variants in the *Endoplasmic reticulum aminopeptidase 2* gene with ankylosing spondylitis susceptibility

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## Research article

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## Abstract

**Background:** *Genetic polymorphisms in the Endoplasmic reticulum aminopeptidase (ERAP) 2 gene* has been attributed with the Ankylosing spondylitis (AS) etiopathogenesis. Here we assessed the association of *ERAP2* gene single nucleotide polymorphisms (SNPs) with AS predisposition in Iranian patients and determined their effect on the inflammatory state of the patients.

**Methods:** For genotyping of rs2548538, rs2287988, and rs17408150 SNPs using Real-time allelic discrimination approach, DNA was extracted from the whole blood of 250 AS patients and 250 healthy subjects. RNA of the peripheral blood mononuclear cells (PBMCs) was separated, cDNA was synthesized, and transcriptional levels of cytokines, including interleukin (IL)-17A, IL-23, IL-10, and transforming growth factor (TGF)- $\beta$  were measured. Enzyme-linked immunosorbent assay (ELISA) was used to measure the serum concentration on the cytokines.

**Results:** Three *ERAP2* gene SNPs were not associated significantly with AS risk. Nonetheless, rs2287988 and rs17408150 SNPs showed statistically significant association with susceptibility to the disease in those AS subjects who were positive to Human leukocyte antigen (HLA)-B27. Transcriptional level and serum concentration of IL-17A and IL-23 were higher, while those of IL-10 were lower in both AS patients and HLA-B27 positive patient group relative to control group. Nevertheless, *ERAP2* gene SNPs in the HLA-B27 positive AS patients did not affect the transcriptional level and serum concentration of cytokines.

**Conclusions:** *ERAP2* gene rs2287988 and rs17408150 SNP are associated with susceptibility to AS, but they probably are not determining the levels of IL-17A, IL-23, and IL-10 in this disease.

## Background

Ankylosing spondylitis (AS) is a chronic autoimmune disease and is characterized by involvement of spine and sacroiliac joints that causes spine deformities, increased disability, and mortality (1). A bulk of research has suggested a significant role for genetic variations in the etiology and pathogenesis of AS (2, 3), in spite of the remarkable involvement of environmental factors as well as aberrant epigenetic regulations (4-8). The pathogenesis of AS is complicated, and earlier research concentrated on the misfolding of human leukocyte antigen (HLA)-B27 molecule in the disease susceptibility; however, genetic studies have proposed that HLA-B27 accounts for a small part of the overall AS risk (9). Epidemiological investigations has shown that HLA-B27 gene is carried by almost 90% of AS patients, while only 1-5% of individuals carrying the HLA-B27 gene will be affected with AS in the future (10). These observations imply to the involvement of non-HLA genes in the AS risk.

Endoplasmic reticulum aminopeptidase (ERAP) 2 is an enzyme that belongs to zn-metallopeptidase family and the corresponding gene is located on the chromosome 5q15. This enzyme is found within the endoplasmic reticulum (ER) that is involved in priming peptides during the antigen presentation pathway via the major histocompatibility complex (MHC) class I (11). In comparison to ERAP1, data lack about the attribution of *ERAP2* polymorphisms with AS predisposition (4, 12, 13). A number of genetic

polymorphisms in the *ERPA2* gene has been attributed to the alterations in the protein structure and function. The AS protective *ERAP2* gene SNP, rs2248374 (14), alters the splicing site in the exon 10, leading to synthesis of a lengthy exon 10 transcript (15). As a loss-of enzyme polymorphism, rs2248374 cause no protein expression of ERAP2 and has been attributed with downregulation of MHC class I molecule levels on the cell surface (15). Another variant is rs2549782 SNP, that confers a modulation in the specificity as well as functional velocity of the enzymatic activity of ERAP2 (14, 16). *ERAP2* gene rs2549782 SNP shows a linkage disequilibrium (LD) with other *ERPA2* SNPs, including rs2548538, rs2287988, rs1056893, and rs2248374, which are marker SNPs that constitute A and B haplotypes that are associated with protein expression of ERAP2 (15). In addition, *ERAP2* gene rs17408150 leads to substitution of a T with an A at codon 669 (p.Leu669Gln) that alters the leucine residue to glutamine, resulting in a significant effect on the ERAP2 enzyme function (17).

Association of *ERAP1* SNPs has already been reported with AS susceptibility in Iranian patients during our previous works (18-21). Furthermore, we recently indicated the association *ERAP2* gene SNPs AS susceptibility in the HLA-B27 positive subjects (22). With respect to the involvement of genetic variations in the alteration of ERAP2 enzyme, it seems that evaluation of such SNPs worth studying. Hence, this study aims to determine the association *ERAP2* gene rs2548538, rs2287988, and rs17408150 SNPs, for the first time to the best of our knowledge, in an Iranian AS population. Furthermore, the possible role of these SNPs was investigated in the controlling of inflammatory and immunomodulatory mediators in the AS disease.

## Methods

### *Study participants*

In this investigation, 250 individuals with AS disease and 250 persons as healthy controls were included (Table 1). The 1984 modified New York Criteria was exerted to diagnose AS disease (23). Healthy controls had no background diseases as well as history of AS or other autoimmune diseases neither in them nor family members and were matched for age and gender with the case group. AS patients were selected from the individuals recruited to Shahid Rajaee and Emam Reza Hospitals affiliated with Alborz and Tabriz University of Medical Sciences, Iran and outpatient Rheumatology Clinics of the Tabriz University of Medical Sciences during 2015 to 2020. The approvement of the study protocol was received from the local Ethical Review committee in Alborz University of Medical Sciences (Permission No. IR.ABZUMS.REC.1399.051). Prior to sampling, the written informed consent forms were obtained from all AS patients and healthy individuals. All study subjects were assessed for the HLA-B27 positivity and the clinical condition of the patients were determined by Bath AS Disease Activity Index (BASDAI), Bath AS Functional Index (BASFI), Bath AS Metrology Index (BASMI), Bath AS Global Score (BASG), and AS quality of life (ASQoL). Using venipuncture, about 10 ml of venous blood was taken from all participants.

### *ERAP2 SNPs genotyping*

To obtain DNA from venous blood samples, the QIAamp DNA Mini Kit (Qiagen, Germany) was used based on the company's instructions. Afterwards, in order to genotype the study participants for *ERAP2* gene rs2548538, rs2287988, and rs17408150 SNPs, Real-time was exerted using TaqMan assays (Applied Biosystems, Foster City, USA) and StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, USA). To perform Real-time genotyping, the PCR reactions mixture was comprised of 5 µl TaqMan Master Mix (containing Taq DNA polymerase and dNTPs; Applied Biosystems, Foster City, USA), 0.5 µl TaqMan Genotyping Assay mix containing primers and probes (Applied Biosystems, Foster City, USA), 2 µl of genomic DNA (20 ng/ µl), and distilled H<sub>2</sub>O (final volume of 15 µl in each tube). The thermocycling settings of the PCR amplification were initial heating for 60°C for 45 seconds followed by 95°C for 10 mins, then 40 amplification cycles in 95°C for 15 seconds and 60°C for 60 seconds, and finally 60°C for 30 seconds.

### ***PBMC separation, RNA isolation, and cDNA synthesis***

PBMCs were isolated from the peripheral blood using Ficoll/Hyphaque (Lymphodex, inno-Train, Kronberg, Germany) density-gradient centrifugation. Extraction of RNA from PBMCs was carried out exerting the Trizol total RNA extraction kit (GeneAll, Korea) based on the producer's guidelines. Synthesizing of the complementary DNA (cDNA) from the extracted RNA samples was conducted by the BioFact™ RT Series cDNA Synthesis Kit (Daejeon, Korea), conforming the company's protocols.

### ***Quantitative Real-time PCR***

In order to assess the mRNA expression levels of inflammatory and immunomodulatory cytokines, including IL-17A, IL-23, IL-10, and transforming growth factor (TGF)-β, quantitative Real-time PCR was exerted using the Rotor-Gene Q Real-time PCR System machine (Qiagen, USA) by the SYBR Green PCR Master Mix. The characteristics of the primers employed for Real-time mRNA transcript quantification are listed in table 2. We randomly selected 80 AS patients and 80 control subjects for mRNA expression analysis. For quantitative Real-time, each reaction mixture contained SYBR Green Master Mix 12.5 µl, cDNA 4.5 µl, forward and reverse primer 1 µl each, and H<sub>2</sub>O 6 µl to reach a final volume of 25 µl. The thermocycling PCR conditions were: 50 °C for 2 minutes, 95 °C for 10 minutes, then 40 cycles of 95 °C for 30 seconds, 60 °C 30 seconds, and 72°C for 30 seconds. To compute the relative mRNA transcript of target genes, the comparative C<sub>T</sub> method, as explained by Schmittgen and Livak (24), was used. The relative expression levels of target genes were determined through normalization in accordance to the mRNA level of the corresponding housekeeping gene (β-Actin). The mathematical formula to compute the relative mRNA expression in each subject was 2<sup>-DDCt</sup>.

### ***Serum concentration of cytokines***

Like quantitative mRNA expression analyses, serum samples from the venous blood of the same subjects were used to determine the concentration of the cytokines. Hence, the enzyme linked immunosorbent assay (ELISA) technique was applied to determine the serum concentrations of IL-23, IL-17A, IL-10, and TGF-β in 80 patients and 80 controls. The optical density was determined using commercial kit

(Invitrogen, Thermo Fisher Scientific, San Diego, CA, USA) and determination of the optical density (OD) in each well by an ELISA reader (Tecan Spectra, Austria).

### **Statistical analysis**

The baseline features of the patient and control groups were determined by descriptive statistical analysis. Determination of the associations between the *ERAP2* gene SNPs and risk of AS was analyzed by Pearson's chi square ( $\chi^2$ ) as well as Logistic Regression, and the association level was determined through measuring the odds ratios (OR) and corresponding 95% confidence intervals (CI). The LD for SNP pairs, the haplotypic analysis, and examination of the genotype distribution of SNPs in the control group to meet the Hardy–Weinberg Equilibrium (HWE) were carried out using the SHEsis online tool (25). Adjusting of the *P* values was carried out by Benjamini-Hochberg Method. Testing for the normal distribution of scale data (mRNA expression, serum concentration, and clinical indexes) was accomplished by the Kolmogorov-Smirnov test. To approximate the significance of difference in the mRNA expression and serum concentration of the cytokines between study groups, the mean comparisons were done by the non-parametric Mann-Whitney *U*-test. Furthermore, the Kruskal-Wallis test was used to perform mean comparison of data among patients with three genotypes for SNPs. Data presentation were done through average  $\pm$  standard deviation (SD) for scale data or by percentage for nominal data. Data analysis was accomplished by SPSS software v.25.0 (IBM SPSS Inc., USA) and graphing of data by bar charts was conducted using GraphPad PRISM software (version 8.00; GraphPad Software, Inc., San Diego, CA, USA).

## **Results**

### **Baseline features of the participants**

The baseline and laboratory indexes of the study population are listed with detail in the Table 1. The male/female distribution of the study subjects in the AS and control groups was 53 (21.2%)/197 (78.8%) and 45 (18%)/205 (82%), respectively. The age of the AS and control groups was  $38.50 \pm 8.80$  and  $37.62 \pm 7.40$ , respectively. The matching of two study groups for age ( $P > 0.05$ ) and sex ( $P > 0.05$ ) was observed. HLA-B27 was observed to be positive in 202 (80.8%) AS patients and 18 (7.2%) healthy controls ( $P < 0.0001$ ). Moreover, the C-reactive protein (CRP) level in the case group ( $3.14 \pm 2.27$ ) was significantly higher ( $P < 0.0001$ ) relative to the control group ( $1.27 \pm 0.95$ ).

### **Association test of *ERAP2* polymorphisms**

The frequency distribution pattern of the genotypes for *ERAP2* gene rs2548538 ( $P=0.830$ ), rs2287988 ( $P=0.443$ ), and rs17408150 ( $P=0.165$ ) SNPs in the healthy control group adhered to the HWE. In the total population analysis, results revealed that all three SNPs did not associate significantly with risk of AS (Table 3). That notwithstanding, when the HLA-B27 positive AS patients and the control group were compared, rs2287988 and rs17408150 SNPs showed statistically significant association with AS risk (Table 4).

For rs2287988, there was significant association in the minor G allele and GG genotype. The G allele was represented in 45.7% of the HLA-B27 positive AS patients and in 38.4% of the healthy controls (OR= 1.35, 95%CI= 1.03-1.76,  $P$ = 0.023). The GG genotype was frequently presented in the HLA-B27 positive AS patients relative to controls (20.3% vs. 13.6%), and the difference was statistically significant (OR= 1.91, 95%CI= 1.09-3.35,  $P$ = 0.022).

In case of rs17408150, it was observed that the C allele had significant association with higher risk of AS in the HLA-B27 positive patients (OR= 1.39, 95%CI=1.06-1.81,  $P$ = 0.013). The AA genotype was represented in the HLA-B27 positive AS group (21.8%) higher than the controls (17.2%); hence the AA genotype was associated significantly with increased AS risk (OR= 2.13, 95%CI=1.19-3.82,  $P$ = 0.010). The heterozygote AT genotype had statistically significantly difference in the distribution between HLA-B27 positive AS cases versus control group (OR= 1.91, 95%CI=1.19-3.06,  $P$ = 0.007). It was observed that the dominant model of AA+AT vs. TT for rs17408150 SNP had significant association with the higher AS risk (OR= 1.96, 95%CI=1.24-3.10,  $P$ = 0.003) in the AS group with positive HLA-B27.

### ***Frequency of the haplotype***

Regarding the haplotypic analysis (rs2548538 A/T, rs2287988 A/G, and rs17408150 A/T), it was detected that four haplotypes had significant association with AS risk. While AAA (OR= 2.13, 95%CI= 1.25-4.31,  $P$ = 0.014) and AAT (OR= 2.85, 95%CI= 1.50-05.40,  $P$ = 0.0083) haplotypes were associated with increased AS risk, the AGA (OR= 0.86, 95%CI= 0.47-0.82,  $P$ = 0.005) and TAT (OR= 0.57, 95%CI= 0.31-0.75,  $P$ = 0.004) haplotypes were associated with decreased risk of AS (Table 5).

### ***Linkage disequilibrium test***

The structure of SNP pairs in the LD block according to the SNP sequence of rs2548538, rs2287988, and rs17408150 are shown in the figure 1. The  $D\Delta$  value between rs2548538 and rs2287988 SNPs was 55% and between rs2548538 and rs17408150 SNPs was 81%. Nonetheless, a partially stronger linkage was detected when only the HLA-B27 positive patients were included in the analysis ( $D\Delta$ = 56% and 87%, respectively, for rs2548538-rs2287988 and rs2548538-rs17408150). However, the  $r^2$  values indicated no remarkable LD between SNP pairs.

### ***mRNA expression of cytokines***

The mRNA transcription level of IL-17A (fold change [FC]= 2.85,  $P$ =0.0001, Figure 2.a) and IL-23 (FC=1.63,  $P$ =0.0084, Figure 2.b) had upregulated levels in the PBMCs obtained from AS patients in relation to healthy subjects. However, there was significant underexpression of the mRNA of IL-10 (FC=0.43,  $P$ =0.0024, Figure 2.c) in the PBMCs obtained from AS patients relative to the healthy subjects. The mRNA expression of TGF- $\beta$  was lower non-significantly in the case group relative to the control group (FC= 0.89,  $P$ > 0.05, Figure 2.d). The mRNA expression of all four cytokines had no significant difference among AS patients with three genotypes for rs2287988 and rs17408150 SNPs (Figure 2 and Supplementary Table 1).

Transcript levels of IL-17A ( $FC=3.00$ ,  $P=0.0001$ , Figure 3.a) and IL-23 ( $FC=1.70$ ,  $P=0.0084$ , Figure 3.b) had upregulated levels in the PBMCs obtained from AS patients positive for HLA-B27 gene relative to the healthy subjects. Conversely, the transcript level of IL-10 ( $FC=0.41$ ,  $P=0.001$ , Figure 3.c) was significantly lower in the PBMCs obtained from AS patients positive for HLA-B27 relative to healthy subjects. The mRNA expression of TGF- $\beta$  had no significant difference between HLA-B27 positive AS patients and the normal controls ( $FC=0.82$ ,  $P>0.05$ , Figure 3.d). The mRNA expression of all cytokines had no significant difference among the AS patients positive for HLA-B27 carrying three different genotypes for rs2287988 and rs17408150 SNPs (Figure 3 and Supplementary Table 1).

### ***Serum concentration of cytokines***

The serum concentrations of IL-17A ( $46.50\pm13.10$  vs.  $19.56\pm6.48$ ,  $P=0.0001$ , Figure 4.a) and IL-23 ( $388.9\pm38.78$  vs.  $200.2\pm24.88$ ,  $P=0.0001$ , Figure 4.b) were increased in the AS group relative to normal control group. However, the serum concentration of IL-10 was significantly lower in the AS group compared with the control group ( $1.89\pm0.18$  vs.  $3.68\pm1.10$ ,  $P=0.0015$ , Figure 4.c). However, the difference in the serum concentration of TGF- $\beta$  was not statistically significant between AS group and the control group ( $20.4\pm4.51$  vs.  $23.4\pm4.21$ ,  $P>0.05$ , Figure 4.d). The serum concentration of all cytokines had no significant difference among the AS cases positive for HLA-B27 harboring three different genotypes for rs2287988 and rs17408150 SNPs (Figure 4 and Supplementary Table 2).

The serum concentrations of IL-17A ( $49.11\pm14.21$  vs.  $19.56\pm6.48$ ,  $P=0.0001$ , Figure 5.a) and IL-23 ( $395.4\pm39.7$  vs.  $200.2\pm24.88$ ,  $P=0.0001$ , Figure 5.b) were higher in the AS patients positive for HLA-B27 relative to normal controls. In contrast, the serum concentration of IL-10 was significantly lower in the HLA-B27 positive AS group compared with the control group ( $1.75\pm0.14$  vs.  $3.68\pm1.10$ ,  $P=0.0017$ , Figure 5.c). However, the difference in the serum concentration of TGF- $\beta$  was not statistically significant between HLA-B27 positive AS cases and the control group ( $19.7\pm4.47$  vs.  $23.4\pm4.21$ ,  $P>0.05$ , Figure 5.d). The serum concentration of all cytokines had no significant difference among the AS cases positive for HLA-B27 carrying three different genotypes for rs2287988 and rs17408150 SNPs (Figure 5 and Supplementary Table 2).

### ***Association of the genotypes and clinical manifestations***

The *ERAP2* gene rs2548538, rs2287988, and rs17408150 polymorphisms were investigated in association with clinical features of AS patients. It was observed that none of the characteristics of the AS patients, including CRP, disease duration, BASDAI, BASFI, BASG, and ASQoL scores had significant association with *ERAP2* gene polymorphisms (Table 6). Moreover, analysis of the association of the *ERAP2* gene rs2548538, rs2287988, and rs17408150 SNPs with the mentioned clinical manifestations in the HLA-B27 positive AS patients, resulting in no significant association.

## **Discussion**

To date, large scale analyses have found over 60 genetic loci for AS risk, even though a bulk of investigations have assigned a large proportion of genetic-related risk of AS to the HLA-B27 gene (3, 26). There is paucity of investigations on the SNPs of the *ERAP2* gene in the context of AS risk. Herein, the second survey by our group is trying to investigate the associations of *ERAP2* gene polymorphisms with the risk of AS in an Iranian population. Additionally, the possible alteration of inflammatory and immunomodulatory cytokines by the *ERAP2* gene rs2548538, rs2287988, and rs17408150 polymorphisms was investigated.

*ERAP2* gene association with AS was detected after identification of *ERAP1* gene association with AS (12). Both *ERAP1* and *ERAP2* genes are found on the chromosome 5, which are structured in an inverse direction and share a common intergenic sequence (27). Studies have found a haplotype in association with AS that are constituted with both *ERAP1* and *ERAP2* polymorphisms (28, 29). On the other hand, ERPA2 polymorphisms generate two main haplotypes, namely haplotype A and B; the haplotype A results in protein expression ERAP2, while the haplotype B cause no protein expression of ERAP2. The prevalence of both haplotypes A and B have been reported to be similar (approximately 50%), and therefore about 25% of the individuals are haplotype B homozygous and have no expression of ERAP2 (15). Lack of ERAP2 expression in haplotype B is because of the *ERAP2* gene rs2248374 polymorphism that influences on the RNA stability (15). It has been reported that there is a strong LD between haplotypes A and B of *ERAP2* SNPs, which can be involved in the immune evasion of trophoblasts (30). Lack of ERAP2 expression has been proposed to be protective in AS and other inflammatory disorders (31). Currently, there are little data with respect to the association of *ERAP2* gene rs2548538, rs2287988, and rs17408150 polymorphisms in AS disease, and our analyses revealed no association of these SNPs with AS risk. However, we noticed that, while AAA (OR= 2.13) and AAT (OR= 2.85) haplotypes were associated with higher AS risk, the AGA (OR= 0.86) and TAT (OR= 0.57,) haplotypes were associated with lower risk of AS. In addition, there was a LD between rs2548538 and rs2287988 SNPs and between rs2548538 and rs17408150 SNPs. Interestingly, a partially stronger linkage was detected when only the HLA-B27 positive AS group was included in the analysis.

According to studies, the association of ERAP2 with AS seems to be independent of HLA-B27 (14, 32). ERAP2 has been reported to impress directly the B\*2705 peptidome, removing a number of ligands containing N-terminal basic residues, leading to promoted levels of nonamers by the increased activity of ERAP1 (33). Based on the level of ERAP1 trimming activity, the influences of ERAP2 on B27 peptidome could be altered (34). A study indicated that *ERAP2* gene rs2248374 SNP was particularly associated with Psoriatic arthritis risk in the subjects negative for HLA-B27 (35). We observed that *ERAP2* gene rs2287988 and rs17408150 polymorphisms were associated with increased risk of the disease in the AS patients positive for HLA-B27. In addition, we previously reported the *ERAP2* gene rs2910686 SNP association with AS risk in patients positive for the HLA-B27 (22). Further evidence is required to conclusively determine the *ERAP2* involvement in collaboration with HLA-B27 in AS pathogenesis.

The overall effect of ERAP2 on the conformation of HLA-B27 have not fully been determined. A study reported that ERAP2 expression did not significantly impress the expression of the folded as well as

unfolded HLA-B27 molecules, markers of ER stress, and the expression of proinflammatory cytokines (36). On the other side, a study demonstrated that lack of ERAP2 triggered upregulation of free heavy chain (FHC)-B27 molecule and promotion of the unfolded protein response (UPR) pathway in patients (37). The FHC-B27 molecules has been reported to stimulate UPR in the antigen presenting cells (APCs) like macrophages, which may result in increased generation and release of IL-23 (38). APCs presenting FHC-B27 molecules can promote the differentiation and expansion of T helper (Th) 17 cells. Cells presenting FHC-B27 molecules can trigger the release of IL-17A from Th17 cells. IL-23 produced by APCs may bind to the IL-23 receptor (IL-23R) on the Th17 cells, resulting in further production of IL-17 (39). Our previous investigation indicated that the transcriptional levels as well as serum concentrations of IL-17A, IL-23, tumor necrosis factor (TNF)- $\alpha$ , and interferon (IFN)- $\gamma$  were not different among AS subjects positive for HLA-B27 carrying different genotypes of *ERAP2* gene rs2910686 polymorphism (which had association with higher AS risk in the AS group positive for HLA-B27) (22). As such, the current study indicated that transcriptional levels as well as serum concentrations of IL-17A, IL-23, IL-10, and TGF- $\beta$  were not significantly different among AS subjects positive for HLA-B27 harboring different genotypes of the AS-associating rs2287988 and rs17408150 SNPs of *ERAP2* gene. In addition, no association was found between clinical manifestations of the AS patients and *ERAP2* gene polymorphisms. Taken together, it appears that although HLA-B27 affects on the association of *ERAP2* gene rs2287988 and rs17408150 SNPs with AS risk, these *ERAP2* polymorphisms might not be involved in the alteration of HLA-B27 and, hence, the inflammatory settings in the AS disease.

## Conclusion

All in all, here we, for the first time, evaluated the association between *ERAP2* gene rs2548538, rs2287988, and rs17408150 SNPs and the risk of AS in the Iranian patients. The results indicated that although *ERAP2* gene SNPs had no association with AS risk, but rs2287988 and rs17408150 SNPs had association with promoted AS risk in the subjects positive for HLA-B27. That notwithstanding, these SNPs were not associated with the transcriptional levels or serum concentrations of the inflammatory or immunomodulatory cytokines. Although we tried to evaluate the involvement of *ERAP2* polymorphisms in AS susceptibility in two studies, further evaluations of this gene, particularly in the haplotype analysis, need to be carried out to clarify the bona fide involvement of *ERAP2* SNPs in the etiology and pathogenesis of AS.

## Declarations

### *Ethics approval and consent to participate*

The approvement of the study protocol was received from the local Ethical Review committee in Alborz University of Medical Sciences (Permission No. IR.ABZUMS.REC.1399.051) and written informed consent forms was taken by all subjects.

### *Research involving human subjects and/or animals*

Research carried out here were in compliance with the Helsinki Declaration. The protocol of this study was approved by the Human Research Ethics Committee from the Alborz University of Medical Sciences, Karaj, Iran (Permission No. IR.ABZUMS.REC.1399.051). Written informed consent forms were obtained from patients and healthy controls before blood taking.

#### ***Consent to publish***

All authors read the manuscript and consent for its publication.

#### ***Availability of data and materials***

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions. All data generated or analyzed during this study are included in this published article.

#### ***Competing interests***

The authors declare that they have no conflict of interest to report.

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#### ***Authors' Contributions***

**Mehrdad Ebrazeh;** Performed the experiments, participated in manuscript preparation, and read the manuscript critically.

**Fatemeh Ezzatifar;** Performed the statistical analysis, participated in manuscript preparation, and read the manuscript critically.

**Shahram Torkamandi;** Contributed in performing the experiments, participated in manuscript preparation and read the manuscript critically.

**Fatemeh Sadat Mohammadi;** Introduced the patients, participated in manuscript preparation, and read the manuscript critically.

**Sevda Salimifard;** Concucted graphical illustrations, participated in manuscript preparation and read the manuscript critically.

**Maryam Hemmatzadeh;** Participated in manuscript preparation and read the manuscript critically.

**Saeed Aslani;** Participated in manuscript preparation and read the manuscript critically.

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**Arezoo Gowhari-Shabgah**; Participated in manuscript preparation and read the manuscript critically.

**Hamed Mohammadi**; Developed the main idea, take the financial support, participated in manuscript preparation and read the manuscript critically.

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## **Abbreviations**

SNPs; Single nucleotide polymorphisms, ERAP; Endoplasmic reticulum aminopeptidase, PBMCs; Peripheral blood mononuclear cells, IL; Interleukin, TGF; Transforming growth factor, HLA; human leukocyte antigen, AS; Ankylosing spondylitis, ER: Endoplasmic reticulum, MHC; Major histocompatibility complex, LD; Linkage disequilibrium, BASDAI; Bath AS Disease Activity Index, BASFI; Bath AS Functional Index, BASMI; Bath AS Metrology Index, BASG; Bath AS Global Score, ASQoL; AS quality of life, ELISA; Enzyme linked immunosorbent assay,  $\chi^2$ ; Chi square, OR; Odds ratios, CI; Confidence intervals, HWE; Hardy–Weinberg Equilibrium, SD; Standard deviation, FHC; Free heavy chain, UPR; Unfolded protein response, APCs; Antigen presenting cells, Th; T helper, IL-23R; IL-23 receptor, TNF; Tumor necrosis factor, IFN; Interferon

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## Tables

**Table 1**  
**Baseline characteristics and clinical manifestations of AS patients and healthy controls.**

Characteristic	AS Patients (n = 250)	Healthy controls (n = 250)	P value
Age (years)	38.50 ± 8.80	37.62 ± 7.40	> 0.05
Female/Male, n (%)	53 (21.2%)/197 (78.8%)	45 (18%)/205 (82%)	> 0.05
HLA-B27 positive, no (%)	202 (80.8%)	18 (7.2%)	< 0.0001
CRP (mg/L)	3.14 ± 2.27	1.27 ± 0.95	< 0.0001
Disease duration (years)	10.79 ± 7.88	-	-
BASDAI score	5.34 ± 3.57	-	-
BASFI score	3.45 ± 2.36	-	-
BASG score	4.45 ± 2.08	-	-
ASQoL score	7.74 ± 4.21	-	-

HLA; Human leukocyte antigen, CRP; C-reactive protein, BASDAI; Bath ankylosing spondylitis disease activity index, BASFI; Bath ankylosing spondylitis functional index, BASG; Bath ankylosing spondylitis global score, ASQoL; Ankylosing spondylitis quality of life

Table 2  
Sequence of the primers used for mRNA expression of cytokines.

Gene name		Sequence of nucleotides (5'-3')	Size (bp)
IL-23	Forward	AGTGGAAAGTGGGCAGAGATT C	115
	Reverse	CAGCAGCAACAGCAGCATTAC	
IL-17A	Forward	CTCTGTGATCTGGGAGGCAAAG	196
	Reverse	GACAATCGGGGTGACACAGG	
IL-10	Forward	AGCTGAGAACCAAGACCCAGAC	72
	Reverse	AAGAAATCGATGACAGCGCC	
TGF-β	Forward	GCAACAATT CCTGGCGATA CCT	222
	Reverse	TCCCCTCCACGGCTCAAC	
β-Actin	ACTB - Forward	GGTCCTCACTGCCTGTCC	140
	ACTB - Reverse	CTCGTCATACTCCTGCTTGCT	

Table 3

Allele and genotype frequencies of *ERAP2* gene rs2548538, rs2287988, and rs17408150 SNPs in AS patients and healthy controls and corresponding association analyses.

SNP*	Allele /Genotype	AS* (n = 250)	Control (n = 250)	OR* (95% CI*)	P	Adjusted P**
		N%	N%			
rs2548538	T vs. A	186 (37.2)	197 (39.4)	0.91 (0.70–1.17)	0.47	-
	A (Reference)	314 (62.8)	303 (60.6)	-	-	-
	TT vs. AA	32 (12.8)	38 (15.2)	0.79 (0.46–1.38)	0.42	0.81
	TA vs. AA	122 (48.8)	121 (48.4)	0.95 (0.65–1.39)	0.81	0.81
	TT vs. TA + AA	32 (12.8)	38 (15.2)	0.81 (0.49–1.35)	0.43	0.81
	TT + TA vs. AA	154 (61.6)	159 (63.6)	0.91 (0.63–1.31)	0.64	0.81
	AA (Reference)	96 (38.4)	91 (36.4)	-	-	-
HWE*			P = 0.830			
rs2287988	G vs. A	217 (43.4)	192 (38.4)	1.23 (0.95–1.58)	0.11	-
	A (Reference)	283 (56.6)	308 (61.6)	-	-	-
	GG vs. AA	47 (18.8)	34 (13.6)	1.58 (0.93–2.71)	0.66	0.66
	GA vs. AA	123 (49.2)	124 (49.6)	1.47 (0.77–1.68)	0.50	0.66
	GG vs. GA + AA	47 (18.8)	34 (13.6)	0.80 (0.90–2.37)	0.12	0.48
	GG + GA vs. AA	170 (68.0)	158 (63.2)	1.23 (0.85–1.79)	0.25	0.50
	AA (Reference)	80 (32.0)	92 (36.8)	-	-	-
HWE			P = 0.443			
rs17408150	A vs. T	242 (48.4)	220 (44.0)	1.98 (0.93–1.53)	0.16	-
	T (Reference)	258 (51.6)	280 (56.0)	-	-	-
	AA vs. TT	49 (19.6)	43 (17.2)	1.45 (0.85–2.49)	0.17	0.20
	AT vs. TT	144 (57.6)	134 (53.6)	1.37 (0.90–2.09)	0.14	0.20

\*SNP; single nucleotide polymorphism, AS; ankylosing spondylitis, OR; odds ratio, 95% CI; 95% confidence interval, HWE; Hardy-Weinberg equilibrium

\*\*FDR correction for multiple comparisons by Benjamini-Hochberg

SNP*	Allele /Genotype	AS* (n = 250)	Control (n = 250)	OR* (95% CI*)	P	Adjusted P**
		N%	N%			
	AA vs. AT + TT	49 (19.6)	43 (17.2)	1.31 (0.86–1.99)	0.20	0.20
	AA + AT vs. TT	193 (77.2)	177 (70.8)	1.39 (0.93–2.08)	0.10	0.20
	TT (Reference)	57 (22.8)	73 (29.2)	-	-	-
HWE		$P = 0.165$				

\*SNP; single nucleotide polymorphism, AS; ankylosing spondylitis, OR; odds ratio, 95% CI; 95% confidence interval, HWE; Hardy-Weinberg equilibrium

\*\*FDR correction for multiple comparisons by Benjamini-Hochberg

Table 4

Allele and genotype frequencies of *ERAP2* gene rs2548538, rs2287988, and rs17408150 SNPs in HLA-B27 positive AS patients and healthy controls.

SNP*	Allele /Genotype	HLA-B27 Positive AS* (n = 202)	Control (n = 250)	OR* (95% CI*)	P	Adjusted P**
		N%	N%			
rs2548538	T vs. A	167 (41.3)	197 (39.4)	1.08 (0.82– 1.41)	0.465-	
	A (Reference)	237 (58.7)	303 (60.6)	-	-	-
	TT vs. AA	27 (13.3)	38 (15.2)	1.04 (0.57– 1.88)	0.8770.87	
	TA vs. AA	113 (55.9)	121 (48.4)	1.37 (0.90– 2.07)	0.1340.40	
	TT vs. TA + AA	27 (13.3)	38 (15.2)	0.86 (0.50– 1.46)	0.5740.76	
	TT + TA vs. AA	140 (69.3)	159 (63.6)	1.29 (0.87– 1.91)	0.2010.40	
	AA (Reference)	62 (30.7)	91 (36.4)	-	-	-
HWE*			P = 0.830			
rs2287988	G vs. A	185 (45.7)	192 (38.4)	1.35 (1.03– 1.76)	0.023-	
	A (Reference)	219 (54.2)	308 (61.6)	-	-	-
	GG vs. AA	41 (20.3)	34 (13.6)	1.91 (1.09– 3.35)	0.0220.08	
	GA vs. AA	103 (50.9)	124 (49.6)	1.31 (0.86-2.00)	0.1910.19	
	GG vs. GA + AA	41 (20.3)	34 (13.6)	1.61 (0.98– 2.66)	0.0550.09	

\*SNP; single nucleotide polymorphism, HLA-B27; Human leukocyte antigen-B27, AS; ankylosing spondylitis, OR; odds ratio, 95% CI; 95% confidence interval, HWE; Hardy-Weinberg equilibrium

\*\*FDR correction for multiple comparisons by Benjamini-Hochberg

SNP*	Allele /Genotype	HLA-B27 Positive AS* (n = 202)	Control (n = 250)	OR* (95% CI*)	P	Adjusted P**
		N%	N%			
	GG + GA vs. AA	144 (71.3)	158 (63.2)	1.45 (0.97– 2.15)	0.071	0.09
	AA (Reference)	58 (28.7)	92 (36.8)	-	-	-
HWE			P = 0.443			
rs17408150A vs. T		211 (52.2)	220 (44.0)	1.39 (1.06– 1.81)	0.013	-
	T (Reference)	193 (47.3)	280 (56.0)	-	-	-
AA vs. TT		44 (21.8)	43 (17.2)	2.13 (1.19– 3.82)	0.010	0.01
AT vs. TT		123 (60.9)	134 (53.6)	1.91 (1.19– 3.06)	0.007	0.01
AA vs. AT + TT		44 (21.8)	43 (17.2)	1.34 (0.83– 2.14)	0.220	0.22
AA + AT vs. TT		167 (82.7)	177 (70.8)	1.96 (1.24– 3.10)	0.003	0.01
TT (Reference)		35 (17.3)	73 (29.2)	-	-	-
			P = 0.165			

\*SNP; single nucleotide polymorphism, HLA-B27; Human leukocyte antigen-B27, AS; ankylosing spondylitis, OR; odds ratio, 95% CI; 95% confidence interval, HWE; Hardy-Weinberg equilibrium

\*\*FDR correction for multiple comparisons by Benjamini-Hochberg

Table 5  
haplotype association of *ERAP2* gene rs2548538, rs2287988, and rs17408150 SNPs according to Haplovview.

RowBlock 1 Haplotypes				Frequencies		OR* (95% CI*)	P
	rs2548538	rs2287988	rs17408150	Haplotype Frequency <sup>¶</sup> (250 AS patients)	Haplotype Frequency (250 control subjects)		
				N (%)	N (%)		
1 A A A				74 (14.8)	65 (13.7)	2.13 (1.25– 4.31)	0.014\$
2 A A T				36 (7.2)	13 (2.7)	2.85 (1.50– 5.40)	0.0083
3 A G A				227 (45.4)	303 (60)	0.86 (0.47– 0.82)	0.005
4 A G T				59 (311.9)	78 (15.7)	0.73 (0.51– 1.05)	0.433
5 T A T				39 (7.8)	77 (15.4)	0.57 (0.31– 0.75)	0.004
6 T G T				49 (9.9)	41 (9.1)	1.07 (0.77– 2.88)	0.517

¶ Frequencies less than 0.03 were excluded  
 \*OR; odds ratio, 95% CI; 95% confidence interval  
 \$ Values in bold imply to significant P

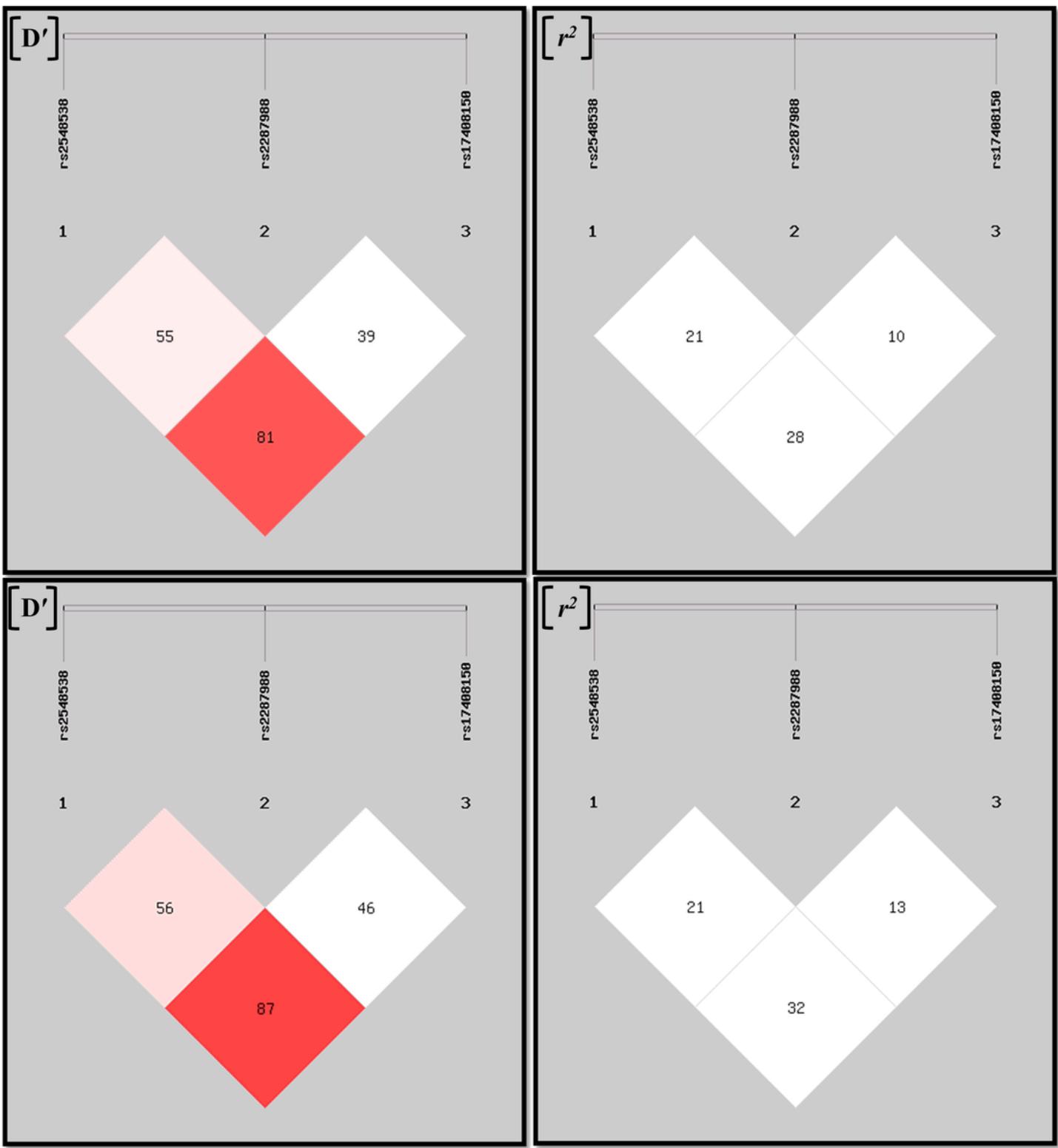
Table 6

Association of *ERAP2* gene rs2548538, rs2287988, and rs17408150 SNPs with clinical manifestations of AS patients (n = 250).

Characteristic	rs2548538 (TT)	rs2548538 (TA)	rs2548538 (AA)	P value
Disease duration (years)	10.37 ± 2.91	10.71 ± 3.63	10.55 ± 3.05	0.388
CRP (mg/L)	3.25 ± 3.10	3.11 ± 3.41	3.44 ± 3.75	0.655
BASDAI score	5.21 ± 3.44	4.46 ± 2.88	4.46 ± 2.43	0.266
BASFI score	3.11 ± 2.49	3.56 ± 2.16	3.64 ± 2.15	0.385
BASG score	4.05 ± 3.11	5.28 ± 2.43	4.55 ± 2.76	0.455
ASQoL score	6.89 ± 3.28	7.80 ± 4.15	7.86 ± 3.54	0.390
	rs2287988 (GG)	rs2287988 (GA)	rs2287988 (AA)	P value
Disease duration (years)	9.88 ± 4.14	10.74 ± 3.75	10.22 ± 4.69	0.256
CRP (mg/L)	3.15 ± 2.57	3.62 ± 2.41	3.11 ± 2.50	0.399
BASDAI score	4.66 ± 2.19	4.11 ± 2.45	5.74 ± 2.48	0.313
BASFI score	3.85 ± 2.08	3.18 ± 2.39	3.44 ± 2.76	0.746
BASG score	4.57 ± 2.30	4.61 ± 2.20	5.18 ± 2.47	0.287
ASQoL score	7.18 ± 3.65	6.98 ± 3.27	7.55 ± 5.40	0.818
	rs17408150 (AA)	rs17408150 (AT)	rs17408150 (TT)	P value
Disease duration (years)	10.24 ± 3.79	9.70 ± 3.87	10.47 ± 4.08	0.213
CRP (mg/L)	3.28 ± 2.23	3.07 ± 2.63	4.54 ± 2.75	0.711
BASDAI score	5.41 ± 2.16	4.12 ± 2.44	4.85 ± 2.11	0.570
BASFI score	3.25 ± 2.61	2.82 ± 2.85	3.35 ± 2.84	0.597
BASG score	4.11 ± 2.38	5.14 ± 2.42	5.71 ± 2.38	0.423
ASQoL score	7.55 ± 4.82	7.35 ± 5.61	6.47 ± 2.63	0.646

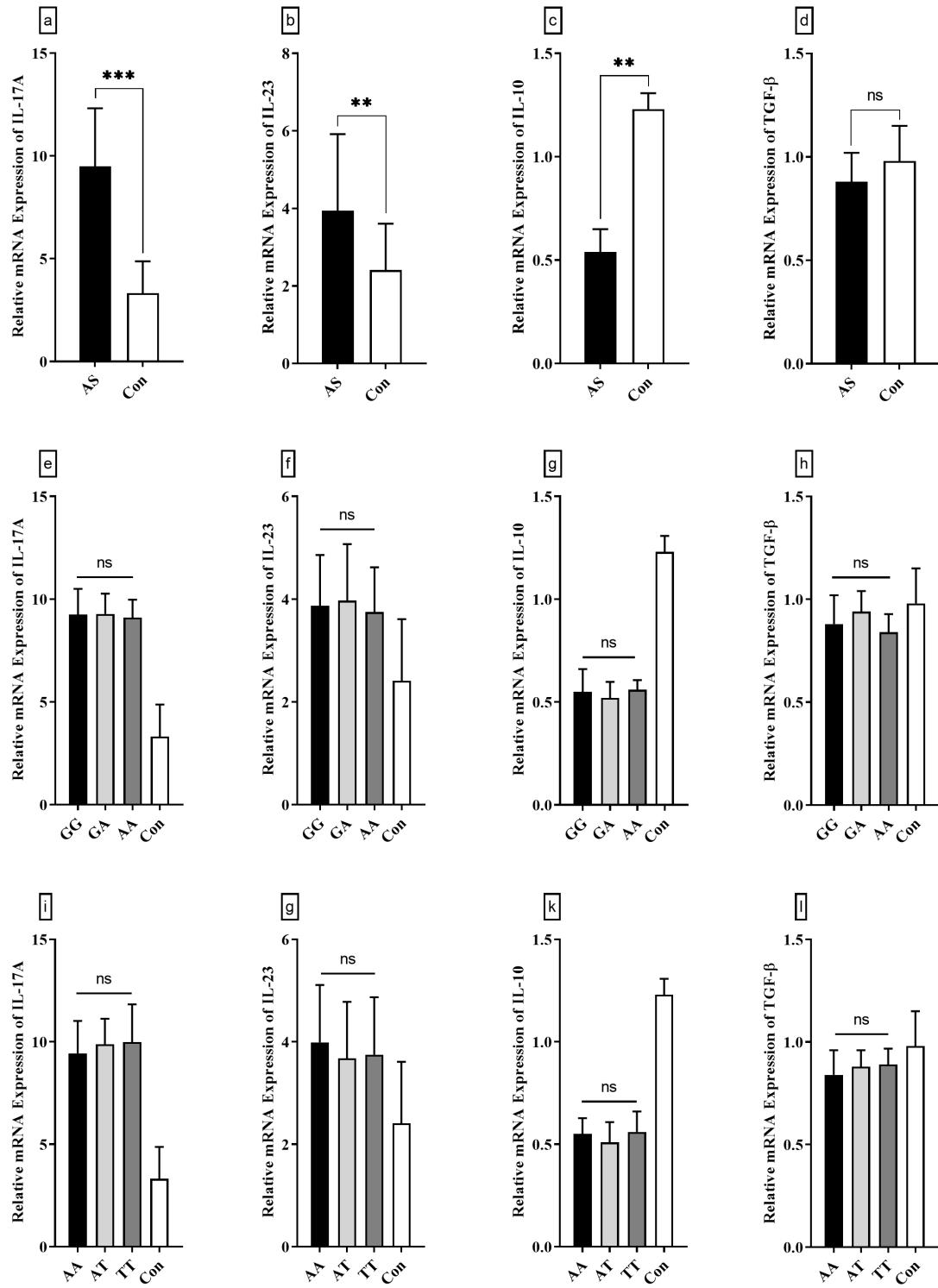
CRP; C-reactive protein, BASDAI; bath ankylosing spondylitis disease activity index, BASFI; bath ankylosing spondylitis functional index, BASG; bath ankylosing spondylitis global score, ASQoL; ankylosing spondylitis quality of life

## Figures



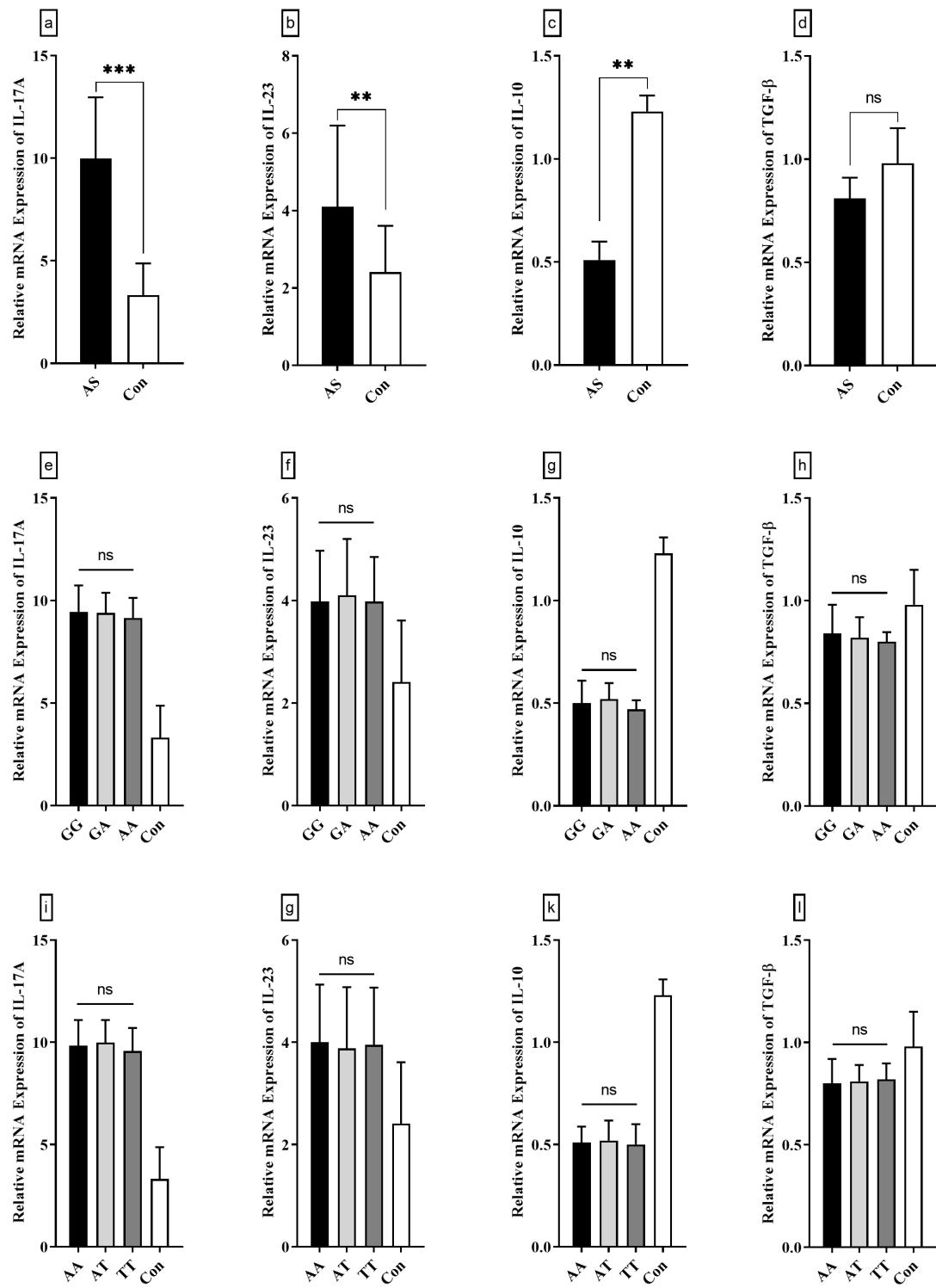
**Figure 1**

Bar charts show the relative mRNA amounts of IL-17A, IL-23, IL-10, and TGF- $\beta$  in the study groups (a, b, c, d). The comparison of the relative mRNA levels of cytokines in the AS patients with three genotypes for rs2287988 SNP (e, f, g, h). Relative transcription level of the cytokines in the AS patients harboring three different genotypes for rs17408150 SNP (l, j, k, l). The mean comparisons were conducted by statistical tests of Mann-Whitney's U or Kruskal-Wallis (\*\* shows  $P<0.01$ , \*\*\* shows  $P<0.001$ ).



**Figure 2**

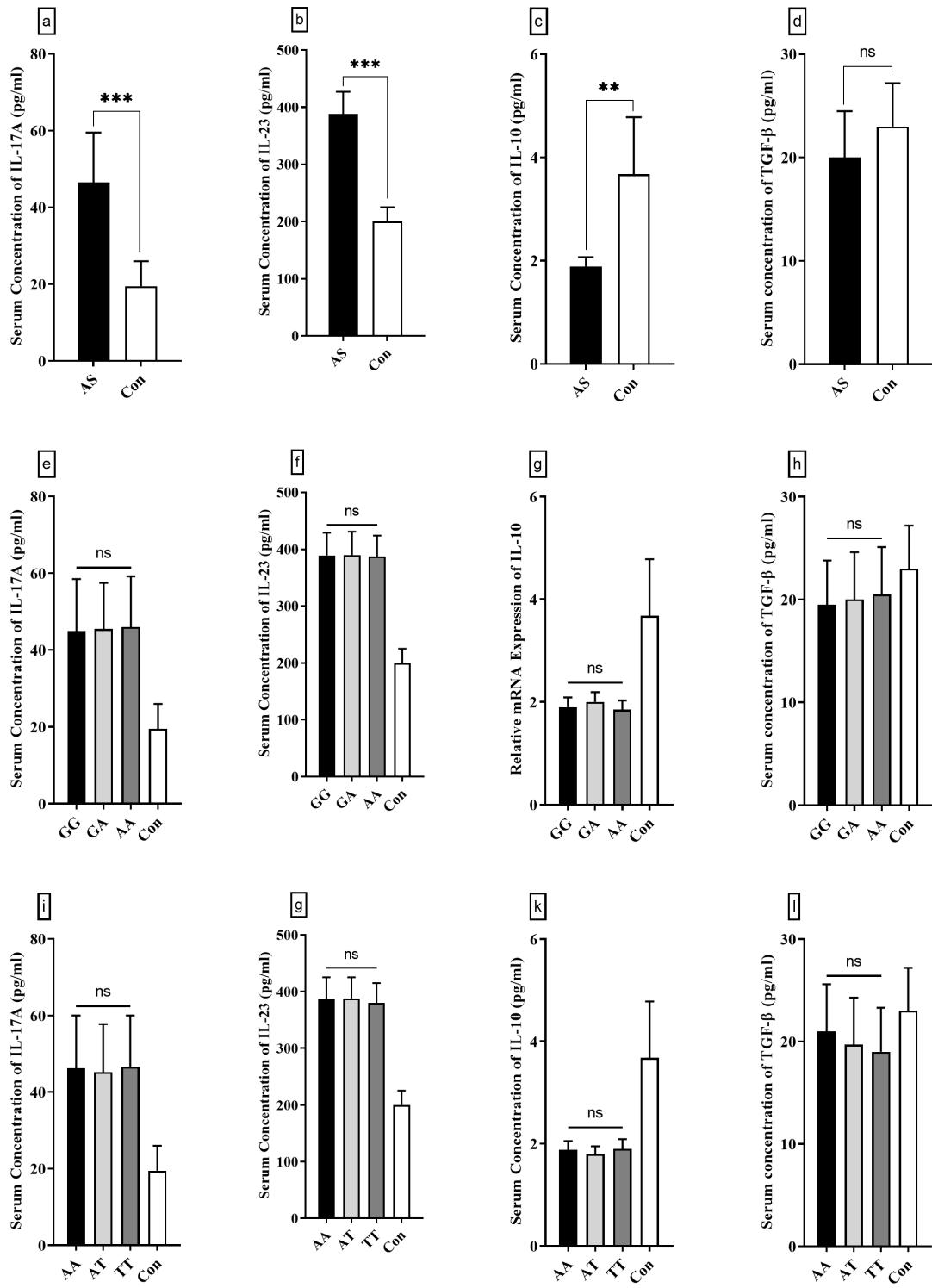
Bar charts show the relative mRNA amounts of IL-17A, IL-23, IL-10, and TGF- $\beta$  in the study groups (a, b, c, d). The comparison of the relative mRNA levels of cytokines in the AS patients with three genotypes for rs2287988 SNP (e, f, g, h). Relative transcription level of the cytokines in the AS patients harboring three different genotypes for rs17408150 SNP (i, j, k, l). The mean comparisons were conducted by statistical tests of Mann-Whitney's U or Kruskal-Wallis (\*\* shows P<0.01, \*\*\* shows P<0.001).



**Figure 3**

Bar charts show the relative transcriptional amounts of IL-17A, IL-23, IL-10, and TGF- $\beta$  mRNAs in the AS patients positive for HLA-B27 and healthy controls (a, b, c, d). The comparison of the relative mRNA amounts of cytokines in the AS patients positive for HLA-B27 harboring three different for rs2287988 SNP (e, f, g, h). Relative transcription level of the cytokines in the AS patients positive for HLA-B27 harboring three different genotypes for rs17408150 SNP (i, j, k, l). The mean comparisons were carried

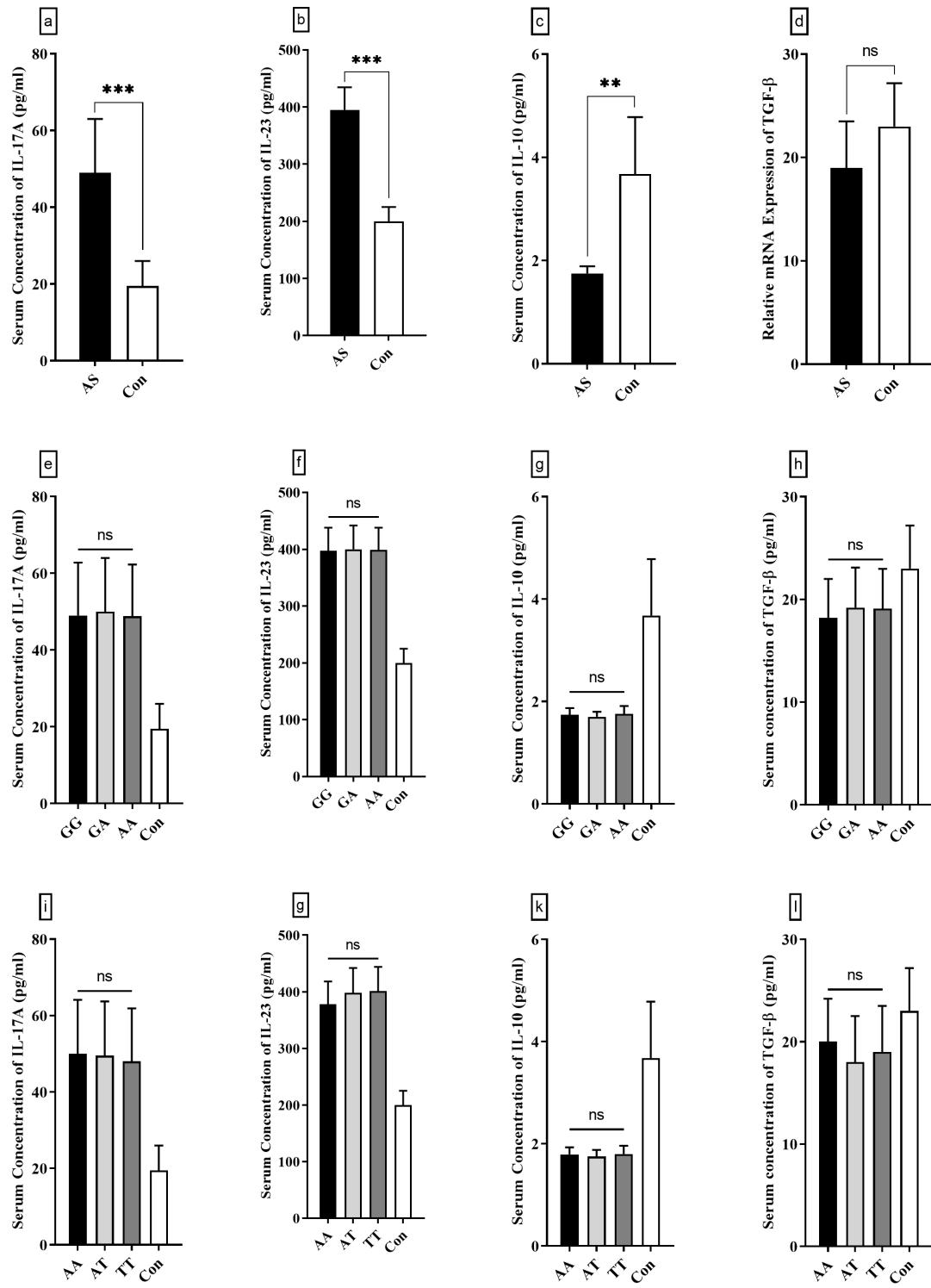
out through the statistical tests of Mann-Whitney's U or Kruskal-Wallis (\*\* shows P<0.01, \*\*\* shows P<0.001).



**Figure 4**

Bar charts depict the serum levels of IL-17A, IL-23, IL-10, and TGF- $\beta$  in the AS patients and normal controls (a, b, c, d). The comparison of the serum levels of cytokines in the AS patients harboring three different genotypes for rs2287988 SNP (e, f, g, h). Serum concentrations the cytokines in the AS patients

with three genotypes for rs17408150 SNP (l, j, k, l). The mean comparisons were done by statistical tests of Mann-Whitney's U or Kruskal-Wallis (\*\* shows P<0.01, \*\*\* shows P<0.001).



**Figure 5**

Bar graphs show the levels of IL-17A, IL-23, IL-10, and TGF- $\beta$  in the serum of the AS patients positive for HLA-B27 and normal controls (a, b, c, d). The comparison of the serum levels of cytokines in the AS patients positive for HLA-B27 harboring three different genotypes for rs2287988 SNP (e, f, g, h). Serum

concentrations of the cytokines in the AS patients positive for HLA-B27harboring three different genotypes for rs17408150 SNP (I, j, k, l). The mean comparisons were done using the statistical tests of Mann-Whitney's U or Kruskal-Wallis (\*\* shows P<0.01, \*\*\* shows P<0.001).

## Supplementary Files

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- [SupplementaryTable.docx](#)