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ORIGINAL ARTICLE

Aryl hydrocarbon receptor gene expression in ankylosing spondylitis and its correlation with interleukin-17, RAR-related orphan receptor gamma t expression, and disease activity indices

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ABSTRACT

Objectives: Considering the role of T helper (Th)17 cells in the pathogenesis of ankylosing spondylitis (AS), the aim of this study was to determine the correlation between aryl hydrocarbon receptor (AHR) gene expression and the expression of Th17-related genes including interleukin (IL)-17 and RAR-related orphan receptor gamma t (RORγt) transcription factor.

Patients and methods: Thirty patients with AS (26 males, 4 females; mean age: 36.1±8.1 years) and 30 age- and sex-matched healthy individuals (26 males, 4 females; mean age: 36.2±14.6 years) were recruited for the case-control study between June 2021 and January 2022. Ribonucleic acid (RNA) was extracted from peripheral blood cells and expression levels of AHR, IL-17, RORγt, and AHR repressor (AHRR) genes were evaluated using real-time polymerase chain reaction technique. The serum level of IL-17 was evaluated with enzyme-linked immunosorbent assay.

Results: The results showed a nonsignificant elevation of AHR, IL-17, and RORγt gene expression in the patient group compared to the control. There was a direct correlation between AHR gene expression and IL-17 and RORγt genes and a negative correlation between AHR and AHRR expression. Moreover, AHR gene expression showed a weak correlation with disease activity indices, including Bath Ankylosing Spondylitis Disease Activity Index, Bath Ankylosing Spondylitis Functional Index, Bath Ankylosing Spondylitis Metrology Index, Bath Ankylosing Spondylitis Global Score, and Ankylosing Spondylitis Quality of Life. Moreover, the serum level of IL-17 was higher in AS patients compared to the healthy group (p=0.02).

Conclusion: Upregulated expression of the AHR gene in ankylosing spondylitis and its correlation with IL-17 and ROR-γ t gene expression suggests that it could be a potential diagnostic and therapeutic target for AS.

Keywords: Ankylosing spondylitis, aryl hydrocarbon receptor, interlukin-17, T helper 17.

Ankylosing spondylitis (AS) is a chronic inflammatory disorder of the axial skeleton causing inflammatory back pain and progressive stiffness of the spine. AS is a prototype of immune-mediated inflammatory rheumatic disorders and the most prevalent member of spondyloarthropathies.[1,2] The disease is typically diagnosed in the third decade of life and is more frequently observed in males than in females. There are many geographical and racial

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variations in the prevalence of AS, probably due to the different spread of HLA (human leukocyte antigen)-B27 alleles, the most significant genetic risk factor contributing to AS. Despite significant advances in the diagnosis and treatment of AS, the role of genetic and environmental factors in disease pathogenesis has not yet been fully understood.[3]

Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that makes a link between environmental or intrinsic factors and genetic modifications.[4] Recent findings have demonstrated the expression of AHR in a variety of immune cells.[5] It appears that AHR regulates some immune events in response to certain environmental factors, boosting the development of many immune-related diseases ranging from inflammatory to neoplastic disorders.[6,7] AHR binds to various exogenous ligands, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), as well as endogenous ligands, for instance, tryptophan-derived metabolites, particularly 6-formylindolo [3,2-b] carbazole (FICZ) and kynurenine.[8,9] The AHR/ligand complex binds the regulatory regions of genes involved in inflammation, such as STAT (signal transducer and activator of transcription), nuclear factor kappa B, interleukin (IL)-22, and IL-17, and modulates their expression.[10,11]

Previous studies have shown that IL-17 and T helper (Th)17 cells play a considerable role in the pathogenesis of AS and other spondyloarthropathies.[12] Th17 cells express AHR at a high level, whereas naïve T cells, Th1 cells, and Th2 cells express none or negligible amounts. AHR expression is upregulated during the differentiation of the Th17 subset and seems to enhance the expression of IL-17A, IL-17F, and IL-22 genes. For instance, it has been shown that AHR facilitates the recruitment of RAR-related orphan receptor gamma t (RORγt) to the IL-22 gene promoter region.[13,14] In addition, the exposure of healthy people to TCDD during the Vietnam War resulted in an upregulated expression of AHR, IL-1β, tumor necrosis factor, and IL-6 genes, which enhanced Th17 cell differentiation and IL-17 production.[8] Recently, a study on 56 AS patients showed that semaphorin4D promoted the differentiation of Th17 cells by increasing the gene expression of RORγt, IL-17, and IL-22 in an AHR-dependent manner.[15]

To date, there are a few studies about AHR gene expression in AS patients; however, in the murine models of autoimmune disorders like rheumatoid arthritis (RA), it has been demonstrated that AHR knockdown in collagen-induced arthritis (CIA) mice reduced serum concentration of the proinflammatory cytokines matrix metalloproteinase-3, IL-1β, and IL-6, resulting in decreased disease severity.[7] Furthermore, in AHR-/- CIA mice, serum levels of IL-6 and IL-1 were reduced, and the frequency of Th17 cells were significantly lower than control animals.[16] Moreover, some studies have shown higher expression of AHR in the synovial tissue of RA patients and inguinal lymph node cells of CIA mice.[8] Likewise, the percentage of AHR-positive cells in peripheral blood mononuclear cells, as well as AHR and CYP1A1 gene expression were higher in RA patients compared to the healthy controls.[17] It appears that upregulation of the AHR gene escalates inflammation and bone destruction in RA through the activation of fibroblast-like synovial cells, dendritic cells, macrophages, and osteoclasts and inhibition of osteoblasts.[18] Accordingly, cotreatment of fibroblast-like synovial cells with the AHR antagonist, GNF351, inhibited the recruitment of AHR to the promoter regions of IL-1β and IL-6 genes and reduced proinflammatory cytokine expression.[17,19] In addition, intranasal exposure to particulate matter samples enhanced Th17 cell differentiation through AHR activation and deteriorated an experimental encephalomyelitis model of mice.[20,21] A significant expansion of Th17 and Th22 cells and an impaired expression of transforming growth factor beta 1 in T cells have been reported in active systematic lupus erythematosus (SLE) patients, which was associated with excessive activation of the AHR pathway.[22,23] Moreover, the upregulated expression of AHR in SLE patients was correlated with disease activity scores. Of note, the disease course in the experimental model of SLE altered by modulating AHR activity.[24]

According to these and other findings suggesting a role for AHR in the differentiation of pathogenic Th17 cells and the implication of these cells in the development of rheumatoid diseases, the present study aimed to evaluate the expression level of AHR and Th17-related genes in AS patients and investigate their correlation.

Furthermore, the expression of the AHR repressor (AHRR) gene as a probable therapeutic target and the serum level of IL-17 as a potential marker of disease activity were assessed. Finally, to evaluate the possibility of applying these results in the determination of disease prognosis, the correlation between molecular findings and clinical data, including Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI), Bath Ankylosing Spondylitis Metrology Index (BASMI), Bath Ankylosing Spondylitis Global Score (BAS-G), and Ankylosing Spondylitis Quality of Life (ASQOL), were analyzed.

PATIENTS AND METHODS

Thirty AS patients (26 males, 4 females; mean age: 36.1±8.1 years) from a single center were enrolled in the case-control study. The study was conducted at Department of Rheumatology, Imam Khomeini Hospital, Tehran University of Medical Sciences between June 2021 and January 2022. Diagnosis of AS was based on the modified New York Criteria according to the patient's symptoms, clinical examination, laboratory results, and radiological findings. Patients with interfering comorbidities or disability and hospitalized patients were excluded from the study. The control group of 30 individuals (26 males, 4 females; mean age: 36.2±14.6 years) was selected from age-and sex-matched healthy volunteers without any sign or symptom of inflammatory disorders (e.g., autoimmune diseases, allergy, asthma, and acute or chronic infection) and with a negative family history of rheumatic diseases.

Ribonucleic acid extraction from peripheral blood mononuclear cells and complementary deoxyribonucleic acid synthesis

Blood samples were collected from patients and controls. Ribonucleic acid (RNA) was isolated using the High Pure RNA Isolation Kit (ROJE Technologies, Tehran, Iran) according to the manufacturer's instructions. RNA quality was assessed with a spectrophotometer (NanoDrop ND1000; Thermo Scientific, Waltham, MA, USA); samples were considered acceptable if the A260/A280 absorbance ratio was within the range of 1.8 to 2.2 and the A260/A230 ratio was 2 to 2.2. Reverse transcription of RNA to complementary deoxyribonucleic acid (cDNA) was performed with the Transcriptor First Strand cDNA Synthesis Kit (ROJE Technologies, Tehran, Iran). The cDNA samples with A260/A280 ratios of 1.7 to 2 were stored at a –70°C freezer until use.

Real-time polymerase chain reaction

For quantification of relative gene expression, SYBR-Green gene expression assay was performed using real-time polymerase chain reaction (RT-PCR). The internal control gene was glyceraldehyde 3-phosphate dehydrogenase. Forward and reverse primers of target genes are listed in Table 1. To perform the test, 10 µL of master mix (RealQ Plus Green; Ampliqon,

Table 1. Forward and reverse primers of target genes

Primer	Sequence	Company
AhR-F	5'-AGC AAG TTC ACA TGG AGG CA-3'	Metabion
AhR-R	5'-CGT GGC AGC ACC CTT TCT AT-3'	Metabion
RORγt-F	5'-TGG AAG TGG TGC TGG TTA GGA TG-3'	Metabion
RORγt-R	5'-GGA GTG GGA GAA GTC AAA GAT GGA-3'	Metabion
IL-17A-F	5'-CGG ACT GTG ATG GTC AAC CTG A-3'	Metabion
IL-17A-R	5'-GCA CTT TGC CTC CCA GAT CAC A-3'	Metabion
AhRR-F	5'-GCG CCT CAG TGT CAG TTA CC-3'	Metabion
AhRR-R	5'-GAA GCC CAGATA GTC CAC GAT-3'	Metabion
GAPDH-F	5'-GTC TCC TCT GAC TTC AAC AGC G-3'	Metabion
GAPDH-R	5'-ACC ACC CTG TTG CTG TAG CCA A-3'	Metabion

Odense, Denmark), 1 µL of assay mix, including forward and reverse primers, 7 µL of distilled water, and 2 µL of diluted sample cDNA (5 ng/µL) were added to the wells. The reaction cycles included 2 min at 50°C, 10 min at 95°C, 45 cycles of 15 secs at 95°C, and 60 secs at 60°C by StepOnePlus™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). All tests were performed in a duplicate form and analyzed by relative quantification. Two nontemplate controls were included in each run. The threshold cycle number was used to calculate the relative expression between samples. Afterward, the relative expression for each sample was calculated using the following equation: relative mRNA expression=$(2^{-\Delta Ct})\times 10^3$.

Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay was performed using Human interleukin 17 ELISA kit ZB-10142C-H9648; (ZellBio GmbH, Lonsee, Germany) according to the manufacturer's instruction. All samples were examined in duplicate form. Absorbance was read by the Hiperion MPR4 ++ Microplate Reader (Medizintechnik GmbH & Co. KG, Roedermark, Germany). The calibration curves were drawn to determine the serum concentration of IL-17.

Statistical analysis

Data were analyzed using IBM SPSS version 26.0 (IBM Corp., Armonk, NY, USA) Data were presented as mean±standard deviation or median and interquartile range (IQR). Quantitative variables were evaluated for normality with the Kolmogorov-Smirnov test. To compare the variables with normal distribution, the independent samples t-test was used. In the case of nonnormal distribution, the Mann-Whitney U test was applied. To evaluate the correlation between two variables, Spearman's correlation coefficient (in nonnormal distribution of one or both quantitative variables) and Pearson's correlation coefficient (in normal distribution of both quantitative variables) were used. A p-value <0.05 was considered statistically significant.

RESULTS

The demographic and clinical data of the participants are presented in Table 2. The relative expression of AHR gene in the AS patients' group was slightly higher than in the healthy control group; however, this difference was not significant (p=0.42). The median of gene expression in AS patients and healthy controls were 1.86 (IQR: 0.41-3.1) and 1.48 (IQR: 0.2-2.76), respectively (Figure 1).

The gene expression of AHRR in the patient group was reduced compared to the healthy controls, but it did not reach significance (p=0.24). Indeed, it was undetectable in 16 (53.3%) of the patients and 11 (36.6%) of the control group. The median of AHRR mRNA level in the healthy individuals was 0.035 (IQR: 0.0-5.8), while it was 0 (IQR: 0.0-1.25) in the patient group (Figure 2).

The relative gene expression of RORγt was greater in the patient group. The median of relative expression of the gene in the patient and control groups were 1.46 (IQR: 0.8-2.1) and 0.44 (IQR: 0.23-2.0), respectively (p=0.08). IL-17 gene expression of AS patients was also increased; however, the difference between groups was not statistically significant (p=0.23). The median IL-17 gene expression in the patient and control groups were 4.41 (IQR: 0.01-11.8) and 0.43 (IQR: 0.05-3.6), respectively (Figure 3).

A weak direct correlation was observed between AHR and IL-17 gene expression (correlation coefficient: 0.14; R: 2%). There was also a direct weak correlation between AHR and RORγt gene expression (correlation coefficient: 0.2; R: 4%), but neither of them were statistically significant (Figure 4).

There was a weak negative correlation between AHR and AHRR genes expression (correlation coefficient: 0.07; R: 0.5%); however, it did not reach a significant level (p=0.6) (Figure 5).

A weak direct correlation was observed between the AHR gene expression and BAS-G (correlation coefficient: 0.055), ASQOL (correlation coefficient: 0.2), BASMI (correlation coefficient: 0.08), BASFI (correlation coefficient: 0.2), and BASDAI (correlation coefficient: 0.2) indices; however, none were statistically significant.

The serum level of IL-17 in AS patients showed a significant elevation compared to the healthy individuals (77.2±42.8 *vs.* 36.4±27.8, p=0.01, Figure 6).

Table 2. Demographic and clinical data of study population

	AS patients (n=30)			Healthy controls (n=30)			
	n	%	Mean±SD	n	%	Mean±SD	*p*
Age (year)			36.1±8.1			36.2±14.6	NS
Sex							NS
Male	26	86.7		26	86.7		
Female	4	13.3		4	13.3		
Smoking							
Yes	9	30		5	16.7		NS
No	21	70		25	83.3		NS
Disease duration (year)			7.16±6.23			-	-
Positive							
HLA-B27	9	30		-	-		-
Negative	15	50		-	-		-
Unknown	6	20		-	-		-
Indices of disease activity							
BASDAI-Index			4.84±3.18			-	-
BASMI-Index			3.6±3.54			-	-
BASFI-Index			4.14±2.94			-	-
BAS-G-Index			5.46±3.49			-	-
ASQoL-Index			9.13±6.17			-	-
Family history							
Yes	6	20		-	-		-
No	24	80		-	-		-
Drugs							
NSAIDs	5	16.7		-	-		-
DMARDs	3	10		-	-		-
Anti-TNF-α	7	23.3		-	-		-
NSAIDs + DMARDs	4	13.3		-	-		-
NSAIDs + Anti-TNF-α	1	3.3		-	-		-
DMARDs+ Anti-TNF-α	3	10		-	-		-
NSAIDs + DMARDs + Anti-TNF-α	2	6.7		-	-		-
No drug (new patient)	5	16.7		-	-		-
Anti-TNF-α							
Yes	13	43.3		-	-		-
No	17	56.7		-	-		-

AS: Ankylosing spondylitis; SD: Standard deviation; HLA: Human leukocyte antigen; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; BASMI: Bath Ankylosing Spondylitis Metrology Index; BAS-G: Bath Ankylosing Spondylitis Global score; ASQoL: Ankylosing Spondylitis Quality of Life; NSAID: Non-steroidal anti-inflammatory drugs; DMARD: Disease-modifying anti-rheumatic drugs; TNF-α: Tumor necrosis factor alpha; NS: Not significant.

DISCUSSION

Ankylosing spondylitis is a chronic inflammatory disease characterized by axial and peripheral arthritis, neo-ossification, enthesitis, and certain extra-articular manifestations, such as uveitis, inflammatory bowel diseases, pulmonary restriction, and cardiovascular complications.[25] It usually presents with low back pain and morning stiffness that if not treated could result in spinal deformities and disability. Since AS prominently affects young males, long-term complications might pose a significant burden of disease on the affected community.[26] Despite improvements in the diagnosis and treatment of AS, the molecular mechanisms of pathogenesis are still under study because it appears that a combination of environmental (e.g., diet, physical stress, air

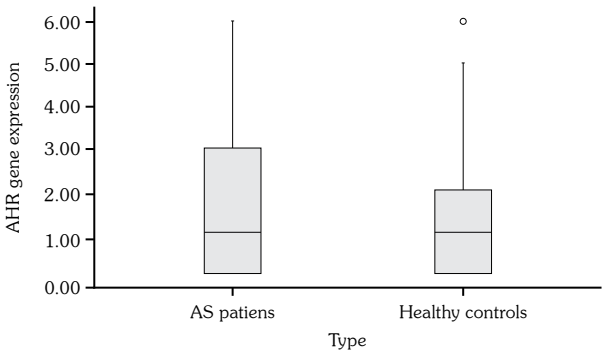

Figure 1. Comparable AHR gene expression in patient and control groups.
AHR: Aryl hydrocarbon receptor.

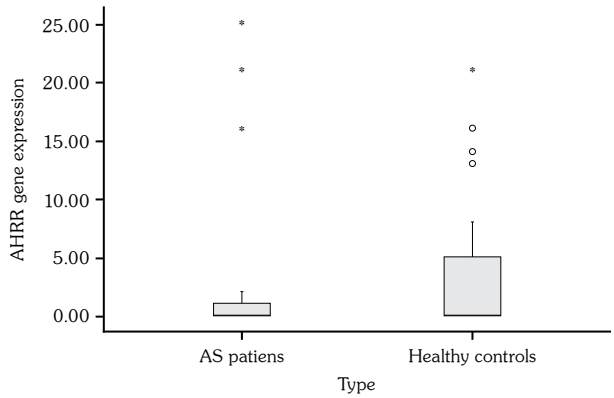

Figure 2. AHRR gene expression in patient and control groups.
AHRR: Aryl hydrocarbon receptor repressor.

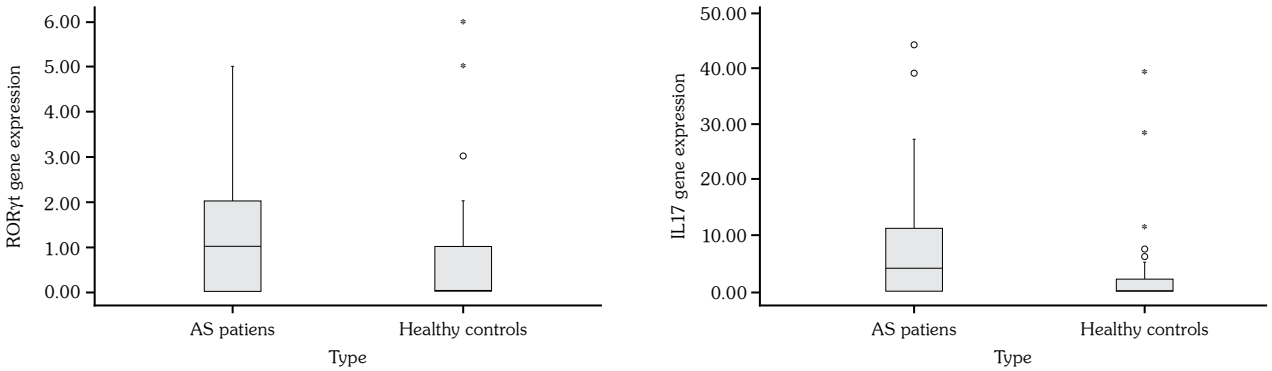

Figure 3. Increased expression of RORγt and IL-17 genes in AS patients compared to the control groups.
RORγt: Related orphan receptor gamma t; IL: Interleukin; AS: Ankylosing spondylitis.

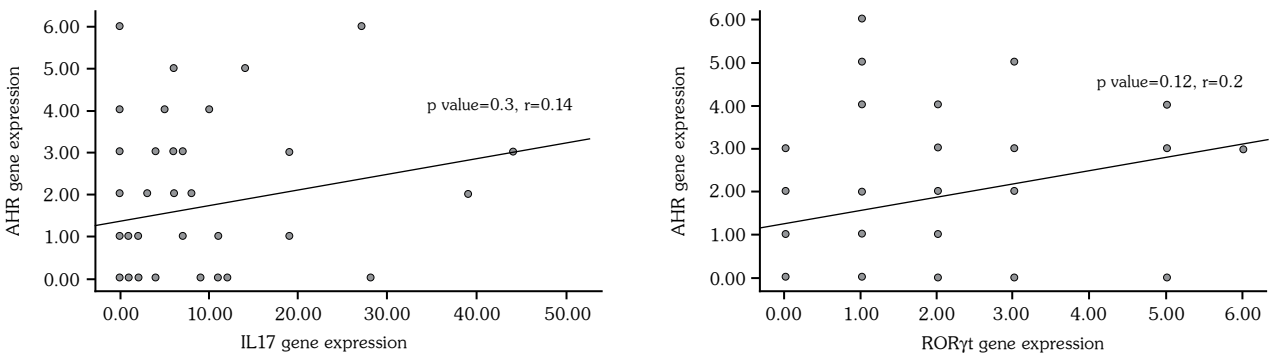

Figure 4. Correlation between genes expression of AHR and RORγt/IL-17.
AHR: Aryl hydrocarbon receptor; RORγt: Related orphan receptor gamma t; IL: Interleukin.

pollution) and genetic factors are responsible for the development and progression of AS. In line with this, recent findings suggest dysregulated expression of cytokines and transcription factors as a risk factor in the pathogenesis of AS.[27]

Aryl hydrocarbon receptor is a ligand-activated transcription factor that controls the expression of a diverse set of genes in a ligand-dependent manner.[28] Recently, the role of this molecule in the regulation of immune responses in the

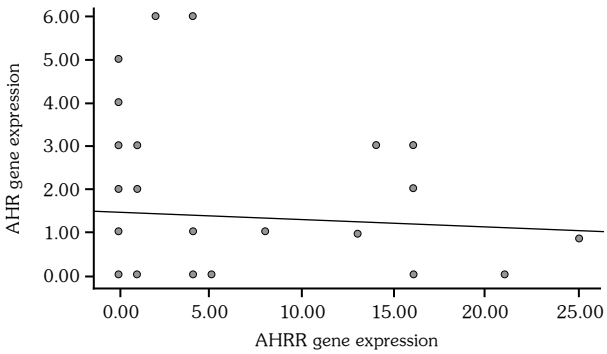

Figure 5. Correlation between AHR and AHRR genes expression.

AHR: Aryl hydrocarbon receptor; AHRR: Aryl hydrocarbon receptor repressor.

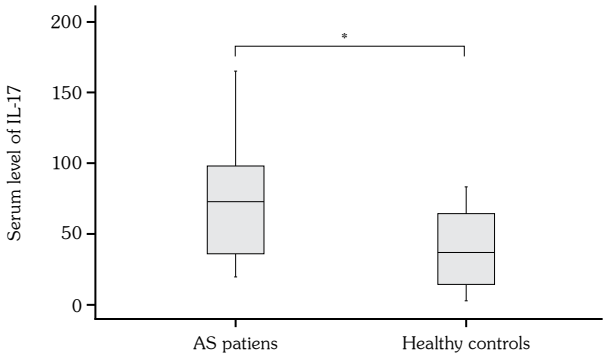

Figure 6. Higher serum level of IL-17 in AS patients compared to the healthy controls (* p=0.01).

IL: Interleukin; AS: Ankylosing spondylitis.

differentiation and proliferation of immune cells has been considered.[6,7] For instance, it has been shown that binding certain ligands to the AHR could promote naïve T cell differentiation to regulatory T cells, such as TCDD, whereas other ligands, such as FICZ, increase Th17 cell differentiation.[29] According to the implication of Th17 cells and IL-17 in the pathogenesis of AS, any correlation between this subtype and AHR expression could be of significance. A growing body of evidence is indicative of the role of Th17 cells in exacerbation of the inflammatory process in AS; for example, higher percentages of Th17 cells have been reported in AS patients compared to the healthy controls.[30] Moreover, a significant imbalance is observed between Th17 and regulatory T subsets in the peripheral blood of these patients.[31] IL-17, mainly produced by Th17 cells, has also been demonstrated to be involved in the pathogenesis of AS. The elevated level of IL-17 has been demonstrated in the serum, synovial fluid, and joint tissue of patients with AS.[32,33] These and other findings paved the way for developing anti-IL-17 antibodies to treat refractory patients.[34] The anti-IL-17 monoclonal antibody secukinumab has been successfully applied to treat complicated patients with axial spondyloarthritis.[35] Our study also confirmed the previous findings about the enhanced expression and elevated serum level of IL-17 in AS patients compared to healthy people.

The expression of RORγt is increased in invariant natural killer T and gamma delta T cells infiltrated in the inflamed joint of AS patients.[36] Therefore, it appears that RORγt inhibition might reduce the activity of these subsets. Accordingly, the administration of guluronic acid (G2013) reduced the gene expression of RORγt, IL-22, and AHR in AS patients while increasing the expression of FOXP3 (forkhead box P3).[37] Similarly, our study showed an upregulated expression of AHR and RORγt genes in the patients group. Although not significant, the present study demonstrated a positive correlation between AHR expression and IL-17/RORγt gene expression, which is suggestive of the involvement of AHR in the differentiation or maintenance of the Th17 subset. Given the accumulating evidence of the efficacy of AHR antagonists in the treatment of experimental models, these results may provide further impetus for the development of human studies about pharmacological aspects of AHR antagonists in autoimmune disorders.[38] Nonetheless, it is worth noting that despite all these findings about the pathological effects of AHR on RA, AS, and SLE, there are studies indicating the protective effect of AHR signaling in some other autoimmune diseases, such as multiple sclerosis, autoimmune uveitis, Behçet's disease, myasthenia gravis, and type 1 diabetes.[39,40] These studies have focused on the opposite role of AHR in the differentiation of regulatory T cells consequent to binding to a different set of ligands.[41]

The other gene evaluated in the present study was AHRR. AHRR is a bHLH/PAS family member that is missing the second PAS domain (PAS-B) implicated in ligand binding by

the AHR. It dimerizes with the AHR nuclear translocator (ARNT) and competes with AHR/ARNT complexes for binding to dioxin-responsive elements.[42] Therefore, it is presumed to have a regulatory effect on the AHR activity through a negative feedback mechanism.[43] The results of our study showed a negative correlation between AHR and AHRR gene expression in the subjects. Moreover, AHRR gene expression was slightly downregulated in the AS patients compared to the healthy individuals. Regarding the recent interest in exploiting the potential of the AHR antagonist in correcting dysregulated immune responses, particularly chronic inflammation, the AHRR might be considered a potential target to inhibit AHR activation.

Correlation analysis between AHR expression and disease activity indices also showed a weak positive correlation between AHR and BASG, BASMI, BASDAI, BASFI, and ASQOL indices, but none of the correlations reached a significant level. Finding molecules that significantly reflect the disease progression or severity might be helpful in the determination of prognosis and disease monitoring; however, considering our results, AHR does not seem to be an appropriate option for this purpose.

Taken together, our findings suggest an enhanced expression of AHR and Th17-related genes in AS patients compared to healthy individuals and imply a positive correlation between them although none was statistically significant. The small size of study population and the heterogeneity of the patients might be the reason for nonsignificant results. Indeed, the lower prevalence of AS in Iran (0.12%, CI: 0.08-0.18)[44] compared to the other countries (between 0.2% and 0.5%)[45] makes it difficult to recruit large number of participants, particularly new or stable patients. Furthermore, it was preferred to enroll the patients under similar treatment regimens and comparable disease durations, but the limited number of stable patients prevented from studying a homogenous population or subgroup analysis. Interfering comorbidities, such as type 2 diabetes, cardiovascular disorders, and recent COVID-19 (coronavirus disease 2019) infection, also excluded a number of volunteers. However, the evidence of AHR gene expression in AS is so scarce that this amount of data might be helpful for designing future studies. Therefore, it is suggested to conduct studies with a larger sample size, preferentially in newly diagnosed patients who have not received immunosuppressive therapy. Moreover, despite the very low or no expression of some genes, such as AHRR, it might be valuable to evaluate the protein expression of the studied genes with the western blot technique or to assess the expression level of AHR in inflamed tissue.

In conclusion, the present study demonstrated a slight upregulation of the AHR gene expression in AS patients compared to healthy individuals. AHR expression was positively correlated with the expression of RORγt and IL-17 genes, while showed a negative correlation with AHRR expression. AHR gene expression levels also displayed a weak positive correlation with disease activity indexes.

Ethics Committee Approval: The study protocol was approved by the Tehran University of Medical Sciences Ethics Committee (date: 20.03.2022, no: IR.TUMS.CHMC.REC.1400.316). 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 participant.

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

Author Contributions: Data collection and processing: M.A.; Idea, concept, design, and literature review: N.S.; Design and data collection: A.R.; Materials and processing: M.S.; Analysis and interpretation: H.M.; Data collection: A.M.; References, funding and critical review: M.H.N.; Writing the article, control/supervision: S.A.

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

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