

Determining what happens to the genes FOXP3, ROR γ t, SOCS1, STAT3, STAT5, and SMAD3 in patients with ankylosing spondylitis who received anti-TNF therapy

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Article

Keywords: ankylosing spondylitis, antitumor necrosis factor therapy, (FOXP3, ROR γ t, SOCS1, STAT3, STAT5, SMAD3) genes

Posted Date: March 13th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2627061/v1>

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Abstract

Background/Objective: Ankylosing spondylitis (AS) is a common inflammatory rheumatic disease that affects the axial skeleton, causing characteristic inflammatory back pain. We aim to examine the genes FOXP3, ROR γ t, SOCS1, STAT3, STAT5, and SMAD3 in patients with ankylosing spondylitis who receive anti-TNF therapy to understand the progression of the disease in a clinically improved or failed response to anti-TNF treatment.

Method: A prospective case-control study was conducted at Basrah Teaching Hospital in southern Iraq. Eighty-one AS patients were divided into two groups: 67 were treated with anti-TNF therapy; 14 were newly diagnosed patients as positive controls, and 65 were healthy individuals. Disease activity was assessed using the AS Bath Disease Activity Index (BASDAI). Conventional radiography and MRI are used to measure the severity of the disease. Blood samples were homogenized, and mRNA was isolated using a total RNA extraction kit. Reverse transcription (RT) was performed. The gene expression of ROR γ t, FOXP3, SOCS1, SAMD1, SMAD3, STAT3, and STAT5 was detected by implementing a real-timePCR system.

Result: There was significant downregulation of the FOXP3 gene in anti-TNF-treated AS patients and biologically naïve AS patients compared to healthy control people, which may be due to how the disease affects the expression and activity of the FOXP3 gene, which does not change with the type of therapy ($P = 0.001$). ROR γ t expression was higher in both established and newly diagnosed AS patients ($p 0.001$) than in healthy controls. The SOCS1 gene was expressed at a low level in patients with AS and positive control patients compared to the SOCS1 gene in healthy control individuals ($p 0.780$). STAT3 expression was lower in established and biologically naïve AS patients than in healthy subjects. Simultaneously, biologically normal AS patients had higher levels of STAT5 in this group. SMAD3 was underexpressed in established and biologically naïve AS patients compared to healthy subjects.

Conclusion: The progression of AS in patients treated with anti-TNF therapy is linked to a secondary change in the expression of the genes FOXP3, ROR γ t, SOCS1, STAT3, STAT5, and SMAD3 that leads to the induction of exTh17cells.

Introduction

Ankylosing spondylitis (AS) is a common inflammatory rheumatic disease that affects the axial skeleton, causing characteristic inflammatory back pain and leading to structural and functional impairments and decreased quality of life. New imaging techniques and therapies have substantially changed the management of this disease over the past decade [1]. How AS is treated has dramatically changed since tumor necrosis factor (TNF)-blocking therapy was developed. Before the availability of these drugs, nonsteroidal anti-inflammatory drugs (NSAIDs) were the main treatment options available. Serologic biomarkers include C reactive protein (CRP), cytokines, adipokines, matrix metalloproteinases (MMPs), calprotectin, CD74 antibodies, bone turnover markers, and circulating protein fragments of cartilage and

connective tissue degradation, as well as other biomarkers used in tracking the activity and diagnosis of AS [2], but they cannot determine the cause of the disease's nonresponse to TNF inhibitors and the cause of progression despite treatment.

Th17 and Treg cells are characterized by the selective expression of distinct transcription factors. FOXP3 is a forkhead transcription factor expressed in Th17 and Treg cells [3–5]. IL-6, IL-21, and IL-23 activate signal transducers and activators of the transcription of STAT3, which is critical for Th17-cell differentiation. In contrast, the IL-2 STAT5 pathway has been shown to inhibit Th17 differentiation [4, 6, 7]. TGF- β activates SMAD2 and Mad-2-associated proteins, as well as SMAD3 and Mad-3-associated proteins, both of which are required for FOXP3 gene activity and histone acetylation [8, 9]. Cytokine signaling proteins and suppressor of cytokine signaling (SOCS) modulate the Th17/Treg balance. SOCS1 decreases the number of Tregs by controlling the IL-2-mediated STAT5 signaling pathway, but it also keeps FOXP3 from being lost and Tregs from turning into Th1 or Th17 cells [10]. While 54% of patients will have stopped taking their first tumor necrosis factor inhibitor (TNFi) within five years, 63% remain on biological therapy [11]. Patients with AS who do not respond to TNFi must be closely monitored to determine why they do not react and what other treatments can be used. This study aimed to determine how gene expression was affected, which could lead to a lack of response to TNFi or the progression of AS even after receiving drugs.

Participants In The Study

The Ministry of Higher Education and Scientific Research; the University of Basrah College of Medicine, 030403/034/2022; and the Basrah Health Department at the Ministry of Health reviewed and ethically approved the study protocol. Before the data were collected, each participant signed a permission form. This was done after the study's purpose was explained, and they were ensured that the data would be safe. Relevant guidelines and regulations were followed for all methods. A prospective case-control study was conducted at Basrah Teaching Hospital in southern Iraq. The study reviewed 81 AS patients from two groups: 67 treated with anti-TNF therapy and 14 newly diagnosed patients who were not given treatment (biologically naïve). All patients were assessed according to the Assessment of SpondyloArthritis International Society (ASAS) criteria for a pivotal treatment center included in the rheumatology and biological therapy clinics at Basrah Teaching Hospital. The patients received anti-TNF therapy [originator infliximab, etanercept, Remsima (biomimetic infliximab), and Amjevita (adalimumab-atto)] for more than three months of sample collection. In addition, 65 healthy individuals were enrolled in the study as a control group (Fig. 1), and disease activity was assessed using the AS Bath Disease Activity Index (BASDAI) [12], according to which patients' BASDAI scores < 4 indicate inactive disease, and BASDAI scores \geq 4 indicate active disease. We used conventional radiography and sacroiliac joint (SIJ) MRI, including the following sequences: semicoronal T1 fat-saturated (T1FS) and semiaxial short tau inversion recovery (STIR) to determine the staging of sacroiliitis.

Materials And Methods

Ethical statement

All patients provided their written informed consent. The study was approved by the local ethics committee of the University of Basrah (approval number 030403/034/2022) and conducted in accordance with the ethical principles for medical research formulated by the Ministry of Higher Education and Scientific Research of Iraq.

Study samples

A total of 3 mL of blood samples were obtained from patients with AS and healthy controls. They were placed in an EDTA tube for gene expression detection by quantitative real-time polymerase chain reaction (qRT–PCR). Blood samples were homogenized, and mRNA was isolated using a total RNA extraction kit, GENEzol™ TriRNA Pure Kit (Geneaid), which is manufactured in Taiwan. Reverse transcription (RT) was performed according to the addScript RT Master kit (2x conc.) through ISO 9001 and 13485 by executing conventional PCR (Kyratec Life Sciences, Woolim Lions Valley B-308 Gasan-dong Geumcheon gu, Seoul, Republic of Korea, 153-803). The gene expression of ROR γ t, FOXP3, SOCS1, SMAD3, STAT3, and STAT5 was detected by implementing a 7500 Real-Time PCR System (Chai Biotechnologies for an Open-Source Real-Time PCR Thermocycler).

Quantitative reverse transcriptase polymerase chain reaction (qRT–PCR)

Sample homogeneity and analysis

The samples were prepared at room temperature.

1. A liquid sample of 200 μ L was placed in a 1.5-mL RNase-free microcentrifuge tube.
2. Six hundred microliters of GENEzol™ reagent was added and mixed well by vortexing.
3. The mixture was incubated for 5 minutes at room temperature.

RNA binding

1. The sample was centrifuged at 16,000 \times g for 1 min to remove cell debris and then transferred to a new 1.5-mL microcentrifuge tube (RNase-free).
2. One volume of absolute ethanol was added directly to one volume of the sample mixture (1:1) in GENEzol® reagent.
3. The sample mixture was mixed well by vortexing, and the RB column was placed into a 2-mL collection tube.
4. The RNA binding step was performed again by placing the rest of the sample mixture on an RB column.

5. The tube was centrifuged at 16,000 x g for 1 min, and the supernatant was discarded. The RB column was placed into a new 2-mL collection tube.

RNA washing

1. Four hundred microliters of prewash buffer was added to the RB column and then centrifuged at 16,000 × g for 30 sec.
2. The RB column was then placed back into the 2-mL collection tube.
3. A volume of 600 µl of washing buffer was added to the RB column.
4. The tube was centrifuged at 16,000 x g for 30 sec, and the supernatant was discarded. The RB column was placed back into the 2-mL collection tube.
5. Then, 600 µl of washing buffer were added to the RB column.
6. The column was centrifuged at 16,000 x g for 30 sec, and the supernatant was discarded.
7. The RB column was placed back into the 2-mL collection tube. The RB column was rewashed with 600 µl of wash buffer.
8. The column was centrifuged at 16,000 x g for 3 min to dry the column matrix.

RNA elution

1. The dry RB column was placed in a clean 1.5-mL (RNase-free) centrifuge tube.
2. Twenty-five milliliters of RNase-free water was added to the medium of the column matrix.
3. The column was left alone for at least 3 minutes to ensure that the matrix absorbed all RNase-free water.
4. The tube was then centrifuged at 16,000 x g for 1 min to filter the purified RNA.

RNA quantification

The concentration of the extracted RNA was determined using a NanoDrop spectrophotometer by UV absorption. Eluted viral RNA samples were either processed directly for RT–PCR or preserved at –20 °C until further use.

Complementary DNA (cDNA) synthesis

cDNA synthesis was performed according to the addScript RT Master (2x conc.) kit, which was manufactured through the ISO 9001 and 13485 system. The components of the cDNA reaction with their volumes and final concentrations were as follows: 10 µl of the reaction mix and 25 ng of template RNA

were used as starting materials, accompanied by 1 µl of random primers. The volume was brought to 20 mL with nuclease-free water. The PCR tubes were spun briefly to collect the solution at the bottom of the tube.

The PCR tubes were placed on a thermal cycler (Kyratec, Australia), incubated at 50 °C for 60 min and 80 °C for 5 min, and then cooled to 4 °C. The samples were then held at -20 °C until use.

Quantitative real-time polymerase chain reaction (qRT PCR)

a) All components were thawed at room temperature. The samples were mixed vigorously and centrifuged to collect the contents at the bottom of the tubes.

b) In nuclease-free water, the primers were diluted to 1:10.

c) In a sterile nuclease-free microcentrifuge tube, the following reagents were mixed: each reaction contained 1 µl of 5 µM of each primer, 10 µl of 2 x iQ™ SYBR Green Supermix, and 7 µl of nuclease-free water (total 19 µl). They were mixed gently by flicking the tube and then spun briefly.

d) In each well of the 16-well PCR plate, 19 µl of the above mixture was added to the bottom of the tube, and 1 µl of cDNA was added to each tube.

e) The reaction was mixed gently by flicking the tube, and the plate was spun briefly.

f) The PCR plate was placed in Chai Biotechnologies for real-time PCR detection.

Amplification curve of PCR (thermal profiling)

The cDNA was denatured for three minutes at 95 °C during stage 1. Stage 2 consisted of fifty cycles of denaturing for 20 seconds at 95 °C and annealing of cDNA with primers, followed by 20 seconds at 60 °C, for FOXP3, STAT3, STAT5, SOCS1, and SMAD-3. RORyt requires an annealing temperature of 58 °C. In this study, the beta-actin gene was used as a reference gene to help measure the target gene's fold expression.

Statistical analysis

All analyses were performed using SPSS software, version 21.0 (IBM, Chicago, IL, USA), illustrating the basic features of invoking SPSS Statistics from an external Java application. The Shapiro-Wilk test was used to determine the normality of the variables. The differences between parameters that were not normally distributed were analyzed with the Kruskal-Wallis test alternative to one-way ANOVA and Friedman's test as an alternative to two-way ANOVA, and they were defined as the mean rank. Categorical variables were compared using the chi-square test and Fisher's exact test.

The Delta-Delta Ct or Livak method was the preferred method for qPCR data analysis. The ΔCt is the difference in Ct values for the gene of interest and the housekeeping (β actin) gene for a sample.

- 1) $\Delta Ct = Ct(\text{gene of interest}) - Ct(\text{housekeeping gene})$.
- 2) The average of the healthy control samples is calculated.
- 3) The $\Delta\Delta Ct$ relative to the average ΔCt of healthy controls was calculated.
- 4) $\Delta\Delta Ct = \Delta Ct(\text{established or biologically naïve AS patient}) - \Delta Ct(\text{average healthy controls})$
- 5) Gene expression fold = $2^{-(\Delta\Delta Ct)}$

Results

The FOXP3 gene was expressed at a low level in patients with AS and positive control patients with means of 0.185 ± 0.728 and 0.247 ± 0.559 , respectively, and 95% confidence intervals of 0.268-0.126 and 0.545-0.117, respectively, compared to the FOXP3 gene in healthy control individuals (Figure 2).

The RORyt gene expression level increased by approximately twofold and half in patients with AS and with positive control means of 2.549 ± 1.888 and 2.666 ± 1.473 , respectively, and confidence intervals of 3.041-2.107 and 3.527-1.930 compared to healthy control individuals (Figure 3).

The SOCS1 gene was expressed at a low level in patients with AS and positive control patients with means of 0.768 ± 1.227 and 0.850 ± 0.751 , respectively, and confidence intervals of 1.043-0.553 and 1.212-0.656, respectively, compared to the FOXP3 gene in healthy control individuals (Figure 4).

The STAT3 gene was expressed at a low level in patients with AS and positive control patients with means of 0.219 ± 0.375 and 0.3 ± 0.482 , respectively, and confidence intervals of 0.302-0.145 and 0.501-0.196, respectively, compared to the STAT3 gene in healthy control individuals (Figure 5).

The STAT5 gene expression level increased by approximately one-half-fold in patients with naïve AS but decreased by one-third-fold in the established AS group, with means of 1.418 ± 0.710 and 0.679 ± 0.977 , respectively, and confidence intervals of 1.816-1.118 and 0.911-0.504, respectively, compared to gene expression in healthy individuals (Figure 6).

The SMAD3 gene was expressed at a low level in patients with AS and positive control patients with means of 0.214 ± 0.310 and 0.272 ± 0.272 , respectively, and confidence intervals of 0.282-0.155 and 0.437-0.174, respectively, compared to the SMAD3 gene in healthy control individuals (Figure 7).

There was a significant correlation of anti-TNF therapy and NSAIDs in AS patients with the gene expression of FOXP3, RORyt, STAT3, and SMAD3 in T cells compared to healthy individuals (Kruskal-Wallis test, $P = 0.001$, significant). As shown in **Table 1**, the other genes, SOCS1 and STAT5, showed no significant correlations. **Table 2** shows the locations of significant correlations between different types of drugs that affect gene expression.

Discussion

FOXP3 positive regulatory T cells (Tregs) are essential for maintaining self-tolerance, and problems with Tregs have been linked to several autoimmune diseases. No one knows how Tregs contribute to the development of ankylosing spondylitis (AS).

In this study, the significant downregulation of the FOXP3 gene in anti-TNF-treated AS patients and biologically naïve AS patients compared to healthy control people could be due to how the disease affects the expression and activity of the FOXP3 gene, which does not change with the type of therapy ($P = 0.001$), whether NSAIDs or anti-TNF drugs. This result is similar to what Guo et al. [13] found. They found that the mean fluorescence intensity (MFI) of FOXP3 in classical Tregs in the peripheral blood of patients with active AS was significantly lower than that in healthy controls ($p = 0.0054$).

ROR γ t is required for Th17 cell lineage commitment and is activated early in the differentiation of Th17 cells in response to IL-6 and TGF- β stimulation [14, 15]. ROR γ t binds to and regulates TH-17-cell-associated genes, such as *Il17a*, *Il17f*, and *Il23r*, by activating their transcription in coordination with other transcription factors [16].

The current study showed high expression of ROR γ t in both established and biologically naïve AS patients ($p = 0.001$) compared with healthy controls, resulting in increased IL-17A expression in effector Th17 cells, enhanced inflammation in AS patients and progression of joint destruction even in patients treated with anti-TNF therapy. In contrast, in rheumatoid arthritis patients, impaired function of Treg cells, which recover after treatment with anti-TNF agents, has been reported [17]. Although TNF inhibition seems to reduce the number of neutrophils in the synovium and the number of ILC3s in the gut of people with AS [18, 19] and it stops IL17R expression in blood cells [20], it does not seem to change the amount of IL-23 or prostaglandin E2 (PGE2) in the blood [21].

Suppressors of cytokine signaling (SOCS) proteins are intracellular inhibitors of cytokine signaling that activate cells through the JAK-STAT pathway (Janus kinase-signal transducers and activators of transcription). The current study showed no significant differences in SOCS1 gene expression among established AS patients, biologically naïve AS patients, and healthy controls. There was a tendency toward lower cellular SOCS1 expression in the AS patients than in the healthy controls ($p = 0.780$). Chen et al. [22] found that cellular SOCS1 expression did not show significant differences between AS patients and controls, but SOCS3 expression increased significantly in AS patients compared to controls in PBMCs ($p = 0.025$), T cells ($p = 0.003$), and monocytes ($p = 0.009$).

Signal transducers and activators of transcription (STATs) play prominent roles in Th17 differentiation [23]. This family of DNA-binding proteins participates in the transmission of signals from cytokine receptors. STAT3 stimulates the differentiation of Th17 cells, while STAT5 contributes to the sending of IL-2 signaling, leading to the inhibition of Th17 differentiation [7].

In the current study, a significant difference was found in the expression of STAT3 and STAT5 in the peripheral blood of established AS patients and biologically naïve AS patients compared to healthy control subjects, whereas STAT3 lowered the expression level in the established and biologically naïve AS patients compared to healthy subjects. At the same time, biologically naïve AS patients were more likely to have more STAT5 in these groups. To our knowledge, the present study is the first to provide an analysis of STAT3 and STAT5 expression in AS patients who used anti-TNF therapy. STAT5 was overexpressed in biologically naïve AS patients. This might result from increasing neutrophils and eosinophils in the whole blood of biological naïve cells, which express both STAT5A and STAT5B isoforms [24]. In addition, in Treg cells, was found STAT5, a critical positive regulator of FOXP3 [3, 25].

This study found that SMAD3 was underexpressed in established and biologically naïve AS patients compared to healthy subjects. One of the critical downstream mediators of the transforming growth factor- β (TGF- β) signaling pathway is SMAD3, which plays a crucial role in joint homeostasis and cartilage repair. TGF- β alone activates SMAD2 and SMAD3, which are required for FOXP3 gene activity and FOXP3 histone acetylation [8, 9]. Furthermore, the crosstalk between SMAD3 and STAT3 could regulate SMAD3 functions, suggesting that one or more unknown factors determine the outcomes of their cooperation. Some studies have suggested that peripheral T regulatory 17 (Tr17) cells are more regulated than classical Tregs [26, 27]. Tr17 cells are activated Treg cells that control the autoimmunity caused by Th17 cells in a way that requires STAT3.

In the present study, FOXP3 gene expression was decreased in established AS patients treated with infliximab and etanercept; ROR γ t gene expression was increased by infliximab, etanercept, amjevita, and NSAIDs; STAT3 expression was decreased in AS patients treated with NSAIDs, infliximab, and etanercept; and SMAD3 expression was reduced in AS patients treated with infliximab and etanercept. Infliximab and etanercept also altered the balance of Foxp3 and ROR γ t expression in peripheral blood toward induced exTh17 cells. The same drugs decreased STAT3 and SMAD3, which play essential roles in decreased osteoclast differentiation, decreased neoangiogenesis, and decreased AS disease activity. A previous study identified a link between changes in circulating Th17 cells evaluated by cellular assays and resolution of synovial inflammation and vascularity during anti-TNF treatment in rheumatoid arthritis [28].

Conclusion

In conclusion, according to our data, the progression of AS patients treated with anti-TNF therapy was associated with a secondary alteration in the expression of the genes FOXP3, ROR γ t, SOCS1, STAT3, STAT5, and SMAD3 toward the induction of exTh17 cells, where the originator infliximab and etanercept altered the balance of Foxp3 and ROR γ t expression in peripheral blood toward the induction of exTh17 cells. The same drugs decreased STAT3 and SMAD3, which are essential for decreasing osteoclast differentiation, angiogenesis, and AS disease activity. The biosimilar Amjevita increased exTH17 cell expression significantly, whereas Remsima had no genetic effect.

Abbreviations

AS: ankylosing spondylitis, TNFi: tumor necrosis factor inhibitor, NSAIDs: nonsteroidal anti-inflammatory drugs, FOXP3: forkhead transcription factor, RORyt: transcription factor of the RAR-related orphan nuclear receptor, STAT3: signal transducer and activator of transcription 3, STAT5: signal transducer and activator of transcription 5, SOCS: suppressor of cytokine signaling, SMAD2: Sma and Mad-2, SMAD3: Sma and Mad-3, Tregs: regulatory T cells, qRT-PCR: quantitative real-time polymerase chain reaction.

Declarations

Acknowledgements

The authors would like to thank the patients for their assistance in completing this research.

Author contributions

Husham A. Aldaoseri, Naael Hussein Ali, and Firas Al-Mubarak collected the data. Naael Hussein Ali and Firas Al-Mubarak reagents and quality control were provided. Aldaoseri performed the experiments and analyzed the data. All of the authors contributed to the article and approved the final version. All authors read and approved the final manuscript.

Funding

The research was not funded.

Availability of data and materials

All the data generated or analyzed during the current study are available from the corresponding author. If a reasonable request is made, the corresponding author will respond.

Consent for publication

Not applicable

Competing interests

All authors have no relevant financial or nonfinancial interests to disclose.

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Tables

Table 1 Distribution of gene expression across types of therapy in patients with established and naïve AS in comparison to healthy individuals.

Distribution of gene expression across to type of therapy for AS patients in comparison to healthy control	P value
FOXP3	0.001
RORyt	0.001
SOCS1	0.551
STAT3	0.001
STAT5	0.160
SMAD3	0.001

Table 2 pairwise comparison between anti-TNF- α therapy for established AS patients, NSAIDs for biological naïve AS patients (positive controls), and healthy people (no drug) that affect the distribution of specific gene expression

Gene	Drug1-Drug2	P value	Adj. P value
FOXP3			
	Infliximab- non drug	0.001	0.001
	Etanercept- non drug	0.001	0.001
	Amjevita- non drug	0.018	0.374
	Remsima- non drug	0.393	0.998
	NSAIDs- non drug	0.023	0.484
RORyt			
	Non drug - Infliximab	0.001	0.028
	Non drug - Etanercept	0.001	0.001
	Non drug - NSAIDs	0.001	0.001
	Non drug -Amjevita	0.002	0.05
	Non drug - Remsima	0.043	0.893
STAT3			
	Infliximab – non drug	0.001	0.001
	NSAIDs – non drug	0.001	0.011
	Etanercept – non drug	0.001	0,001
	Amjevita – non drug	0.008	0.170
	Remsima - non drug	0.078	0.962
SMAD3			
	Infliximab – non drug	0.001	0.001
	NSAIDs – non drug	0.001	0.011
	Etanercept – non drug	0.001	0.001
	Amjevita – non drug	0.006	0.129
	Remsima – non drug	0.102	0.976

Non-drug users were healthy people.

Figures

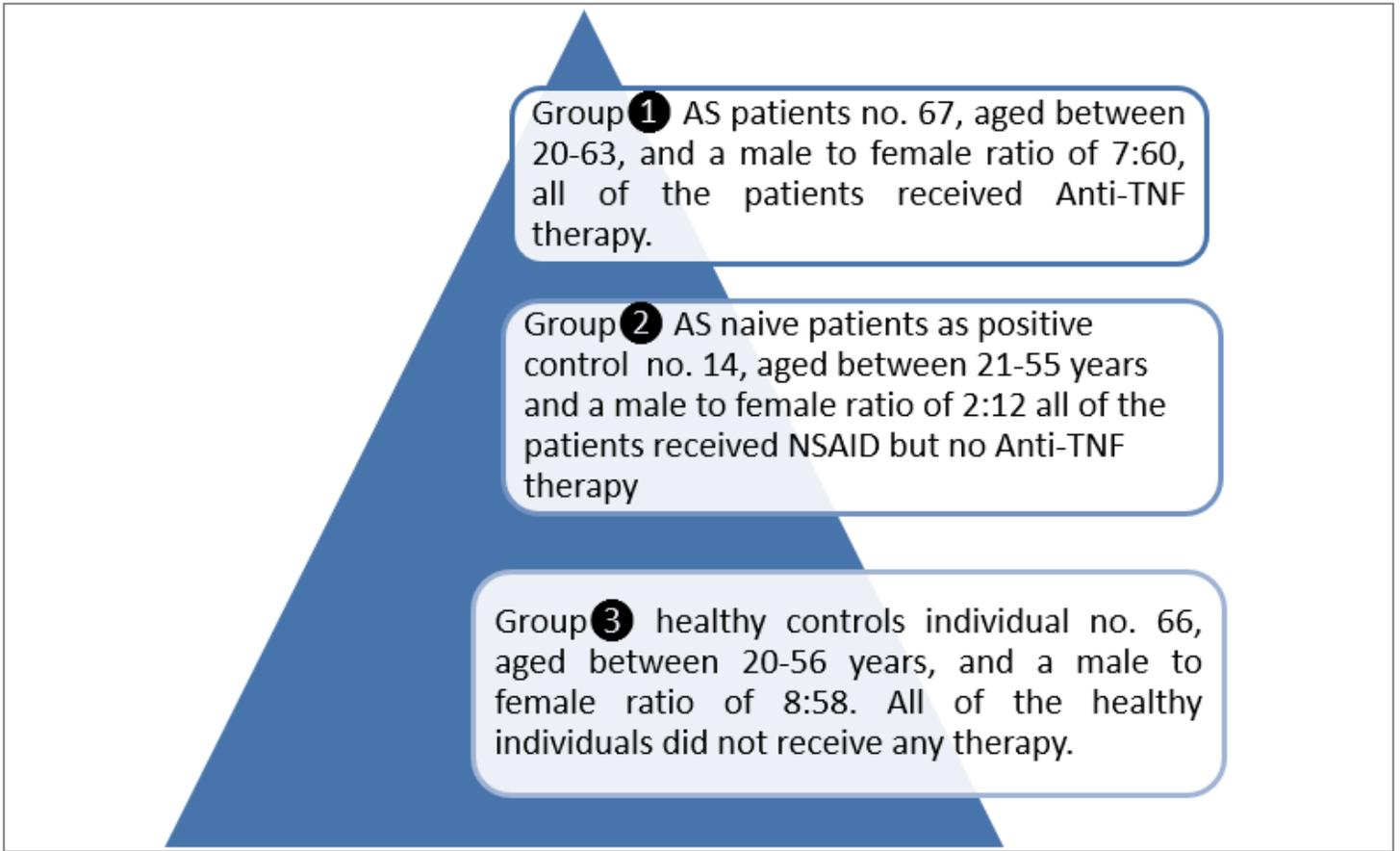


Figure 1

Divides the general characteristics of ankylosing spondylitis patients and healthy people into three categories.

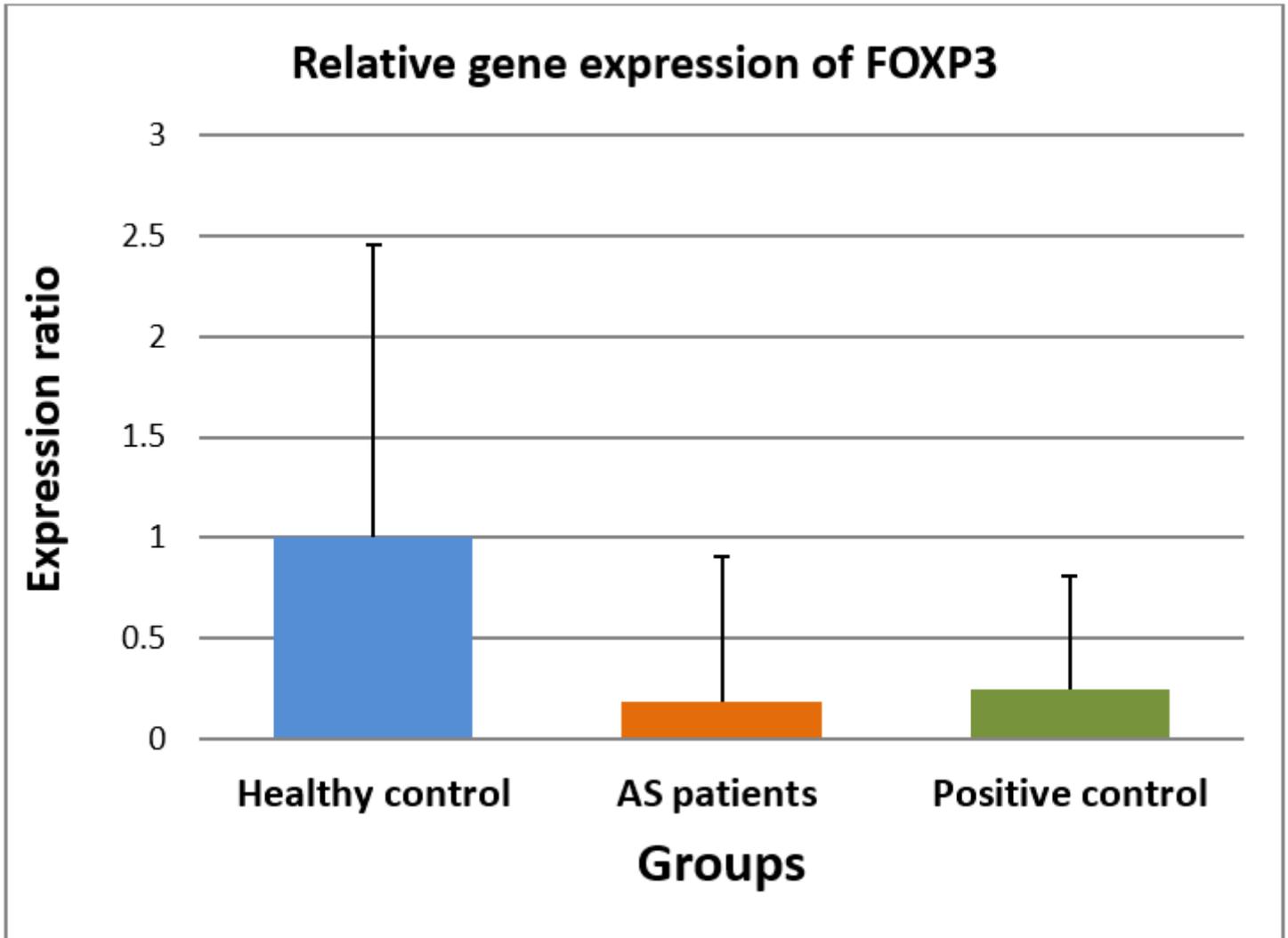


Figure 2

FOXP3 relative gene expression in patients with AS, positive control patients, and healthy control individuals. All RNA was extracted, reverse transcribed, and the synthesized cDNA was used as a template for a qRT-PCR relative expression assay using SYBR green master mix. The FOXP3 gene showed decreased expression by about 0.88% of one-fold in patients with AS and 0.75% in positive controls compared with control. (B): CTs were used to analyze the data, which was normalized to the actin housekeeping gene, which was equalized to one. (Kruskal-Wallis test $P = 0.001$, significant).

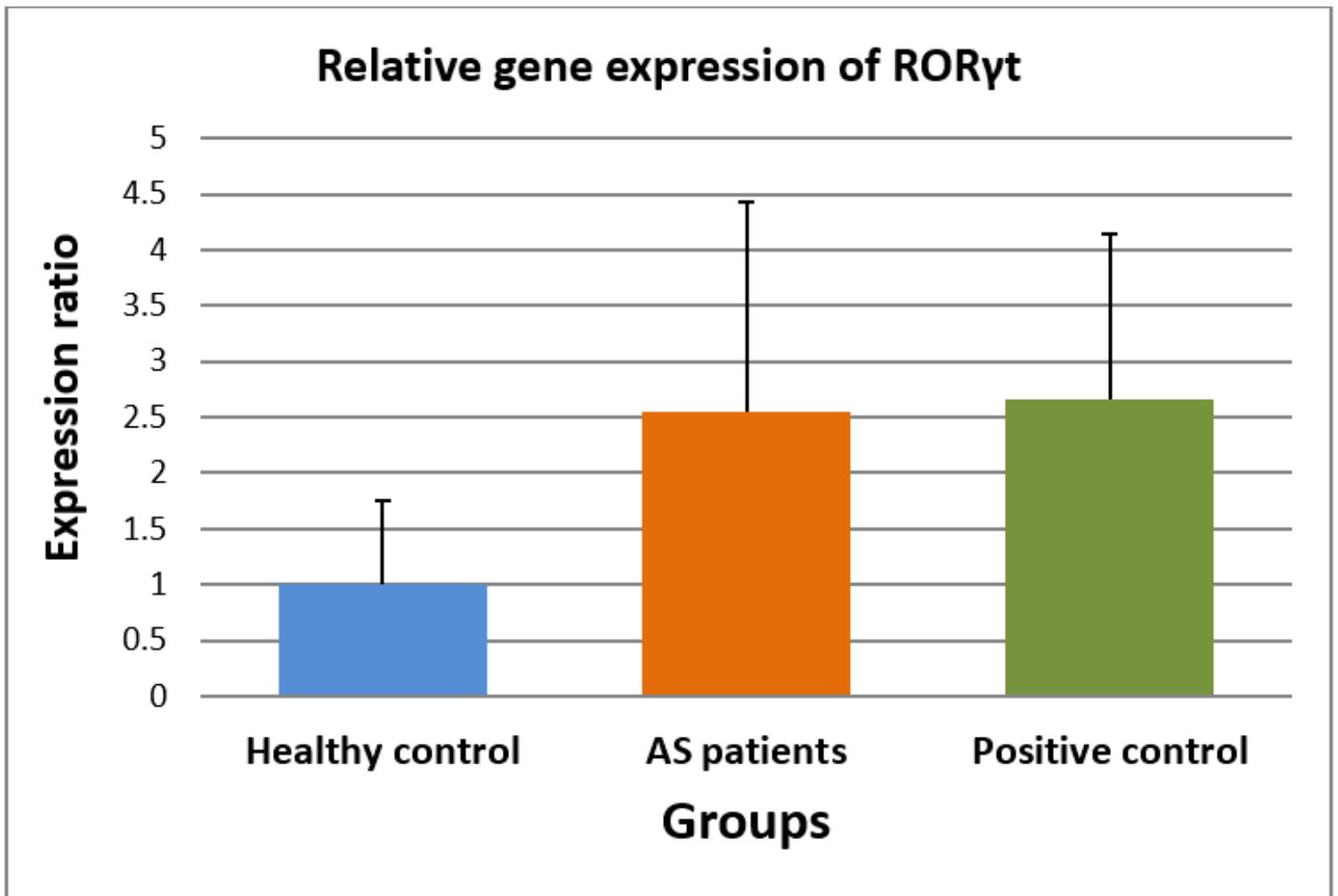


Figure 3

RORyt relative gene expression in patients with AS, positive control patients, and healthy control individuals. All RNA was extracted, reverse transcribed, and the synthesized cDNA was used as a template for a qRT-PCR relative expression assay using SYBR green master mix. The FOXP3 gene showed decreased expression by about 0.30% of one-fold in patients with AS and increased approximately twofold in patients with naive AS compared with control. (B): CTs were used to analyze the data, which was normalized to the actin housekeeping gene, which was equalized to one. (Kruskal-Wallis test $P = <0.001$, significant).

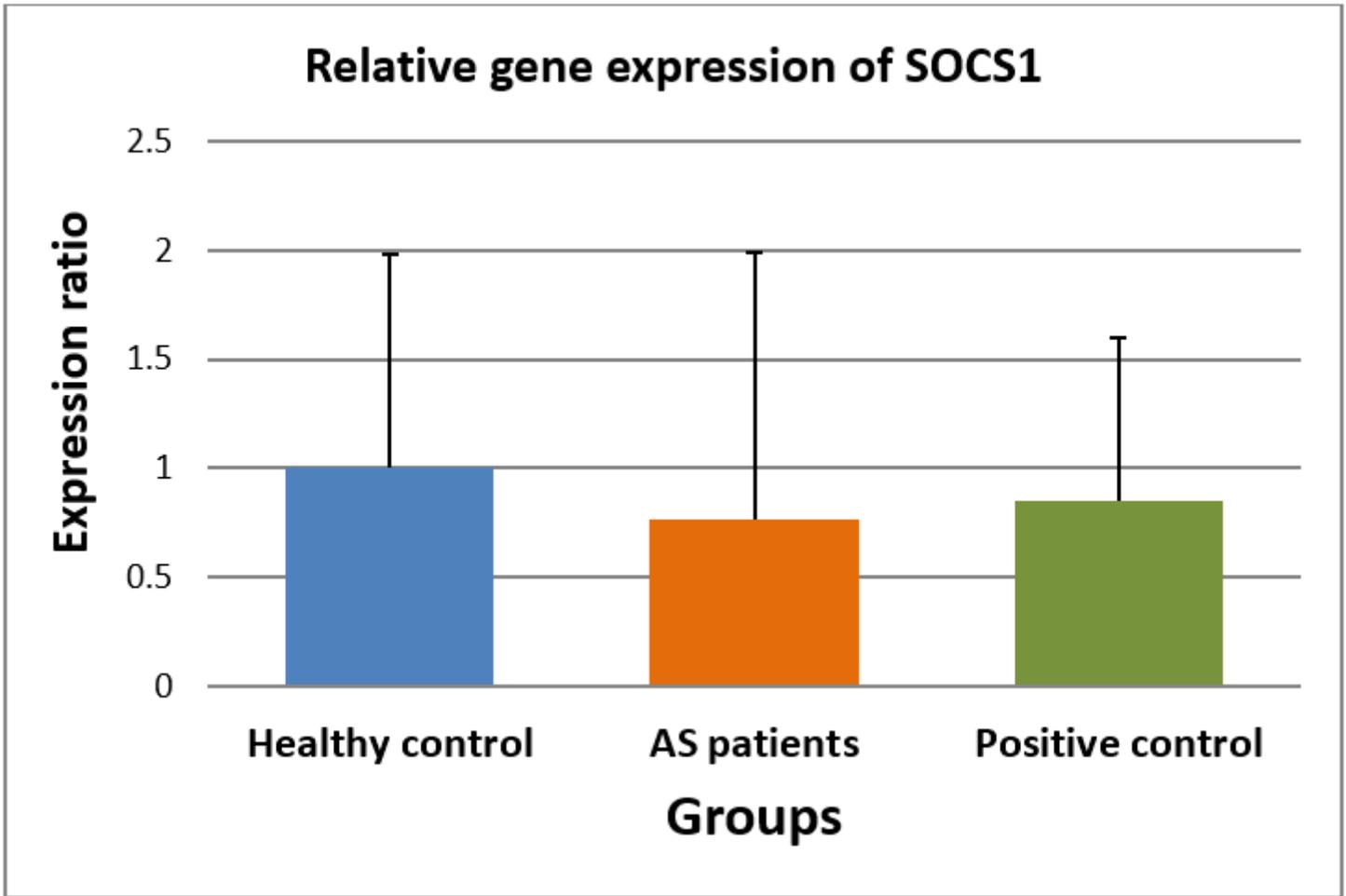


Figure 4

SOCS1 relative gene expression in patients with AS, positive control patients, and healthy control individuals. All RNA was extracted, reverse transcribed, and the synthesized cDNA was used as a template for a qRT-PCR relative expression assay using SYBR green master mix. The SOCS1 gene showed decreased expression by about 0.25% of one-fold in patients with AS and decreased by 25% in patients with naive AS compared with control. (B): CTs were used to analyze the data, which was normalized to the actin housekeeping gene, which was equalized to one. (P = 0.780, no significant Kruskal-Wallis test).

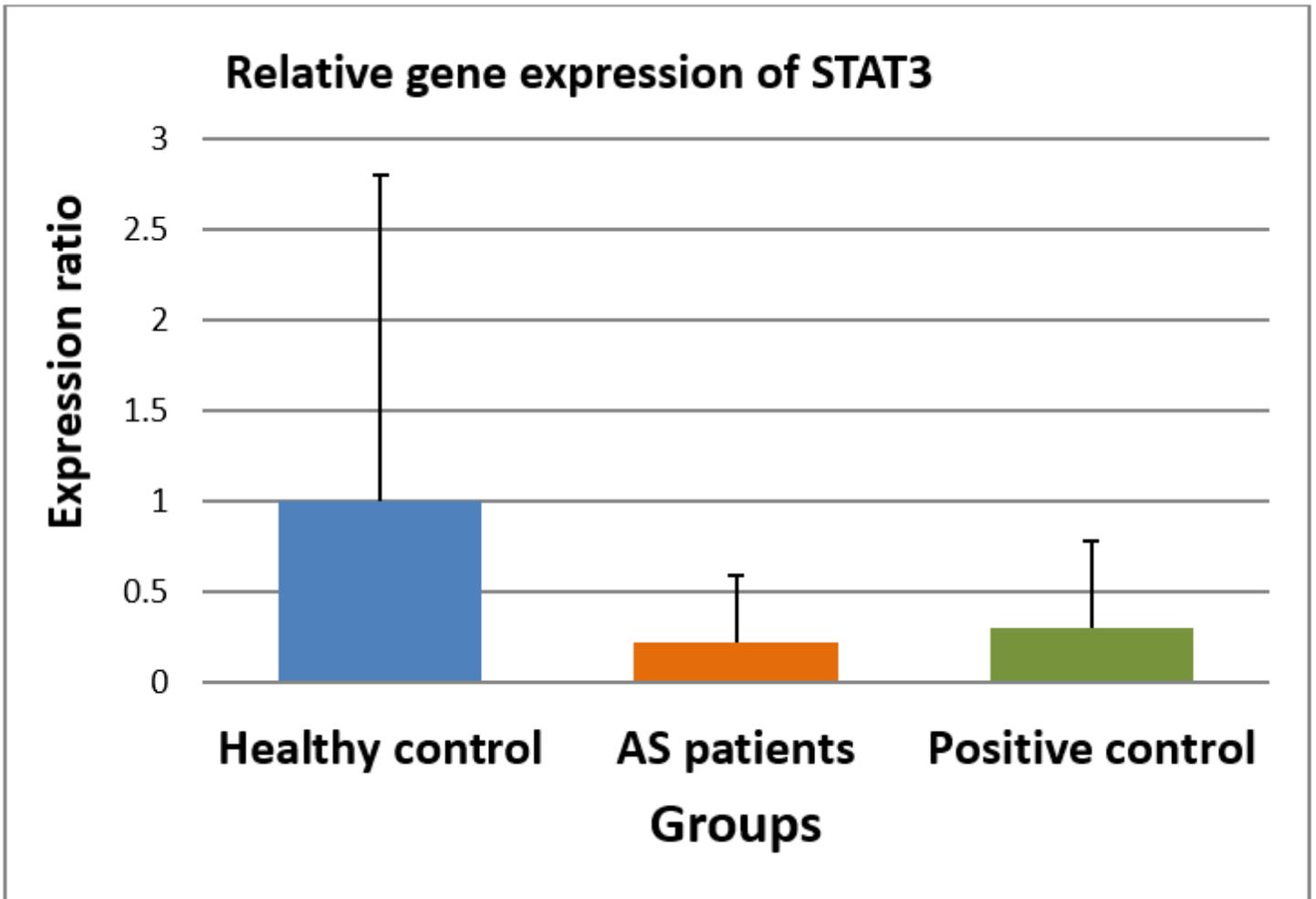


Figure 5

STAT3 relative gene expression in patients with AS, positive control patients, and healthy control individuals. All RNA was extracted, reverse transcribed, and the synthesized cDNA was used as a template for a qRT-PCR relative expression assay using SYBR green master mix. The STAT3 gene showed decreased expression by approximately 0.80% of one-fold in patients with AS and 0.70% in positive controls compared with control. (B): CTs were used to analyze the data, which was normalized to the actin housekeeping gene, which was equalized to one. (Kruskal-Wallis test $P = <0.001$, significant).

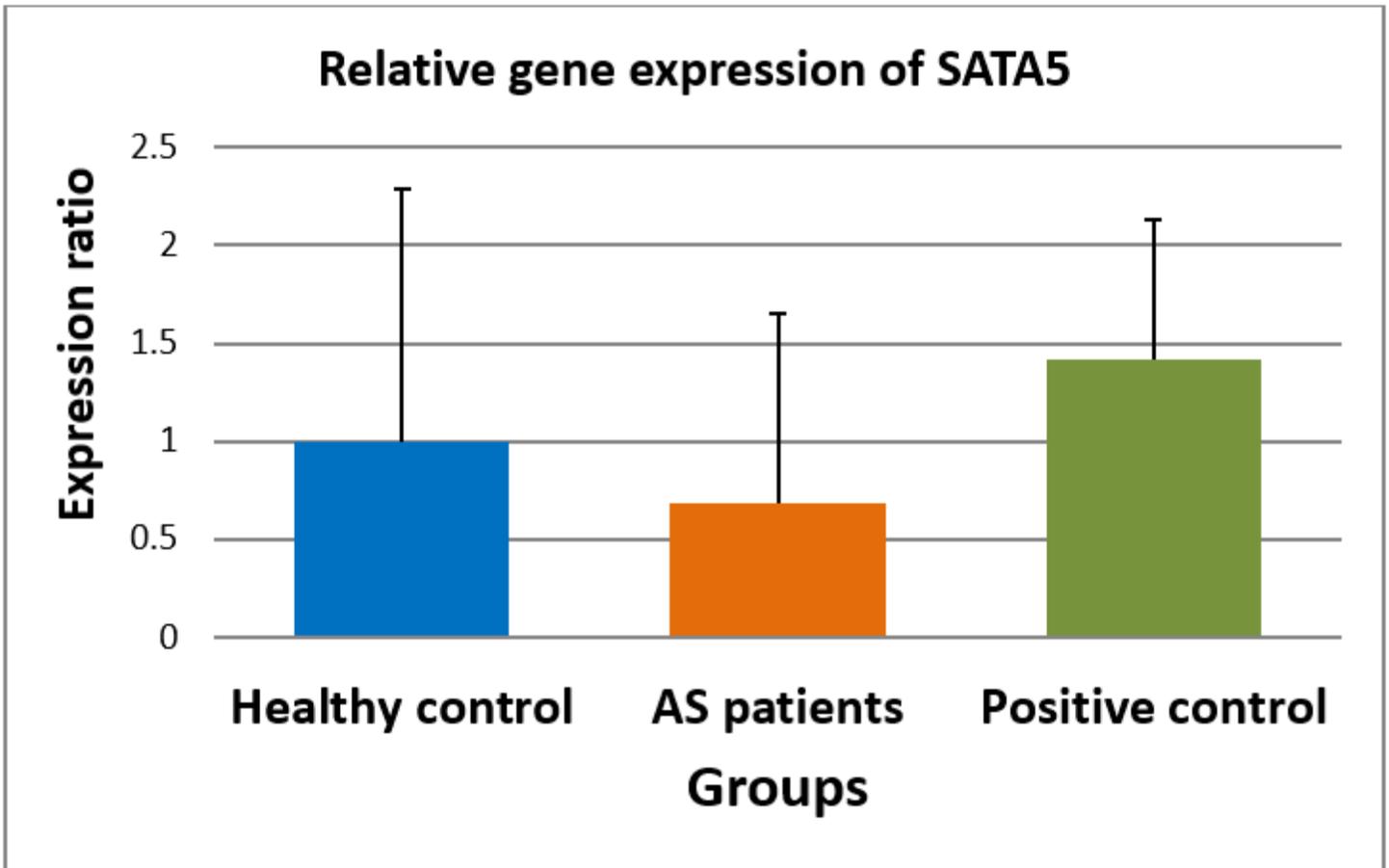


Figure 6

STAT5 relative gene expression in patients with AS, positive control patients, and healthy control individuals. All RNA was extracted, reverse transcribed, and the synthesized cDNA was used as a template for a qRT-PCR relative expression assay using SYBR green master mix. The STAT5 gene showed decreased expression by about 0.30% of one-fold in patients with AS and increased approximately one-half fold in patients with naive AS compared with control. (B): CTs were used to analyze the data, which was normalized to the actin housekeeping gene, which was equalized to one. (P = 0.025, significant Kruskal-Walis test).

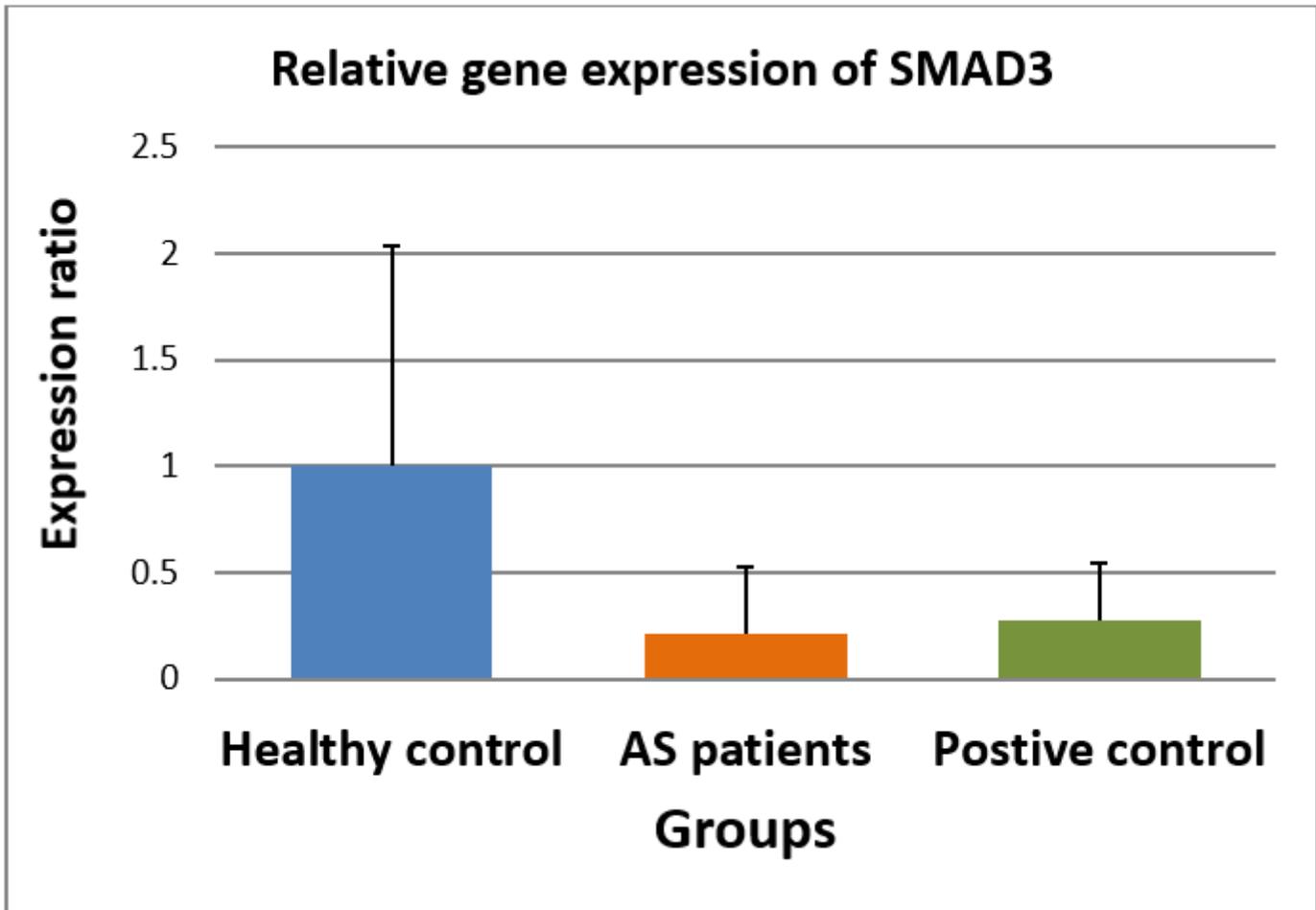


Figure 7

SMAD3 relative gene expression in patients with AS, positive control patients, and healthy control individuals. All RNA was extracted and reverse transcribed, and the synthesized cDNA was used as a template for a qRT-PCR relative expression assay using SYBR green master mix. The SMAD3 gene decreased expression by approximately two-thirds of one-fold in patients with AS and 0.70% in positive controls compared with control. (B): CTs were used to analyze the data, which was normalized to the actin housekeeping gene, which was equalized to one. (Kruskal-Wallis test $P = <0.001$, significant).