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Research article

Jianpi Qingre Tongluo Decoction exerted an anti-inflammatory effect on AS by inhibiting the NONHSAT227927.1/JAK2/STAT3 axis

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ABSTRACT

Purpose: This study aims to determine whether Jianpi Qingre Tongluo Decoction (JQP) alleviates ankylosing spondylitis (AS) inflammation via the NONHSAT227927.1/JAK2/STAT3 axis.
Methods: The effect of JQP on immune-inflammatory indicators in AS patients was explored through a combination of data mining, association rule analysis, and random walk model evaluation. Subsequently, network pharmacology and molecular docking were performed to screen out the potential signaling pathway. ELISA, PCR and wb were used to evaluate the effect of JQP on AS-FLS activity and inflammatory factors. The role of NONHSAT227927.1/JAK2/STAT3 combination in inflammation was studied by editing NONHSAT227927.1 and adding the JAK2/STAT3 inhibitor AG490. Involvement of the JAK2/STAT3 pathway was detected by PCR, WB, or immunofluorescence analysis.
Results: Retrospective data mining results show that JQP can effectively reduce the immune inflammatory response in AS patients. Through network pharmacology and molecular docking, it is speculated that JQP exerts its effect on AS through the JAK2/STAT3 pathway. Overexpression of NONHSAT227927.1 activated the JAK2/STAT3 pathway and promoted the expression of inflammatory factors, while serum containing JQP reversed the effects of NONHSAT227927.1 overexpression. NONHSAT227927.1 silencing inhibits the proliferation of AS-FLSs, inhibits the levels of inflammatory factors, and reduces the expression of JAK2/STAT3 protein. After adding the pathway blocker AG490, it was observed that the cell viability of AS-FLSs was reduced by inflammatory factors and the levels of JAK2/STAT3 were inhibited. , and overexpression of NONHSAT227927.1 can reverse this trend.
Conclusions: JQP exerted an anti-inflammatory effect on AS by inhibiting the NONHSAT227927.1/JAK2/STAT3 axis.

1. Introduction

Ankylosing spondylitis (AS) is a chronic rheumatic disorder with inflammatory low back pain as the most common presenting

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symptom, mainly affecting the sacroiliac joints and spine, but also concerning peripheral joints and other organs [1,2]. AS alters the biomechanical properties of the spine through chronic inflammatory responses, leading to unstable spine fractures after minor trauma [3]. The synovium is a highly specialized tissue that can secrete joint synovial fluid to lubricate and protect articular cartilage. Synovial hyperplasia and bone erosion are the characteristic features of many inflammatory joint disease including AS [4] Fibroblast-like synoviocytes (FLSs), a heterogenous cell population of the synovium, contribute actively to inflammatory responses and play a decisive role in the pathophysiological process of AS. FLSs secrete a variety of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) [5] and interleukin-6 (IL-6) to trigger and exacerbate inflammatory signaling cascades [6,7], leading to local inflammation and joint destruction [8]. At present, the main goals of AS management are to control inflammation, promote functional recovery, and reduce deformity [9]. Despite the favorable overall efficacy of the available AS treatment regimens, the clinical symptoms of many patients have not achieved acceptable improvement, and pain still severely compromises the health and daily life of patients. Therefore, developing new drugs to effectively relieve AS symptoms remains an urgent issue to be solved. Encouragingly, traditional Chinese medicine has shown great potential in the treatment of AS. Domestic experts have basically reached a consensus that traditional Chinese medicine can be applied to the clinical treatment of AS [9,10].

TCM has potential values in treating AS. In line with the tenets of traditional Chinese medicine, AS belongs to the category of "Bi Syndrome." Chinese clinicians are more inclined to a combination of traditional Chinese and Western medicine to provide personalized treatment for patients based on syndrome differentiation. The traditional Chinese medicine compounds Xinfeng Capsule (XFC, patent number ZL201310011369.8) and Huangqin Qingre Chubi Capsule (HQC, patent number ZL201110095718.X) are prepared under the guidance of Xinan medical theory and combined to form Jianpi Qingre Tongluo Decoction (JQP). XFC was prepared from Huang Qi (Astragalus flavone; *A. membranaceus (Fisch.) Bge.,* root), Yi Yi Ren (coix seed; *Coix lacryma-jobi L.var.mayuen (Roman.) Stapf,* kernel), Lei Gong Teng (tripterigium wilfordii; *Tripterygium wilfordii Hook. f.,*root),Wu Gong (centipede; *Scolopendridae,* Dried body) at a ratio of 20:20:20:1 [11]. The content and stability of the four core chemical components of traditional Chinese medicine in XFC were analyzed by ultra-high performance liquid chromatography-tandem mass spectrometry. Through testing, the method of reproduction of astragaloside IV, formononetin, and verbascoside was established, and a directional quantitative analysis method was established [12]. HQC is prepared from Huang Qin (s.baicalensis; *Scutellaria baicalensis Georgi*; root); Wei Ling Xian (chinese clematis root; *Clematis chinensis Osbeck*; root); Zhi Zi(gardenia jasminoides; *Gardenia jasminoides J. Ellis*; fruit) Yi Yi Ren (coix seed; *Coix lacryma-jobi L.var. mayuen (Roman.) Stapf*, kernel); Tao Ren (peach seed; *Prunusdavidiana (Carr.)Franch;* kernel) in a ratio of 10:10:9:30:5. The six core chemical components of traditional Chinese medicine in HQC were identified through fingerprints, proving that baicalin, gardeniposide, oleanolic acid, amygdalin, and coixin The content is relatively high, and the similarity between the HQC fingerprint and the monomer reference substance is above 0.9, indicating that the quality of HQC used in clinical applications is stable [13]. Western medical treatment has the advantages of rapid onset of action and significant improvement in indicators, but has unavoidable drawbacks, such as prolonged use of hormones that may lead to necrosis of the femoral head [14], decreased immunity, and increased risk of infections [15], gastrointestinal problems and hepatotoxicity associated with nonsteroidal anti-inflammatory drugs (NSAIDs), the high price of biologics. Therefore, TCM as a complementary therapy can achieve the goal of reducing toxicity, improving therapeutic efficacy as well as enhancing synergistic effects. JQP has been used for the treatment of damp-heat syndrome of AS for more than 20 years, showing remarkable therapeutic effects**.** The HPLC-generated fingerprint has verified JQP's safety, stability, and efficacy in medical treatments [16,17]. JQP contributes to reducing AS inflammation and redox reactions [18], improving bone metabolism, relieving extra-articular symptoms, inhibiting platelet activation [19], and improving cardiac function [20]. Moreover, JQP can also notably alleviate the anxiety and depression state of AS patients.

Long non-coding RNAs (LncRNAs) are generally defined as non-coding RNA transcripts with a transcript length of more than 200

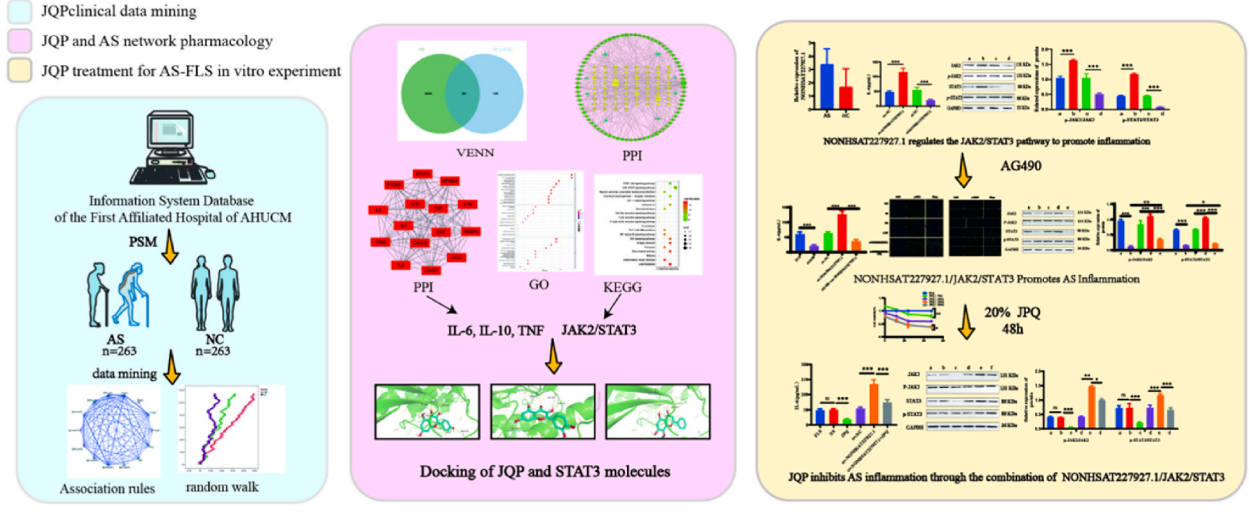


Fig. 1. Flow chart of study.

nucleotides, which are extensively implicated in a variety of biological processes [21,22]. Aberrant lncRNA expression pattern has been associated with the pathogenesis of AS [23]. For example, up-regulation of lncRNA NEF is indicative of AS recurrence and dismal outcomes [24]. LncRNA MEG3 is down-regulated in AS and holds predictive value concerning disease duration, disease severity, and length of hospital stay [25]. LncRNA MEG3 subsides AS inflammation partially by targeting miR-146a [26]. High-throughput sequencing and bioinformatics analysis previously conducted by our team has revealed the involvement of lncRNA NONHSAT227927.1 in AS inflammation.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is a common pathway of cytokine signal transductions, and JAK/STAT pathway dysregulation precedes numerous inflammatory and autoimmune diseases [27,28]. Pro-inflammatory cytokine activation of the JAK/STAT pathway is a critical event in the initiation and progression of AS. For example, it is reported that the JAK/STAT signaling cascade plays a potential role in the pathogenesis of AS by regulating the activation and proliferation of IL17+ effector memory T cells [29,30]. Among different JAK/STAT isoforms, JAK2/STAT3 has been studied most intensively. Accordingly, we speculated that AS inflammation could be partially attributed to the dysregulation of the JAK2/STAT3 pathway.

In this study, we validated the beneficial impact of JQP on immune and inflammatory markers in AS patients in a laboratory setting. Additionally, our in vitro experiments supported the notion that JQP may suppress AS inflammation by modulating the NONHSAT227927.1/JAK2/STAT3 pathway. These findings contribute to the potential therapeutic implications of JQP for ankylosing spondylitis. (Fig. 1).

2. Materials and methods

2.1. Drug source

JQP consists of XFC and HQC, XFC, patent No. ZL201310011369.8, Approval number Z20050062; HQC, patent No. ZL201110095718.X, Approval number Z20200001, Both drugs were purchased from Anhui Provincial Hospital of Traditional Chinese Medicine.

2.2. Clinical data for diagnosis of AS

The data for inpatient AS patients in the Department of Rheumatology from June 2012 to June 2021 was sourced from the Information System Database of the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine [31]. Exclusion criteria: incomplete data, severe diseases affecting immune globulin and complement such as diseases of the circulatory system, blood system, and respiratory system, pregnancy, and lactation. Non-steroidal anti-inflammatory drugs and other fundamental treatments were regularly administered to all the patients included. The clinical data that was gathered encompassed age, gender, disease progression, and clinical laboratory markers. The clinical laboratory indicators included erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), immunoglobulin A (IgA), immunoglobulin M (IgM), immunoglobulin G (IgG), complement component 3 (C3), and complement component 4 (C4). After screening and deduplication, After collecting the clinical data of 705 AS patients, they were split into the JQP group and the non-JQP group based on whether they had been given JQP treatment. Out of the total, 263 individuals were given JQP treatment while 4423 were not given JQP treatment (Review number: 2020AH-208).

2.3. Association rule analysis

An Apriori algorithm-based association rule analysis was performed for mining association relationships among items. JQP and laboratory parameters were used as variables. The use of JQP in AS patients was scored as 1, and 0 otherwise; any improvement in laboratory parameters was assigned as 1, and 0 otherwise, with minimum support of 30 %, minimum confidence of 50 %, and a lift of >1.

2.4. Random walk model for laboratory indicators

The ORACLE 10 g tool was employed to assess the random walk model of immune-inflammatory indicators between the two groups in order to measure the effectiveness of JQP treatment on laboratory indicators in AS patients numerically. Randomized stroll models can be used to determine whether JQP treatment is associated with improved immune inflammatory markers in the long run, and to determine improvements in overall patient outcomes.

2.5. Acquisition of active ingredients, potential targets, and signaling pathways

The active ingredients and corresponding targets of each TCM in the core prescription were screened from the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (https://tcmspw.com) by the criteria of drug likeness (DL) $\geq$ 0.18 and oral availability (OB) $\geq$ 30 %. Gene/pathway-protein data were obtained via UniProt (https://www.uniprot.org/) with conditions set to Popular Organism: Human and Status: Review (Swiss-Prot). Through integration, the information of active ingredients-target genes-pathways was finally obtained.

With “ankylosing spondylitis” as keywords, AS-related target genes were obtained from 5 disease databases including Therapeutic

Target Database (http://db.idrblab.net/ttd/), Human Online Mendelian Inheritance (https://www.omim.org/), Drug Database (https://www. drugbank.ca/), Gene Cards (https://www.genecards.org/), and DisGeNET (https://www.disgenet.org/).

2.6. Construction of PPI network

Using the Search Tool for the Retrieval of Interacting Genes (STRING) and setting the species to "Homo sapiens" with a minimum interaction score >0.4, the PPI network was constructed. For PPI network visualization, the analysis results were installed in Cytoscape 3.7.2 after being exported to a "TSV" format file. For maximum group centrality (MCC) screening, the degree value is utilized, and the top 20 target genes were chosen as core target genes.

2.7. KEGG analysis

The potential target genes were imported into Metascape (https://metascape.org/) for customized analysis, and the species was limited to "Homo sapiens". The P-value cutoff for KEGG analysis was set at 0.05. Bioinformatics (http://www.bioinformatics.com.cn/), an online bioinformatic analysis and visualization cloud platform, was used for visualization.

2.8. Preparation of JQP-medicated serum

After a week of adaptive feeding, 20 male Sprague-Dawley (SD) rats, specifically pathogen-free and weighing 180–220 g (Lscxk 2017-001, Experimental Animal Center of Anhui Medical University), were randomly assigned to the usual group and the JQP group. JQP (32.4 mg/100g) was administered intragastrically at a dose of 20 times the clinical equivalent, which was calculated using a conversion coefficient based on body surface area from human to rat. For seven days in a row, the same amount of solvent was administered once daily to the usual group. Rats' abdominal aortas were used to harvest blood 1 h following the previous injection. The blood was then centrifuged for 10 min at 3000 r/min to extract serum, and the samples were kept in a water bath at 56 °C for 30 min for inactivation, separated at −80 °C for aseptic operation, and stored for use. The study adheres to the 3R guidelines, namely Replacement, Reduction and Refinement. This study was approved by the Experimental Animal Ethics Committee of Anhui University of Traditional Chinese Medicine (Review number: AHUCM-rats-2023020).

2.9. Molecular docking of JQP core components with STAT3

The common targets of JQP intervention in AS disease were imported into Cytoscape 3.7.2 to construct a "drug-active ingredient-target" network. The core components of JQP were sorted according to the degree value, and the top three components (quercetin, kaempferol, and wogonin) were selected for molecular docking with the pathway proteins. The Protein Data Bank (PDB) format file of the STAT3 protein was searched in the PDB database (https://www.rcsb.org/), and the Mol2 format file of the structure of quercetin, kaempferol, and wogonin was downloaded from the TCMSP database. Before molecular docking, the target protein receptor molecules were dehydrated and the ligand small molecules were removed using PyMOL software. The target protein was hydrogenated using Auto Dock 4.2.6 software. Finally, the receptor proteins were molecularly docked with ligand small molecules using Auto Dock Vina 1.1.2 software and visualized using PyMOL 2.3.0 software. The binding energy between receptor proteins and ligand small molecules was less than −5 kcal/mol, indicating a strong binding force.

2.10. Cultivation of AS-FLSs

Purchased from Subikang iCell Bioscience Inc. (Shanghai, China), AS-FLSs (NO: JDBG200752) were cultured at 37 °C with 5 % CO2 in a standard cell culture medium supplemented with 10 % fetal bovine serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Twice a week, the medium was changed until a sufficient number of cells were present. The cultures were then separated and kept in storage at −80 °C.

2.11. Reagent handling and cell transfection

The JAK2/STAT3 pathway inhibitor AG-490 (10 μm) (MCE, No.: HY-12000) was applied to AS-FLSs for a duration of 24 h. To overexpress NONHSAT227927.1, the NONHSAT227927.1 gene was introduced into the pcDNA3.1 plasmid (GenePharma, Shanghai, China). Additionally, an empty pcDNA3.1 vector was created. Furthermore, three si-RNA sequences that target NONHSAT227927.1 were created in order to mute the latter. The following primers were utilized: si-RNA-NC (forward sequence: 5′-UUCUCCGAACGUGUCACGUTT-3′ and reverse sequence: 5′-ACGUGACACGUUCGGAGAATT-3′), si-NONHSAT227927.1.1 (forward sequence: 5′-CGACUGACUCGAUCUUUGAAG-3′ and reverse
sequence: 5′-ACGUGACACGUUCGGAGAATT-3′), si-NONHSAT227927.1.2.
(forward sequence: 5′-CGACUGACUCGAUCUUUGAAG-3′ and reverse
sequence: 5′-UCAAAGAUCGAGUCAGUCGGG-3′), and si-NONHSAT227927.1.3.
(forward sequence: 5′-AGAGGUGUUCAUUAUGGAAUC-3′ and reverse
sequence: 5′-UUCCAUAAUGAACACCUCUGA-3′). Cell transfection was performed using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, California, USA). The cells were harvested for the following detection 48 h after the transfection.

2.12. Enzyme-linked immunosorbent assay (ELISA)

AS-FLS culture supernatant was collected, seeded into a microtiter plate (100 μL per well), and incubated at 37 °C for 1.5 h. The levels of IL-6 (JYM0140Hu), IL-17 (JYM0082Hu), IL-10 (JYM0155Hu), and TNF-α (JYM0110Hu) were found in complete accordance with the ELISA kits' recommendations. Using a microplate reader, the optical density (OD) at 490 nm was determined. Every sample underwent three independent tests.

2.13. Real-time quantitative polymerase chain reaction (RT-qPCR)

The RNAiso Plus Reagent (TaKaRa) was utilized to extract total RNA. β-actin was used as the internal control when creating the cDNAs using the PrimeScriptTMRT Reagent Kit with gDNA Eraser (TaKaRa, Japan). To determine the relative expression values, the 2-ΔΔCt method was used.

2.14. Cell counting kit-8 (CCK-8) assay

The CCK-8 assay was used to detect the effect of different concentrations of JQP on the viability of AS-FLSs at 24, 48, and 72 h. AS-FLSs were digested with trypsin, and 100 μL cell suspension was added to each well of a 96-well culture plate. Upon reaching 80 % confluence, the cells were supplemented with the pre-prepared JQP-containing serum at different concentrations and incubated for 12, 24, 48, and 72 h. Thereafter, 10 μL CCK-8 solution was added into each well for another 1.5-h incubation. The absorbance of each well at 450 nm was detected using a microplate reader to evaluate the effect of different concentrations of JQP on the viability of AS-FLSs.

2.15. Western blot (WB)

The cells were subjected to radio-immunoprecipitation assay cell lysis buffer to extract the total protein, which was then quantified, loaded with samples, electrophoresis, and transferred to the membrane. The membrane was treated with 5 % skim milk at room temperature for 2 h, followed by an overnight incubation at 4 °C with primary antibodies against phosphorylation p-STAT3 (1:1000), *p*-JAK2 (1:500), JAK2 (1:500), and STAT3 (1:1000). Subsequently, the membrane underwent incubation with the horseradish peroxidase-labeled secondary antibody (diluted at a 1:1 × 104 ratio) at ambient temperature for a duration of 2 h. All protein bands were observed through electrochemiluminescence. The relative expressions of target proteins were computed by dividing the absorbance of LaminB1 by that of glyceraldehyde-3-phosphate dehydrogenase.

2.16. Statistical analysis

This study adopted a retrospective self-controlled case series design. Data were analyzed using SPSS 22.0 software (IBM Corp., USA) and expressed by median and quartile (P25, P75). Categorical variables (gender) were compared using the chi-square test. The paired *t*-test or Kruskal-Wallis nonparametric test was used to evaluate the significance of differences between the two groups. A value of $P < 0.05$ was indicative of significant differences. The plant names mentioned in the article have been checked through World Floraonline (www.worldfloraonline. org). Map plotting was performed with Graphpad Prism 8.0, and puzzles were made with Adobe Illustrator 2022.

Table 1
Baseline matching of AS patients.

	Before PSM			After PSM		
	JQP group (n = 263)	Non-JQP group (n = 442)	P	JQP group (n = 263)	Non-JQP group (n = 263)	P
Age	43 (34,52)	37 (30,48)	**<0.01**	43 (34,52)	41 (31,52)	0.54
Gender, n						
Male	70	125	0.62	70	70	1
Female	193	317		193	193	
Disease duration, years	9 (8,11)	8 (7,10)	**<0.01**	9 (8,11)	9 (7.50,10.00)	0.06
ESR, mm/h	25 (14,43)	26 (14,54)	0.35	25 (14,43)	26 (13,51)	0.47
CRP, mm/L	18.87 (5.91,37.55)	9.83 (1.39,31.47)	0	18.87 (5.91,37.55)	11.24 (1.50,34.67)	0.65
IgA, mmol/L	2.42 (1.92,3.22)	2.1 (1.65,2.80)	**0.01**	2.42 (1.92,3.23)	2.27 (1.79,3.01)	0.23
IgG, mmol/L	12.6 (10.50,15.23)	11.7 (9.87,14.18)	0.87	12.6 (10.50,15.26)	12.07 (10.50,15.01)	0.52
IgM, mmol/L	1.03 (0.80,1.39)	1.11 (0.80,1.48)	**<0.01**	1.03 (0.80,1.39)	1.09 (0.80,1.46)	0.14
C3, g/L	1.24 (1.08,1.40)	1.17 (1.05,1.35)	**<0.01**	1.24 (1.08,1.40)	1.18 (1.03,1.36)	0.23
C4, g/L	0.31 (0.26,0.36)	0.29 (0.26,0.35)	**<0.01**	0.31 (0.26,0.36)	0.28 (0.24,0.34)	0.63

Notes:: Chi-square test was used for gender comparison, and Mann-Whitney nonparametric test was used for age, disease duration, and laboratory parameter comparisons.

3. Results

3.1. Changes of laboratory parameters in AS patients after JQP treatment

The data of 705 AS patients admitted to the Rheumatology Department of Anhui Provincial Hospital of Traditional Chinese Medicine between June 2012 and June 2020 was gathered. The JQP group and the non-JQP group were the two groups to which these patients were allocated. The patient's baseline data underwent Propensity Score Matching (PSM) in a 1:1 ratio to mitigate any potential selection biases. The volume difference that was matched was set to 0.01, and the fixed covariates were age, gender, disease duration, number of hospitalizations, and clinical laboratory parameters. After PSM, 526 AS patients were included in the study, with 263 from the JQP group and 263 from the non-JQP group (Table 1). Following the comparison, there was no significant disparity in the initial demographic attributes and clinical and laboratory parameters between the two cohorts.

3.2. Effect of JQP on clinical laboratory parameters in patients with AS after PSM

The levels of ESR, CRP, IgA and C4 in the JQP group were decreased after treatment compared with those before treatment ($P < 0.01$). The levels of IgG, IgM, and C3 in the JQP group were not statistically significant before and after treatment ($P > 0.05$). The levels of ESR, CRP, IgA and IgG in the non-JQP group were decreased significantly after treatment ($P < 0.01$), but the changes in the levels of IgM, C3 and C4 were not statistically significant. In addition, Comparing the two groups before and after treatment, the ESR, CRP and IgA indicators of the JQP group after treatment were significantly improved compared with the non-JQP group. The non- JQP group improved C4 better than the JQP group, suggesting that JQP has advantages over the non-JQP group in improving AS immune and inflammatory indicators.($P < 0.05$; Table 2).

3.3. Examining the correlation between JQP and clinical laboratory indicators through association rule analysis

The former item was set as JQP and the latter item as laboratory indicator, with a minimum confidence of 55 % and a minimum support of 30 %. The Apriori algorithm-based association rule analysis showed that JQP improved the confidence level of ESR to 81.89 %, CRP to 70.94 %, IgG to 69.81 %, and C4 to 60.75 %. The improvement degree of the above indicators was all greater than 1 (Fig. 2). These results suggested that JQP was closely associated with the improvement of immune-inflammatory indicators in AS patients.

3.4. Random walking evaluation model of laboratory indicators after JQP treatment

Random walking was performed for the indicators with statistical differences before and after treatment in the JQP group, and the random walk steps of ESR, CRP, and C4 were 48, 63, and 18, respectively. The random positive growth rates of ESR, CRP, and C4 were 0.24 ± 0.10, 0.31 ± 0.14, and 0.33 ± 0.12, respectively. The number of comprehensive evaluation records for ESR, CRP, and C4 were 114, 319, and 313, respectively (Fig. 3). The clinical significance of the above data indicates that every time the comprehensive evaluation of ESR, CRP, and C4 indicators is improved, the patient needs to take 11.74, 9.42, and 4.64 steps, respectively.

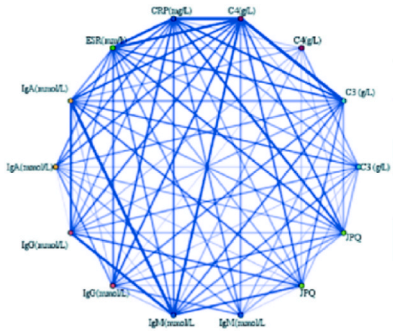
Network pharmacology and molecular docking of the main components of JQP with STAT3 protein molecule.

From TCMSP database, coix seed (Yi Yi Ren: YYR), Astragalus flavone (Huang Qi: HQ), tripterygium wilfordii (Lei Gong Teng: LGT), centipede (Wu Gong: WG), gardenia jasminoides (Zhi Zi:ZZ), Chinese clematis root (Wei Lin Xian:WLX), s.baicalensis(Huang-Qin:HQ) and peach kernel (Tao Reng:TR) were retrieved from the database. 163 active compounds and 218 potential target compounds were obtained. Through the screening of genecards database and OMIM database, 2073 as targets were obtained. By examining the intersection of drug and disease targets, 69 overlapping genes were identified (Fig. 4A). The above results show that these 69 Chinese medicine candidate targets are involved in the occurrence process of AS (Fig. 4B). They were screened by degree value through

Table 2
Effect of JQP on clinical laboratory parameters of AS patients.

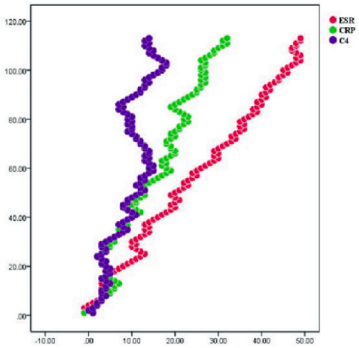
	JQP group (n = 263)			Non-JQP group (n = 263)			
	Before treatment	After treatment	Difference	Before treatment	After treatment	Difference	P
ESR, mm/h	25 (14,43)	12(7.21)	7(0,22)[c]	26 (13,51)	19 (11,34)	1(0,14)[b]	**<0.01**
CRP, mg/L	18.87 (5.91,37.55)	5.74(2.04,14.28)	7.25(0.78,22.83)[c]	11.24 (1.50,34.67)	1.94 (0.50,10.31)	2.96(0,21.67)[b]	**<0.01**
IgA, mmol/L	2.42 (1.92,3.23)	2.22(1.71,3.10)	0(0,0.24)[a]	2.27 (1.79,3.01)	2.24 (1.72,2.98)	0(0,0.14)[a]	**<0.01**
IgG, mmol/L	12.6 (10.50,15.26)	12.4 (10.30,15.00)	0(0,0.07)	12.07 (10.5,15.01)	11.9 (10.23,14.28)	0(0,0.01)[a]	0.08
IgM,mmol/L	1.03 (0.80,1.39)	1.04 (0.79,1.37)	0(0,0)	1.09 (0.80,1.46)	1.09 (0.77,1.46)	0(0,0.01)	0.53
C3, g/L	1.24 (1.08,1.40)	1.20 (1.06,1.35)	0(0,0.13)	1.18 (1.03,1.36)	1.14 (0.99,1.3)	0(0,0.09)	0.51
C4, g/L	0.31 (0.26,0.36)	0.26 (0.20,0.31)	0.03(0,0.09)[b]	0.28 (0.24,0.34)	0.27 (0.22,0.32)	0(0,0.04)	**<0.01**

Notes.
The P-value was the difference between the JQP group and the non-JQP group after treatment, and the Mann-Whitney nonparametric test was the statistical method used.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.



Items(LHS→RHS)	Support %	Confidence %	Lifts
JQP →CRP	81.89	41.1	1.27
JQP→ESR	70.94	35.61	1.19
JQP →C4	69.81	35.04	1
JQP →IgG	60.75	30.49	1.28

Fig. 2. Examining the correlation between JQP and clinical laboratory indicators through association rule analysis.



	Maximum value of random fluctuation	Random walk steps	Walking positive growth rate	Improvement factor	Stochastic volatility power law value	Comprehensive evaluation record	Ratio
ESR	49	322	0.24±0.10	0.22	0.09	114	11.74
CRP	63	322	0.31±0.14	0.28	0.08	319	9.42
C4	18	322	0.33±0.12	0.32	0.19	313	4.64

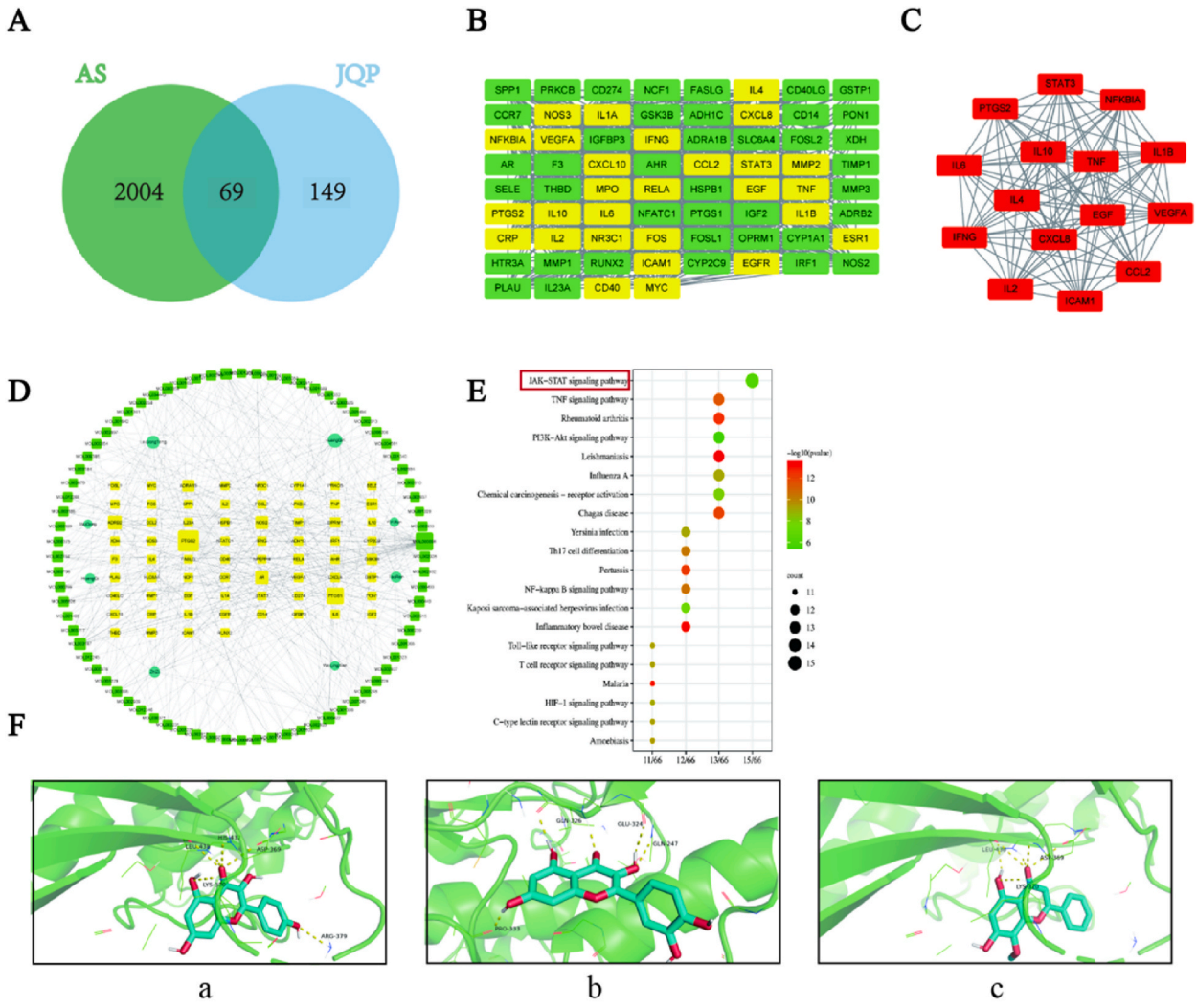
Fig. 3. Random walk model of inflammatory indicators in AS patients treated with JQP. Note: Time changes are indicated by the horizontal line's increasing length as the number of walks increases. As the intervention bears effect, the vertical line's height rises, signifying that the indicator is becoming healthier.

the PPI network, and the top 20 proteins were finally screened out (Fig. 4C), which are the targets of Chinese medicine against AS immune inflammation. In addition, we used Cytoscape 3.7.2 software to draw the drug active ingredient-target gene network (Fig. 4D), and KEGG further analyzed 69 candidate targets. The results showed that the JAK2/STAT3 pathway related to AS was involved in the top 20 candidate targets (Fig. 4E).

KEGG results revealed the importance of the JAK2/STAT3 signaling pathway for AS. Based on Cytoscape 3.7.2 software drug compound target gene network in the degree value of the ranking, the top three core compounds are quercetin, kaempferol and wogonin (Fig. 4D), that is, the above components were selected for molecular docking with the pathway proteins. we selected the top three core drug active ingredients (quercetin, kaempferol, and wogonin) for molecular docking with the STAT3 (4ZIA) protein (Fig. 4F). Use PyMOL 2.1 software to visualize the compounds formed after docking with the protein, and obtain the binding mode of the compound and the protein. Based on the binding mode, the amino acid residues and protein pockets of the compounds can be clearly seen. The active amino acid residues that quercetin binds to the STAT3 target protein are ARG-379, ASP-369, HIS-437, LEU-438 and LYS370. The active amino acid residues that kaempferol binds to STAT3 target protein are GLN-326, GLU-324 and GLN-247. The active amino acid residues that wogonin binds to STAT3 target protein are LEU-438, ASP-369 and LYS-370. The compound exhibits a robust reactivity with the aforementioned amino acid residues, enhancing its stability within the JAK2/STAT3 protein pocket and potentially functioning as a potent small molecule.

3.5. Effects of NONHSAT227927.1 on cell viability, inflammatory cytokines and inflammatory pathways

In previous studies, we found that NONHSAT227927.1 was highly expressed in AS patients, and the ROC curve results showed that NONHSAT227927.1 has significant diagnostic value [32] for AS. In order to explore the impact of NONHSAT227927.1 expression on AS inflammatory response, we constructed 3 si-RNAs targeting NONHSAT227927.1, and selected si-NONHSAT227927.1#3 with the best transfection efficiency for subsequent experiments (Fig. 5A). RT-PCR and CCK-8 detection showed that overexpression of NONHSAT227927.1 enhanced the activity and expression of AS-FLS, while silencing NONHSAT227927.1 decreased the activity and expression of AS-FLS (Fig. 5B–C). Increased levels of IL-6, IL-17, and TNF-α and decreased levels of IL-10 were observed in the overexpression NONHSAT227927.1 group. On the contrary, silencing NONHSAT227927.1 showed the opposite trend (Fig. 5D–G). The results showed that, NONHSAT227927.1 promotes the progression of inflammation. To further verify the regulation of the JAK2/-STAT3 pathway by NONHSAT227927.1. RT-PCR, WB and IF showed that overexpression of NONHSAT227927.1 increased the levels and proteins of JAK2, STAT3, *p*-JAK2 and p-STAT3, while silencing NONHSAT227927.1 showed the opposite trend (Fig. 5H–K),



Traditional Chinese medicine	Activeingredients	MoleculeName	Degree	Core targets	Binding energy
Astragalus + Gardenia	MOL000098	quercetin	254	STAT3(4ZIA)	-7.9kcal/mol
Astragalus + Tripterygium + Gardenia	MOL000422	kaempferol	150	STAT3(4ZIA)	-7.3kcal/mol
Scutellaria	MOL000173	wogonin	77	STAT3(4ZIA)	-7.1kcal/mol

Fig. 4. Network pharmacology and molecular docking of the main components of JQP with the STAT3 protein molecule. A. Venn diagram of candidate target genes of JQP and AS. B. Protein-protein interaction network, the darker the color, the higher the importance. C. Degree screening of the top 20 gene targets. D. Cytoscape 3.7.1 The software draws the active ingredient-target gene network of TCM, in which the yellow square represents the target gene, the green circle represents the traditional Chinese medicine, and the green square represents the active ingredient of the traditional Chinese medicine. E. Bubble chart of KEGG pathway enrichment analysis of candidate AS targets. The size of the dots indicates the number of selected genes, and the color indicates the p value of the enrichment analysis. F. Visualization of molecular docking of active ingredients and core protein targets in the core formulation. (a) Docking diagram of quercetin (MOL000098) and STAT3. (b) Docking diagram of kaempferol (MOL000422) and STAT3. (c) Docking diagram of wogonin (MOL000173) and STAT3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

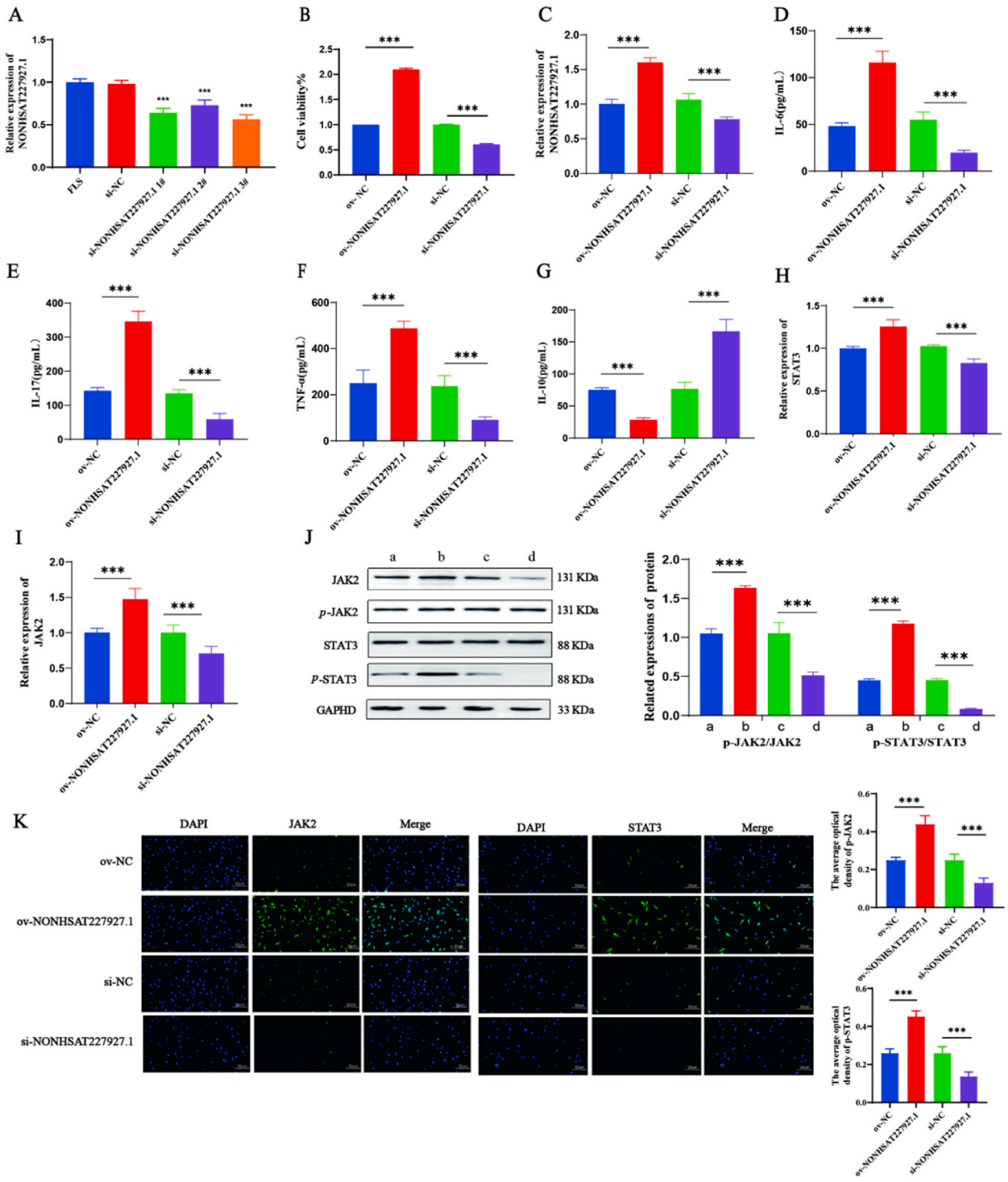


Fig. 5. Effects of NONHSAT227927.1 on cell viability, inflammatory cytokines and inflammatory pathways.A, Screening of NONHSAT227927.1 small interfering RNA model. B, CCK-8 method was used to detect the cell viability of AS-FLS. C, RT-qPCR was used to detect the expression of NONHSAT227927.1 in AS-FLS. D-G, ELISA was used to detect the levels of IL-6, IL-17, TNF-α, and IL-10 in AS-FLS. H–I, RT-qPCR was used to detect the levels of JAK2 and STAT3 in AS-FLS. J, WB was used to detect Levels of JAK2, STAT, *p*-JAK2 and p-STAT3 in AS-FLS. K, IF was used to detect the expression of JAK2 and STAT3 in AS-FLS. All experiments were repeated three times.a: ov-NC, b: ov-NONHSAT227927.1, c: si-NC, d: si-NONHSAT227927.1. ***P $<$ 0.001.

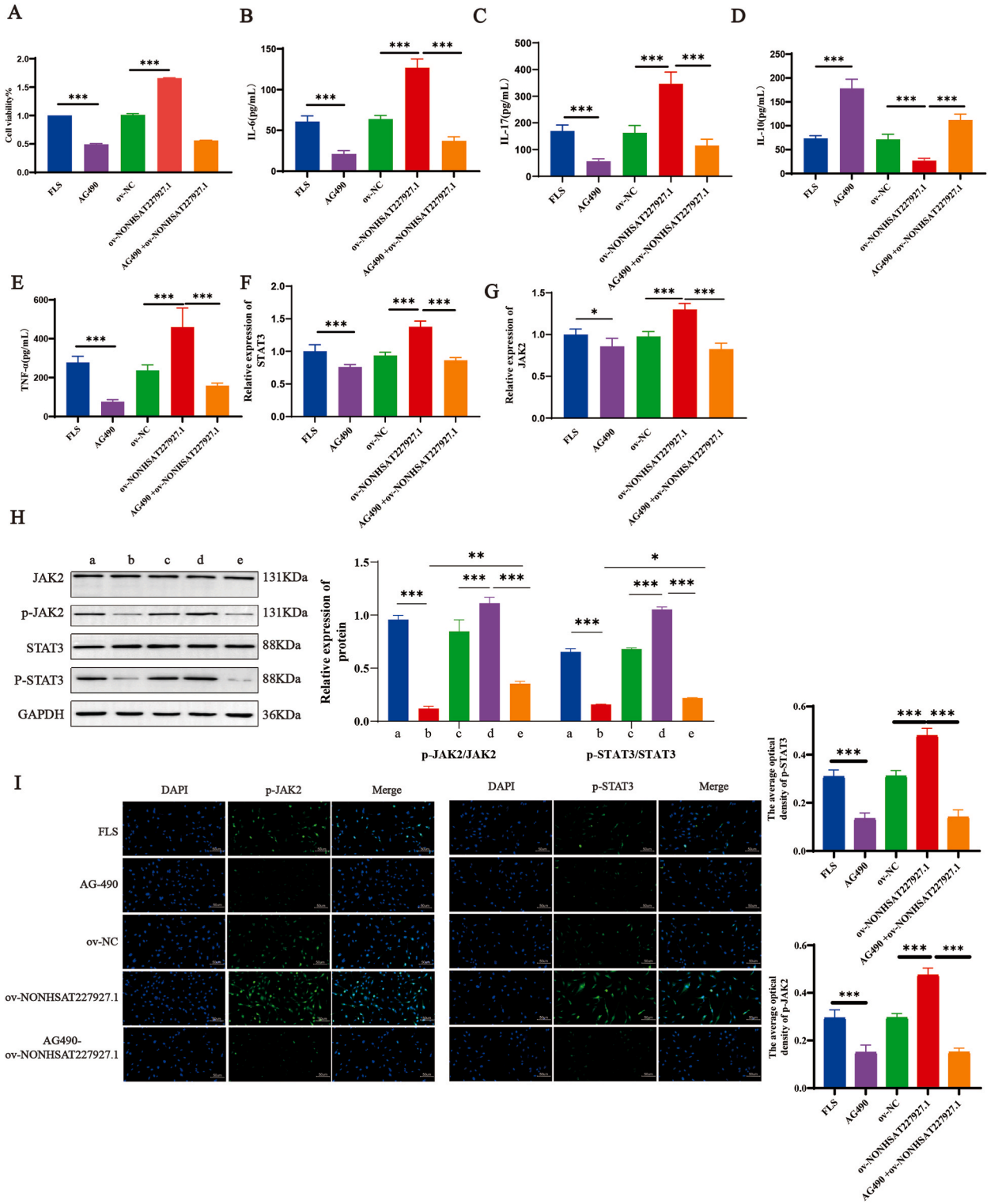


Fig. 6. NONHSAT227927.1/JAK2/STAT3 combination regulates the activity of AS-FLS and the release of inflammatory factors. A, CCK-8 method was used to detect the cell viability of AS-FLSs. B-E, ELISA method was used to detect the levels of IL-6, IL-17, TNF-α, and IL-10 in AS-FLS. FG, RT-qPCR was used to detect Levels of JAK2 and STAT3 in AS-FLS. H, WB was used to detect the levels of JAK2, STAT, *p*-JAK2 and p-STAT3 in AS-FLS. I, IF was used to detect the expression of JAK2 and STAT3 in AS-FLS. All experiments were repeated three times. a: FLS, b: AG490, c: ov-NC, d: ov-NONHSAT227927.1, e: AG490 + ov-NONHSAT227927.1.*P < 0.05,**P < 0.01,***P < 0.001.

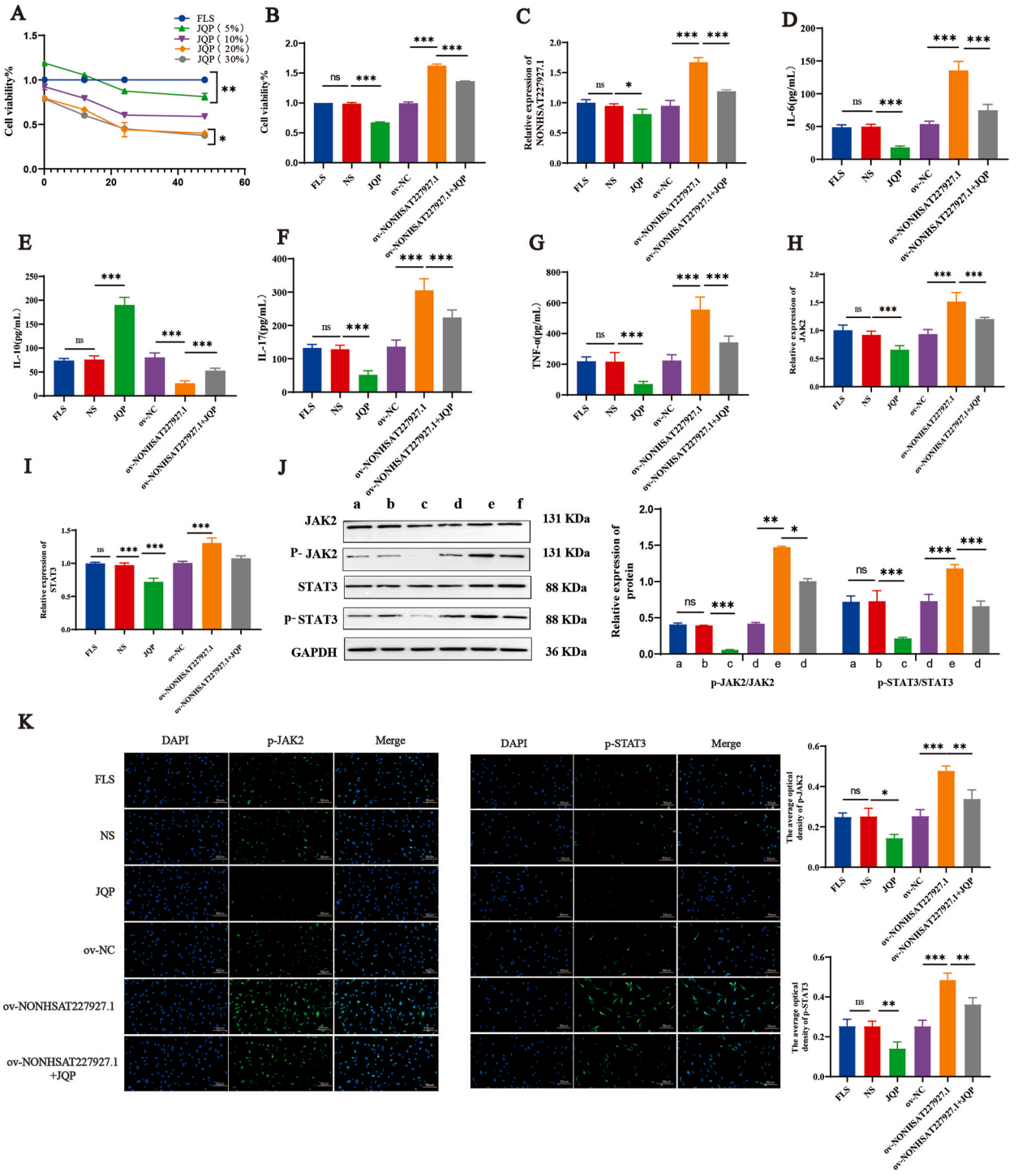


Fig. 7. JQP reversed the effects of NONHSAT227927.1 overexpression on AS-FLS viability, inflammatory factors and JAK2/STAT3 pathway. A, CCK-8 method was used to screen the optimal concentration of JQP-containing serum. B. Use CCK-8 method to detect the cell viability of AS-FLSs. C. RT-qPCR detects the expression of NONHSAT227927.1. D-G used ELISA to detect the levels of IL-6, IL-17, TNF-α, and IL-10. HI, RT-qPCR was used to detect the expression of JAK2 and STAT3. J. WB detects the expression of JAK2, STAT3, *p*-JAK2 and p-STAT3 proteins. K. Immunofluorescence method detects the expression of *p*-JAK2 and p-STAT3 proteins. a: FLS, b: NS, c: JQP, d: ov-NC, e: ov-NONHSAT227927.1, f: ov-NONHSAT227927.1 + JQP. The data is displayed in the form of the average value plus or minus the standard deviation. The experiments were conducted thrice.*P < 0.05,**P < 0.01,***P < 0.001.

suggesting that the NONHSAT227927.1/JAK2/STAT3 combination can inhibit the inflammatory response of AS-FLS.

3.6. NONHSAT227927.1/JAK2/STAT3 regulated AS-FLS viability and inflammatory cytokine release

To investigate whether NONHSAT227927.1 affected the inflammatory response of AS-FLSs via JAK2/STAT3, We first constructed the NONHSAT227927.1 small interfering plasmid and overexpression plasmid (Fig. 6A–B). we used a specific STAT3 inhibitor AG-490 to inhibit STAT3 activation. RT-qPCR and ELISA results showed that AG490 reduced AS-FLS viability and decreased inflammatory cytokines. Overexpression of NONHSAT227927.1 significantly enhanced AS-FLS viability and elevated inflammatory cytokines, while the addition of AG490 reversed the effect of NONHSAT227927.1 overexpression on AS-FLS viability and inflammatory cytokines (Fig. 6C–F). Further, we performed immunofluorescence and Western blot to explore the effect of AG490 on JAK2, STAT3, *p*-JAK2, and p-STAT3 proteins. The results revealed that overexpression of NONHSAT227927.1 significantly increased JAK2, STAT3, *p*-JAK2, and p-STAT3 proteins, while adding AG490 reversed the effect of NONHSAT227927.1 overexpression on the JAK2/STAT3 pathway. It was indicated that NONHSAT227927.1 induced inflammation in AS-FLSs through JAK2/STAT3(Fig. 6F–I).

3.7. JQP reversed the effect of NONHSAT227927.1 overexpression on AS-FLS viability, inflammatory factors, and JAK2/STAT3 pathway

To determine whether JQP inhibits the inflammatory response to AS through NONHSAT227927.1. We first screened for the optimal concentration of JQP-containing serum. we established the culture medium containing 5 %, 10 %, 20 %, and 30 % JQP-containing serum and detected the viability of AS-FLSs at 0, 12, 24, 48, and 72 h. The results showed that the 20 % JQP-containing serum group had the strongest inhibitory effect on cell viability at 48 h ($P < 0.01$, Fig. 7A). Consequently, a serum containing 20 % JQP was chosen for the subsequent experiments. CCK-8, RT-qPCR, and ELISA were used to evaluate the effect of JQP on AS-FLS viability, NONHSAT227927.1 expression, and inflammatory cytokines. The results showed that JQP-containing serum decreased AS-FLS viability, NONHSAT227927.1 expression, as well as IL-17, IL-6, and TNF-α levels, but increased IL-10 expression. Meanwhile, overexpression of NONHSAT227927.1 enhanced AS-FLS viability and increased inflammatory cytokines (Fig. 7A). In order to establish if the influence of JQP on AS-FLSs was attained through the regulation of NONHSAT227927.1 expression, functional rescue experiments were executed. The findings showed that JQP treatment partially reduced the impact of NONHSAT227927.1 overexpression on the concentrations of IL-6, IL-17, and TNF-α in AS-FLSs. ($P < 0.01$, Fig. 7B–G). Furthermore, IF and WB showed that JQP-containing serum reduced the expressions of JAK2, STAT3, *p*-JAK2, and p-STAT3, and overexpression of NONHSAT227927.1 increased the expressions of JAK2, STAT3, *p*-JAK2, and p-STAT3. The promotion effect of NONHSAT227927.1 overexpression on the pathway proteins was interrupted by JQP treatment (Fig. 7H–K). In conclusion, the results showed that JQP inhibited the inflammatory response of AS-FLSs via NONHSAT227927.1/JAK2/STAT3.

4. Discussion

Since AS is an immune-mediated arthritis characterized by chronic inflammation, the main goal of currently available treatments is to mitigate the inflammatory burden [33]. The international guidelines for AS management have also highlighted the importance of inflammation control, progressive joint damage prevention, and bodily function maintenance [34,35]. However, conventional synthetic disease-modifying antirheumatic drugs (DMARDs) or long-term corticosteroids yield limited effects for alleviating AS symptoms [36,37]. Compared with other rheumatoid diseases such as rheumatoid arthritis and psoriatic arthritis, AS has fewer treatment options [38]. Traditional Chinese medicine has served as complementary medicine for AS treatment for a long time, with the characteristics of multi-component, multi-target, and multi-channel.

At first, this study conducted retrospective data mining and assigned 705 AS patients to the JQP group and non-JQP group, and 263 AS patients treated with JQP were eventually selected after PSM. Compared with the non-JQP group, the JQP group exhibited a better improvement effect on immune-inflammatory indicators. Association rule analysis showed that JQP was strongly associated with ESR, CRP, and IgG. It was suggested that JQP was closely related to the improvement of immune-inflammatory markers in AS patients. The random walk model considers each patient's individual treatment outcome as a single point and gauges the efficacy of the treatment by calculating the proportion of the cumulative fluctuation value of the random walk to the random walk point, which is the random positive increase rate [39]. The evaluation of the random walk model showed that JQP was linked to a long-term amelioration of immune inflammation in individuals with AS. Then, we performed KEGG analysis on JQP and AS to find out the potential pathway of JQP acting on AS. The top three core components of JQP were molecularly docked with STAT3 protein, and the results demonstrated that quercetin, kaempferol, and wogonin had a strong targeting relationship with the STAT3 protein. The JAK2/STAT3 signaling pathway serves as a major regulator of inflammatory responses in AS [40]. JAKs, together with several STAT proteins, mediate the signal transduction of various extracellular cytokines and impact a wide range of cellular functions. Among them, STAT3 has been reported to participate in the IL-6-dependent induction of acute phase response genes [41].

Growing evidence has indicated the vital role of lncRNAs in the pathology of autoimmune diseases, including AS [42]. LncRNAs can predict disease recurrence and poor outcomes of AS patients, functioning as promising biomarkers for AS [43,44]. Under the pressure of the internal and external environment of the body, non-specific immune cells can specifically express related lncRNAs to regulate the differentiation and function of immune cells, thereby regulating the host immune response [45] have found that lncRNA-AK001085 is poorly expressed in the serum of AS patients and has a negative correlation with immune-inflammatory markers including CRP and ESR. Ding et al. [46]. have also identified 35 lncRNAs aberrantly expressed in spondyloarthropathy. Our team previously performed high-throughput sequencing on AS-PBMCs and screened lncRNAs enriched in immune-inflammatory responses

with fold-difference (fold-difference ≥2, $P \leq 0.05$) for RT-qPCR validation. LncRNA NONHSAT227927.1 with a distinctly abnormal expression pattern was selected for further study. To verify whether NONHSAT227927.1 promoted the imbalance of cytokine network in AS by activating the JAK2/STAT3 pathway, we detected the expression of NONHSAT227927.1 in PBMCs of 30 AS patients and 30 normal individuals and found that NONHSAT227927.1 was highly expressed in AS patients. The ROC curve confirmed the diagnostic significance of NONHSAT227927.1 for AS. Thereafter, NONHSAT227927.1 was overexpressed or silenced in AS-FLSs, and it was found that NONHSAT227927.1 reduced AS-FLS viability and exacerbated inflammatory cytokine release. To verify whether NONHSAT227927.1 played a role in AS-FLSs by activating the JAK2/STAT3 pathway, we added the STAT3 inhibitor AG-490 into NONHSAT227927.1 overexpression-treated FLSs and found that the addition of AG490 reversed the promoting effect of NONHSAT227927.1 overexpression on JAK2/STAT3. Briefly, these results confirmed that the NONHSAT227927.1/JAK2/STAT3 axis promoted inflammatory responses in AS-FLSs.

JQP is composed of two Chinese patent medicines, XFC and HQC. Accumulating clinical studies have reported the promising efficacy and safety of JQP in treating arthritis patients, mainly manifested by reducing joint inflammation, improving joint function, and relieving pain [20,22]. In the HQC prescription, Huang Qin and Zhi Zi are the monarch drugs, which together play the role of clearing the lungs and detoxifying. Baicalin has anti-tumor, antibacterial, diuretic and other functions [47]. Zhi Zi has the functions of clearing away heat and diuresis, purging fire and relieving troubles. Among them, gardeniposide has more It has strong anti-inflammatory and analgesic effects [48]. The ministerial drug Wei Ling Xian has the effects of dispelling wind, removing dampness, unblocking collaterals and relieving pain, and can strengthen the treatment of rheumatic paralysis. Yi Yi Ren is used as an adjuvant to diuretic and dampness, invigorate the spleen and stop diarrhea, remove paralysis and detoxify [49]. Tao Ren mainly activates blood circulation and removes blood stasis, relaxes the menstrual flow, activates blood circulation, removes blood stasis and regenerates [50]. XFC is composed of the royal medicine HuangQi, which can replenish qi, strengthen the spleen, and strengthen the body [51]. The astragalus polysaccharide in it can enhance immune function and regulate body metabolism [52]. Yi Yi Ren can remove dampness and remove paralysis. Coix seed oil can promote cell apoptosis and inhibit cell apoptosis. Tumor cell processes. Lei Gong Teng can unblock the meridians and activate meridians, and triptolide can inhibit the expression of vascular endothelial growth factor and interleukin (IL-6) in synovial tissue. Centipede can dispel wind and relieve spasm. It has the functions of regulating the body's immunity and relieving airway spasm, and is widely used in the treatment of allergic diseases [53]. Network pharmacology shows that the core drug target of JQP in treating AS are quercetin, kaempferol, and wogonin. Quercetin can alleviate joint symptoms in arthritic mice, reduce inflammatory cytokines [54], and also affect the development and function of osteoblasts and osteoclasts [55]. Kaempferol reduces the severity of arthritis in collagen-induced arthritis mice and inhibits osteoclast differentiation in vitro and in vivo [56]. Wogonin can significantly improve the changes in the levels of oxidative stress markers, antioxidant proteins, and inflammatory cytokines in rheumatoid arthritis [57]. Consistently, we speculated that JQP exerted its anti-inflammatory effect on AS by inhibiting the JAK2/-STAT3 pathway. Through a series of experiments, we found that JQP reduced NONHSAT227927.1 expression, suppressed inflammatory factors, and inhibited the activation of the JAK2/STAT3 pathway. Furthermore, JQP-containing serum decreased

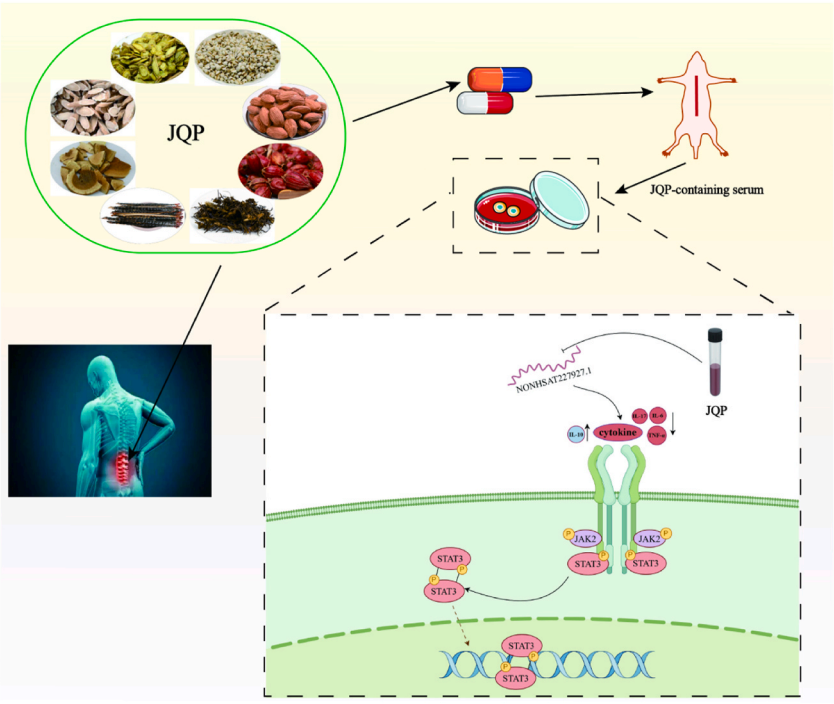


Fig. 8. JQP exerts an anti-inflammatory effect on AS by inhibiting the NONHSAT227927.1/JAK2/STAT3 axis.

NONHSAT227927.1, reduced JAK2 and STAT3 proteins, decreased IL-6, IL-17, and TNF-α, and increased IL-10. The performance of functional rescue experiments aimed to establish that the influence of JQP on AS-FLSs was mediated by NONHSAT227927.1. The results confirmed that the intervention of JQP-containing serum inhibited the activation of JAK2/STAT3 pathway and the enhancement of inflammation caused by overexpression of NONHSAT227927.1.

Nevertheless, there are still deficiencies in this paper, which need to be further studied in the future. This experiment in the patient body for the mechanism of the study could not be done, although the efficacy of JQP was proved in the clinical part, but for the mechanism of the part of the study was not supplemented by animal experiments, this part of the content is an important part of the team to be carried out in the future; for the fingerprint of JQP should be supplemented in order to accurately network pharmacological target selection; in vitro experiments did not design a blank serum group in order to exclude the therapeutic effect of rat serum. In the future, we will conduct in-depth research on the above deficiencies and analyze the specific mechanism of JQP to improve AS in depth.

5. Conclusions

To conclude, JQP exerts an anti-inflammatory effect on AS by inhibiting the NONHSAT227927.1/JAK2/STAT3 axis. JQP may be a promising drug candidate, providing more possibilities for regulating and suppressing inflammatory responses in AS and other autoimmune diseases (Fig. 8).

Data availability statement

Data from this study can be obtained from the corresponding authors upon reasonable request.

Ethical statement

The animal experiment part of this study was approved by the Experimental Animal Ethics Committee of Anhui University of Traditional Chinese Medicine (AHUCM-rats-2023020), and the clinical patient data was approved by the Ethics Committee of the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine (2020AH-208).

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CRediT authorship contribution statement

Xiang Ding: Writing – original draft, Visualization, Data curation. **Jian Liu:** Investigation, Funding acquisition, Formal analysis. **Yanqiu Sun:** Visualization, Data curation. **Xiaolu Chen:** Formal analysis, Data curation. **Xianheng Zhang:** Validation, Methodology, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34634.

Abbreviations

JQP	Jianpi Qingre Tongluo Decoction
AS	ankylosing spondylitis
C3	complement C3
C4	complement C4
CRP	C-reactive protein
DL	drug likeness
ESR	erythrocyte sedimentation rate
FLS	Fibroblast-like synoviocytes
GO	Gene Ontology
HQC	huangqin Qingre Chubi Capsule

XFC	xinfeng Capsule
IgA	immunoglobulin A
IgG|	immunoglobulin G
IgM	immunoglobulin M
IL-	interleukin
JAK/STAT	Janus kinase/signal transducer and activator of transcription
KEGG	kyoto encyclopedia of genes and genomes
LHS	left hand side
LncRNAs	long non-coding RNAs
MCC	maximum group centrality
OB	oral availability
OD	optical density
PBMC	peripheral blood mononuclear cells
PDB	protein data bank
PSM	propensity Score Matchin
RHS	right hand side
SD	sprague-dawley
STRING	search tool for the retrieval of interacting Genes
TCM	traditional Chinese Medicine
TCMSP	Traditional Chinese Medicine Systematic Pharmacology database and analysis platform
TNF-α	tumor necrosis factor alpha

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