Clinical Nutrition ESPEN 50 (2022) 124–132

Contents lists available at ScienceDirect

Clinical Nutrition ESPEN

journal homepage: http://www.clinicalnutritionespen.com

Original article

Metabolomic profiling in ankylosing spondylitis using time-of-flight mass spectrometry

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ARTICLE INFO

Article history:
Received 28 October 2021
Accepted 15 June 2022

Keywords:
Ankylosing spondylitis
Fatty acid
Metabolomics
Phosphatidylcholine
UPLC Q-TOF/MS
Plasma

SUMMARY

Background & aims: Ankylosing spondylitis (AS) is an inflammatory disease associated with destructive changes in the skeleton and joints. The exact molecular mechanism of the disease has not been fully elucidated. This study aimed to determine metabolic differences between active AS patients and healthy controls to understand the molecular mechanism of AS.

Patients and methods: The study included 38 subjects, comprising 18 patients with active AS and 20 healthy controls. Metabolic profiling of the plasma was performed using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC Q-TOF/MS). Data acquisition, classification, and identification were achieved with the METLIN (https://metlin.scripps.edu/) database and XCMS (https://xcmsonline.scripps.edu).

Results: Significant alterations were identified in the unsaturated fatty acids (FA), linoleic acid, alpha-linolenic acid, FA degradation, and FA biosynthesis pathways. Down -regulations were observed in phosphatidylcholine (PC) (16:0/0:0), beta-D-Fructose, stearic acid, trimipramine N-Oxide and muconic acid, and up-regulation were detected in PC (18:2/0:0), 3-Methylindole, palmitic acid (PA), alpha-Tocotrienol, and beta-D-glucopyranoside in active AS patients compared to the healthy control subjects.

Conclusion: Pathway analysis revealed that dysregulation in FA metabolism is associated with AS, and therefore, modulation of diet according to PA and PC may be potential therapeutic targets.

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1. Introduction

Ankylosing spondylitis (AS), a type of spondylarthritis, is a chronic, painful, and debilitating inflammatory disease, mainly affecting the axial skeleton and sacroiliac joints [1]. The main symptoms of the disease are inflammatory back pain and spinal stiffness. In severe cases, permanent motion limitations in the spinal column can also be observed. Due to the difficulties in diagnosis, the exact prevalence of the disease is unknown, but it has been reported to reach 49 per 10.000 [2]. Although endoplasmic reticulum (ER) unfolded protein response, human leukocyte antigen (HLA)-B27 positivity, ER aminopeptidases, arthritogenic peptides, and gut microbiome changes have been postulated to be associated with the pathogenesis of AS, the exact molecular mechanism is still not widely understood [3,4].

In recent years, omics platforms have been used for structural characterization and quantitation of a broad spectrum of small molecules. Metabolomics, which can be performed with either a targeted or untargeted approach, is one of the most critical and emerging parts of omics [5]. Untargeted metabolomics enables the detection of all possible metabolites simultaneously in cells, tissues, and biofluids. Therefore, untargeted metabolomics analyses help to identify novel biomarkers, determine new therapeutic targets, and understand the disease mechanism [6]. Nuclear magnetic resonance (NMR) spectroscopy and high resolution and highly accurate quadrupole time-of-flight (QTOF) mass spectrometry (MS) are the two most used techniques in untargeted metabolomics. Compared with QTOF-MS, NMR-based techniques have the disadvantages of lower sensitivity and discrimination capacity of the metabolites

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https://doi.org/10.1016/j.clnesp.2022.06.011
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[6–8]. Although many different sample types can be used in metabolomics analysis, serum and plasma are the most used sample types due to higher detectable metabolites than other biological fluids [6]. Recent studies have detected a few metabolites using targeted and untargeted metabolomics analyses in body fluids to understand disease pathogenesis [9–11], but these have not yet been proved to be helpful in routine practice.

This study conducted untargeted metabolomics analyses to investigate the plasma metabolic profile in AS patients and healthy control subjects using QTOF-MS. The study aimed to identify metabolites that could serve as biomarkers to help understand the metabolic changes in AS. To date, only a few metabolomics studies have been performed using NMR and MS techniques in patients with AS, and although novel information has been obtained, the molecular mechanism of the disease has not been dealt with in-depth. This study can be considered to contribute to the existing literature regarding AS.

2. Material and methods

2.1. Study design, recruitment, and sample collection

This prospective study was conducted in the Department of Clinical Biochemistry and the Department of Physical Medicine and Rehabilitation in Sivas Cumhuriyet University from July 2019 to August 2021. A total of 38 subjects were enrolled, comprising 20 healthy controls and 18 patients diagnosed with AS according to the modified New York criteria [12], clinical manifestations, laboratory findings, and MRI. For healthy control group, samples collected from health care workers working at the Sivas Cumhuriyet University Hospital. The exclusion criteria for control group included a clinical suspicion of infections or the presence of liver disease, kidney disease, any other rheumatic disease, chronic disease, malignancy, pregnancy, receiving any drug for the last 6 months or smoking. For patient group, the exclusion criteria included the presence of diabetes mellitus, hypertension, coronary artery disease, autoimmune disease, liver disease, kidney disease, other rheumatic diseases, and malignancy. None of the patients had previous history of treatment with biologic agent. However, patients were not excluded for a history of taking nonsteroidal anti-inflammatory drugs. The activity of the disease was assessed by Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) index. The index consists of 6 measurements related with the fatigue, spinal and peripheral joint pain, sensitivity, and morning stiffness. Total score above four or more are considered active disease [12]. After evaluating the disease activity, fasting blood samples were collected in green top tubes (Greiner, Kremsmünster, Austria) with lithium heparin. After centrifugation (3500 rpm, 15 min, 4 °C), the supernatant was immediately aliquoted and stored at −80 °C until analysis. All patients had ≥4 BASDAI scores. The mean BASDAI score was 6.53 ± 1.36. We also evaluated the disease activity using the criteria of Bath Ankylosing Spondylitis Functional Index (BASFI) and Ankylosing Spondylitis Disease Activity Score (ASDAS). The mean scores of BASFI and ASDAS in the patient group were 8 ± 4 and 3.66 ± 2.46, respectively. C-reactive protein (CRP) analyses were performed by using the turbidimetric method (Roche Cobas c 702), and erythrocyte sedimentation rate (ESR) was determined by using Wester green method (Symex Starrsed Interliner, Netherland). The patients' median CRP and ESR values were 5.5 (3–17.25) mg/L and 4.19 (1.94–13.65) mm/hour, respectively.

The procedures were approved by the Ethics Committee of Sivas Cumhuriyet University in accordance with the ethical standards established by the institution where the experiments were performed or in accordance with the Helsinki Declaration. All the subjects gave written informed consent before enrolment into the study (Decision number:2019-02-01).

2.2. Sample, quality control, and internal standard preparation

200 μL of thawed plasma samples homogenized in a vortex mixer were mixed with 600 μL of acetonitrile and incubated. The sample was vortexed and then stored in a refrigerator for 10 min then centrifuged (Nüve NF 1200R, Ankara, Akyurt) for 5 min at 16.900×*g*. The resulting supernatant was removed and evaporated to dryness for 12 h using a vacuum concentrator (Thermo Fisher, Scientific, Waltham, MA, USA). The dry extracts were then reconstituted in 200 μL of acetonitrile: water (1:1, v/v) containing 0.1% formic acid and centrifuged at 13.000 rpm (15,000×*g*) for 15 min at 4 °C to remove insoluble debris. The supernatants were transferred to 96-well plates. Glycine d5 and citric acid d4 were selected as the internal standard [IS] to improve data quality and check the autonomous integration success of the software. To guarantee mass accuracy, a reference solution was directly infused into the source, enabling continuous internal calibration during analysis, and ensuring accuracy and reproducibility. For that purpose, purine (*m*/*z* 121.0508) and hexakis phosphazinen (*m*/*z* 922.0097) signals were used. The reproducibility standard deviation of each internal standard was <20%. The blank sample was prepared with a mobile phase mixture containing only ISs. In addition, quality control (QC) samples were prepared by mixing 5 μL of extracted solution from each sample to ensure system stability and performance. The blank sample was injected to observe any interference from the instrument or media. QC samples were injected into the liquid chromatography quadrupole time of flight mass spectrometry (LC/Q-TOF/MS) system to remove false positives from the system. Analyses were performed immediately after the sample preparation.

2.3. LC- MS analysis

The LC/Q-TOF/MS system consisted of an Agilent 1290 Infinity LC system coupled with an Agilent 6530 Accurate-Mass Q-TOF mass spectrometer (Agilent, USA) and ZORBAX RRHD Eclipse Plus C18, 95 Å, 2.1 × 100 mm, 1.8 μm (Agilent, USA). The mobile phase system consisted of 0.1% formic acid in water (A) and acetonitrile (B) using a gradient elution as follows: A mobile phase flow rate of 0.4 mL/min was employed for gradient elution at a total 12-min run time as follows: 0–0.5 min, 2% B; 0.5–4 min, 20% B; 4–8 min, 50% B; 4–6 min, 95% B; 8–9 min, 95% B; 9–9.25 min 2% B and 9.25–12 min, 2% B for equilibration of the column. The column temperature was set at 55 °C during the analysis. The injection volume was 4.0 μL. The mass range was between 50 and 1000 *m*/*z*. 3.5-kV capillary voltage was applied during the study. Mass scanning ranged from 50 to 1000 *m*/*z*. The ion source was Electron Spray Ionization (ESI). The MS absorbance threshold was set at 2000. The instrument acquired data through the optimized parameters of drying gas temperature, 350 °C; drying gas flow, 11 L/min; nebulizer, 40 psi; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min. Fragmentor voltage was 275V. All samples and quality controls were analyzed using this method.

2.4. LC-MS/MS analysis

Quality control samples were analyzed using the MS/MS method. The chromatographic conditions were the same as in the Q-TOF MS analysis. Collision energy was set at 10V. Iterated- Data dependent Acquisition (I-DDA) workflow was followed [13]. Mass ranges were arranged as 50–270, 270–500, 500–750, and 750–1000 *m*/*z* for precursors.

2.5. Chemometric and statistical analysis

The total Ion chromatogram of the study is presented in Fig. 1. Such complex chromatograms mostly include thousands of peaks with huge intensity alterations across the spectrum. Therefore, several algorithms have been developed for peak detection. The XCMS (https://xcmsonline.scripps.edu/) centWave algorithm was selected for peak detection in this study. All samples were imported into XCMS online, peak detection was applied, and the Obiwarp algorithm of the software was used for peak alignment. The retention time of each chromatogram was optimized using this approach. Then, peak grouping was applied to obtain a unique retention time and *m*/*z* value called 'feature' for the analysis. Finally, an Excel sheet was generated for all samples, including retention time, unique *m*/*z* values, and intensity for each feature. This Excel sheet was imported into MATLAB for chemometrics analysis. Features with low reproducibility in QCs and detected in blanks were removed. Peaks that were not detected in half of the quality control samples were eliminated. All features lower than 20% standard deviation was considered for the chemometrics analysis. The batch effect was removed with the R package called 'stattarget2'. Quality control-based signal drift correction was maintained by following the workflow [14]. According to batch drift correction, hundreds of features were recovered in terms of %RSD value (Fig. 2). After cleaning the data, classification was performed by importing the data into PLS Toolbox 8.5, and Partial Least Square- Discriminant Analysis (PLS-DA) was implemented for each group. Three latent variables were selected to explain the model. The LV1-LV2-LV3 score plots are shown in Fig. 3, which shows the alteration of metabolites between each group. As seen in Fig. 4, groups were definitively separated. ROC analyses were also applied to evaluate the selectivity and sensitivity of the model and cross-validation data set. The area under the curve (AUC) values for the dataset and cross-validation were calculated to be 1.00 and 0.99, respectively. The specificity vs. selectivity graph shows that the model is not over-fitted, and chance correlation is not possible. Figure 4 is plotted by MATLAB pls Toolbox. Regression coefficients for both the model and cross-validation dataset were 0.98 and 0.85, respectively. These values confirmed both the goodness of fit and predictivity power of the PLS-DA model [15]. Fifty permutations were proceeded to 3 components for the permutation test in Fig. 4. According to Fig. 5, permuted Q2 [blue] and R2 values were significantly lower than the original data. It also explained the discriminatory ability of the model. A Variable in Projection (VIP) graph was also plotted (Fig. 6). VIP scores $\geq$1 were accepted as statistically significant for this model. Therefore, features with a VIP score $\geq$1 was considered for metabolite annotation. A total of 40 metabolites were annotated and 13 of them were significantly different between groups (VIP score $\geq$1). We analyzed the correlation between ASAS, BASFI, BASDAI, CRP, ESR, and annotated metabolites using the Spearmen method.

3. Results

The evaluation was made of the metabolic profiles of 18 AS-active patients with a mean age of 37 ± 9 years, and 20 healthy control subjects with a mean age of 36 ± 10 years ($p > 0.05$). The female to male ratios were 0.56 and 0.66 in the patient and control groups, respectively ($p > 0.05$). A total of two patients had HLA-B27 positivity. After data cleaning and chemometric analysis, several features were selected for the annotation part. Hundreds of *m*/*z* values were selected according to the VIP scores. Their *m*/*z* scores and retention times were listed for Q-TOF analysis. These values were matched with the MS/MS analysis of quality control samples. In this way precursor and fragment ions for the selected features were determined. Then, the MS/MS spectrums of selected ions were compared with the reference standards of METLIN (https://metlin.scripps.edu/) and the Human Metabolome Database. For MS/MS analysis, collision energy was set as 10V. [M+H] ions were considered for negative and positive ion modes. 20-ppm mass tolerance was determined, and three matches for product ion were accepted for annotation. The i-DDA algorithm was applied to match the MS and MS/MS spectra of the samples with the reference spectra of KEGG, HMDb, and METLIN databases. Thirteen metabolites were annotated (Table 1). Metaboanalyst 5.0 software was used for pathway analysis. According to

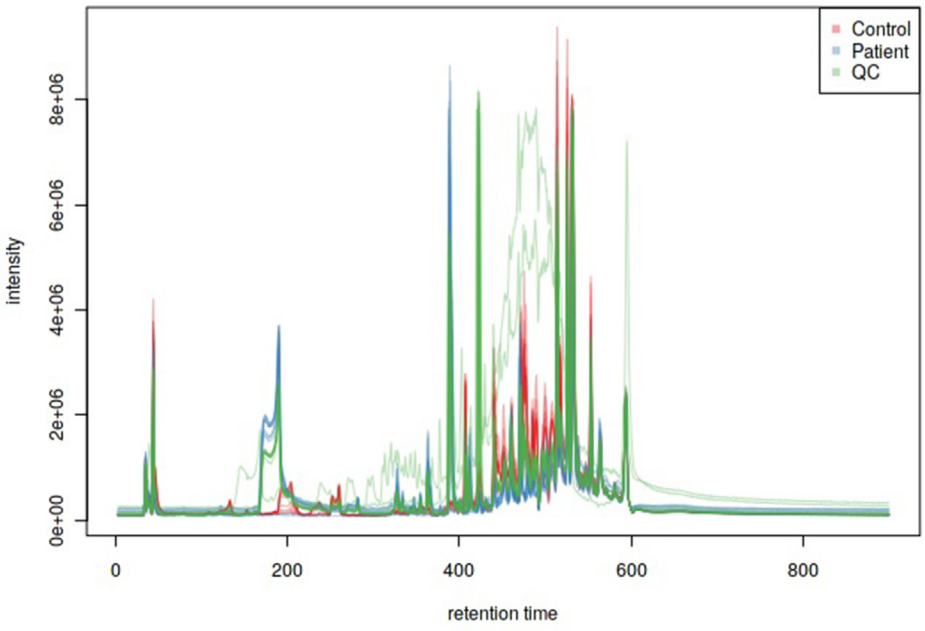


Fig. 1. Total ion chromatograms of each sample via Q-TOF MS/MS. Q-TOF MS/MS, quadrupole time of flight tandem mass spectrometry for positive [a] and negative [b] ion modes.

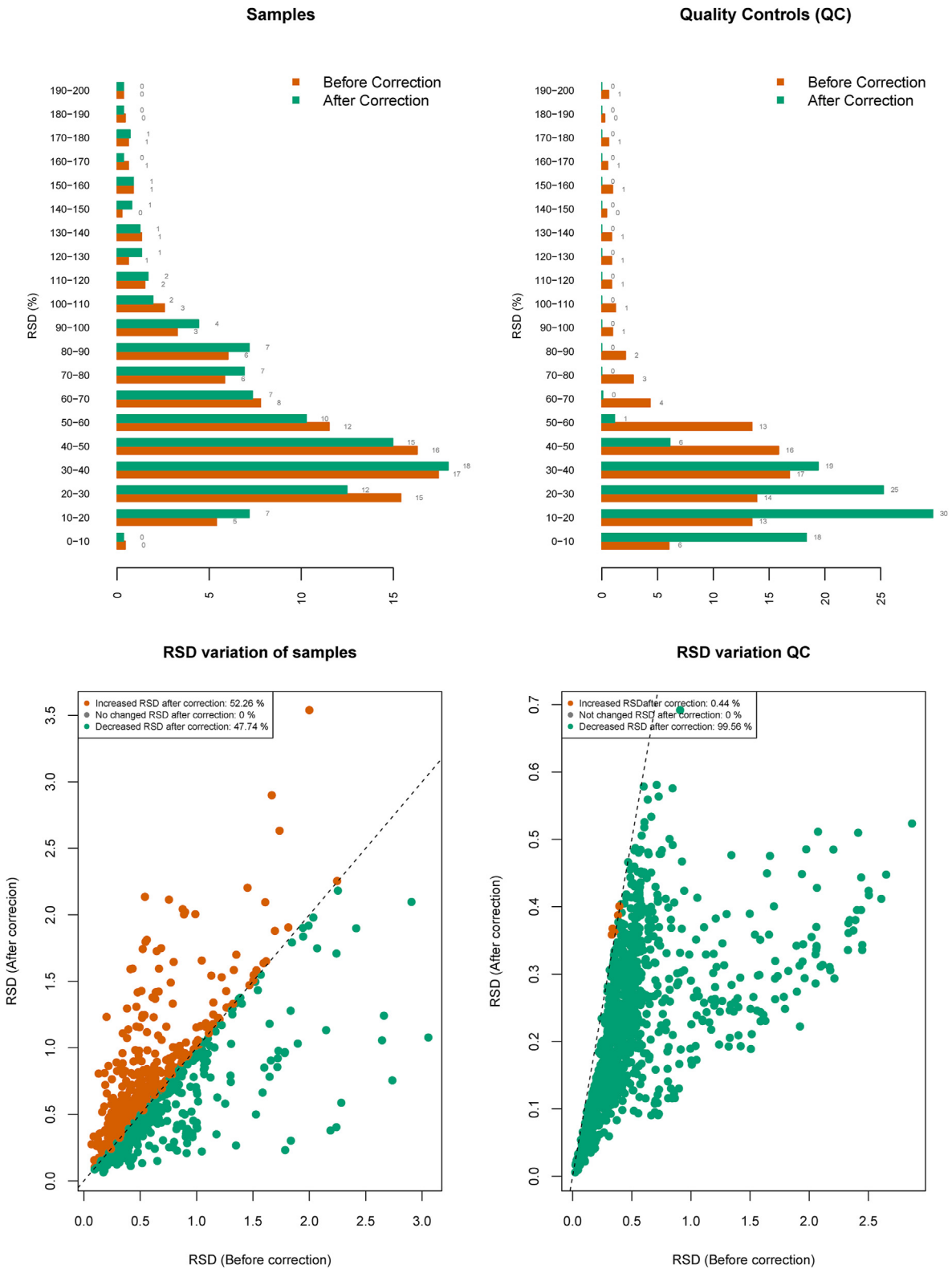


Fig. 2. RSD variations of samples and QC.

the data, nine different pathways were significantly affected by the AS cases, as summarized in Fig. 7. The serum metabolites which significantly differentiated AS patients from healthy control subjects are shown in Table 1. CRP was statistically significant correlated with PC (16:0/0:0) ($p = 0.035$, $r = -0.498$) and BASFI was statistically significant correlated with PC (18:2/0:0) ($p = 0.048$, $r = 0.471$).

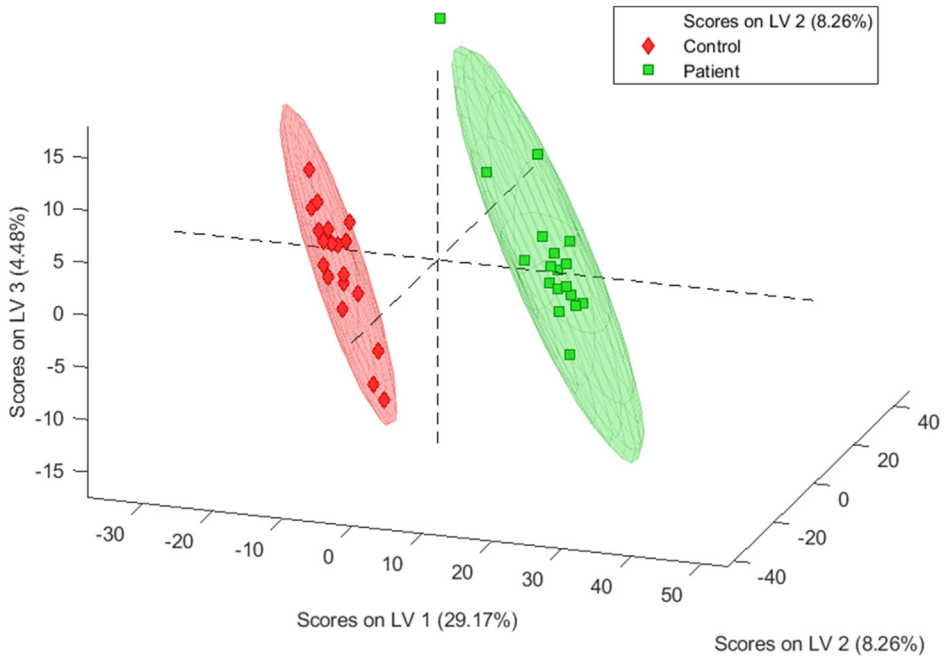


Fig. 3. Score Plot of Latent Variables in accordance with the PLS-DA model.

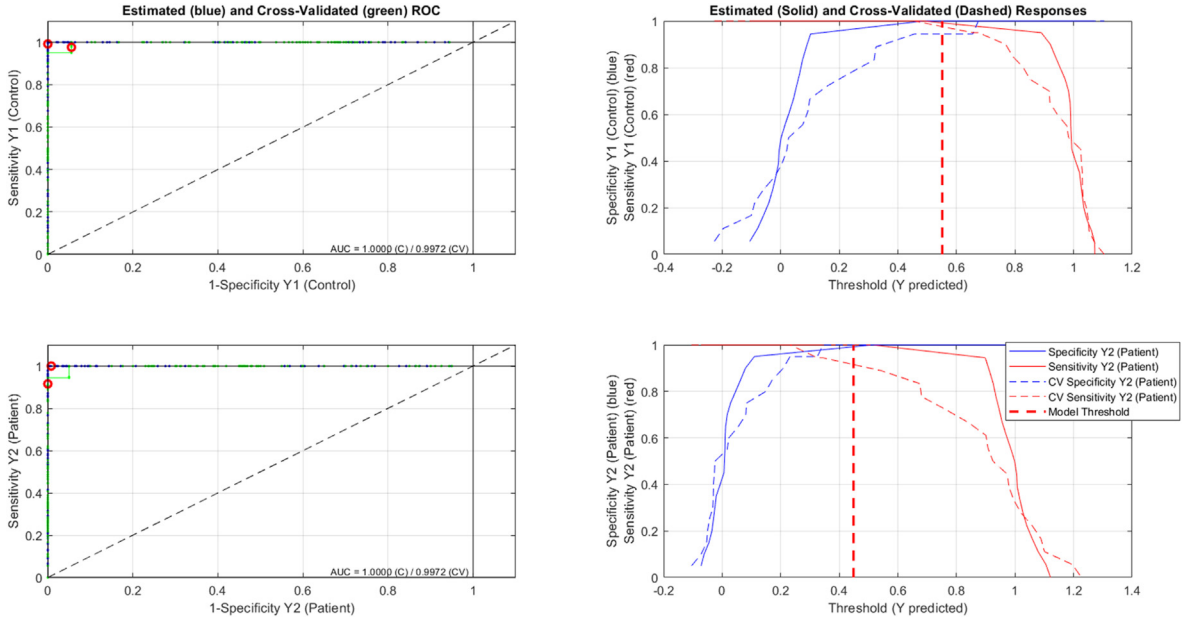


Fig. 4. Permutation test for the PLS-DA model.

4. Discussion

In the present study, dysregulation was mainly observed in the fatty acid (FA) and phospholipid metabolisms in active AS patients compared to healthy control subjects. Down-regulations were observed in PC (16:0/0:0), beta-D-Fructose, stearic acid, trimipramine N-Oxide, and muconic acid, and up-regulation were detected in PC (18:2/0:0), 3-Methylindole, palmitic acid (PA), alpha-Tocotrienol, and beta-D-glucopyranoside in active AS patients compared to healthy control subjects.

Fatty acids are one of the most abundant organic molecules and essential components of biological systems due to their roles in energy storage, biological membrane, and cellular communication [16]. In addition, FAs are associated with the regulation of inflammation [17]. Since uncontrolled inflammation has a central role in the pathogenesis of rheumatological disorders, the role of FAs in the pathogenesis and clinical outcomes of certain rheumatological diseases, including rheumatoid arthritis [RA], systemic lupus erythematosus, systemic sclerosis, and idiopathic inflammatory myopathy, has been widely evaluated in previous studies [18]. Abnormalities in FA metabolism have been related to the cartilage and bone destruction in RA [19–21]. There was dysregulation in the biosynthesis of unsaturated FA, linoleic acid metabolism, alpha-linolenic acid metabolism, FA degradation, and FA biosynthesis in the current study.

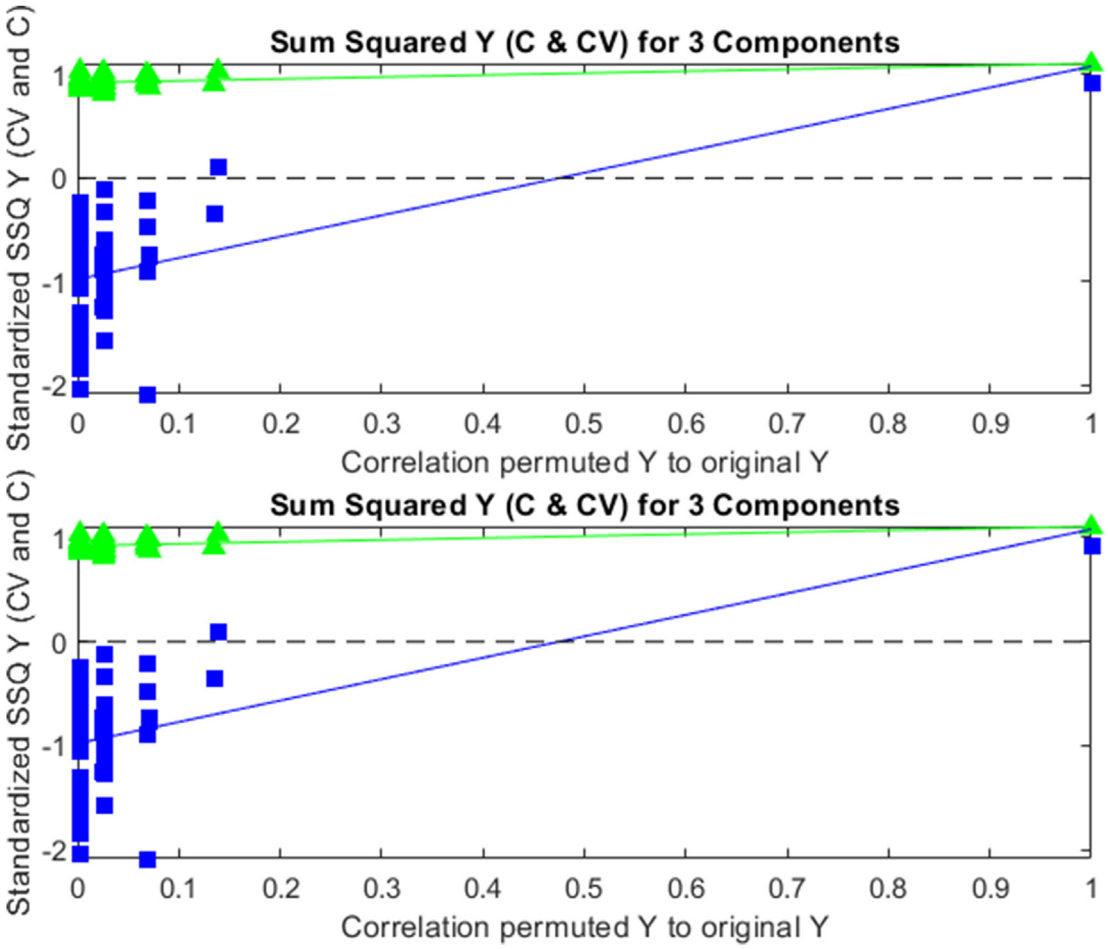


Fig. 5. ROC curve for the model and cross-validation datasets.

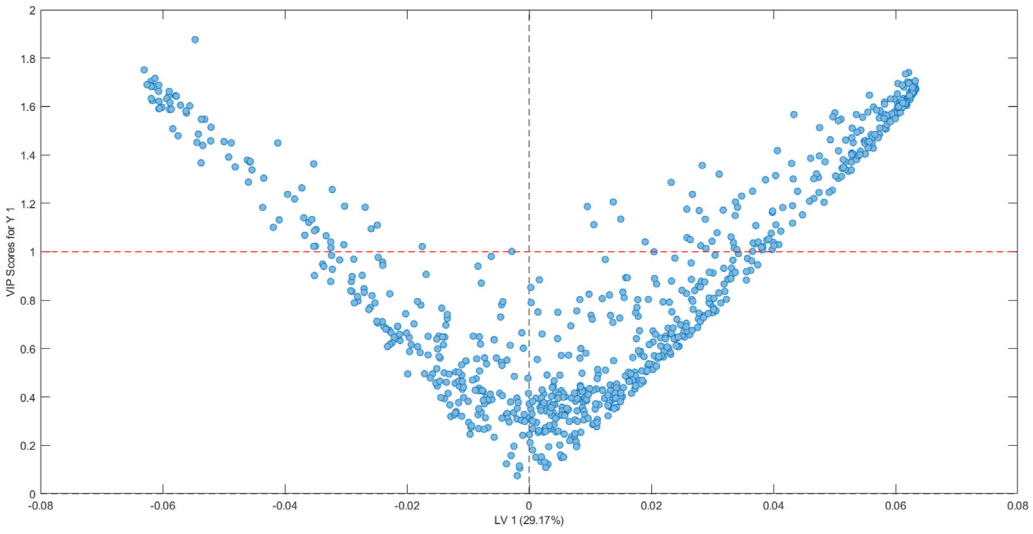


Fig. 6. Variables in projection [VIP] scores for the PLS-DA model.

In contrast to the above-mentioned rheumatological diseases, few researchers have addressed FA metabolism in patients with AS [22–24]. High FA oxidation has been reported in AS patients, and this has been associated with the progression of AS [22]. Ou et al. concluded that PC (18:1(9Z)/18:1(9Z)) and 1-palmitoylglycerol could be novel biomarkers for distinguishing healthy control subjects from AS patients with the higher area under the curve values [23]. Chen et al. reported significant differences between AS patients and a healthy control group in terms of several free and esterified FAs [24]. Considering previous studies and our findings,

Table 1
List of the significantly different metabolites identified in patient vs control group.

KEGG ID	HMDB ID	Compound	Exact Mass	Fold	Retention Time	p-value	Regulation
C00157	HMDB0000564	PC(16:0/0:0)	496.3314	1.53	9.37	7.6×10^{-4}	DOWN
Not Available	HMDB0008039	PC(18:2/0:0)	519.333	2.82	7.85	0.03	UP
C00791	HMDB0000562	Creatinine	114.066	2.13	0.63	0.04	DOWN
C01046	HMDB0062660	N-methyl Glutamine	161.096	2.21	7.06	7.42×10^{-6}	
C08313	HMDB0000466	3-Methylindole	132.081	1.60	1.65	0.045	UP
C02336	HMDB0000660	beta-D-Fructose	179.056	9.86	5.53	7.87×10^{-5}	DOWN
C00249	HMDB0000220	Palmitic acide	256.340	2.27	1.74	3.8×10^{-5}	UP
C01530	HMDB0000827	Stearic acid	283.420	3.47	7.00	7.08×10^{-6}	DOWN
C00864	HMDB0000210	Vitamin B5	220.1076	1.56	5.45	3.36×10^{-6}	UP
C01104	HMDB0000925	Trimethylamine N-Oxide	311.212	2.12	8.59	1.19×10^{-3}	DOWN
C14153	HMDB0006327	alpha-Tocotrienol	425.640	1.68	8.52	1.28×10^{-4}	UP
C00221	HMDB0029965	beta-D-glucopyranoside	629.330	1.50	9.46	1.38×10^{-4}	UP
C02480	HMDB0002349	Muconic acid	143.089	21.97	5.91	7.64×10^{-8}	DOWN

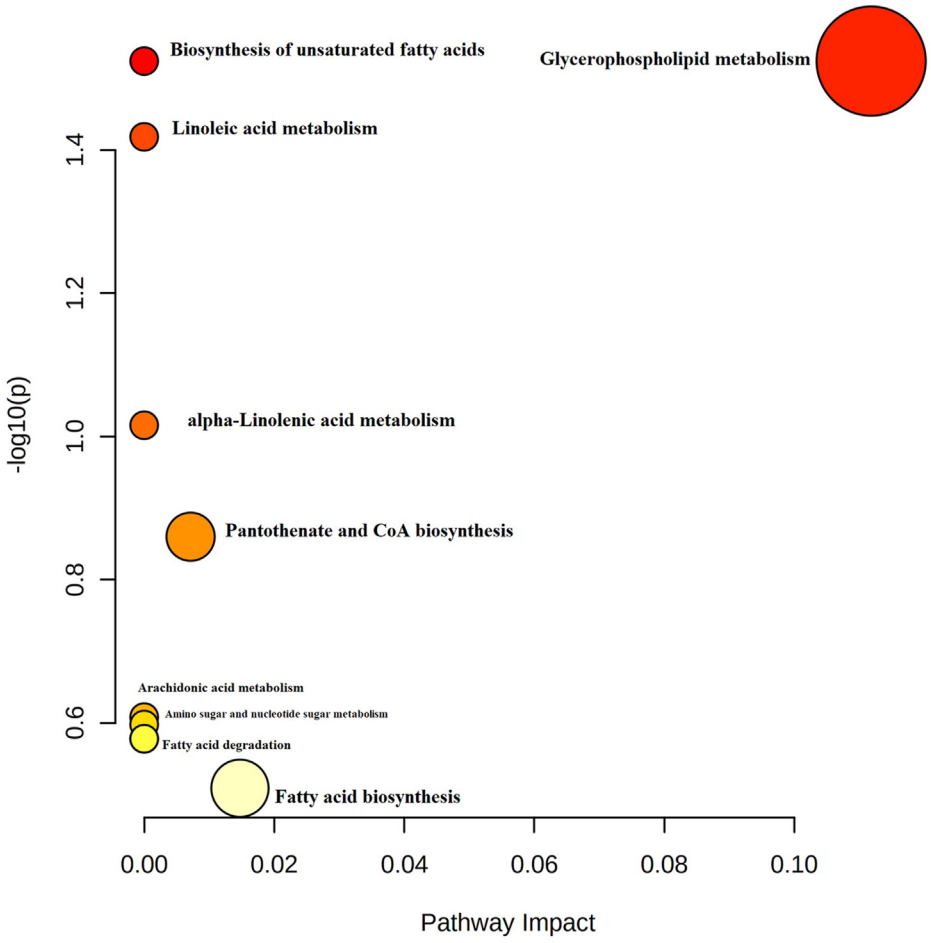


Fig. 7. Pathway analysis generated by MetaboAnalyst online: major pathways varied in the patient and control groups.

changes in FA composition and metabolism can be considered to play critical roles in AS. Therefore, evaluating FA metabolism may be valuable to understand the pathogenesis of the disease and determine its therapeutic potentials.

In the current study, down-regulation was observed in the PC (16:0/0:0), 1-Acyl-sn-glycero-3-phosphocholine, in the patients compared to healthy controls. However, up regulation was found in the PC (18:2/0:0) in patients. Therefore, we think that the dysregulation in PC may have a role in the molecular mechanism of the disease. PC (16:0/0:0) is a lysophospholipid produced by the activation of the phospholipase A2 from PC in the endoplasmic reticulum (ER) membrane [25]. Down-regulation was also determined of trimethylamine N-Oxide synthesized endogenously from trimethylamine, which can be derived from PC [26]. Taking these two findings together, the downregulation of PC (16:0/0:0) and trimethylamine N-Oxide can be related to the decreased levels of PC in patients. In a metabolomics study using LC-MS and GC–MS techniques, Gao et al. determined reduced PC levels in AS patients compared to a healthy control group [27]. This finding supports the current study hypothesis.

Previous in vitro studies have shown the anti-inflammatory effect of PC. The protective role of PC has been found on

intestinal epithelial cells exposed to LPS-induced injury by inhibiting the TNF-α secretion and mitogen-activated protein kinase (MAPK) signaling pathways [28]. After exogenous PC application, blocked pro-inflammatory signaling was observed in the TNF-α induced pro-inflammatory response of subcolon Caco-2 cells [29]. PC supplementation was seen to exert beneficial effects on the morphological, functional, and microcirculatory characteristics in an experimental chronic arthritis model performed on male DBA1/J mice [30]. Inflammatory reactions driven by TNF-α have a critical role in joint inflammation and bony effects in AS. Hence, one of the novel strategies in AS patients is blocking the TNF-alpha effects [3]. A randomized, placebo-controlled study demonstrated that oral PC supplementation effectively diminished the inflammatory activity caused by ulcerative colitis [31]. Therefore, it is thought that detailed targeted analyses should be performed in AS patients to detect the changes in PC metabolism. This study also detected a negative correlation between CRP and PC (16:0/0:0). CRP level is a well-known indicator of inflammation, and it was correlated both with activity and radiographic sacroiliitis progression in AS [32]. Accordingly, we think that PC (16:0/0:0), supplementation may also help to decrease joint and bone damage by inhibiting the pro-inflammatory response in AS.

In the present study, up-regulation was found in patients than in the healthy controls in terms of PA, a saturated FA obtained by diet or synthesized endogenously. Several previous studies have shown that PA causes inflammatory responses by triggering pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1, TNF-α, and NF-κB [33–35]. A link has also been revealed between PA and a deleterious influence on joint and cartilage tissues [36–38]. Tan et al. demonstrated that palmitic acid increased cartilage lesions and induced the unfolded protein response and ER stress pathways in mouse knee joints [36]. It has been reported that palmitate-induced chondrocyte apoptosis and articular cartilage breakdown act as pro-inflammatory and catabolic factors [37]. Nazli et al. showed that palmitate-induced ER stress affected chondrocytes and cartilage functions by activating unfolded protein response [38]. The ER unfolded protein response is one of the crucial factors in AS pathogenesis [3]. Therefore, we think that up-regulation of PA involves the molecular mechanisms of increased inflammatory response-related bone and joint damage and unfolded protein response in AS patients.

In this study, we compared active patients and healthy controls in terms of metabolome profile. The differences between before and after treatment were not evaluated. Besides, our study population had not the inactive patient group. Besides, we did not have the chance to use a separate validation cohort to validate our findings. However, to validate our models, we applied ten-fold cross-validation. In this way, we had the chance to see that our models work in different resamples of the dataset. These are the main limitations of our study.

In conclusion, the LC-MS-based untargeted metabolomics approach was performed to determine the plasma metabolite profile changes in active AS patients in this study. The study results have led to a deeper understanding of the metabolic changes in AS. Thirteen metabolites and nine pathways were significantly altered in AS patients compared to age and gender-matched healthy control subjects. The pathways mainly affected were unsaturated FAs, linoleic acid metabolism, alpha-linolenic acid metabolism, FA degradation, and FA biosynthesis. These findings bring a new perspective to the molecular mechanism by which certain changes in lipid metabolism might contribute to AS disease pathogenesis. PA and PC (16:0/0:0) were identified as potential metabolites playing a role in molecular mechanisms in AS. Accordingly, we think that modification in the diet in terms of PA and PC may attenuate or even reverse the development of detrimental effects of the disease. Finally, future studies regarding the targeting analyses of the PA, PC, and other FAs may contribute to better understanding the pathogenesis of the disease and determine novel treatment strategies in AS. Additionally, novel studies with the different patient groups such as pre and post-therapy groups and the inactive group will help to reveal the effects of the changes in these metabolites more clearly in AS.

Author contributions

Halef Okan Doğan: Conceptualization, Funding, Investigation, Writing **Onur Şenol:** Methodology, Investigation, Reviewing final version, Visualization **Ahmet Karadağ:** Methodology, Investigation, Reviewing final version and **Şeyma Nur Yıldız:** Methodology.

Ethical approval

The procedures were approved by the Ethics Committee of Sivas Cumhuriyet University in accordance with the ethical standards established by the institution where the experiments were performed or in accordance with the Helsinki Declaration (Decision number:2019-02-01).

Funding

The study was supported by Sivas Cumhuriyet University, Turkey with the grant numbers of T-883 and RGD-036.

Declaration of competing interest

The authors have no conflict of interests to declare.

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