Untargeted Lipidomics Reveals Specific Lipid Abnormalities in Ankylosing Spondylitis

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Abstract

Objective.

To identify potential biomarkers, we used lipidomics to study the changes in serum lipids of patients with ankylosing spondylitis (AS).

Methods.

Serum samples were collected from 115 patients with AS and 108 healthy controls (HCs). Ultrahigh-performance liquid chromatography coupled with Q Exactive spectrometry was used to explore the serum lipid changes and identify potential lipid biomarkers for the clinical diagnosis of AS.

Results.

Lipid phenotypes associated with disease activity were detected in the serum of patients with AS, with increased levels of triacylglycerol (TAG) (18:0/18:1/20:0) and decreased levels of phosphatidylcholine (PC) (16:0e/26:4) and PC (18:1/22:6). These three lipids can be used as potential biomarkers for the clinical diagnosis of AS. The diagnostic efficacy was evaluated by receiver operating characteristic (ROC) analysis.

Conclusion.

Our findings identified serum lipid biomarkers for AS and shed light on its diagnosis in the clinic.

Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory arthritis characterized by inflammatory back pain that can lead to spinal structural damage. AS mainly affects the axial skeleton, leading to severe stiffness and pain and even loss of spinal activity, which seriously damages the quality of life of patients [2]. In addition, AS may involve the surrounding joints, eye, skin and intestinal system, showing extra-articular symptoms, including inflammatory bowel disease (IBD) and uveitis. Because early AS is mostly asymptomatic or has no obvious pathological changes, untimely diagnosis is common. Therefore, early diagnosis is very important for the treatment and inhibition of the progression of AS [5].

The Bass Ankylosing Spondylitis Disease Activity Index (BASDAI) and/or Ankylosing Spondylitis Disease Activity Score (ASDAS) are mainly used to evaluate the disease activity of AS. The BASDAI involves six aspects, including fatigue, back pain, peripheral joint pain/swelling, enthesitis and morning stiffness. The ASDAS includes the self-reported back pain index, duration of morning stiffness, pain/swelling of peripheral joints, and global assessment of disease activity. In addition, ASDAS includes laboratory test results, such as C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR), and each parameter is

weighted. At present, the diagnosis of AS is based on the comprehensive evaluation of clinical and radiological changes, and the average diagnosis time is 5 to 10 years [8]. Immune, genetic and inflammatory factors play an important role in the pathogenesis of AS, but the exact pathogenesis has not been fully elucidated [9]. In recent years, some serum markers, such as immunoglobulin G (IgG), IgA, CRP, interleukin 17 and 23, matrix metalloproteinases and noncoding RNA, have been widely used to explore the aetiology of AS and evaluate AS activity but have not achieved sufficient diagnostic sensitivity or specificity [10–12].

As a rapidly developing research field in the new era, "omics" technology has become a powerful comprehensive method to evaluate disease changes and their biochemical mechanisms [13]. Lipidomics is a new field that systematically analyses whole lipids. By comparing the changes in the lipid metabolic network in different physiological states, these methods can identify the key lipid biomarkers in metabolic regulation and finally reveal the mechanism of lipids in various biological activities. Although several studies on the application of metabonomics to AS have been reported [14–16], generally speaking, the sample size of most studies is small. In this study, ultrahigh-performance liquid chromatography coupled with Q Exactive (UPLC-QE) spectrometry was used to detect the serum of 115 patients with AS and 108 healthy controls (HCs). We aimed to study the changes in systemic lipid metabolism in patients with AS and to find potential serum markers for the diagnosis of AS.

Methods

Study populations

All procedures were approved by the Institutional Review Board and the Ethics Committee of the First Affiliated Hospital of Nanjing University of Traditional Chinese Medicine and followed the Declaration of Helsinki. After reviewing a written plan of the whole study, all volunteers signed and provided written informed consent.

A total of 223 participants, including HCs and patients with AS from the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, were recruited between September 2017 and January 2019, and all the participants were Asian.

Sample preparation

The samples were first thawed on ice. Then, 225 μ L of ice-cold methanol containing a mixture of internal standards was added to 40 μ L of serum, and the mixture was vortexed for 10 s. Then, 750 μ L of cold methyl tert butyl ether was added, and the mixture was vortexed for 10 seconds and shaken on an orbital mixer at 4°C for 10 min. After the addition of 188 μ L of room temperature LC/MS-grade water, the mixture was vortexed for 20 seconds and centrifuged for 2 min (14,000 rcf, 4°C). The supernatant was transferred to clean new tubes and dried in a vacuum centrifuge. The upper phase lipids were reconstituted with 110 μ L of methanol:toluene (9:1) for UPLC-QE MS analysis. The order in which the samples from the patients

with AS and HCs were analysed was random, and quality control samples were injected once per batch of 10 samples.

Untargeted lipidomic analysis was performed using the Dionex UltiMate 3000 Ultra-Performance Liquid Chromatography (UPLC) system (Santa Clara, CA, USA) coupled via an electrospray ionization source with a Q Exactive mass spectrometer (Thermo Fisher Scientific, USA). For the separation of lipids, 1 μ L of sample solution was injected onto a reversed-phase Waters Acquity UPLC CSH C18 column (100 mm $\times$ 2.1 mm, 1.7 μ m) maintained at 60°C by gradient elution. All MS experiments were performed in positive and negative ion modes using a heated ESI source with a spray voltage of 3 kV (positive).

Data Analysis

The raw data were first preprocessed by MS-DIAL, and then, the variables were identified by the Fiehn Lab database. After removal of the features with missing values > 80%, the data normalization step was completed by MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca/>). The determined blood lipids were further analysed using univariate and multivariate statistical methods. The normalized data were imported into SIMCA software (version 14.1; Umetrics) for orthogonal partial least squares-discriminant analysis (OPLS-DA). Significantly different lipids were identified based on variable importance in the projection (VIP) obtained from the OPLS-DA model and the Wilcoxon rank-sum test (p value) with the Benjamini-Hochberg-based false discovery rate (FDR). The lipids that met the criteria of VIP > 1.0, p value < 0.05 and FDR < 0.05 were identified as differentially abundant lipids.

Candidate differentially abundant lipids were analysed to identify potential diagnostic biomarkers. Based on these lipids, the forward stepwise binary logistic regression method and the Wald test were used to establish the model by SPSS 25.0 software (SPSS, Inc.). Then, the diagnostic efficacy of the regression analysis results was quantified by receiver operating characteristic (ROC) curve analysis, and the area under the ROC curve (AUC) was calculated.

Results

Basic characteristics of the participants

In this study, a total of 223 serum samples from 108 HCs and 115 patients with AS were collected to identify candidate biomarkers. The demographic characteristics and clinical information of the subjects are shown in Table 1.

Table 1
Clinical Characteristics of the Subjects

Characteristic	AS(n = 115)	HCS(n = 108)
Basic characteristics		
Male	71	33
Female	44	75
Age (years), mean $\pm$ SD	42.18 $\pm$ 13.76	30.68 $\pm$ 7.53
BMI(kg/m ²), mean $\pm$ SD	25.12 $\pm$ 3.57	—
Clinical variables		
ESR (mm/h), mean (median)	26.91 (2)	—
CRP (mg/l), mean (median)	16.04 (8.88)	—
ALP (U/L), mean (median)	88.94 (90)	—
ALB (g/mL), mean (median)	41.11(41.3)	—
ASDAS, mean $\pm$ SD	2.05 $\pm$ 0.35	—
HLA-B27	95.83%	—
BMI: body mass index; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; ALP: alkaline phosphatase; ALB: albumin; ASDAS: ankylosing spondylitis disease activity index; HLA-B27: human leukocyte antigen-B27.		

Serum lipid profiling of UPLC-QE/MS

The workflow of this study is shown in Fig. 1. In the untargeted lipid metabolomics analysis, we examined 223 serum samples. The lipid profiling data were acquired in positive and negative ESI modes with two injections, and a total of 586 lipids were determined. We further applied the OPLS-DA (Fig. 2A) analysis model to identify the metabolic profile differences between the AS and HC groups. Without overfitting of the model (Fig. 2B), there was apparent separation between the two groups, indicating that the lipid metabolic pattern was changed. Based on the significant changes in the comparison between the lipid metabolites of the AS and HC groups, multivariate and univariate criteria (VIP > 1, p value < 0.05, and FDR < 0.05) were used to identify 114 metabolites of the AS group compared with the HC group (Fig. 2B).

Definition and verification of potential biomarkers for AS

Significance analysis of microarray (SAM) and random forest (RF) analyses were further used for screening lipid metabolites that changed significantly during disease progression (Fig. 3A, B), and 15 lipids were retained. Details of these metabolites are listed in Table 2. A heatmap showing the relative intensity distribution of these metabolites in the AS and HC groups is shown in Fig. 3C.

Table 2
Identified differential lipid metabolites between the AS and HCs.

Metabolite	^a VIP	^b <i>p</i> Value	^c FDR	^d FC
DAG(16:0/18:2)	2.099	< 0.001	< 0.001	1.520
LPC 20:3(SN2)	1.573	0.001	< 0.001	1.266
PC(16:0e/26:4)	3.123	< 0.001	< 0.001	0.635
PC(16:1e/18:2)	1.559	< 0.001	< 0.001	0.743
PC(18:1/18:2.1)	1.805	< 0.001	0.001	0.814
PC(18:1/22:6)	1.924	< 0.001	0.001	0.759
PC(18:1e/18:2)	2.693	< 0.001	< 0.001	0.745
PC(20:4e/26:4)	1.889	< 0.001	< 0.001	0.666
PE(16:0e/22:6)	1.589	< 0.001	0.001	0.856
TAG(16:0/18:1/22:5)	2.425	< 0.001	< 0.001	1.426
TAG(16:0/18:2/20:4)	1.783	< 0.001	< 0.001	1.494
TAG(16:0/20:4/22:6)	1.864	< 0.001	< 0.001	1.475
TAG(18:0/18:1/18:1)	2.065	< 0.001	< 0.001	1.456
TAG(18:0/18:1/20:4)	3.104	< 0.001	< 0.001	1.726
TAG(18:1/18:2/22:5)	2.684	< 0.001	< 0.001	1.548
^a VIP was obtained from the OPLS-DA model with a threshold of 1.0. ^b <i>p</i> Value were obtained from one-way ANOVA. The value of ^c FDR was obtained from the adjusted <i>p</i> Value in the metaboanalyst 5.0. The value of ^d FC was obtained by comparing those metabolites in patients with SLE with the HCs.				
VIP: variable importance in the projection, FC: fold change, FDR: false discovery rate.				

Subsequently, binary logistic regression was performed using SPSS 25.0 software to further analyse the above 15 differentially altered lipid metabolites. A regression model was established using the forwarding stepwise optimization algorithm (Wald), and TAGs (18:0/18:1/20:0), PC (16:0e/26:4) and PC (18:1/22:6) were determined to be reliable lipid biomarkers. Figure 4 shows that TAG (18:0/18:1/20:0) and PC (16:0e/26:4) levels were significantly increased, while PC (18:1/22:6) levels were decreased in patients with AS. Then, the diagnostic potential of the three lipids was evaluated (Fig. 5). For the AS and HC groups, TAGs (18:0/18:1/20:0), PC (16:0e/26:4), PC (18:1/22:6) and their combination showed AUCs of 0.761, 0.736, 0.651 and 0.836, sensitivities of 65.22%, 61.74%, 57.39% and 65.22%, and specificities of 83.33%, 76.85%, 70.37% and 89.81%, respectively.

Finally, correlations between TAGs (18:0/18:1/20:0), PC (16:0e/26:4), PC (18:1/22:6) and AS disease activity were determined (Fig. 6). The combination of TAGs (18:0/18:1/20:0), PC (16:0e/26:4) and PC (18:1/22:6) distinguishes remission AS from active AS and HCs, with coincidence rates of 65.21% and 85.71%, respectively. In addition, 0.322 as the cut-off value could differentiate between patients with active AS and HCs. Therefore, the combination of TAGs (18:0/18:1/20:0), PC (16:0e/26:4) and PC (18:1/22:6) is an ideal lipid biomarker to differentiate between patients with AS and HCs.

Discussion

AS is a chronic rheumatoid arthritis characterized by inflammatory back pain, which can lead to spinal structural injury. In addition, AS affects the surrounding joints, eye, skin and intestinal system, manifesting itself as extra-articular symptoms such as inflammatory bowel disease and uveitis [16]. At present, clinical monitoring is mainly based on X-ray diagnosis of AS, but the process is lengthy [17]. Because early AS is usually asymptomatic or has no obvious pathological changes, early diagnosis of AS is still a difficult problem. Some biomarkers, such as immunoglobulin G (IgG), IgA or C-reactive protein, have not reached sufficient diagnostic sensitivity or specificity [18]. At present, omics technology has become a powerful biochemical analysis tool, providing important insights into the process of various diseases. As an important branch of metabonomics, lipidomics has been used in the early diagnosis of various diseases, such as Sjogren's syndrome [19], systemic lupus erythematosus [20] and ulcerative colitis [21]. However, there are few studies on lipidomics for AS. We used HPLC-QE to detect the lipid profile of patients with AS and obtained 586 kinds of lipids. Finally, the lipid types related to the activity of AS disease were determined and verified.

In patients with AS, the characteristics of metabolic syndrome have been found [23]. Metabolic syndrome is a series of metabolic disorders, including obesity, insulin resistance, impaired glucose tolerance, hypertension and dyslipidaemia [24]. Compared with healthy people, patients with AS have higher overall mortality and cardiovascular disease (CVD)-related mortality [25, 26]. Chronic inflammation, impaired lipid metabolism, enhanced lipid peroxidation, early endothelial dysfunction and excessive activation of coagulation cascades can lead to AS-related atherosclerotic cardiovascular disease (ACD) [27, 28]. We examined the lipid profile of patients with AS and screened for differentially abundant lipids and found that serum TAG (18:0/18:1/20:0) levels were increased. According to recent studies, high serum TAG and low HDL levels are considered independent risk factors for ACD [29, 30]. Our results showed that TAGs (18:0/18:1/20:0) can be used as a potential biomarker for the diagnosis of AS, with a diagnostic potential of 0.761.

PC is an important component of the cell membrane, alveolar surfactant, lipoprotein and bile and is also the source of lipid messengers such as lysophosphatidylcholine, phosphatidic acid, diglyceride, lysophosphatidic acid and arachidonic acid. Researchers have long confirmed the positive protective effect of PC on the heart by regulating the content of cholesterol in the body and effectively reducing the incidence of cholesterol, hyperlipidaemia and coronary heart disease. The role of PC in emulsifying and decomposing lipids can improve blood circulation, remove peroxides, reduce the retention time of fat in

the inner wall of blood vessels, and promote the dissipation of atherosclerotic plaques, so it can prevent and treat arteriosclerosis. Our results showed that PC levels are decreased in patients with AS and that PC (16:0e/26:4) and PC (18:1/22:6) may be potential biomarkers of AS disease activity. Based on the above results, abnormal serum lipid metabolism in patients with AS may induce cardiovascular disease. TAG (18:0/18:1/20:0), PC (16:0e/26:4) and PC (18:1/22:6) can be used as effective biomarkers for the diagnosis of AS disease.

In conclusion, a nontargeted HPLC-QE serum lipid method was applied to investigate the lipid characteristics of patients with AS and HCs. TAG (18:0/18:1/20:0), PC (16:0e/26:4) and PC (18:1/22:6) reflect the dynamic process of lipid levels in AS. These three lipids and their combinations can be used as potential biomarkers for the diagnosis of AS and to distinguish different degrees of the disease. Understanding the changes in lipids in AS is an essential step in achieving proper cardiovascular risk management.

Declarations

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Contributions Z.L., W.G., S.J and W.Z. conceived and designed the experiments; Z.L., Y.W., W.J. and B.Q. performed the experiments; Z.L. and Y.W. analyzed the data; Z.L., W.G., Y.W., W.J. and B.Q. contributed reagents/materials/analysis tools; Z.L. and Y.W. wrote the paper.

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Ethics declarations

All procedures were approved by the Institutional Review Board and the Ethics Committee of the First Affiliated Hospital of Nanjing University of Traditional Chinese Medicine and followed the Declaration of Helsinki. After reviewing a written plan of the whole study, all volunteers signed and provided written informed consent.

Conflict of interest

None of the authors have conflict of interest to declare.

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Figures

Figure 1

Design of this study.

Figure 2

Identification of potential lipid biomarkers for the diagnosis of AS. (A) Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot based on HCs and SLE groups in the Discovery Set. (B) 200 permutation tests of the OPLS-DA model.

Figure 3

Lipidomic profiling of serum samples from 15 lipid species that distinguish AS and SLE. (A) The result of the SAM scatter plot of observed scores plotted versus expected scores with a delta value of 0.1. Significant lipid species are represented in green. (B) Significant features identified by random forest. (C) A heatmap of the differential lipid species between HCs and AS.

Figure 4

Serum relative intensities of TAG (18:0/18:1/20:0), PC(16:0e/26:4) and PC(18:1/22:6) in the HCs and AS.

Figure 5

Receiver operating characteristic curve analysis (ROC) of TAG (18:0/18:1/20:0), PC(16:0e/26:4) and PC(18:1/22:6) and their combination. A: PC(16:0e/26:4); B: TAG (18:0/18:1/20:0); C: PC(18:1/22:6). AUC:

area under the curve.

Figure 6

Diagnosis of disease activity in AS using the combination of TAG (18:0/18:1/20:0), PC(16:0e/26:4) and PC(18:1/22:6). (A) Diagnostic coincidence rate for the comparison between remission vs active AS. (B) Diagnostic coincidence rate for the comparison between remission AS vs HCs. (C) Diagnostic prediction rate for the comparison between active AS vs HCs.

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