

Original Article

LC-MS-based lipidomic analysis of serum samples from patients with rheumatoid arthritis

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Abstract: Rheumatoid arthritis (RA) is a common chronic inflammatory disorder characterized by inflammation of the synovium, which resulting in joint damage, chronic pain, and disability. Lipid metabolism in RA patients is biologically complex and is incompletely understood. Identification of reliable biomarkers for early diagnosis is critical for design of a specific RA therapy. In this study, an untargeted lipidomics approach using ultra high performance liquid chromatography coupled to quadrupole-orbitrap high resolution mass spectrometry (UHPLC-Q-E-MS) was developed to profile lipid alterations in RA patients. Multivariate data analysis clearly differentiated the RA group from health controls. 12 metabolites in positive ion mode and 13 metabolites in negative ion mode were identified as potential lipid markers, and findings strongly suggested metabolism of linoleic acid, alpha-linolenic acid, glycerolipid, and glycerophospholipid are perturbed in RA. This provides new insight into underlying mechanisms of RA, and demonstrates LC-MS based lipidomics is a powerful approach for discovery of new biomarkers.

Keywords: Lipidomics, LC-MS, rheumatoid arthritis (RA)

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterized by inflammation of the synovium [1, 2], which leads to progressive joint degeneration, disability, and increased risk of cardiovascular complications [3]. Patients with RA continue to endure pain and disabling fatigue despite controlling objective inflammation [4]. It is hard to cure RA, so the accurate early detection and diagnosis is becoming increasingly important. In addition, the evaluation of the response to therapeutic intervention is necessary for the management of this disease. Metabolites and lipids in serum of RA patients are irregular which may be used as potential biomarkers to predict pathological changes. However, lipid metabolism in RA patients is complex and not well understood.

Metabolomics is an emerging technology which enables global assessment of metabolites and their biologic significance in diseases. There have been several recent metabolomic studies on RA [5-7]. Zabek and co-workers monitored

female patients with rheumatoid arthritis using NMR-based metabolomics, and twelve metabolites were found to be of importance in distinguishing rheumatoid arthritis from healthy controls (HC) [6]. Yang and colleagues analyzed synovial fluid samples from RA patients using proteomic and metabolomic approaches [8]. Lipidomics, as a branch of metabolomics focusing on profiling, quantification and identification of lipids in a biologic system, has also been applied in screening RA biomarkers in synovial fluid [9]. However, it has not been extensively applied in investigation of serum samples from RA patients. In a recently published lipidomic study on RA, a triple quadrupole based mass spectrometer was exploited to determine lipid species in plasma from fasting RA patients [10]. However, identification of new lipid markers with a QTRAP mass spectrometer as was used in that study is difficult due to the low resolution and low mass accuracy of this technique.

In this study, an untargeted lipidomics approach using ultra high performance liquid chromatog-

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Table 1. Demographic information on the study subjects

Sample	Number	Mean age (\pm SD)	Female	Male
RA	10	56.8 \pm 12.7	8	2
Normal	10	56.4 \pm 12.4	8	2

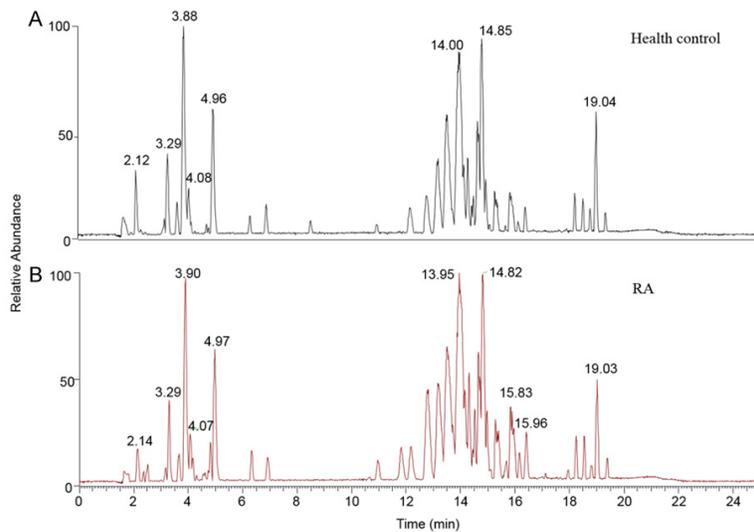


Figure 1. Full scan base peak mass chromatograms in positive mode: (A) Health control and (B) RA group.

raphy coupled to quadrupole-orbitrap high resolution mass spectrometry (UHPLC-Q-E-MS) was developed to profile lipid alterations in RA patients. The RA group and health controls were clearly distinguished by multivariate data analysis, which included principal component analysis (PCA) and partial least squared discriminant analysis (PLS-DA). Lipids with significant alterations were identified with high resolution MS and MS/MS data, and perturbed metabolic pathways were analyzed. This approach may be helpful for further understanding of the molecular mechanism of RA.

Materials and methods

Chemicals and reagents

Formic acid, HPLC grade methanol, acetonitrile (ACN) and isopropanol (IPA) were obtained from Fisher Scientific. Chloroform was obtained from Tong Guang Fine Chemicals Company (Beijing, China). Free fatty acid (FFA) 19:0 and ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water was supplied by a Millipore system (Millipore, Billerica, MA, USA).

Sample collection

A total of 10 RA patients and 10 age and sex matched health controls were recruited from the Sino-Japanese Friendship Hospital (Beijing, China). The demographic information on the study subject is shown in **Table 1**. This study was approved by the Ethics Committee of the Sino-Japanese Friendship Hospital, and all subjects provided written informed consent prior to participation.

Sample processing

Lipids were extracted from serum samples by a modified Folch method [11, 12]. Typically, 100 μ L of serum were aliquoted into a 0.6 ml Eppendorf tube and mixed with 400 μ L of chloroform/methanol (2:1, V/V) containing 20 μ g/ml of free fatty acid

19:0 as an internal standard. After vortexing for 10 min, the mixture was centrifuged at 13000 rpm at 4°C for 20 min. The lower lipid containing chloroform phase was evaporated with a speed vacuum, and the residue was stored at -80°C for further analysis. All samples were processed in the same laboratory to avoid bias.

LC-MS analysis

An Ultimate 3000 ultra high performance liquid chromatography (UHPLC) system coupled to Q-Exactive MS (Thermo Scientific) was used for lipid separation and detection. Samples were reconstituted in 20 μ L chloroform/methanol (1:1, V/V) and diluted three times in IPA/ACN/water (2:1:1, V/V/V). After centrifugation at 12000 rpm for 15 min, 5 μ L of supernatant were injected for LC-MS/MS analysis.

Chromatographic separation was performed on a reversed phase X select CSH C18 column (4.6 mm \times 100 mm, 2.5 μ m, Waters, USA), which is consistent with what has been reported [13]. Two solvents were used for gradient elution: (A) ACN/water (3:2, V/V), (B) IPA/ACN (9:1, V/V). Both A and B contained 10 mM

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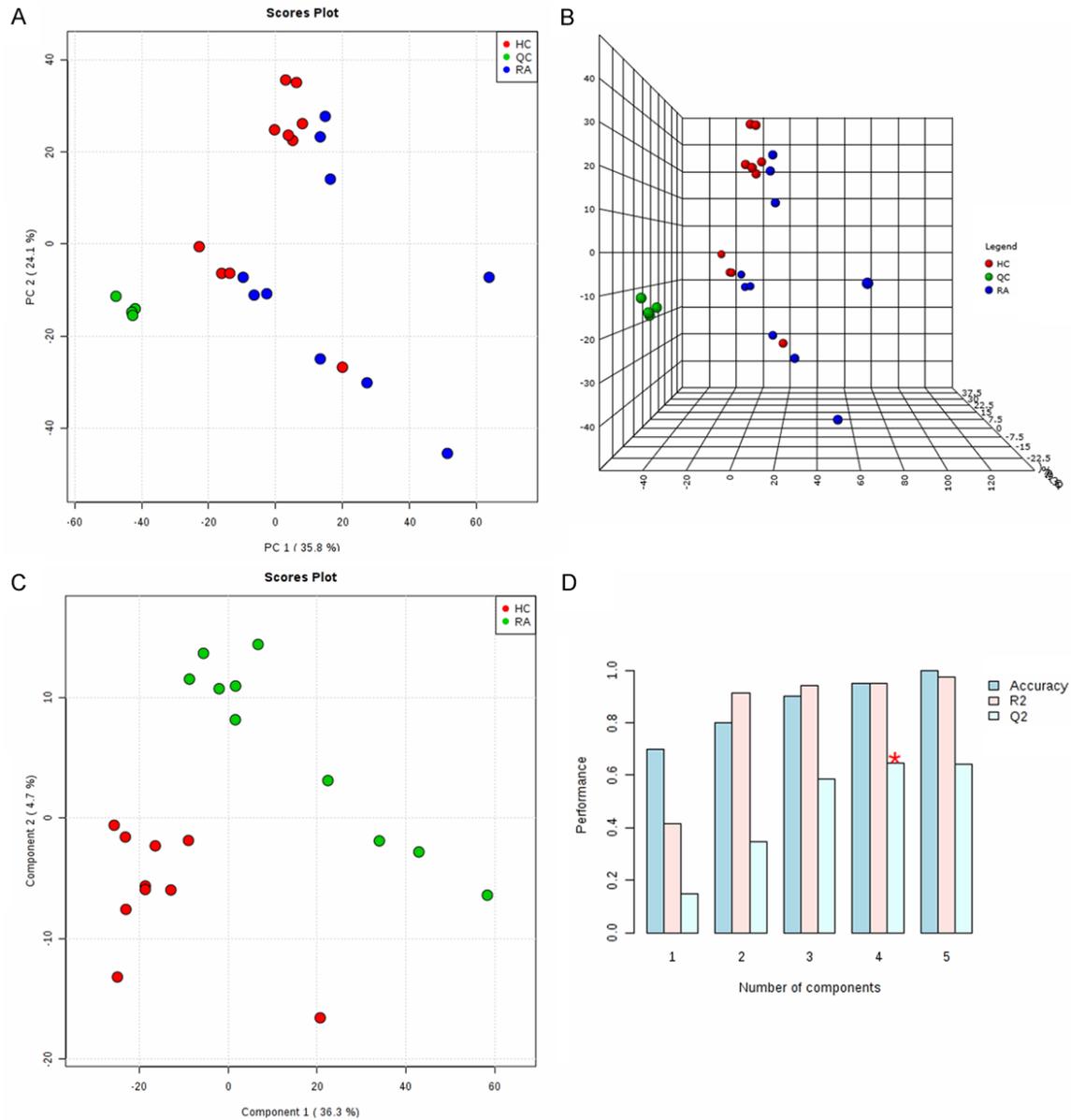


Figure 2. (A) 2D PCA and (B) 3D score plot of positive ion mode. (C) PLS-DA score plot of positive ion mode. (D) Cross validation of the PLS-DA models.

ammonium acetate and 0.1% formic acid. The gradient program was: 0 min-40% B; 2 min-43% B; 2.1 min-50% B; 12 min 60% B; 12.1-75% B; 18 min-99% B; 19 min-99% B; 20 min-40% B; 25 min-40% B. The column temperature was maintained at 50°C, and the flow rate was set to 0.6 ml/min.

Mass spectrometric detection was performed by electrospray ionization in both positive ion mode and negative ion mode. The source voltage was maintained at 3.3 kV in the positive ion

mode and 2.8 kV in the negative ion mode. All other interface settings were identical for both positive ion mode and negative ion mode. The capillary temperature, sheath gas flow and auxiliary gas flow were set at 320°C, 40 arb and 10 arb, respectively. Data were collected in a data-dependent top 10 scan mode. Survey full-scan MS spectra (mass range m/z 80 to 1200) were acquired with resolution $R = 70,000$ and AGC target $1e^6$. MS/MS fragmentation was performed using high-energy c-trap dissociation (HCD) with resolution $R = 17,500$ and AGC tar-

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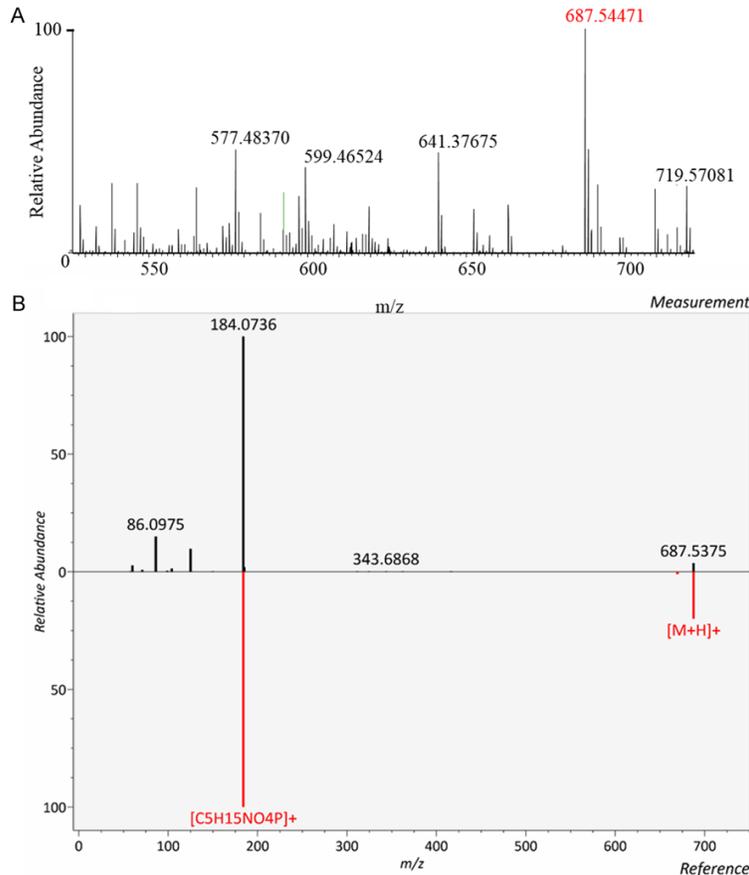


Figure 3. A. High resolution MS spectrum in positive ion mode at retention time of 9.86 min. B. Upper, the measured MS/MS spectrum of m/z 687.54; lower, the MS/MS spectrum of SM 33:2 in database.

get $1e^5$. The stepped normalized collision energy (NCE) was set to 15, 30, and 45, respectively. External mass calibration was applied before every sequence run.

The stability of retention time, mass accuracy and intensity is essential in LC-MS based lipidomic analysis. A pooled serum sample was therefore prepared as quality control (QC) to assess the stability of the instrument and ensure the reliability of the data. QC sample was run before and after the sequence and in every 10 sample runs in the sequence in order to ensure the reproducibility of the data.

Data processing and analysis

The acquired raw data were processed using MS-DIAL [14]. Statistical significance was calculated using the Student's *t*-test, and molecular features were chosen when their *P*-value was < 0.05 and fold change was > 2 or < 0.5 . The

retention time and intensities of ions from both positive ion mode and negative ion mode were imported into SIMCA-P (version 11.5) program and treated separately for multivariate analysis. Principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) were calculated by Pareto scaling. Pathway analysis was carried out with the Metabo Analyst 3.0 Web service [15, 16].

Results

Serum lipidomic profiles

Samples were analyzed with UHPLC-MS in a data-dependent scan mode, and a number of lipid classes and subclasses were detected, including free fatty acids (FFA), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM), monoglyceride (MG) and triacylglycerol (TG). The analytical performance of the LC-MS based lipidomics method was evaluated using pooled QC samples. All samples were run in a single sequence and QC samples were inserted every 10 sample injections. The QC samples were tightly clustered in the principal component analysis (PCA) score plot, as shown in **Figure 2A**. There were no retention shifts and the RSD values of peak intensities were less than 5% (see **Figure S1** in supporting material). These results indicated that this serum lipidomics method was stable and reliable.

Typical full scan base peak ion chromatograms of the RA group and health controls (HC) in positive ion mode are presented in **Figure 1**.

Multivariate analysis

Raw data were processed on MS-DIAL software according to the instructions in the software tutorial [14]. Datasets containing m/z values,

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Table 2. Significant metabolites identified in positive ion mode

Metabolites	<i>m/z</i>	RT (min)	Fragment ions	<i>P</i> -value	Fold change	Change trend
TG 50:1	855.7443	17.05	599.51, 81.07, 261.22	9.46E-03	6.27	↑
TG 50:2	853.7308	16.73	597.48, 184.07	2.88E-02	4.69	↑
TG 54:1	911.8045	19.41	607.56	6.77E-03	2.51	↑
lysoPE 22:5	528.3109	3.43	100.11, 387.29	5.14E-03	2.44	↑
PC 16:0/20:5	780.556	11.84	184.07	1.13E-02	2.38	↑
PC 44:4	894.6966	16.36	86.10, 184.07	9.19E-03	2.24	↑
PC 32:2	730.5391	11.46	86.10, 184.07	3.03E-02	2.20	↑
PC 36:6	778.5396	10.38	86.10, 184.07	3.48E-02	2.13	↑
PC 40:7	832.5856	12.34	86.10, 184.07	1.17E-02	2.10	↑
SM 33:2	687.5456	9.86	184.07	4.20E-04	2.07	↑
Cer_NDS 41:0	638.6454	17.04	620.63, 284.29	1.22E-04	0.34	↓
Cer_NDS 42:0	652.6646	17.26	634.64, 284.29	2.69E-05	0.17	↓

Table 3. Significant metabolites identified in negative ion mode

Metabolites	<i>m/z</i>	RT (min)	Fragment ions	<i>P</i> -value	Fold change	Change trend
lysoPE 24:0	564.4037	10.89	367.36, 196.04	3.29E-02	3.97	↑
PC 16:0/20:5	838.5612	11.86	255.23, 301.22, 764.12	9.56E-03	2.99	↑
PG 36:3	771.52	10.98	281.25, 152.99	2.64E-03	3.81	↑
PI 32:1	807.5046	10.87	255.23, 553.28	2.30E-02	3.95	↑
PI 34:3	831.506	10.16	255.23, 533.27	3.95E-02	8.68	↑
PI 36:2	861.551	13.77	283.27, 581.30	4.50E-03	2.40	↑
PI 36:4	857.521	10.95	255.23, 553.27	2.26E-02	3.72	↑
PI 37:4	871.5348	12.16	269.25, 567.30	6.93E-04	3.15	↑
PI 38:3	887.5669	14.24	283.27, 581.32	1.16E-02	2.01	↑
PI 38:5	883.5362	11.14	255.23, 329.25, 553.27	2.10E-03	3.61	↑
PI 38:6	881.5219	10.33	255.23, 78.96, 152.99	2.58E-02	2.21	↑
PI 40:6	909.5532	12.72	283.26, 581.31	2.41E-02	2.29	↑
PI 40:7	907.5368	10.58	281.25, 579.29	6.30E-03	5.60	↑

retention time, and peak area were exported as an Excel file, and then the Excel file was imported into the SIMCA-P program (version 11.5) for multivariate analysis. The 2D and 3D PCA score plot containing RA, HC and QC groups in positive ion mode are shown in **Figure 2A** and **2B** respectively. All samples scattered in the figure fell into the 95% confidence interval basically. The RA and HC groups were clustered together and separated from each other, which suggested that the involved biochemical pathway was significantly perturbed in the RA group. QC samples clustered together tightly, reflecting the stability of this instrument and reproducibility of this method.

PLS-DA is a supervised statistical algorithm, and this algorithm was applied to the classification and differentiation of the RA and HC groups

for further analysis. The score plot in the PLS-DA showed better clustering tendency between the RA and HC groups, as shown in **Figure 2C**. The fraction of the sum of squares R²Y was 0.965, and the predicted residual sum of squares Q² (cum) was 0.791 in positive ion mode. Cross validation showed no overfitting of the classification model (**Figure 2D**). These multivariate analyses indicated significant metabolic differences between the RA and HC groups.

Identification of potential biomarker between RA and HC groups

Identification of metabolites is challenging and time-consuming. Acquired raw data were processed using MSDIAL. Molecular features which met the conditions of *P*-value < 0.05, and

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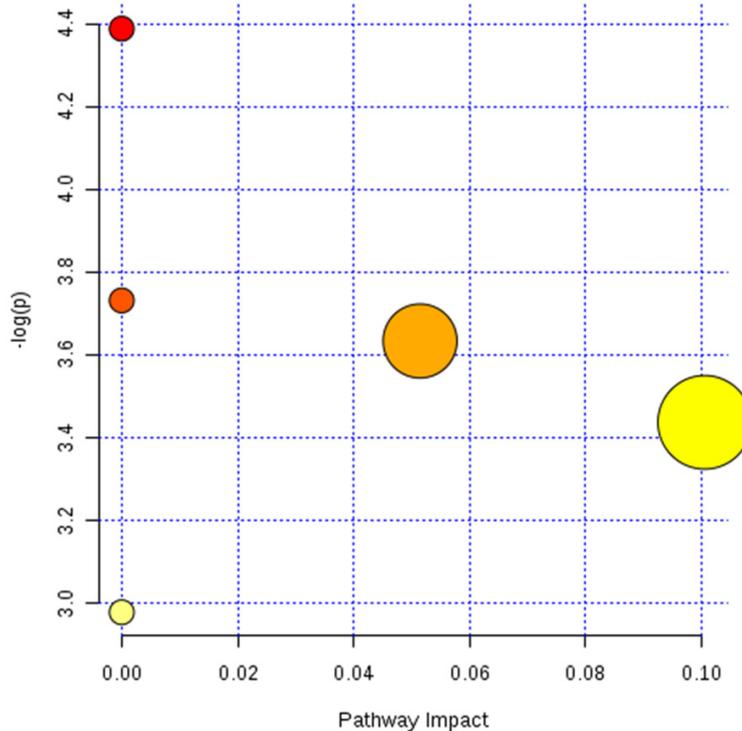


Figure 4. Summary of pathway analysis.

fold change > 2 or < 0.5 were used to interrogate the build-in database in MSDIAL for matches and identification. The accurate mass tolerances of MS and MS/MS were set to 0.01 and 0.05 Da respectively.

The identification process is briefly illustrated below by using a molecular feature at retention time 9.86 min with $[M + H]^+$ ion m/z 687.54 in ESI positive ion mode as an example. **Figure 3A** illustrates the high resolution full scan MS spectrum, and the accurate m/z value was obtained. Searching on lipidmap with this value yielded two possible compounds: SM 33:2 and PE-Cer 36:2. The MS/MS was further performed on this ion, and its structural information was interpreted using the resultant fragmentation pattern. **Figure 3B** (upper) shows the ESI-MS/MS spectrum of the $[M+H]^+$ ion at m/z 687.54471, and the lower panel shows the theoretical fragmentation pattern of SM 33:2. The m/z 184.07 daughter ion was produced by loss of the phosphorylcholine head group, which was frequently observed from sphingomyelin and thus serves as a diagnostic ion [17, 18]. The metabolite of m/z 687.54 at retention time 9.86 min was therefore determined to be SM 33:2.

Using the method above, 12 significantly changed metabolites in positive ion mode and 13 significantly changed metabolites in negative ion mode were tentatively identified with accurate mass spectra and MS/MS spectra. **Tables 2** and **3** list these metabolites with their retention time, accurate m/z value, fragment ions, fold change, and p -value in positive ion mode and negative ion mode respectively. As shown in **Tables 2** and **3**, the majority of the significant metabolites were increased in the RA group as compared to HC group. Although most of these lipids were upregulated including three TGs, five PCs, and ten PIs, two Cer_NDSs which belong to a ceramide subclass [19, 20] were down regulated. Interestingly, PC 36:5 was identified in both

positive ion mode with $[M + H]^+$ and negative ion mode with $[M + Ac]^-$. Although the peak intensity of $[M + Ac]^-$ was much lower than that of $[M + H]^+$ due to the chemical properties of PC [21], the fact that PC 36:5 was identified in both positive and negative ion modes allowed greater confidence in the identification function of MSDIAL.

Pathway analysis

To determine which pathways are dysregulated in RA patients, lipids identified with the methodology as described were analyzed using the Metabo Analyst 3.0 website for pathway analysis. Results showed several pathways involving metabolism of linoleic acid, alpha-linolenic acid, glycerolipid, glycerophospholipid, and arachidonic acid may be perturbed in patients with RA. A summary of pathway analysis is shown in **Figure 4** and is listed in **Table 4** in detail. The specific mechanisms underlying these perturbations including the enzymes and genes which are involved require further investigation. However, this study provides a new link between linoleic acid metabolism, glycerolipid metabolism, and RA. This metabolic disturbance may play a crucial role in the pathogenesis of RA, and this in turn may provide a new approach for diagnosis of RA.

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Table 4. Results from pathway analysis

	Total	Expected	Hits	Raw p	-LOG (p)	Holm adjust	FDR	Impact
Linoleic acid metabolism	15	0.01	1	1.24E-02	4.39E+00	9.94E-01	6.43E-01	0.00
Alpha-Linolenic acid metabolism	29	0.02	1	2.40E-02	3.73E+00	1.00E+00	6.43E-01	0.00
Glycerolipid metabolism	32	0.03	1	2.64E-02	3.63E+00	1.00E+00	6.43E-01	0.05
Glycerophospholipid metabolism	39	0.03	1	3.22E-02	3.44E+00	1.00E+00	6.43E-01	0.10
Arachidonic acid metabolism	62	0.05	1	5.09E-02	2.98E+00	1.00E+00	8.14E-01	0.00

Discussion

Rheumatoid arthritis (RA) is a chronic autoimmune disorder. Immune-mediated increases in autoantibodies or inflammatory factors in RA patients can lead to changes in nutrient metabolism, particularly in protein and lipid metabolism. It is well known that the leading cause of death in RA patients is cardiovascular disease (CVD), especially coronary atherosclerosis [22]. Patients with persistent RA had a more abnormal balance in lipids circulating in the blood as compared with controls that did not have RA [23]. This dyslipidemia in RA patients may be due to the fact that cytokines (such as TNF- α , IL-1, IL-6, and so on.) interfere with lipid metabolism in RA patients [24]. In summary, there are lipid-spectrum abnormalities in RA patients, and such dyslipidemia may be associated with disease activity. Identifying the differences in lipid metabolism between RA patients and controls is therefore critical for obtaining new RA biomarkers and for characterizing mechanisms of dyslipidemia in RA.

Consistent with many previous studies [25, 26], we observed that the RA group showed significantly elevated levels of TG. Increased plasma TG has been known to be a risk factor for cardiovascular disease and hypertriglyceridemia, which are associated with inflammatory progression [27]. It is therefore reasonable to suppose that elevation of TG in RA patients may contribute to increased risk of CVD.

Phospholipids are ubiquitous in nature and constitute important components of the lipid bilayer of cells and play major roles in cell signaling and metabolism. In this study, the increased levels of phospholipid metabolites indicated a marked perturbation in phospholipid metabolic pathways in RA. The majority of these phospholipids were phosphatidylinositols (PI). Phosphorylated forms of PI are called

phosphoinositides and play important roles in lipid signaling, cell signaling and membrane trafficking. PI3K, which is the kinase for PI, is known to be associated with many steps in the mechanism of inflammatory reaction [28]. One study confirmed the therapeutic potential of PI3K inhibition in a mouse model of RA [29]. Lyso-PE is an important phospholipid synthetic pathway intermediate, and abnormalities of this metabolite reflect the impact of RA on the phospholipid metabolic pathway. PC was another metabolite in the present study which shows significant alteration. Antibodies against PC have been implicated in RA-related atherosclerosis and may find use in establishing stratification algorithms for CV risk [30].

Conclusion

Lipidomics has become increasingly promising in the search for clinical biomarkers, as well as for identification of lipid-related pathways which are disturbed in various pathologic and physiologic conditions. In this study, we developed an untargeted analytical approach based on UHPLC-Q-Exactive MS to profile lipid alterations in serum samples of rheumatoid arthritis patients. Patients with RA were distinguished from health control individuals with the help of multivariate statistical analysis such as PCA and PLS-DA, which shows significant lipid perturbation in RA. Potential biomarkers including TGs, PCs, and PIs were identified tentatively with the assistance of high accuracy of MS and MS/MS spectra. Pathway analysis demonstrated that several metabolic pathways such as linoleic acid metabolism were dysregulated in RA. The pathophysiologic significance of these lipid alterations was also addressed. This study may provide new insight into underlying mechanisms of RA and shows that LC-MS based lipidomic profile is a powerful approach for discovery of new potential biomarkers in clinical research.

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Disclosure of conflict of interest

None.

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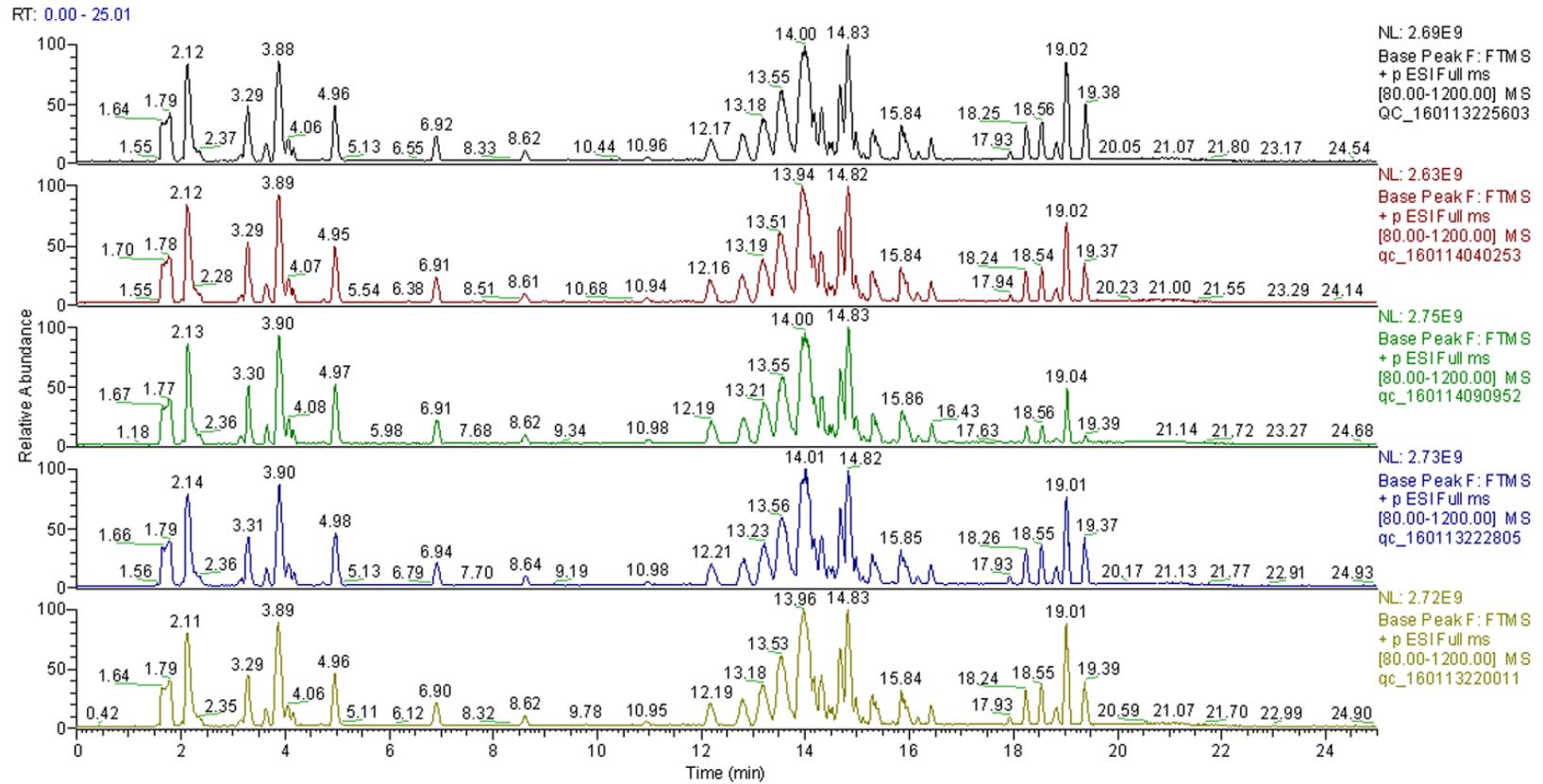


Figure S1. Full scan base peak mass chromatograms of QC samples.