Downregulation of Tryptophan-related Metabolomic Profile in Rheumatoid Arthritis Synovial Fluid

Kwi Young Kang, Soo Hyun Lee, Seung Min Jung, Sung-Hwan Park, Byung-Hwa Jung, and Ji Hyeon Ju

ABSTRACT. ***Objective.*** Synovial fluid (SF) is one of the most important materials that reflect the pathophysiological process of arthritis. A metabolomic and lipidomic study of SF was performed with the aim of identifying tentative diagnostic markers or therapeutic candidates for rheumatoid arthritis (RA).

Methods. SF was aspirated from 10 patients with RA and 10 patients with osteoarthritis (OA). RA SF and OA SF were collected and analyzed by ultraperformance liquid chromatography quadruple time-of-flight mass spectrometry. Associations among clinical variables, laboratory results, and metabolic profiles were investigated.

Results. The metabolic pathways for carnitine, tryptophan, phenylalanine, arachidonic acid, and glycophospholipid were significantly upregulated in OA SF. The metabolic pathways for taurine, cholesterol ester, and the β-oxidation of pristine acid, linolenic acid, and sphingolipid were activated more in RA SF than in OA SF. In particular, the tryptophan pathway, which comprises kynurenine, indoleacetic acid, indole acetaldehyde, and N′-formylkynurenine, was downregulated. Interestingly, the levels of tryptophan metabolites kynurenine and N′-formylkynurenine, which are involved in immune tolerance, were significantly lower in RA SF compared with OA SF ($p < 0.05$), but the opposite pattern was observed for erythrocyte sedimentation rate ($p < 0.01$) and the levels of C-reactive protein (CRP; $p < 0.01$), rheumatoid factor ($p < 0.01$), and anticyclic citrullinated peptide antibody ($p < 0.05$). Kynurenine concentration correlated inversely with CRP concentration in RA SF but not in OA SF ($r -0.65$, $p < 0.05$).

Conclusion. Advances in metabolomic techniques enabled us to delineate distinctive metabolic and lipidomic profiles in RA SF and OA SF. RA SF and OA SF showed distinct metabolic profiles. (J Rheumatol First Release September 1 2015; doi:10.3899/jrheum.141505)

Key Indexing Terms:

TRYPTOPHAN	RHEUMATOID ARTHRITIS	SYNOVIAL FLUID
OSTEOARTHRITIS	MASS SPECTROMETRY	METABOLOMICS

Rheumatoid arthritis (RA) is characterized by persistent synovitis, systemic inflammation, and autoantibodies[1]. Inflammation in the synovium caused by the infiltration of immune cells and the local production of proinflammatory cytokines play significant roles in disease progression[2,3]. Osteoarthritis (OA) is a progressive disorder characterized by the degradation of cartilage, osteophyte formation, narrowing of the joint space, and subchondral sclerosis[4]. The differential diagnosis of RA from other kinds of arthritis, such as OA, is difficult in the early disease state.

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Synovial fluid (SF) is a serum filtrate found in synovial joints, where it also receives protein contributions from the surrounding tissues, articular cartilage, synovial membrane, and bone. Because SF is in direct contact with the joint, it may be an excellent source for the discovery of biomarkers[5]. Analyzing metabolites in SF is more beneficial than analyzing those in serum when studying arthritis. Because SF is proximal to the site of disease, it reflects disease activity and provides information about disease progression in the most relevant compartment (the joint). In addition, alterations in the joint cavity caused by disease may be reflected in the composition of SF and might correlate with disease severity and progression[6].

Metabolomics can provide information about the distinctive characteristics and physiological status of a biological system and may help identify new targets for diagnosis, prognosis, and therapeutic intervention in important human diseases. A metabolomic approach is particularly helpful for unbiased monitoring of changes in endogenous metabolism-related physiological processes[7] and for understanding the relevant pathophysiological changes in a disease state[8]. Previous studies have confirmed that chronic inflammation in RA is associated with proinflammatory cytokines that affect the whole body systematically, including various effects on metabolism. Thus, the extent of the metabolic changes and the types of metabolites may provide markers of cytokine-mediated inflammatory processes in RA, which can be examined using a metabolomic study[9].

In previous metabolomic studies of RA, proteome analysis of SF was used mainly to identify proteins that distinguish RA from OA[10,11,12]. Metabolomic analysis of SF from RA and OA has used nuclear magnetic resonance spectroscopy, but the number of SF samples (4 RA samples and 15 OA) was too small to identify differences in the metabolic profiles between RA and OA[13].

The objective of our study was to examine the relative metabolic profiles in the SF of patients with RA and to compare these with the profiles from patients with OA. We used liquid chromatography/mass spectrometry to analyze these samples, and we performed correlational analysis to examine the relationships between metabolites in SF and clinical variables in patients with RA.

MATERIALS AND METHODS

Study patients. SF was obtained from 10 patients with RA and 10 patients with OA. All 20 patients were women; we recruited only women because sex differences can affect metabolites[14]. RA was diagnosed by a rheumatologist according to the American College of Rheumatology 1987 revised criteria[15]. OA was diagnosed according to clinical and radiological criteria. Laboratory data were measured at the time of SF aspiration. The OA radiological score was evaluated with the Kellgren–Lawrence (KL) grading scale[16].

Sampling of SF. When aspiration was indicated as part of routine clinical practice, SF samples were obtained by sterile aspiration from the knees of all the patients studied. SF samples were aspirated using a 21-gauge needle through the lateral infrapatellar approach. Total white blood cell (WBC) count was determined in the SF samples. The samples were stored at –70°C until analyzed.

Materials for metabolomics. Formic acid, leucine enkephalin, sodium formate, caffeine, acetaminophen, reserpine, hippuric acid, glycocholic acid, adipic acid, and methanol (HPLC grade) were purchased from Sigma-Aldrich Chemical Co. Ultrapure water (18.2 MW) was obtained using a Milli-Q apparatus from Millipore.

Sample preparation for metabolomics. The SF samples were prepared using cold methanol precipitation. The prepared sample was transferred to autosampler vials and injected into the ultraperformance liquid chromatography quadruple time-of-flight mass spectrometer (UPLC-QTOF-MS).

A quality control (QC) sample was prepared by mixing equal amounts of each sample and processed using the same method used for sample preparation[17]. The QC sample was used for column conditioning and method validation[18,19] (Supplementary Tables 1, 2, and 3 available online at jrheum.org).

UPLC-QTOF-MS analysis. An ACQUITY UPLC (Waters) was connected directly to a QTOF-MS (SYNAPT G2; Waters). The separations were performed using an ACQUITY UPLC BEH C18 column (1.7 mm particle size, 2.1 mm inner diameter, 100 mm length; Waters) at 50°C. Initially, 20 ml QC samples were injected 15 times before the actual sample analysis using a short gradient program to condition the column and to optimize the system[20]. Gradient elution was performed using a mixture of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol) at a flow rate of 0.4 ml min^{-1}. The starting conditions for the short gradient were 90% A and 10% B (v/v), shifting to 100% B in a linear gradient over 3 min. This solvent composition was maintained for 2.5 min, followed by a return to the starting conditions. Reequilibration was performed for 2.5 min until the next conditioning injection. For the analysis of the samples, a longer gradient program was used with the same solvent system as the conditioning gradient. The starting conditions were 90% A and 10% B (v/v) for 0.5 min, shifting to 80% A and 20% B over 3 min, to 30% A and 70% B at 6 min, and to 100% B at 12 min. The solvent composition was held at 100% B for 2 min before returning to the starting conditions.

Reequilibration of the system for 1.5 min using 90% A and 10% B (v/v) was performed until the next injection. Ten-ml samples were injected. All of the samples were stored at 4°C during the analysis. To eliminate the effect of sample order, the selection of injected samples was alternated between the samples from OA and RA groups. One QC sample was run every 10 samples, and the test mixture was injected 3 times, at the beginning, middle, and end of the analysis, to verify the reproducibility and the repeatability within and among the sets of analytical samples.

MS based on MS^E data acquisition was performed in both the positive and negative ionization modes using an electrospray ionization source interface. In MS^E, the collision cell was fast switched between low and high collision energy, to produce alternate precursor and fragment ion spectra in a single analytical run[21]. Thus, in MS^E mode all spectral data were obtained in the same MS variables except collision energy. The capillary voltage was set at 3200 V and 2500 V for the positive and negative ionization modes, respectively, and the cone voltage was 40 V for both ionization modes (Supplementary Figure 1, available online at jrheum.org). The disolvation and cone gas was nitrogen at a flow rate of 600 l h^{-1} and 100 l h^{-1}, respectively. The source temperature was 120°C and the dissolution temperature was 350°C. Leucine–enkephalin (0.2 mg L^{-1} in 50% methanol) was used as the lock mass [mass-to-charge ratio (m/z) 556.2771 for the positive ionization mode and 554.2615 for the negative ionization mode] at a flow rate of 20 ml min^{-1}. The full-scan data were collected at a range of 50–1200 m/z over a period of 15 min using a scan time of 0.5 s and an interscan delay of 0.1 s in the resolution mode. All of the acquired spectra were corrected automatically during acquisition based on the lock mass. The MS data were collected into 2 separate data channels using collision energies alternating between 0 eV (low-energy scans) and 30 eV (high-energy scans) in the centroid mode. Preceding the analysis, the MS was calibrated using a 0.2 mM sodium formate solution.

Data analyses. The raw MS data from all of the samples were processed using MarkerLynx XS software version 4.1 (Waters). This application identifies the mass retention time pairs (RT-m/z pair) and the intensities of the peaks eluted from at least 2 of the samples. After profiling, the areas of these peaks, representing the ion intensities, were normalized to the summed total of the ion intensities of each chromatogram. The sample name, the identified RT-m/z pair, and the normalized ion intensity were used for statistical analysis[22].

The multivariate analysis method principal component analysis (PCA) was performed. It identifies inherent group clustering and highlights the factors responsible for the clustering (Supplementary Figure 2, available online at jrheum.org). The sample list, factor list, and PCA results were displayed in the MarkerLynx browser. The MarkerLynx software processed the chromatographic full-scan data acquired in the centroid mode. Orthogonal partial least-squares–discriminant analysis (OPLS-DA), a multivariate analysis method, was performed using EZinfo software (Umetrics Inc.). To generate the OPLS-DA plots, the Pareto scale was used for the SIMCA-P analysis. The quality of each OPLS-DA model was examined using the R^2 and Q^2 values to confirm that the models were not overfitted and to evaluate the predictive ability of each model. R^2 represents the goodness-of-fit variable, defined as the proportion of variance in the data described by the model. Q^2 represents the predictive ability variable, defined as the proportion of variance in the data predicted by the model. An S-plot is a plot of the covariance, p, against the correlation, p (corr), in conjunction with the variable trend plots. S-plots reveal potentially significant variables based on their reliability and their contributions to the differences found in the OPLS-DA models. The variables exhibiting the most significant differences are located at the bottom or the top of the plot, and those variables exhibiting less significant differences are in the middle. The significance of differences in the variables and in serum biochemical variables between 2 groups was determined using paired-sample t tests. P values < 0.05 were considered significant.

The peak identification of metabolite biomarkers was performed by verifying the accuracy of the mass measurements, the peak RT, and the tandem MS results against an in-house database and online databases, including METLIN (http://metlin.scripps.edu), HMDB (www.hmdb.ca), ChemSpider (www.chemspider.com), and KEGG (www.kegg.com). An in-house database included the information on authentic chemical standards for various metabolite molecules when authentic chemical standards were analyzed with the same method in the present study; exact mass precursor and fragment ion as well as RT. An in-house database was applied as a substitute for a separate validation sample set in our study.

Correlation analysis was used to evaluate the overall correlations between various factors. The distance was measured using the Pearson correlation coefficient (Rp) in MetaboAnalyst (www.metaboanalyst.ca). The Rp is a value ranging between +1 and –1. Values between 0 and 0.3 (or 0 and –0.3) were interpreted as a weak positive (or negative) linear relationship between the 2 variables.

Statistical analysis. Mean and SD were calculated for continuous data, and frequencies were recorded for dichotomous and ordinal data. The chi-squared test and Mann–Whitney U test were used to compare differences in clinical data between patients with RA and patients with OA. All analyses were performed using SPSS 18.0 (IBM). Levels < 0.05 were considered significant.

Ethics statement. This study protocol was approved by the institutional review board of The Catholic University of Korea (KC12TNSI0071). We acquired consent forms from all the patients.

RESULTS

Characteristics of the patients. The characteristics of the patients are shown in Table 1. All patients were women. There were no significant differences in body mass index, uric acid, and serum lipid profile, including the concentrations of total cholesterol, triglyceride, low-density lipoprotein cholesterol. The mean ages of the patients with RA and patients with OA were 52 ± 11 and 72 ± 11, respectively, (p = 0.002). Serum erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) concentration were significantly higher in the patients with RA versus in the patients with OA (p = 0.004 and p = 0.001, respectively). Rheumatoid factor (RF) and anticyclic citrullinated peptide antibodies (anti-CCP) were not detected in the SF from all patients with OA. The mean SF WBC count was higher in the patients with RA than in the patients with OA: 21,480 ± 16,640 cells/mm^3 and 118 ± 28 cells/mm^3, respectively, (p = 0.036). The WBC count in SF was < 2000/mm^3 in all patients with OA, indicating that the underlying disease was noninflammatory arthritis. All patients with RA received prednisolone at a dosage of 2.5–7.5 mg/day, whereas only 1 patient with OA received prednisolone at 2.5 mg/day. Methotrexate (MTX) monotherapy was used to treat 4 patients with RA and combination therapies of MTX and other disease-modifying antirheumatic drug (DMARD) were used to treat 6 patients with RA. No patients with OA received MTX or DMARD.

Table 1. Characteristics of the female patients with RA and OA. Data are mean ± SD unless otherwise indicated.

Variables	Patients with RA, n = 10	Patients with OA, n = 10	p
Age, yrs	52 ± 11	72 ± 11	0.002
Disease duration, yrs	9 ± 8	9 ± 7	1.000
Diabetes mellitus, n	0	3	0.211
Height, cm	158 ± 2	152 ± 6	0.065
Weight, kg	58 ± 6	57 ± 11	0.837
BMI, kg/m^2	23 ± 3	25 ± 2	0.394
Laboratory findings			
ESR, mm/h	69 ± 24	25 ± 13	0.004
CRP, mg/dl	6.8 ± 8.3	0.1 ± 0.1	0.001
Rheumatoid factor	215 ± 245	3 ± 2	< 0.001
Anti-CCP antibody, IU	214 ± 152	1 ± 1	0.019
Uric acid, mg/dl	4.0 ± 1.2	4.9 ± 0.5	0.279
Total cholesterol, mg/dl	174 ± 29	164 ± 23	0.456
Triglyceride, mg/dl	99 ± 70	158 ± 74	0.066
LDL cholesterol, mg/dl	95 ± 30	88 ± 17	0.710
SF WBC count/mm^3	21480 ± 16640	118 ± 28	0.036
Kellgren-Lawrence grading scale*		2.8 ± 0.6	
Treatment agents, n (%)			
NSAID	10 (100)	10 (100)	1.000
Prednisolone	10 (100)	1 (10)	< 0.001
Methotrexate	10 (100)	0 (0)	< 0.001
Hydroxychloroquine	1 (10)	0 (0)	1.000
Sulfasalazine	1 (10)	0 (0)	1.000
Leflunomide	2 (20)	0 (0)	0.474

*Kellgren-Lawrence grading scale: measurements taken from knee radiograph, available in all patients with OA. RA: rheumatoid arthritis; OA: osteoarthritis: BMI: body mass index; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; anti-CCP: anticyclic citrullinated peptide; LDL: low-density lipoprotein; SF: synovial fluid; WBC: white blood cell; NSAID: nonsteroidal antiinflammatory drugs.

Multivariate analyses of the metabolic profiles. Typical base

peak ion chromatograms from the SF samples of each group are shown in Figure 1. In the multivariate statistical analysis, OPLS-DA was used to analyze the differences in the chromatographic data between the OA and RA groups. For the OPLS-DA score plots, the OA group was separated in the P1 component direction and the RA group was separated in the P2 component direction (Figure 2). R^2 and Q^2 values > 0.5 indicate a high-quality OPLS-DA model[23]. In all analyses, the models displayed 99% goodness-of-fit (R^2 Y = 0.99) and a predictive ability > 58% (Q^2 Y = 0.58), which demonstrated that the OPLS-DA models in our present study were well fitted and displayed an acceptable predictive ability. These results suggested that these models could discriminate between the patterns of metabolites in both groups.

To identify potential variables contributing to the detected differences, S-plots from the OPLS-DA models were constructed. Cutoff values for the correlation of p (corr) ≥ 0.5 were used in every OPLS-DA model. Additionally, a variable importance in the projection (VIP) value > 1.0 was applied

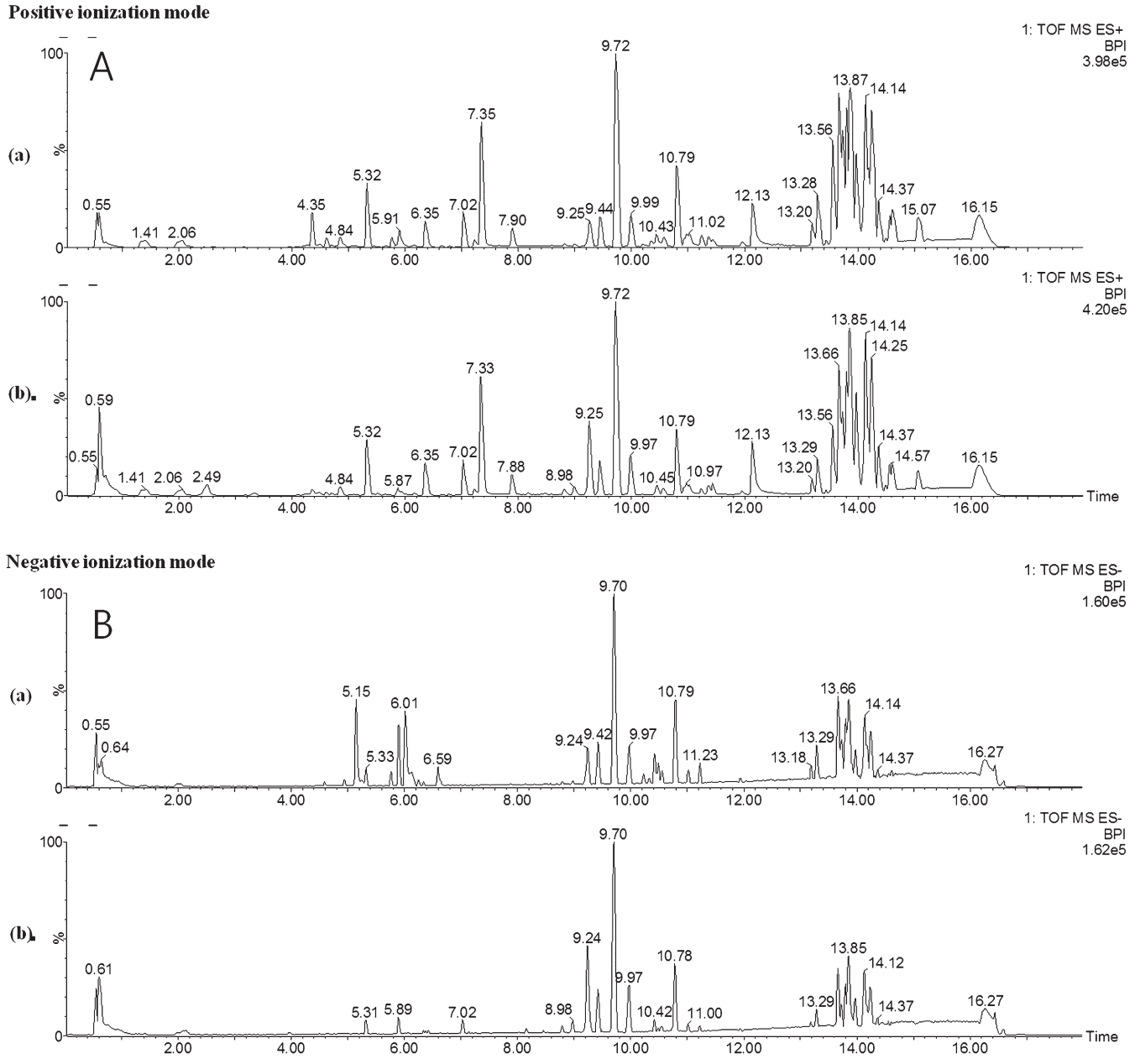

Figure 1. Typical base peak intensity chromatograms of SF samples from the RA (A) and OA (B) groups. A. Chromatograms detected in positive ionization modes. B. Chromatograms detected in negative ionization modes. RA: rheumatoid arthritis; OA: osteoarthritis; TOF: time-of-flight; MS: mass spectrometer; BPI: base peak ion.

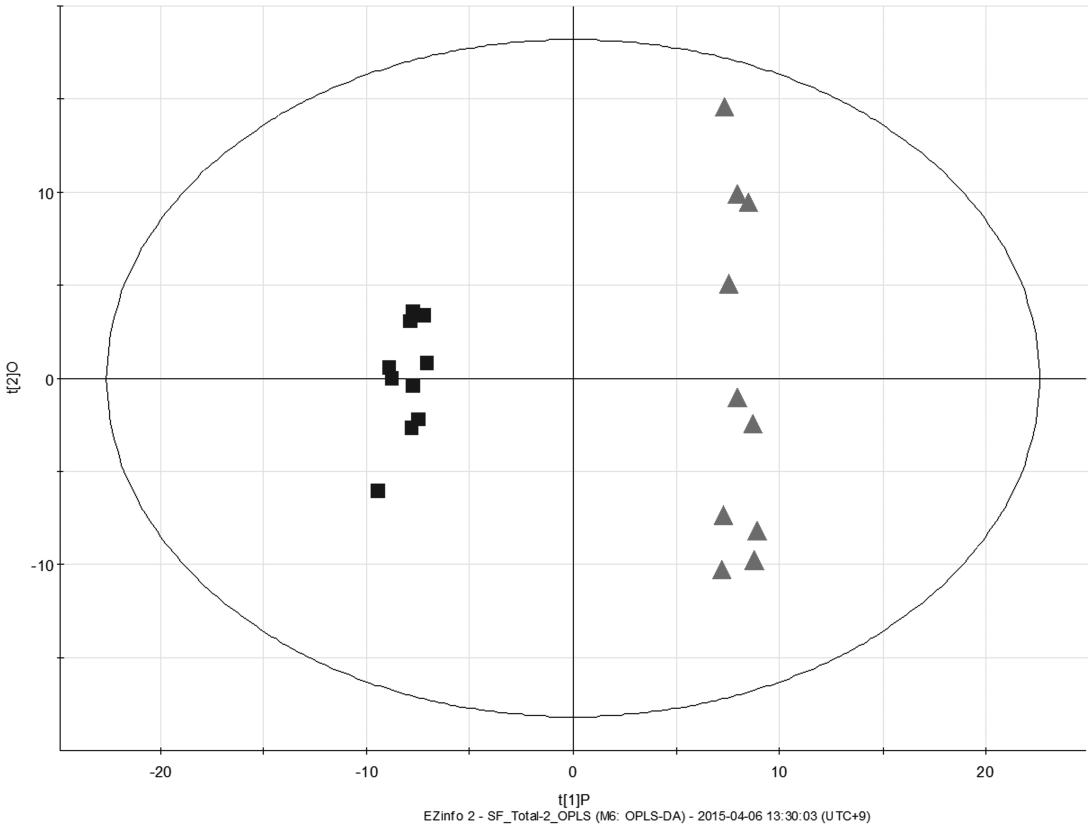

Figure 2. OPLS-DA score plots of SF metabolic profiling of the OA (boxes) and RA (triangles) groups. OPLS-DA was performed to analyze the differences in the chromatographic data between the OA and RA groups using EZinfo software (Umetrics Inc.). To generate the OPLS-DA plots, the Pareto scale was used for the SIMCA-P analysis. For the OPLS-DA score plots, the OA group was separated in the P1 component direction and the RA group was separated in the P2 component direction. OPLS-DA: orthogonal partial least-squares–discriminant analysis; SF: synovial fluid; OA: osteoarthritis; RA: rheumatoid arthritis.

to restrict the potential variables. According to these processes, 81 metabolites of 661 variables (total variables detected in both the positive and negative ionization modes) were identified as distinct metabolites that differed between OA and RA.

Identification of potential metabolites. The UPLC-Synapt G2 analysis provided the RT, the exact molecular mass, and the MS/MS spectra used for the structural identification of the metabolites. The molecular masses were detected precisely through QTOF with narrow measurement errors (< 5 mDa). The potential elemental composition and mass fragment information for each metabolite were also collected using the MS and MS/MS spectra. The potential metabolites were identified by database searches and then confirmed by the authentic chemical references. Based on these processes, 21 out of 81 metabolites were identified (Table 2). The significance of differences in the identified metabolites between the 2 groups was determined based on independent sample t tests.

Significant differences in amino acid metabolism, purine metabolism, and several factors of lipid metabolism were confirmed between OA and RA. The SF levels of kynurenine, N′-formylkynurenine, indolelactic acid, and indole acetaldehyde in tryptophan metabolism were lower in RA than in OA. The levels of N6,N6,N6-trimethyl-L-lysine and carnitine in carnitine metabolism and the levels of 3-hydroxymandelic acid and phenylactic acid were also lower in RA. The levels of 5-L-gluygutamyltaurine and taurine in taurine metabolism were higher in RA. The levels of (S)-ureidoglycolic acid and methylguanine in purine metabolism were higher in RA. Some lipid metabolites, including arachidonic acid metabolites [trihydroxyeicosatrienoic acid/13,14-dihydro-lipoxin A4, and 12,20-dioxo-leukotriene B4), corticosterone metabolite, and glycophospholipid [lysophosphatidylcholine (lysoPC; 18:1)] were lower in RA. The levels of cholesterol ester [CE(24:1[15Z])], β-oxidation product (trimethyltridecanoic acid), and sphingolipid metabolite were higher.

Correlation between clinical variables and validated metabolites. The relationships between the clinical variables and the validated metabolites were examined using correlational analysis (Table 3). ESR and CRP concentrations were strongly inversely correlated with kynurenine and L-carnitine levels and strongly positively correlated with CE[24:1(15Z)].

Table 2. Identified metabolites that displayed significant differences between the OA and RA groups.

RT-m/z (ionization mode)	Molecular Formula	Fragmentation	Metabolite	Peak Intensity OA	Peak Intensity RA	p	Related Metabolism
0.55_189.17 [M+H]	$C_9H_{20}N_2O_2$	$C_6H_{14}N(-CO_2)$	N6,N6,N6-Trimethyl-L-lysine	2.83	1.59	0.046	Carnitine
0.59_150.02 [M+2Na]	$C_7H_{15}NO_3$	$C_4H_7O_3(-C_3H_9N)$	5-L-Glutamyltaurine	5.63	7.95	0.036	Taurine
0.60_162.12 [M+H]	$C_7H_{15}NO_3$	$C_4H_7O_3(-C_3H_9N)$	L-Carnitine	72.06	57.16	0.039	Carnitine
0.62_169.05 [M+H]	$C_8H_8O_4$	$C_7H_7O_2(-CO_2H)$	3-Hydroxymandelic acid	32.79	26.17	0.006	Phenylalanine and tyrosine
0.63_124.01 [M–H]	$C_2H_7NO_3S$	$C_2H_6NO_2S$ $(-H_2O)$	Taurine	10.66	14.58	0.044	Taurine
0.72_135.01 [M+H]	$C_3H_6N_2O_4$	$C_3H_6N_2O_4(-COH_2N)$	(S)-Ureidoglycolic acid	34.58	58.87	0.003	Purine
1.39_166.09 [M+H]	$C_6H_7N_5O$	$C_5H_4N_5O(-CH_3)$	Methylguanine	18.64	25.12	0.016	Purine
3.17_145.06[M–$H_2$0–H]	$C_{10}H_{12}N_2O_3$	$C_9H_{11}N_2O(-CO_2H)$	Kynurenine	9.39	5.07	0.024	Tryptophan
4.47_204.06 [M–H]	$C_{11}H_{11}NO_3$	$C_{10}H_{10}NO(-CO_2H)$	Indolelactic acid	30.53	17.99	0.046	Tryptophan
4.48_158.059 [M–H]	$C_{10}H_9NO$	$C_{10}H_8N(-OH)$	Indoleacetaldehyde	4.94	2.70	0.039	Tryptophan
4.77_130.07 [M+H]	$C_{11}H_{12}N_2O_4$	$C_{10}H_9NO_2(-CO_2H_3N)$	N′-Formylkynurenine	5.65	2.22	0.050	Tryptophan
6.27_167.07 [M+H]	$C_9H_{10}O_3$	$C_7H_7(-C_2O_3H_3)$	Phenylactic acid	3.73	2.67	0.010	Phenylalanine and tyrosine
6.35_119.09 [M+3H]	$C_{20}H_{34}O_5$	$C_{17}H_{30}O_3(-C_3H_4O_2)$	Trihydroxyeicosatrienoic acid/13,14-Dihydrolipoxin A4	8.22	6.03	0.039	Arachidonic acid
6.68_197.09 [M+2Na]	$C_{20}H_{28}O_5$	$C_{17}H_{24}O_3(-C_3H_4O_2)$	12,20-Dioxoleukotriene B4	2.34	1.66	0.029	Arachidonic acid
7.25_211.11 [M+2Na]	$C_{22}H_{32}O_5$	$C_{19}H_{27}O_2(-C_3O_3H_5)$	11β-Hydroxy-3,20-dioxopregn-4-en-21-oic acid	7.85	5.84	0.006	Corticosterone
9.99_280.66 [M+H+K]	$C_{26}H_{54}NO_7P$	$C_5H_{13}O_5P$ $(-C_{21}H_{42}NO_2)$	LysoPC(18:1)	19.00	15.38	< 0.001	Glycophospholipid
10.35_260.66 [M+2H]	$C_{51}H_{90}O_2$	$C_{27}H_{46}O(C_{24}H_{44}O)$	CE[24:1(15Z)]	1.72	5.98	< 0.001	Cholesterol ester
11.01_255.23 [M–H]	$C_{16}H_{32}O_2$	$C_{15}H_{31}$ $(-CO_2H)$	Trimethyltridecanoic acid	95.03	117.79	0.009	Beta-oxidation of pristanic acid
11.01_279.24 [M+Na]	$C_{16}H_{32}O_2$	$C_{15}H_{31}$ $(-CO_2H)$	Trimethyltridecanoic acid	18.75	24.22	0.011	Beta-oxidation of pristanic acid
11.95_285.29 [M+2Na+H]	$C_{22}H_{34}O_2$	$C_{21}H_{33}$ $(-CO_2H)$	Docosapentaenoic acid	7.26	9.51	0.040	Linolenic acid and linoleic acid
13.64_722.56 [M+Na]	$C_{40}H_{77}NO_8$	$C_6H_{11}(-C_{34}H_{66}NO_8)$	Galactosylceramide (d18:1/16:0)/Glucosylceramide (d18:1/16:0)	9.05	10.64	0.044	Sphingolipid

OA: osteoarthritis; RA: rheumatoid arthritis.

Table 3. Correlations between tryptophan metabolites and ESR and levels of CRP, RF, and anti-CCP.

Metabolite	ESR		CRP	
	Rp*	p	Rp*	p
Kynurenine	–0.58	0.007	–0.60	0.005
L-Carnitine	–0.45	0.045	–0.62	0.004
CE[24:1(15Z)]	0.61	0.004	0.70	< 0.001
Metabolite	RF		Anti-CCP	
	Rp*	p	Rp*	p
N′-Formylkynurenine	–0.50	0.025	–0.54	0.015
L-Carnitine	–0.67	0.001	–0.55	0.013
3-Hydroxymandelic acid	–0.61	0.004	–0.61	0.004
11β-Hydroxy-3,20-dioxopregn-4-en-21-oic acid	–0.57	0.009	–0.63	0.003
5-L-Glutamyltaurine	0.57	0.008	0.70	< 0.001
Methylguanine	0.56	0.010	0.71	< 0.001
Galactosylceramide (d18:1/16:0)/ Glucosylceramide (d18:1/16:0)	0.50	0.024	0.50	0.027
(S)-Ureidoglycolic acid	0.74	< 0.001	0.92	< 0.001
CE[24:1(15Z)]	0.76	< 0.001	0.73	< 0.001
Taurine	0.57	0.09	—	—
LysoPC(18:1)	—	—	–0.53	0.016
Indoleacetaldehyde	—	—	–0.49	0.030
Docosapentaenoic acid		—	0.46	0.039

*Rp: Pearson correlation coefficients. The correlation coefficient (γ) takes on values ranging between +1 and –1. Values between 0 and 0.3 (0 and –0.3) were interpreted as a weakly positive (negative) linear relationship between 2 variables; values between 0.3 and 0.5 (–0.3 and –0.5) were interpreted as a moderately positive (negative) linear relationship; and values between 0.5 and 1 (–0.5 and –1) were interpreted as a strongly positive (negative) linear relationship. ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; anti-CCP: anticyclic citrullinated peptide; RF: rheumatoid factor; lysoPC: lysophosphatidylcholine.

RF and anti-CCP levels correlated negatively with the levels of N′-formylkynurenine, L-carnitine, 3-hydroxymandelic acid, and 11b-hydroxy-3,20-dioxopregn-4-en-21-oic acid. CE[24:1(15Z)], (S)-ureidoglycolic acid, 5-L-glutamyltaurine, and methylguanine were strongly positively correlated with RF and anti-CCP levels. The levels of ESR and CRP were elevated more in RA SF than in OA SF, but the kynurenine level was significantly lower in RA SF than in OA SF ($p < 0.05$; Figure 3A). RF and anti-CCP antibody levels were higher but N′-formylkynurenine level was lower in RA SF ($p < 0.05$; Figure 3B). Interestingly, kynurenine level was inversely correlated with CRP in RA SF (r –0.65, $p < 0.05$; Figure 3C) but not in OA SF (r –0.39, $p = 0.39$; Figure 3D).

DISCUSSION

Just as genomics studies the genetic basis of phenotype, and transcriptomics and proteomics study the products of these genes, metabolomics seeks to understand the downstream effects caused by the action of proteins and enzymes in the context of energy and metabolite consumption and regulation. A hypothesis-forming approach, it is driven by the nondiscriminant analysis of the low-molecular-weight metabolite component of target samples[24].

Of the 661 metabolites that we analyzed, the expression of 21 metabolites differed between RA SF and OA SF. Our results show that the levels of lipid metabolites tended to be higher in RA SF than in OA SF. Lipid species such as prostaglandins (PG) and lipid mediators play important roles in the regulation of inflammation. PG and leukotrienes are considered crucial in the onset and development of arthritic diseases[25]. It is known that SF lipid levels are higher in patients with RA than in patients with OA[26]. In our study, the SF from patients with RA had higher concentrations of most phospholipid classes except lysoPC. This finding may be associated with the fact that the proliferation of cells such as fibroblast-like synoviocytes mediates the synthesis and

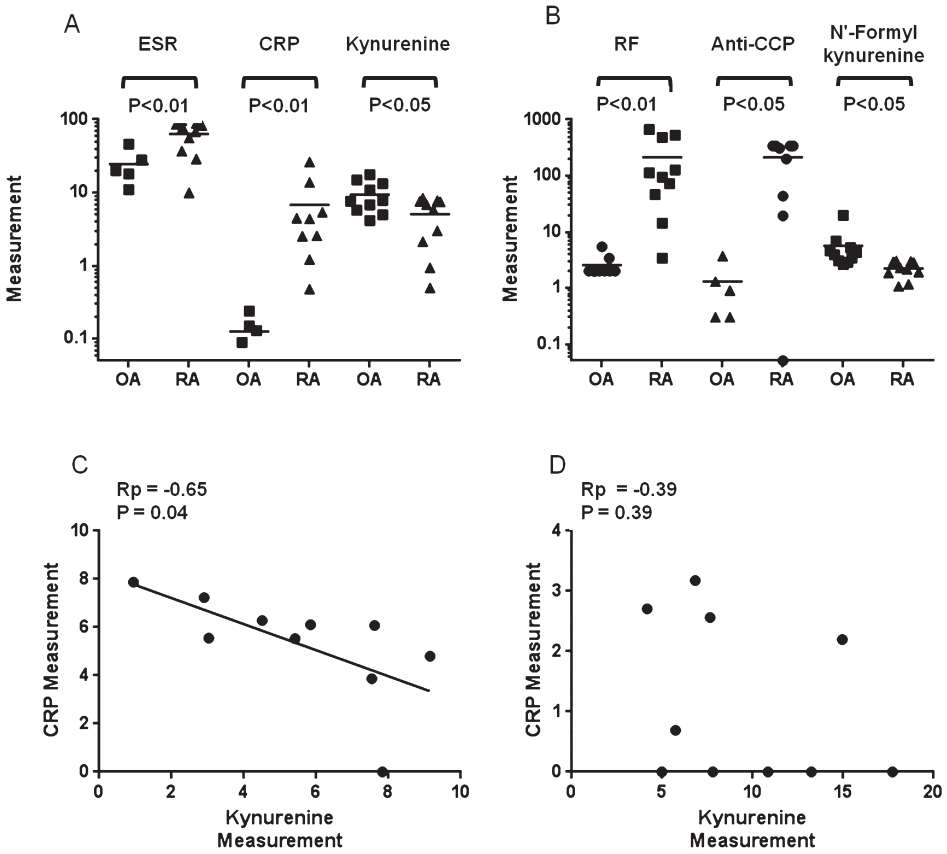

Figure 3. Correlation between tryptophan metabolites and ESR, and levels of CRP, RF, and anti-CCP. A. Serum ESR and CRP levels were significantly higher in RA SF than in OA SF ($p < 0.01$). Kynurenine level was lower in RA SF than in OA SF ($p < 0.05$). B. Serum levels of RF and anti-CCP antibody were significantly higher in RA SF than in OA SF ($p < 0.05$, $p < 0.01$). N′-formylkynurenine was also lower in RA SF ($p < 0.05$). C. Kynurenine level correlated inversely with the serum level of the inflammatory marker CRP in RA (r –0.65, $p < 0.05$). D. In OA SF, kynurenine level did not correlate significantly with CRP level (r –0.39, $p = 0.39$). ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; RF: rheumatoid factor; anti-CCP: anticyclic citrullinated peptide antibodies; SF: synovial fluid; OA: osteoarthritis; RA: rheumatoid arthritis.

release of phospholipids[27].

LysoPC level was lower in RA SF than in OA SF, a finding that has been reported before[28]. LysoPC plays an important role in inflammation. LysoPC is generated by the hydrolysis of membrane PC by phospholipase A2 and acts as a chemoattractant at the sites of inflammation, where it promotes inflammation[29]. We postulate that prednisolone treatment in patients with RA affects the activity of phospholipases[30], leading to the reduction in downstream metabolite levels in SF from patients treated with prednisolone.

Interestingly, the concentrations of tryptophan metabolites differed significantly between RA SF and OA SF in our study. Tryptophan is an exogenous amino acid that must be provided in the diet. Tryptophan and its catabolic metabolites generated through the kynurenine pathway including kynurenine are involved in the inflammation. Kynurenine has known antiinflammatory effects that are toxic to T cells and induce cell death by apoptosis[31,32]. Kynurenine is formed from tryptophan by the activity of indoleamine 2, 3-dioxygenase (IDO). The activation of IDO is actively involved in the resolution of arthritis in mice associated with an increase in kynurenine metabolites[33]. Kynurenine itself has been identified as a ligand for the aryl hydrocarbon receptor, which is important in the maturation of immune cells, and its addition promotes the differentiation of regulatory T cells and suppresses the differentiation of pathogenic Th17 cells[34].

This study has a few limitations. We compared metabolites in RA SF with those in OA SF. Although OA is a noninflammatory arthritis, it may also involve mild synovitis. It would be more appropriate to use SF from healthy controls for analyzing RA-specific metabolites. However, the identification of metabolites in SF from healthy controls is not possible for ethical reasons. Another limitation was the characteristics of the study subjects. The OA group included 3 patients with OA who had a history of diabetes mellitus (DM), which can induce metabolic alteration. To confirm whether a history of DM affected the results, score plot of principal component analysis (PCA) was reviewed. Multivariate analysis methods such as PCA were applied to find meaning in metabolomics datasets. They enable identification of spectral features contributing most to variation or separation. PCA analyzes a data table in which samples (observations) are described by several intercorrelated quantitative dependent variables. Its goal is to extract the important information from the table, to represent it as a set of new orthogonal variables called principal components, and to display the pattern of similarity of the observations and of the variables as points in maps. The score plot in PCA represents a summary of the relationship among the samples and is used for interpreting relations among samples. Thus, the relationship among the samples can be predicted by reviewing the score plot in PCA. If patients with OA who have DM have similarities differentiated from the others, they may be settled close together in the score plot of PCA. In the score plot of PCA for the present study set, 3 samples did not cluster together, comparing relations among the other samples (Supplementary Figure 3, available online at jrheum.org). Therefore it was clear that history of DM did not affect the results obtained in our study. Additionally, the study patients included patients taking different DMARD regimens. Drug treatment did not introduce systematic problems in the metabolomic profiling diagnosis, but there is a possibility that a number of metabolites were mildly affected. It is even possible that the patients' response to treatment may correlate with changes in metabolite levels.

We found that the SF from patients with RA could be differentiated from the SF of those with OA. The differentiation was based mainly on elevated levels of lipid mediators and lower levels of metabolites associated with tryptophan degradation. In particular, the levels of tryptophan metabolites in SF were associated with the levels of inflammatory markers, RF, and anti-CCP antibody. These may be potential biomarkers for the diagnosis of RA.

ONLINE SUPPLEMENT

Supplementary data for this article are available online at jrheum.org.

REFERENCES

1. Scott DL, Wolfe F, Huizinga TW. Rheumatoid arthritis. Lancet 2010;376:1094-108.
2. Andersson AK, Li C, Brennan FM. Recent developments in the immunobiology of rheumatoid arthritis. Arthritis Res Ther 2008;10:204.
3. Huber LC, Distler O, Tarner I, Gay RE, Gay S, Pap T. Synovial fibroblasts: key players in rheumatoid arthritis. Rheumatology 2006;45:669-75.
4. Attur M, Krasnokutsky-Samuels S, Samuels J, Abramson SB. Prognostic biomarkers in osteoarthritis. Curr Opin Rheumatol 2013;25:136-44.
5. Ruiz-Romero C, Blanco FJ. Proteomics role in the search for improved diagnosis, prognosis and treatment of osteoarthritis. Osteoarthritis Cartilage 2010;18:500-9.
6. Hui AY, McCarty WJ, Masuda K, Firestein GS, Sah RL. A systems biology approach to synovial joint lubrication in health, injury, and disease. Wiley Interdiscip Rev Syst Biol Med 2012;4:15-37.
7. Priori R, Scrivo R, Brandt J, Valerio M, Casadei L, Valesini G, et al. Metabolomics in rheumatic diseases: the potential of an emerging methodology for improved patient diagnosis, prognosis, and treatment efficacy. Autoimmun Rev 2013;12:1022-30.
8. Johnson CH, Gonzalez FJ. Challenges and opportunities of metabolomics. J Cell Physiol 2012;227:2975-81.
9. Young SP, Kapoor SR, Viant MR, Byrne JJ, Filer A, Buckley CD, et al. The impact of inflammation on metabolomic profiles in patients with arthritis. Arthritis Rheum 2013;65:2015-23.
10. Balakrishnan L, Bhattacharjee M, Ahmad S, Nirujogi RS, Renuse S, Subbannayya Y, et al. Differential proteomic analysis of synovial fluid from rheumatoid arthritis and osteoarthritis patients. Clin Proteomics 2014;11:1.
11. Uchida T, Fukawa A, Uchida M, Fujita K, Saito K. Application of a novel protein biochip technology for detection and identification of rheumatoid arthritis biomarkers in synovial fluid. J Proteome Res 2002;1:495-9.
12. Mateos J, Lourido L, Fernández-Puente P, Calamia V, Fernández-López C, Oreiro N, et al. Differential protein profiling of synovial fluid from rheumatoid arthritis and osteoarthritis patients

using LC-MALDI TOF/TOF. J Proteomics 2012;75:2869-78.
13. Hügle T, Kovacs H, Heijnen IA, Daikeler T, Baisch U, Hicks JM, et al. Synovial fluid metabolomics in different forms of arthritis assessed by nuclear magnetic resonance spectroscopy. Clin Exp Rheumatol 2012;30:240-5.
14. Slupsky CM, Rankin KN, Wagner J, Fu H, Chang D, Weljie AM, et al. Investigations of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. Anal Chem 2007; 79:6995-7004.
15. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
16. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957;16:494-502.
17. Sangster T, Major H, Plumb R, Wilson AJ, Wilson ID. A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. Analyst 2006;131:1075-8.
18. Gika HG, Theodoridis GA, Wilson ID. Liquid chromatography and ultra-performance liquid chromatography-mass spectrometry fingerprinting of human urine: sample stability under different handling and storage conditions for metabonomics studies. J Chromatogr A 2008;1189:314-22.
19. Gika HG, Theodoridis GA, Wingate JE, Wilson ID. Within-day reproducibility of an HPLC-MS-based method for metabonomic analysis: application to human urine. J Proteome Res 2007; 6:3291-303.
20. Lai L, Michopoulos F, Gika H, Theodoridis G, Wilkinson RW, Odedra R, et al. Methodological considerations in the development of HPLC-MS methods for the analysis of rodent plasma for metabonomic studies. Mol Biosyst 2010;6:108-20.
21. Plumb RS, Johnson KA, Rainville P, Smith BW, Wilson ID, Castro-Perez JM, et al. UPLC/MS(E); a new approach for generating molecular fragment information for biomarker structure elucidation. Rapid Commun Mass Spectrom 2006;20:1989-94.
22. Xing J, Chen X, Sun Y, Luan Y, Zhong D. Interaction of baicalin and baicalein with antibiotics in the gastrointestinal tract. J Pharm Pharmacol 2005;57:743-50.
23. Bylesjö M, Eriksson D, Sjödin A, Jansson S, Moritz T, Trygg J. Orthogonal projections to latent structures as a strategy for microarray data normalization. BMC Bioinformatics 2007;8:207.
24. Fitzpatrick M, Young SP. Metabolomics—a novel window into inflammatory disease. Swiss Med Wkly 2013;143:w13743.
25. Chen M, Lam BK, Kanaoka Y, Nigrovic PA, Audoly LP, Austen KF, et al. Neutrophil-derived leukotriene B4 is required for inflammatory arthritis. J Exp Med 2006;203:837-42.
26. Oliviero F, Lo Nigro A, Bernardi D, Giunco S, Baldo G, Scanu A, et al. A comparative study of serum and synovial fluid lipoprotein levels in patients with various arthritides. Clin Chim Acta 2012;413:303-7.
27. Schwarz IM, Hills BA. Synovial surfactant: lamellar bodies in type B synoviocytes and proteolipid in synovial fluid and the articular lining. Br J Rheumatol 1996;35:821-7.
28. Kosinska MK, Liebisch G, Lochnit G, Wilhelm J, Klein H, Kaesser U, et al. A lipidomic study of phospholipid classes and species in human synovial fluid. Arthritis Rheum 2013;65:2323-33.
29. Fuchs B, Schiller J, Wagner U, Häntzschel H, Arnold K. The phosphatidylcholine/lysophosphatidylcholine ratio in human plasma is an indicator of the severity of rheumatoid arthritis: investigations by 31P NMR and MALDI-TOF MS. Clin Biochem 2005;38:925-33.
30. Masferrer JL, Seibert K. Regulation of prostaglandin synthesis by glucocorticoids. Receptor 1994;4:25-30.
31. Fallarino F, Grohmann U, Vacca C, Bianchi R, Orabona C, Spreca A, et al. T cell apoptosis by tryptophan catabolism. Cell Death Differ 2002;9:1069-77.
32. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med 1999;189:1363-72.
33. Williams RO. Exploitation of the IDO pathway in the therapy of rheumatoid arthritis. Int J Tryptophan Res 2013;6 Suppl 1:67-73.
34. Nguyen NT, Kimura A, Nakahama T, Chinen I, Masuda K, Nohara K, et al. Aryl hydrocarbon receptor negatively regulates dendritic cell immunogenicity via a kynurenine-dependent mechanism. Proc Natl Acad Sci U S A 2010;107:19961-6.