Variation in the synovial fluid metabolome according to disease activity of rheumatoid arthritis

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Abstract

Objective

Because genetic and environmental factors both contribute to rheumatoid arthritis (RA), metabolomics could be a very useful tool to elucidate the pathophysiology of RA, and to predict response to treatment. This study was carried out to investigate synovial fluid (SF) metabolic perturbation in RA patients according to the degree of disease activity using gas chromatography/time-of-flight mass spectrometry (GC/TOF MS).

Methods

SF samples were obtained from 48 RA patients. Disease activity was assessed using DAS28-ESR(3). SF metabolomic profiling was performed using GC/TOF-MS, in conjunction with multivariate statistical analyses and pathway analyses.

Results

Significant discrimination of metabolite profiles between moderate and high disease activity groups was shown by PLS-DA, which provided evidence that SF metabolic profiles predicted disease activity. We found the significant correlation between DAS28-ESR(3) value and the intensities of 12 metabolites. The intensities of glycocyamine and indol-3-lactate positively correlated with DAS28-ESR(3) value. On the other hand, β-alanine, asparagine, citrate, cyano-L-alanine, leucine, nicotinamide, citrulline, methionine, oxoproline, and salicylaldehyde negatively correlated with DAS28-ESR(3) value. We found fifteen pathways that were significantly associated with disease activity in RA and that the higher the disease activity, the more amino acid metabolic processes were affected.

Conclusion

We found the SF metabolic alterations in RA patients according to disease activity by using GC/TOF MS and identified 12 candidate metabolic biomarkers that may well reflect the disease activity of RA. SF metabolomic approaches based on GC/TOF MS might provide additional information relating to monitoring disease activity in RA.

Key words

rheumatoid arthritis, metabolomics, synovial fluid, gas chromatography-mass spectrometry, disease activity

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Introduction

Rheumatoid arthritis (RA), affecting nearly 1% of the populations, is a chronic autoimmune joint disease characterised by persistent inflammation in the synovial lining and synovial hyperplasia with heterogeneous course and different pathogenic mechanisms, which can reduce quality of life and increase morbidity and mortality (1, 2).

Synovial fluid (SF) normally functions as a biological lubricant as well as a biochemical pool through which nutrients and regulatory cytokines traverse. Because of the anatomic relationship of the tissue in the joint, events such as inflammation and enzyme-mediated degradation within the synovium and cartilage are reflected in changes in the cellular and chemical composition of SF. Also, SF is known to be one of the most important biofluids that reflects the pathologic conditions of inflammatory arthritis (3). In clinical practice, SF analysis has found to be a valuable adjunct to conventional investigations and is routinely advised in most cases of arthritis. Evaluation of synovial fluid will not only provide a specific biomarker of diagnostic and prognostic utility but will also enable us to have a better understanding about the progression of RA (4).

Metabolomics, an omic science in systems biology, encompasses the comprehensive profiling of metabolic changes, including the study of metabolic pathways and quantification of unique biochemical molecules, within living systems (5, 6). This holds huge potential in the analysis of biofluids to characterise the global metabolic profiles of various pathologies. Alterations in biological pathways could provide insight into the mechanisms that underlie various physiological and pathological conditions (6). Because RA is thought to be multifactorial, with genetic factors and environmental factors playing important roles, metabolomics could be a very useful tool to elucidate the pathophysiology of RA. Metabolomics in RA would be helpful in providing new insights into the pathogenesis of the disease, discriminating between RA and other inflammatory arthritis, monitoring the disease activity and predicting response to treatment (7-9). Moreover, SF metabolomic approaches may help to uncover an unknown pathogenesis of RA through an understanding of the changes in SF metabolism in RA because SF is in close proximity to articular tissues primarily altered during joint pathology. The extent of the metabolic changes and the types of metabolites found in SF may be good markers reflecting the disease activity of rheumatoid joints, which would provide an important new tool for optimising patient care. Few metabolomic studies on rheumatoid synovial fluid have been attempted to find SF metabolic perturbation and potential metabolic biomarkers reflecting the disease activity of RA.

Here, we performed metabolome profiling of SF samples from RA patients using gas chromatography-time of flight/mass spectrometry (GC/TOF MS). The aim of the present study was to investigate RA disease activity-related metabolic alterations by analysing the SF metabolic profiling of RA by GC/TOF MS.

Materials and methods

Study patients and synovial fluid collection

We collected SF samples from 48 patients with RA, and 18 patients with gout. All patient samples were drawn from the rheumatology clinic at the Samsung Medical Center and Kangbuk Samsung Hospital in Seoul, Korea. Of those, patients with gout were not included. The patients with RA met all recruitment criteria following the 1987 American College of Rheumatology classification criteria for RA (10) or ACR-EULAR classification criteria for RA (11). Gout was diagnosed on the basis of 2015 ACR/EULAR classification criteria (12).

Medical records were reviewed for age, sex, and laboratory findings such as erythrocyte sedimentation rate (ESR, mm/hr), C-reactive protein (CRP), rheumatoid factor, and anti-cyclic citrullinated peptide (anti-CCP) antibody. Disease activity score (DAS) is based on information of clinical findings in a combination of different laboratory measures. In this study, the disease activity was evaluated by 28-joint Disease Activity

Score-Erythrocyte Sedimentation Rate without patient's global assessment (DAS28-ESR(3)). A rheumatologist performed the assessment of 28-swollen and tender joint count, which was calculated using the formula described in previous report (13). The disease activity status of RA was defined according to DAS28-ESR(3) score: DAS28-ESR(3) score <3.2, low disease activity; 3.2 ≤DAS28-ESR(3) score ≤5.1, moderate disease activity; DAS28-ESR(3) score ≥5.1, high disease activity.

Joint effusions from patients were aspirated from knee as clinically indicated using a standard sterile procedure. SF samples were transferred to the laboratory for cell count analysis and examined on the same day. For metabolomic analysis, SF samples were centrifuged at 14,000g for 10 min at 4°C immediately after collection and the clarified supernatants were aliquoted into Eppendorf tubes and stored at -80°C before performed the experiments. The experimental protocols used in this study were approved by the Samsung Medical Center (#2011-07-054-004), and Kangbuk Samsung Hospital institutional review board (#2014-01-082) and written informed consent was obtained from each patient included in this study. This study was in compliance with the Helsinki Declaration.

Metabolite extraction from synovial fluid

Metabolite samples were prepared from SF using a previously published method with a slight modification (14). Briefly, SF samples were centrifuged at 500 x g at 4°C for 5 min, and 100 μl of the supernatant was collected. The supernatant was mixed with 900 μl of pure methanol at -20°C, and the mixture was vortexed for 3 min at room temperature. The extract was centrifuged at 16,100 x g for 5 min at 4°C, and 500 μl of the supernatant was then concentrated to complete dryness in a vacuum concentrator (Labconco, Kansas City, MO). The dried samples were kept at -80°C until derivatisation and GC/TOF MS analysis.

Metabolite analysis with GC/TOF MS

For metabolite analysis by GC/TOF MS, dried metabolite samples were derivatised by methoximation and silylation. First, metabolite samples were incubated with 10 μl of 40 mg/ml methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO) in pyridine at 30°C for 90 min for the methoxymation. Second, the samples were then incubated with 50 μL of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (Fluka, Buchs, Switzerland) at 37°C for 30 min for the silylation. Then, 2 μl of a mixture of methyl esters of C8, C9, C10, C12, C14, C16, C18, C20, C22, C24, C26, C28, and C30 fatty acids (Sigma-Aldrich) was added to the derivatised metabolite samples as retention index markers.

For the identification and the relative quantification of metabolites, an Agilent 7890B GC (Agilent Technologies, Santa Clara, CA) coupled with a Pegasus HT–TOF MS (LECO, St. Joseph, MI) was used. An aliquot of 0.5 μL of derivatised sample was injected into GC in splitless mode. The injected metabolites were separated on an RTX-5Sil MS column (30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness; Restek, Bellefonte, PA) with an additional 10 m guard column. The initial oven temperature was set at 50°C for 1 min, ramped to 330°C at a rate of 20°C/min, and held at 330°C for 5 min. Mass spectra were recorded in a mass range of 85–500 m/z at an acquisition rate of 16 spectra/s. Temperatures of the ion source and the transfer line of TOF MS were set at 250°C and 280°C, respectively. The ionisation of metabolites was performed by electron impact at 70 eV.

Data processing for GC/TOF MS, quality control, and statistical analyses

For the detection and deconvolution of raw data, LECO Chroma TOF software (C version; LECO) was used for preprocessing. Pre-processed data were further processed using BinBase (15). Metabolites were identified by referring their mass spectra and retention indices of peaks to Fiehn, NIST and in-house libraries (15-17). Compared to the peaks of the authentic standards, the peaks with mass spectral similarity above 700 were regarded as authentic metabolites. Relative quantities of identified metabolites were reported as peak heights of their unique ion intensity. To process missing values, the lowest background intensity was subtracted from the intensity of the quantified ion in its retention time region of ± 5 s using the MZmine software (15).

For an accurate analysis of metabolites without external interference, daily quality control was performed (17). Two blank method samples and four calibration curve samples consisting of 31 pure reference compounds including various amino acids, organic acids, and sugars were derivatised by the same procedures. To avoid batch effects, samples were randomly ordered and then analysed all at once.

For statistical analyses, the peak intensity of the identified metabolites was normalised by the median of the sum of the peak intensities of all identified metabolites in each sample. For Spearman correlation analysis, STATISTICA (v. 7.1; StatSoft, Tulsa, OK) was used. The multivariate analysis of the partial least squares discriminant analysis (PLS-DA) and the orthogonal partial least squares discriminant analysis (OPLS-DA) as well as their permutation tests (leaves out 1/7th of the data) and receiver operating characteristic (ROC) curve analysis were all performed using SIMCA-P+ (v. 14.1; Umetric AB, Umea, Sweden). The metabolite set enrichment analysis (MSEA) was performed using the Web-based MetaboAnalyst (http://www.metaboanalyst.ca) (18). Heat map was visualised using a MultiExperiment Viewer (19).

Results

Patient characteristics

The baseline characteristics and laboratory findings of the RA patients at the time of arthrocentesis are presented in Table I. Forty eight patients were categorised into moderately active (n=35) and highly active (n=13) groups, as assessed by DAS28-ESR(3). There were no significant differences in demographics including age and medications except prednisolone used between RA patients with moderate disease activity and those with high disease activity. Acute phase reactants such as ESR or CRP and white blood cell (WBC) counts in SF were higher in the high

Table I. Baseline characteristics of patients with RA.

Disease activity	Moderate (n=35)	High (n=13)	*p*-value
Demographics			
Male (%)	8 (22.9)	5 (38.5)	0.298
Age (mean ± SD)	54.4 ± 15.1	58.9 ± 10.0	0.324
Laboratory findings			
RF positive	26 (74.3)	12 (92.3)	0.248
ACPA positive	25 (71.4)	12 (92.3)	0.246
SF WBC (/mm^3) (n=42)	8,068.7 ± 7095.1	20,362.9 ± 19,188.1	0.042
CRP (mg/dl)	1.68 ± 1.78	6.38 ± 4.20	0.002
ESR (mm/hr)	52.42 ± 26.67	96.62 ± 23.74	<0.001
DAS28-ESR(3)	4.14 ± 0.46	5.62 ± 0.32	<0.001
Medications			
MTX user	25 (71.4)	6 (46.2)	0.173
LEF user	8 (22.9)	0 (0.0)	0.088
SSZ user	4 (11.4)	2 (15.4)	0.656
HCQ user	8 (22.9)	0 (0.0)	0.088
Pd user	26 (74.3)	5 (38.5)	0.039
Pd equivalent (mg)	4.0 ± 3.3	3.3 ± 4.7	0.615
Biologics user	7 (20.0)	1 (7.7)	0.418

Data are expressed as mean ± standard deviation, or number (percentage) unless otherwise indicated. SD: standard deviation; RF: rheumatoid factor; SF: synovial fluid; WBC: white blood cells; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; DAS28-ESR(3): disease activity score-28 joints with three variables; MTX: methotrexate; LEF: Leflunomide; SSZ: sulfasalazine; HCQ: hydroxychloroquine; Pd: prednisolone.

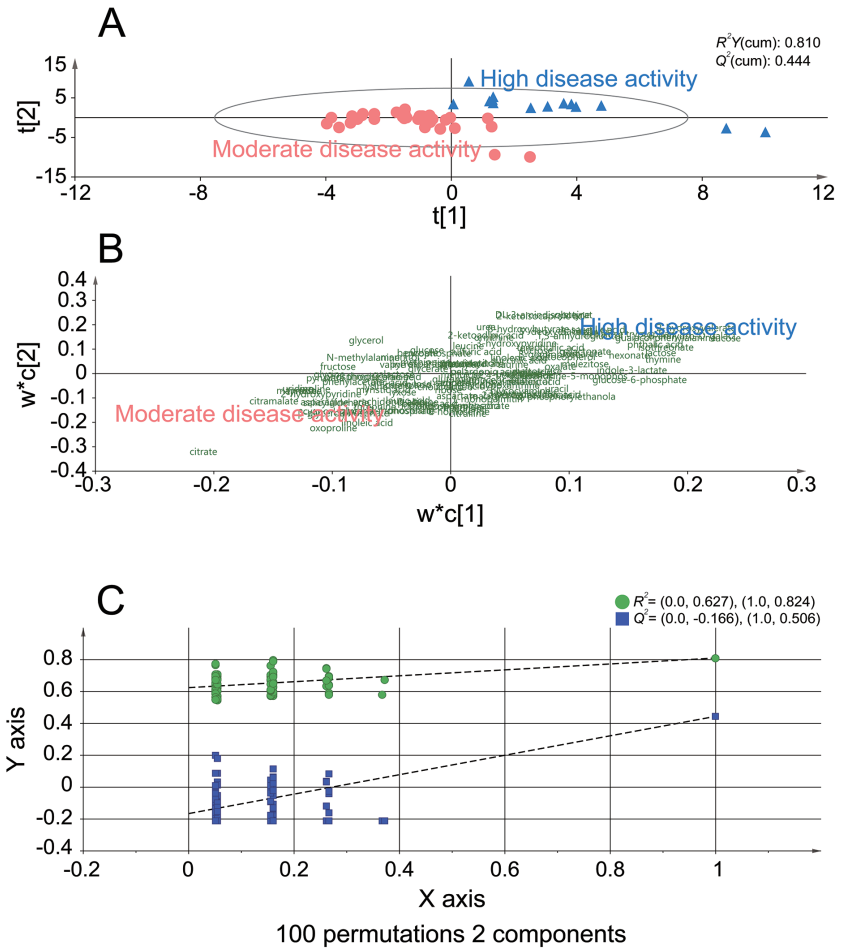

Fig. 1. The score plot (**A**), loading plot (**B**), and permutation tests (**C**) of the PLS-DA using 125 metabolites. Rheumatoid arthritis (RA) patients with moderate (n=35) and high disease activity (n=13) were used. In the permutation tests with 100 iterations (**C**), the Y-axis intercepts of R^2 and Q^2 are 0.624 and -0.166, respectively, indicating that the original model is valid.

disease activity group compared to moderate disease activity group.

Identification of metabolites

SF samples from 48 patients with RA were analysed by GC/TOF MS. A total of 125 metabolites were identified in the SF samples. Identified metabolites were classified into several chemical classes based on MeSH Tree, consisting of amino acids (21.6% of identified metabolites), organic acids (20.8%), sugar and sugar alcohols (18.4%), fatty acids (14.4%), amines (11.2%), and phosphates (5.6%), and miscellaneous (8.0%) (Supplementary Table S1).

Distinct metabolomic profiles in SF samples of RA patients according to disease activity

We applied supervised PLS-DA to capture the distinctive metabolic phenotypes and to maximise the discrimination between patients with moderate disease activity and those with high disease activity by means of all the 125 identified metabolites. The PLS-DA score plot showed that the cluster of RA patients with high disease activity is well separated from those with moderate disease activity in t[1] (Fig. 1A). The loading plot of PLS-DA showed the brief overview of contribution of each metabolite to the PLS-DA model, by which metabolites responsible for discrimination in the score plots of PLS-DA could be visualised (Fig. 1B and Suppl. Table S2). The variation values of the PLS-DA model are 0.810 of R^2Y, and 0.444 of Q^2, respectively, indicating the high explanation and prediction capabilities of the PLS-DA model. To prevent the PLS-DA model overfitting, permutation tests with 100 iterations for 2 components were performed. All points of permuted R^2 values were located on the lower side than the original point, and the regression line of Q^2 with value of -0.166 had a negative intercept (Fig. 1C). These results strongly indicated the reasonable validity of the PLS-DA model.

Synovial fluid metabolic perturbations reflecting disease activity in RA

We found 12 metabolites that were significantly associated with the RA dis-

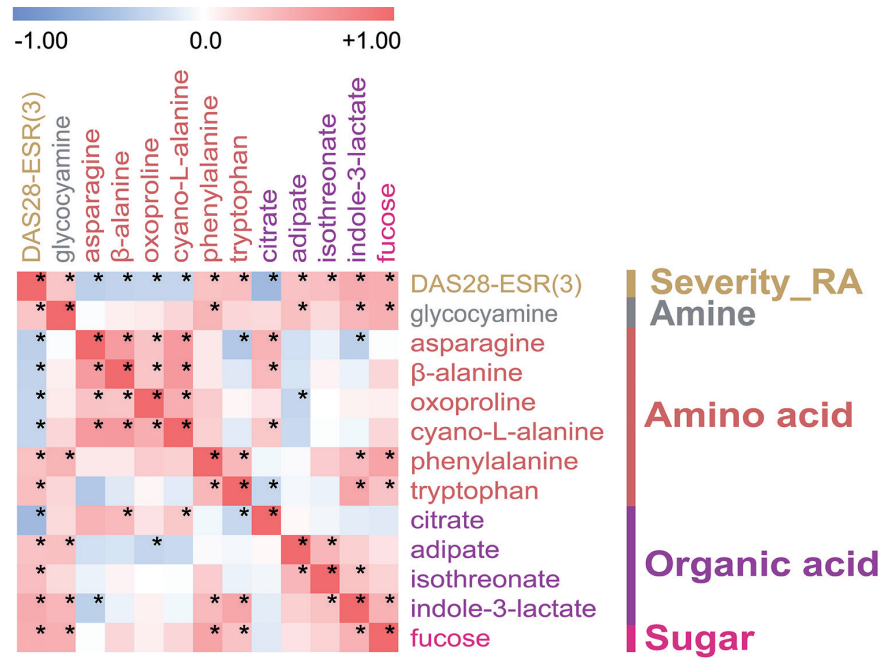

Fig. 2. Correlation map for relation between 12 metabolites abundance and DAS28-ESR(3) value in patients with rheumatoid arthritis (RA).
*Represents significant correlation between two variables (p<0.05).

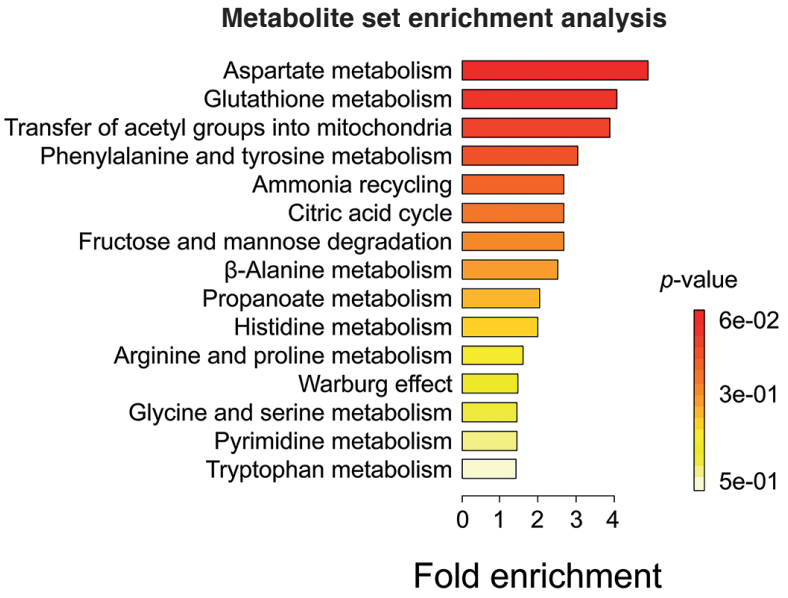

Fig. 3. Metabolite set enrichment analysis of 12 significant metabolites that are associated with disease severity. Each bar represents one type of metabolism. The length of the bar represents the degree of enrichment. The color of the bar represents the degree of significance of the change.

ease activity based on a DAS28-ESR(3) (Fig. 2 and Suppl. Table S3). We indicated statistical significant correlation between DAS28-ESR(3) value and the intensities of 12 metabolites (adipate, asparagine. citrate, cyano-*L*-alanine, fucose, glycocyamine, indole-3-lactate, isothreonate, oxoproline, phenylalanine, tryptophan, and β-alanine). The intensities of adipate, fucose, glycocyamine, indole-3-lactate, isothreonate, phenylalanine, and tryptophan showed statistically significant positive correlation with DAS28-ESR(3) value. On the other hand, asparagine, citrate, cyano-*L*-alanine, oxoproline, and β-alanine showed statistically significant negative correlation with DAS28-ESR(3) value.

MSEA using 12 metabolites with significant changes corresponding to disease severity

To investigate whether metabolism is affected by disease activity in RA, we performed MSEA using the 12 metabolites which are significantly associated with RA disease activity. As shown in Figure 3, we found fifteen unique pathways that were altered, corresponding to disease activity. We found that the higher the disease activity, the more amino acid metabolic processes were affected.

Comparison of SF metabolic perturbations according to DAS28-ESR(3) value and SF WBC count in RA

Because the SF metabolic perturbation of RA according to the disease activity may be affected by the SF WBC count, we investigated the changes in SF metabolome according to SF WBC count in RA patients (n=42; Fig. 4 and Suppl. Table S4). Twelve metabolites were significantly associated with SF WBC count. Of those, there are only two metabolites, asparagine and citrate, that are commonly correlated with the increase in DAS28-ESR(3) value and SF WBC count in RA patients (Fig. 4). The remaining 10 metabolites show the correlation with SF WBC count, not DAS28-ESR(3) value. In addition, to create a discrimination model for RA patients according to disease activity, we performed OPLS-DA using 10 metabolites which are specifically related with RA severity (Suppl. Fig. 2). The OPLS-DA model distinguished RA patients with high disease activity from those with moderate disease activity (Suppl. Fig. 2A, B). Also, the ROC curve of the OPLS-DA model showed AUC score of 0.978, indicating that the model is suitable for the discrimination of severity of RA patients (Suppl. Fig. 2C).

We compared metabolic differences between RA patients and gout patients. The demographics and laboratory findings of the patients with gout at the time of arthrocentesis are presented in Supplementary Table S5. The SF metabolic profile of RA assessed by PLS-DA was distinguishable from that of the SF samples obtained from gout arthritis, which is the most common form of

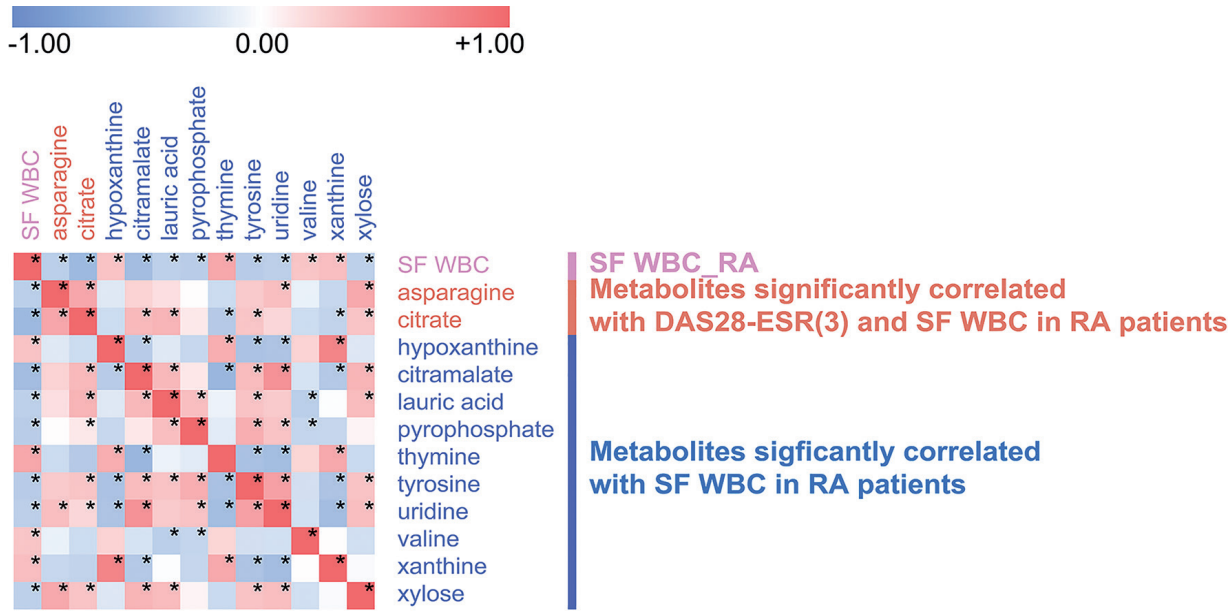

Fig. 4. Correlation map for relation between 12 metabolites abundance and synovial fluid white blood cell count (SF WBC) in patients with rheumatoid arthritis (RA).
*Represents significant correlation between two variables ($p<0.05$).

inflammatory arthritis in men (Suppl. Fig. 1 and Suppl. Table S4). This result shows that SF metabolomic perturbation according to RA disease activity is not associated with that according to SF WBC count and is disease-specific metabolic configuration.

Discussion

To optimise treatment options for RA, it is important to accurately assess the disease activity in RA. Many measurement tools have been developed for this purpose. Few studies have attempted to predict disease activity using metabolomic profile of SF in RA. We investigated whether GC/TOF-MS-based metabolomics could detect metabolic alterations according to disease activity in SF samples from RA patients and identify candidate metabolic biomarkers that may well reflect the disease activity of RA. The SF metabolic profiles of RA patients with high disease activity were significantly different from that of RA patients with moderate disease activity. We identified a set of 12 metabolites responsible for the discrimination of disease activity of RA, as assessed by DAS28-ESR(3). This is a meaningful study suggesting that metabolic perturbations in SF samples from RA may provide additional information for monitoring disease activity and subsequently evaluating the drug response in the future.

Metabolomics reflects both physiological and pathological states. Any pathophysiological mechanism caused by a disease in a biological system will inevitably lead to related changes in the concentrations of specific metabolites. Metabolomic approach in patients with various rheumatic diseases may provide a better understanding of the disease-associated changes. On that basis, there have been many recent metabolomic studies for evaluating clinical diagnosis and therapeutic response in RA (7, 20, 21).

Biochemically, the SF is an ultrafiltrate of plasma across the synovial membrane enriched with various compounds produced by the fibroblast-like synoviocytes (FLS). In pathological conditions, SF evaluation provides information about the pathology of the rheumatoid joints. From these, it may be thought that the metabolomics of one joint may reflect the whole body state. In this study, metabolomic investigations are shown to be distinctive metabolic profiles splitting into moderately active group and highly active group in RA. This observation indicates that the severity of inflammation in RA patients is reflected in the GC/TOF-MS spectra. In previous study, treatment of rheumatoid FLS with curcumin showed that the metabolic perturbation by TNF-α in rheumatoid FLS could be reversed to that of the normal control group (22). Taken together, optimal treatment in the highly active group may be changed into the metabolic profile of patients with remission or low disease activity, suggesting that it may be useful for monitoring the response of therapy. Of a total of 125 metabolites identified from SF of RA patients, 12 metabolites responsible for discriminating RA with high disease activity group and moderate disease activity group were selected: amino acids (asparagine, β-alanine, oxoproline, cyano-*L*-alanine, phenylalanine, and tryptophan), organic acids (citrate, adipate, isothrenoate, and indole-3-lactate), amines (glycocyamine), and sugar and sugar alcohols (fucose). These SF metabolites are suggested to serve as useful candidates for monitoring disease activity in RA. Their validity should have tested with large numbers in a multicentre setting.

SF metabolic perturbation was associated with RA pathophysiology. Activated immune cells and FLS in RA have an acute need to generate sufficient energy and biomolecules to support growth, proliferation and the production of pro-inflammatory cytokines (23). Amino acids play a critical role not only in

protein synthesis but also in regulation and energy metabolism. It is assumed that chronic inflammation can alter any of the amino acid metabolisms. Previously, the observed metabolic perturbations between RA patients and healthy controls were alanine, aspartate and glutamate metabolism and arginine and proline metabolism, pentose phosphate pathway, and fatty acid metabolism (24). It was reported that the alteration of tryptophan metabolism could be a surrogate for poor immunologic tolerance and consequently more inflammation in the serum (25, 26). The plasma levels of 20 amine metabolites were reported to be significantly decreased in collagen-induced arthritis model, which suggest that a disordered amine response is linked to RA-associated muscle wasting and energy expenditure (27). Our study also showed changes in mainly amino acid metabolisms profiles under highly active disease status in RA. Put together, the pathways that were clearly affected to provide the sufficient energy and proinflammatory molecules required under chronic inflammatory condition were suggested to be amino acid metabolism.

RA synovium and synovial cavity was known to be hypoxic condition as a consequence of the increased cellular demand for oxygen during the inflammatory response. Hypoxia has been implicated in the pathogenesis of RA (28). Hypoxic condition in RA joints leads to the stabilisation and increased activity of hypoxia-inducible factor-1α (HIF-1α), which is involved in the persistence of inflammation, synovial invasiveness and progression of neovascularisation during RA (29-31). Once stabilised, HIF-1α transactivate target genes that support the Warburg effect (23). It refers to the observation that cancer cells tend to favour metabolism via aerobic glycolysis rauther than the much more efficient oxidative phosphorylation pathway (32). Also, increased proliferation and rapid activation of immune cells during inflammation may result in a metabolic switch in favour of glycolysis over oxidative phosphorylation. In the MSEA, we found the Warburg effect has a biologically meaningful metabolic pathway in the context of RA disease activity. Taken together, the inflammation becomes worse and activated immune cells and FLS becomes increases under high disease activity, so that the hypoxia can be expected to worsen. The distinctive Warburg effect in this study may be explained due to hypoxic condition in RA joints during high disease activity period.

It is well known that SF metabolic perturbation of RA is significantly different that of osteoarthritis (33, 34). SF metabolic perturbation due to disease activity would call into question whether it is a nonspecific change due to inflammatory arthritis such as gout or increased WBC count. As shown in Figure 4 and Supplementary Figure 1, SF metabolic perturbation according to RA disease activity was different from those according to SF WBC count or by gout, which suggests specific metabolic changes according to disease activity in RA. As shown in Supplementary Figure 2, the distinctive signature with the ten metabolites achieved the highest AUC value and significantly increased the discrimination performance for RA patients according to disease activity. We will expand the number of samples and analyse samples using a different data set to validate the applicability and stability of the distinctive model in future studies.

One of the limitations of our study is the absence of negative control. The lack of group with low disease activity or remission may limit our ability to determine distinctive metabolic perturbation according to the disease activity. Most RA patients requiring arthrocentesis have moderate to high disease activity, so we have difficulty in enrolling patients with low disease activity or remission state. This preliminary study was considered meaningful in that it is conducted to evaluate its feasibility of SF metabolic perturbation in monitoring RA disease activity. Second, we used DAS28-ESR(3) as a tool for RA disease activity in this study. The original DAS was based on ESR. DAS28 based on ESR may be calculated using three or four variables. Unlike DAS28-ESR(3), DAS28-ESR(4) additionally includes patient global assessment. Moreover, even if various DAS indices have been extensively developed for RA, many rheumatologists are currently using DAS28-ESR(4). However, patient global assessment is not available in all patients due to the limitation of this retrospective study. On the other hand, it may be less influenced by subjective patient's mood. It was also reported that DAS28-ESR(3) and DAS28-ESR(4) do not have much difference in disease activity measurement (35, 36). Hence, DAS28-ESR(3) may seem more feasible than DAS28-ESR(4).

We found the metabolic alterations in synovial fluids from RA patients according to disease activity using GC/TOF MS and identified 12 candidate metabolic biomarkers that may well reflect the disease activity of RA. It is suggested that synovial metabolite perturbations, especially perturbation in amino acid metabolism, are correlated with disease activity of RA. GC/TOF MS-based metabolic profile of SF samples in patients with RA may be a potential alternative for monitoring disease activity and, subsequently, therapeutic response in RA. Our metabolomic approach would provide complementary information to current measurement tools for the disease activity of RA such as DAS28-ESR.

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