ABSTRACT: Despite osteoarthritis (OA) and rheumatoid arthritis (RA) being typically age-related, their underlying etiologies are markedly different. We used 1H nuclear magnetic resonance (NMR) spectroscopy to identify differences in metabolite profiles in low volumes of OA and RA synovial fluid (SF). SF was aspirated from knee joints of 10 OA and 14 RA patients. 100 μL SF was analyzed using a 700 MHz Avance IIIHD Bruker NMR spectrometer with a TCI cryoprobe. Spectra were analyzed by Chenomx, Bruker TopSpin and AMIX software. Statistical analysis was undertaken using Metaboanalyst. 50 metabolites were annotated, including amino acids, saccharides, nucleotides and soluble lipids. Discriminant analysis identified group separation between OA and RA cohorts, with 32 metabolites significantly different between OA and RA SF (false discovery rate (FDR) < 0.05). Metabolites of glycolysis and the tricarboxylic acid cycle were lower in RA compared to OA; these results concur with higher levels of inflammation, synovial proliferation and hypoxia found in RA compared to OA. Elevated taurine in OA may indicate increased subchondral bone sclerosis. We demonstrate that quantifiable differences in metabolite abundance can be measured in low volumes of SF by 1H NMR spectroscopy, which may be clinically useful to aid diagnosis and improve understanding of disease pathogenesis.

KEYWORDS: synovial fluid, metabolomics, osteoarthritis, rheumatoid arthritis, nuclear magnetic resonance
are marked by inflammation of the synovium and destruction of articular cartilage and underlying bone. The RA synovium becomes hyperplastic with infiltration by a variety of immunocompetent cells (activated neutrophils in particular). RA synovial fluid is enriched with cytokines, inflammatory mediators such as leukotrienes, and proteases that degrade the extracellular matrix and hyaluronic acid.

SF is located within the articular joint cavity, providing a pool of nutrients for surrounding tissues but primarily serving as a biological lubricant, containing molecules with low-friction and low-wear properties to articular surfaces. As a serum filtrate, not only does SF contain systemic protein/metabolite markers of disease, but also due to its close contact and near proximity to numerous tissues found to be primarily altered during joint pathology (including synovial membrane, cartilage, and bone), this biofluid holds significant potential in the discovery of biomarkers whose levels are altered at early stages of disease progression.

Metabolomics systematically and comprehensively profiles metabolic changes within biological systems, including metabolic pathway analysis and abundance of unique biochemical molecules. The small molecules investigated include secondary metabolites, metabolic intermediates and hormones, as well as other molecules involved in cellular signaling.

Few studies have investigated the whole profile of metabolites within human SF. A recent investigation analyzing 10 mL of each SF sample by liquid chromatography coupled with mass spectrometry (MS), identified 21 differentially expressed metabolites between OA and RA SF. These included a generally elevated level of lipid metabolites and phospholipids in RA patients compared to OA patients, although decreased levels of the phospholipid lyso- phosphatidylcholine was observed in OA.

Nuclear magnetic resonance (NMR) spectroscopy is complementary to MS analysis and may offer advantages over MS in the metabolomics analysis of native samples, with a minimal level of sample preparation using a noninvasive and nondestructive method, subsequently producing results which are more reproducible and robust. One limiting factor of NMR spectroscopy of SF has previously been the minimum volume required for analysis, with difficulties in analyzing SF from normal human joints in which <200 μL can be aspirated. Using the methods described in this study we show that analyzable and reproducible 1H NMR spectra can be produced from just 100 μL of SF, thus increasing the potential applications of this technique when sample volume is limited.

The aim of this study was to apply 1H NMR to study human SF metabolics, in order to provide novel insights on the underlying pathogenesis of OA and RA, and to optimize protocols for clinically relevant volumes of SF typically available in a clinical setting. In this study we describe protocols for human SF sampling and processing and present a novel approach to inflammation analysis, carrying out full profile metabolite NMR examination on low volumes (100 μL) of SF. This method of biofluid analysis enables further understanding of disease pathogenesis, inflammatory signatures and biomarkers, and may aid in both our comprehension of these conditions and progress toward an earlier diagnosis and/or prognostic indicators to stratify patients to treatment.

## EXPERIMENTAL METHODS

### Sample Cohorts

SF samples from patients with OA and RA were collected in accordance with the declaration of Helsinki. The study that collected OA SF was approved by Maastricht UMC Medical Ethical Committee, approval number 08 – 4 – 028, according to Dutch law. The study that collected the RA SF was approved by Sefton Adult Ethics Committee; all patients gave written, informed consent. RA patients (mean age 65.4 years) fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA and were seropositive for rheumatoid factor (RF). OA patients (mean age 67.4 years) were diagnosed by radiographs using Kellgren and Lawrence score, and general orthopedic guidelines carried out by an experienced orthopedic knee surgeon. A diagnosis of RA was excluded for all OA patients.

OA and RA groups were sex and age-matched to within 5 years of age. RA patients were receiving multiple therapies including disease-modifying antirheumatic drugs (DMARDs, typically methotrexate), TNF inhibitors and/or prednisolone and had a mean disease duration of 13 years (range 0–35 years). OA patients were typically receiving nonsteroidal anti-inflammatory drugs (NSAIDs) and in end-stage disease.

### Sample Collection

OA and RA SF from knee joints was aspirated into heparinized tubes and processed within 1 h. Aliquots of whole SF were centrifuged at >2000g for 5 min and cell-free SF was decanted and frozen at −20 °C.

### Sample Preparation for NMR

100 μL of SF was thawed out over ice and diluted to a final volume containing 50% (v/v) SF, 40% (v/v) ddH2O (18.2 Ω), 10% (v/v) 1 M PO4− pH 7.4 buffer (Na2HPO4·VWR, Pennsylvania, US; NaH2PO4 Sigma-Aldrich, Gillingham, UK) in deuterium oxide (D2O) and 0.0025% (v/v) sodium azide (NaN3 Sigma-Aldrich, Gillingham, UK). Samples were vortexed for 1 min, centrifuged at 13 000g and 4 °C for 2 min and 190 μL transferred into 3 mm outer diameter NMR tubes using a glass pipet.

### NMR Setup Acquisition and Processing

SF was analyzed using 1H NMR spectroscopy on a 700 MHz NMR spectrometer Bruker Avance III HD with a TCI cryo-probe and chilled SampleJet autosampler. Software for acquisition and processing was carried out using Topspin 3.1 and IconNMR 4.6.7. 1D 1H standard CPMG-type metabolomics experiments, with optimal water suppression via presaturation, were acquired with the cpmgpr1d filters for small molecules via a Carr–Purcell–Meiboom–Gill (CPMG) sequence. Spectra were acquired at 37 °C with 32 transients a 15 ppm spectral width, 32 K points, 9.6 ms echo time and a 3.1 s interscan delay. Metabolomics data have been deposited to the EMBL-EBI MetaboLights database with the identifier MTBLS564. The complete data set can be accessed at https://www.ebi.ac.uk/metabolights/MTBLS564.

### Spectra Processing and Quality Control

Spectra were assessed to conform to minimum reporting standards as outlined by the Metabolomics Society to ensure consistent line widths, baseline corrections and water suppression. As commonly used internal standards (TSP and DSS) are known to bind to some proteins including albumins, common in protein-rich fluids such as SF, we indirectly referenced the spectra via an intrinsic metabolite whose shifts are unaffected by protein presence (glucose). All spectra passing appraisal were then divided into “buckets” that were defined globally for all spectra by the peak limits using Amix software. Spectra were then prepared for statistical analysis by bucketing/binning according to spectral features or peaks; all
peaks, both identified and unknown, were included in the bucket table.

**Metabolite Annotation and Identification**

Metabolites in the SF $^1$H NMR spectra were initially tentatively annotated using the metabolite discovery software Chenomx (Chenomx, Canada) and, where possible, their identities confirmed using an in-house library of metabolite spectra (further details in Supplementary Table S1).24

**Metabolomics Statistical Analysis**

Statistical analyses were performed using Metaboanalyst (http://www.metaboanalyst.ca/).25 The bucketed experimental data were normalized by the median and additionally Pareto scaled (for multivariate analysis). Univariate analysis was by t test with application of a false-discovery rate (FDR) adjusted p-value of 0.05. Multivariate analysis was via unsupervised principal component analysis (PCA) followed by partial least squares discriminant analysis (PLS-DA) validated via leave one out cross-validation to automatically determine optimal number of components for the model.

**Metabolomics Pathway Analysis**

Pathway analysis was carried out using the list of significantly different metabolites between OA and RA SF using Metaboanalyst and with reference to the KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathway database (http://www.kegg.jp/kegg/pathway.html) for human metabolite pathways.25 Pathway enrichment was determined by Hypergeometric test and reported with an FDR-adjusted p-value.

**RESULTS**

**Metabolite Identification**

NMR spectra for all cohorts showed a consistent set of metabolite signals present. NMR spectra of extracts were divided into individual spectral bins accounting for one or more multiplets. The number of spectral bins in each extract was 171, of which 126 (73.6%) were assigned to 50 metabolites. Quantile plots identifying the global variation of metabolite abundances within each cohort demonstrated much less variation within the OA cohort than the RA cohort, indicating a more homogeneous OA population (Figure 1). Fifty metabolites were annotated in total, including amino acids, saccharides, nucleotides and soluble lipids (Table 1).

**Univariate Comparison of OA and RA SF**

Univariate analysis of the 171 spectral bins identified 91 significantly different bins (including 50 annotated), which corresponded to 32 metabolites which were significantly different (FDR < 0.05) between OA and RA SF. These included citrate, creatinine, glucose, glutamine, glycerol, pyruvate and taurine which were higher in OA (Table 2, Figure 2, Figure 3A). 3-hydroxybutyrate, acetate, isoleucine, leucine, sarcosine and threonine were higher in RA. Acetylated saccharides, an annotation that incorporates commonly acetylated molecules such as heparin and chondroitin sulfate, and monosaccharide subunits N-acetyl glucosamine and N-acetyl galactosamine, were also significantly higher in RA. No significant association was observed between metabolite profile or drug therapy in the RA patients, or with the length of disease activity.

**Multivariate Analysis Comparison of OA and RA SF**

PCA analysis exhibited separation between OA and RA cohorts (Figure 3B). Regression analysis (PLS-DA, partial least-squares discriminant analysis) correctly distinguished between the two groups in 5 components (Figure 3C, R$^2$ 0.99448, Q$^2$ 0.95496).

**Biological Context**

In order to determine the metabolic pathways that were differentially regulated between OA and RA, the list of 32 significantly different metabolites was uploaded into Metaboanalyst for Pathway Analysis. The results of this analysis are shown in Table 3, and include pathways relating to amino acid synthesis and degradation, taurine and hypotaurine metabolism and glycolysis. With reference to current understanding of metabolic pathways,26 we combined the results of the pathway analysis into a single metabolic flow diagram (Figure 3D) to enable interpretation of the interactions between the metabolites and the different metabolic pathways.

Normalized levels of acetylated saccharide in RA SF correlated positively with serum levels of inflammatory markers (Figure 4), including the acute-phase protein C-related protein (CRP, g/L) (Pearson R$^2$ = 0.78, p = 0.008, n = 10), erythrocyte sedimentation rate (ESR, mm/h) (Pearson R$^2$ = 0.62, p = 0.02, n = 13) and RF titer (Pearson R$^2$ = 0.618, p = 0.018, n = 14).

**DISCUSSION**

In this study we measured OA and RA SF metabolomes using $^1$H NMR spectroscopy. This study differs from previous ones in that we have studied the metabolome of SF, the biofluid that is in contact with all the tissues and cells at the site of disease manifestation and joint damage, thereby allowing analysis of the pathophysiology of the whole joint. To date, metabolomic studies in OA and RA have focused on biofluids, including urine, serum and SF using a number of different platforms including gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) and $^1$H NMR spectroscopy.19,22,27–37 These studies have provided insight into the metabolites found within human arthritic SF, and the different underlying cellular pathology of OA and RA joints. The RA synovial environment is characterized by proliferation of synovial fibroblasts and angiogenesis. Infiltration of immune cells including neutrophils, macrophages, and T and B cells drive inflammation within the joint, releasing proteases, cytokines, chemokines and lipid mediators that perpetuate inflammation leading to growth of an invasive, inflammatory pannus across the surface of the cartilage. Further dysregulation of neutrophils and osteoclasts within the pannus leads to degradation of the underlying cartilage matrix and bone erosion.33,14 In contrast, while there is some evidence of mild to moderate inflammation and synovial hyperplasia within OA joints, this is markedly less than the levels observed in RA, with the population of infiltrating immune cells consisting mainly of T cells and macrophages.38,39 Synovitis is rare in OA, and the loss of articular cartilage and bone is caused by a dysregulation in proteases including MMPs leading to destruction of the cartilage extracellular matrix.6,7

A common feature of previous studies investigating OA and RA SF metabolites is the activation of metabolic pathways controlling glycolysis and fatty acid metabolism; however most studies compare OA or RA serum metabolites to sera from healthy individuals. Our study presents a direct comparison of OA and RA metabolite profiles in SF using $^1$H NMR spectroscopy, and provides a novel insight into the differences in metabolic activity within joints of patients with two common forms of arthritis. In addition, we believe this is the first study...
of this kind to make the raw data fully open access via public repository. In this study we identified 50 metabolites in OA and RA SF: 22 amino acids, 5 sugars, 12 fatty and organic acids, 11 others. Multivariate analysis separated patient SF metabolite profiles based on the levels of 32 metabolites that were significantly different between OA and RA SF. These metabolites were associated with metabolic pathways controlling amino acid synthesis, taurine metabolism, glycosphospholipid metabolism, glycolysis and the tricarboxylic acid (TCA) cycle. OA SF had significantly higher levels of substrates for glycolysis and the TCA cycle, including glucose, mannose, pyruvate and citrate. In addition, many amino acids, which feed into glycolysis and the TCA cycle, were higher in OA, including tyrosine, glutamine, proline, histidine, asparagine, taurine and alanine. We have previously identified altered glycolysis pathways in early OA using NMR metabolomics. Furthermore, a number of studies have identified altered status of glycolysis and glucose metabolism glycolytic proteins in OA. A previous study of urine metabolomics found altered TCA cycle activity in OA. The authors hypothesized this was due

Table 1. List of 50 Metabolites Detected in OA and RA SF by \(^1\)H NMR Spectroscopy

| Category          | Metabolites                                                                 |
|-------------------|----------------------------------------------------------------------------|
| Amino Acids       | 2-Aminobutyrate, 5-Aminolevulinate, Alanine, Asparagine, Creatine, Creatinine, Glutamine, Glycine, Guanidoacetate, Histidine, Isoleucine, Leucine, Lysine, Methionine, n-Acetylglutamic acid, Ornithine, Phenylalanine, Proline, Sarcosine, Threonine, Tyrosine, Valine |
| Fatty and Organic Acids | 2-Hydroxybutyrate, 3-Hydroxybutyrate, 3-Hydroxyisovalerate, Acetate, Acetoacetate, Carnitine, Citrate, Formate, Lactate, Malonate, Pyruvate, Taurine |
| Sugars            | Acetylated-saccharide, Glucose, Glycerol, Mannose, Myoinositol               |
| Other             | Acetylcholine, Adenosine, Choline, Dimethylamine, Ethanol, Isopropanol, Mobile-lipid, n-Phosphocholine, Oxypurinol, \(\alpha\)-Glycero-3-phosphocholine, Xanthine |

Figure 1. Quantile Plots of OA and RA spectra depicting the median spectral plot (black line) and variation from the median within each cohort (yellow to red scale) for the full spectral range (8.5–0.5 ppm) and a more detailed region (4.15–3.55 ppm). Peaks of interest are annotated as examples. Note multiple peaks for some metabolites, e.g., glucose.
to changes in cartilage metabolism which resulted in elevations of acetic acid, isotropic acid and citrate in the urine of OA patients. In addition, a reduction in excretion of glutamine was suggestive of altered energy metabolism in chondrocytes. In our study we identified increased citrate and glutamine in RA versus OA SF, while taurine was higher in OA versus RA SF, while adiponic acid was signifi-
cantly higher in RA, supporting this finding.

Table 2. List of 32 Significantly Altered Metabolites between OA and RA SF (FDR < 0.05)

| metabolite                          | HMDB ref    | higher in OA | higher in RA | fold change OA vs RA | −log10 (p-value) | FDR      |
|-------------------------------------|-------------|--------------|--------------|-----------------------|------------------|----------|
| 2-Hydroxybutyrate                   | HMDB00008   | Y            |             | 1.19                  | 3.2495           | 1.70 × 10⁻³ |
| 3-Hydroxybutyrate                   | HMDB00357   | Y            |             | 1.59                  | 2.3288           | 1.02 × 10⁻² |
| 3-Hydroxyisovalerate                | HMDB00754   | Y            |             | 2.22                  | 7.6958           | 6.85 × 10⁻⁷ |
| Acetate                             | HMDB00042   | Y            |             | −1.69                 | 2.6401           | 5.56 × 10⁻³ |
| Acetylene-acid-saccharide           | None        | Y            |             | −1.49                 | 4.5038           | 1.97 × 10⁻⁴ |
| Adenosine                           | HMDB00050   | Y            |             | 1.94                  | 1.6375           | 4.45 × 10⁻² |
| Alanine                             | HMDB00161   | Y            |             | 1.42                  | 2.79             | 4.18 × 10⁻³ |
| Aspartagine                         | HMDB00168   | Y            |             | 1.31                  | 3.8413           | 5.83 × 10⁻⁴ |
| Citrate                             | HMDB00094   | Y            |             | 1.67                  | 5.972            | 1.65 × 10⁻³ |
| Creatinine                          | HMDB00562   | Y            |             | 1.43                  | 4.0608           | 4.48 × 10⁻⁴ |
| Glucose                             | HMDB00122   | Y            |             | 1.82                  | 4.2582           | 3.03 × 10⁻⁴ |
| Glutamine                           | HMDB00641   | Y            |             | 1.80                  | 9.7868           | 9.26 × 10⁻⁹ |
| Glycerol                            | HMDB00131   | Y            |             | 1.42                  | 2.6897           | 5.18 × 10⁻³ |
| Glycine                             | HMDB0123    | Y            |             | −1.64                 | 3.9516           | 5.28 × 10⁻⁴ |
| Guanidoacetate                      | HMDB00128   | Y            |             | 1.19                  | 3.283            | 1.61 × 10⁻³ |
| Histidine                           | HMDB00177   | Y            |             | 1.34                  | 3.5713           | 1.36 × 10⁻³ |
| Isoleucine                          | HMDB00172   | Y            |             | −1.39                 | 4.0006           | 4.88 × 10⁻⁴ |
| Leucine                             | HMDB00687   | Y            |             | −1.20                 | 2.1407           | 1.52 × 10⁻² |
| Mannose                             | HMDB00169   | Y            |             | 1.73                  | 3.5567           | 9.85 × 10⁻⁴ |
| Methionine                          | HMDB00696   | Y            |             | −1.30                 | 2.8572           | 3.69 × 10⁻³ |
| Mobile-lipid                        | None        | Y            |             | 1.96                  | 4.7317           | 1.42 × 10⁻⁴ |
| Myoinositol                         | HMDB00211   | Y            |             | 1.24                  | 2.48             | 7.61 × 10⁻³ |
| n-Acetylamino acid                  | None        | Y            |             | 1.11                  | 2.5075           | 7.24 × 10⁻³ |
| Prolin                              | HMDB00162   | Y            |             | 1.26                  | 3.1978           | 1.85 × 10⁻³ |
| Pyruvate                            | HMDB00243   | Y            |             | 3.44                  | 11.994           | 8.61 × 10⁻⁴ |
| Sarcosine                           | HMDB00271   | Y            |             | −1.56                 | 4.6614           | 1.51 × 10⁻⁴ |
| sn-Glyco-3-phosphocholine           | HMDB00086   | Y            |             | 1.49                  | 3.0967           | 2.27 × 10⁻³ |
| Taurine                             | HMDB00251   | Y            |             | 1.26                  | 2.2132           | 1.32 × 10⁻² |
| Threonine                           | HMDB00167   | Y            |             | −1.22                 | 1.6233           | 4.50 × 10⁻² |
| Tyrosine                            | HMDB00158   | Y            |             | 1.40                  | 2.9942           | 2.78 × 10⁻³ |
| Valine                              | HMDB00883   | Y            |             | 1.25                  | 2.5638           | 6.45 × 10⁻³ |

“Y = yes.”

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3784
disease, whereas in previous studies OA SF were collected from patients at an earlier stage of disease progression.

A number of metabolites increased in RA SF indicate activation of ketosis, possibly due to the low glucose levels observed in RA. Metabolites including leucine, isoleucine and threonine were elevated in RA SF. Ketosis occurs via a shift in metabolism during glucose limitation: in the absence of glucose, e.g., via dietary limitation or decreased mobilization of glycogen stores, or during oxygen limitation, metabolism of stored lipids is stimulated to increase the supply of acetyl CoA. 3-Hydroxybutyrate, a key metabolite in ketosis, was previously identified as being elevated in RA serum compared to healthy controls. In our study we found levels of this metabolite to be lower in RA compared to OA SF. A number of lipid

Figure 2. Boxplots of representative metabolites identified from univariate analysis as significantly different between OA and RA SF (*p < 0.05, **p < 0.01, ***p < 0.001). Y-axis represents normalized peak intensity following median normalization and Pareto scaling.
metabolites have been previously reported to be lower in RA biofluids, with a possible explanation being lipids as a source of energy within the hypoxic joint. Alteration of lipid metabolism is associated with changes in membrane composition/permeability, gene expression and protein distribution and function, as well as in cellular functions such as cell growth, proliferation, differentiation, survival, apoptosis, and chemotaxis, implicated in the RA disease process. Metabolomic analysis of urine from RA patients prior to commencement of anti-TNF therapy identified higher histamine, glutamine and xanthurenic acid, along with lower levels of ethanolamine as biomarkers of a good response to therapy. Serum metabolite biomarkers can also distinguish responders and nonresponders to methotrexate. A number of serum metabolites and metabolic pathways have been shown to correlate with markers of inflammation in RA, e.g., CRP.
Neutrophils. Neutrophils are also the main source of SF exoglycosidases (including β-D-glucuronidase and β-D-N-acetyl glucosaminidase) in RA compared to OA SF. These enzymes are responsible for the degradation of glycosaminoglycans (GAGs) such as heparan sulfate within the cartilage matrix, and of hyaluronic acid within synovial fluid, leading to the reduced viscosity of SF and irreversible damage to cartilage that is a hallmark of RA. Degradation of GAGs by these enzymes results in the liberation of acetylated saccharides from the ECM.

Glycans (GAGs) such as heparan sulfate within the cartilage matrix, and of hyaluronic acid within synovial fluid, leading to the reduced viscosity of SF and irreversible damage to cartilage that is a hallmark of RA. Degradation of GAGs by these enzymes results in the liberation of acetylated saccharides from the ECM. The presence of these metabolites concurs with previous reports that the nature of RA SFs was evident in the spectral analysis. Despite the fact that we were unable to include normal SF, and in particular normal RA SF samples with a high degree of accuracy. In addition, we were unable to include “normal” SF samples from healthy donors in our study. Definition of normal SF, and in particular the ethical collection of such from living donors, is ambiguous.

Table 3. Pathway Analysis Using Metaboanalyst and with Reference to the KEGG Database Predicted the Most Enriched Pathways from the List of Metabolites That Were Significantly Different between OA and RA SF (FDR ≤ 0.1)\(^a\)

| pathway                  | total | hits | −log(p)  | FDR   |
|--------------------------|-------|------|----------|-------|
| Aminoacyl-tRNA biosynthesis | 75    | 12   | 24.693   | \(1.51 \times 10^{-2}\) |
| Nitrogen metabolism      | 39    | 6    | 12.124   | \(2.17 \times 10^{-4}\) |
| Valine, leucine and isoleucine biosynthesis | 27 | 5 | 11.165 | \(3.78 \times 10^{-4}\) |
| Taupine and hypotaurine metabolism | 20 | 4 | 9.3989  | \(1.66 \times 10^{-3}\) |
| Alanine, aspartate and glutamate metabolism | 24 | 4 | 8.6483  | \(2.81 \times 10^{-3}\) |
| Glycine, serine and threonine metabolism | 48 | 5 | 8.2939  | \(3.24 \times 10^{-3}\) |
| Arginine and proline metabolism | 77 | 6 | 8.1678  | \(3.24 \times 10^{-3}\) |
| Galactose metabolism     | 41    | 4    | 6.5415   | \(1.44 \times 10^{-2}\) |
| Glycolysis or Gluconeogenesis | 31 | 3 | 5.0818  | \(5.52 \times 10^{-2}\) |
| Valine, leucine and isoleucine degradation | 40 | 3 | 4.3697  | \(1.02 \times 10^{-1}\) |

\(p\)-values were determined by Hypergeometric test and reported with an FDR-adjusted \(p\)-value. The number of metabolites enriched in our dataset (hits) is shown compared with the total number of metabolites in the KEGG pathway.

One limitation to our study was the heterogeneous nature of the RA patient cohort, which included patients at different stages of disease (from early RA with <1 year diagnosis up to long-standing RA >35 years diagnosis), and on a variety of different treatments including DMARDs such as methotrexate, biologic therapies and glucocorticoids. The heterogeneous nature of RA SFs was evident in the spectral analysis. Despite this, regression analysis (PLS-DA) was able to segregate OA and RA SF samples with a high degree of accuracy. In addition, we were unable to include “normal” SF samples from healthy donors in our study. Definition of normal SF, and in particular the ethical collection of such from living donors, is ambiguous.

Figure 4. Correlation of acetylated saccharide with CRP, ESR and RF titer in RA patients. Levels of acetylated saccharide (median normalized with Pareto scaling) in RA SF correlated positively with serum levels of CRP (Pearson \(R^2 = 0.78\), \(p = 0.008\), \(n = 10\)), ESR (Pearson \(R^2 = 0.62\), \(p = 0.02\), \(n = 14\)) and RF titer (Pearson \(R^2 = 0.618\), \(p = 0.018\), \(n = 14\)).

\(^{a}\)Pathway enrichment was determined by Hypergeometric test and reported with an FDR-adjusted \(p\)-value. The number of metabolites enriched in our dataset (hits) is shown compared with the total number of metabolites in the KEGG pathway.

including metabolites associated with arginine metabolism (arginine and ornithine), tryptophan metabolism (serotonin and tryptophan) and branched-chain amino acids (isoleucine, leucine, and valine). We did not find correlation of these metabolites with markers of inflammation in RA SF in our study; however, we observed significant correlation of acetylated-saccharide with serum CRP levels, ESR and RF titer. Our assignment of this metabolite as “acetylated saccharide” was based on comparison of \(^1\)H \(^{13}\)C 2D spectra to our in-house library of common acetylated molecules including acetylated heparin and acetylated chondroitin sulfate, and to monosaccharide subunits N-acetyl glucosamine and N-acetyl galactosamine (data not shown). While the precise identity of this metabolite as acetylated-saccharide with serum CRP levels, ESR and RF titer.

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CONCLUSION
In summary, we have made a direct comparison of SF from RA and OA and found that the metabolic pathways that differ most between the two forms of arthritis are glycolysis, amino acid biosynthesis and taurine and hypotaurine metabolism. In general, metabolites of glycolysis and the TCA cycle were higher in OA compared to RA; these results concur with higher level of inflammation, synovial proliferation and hypoxia found in RA compared to OA. Levels of taurine were also higher in OA indicating increased subchondral bone sclerosis compared to RA. The study has deepened our understanding of the metabolic differences (and hence pathophysiology) of these two forms of arthritis. This study also demonstrates the feasibility of performing 1H NMR metabolomics on small clinical samples (100 μL), hence widening the applicability of the technology within a clinical setting. We speculate that the discriminative power of our approach to analysis of 100 μL SF using 1H NMR spectroscopy may be sensitive enough to identify changes in disease progression that could inform the use of disease modifying therapies or measure the efficacy of future therapies on disease activity/progression in OA and RA.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00455.

Table S1: List of 50 metabolites detected in OA and RA SF by 1H NMR spectroscopy, assignment levels according to the Metabolomics Society Initiative^23 (PDF)

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Notes
The authors declare no competing financial interest. Metabolomics data have been deposited to the EMBL-EBI MetaboLights database with the identifier MTBLS664. The complete data set can be accessed at https://www.ebi.ac.uk/metabolights/MTBLS664.

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ABBREVIATIONS
ACR, American College of Rheumatology; ADAMTS, A disintegrin and metalloproteinase with thrombospondin motifs; CRP, C-related protein; DMARD, Disease-modifying antirheumatic drug; ECM, Extracellular matrix; ESR, Erythrocyte sedimentation rate; FDR, False discovery rate; GAGs, Glycosaminoglycans; GC−MS, Gas chromatography−mass spectrometry; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC−MS, Liquid chromatography−mass spectrometry; MMP, Matrix metalloproteinases; MS, Mass spectrometry; NMR, Nuclear magnetic resonance; NSAID, Nonsteroidal anti-inflammatory drug; OA, Osteoarthritis; PCA, Principal Component Analysis; PLS-DA, Partial Least Squares Discriminant Analysis; RA, Rheumatoid arthritis; SF, Synovial fluid; TCA, Tricarboxylic acid; TNF, Tumor necrosis factor

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