

■ **SYNOVIUM**

Differences between infected and noninfected synovial fluid

AN OBSERVATIONAL STUDY USING METABOLIC PHENOTYPING (OR "METABONOMICS")

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Aims

The diagnosis of joint infections is an inexact science using combinations of blood inflammatory markers and microscopy, culture, and sensitivity of synovial fluid (SF). There is potential for small molecule metabolites in infected SF to act as infection markers that could improve accuracy and speed of detection. The objective of this study was to use nuclear magnetic resonance (NMR) spectroscopy to identify small molecule differences between infected and noninfected human SF.

Methods

In all, 16 SF samples (eight infected native and prosthetic joints plus eight noninfected joints requiring arthroplasty for end-stage osteoarthritis) were collected from patients. NMR spectroscopy was used to analyze the metabolites present in each sample. Principal component analysis and univariate statistical analysis were undertaken to investigate metabolic differences between the two groups.

Results

A total of 16 metabolites were found in significantly different concentrations between the groups. Three were in higher relative concentrations (lipids, cholesterol, and N-acetylated molecules) and 13 in lower relative concentrations in the infected group (citrate, glycine, glycosaminoglycans, creatinine, histidine, lysine, formate, glucose, proline, valine, dimethylsulfone, mannose, and glutamine).

Conclusion

Metabolites found in significantly greater concentrations in the infected cohort are markers of inflammation and infection. They play a role in lipid metabolism and the inflammatory response. Those found in significantly reduced concentrations were involved in carbohydrate metabolism, nucleoside metabolism, the glutamate metabolic pathway, increased oxidative stress in the diseased state, and reduced articular cartilage breakdown. This is the first study to demonstrate differences in the metabolic profile of infected and noninfected human SF, using a noninfected matched cohort, and may represent putative biomarkers that form the basis of new diagnostic tests for infected SF.

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Keywords: Metabolic profiling, Metabonomics, Nuclear magnetic resonance spectroscopy, Infection, Synovial fluid

Article focus

■ To identify metabolic differences between infected and noninfected human synovial fluid using nuclear magnetic resonance spectroscopy.

Key messages

■ A total of 16 metabolites were found in significantly different concentrations between the infected and noninfected human synovial fluid.

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- Three metabolites were in higher relative concentrations in the infected group (lipids, cholesterol, and N-acetylated molecules).
- A total of 13 were in lower relative concentrations in the infected group (citrate, glycine, glycosaminoglycans, creatinine, histidine, lysine, formate, glucose, proline, valine, dimethylsulfone, mannose, and glutamine).

Strengths and limitations

- This is the first study to identify important metabolic differences between infected and noninfected human synovial fluid.
- The samples in both groups are matched for age, sex, and medical comorbidities, which is a strength.
- The small number of samples (16) used in this study is a limitation.

Introduction

Periprosthetic joint infection (PJI) is one of the most devastating complications of joint arthroplasty, affecting between 0.7% and 2.4% of arthroplasty patients,¹ and remains the commonest indication for revision arthroplasty.² Infection in the native joint is also problematic and, left untreated, can lead to irreversible degenerative changes in the joint. Joint infections can also lead to systemic sepsis, resulting in increased patient morbidity and a risk to life. Therefore, rapid identification of bacterial species in synovial fluid (SF) is important for several reasons. It provides important diagnostic information, critical information for identifying the likely source of infection, and identification of antibiotic resistance and sensitivity that facilitates appropriately targeted antibiotic therapy.³

Microscopy, culture, and sensitivity remain the gold standard for identification of infecting organisms in both septic arthritis and PJI. However, diagnosis is becoming increasingly challenging due to the increased incidence of polymicrobial infections and PJIs due to biofilm-producing bacteria and slow-growing, fastidious organisms.⁴ Serological markers of infection, such as CRP and ESR, have been widely used as screening tests for PJI, due to the ease by which they can be obtained, their low cost, and relatively high sensitivity. Threshold values of 1 mg/dl for CRP and 30 mm/hr for ESR have been widely accepted.⁵ However, their specificity is not high and they are often found elevated in the early postoperative period and in noninfectious inflammatory conditions.

Other markers remain under investigation as potential alternative or complementary markers of infection. These include interleukin-6, procalcitonin, tumour necrosis factor alpha,⁶ D-dimer,⁷ and intercellular adhesion molecule-1.⁸ However, the most promising biomarkers are currently alpha-defensin (α -defensin) and leucocyte esterase. Alpha-defensin has been claimed to be the most accurate single biomarker for PJI.⁹ A systematic review

and meta-analysis demonstrated a pooled diagnostic sensitivity and specificity, from six studies, of 1.00 (95% confidence interval (CI) 0.82 to 1.00) and 0.96 (95% CI 0.89 to 0.99).⁹ However, Synovasure, the on-table quick lateral flow test kit by Zimmer Biomet (Warsaw, Indiana, USA), has mixed results, and one meta-analysis has shown it to be much less accurate than the α -defensin laboratory immunoassay.¹⁰ Therefore, its results must be interpreted carefully. The leucocyte esterase colorimetric strip test also allows for improved accuracy of diagnosis and more timely management of the underlying infection. A systematic review and meta-analysis of five studies demonstrated a pooled diagnostic sensitivity and specificity of 0.81 (95% CI 0.49 to 0.95) and 0.97 (95% CI 0.82 to 0.99).⁹

Recent articles have summarized some of the serum biomarkers for PJI and discuss a shift towards genomics and proteomics as important techniques in identifying putative biomarkers.^{11,12} However, less is known about the role of molecular techniques in identifying putative biomarkers in infected SF. Molecular techniques are a promising frontier in the diagnosis of both septic arthritis and PJI. They are particularly suited for the diagnosis of PJI caused by a biofilm and are culture-independent techniques. Metabolic phenotyping is a novel technique, which studies the metabolites within a cell, tissue, or biofluid using either mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy. This technique has been employed in a number of conditions to influence clinical practice.¹³ Its potential lies in the ability to analyze hundreds or even thousands of small molecules and mobile moieties within macromolecules, such as lipo- and glycoproteins, simultaneously. This can provide not only individual biomarkers for a specific pathological process, but also identify a unique metabolic “signature” consisting of many metabolites that may identify a distinct pathology based on relative concentrations of these molecules.

In contrast to MS, NMR requires little sample preparation and can be performed more rapidly. There are few NMR studies looking for biomarkers of SF infection, all of which contain unmatched cohorts or results that are not statistically significant.^{14–16}

Our hypothesis was that there were differences in the metabolic composition of infected and noninfected SF manifesting as small molecule differences. The aim of this study was therefore to identify these differences using NMR spectroscopy.

Methods

Ethical approval was granted by the local research ethics committee (Project 15/LO/0388) and informed written consent to participate in this study was provided by all patients. SF was harvested from eight patients with microscopy, culture, and sensitivity (MC&S) - proven infection and eight matched patients with noninfected joints at our institution. The infected cohort consisted of

Table 1. Patient demographics. There was no elbow noninfected control and therefore a knee control was used instead.

Characteristic	Infected SF	Noninfected SF (control group)	p-value
Number of patients	8	8	N/A
Joint involved, n			N/A
Elbow	1	0	
Knee	3	4	
TKA	2	2	
THA	2	2	
Responsible organism, n			N/A
Coag-positive <i>Staphylococcus aureus</i>	1	N/A	
Group B <i>Meningococcus</i>	2	N/A	
B-haemolytic <i>Streptococcus</i> group C	1	N/A	
<i>Bacillus species</i>	1	N/A	
<i>Rothia species</i>	1	N/A	
Coag-negative <i>Staphylococcus aureus</i>	2	N/A	
Mean age, yrs (SD)	66.6 (12.0)	58.9 (11.6)	0.223*
Sex (male:female)	6:2	6:2	N/A
Ethnicity, n			N/A
Caucasian	5	4	
Asian	2	2	
Afro-Caribbean	1	2	
Disease, n			
IHD	0	1	1.00†
Hypertension	3	2	1.00†
Hypercholesterolaemia	0	2	0.480†
DM	1	2	1.00†
CVA	1	0	1.00†

*Independent-samples t-test.

†Fisher's exact test.

coag, coagulase; CVA, cerebrovascular accident; DM, diabetes mellitus; IHD, ischaemic heart disease; SF, synovial fluid; THA, total hip arthroplasty; TKA, total knee arthroplasty.

SF from four native knee joints, two prosthetic total knee arthroplasties (TKAs), and two prosthetic total hip arthroplasties (THAs). The noninfected cohort consisted of SF from three native knee joints, one native elbow joint, two prosthetic TKAs, and two THAs undergoing primary joint arthroplasty for end-stage osteoarthritis (ESOA) or revision arthroplasty for aseptic loosening. Similar joints were chosen for the noninfected cohort, as our previous research has demonstrated statistically significant differences between the metabolic profiles of osteoarthritic hip and knee human synovial fluid (HSF).¹⁷

In addition to providing informed consent, all patients completed a questionnaire, which included information on demographics, diet, lifestyle, medical comorbidities, and medications. Comorbidities included seven metabolic diseases (diabetes, hypertension, ischaemic heart disease, gout, osteoporosis, raised cholesterol, and stroke). Exclusion criteria included pregnancy, patients unable to consent, those under the age of 18 years, or a dry aspirate.

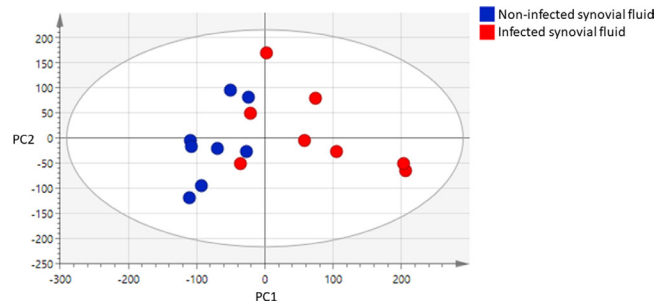


Fig. 1

Principal component (PC) analysis scores plot of PC1 versus PC2, with each data point representing the nuclear magnetic resonance spectrum of an individual human synovial fluid sample and demonstrating separation between the infected and noninfected groups. Data were scaled to unit variance. The percentage variation explained is 38% for PC1 and 21% for PC2.

Sample preparation and metabolic phenotyping. Samples in the septic arthritis cohort were collected during diagnostic aspiration at the bedside or intraoperatively using a standardized protocol. For bedside aspirates, the knee area was prepared with chlorhexidine, and a 16 G needle was inserted in a standard superolateral approach. For intraoperative samples, a posterior approach was used for the hip joint, whereas the knee joint was approached using a medial parapatellar approach through a midline incision.¹⁸ Following skin incision but prior to knee arthroscopy/hip capsulotomy, a 14 G needle and syringe was inserted into the suprapatellar pouch of the knee/along the femoral neck of the hip and SF was aspirated. Samples were divided into two aliquots. The first was sent for routine MC&S and the second was centrifuged at 10,000 g for 15 minutes. The supernatant was aliquoted, thus removing any cellular material or debris. All samples were stored at -80°C for a maximum of six months before analysis.

Samples were defrosted no more than one hour before being assayed. Each sample was prepared for NMR analysis by adding 400 µl of 75 mmol/l sodium dihydrogen phosphate (NaH_2PO_4) buffer at pH 7.4 containing 6.2 mmol/l sodium azide (NaN_3), 4.6 mmol/l of 3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionic acid sodium salt (TSP), and 20% deuterium oxide (D_2O) to an equal amount of sample, as described by Dona et al.¹⁹ To identify and exclude potential contamination at the sample preparation stage, blank samples containing only buffer were run in tandem with SF samples.

Experiments were performed in a Bruker Avance III 600 MHz spectrometer equipped with a Samplejet refrigerated autosampler (Bruker BioSpin, Billerica, Massachusetts, USA). For each sample, one-dimensional (1D) ¹H-NMR spectra were acquired for each sample using the NOESY 1D pulse sequence with gradients for optimized water presaturation, as a sum of 128 free induction decays (FIDs), with 128 k complex data points each, using a mixing time of 10 ms, a delay between two 90

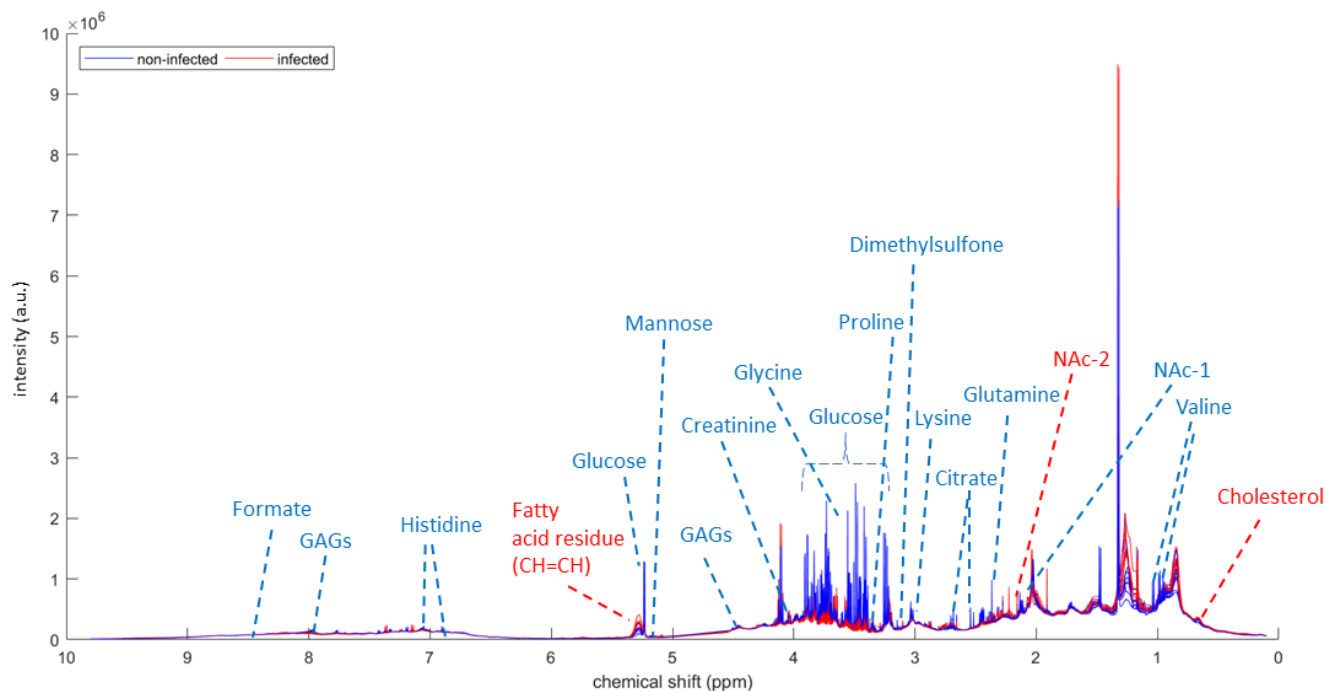


Fig. 2

Stacked spectra of all 16 samples demonstrating the significant metabolites, with those in red having a higher concentration in the infected group and those in blue having a higher concentration in the noninfected group. CH=CH, protons from the unsaturated fatty acid residues; GAG, glycosaminoglycan; NAc, N-acetylated group; ppm, parts per million; a.u., arbitrary units.

radiofrequency pulses of 4 μ s, and a relaxation delay of 4 s.

Each FID was multiplied by an exponential function prior to being Fourier transformed, providing a line broadening of 0.3 Hz. The NMR spectra were automatically phased and baseline corrected and the chemical shifts were internally referenced to the alpha-glucose anomeric doublet at 5.23 ppm. Spectral regions containing only noise and the residual water peak were excluded. Spectra processing was done in Topspin 3.2 (Bruker, Rheinstetten, Germany). The NMR spectra, each consisting of 27,492 data points, were imported to Matlab (Matlab2016b, Mathworks, Natick, Massachusetts, USA). Spectra were aligned to allow comparison of peaks between samples using methodology developed by Veselkov et al.²⁰ Spectra were then normalized using median-fold change normalization methods²¹ and scaled to unit variance (each variable is divided by its SD) in order to avoid a dominance effect of highly intense variables over the less intense variables in multivariate analysis.¹⁷

Metabolite identification. The putative biomarkers derived from the statistical analysis were identified by matching chemical shift and peak multiplicity with information from the literature and Human Metabolome Database (HMDB).²² Statistical total correlation spectroscopy (STOCSY),²³ which highlights statistical correlations of a peak (or variable) with other peaks (or variables) in the spectra belonging to the same molecule or biochemically related molecule, was also used to aid metabolite identification. Two-dimensional NMR spectra, namely

¹H-¹H total correlation spectroscopy (TOCSY) and ¹H-¹³C heteronuclear single-quantum correlation spectroscopy (HSQC), were acquired for representative samples to further confirm the identified metabolites. All metabolites were annotated to level 2 confidence level, according to Metabolomics Standards Initiative.²⁴

Patient demographics. Table I summarizes the demographics of the patients. There was no significant difference in age ($p = 0.223$, independent-samples t -test), sex, or medical comorbidities between the groups.

Statistical analysis. Differences due to medical comorbidities between the two groups were investigated using Fisher's exact test and differences in patient age were tested using the independent-samples t -test.

The NMR dataset, consisting of 27,492 variables per sample, was analyzed using the most commonly applied multivariate analysis techniques, namely principal component analysis (PCA) and orthogonal partial least squares-discriminate analysis (O-PLS-DA).²⁵ In these analyses, the NMR data points can be considered as part of a multidimensional graph representing 'metabolic coordinates'. A more detailed explanation of the methods can be found in the article by Lindon et al.²⁵ PCA provides an overview of the samples, highlights clustering, and identifies outliers. O-PLS-DA was also performed on the dataset. Sample identities were included in the O-PLS-DA modelling. Cross-validation was used to assess model robustness to over-fitting. Multivariate analysis was performed in SIMCA 14 statistical package (Sartorius Stedim Biotech, Umeå, Sweden).

Table II. Metabolites identified from univariate analysis. Three of the metabolites were found in significantly greater quantities in the infected synovial fluid group. The chemical shift indicates which metabolite peaks were integrated and used for metabolite comparison between the two groups.

Metabolite ID	NMR chemical shift (ppm)	Higher in infected/ noninfected HSF	p-value*	FDR
Citrate	2.53	Noninfected	0.001	0.041
Glycine	3.55	Noninfected	0.002	0.053
GAGs	7.97	Noninfected	0.003	0.075
NAC-1	2.02	Noninfected	0.005	0.141
Creatinine	4.05	Noninfected	0.007	0.221
Lysine	3.03	Noninfected	0.008	0.251
Histidine	7.05	Noninfected	0.011	0.326
Formate	8.45	Noninfected	0.012	0.359
Glucose	5.23	Noninfected	0.015	0.449
Fatty acyl residues (CH=CH)	5.28	Infected	0.017	0.500
Cholesterol (C18)	0.65	Infected	0.023	0.679
Proline	3.34	Noninfected	0.023	0.692
Valine	1.035	Noninfected	0.028	0.830
Dimethylsulfone	3.15	Noninfected	0.039	1.00
Mannose	5.18	Noninfected	0.039	1.00
Glutamine	2.45	Noninfected	0.039	1.00
NAC-2	2.04	Infected	0.050	1.00

*Independent-samples t-test.

FDR, false discovery rate; GAGs, glycosaminoglycan; HSF, human synovial fluid; NAC, N-acetylated group; NMR, nuclear magnetic resonance; ppm, parts per million.

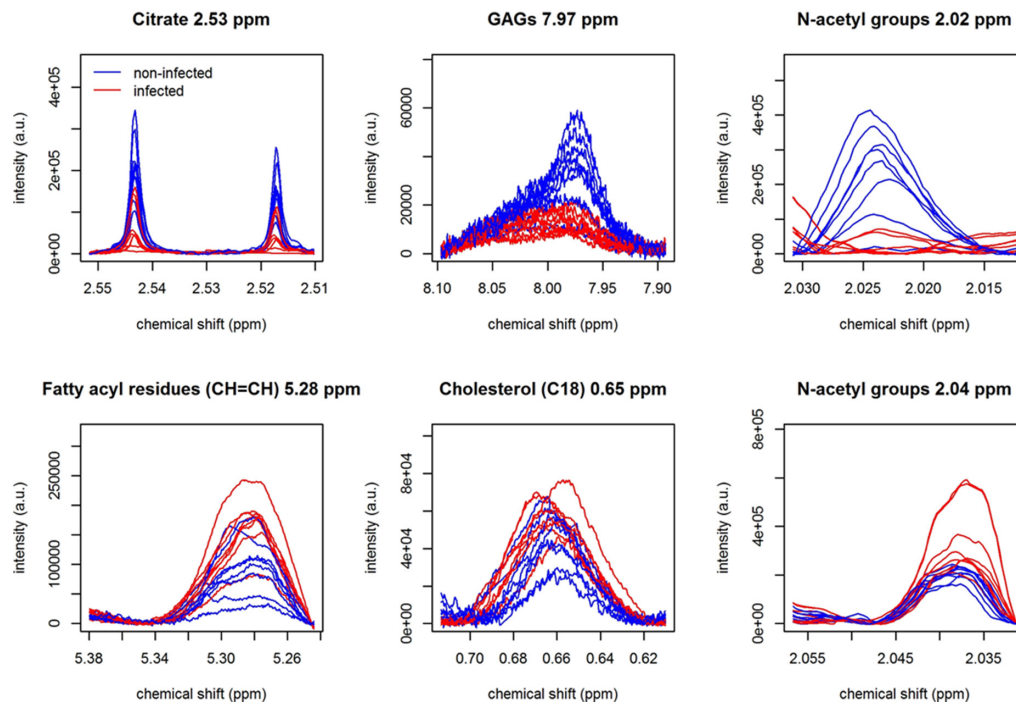


Fig. 3

Metabolite analysis from spectral inspection. Figure demonstrates differences in signal intensity between the infected (red) and noninfected (blue) synovial fluid samples. Citrate and glycosaminoglycans (GAGs) are included as examples of metabolites found in significantly lower concentrations in the infected group compared to the noninfected group. GAGs were considered false negatives after false discovery rate (FDR) correction, although visually, and following statistical testing, differences can be seen between the two groups. Fatty acyl residues, cholesterol, and N-acetylated (NAC) groups were found in significantly greater quantities in the infected group compared to the noninfected group. ppm, parts per million.

NMR peaks of the identified metabolites were separately integrated and further tested by univariate analysis (independent-samples *t*-test) using R statistical software

(R Foundation for Statistical Computing, Vienna, Austria). The resulting p-values were adjusted for false discovery rate (FDR) using the Benjamini–Hochberg method.²⁶

Network analysis was performed using the statistically significant metabolites to further the interpretation of metabolic changes, using the MetaboNetworks software in Matlab.²⁷

Results

PCA analysis of differences between infected and noninfected metabolites. The PCA scores plot (Figure 1) demonstrated separation in PC1 between the ¹H-NMR spectra in the infected and noninfected groups, although there was still some overlap. This suggested that there may be metabolic differences in the SF composition between the two groups. An additional PCA scores plot with the involved joints labelled has been included (Supplementary Figure a), which demonstrated that the separation was independent of the joint type from which the fluid was taken. Furthermore, a third PCA scores plot containing four subgroups (native and prosthetic noninfected SF plus native and prosthetic infected SF) was created (Supplementary Figure b), further demonstrating that the tendency for separation is driven by the presence of infection rather than joint type. The O-PLS-DA model obtained was not significant following cross-validation. This result contrasts with that obtained by PCA, and might reflect the low ratio of samples to variables and a lower strength of metabolite variation between groups. Univariate analysis was conducted to investigate the differences shown by PCA.²⁸

Univariate analysis and metabolite identification. A total of 32 metabolites were identified from the NMR spectra (Supplementary Table i). The integral (area under the curve) of each metabolite peak, which is proportional to the concentration of each metabolite, was tested using the independent-samples *t*-test. Figure 2 depicts all the metabolites that were significantly different between the groups in univariate analysis. After correction for multiple testing, only one metabolite, citrate (decreased in the infected group) remained significant (Table II). However, multiple testing correction can lead to a number of false positive as well as true positive discoveries being discarded, particularly in the presence of a small sample size.²⁸ This is demonstrated in Figure 3, where signals from glycosaminoglycans (GAGs) and N-acetylated group 1 (NAC-1) for instance are clearly different between groups, but were non-significant after FDR correction. Therefore, all significant metabolites will be summarized and discussed.

The infected SF group showed a significantly higher relative concentration of fatty acyl residues (based on the corresponding CH=CH peak; Figure 2), cholesterol and cholesteryl esters from lipoproteins (based on the C18 methyl peak), and N-acetylated groups (based on a NAc peak, NAC-2) compared to the noninfected group. The noninfected SF showed significantly greater levels of citrate, glycine, GAGs (based on a broad amide resonance), N-acetylated groups (based on a different N-acetyl peak, NAC-1), creatinine, lysine, histidine, formate,

glucose, proline, valine, dimethylsulfone, mannose, and glutamine compared to the infected group.

The 7.97 broad peak attributed to the amide protons of GAGs was found to be highly correlated with the NAC-1 peak and therefore NAC-1 is not considered as a separate metabolite in the subsequent analysis. Therefore, it is possible that NAC-1 originates from the N-acetyl groups of GAGs. The NAC-2 peak, which relates to an N-acetyl group, is a broad peak and should be associated with macromolecules, possibly N-acetylated glycoproteins.²⁹ A subgroup analysis comparing the native and prosthetic joints in both the infected and noninfected groups revealed that the concentration of GAGs remained lower in the infected group. This difference was independent of whether the joint was native or prosthetic (Supplementary Figure c).

Evaluation of individual spectra. Two identified metabolites found in lower concentrations in the infected group (citrate and GAGs) are demonstrated in Figure 3. These can be compared to the individual spectral analysis of the three metabolites (lipids (fatty acyl residues), cholesterol, and N-acetylated glycoproteins (NAC-2)) found in higher concentrations in the infected group.

The other fatty acyl residues in the stacked spectra were also visualized to see if there was a difference between the cohorts: the peak at 1.26 ppm from fatty acyl CH₂ groups gave a *p*-value of 0.051 (independent-samples *t*-test), and the peak at 0.85 ppm from fatty acyl CH₃ groups had a *p*-value of 0.068 (independent-samples *t*-test; Supplementary Figure d). While not significant at the arbitrarily chosen cut-off *p*-value, they do show the same trend and reinforce the assignment as lipids (fatty acyl residues).

A network analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, illustrating the connections between the significantly changed metabolites in infected SF and their possible connections in the human metabolic pathway (Figure 4). This network of relationships between metabolites demonstrates the complex relationship between all the metabolites that significantly changed in infected SF. For example, the connections between the metabolites found in lower concentrations in infected SF (as shown by green lines in Figure 4) reveal that a complex relationship might occur through several metabolic pathways.

Discussion

We sought to analyze, by NMR spectroscopy, the small molecule composition of HSF from patients with infected joints and compare these with matched samples from patients with uninfected joints. After appropriate statistical analysis (PCA and univariate analysis of spectra), three metabolites were found in relatively higher concentrations (lipids, cholesterol (C18), and NAC-2) and 13 in relatively lower concentrations in the infected group (citrate, glycine, GAGs, creatinine, histidine, lysine, formate, glucose, proline, valine, dimethylsulfone, mannose, and glutamine). These may reflect different

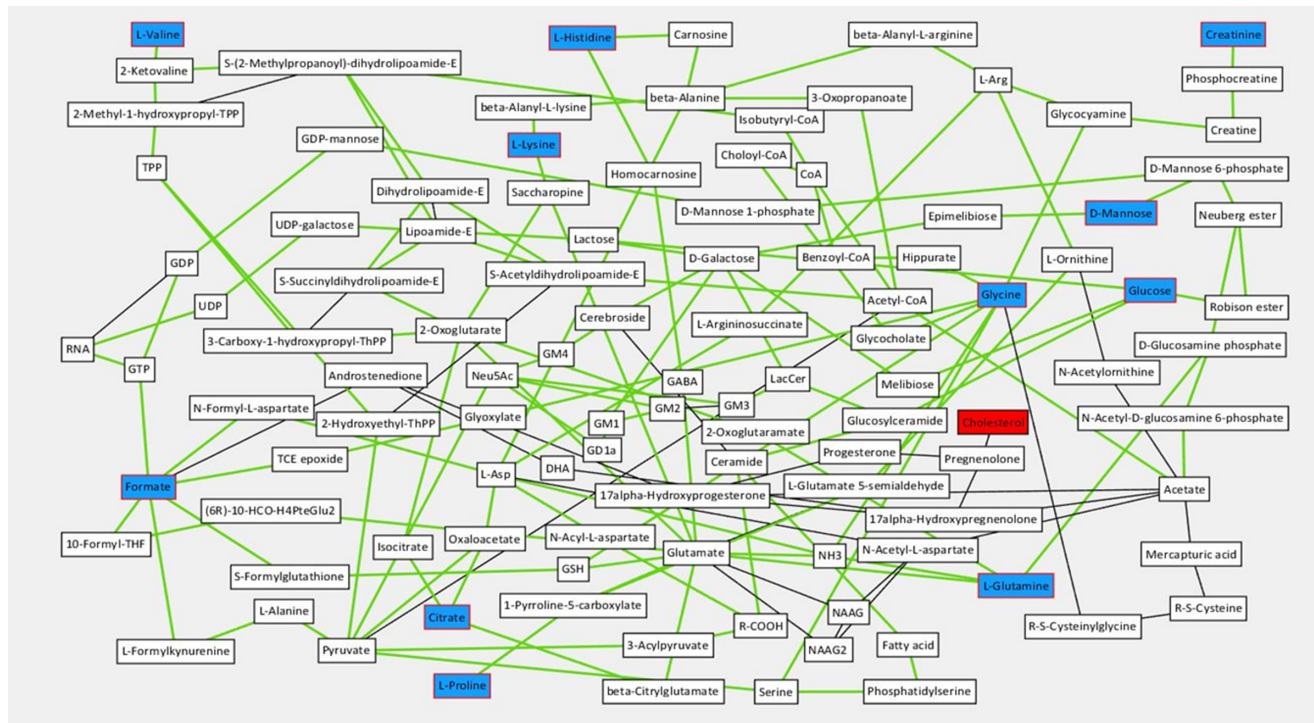


Fig. 4

Network analysis of all the identified metabolites from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database illustrating the potential metabolic pathways. Metabolites found in greater concentrations in infected synovial fluid are shown in red. Metabolites found in lower concentrations in infected synovial fluid are in blue. Metabolic connections between the metabolites found in lower concentrations in infected synovial fluid in green reveal that a complex relationship might occur through several metabolic pathways. The variation in molecules such as lipids (fatty acyls), glycosaminoglycans (GAGs), and glycoproteins (N-acetylated group 2 (NAC-2)) were not taken into account to build this map, as these assignments are unspecific and do not correspond to unique entries in the KEGG database. CoA, coenzyme A; DHA, docosahexaenoic acid; GABA, gamma aminobutyric acid; GDP, guanosine diphosphate; GM1-3, gangliosides GM1-3; GSH, glutathione; GTP, guanosine-5'-triphosphate; NAAG, N-acetylaspartylglutamic acid; NH₃, ammonia; R-COOH, carboxyl group; THF, tetrahydrofuran; TPP, thiamine pyrophosphate; UDP, uridine diphosphate.

Table III. List of identified metabolites found in significantly higher concentrations in infected synovial fluid and their proposed function.

Metabolite ID	Function of metabolite	Reference
NAC-2 (N-acetylated glycoprotein)	NAC-2 may correspond to an acute phase glycoprotein. Such acute phase proteins are markers of inflammation and infection. ³⁰	Naughton et al ³⁰
Unsaturated lipids (CH=CH)	Increased levels of lipoprotein-associated fatty acids are found in inflamed joints. Fatty acids have also been found in the CSF of infected rhesus monkeys.	Wook et al ¹⁶ Wikoff et al ³¹
Cholesterol and cholesterol esters	VLDL-associated Apo B was present in significantly greater concentrations in the CSF of TBM patients. Lipid metabolism related molecules have also been found in increased concentrations in the CSF of TBM patients.	Mu et al ³² Li et al ³³

apo-A1, apolipoprotein A-1; CSF, cerebrospinal fluid; HDL, high-density lipoprotein; NAC, N-acetylated groups; PG, proteoglycan; RA, rheumatoid arthritis; SCFA, short chain fatty acids; SF, synovial fluid; TBM, tuberculosis meningitis; VLDL, very low density lipoprotein.

metabolic processes between infected and noninfected SF.

This is the first study to examine the metabolic differences in SF between infected and noninfected joints where the samples are matched for age, sex, and medical comorbidities. The metabolites found in significantly greater quantities in the infected cohort can broadly be grouped into those having a defensive role against pathogenic microorganisms, a role in lipid metabolism, and the inflammatory response. Those found in significantly reduced concentrations in the infected cohort can broadly be grouped into those involved in carbohydrate metabolism, nucleoside metabolism, the

metabolic pathway of glutamate, increased oxidative stress in the diseased state, and reduced articular cartilage breakdown.

Analysis of metabolites with increased concentrations in infected synovial fluid. The possible functions of the three metabolites found in significantly greater quantities in the infected cohort are listed in Table III.

In comparison with blood serum, acute phase glycoproteins, such as α 1-acid glycoprotein, have N-acetyl signals around 2.00 to 2.05 ppm region.²⁹ There is a possibility that NAC-2 might correspond to an acute phase glycoprotein. Such acute phase proteins are markers of inflammation and infection.³⁰

Table IV. List of identified metabolites found in significantly lower concentrations in infected synovial fluid and their proposed function.

Metabolite ID	Function of metabolite	Reference
Citrate	Citrate is a major intermediary in the TCA cycle, urea cycle, and amino acid and fatty acid metabolism. ⁴⁰	Li et al ³³
Glucose	Increased carbohydrate metabolism has been demonstrated in the CSF of TBM patients. This is thought to be secondary to the increased energy expenditure in the infected SF.	Berg et al ⁴⁰
Mannose		
Glycine	Glycine and glutamine are involved in nucleoside metabolism. Increased nucleoside metabolism has been demonstrated in the CSF of TBM patients. Valine is a BCAA and is also involved in the synthesis of glutamine. This is thought to be secondary to the increased energy expenditure in the infected SF.	Li et al ³³ Wu et al ⁴¹
Glutamine		
Valine	Glutamine, lysine, and proline are involved in the metabolic pathway of glutamate, which bridges the urea cycle with the TCA cycle. Therefore, reduced concentrations of these metabolites may be secondary to increased energy expenditure in the infected SF.	Wu et al ⁴¹ Weiner et al ⁴² Revelles et al ⁴³
Glutamine		
Proline		
Lysine	Reduced concentrations of creatine and histidine were identified in TBM patients. Creatinine is a metabolite of creatine. This may explain the reduced concentration of creatinine in the infected SF group.	Weiner et al ⁴²
Creatinine		
Histidine	Dimethylsulfone is a powerful scavenger of oxygen free radicals, induces macrophage apoptosis, and stimulates granulocyte differentiation. Reduced concentrations in infected SF may be a response to increased oxidative stress, differentiation, and induced apoptosis of macrophages.	Rosenblum et al ⁴⁴ Marthyn et al ⁴⁵ Watson et al ⁴⁶
Dimethylsulfone		
GAGs		
	GAGs are markers of articular cartilage and proteoglycan breakdown, which occur in OA. Staphylococcal infections also lead to GAG breakdown with subsequent destruction of the articular cartilage. The reduced concentration of these metabolites in the infected group may be due to increased articular cartilage degradation in the matched noninfected group, secondary to chronic ESOA versus acute cartilage degradation in the infected group.	Thompson et al ⁴⁷ Schiller et al ⁴⁸ Smith and Schurman ⁴⁹
Formate	Formate is a short-chain fatty acid produced during BCAA catabolism. Such short-chain fatty acids are the major end products of bacterial metabolism in human large intestine. Its role in infected SF is unclear. According to the network analysis performed, using the KEGG database, formate is also involved in nucleotide and amino acid metabolism.	Macfarlane et al ⁵⁰

BCAA, branched chain amino acid; CSF, cerebrospinal fluid; ESOA, end-stage osteoarthritis; GAG, glycosaminoglycan; KEGG, Kyoto Encyclopedia of Genes and Genomes; NAc, N-acetylated groups; OA, osteoarthritis; SF, synovial fluid; TBM, tuberculosis meningitis; TCA, tricarboxylic acid cycle.

Lipids are an important substrate for energy production, both at rest and during muscle activity. They may flow from muscles into the joint space. Therefore, measurement of lipid peak signals may provide important information on effusion mechanisms, improving understanding of disease progression.³⁴ A canine study demonstrated the presence of fatty acyl residues and lipoproteins in SF using NMR spectroscopy at the same chemical shift as that seen in this study.³⁵ Wikoff et al³¹ looked at cerebrospinal fluid (CSF) in a group of rhesus monkeys inoculated with a cell-free stock of simian immunodeficiency virus (SIV). They collected samples before and after inoculation. Using MS, they identified several metabolites in significantly greater quantities in infected CSF, including several fatty acids. This would agree with our findings of increased unsaturated lipids in the infected cohort. The other unsaturated lipids in the stacked spectra were also visualized, but no significant difference was found between the cohorts (Figure 4). This may be due to the unsaturated lipids at 5.28 ppm having the best resolved peak compared to the peaks at 0.85 ppm and 1.26 ppm. However, the p-values were close to significance and with more samples, the resolution of the peaks may improve.

We have found evidence of cholesterol/cholesterol esters originating from SF lipoproteins, increased in the infected group. Although unequivocal attribution of these signals to a particular group of lipoproteins from 1D NMR spectra information is made difficult (due to the overlap between spectral peaks from the different lipids present in the same spectral region), there is evidence of

involvement of lipoproteins in the context of both inflammation and infection.³⁶

High-density lipoprotein (HDL)-associated apolipoprotein A-1 (apo A-1) is a negative acute-phase protein, with a reduction in its levels by at least 25% during acute inflammation. Conditions that result in changes to the plasma concentration of acute-phase proteins include various inflammatory conditions and infection.³⁷ In rheumatoid arthritis (RA), circulated levels of apo A-1 and HDL cholesterol in untreated patients are lower than normal controls.³⁸ However, in the SF of RA patients, apo A-1 levels are increased, although its concentration still remains well below what is found in the plasma.³⁹ This change is accompanied by increased cholesterol levels in the SF, suggesting infiltration of HDL within inflamed joints. As detailed above, it may be that HDL-associated apo A-1 functions to inhibit lymphocyte/monocyte interaction in an attempt to mediate the inflammatory response associated with infection, although this currently remains speculative. A proteomic study demonstrated increased abnormal lipid metabolism in the CSF of patients with tuberculosis meningitis (TBM).³² Apolipoprotein B (Apo B), the major structural protein of very-low-density lipoprotein (VLDL), was present in significantly greater concentrations in the CSF of TBM patients. Using ¹H NMR spectroscopy, a similar study identified three lipid metabolism-related molecules (choline, glycine, and lipoprotein) at increased concentrations in the CSF of TBM patients.³³ The authors suggested that the increased choline and glycine concentrations in TBM patients may

be consequences of greater glycerophospholipid and glycerolipid metabolism to meet the increased demand for energy in TBM.

Analysis of metabolites with decreased concentrations in infected synovial fluid. The possible functions of the metabolites found in significantly decreased concentrations in the infected cohort are listed in Table IV.

Carbohydrates including glucose and mannose, as well as the intermediate metabolite citrate, were present in lower levels in infected HSF. This overall change in carbohydrate metabolism suggests increased metabolism in infected HSF compared with noninfected HSF. Although this has not been demonstrated in SF before, similar findings have been demonstrated in the CSF of bacterial meningitis patients.³³ Nevertheless, other metabolic pathways might be involved, causing changes in these metabolites such as the synthesis of glycolipids (Figure 4). Two metabolites involved in purine metabolism (glycine and glutamine) were also reduced in infected HSF. This suggests altered nucleoside metabolism in the presence of infection. Similar findings were demonstrated in the CSF of bacterial meningitis.³³ Furthermore, as valine is involved in the synthesis of glutamine,⁴¹ it is not unexpected that its concentration is reduced in infected HSF.

Glutamine, lysine, and proline are involved in the metabolic pathway of glutamate, which accounts for reduced concentration of proline in infected fluid.^{41,43} Glutamate bridges the urea cycle with the TCA cycle.⁴¹ Therefore, reduced concentrations of these metabolites may be secondary to increased energy expenditure in the infected SF. Similar findings were also demonstrated in the serum of patients with TB, where reduced concentrations of glutamine were identified in the serum of patients with active TB.⁴² Furthermore, reduced concentrations of histidine and creatine among other amino acids (AAs) were also identified in the serum of patients with TB. As creatinine is a metabolite of creatine, this may explain the reduced concentrations of creatinine in the infected SF group. Amino acid metabolism is complex, involving numerous metabolites. Gluconeogenesis, proteolysis, and oxidative catabolism all contribute to AA balance. Another explanation for reduced concentrations of AAs seen in infected SF may be due to increased protein synthesis secondary to increased bacterial⁵¹ and/or macrophage activity⁵² as part of the immune response.

Dimethylsulfone is derived from dietary sources, endogenous human methanethiol metabolism, and intestinal bacterial metabolism.⁵³ It is a powerful scavenger of oxygen free radicals,⁴⁴ induces macrophage apoptosis,⁴⁵ and stimulates granulocyte differentiation.⁴⁶ Reduced concentrations in infected SF may be a response to increased oxidative stress, differentiation, and induced apoptosis of macrophages.

Four of the patients in the matched noninfected group had end-stage osteoarthritis (ESOA) of the knee. GAGs are markers of articular cartilage and proteoglycan breakdown.⁴⁷ However, *in vitro* animal studies have also

demonstrated that staphylococcal infections lead to GAG breakdown with subsequent destruction of the articular cartilage.⁴⁹ The reduced concentration of this metabolite in the infected group may be due to increased articular cartilage degradation in the matched noninfected group secondary to ESOA, which is greater than the amount of articular cartilage degradation occurring in the infected group. A possible explanation for this could be the greater chronicity of cartilage degradation secondary to ESOA in the noninfected group, compared to the acute and more short-term degradation occurring in the infected group.

Formate is a short-chain fatty acid produced during BCAA catabolism. Such short-chain fatty acids are the major end-products of bacterial metabolism in human large intestine.⁵⁰ However, its role in infected SF remains unclear. According to the network analysis performed, formate can also be involved in nucleotide and AA metabolism (Figure 4).

Potential of NMR in further analysis of SF in health and disease. ¹H NMR is one of the preferred analytical techniques to study complex biological samples, as it produces a comprehensive profile of metabolic signals without derivatization, separation, and preselected measurement parameters.¹³

Over the past decade, ¹H-NMR-based metabolic phenotyping has developed into a powerful tool for the identification of metabolites and biochemical markers for a variety of human disorders.¹³ Metabolic phenotyping of SF provides a direct representation of end-stage biochemistry, making metabolites good candidates for biomarker screening. They are the final product of enzyme catalysis and other biotransformations, as well as being smaller in number than the proteome.¹³ They provide a 'top down' view of a biological system, with the advantage of representing the genetic disease traits but also environmental interactions, as well as being sensitive to gut microbiome activity.¹³ NMR spectroscopy gives sharp well-resolved peaks for small molecule metabolites (usually defined as molecules with a molecular weight < 1500 Daltons), but yields only broad unresolved bands for proteins and other macromolecules. Therefore, enzymes such as α -defensin cannot be detected, which might also be outside the detection limit of NMR. The techniques utilized in this study could be used in larger groups to further identify and analyze molecular biomarkers in PJI and native joint infections, acting as an adjunct to α -defensin.

Clinical relevance. Our study demonstrates the potential of metabolic phenotyping of joint fluid using NMR spectroscopy to identify important metabolites in the context of infection. Some of these metabolites will drive further studies to determine whether they provide biomarkers of infection, leading to the prospect of developing bedside diagnostic tests for joint infection. This observational study is certainly preliminary and the putative biomarkers will require further validation in larger cohorts.

Further studies should utilize a larger group of fluid samples with a more targeted analysis of individual

metabolites for a specific patient group or disease category. The ultimate purpose of these studies would be to identify combinations and concentrations of metabolites for each bacterial species that would provide a 'metabolic fingerprint' for the organism, thus facilitating early diagnosis and expeditious antibiotics and surgical treatment.

This study had several limitations. Despite the samples in both groups being matched for age, sex, and medical comorbidities, the overall numbers were small (eight in each group). However, to our knowledge, this is the largest cohort of infected SF analyzed by NMR, with a noninfected comparison group of matched controls. It would also have been preferable to have an age-/sex-matched nonarthritic, noninfected control group, although ethical constraints would make such samples difficult to acquire. The significance of the identified metabolites could have been further validated by performing additional tests, such as an enzyme-linked immunosorbent assay (ELISA). However, due to a lack of resources, this was not possible at this time.

In conclusion, to our knowledge this is the first article to demonstrate differences in the metabolic profile of infected and noninfected SF with matched controls. Three molecules were found in significantly greater concentrations in the infected cohort (unsaturated lipids, cholesterol/cholesterol esters, and glycoproteins). These have a defensive role against pathogenic microorganisms, a role in lipid metabolism, and the inflammatory response. There were 13 metabolites found in significantly reduced concentrations in the infected cohort (citrate, glycine, GAGs, creatinine, histidine, lysine, formate, glucose, proline, valine, dimethylsulfone, mannose, and glutamine). These can broadly be grouped into those involved in carbohydrate metabolism, nucleoside metabolism, the metabolic pathway of glutamate, increased oxidative stress in the diseased state, and reduced articular cartilage breakdown.

Although more research is required with a larger group of patients, these metabolites may serve as novel biomarkers for the diagnosis of PJI and native joint infection and could be used as adjuncts with other recognized biomarkers, such as α -defensin.

Instagram

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Supplementary material



Principal component analysis scores plots demonstrating whether the differences between the infected and noninfected groups were secondary to the joint from which the fluid was taken, or whether the joint was native or prosthetic; a table listing the metabolites consistently identified in all samples; a box plot comparing the concentrations of glycosaminoglycans between the two groups; and univariate analysis spectra looking for differences in fatty acyl residues at different signal intensities between the two groups.

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- M. K. Jaggard: Contributed substantially to the conception and design of the work, Contributed to the sample collection, assembly of data, and statistical analysis.
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