Synovial fluid metabolomics in different forms of arthritis assessed by nuclear magnetic resonance spectroscopy

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Abstract

Objectives

Currently there are no reliable biomarkers in the synovial fluid available to differentiate between septic and non-septic arthritis or to predict the prognosis of osteoarthritis, respectively. Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique that allows a rapid, high throughput metabolic profiling of biological fluids or tissues.

Methods

Proton (1H)-nuclear magnetic resonance (NMR) spectroscopy was performed in synovial fluid samples from patients with septic arthritis, crystal arthropathy, different forms of inflammatory arthritis or osteoarthritis (OA). The metabolic environment based on the low molecular weight components was compared in disease subsets and principal component analysis (PCA) was performed.

Results

Fifty-nine samples from patients with OA, gout, calcium pyrophosphate disease, spondylarthritis, septic arthritis and rheumatoid arthritis (RA) were analysed. NMR yielded stable and reproducible metabolites over time. Thirty-five different metabolites as well as paracetamol and ibuprofen were identified in synovial fluid. The metabolic profile of septic arthritis assessed by PCA was distinguishable from the other samples whereas no differences were seen in OA compared to crystal-associated arthritis, RA or spondylarthritis.

Conclusion

1H-NMR is a fast analytic tool with possible implications in synovial fluid diagnostics. A distinctive metabolism is observed in septic arthritis whereas metabolites in OA are similar to those in inflammatory arthritis.

Key words

human synovial fluid, nuclear magnetic resonance spectroscopy, NMR, principle component analysis, PCA, metabolomics, arthritis, septic, inflammatory, osteoarthritis, gout

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Introduction

Metabolomics of tissues and biofluids is a rapidly growing field within analytic systems biology (1). In contrast to pure gene or protein expression studies, metabolomics characterises the metabolic environment taking into account environmental, mechanical as well as local influences such as hypoxia.

Nuclear magnetic resonance (NMR) spectroscopy has been established as a tool of metabolic profiling in biofluids (2). NMR is non-invasive and in contrast to mass spectroscopy, it requires little sample preparation and is performed more rapidly. Statistical methods such as principal component analysis (PCA) on NMR spectra permit complex multivariant analyses. Pattern recognition methods are used to reduce multivariate NMR spectral data into 2 or 3 dimensions, such that clustering of samples on the basis of similarities of biochemical profiles can be observed. Proton (1H) NMR spectroscopy is the most sensitive and widely used approach. As NMR-signals are proportional to the relative amounts of the different types of molecules they belong to, quantitative analyses can also be performed.

NMR as a potential diagnostic tool in arthritis has been tested in several studies. Compared to serum, analysis of synovial fluid showed increased amounts of ketone bodies and lactic acid but reduced glucose levels, which is indicative for a hypoxic, anaerobic intra-articular state (3). Triglycerides in synovial fluid are increased in RA compared to osteoarthritis (OA) (4). N-acetyl groups are lower in RA (5). Triglycerides, creatinine and N-acetyl groups all correlated with disease activity in RA (4). In septic arthritis, NMR yielded higher amounts of lactic acid as compared to non-septic arthritis (6). In an OA animal model, NMR spectroscopy has been used to give a multifactorial and sequential picture of degradation in cartilage (7). However, in all of the studies in humans, univariate analysis of synovial fluid was performed and only a few subsets of arthritis were investigated.

In this pilot study we evaluated the accuracy of multivariate metabolic profiling by NMR spectroscopy of synovial fluid in different forms of arthritis. We analysed the composition and stability of synovial fluid focusing on the low molecular weight metabolites by 1H-NMR. Subsequently we compared metabolic profiles of synovial fluid from patients suffering from septic arthritis, gout, calcium pyrophosphate disease (CPPD), RA, spondylarthritis or OA through PCA.

Patients and methods

Study population and synovial fluid collection

Routine arthrocentesis was performed in knee joints of 59 patients with the clinical diagnosis of arthritis. Synovial fluid was analysed routinely by white cell count, polarising microscopy, gram stain and culture. The final diagnosis was made by a rheumatology consultant or infectious disease specialist in case of a septic arthritis. Surplus synovial fluid samples were frozen within 1 hour at -80 degrees and thawed for NMR processing after a maximum freezing time of 12 months. This study was approved by the local ethics committee and informed consent was obtained.

Sample preparation and NMR spectroscopy

Synovial fluid was suspended in sodium phosphate buffer at pH 7.0±0.1 containing 0.025M sodium trimethylsilyl-2,2,3,3,-d_4-propionate (TMSP) as NMR chemical shift standard and 10% D_2O as NMR lock substance. Initial NMR spectra indicated that the samples prepared in this way remained unchanged for several days. In a test series, identical results were obtained between pH values of 7.0 and 7.4, whereas at pH above 7.4 the spectra significantly change.

Bruker Avance III spectrometer operating at 600 MHz of 1H frequency and equipped with a 5 mm TCI-CryoProbe (Bruker BioSpin Switzerland) and BCU-05 temperature regulation system was used for the study. All samples were acquired at 298 Kelvin. The temperature was calibrated with 99.8% MeOD. Two 1-dimensional proton spectra were recorded on each sample. NOESY-1D-^{1}H spectra were recorded with 32 scans using a 4-second presaturation

pulse and gradients for water suppression. A T_2-filtered 1D-^{1}H spectrum using 120 ms Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence to remove unspecific signals from high molecular weight components was recorded with 320 transients in 40 minutes experiment time. The sweep width covered 20 ppm (parts per million), the acquisition time was set to 3 seconds and the recycle delay to 4 seconds. The spectra were zero-filled to 103k points and processed applying an exponential window function with 0.3 Hz line broadening. Phase and baseline corrections were adjusted manually.

Preprocessing of NMR spectra

The software package AMIX 3.9 (Analysis of MIXtures, Bruker BioSpin, Germany) was employed for both spectra reduction and statistical analysis as described below. NMR analysis was performed by two blinded investigators (H.K. and J.M.H.) according the same protocol. Initially, all NMR spectra were manually lined up and scaled using the chemical shift and intensity of the TSMP-signal, respectively. The spectra were then reduced into 0.02 ppm chemical shift bins while excluding the region of the dominant water signal between 4.7 and 5.2 ppm.

Multivariate spectral analysis

PCA as an unsupervised principal component analysis was performed using the AMIX statistics package version 3.9 (Bruker BioSpin, Germany). The first principal component, PC1, describes the largest variation in the data set, the PC2 describes the second-largest variation. Two principal components depicted orthogonal to each other span a scores plot, on which the individual observables are projected. In the case of NMR spectra, a principal component corresponds to the intensity variation of a particular signal. The spectra from different patients line up along the principal component vector depending on the intensity of this signal, which in turn depends on the concentration of the corresponding metabolite. Thus, an anomalous deviation in the concentration of a particular metabolite in a subset of sample spectra (in a subgroup of

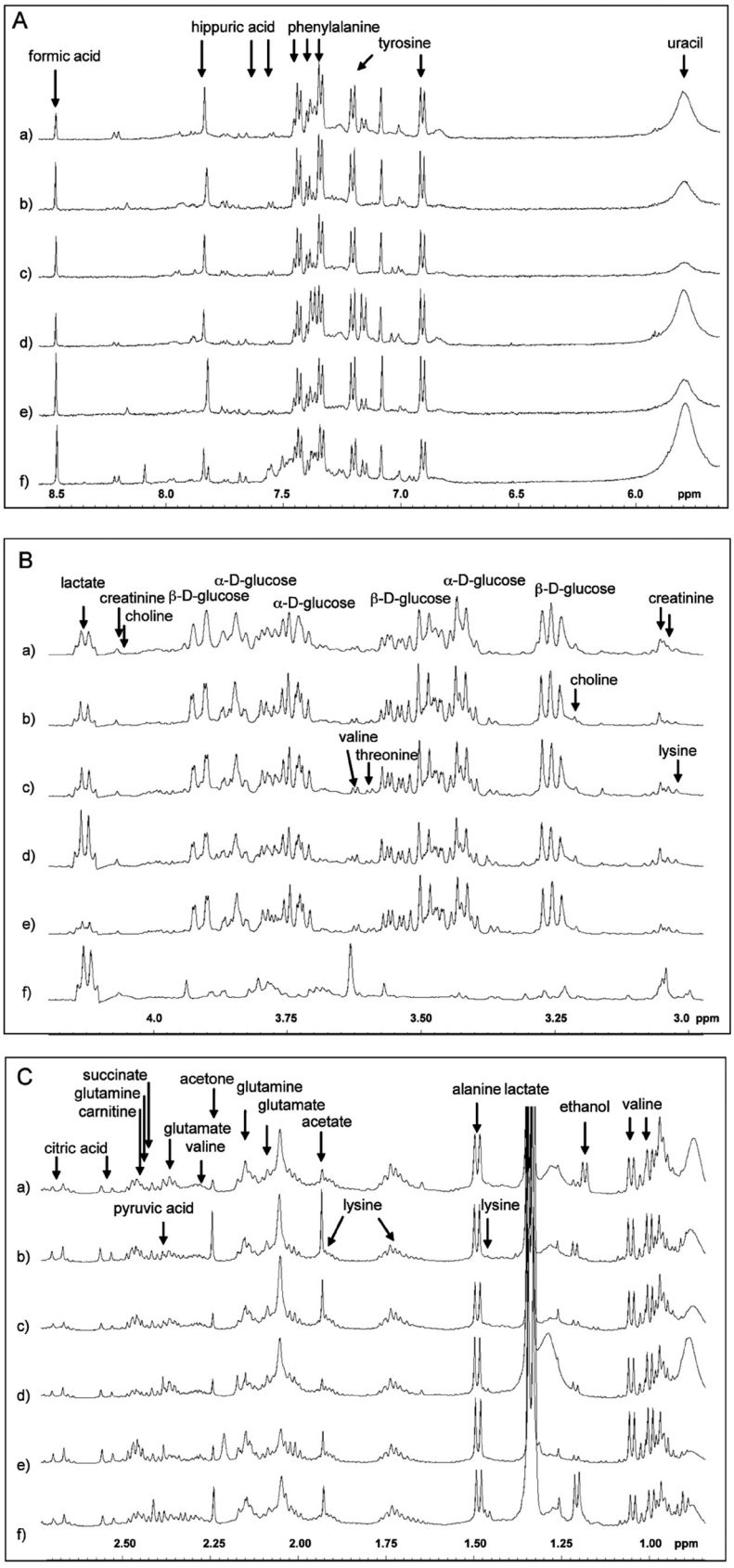

Fig. 1. Representative NMR spectrum in the syvovial fluid from each disease: a) calcium pyrophosphate disease, b) gout, c) spondylarthritis, d) rheumatoid arthritis, e) osteoarthritis, f) septic arthritis. Signals from major metabolites are indicated. Three different spectral regions are shown: **A)** aromatic region 6-8ppm; **B)** sugar region 2–4 ppm; **C)** aliphatic region 1–2ppm on the bottom.

patients) manifests as a clustering of data along the principal component.

Identification of individual metabolites

Synovial fluid spectra were compared with spectra of 500 common metabolites at pH 7.0 in the Bruker BioSpin Biofluid Reference Spectra Database. The data base search was carried out in two different ways. On one hand, automatic routines were used to match signal patterns of metabolites against the synovial fluid NMR spectra. On the other hand, unidentified NMR signals were used to search the metabolite data base for matching patterns. In either case, the results were evaluated visually.

Results

Patients and samples

In total, 59 synovial fluid samples were analysed from patients with gout (18 samples), OA (15 samples), calcium pyrophosphate disease (CPPD) (11 samples), septic arthritis (4 samples), RA (4 samples), reactive arthritis (3 samples), Crohn's disease (2 samples), ankylising spondylitis (1 sample) and psoriasis arthritis (1 sample). Patients with septic arthritis did not receive prior antibiotic treatment. Isolated bacteria were staphylococcus aureus in 3 samples and streptococcus gallolyticus in 1 sample. For PCA, reactive arthritis, Crohn's disease, ankylosing spondylitis (Bechterew) and psoriasis arthritis were grouped together as spondylarthritis; together with RA they were classified as inflammatory arthritis. The median synovial cell count was $92.5x10^9$/l (range 52–133) for septic arthritis, $38.5x10^9$/l (range 15–62) for crystal arthropathies, $19x10^9$/l (range 14–60) for inflammatory arthritis and $0.9x10^9$/l (range 0.7–1) for OA.

Identification of metabolites in 1H NMR spectra

Synovial fluids spectra were compared with spectra of 500 common metabolites in the Bruker BioSpin Biofluid Reference Spectra Database combining automatic search routines and visual inspection. In addition to the signals assigned to metabolites, several unidentified strong peaks were also observed both in the aliphatic and aromatic spectral regions.

Representative spectra with corresponding metabolites are shown in Figure 1 for CPPD, gout, spondylarthritis, RA, OA and septic arthritis. Clearly visible metabolites in a high number of spectra were assigned as major metabolites, whereas metabolites present only in trace amounts or detectable only in some of the spectra were classified as minor metabolites (Table I).

Previously described metabolites in synovial fluid such as formic acid, histidine, phenylalanine, tyrosine, glucose, lactate, creatinine, lysine, citrate, glutamine, alanine, valine, succinate, threonine, hydroxybuturate, acetone, acetate and isoleucin and puruvic acid were also detected in this study (3, 4, 8). We further detected hippuric acid, carnitine, choline, ethanol, uracil, cysteine, glucuronic acid, histidine, homocysteine, inosine, serine, tryptophane, taurine and 1-methyl-uric acid. Trace amounts of paracetamol were found in 6 patients and of ibuprofen in 3 patients. Hyaluronan, which was described previously, could not be detected in this analysis, as high molecular weight components are not monitored in the selected NMR experiment (9).

Statistical analysis

In Figure 2, principal component score plots of principal component 1 (PC1) *versus* principal component 2 (PC2) are indicated representing 1H NMR spectral data from subjects with septic arthritis, OA, gout, CPPD and inflammatory arthritis. A model comprising five principal components explained 95% of the variation of the data. PCA of all samples produced a score plot in which septic arthritis samples could be distinguished from non-septic arthritis (Fig. 2A). One outlier was observed in a sample of an OA patient. In this sample, no bacteria were detected in culture. The first PC vector originated from a lactate methyl signal at 1.33 ppm which was strongly present in the four septic samples. The two signals of lactate, a doublet at 1.33ppm and a quartet are also seen in Figure 1A.

Investigation of the data in terms of scores plots of other combinations of the principal components did not, however, reveal significant clustering of data. No metabolic difference by multivariate analysis could be detected in the PCA of inflammatory arthritis *vs*. OA and crystal arthropathies (Fig. 2B) or OA alone (Fig. 2C). In further analyses, the 4 RA samples did not differ from spondyloarthritis (data not shown). Comparison of synovial fluid from patients suffering of gout to the CPPD samples revealed no metabolic difference between the two groups (Fig. 2D).

Table I. List of metabolites identified in the NMR spectra of synovial fluid.
Signals belonging to the major metabolites are indicated in Figure 1, the minor metabolites were detected only in trace amounts.

Major metabolites	Minor metabolites
D-glucose	cysteine
acetone	glucuronic acid
acetate	histidine
formic acid	homocysteine
hippuric acid	3-hydroxybuturic acid
carnitine	inosine
choline	serine
citric acid	tryptophane
creatinine	taurine
creatine	1-methyl-uric acid
ethanol	threonine
L-glutamate	
L-glutamine	
lactic acid	
L-alanine	
L-valine	
L-threonine	
L-tyrosine	
L-phenylalanine	
lysine	
phenylalanine	
puruvic acid	
succinic acid	
uracil	

Discussion

This study investigated the metabolic fingerprint of synovial fluid in different forms of arthritis by NMR. First, we obtained comprehensive information about the composition of human synovial fluid with respect to its low molecular weight metabolites and drugs. The spectrum of NMR-identified metabolites in the inflamed joint could be expanded to 35 and we detected paracetamol and ibuprofen in several patients. This was achieved by comparison of the spectra to the Bruker metabolomics NMR-spectra database consisting of 500 reduced spectra and common medical substances.

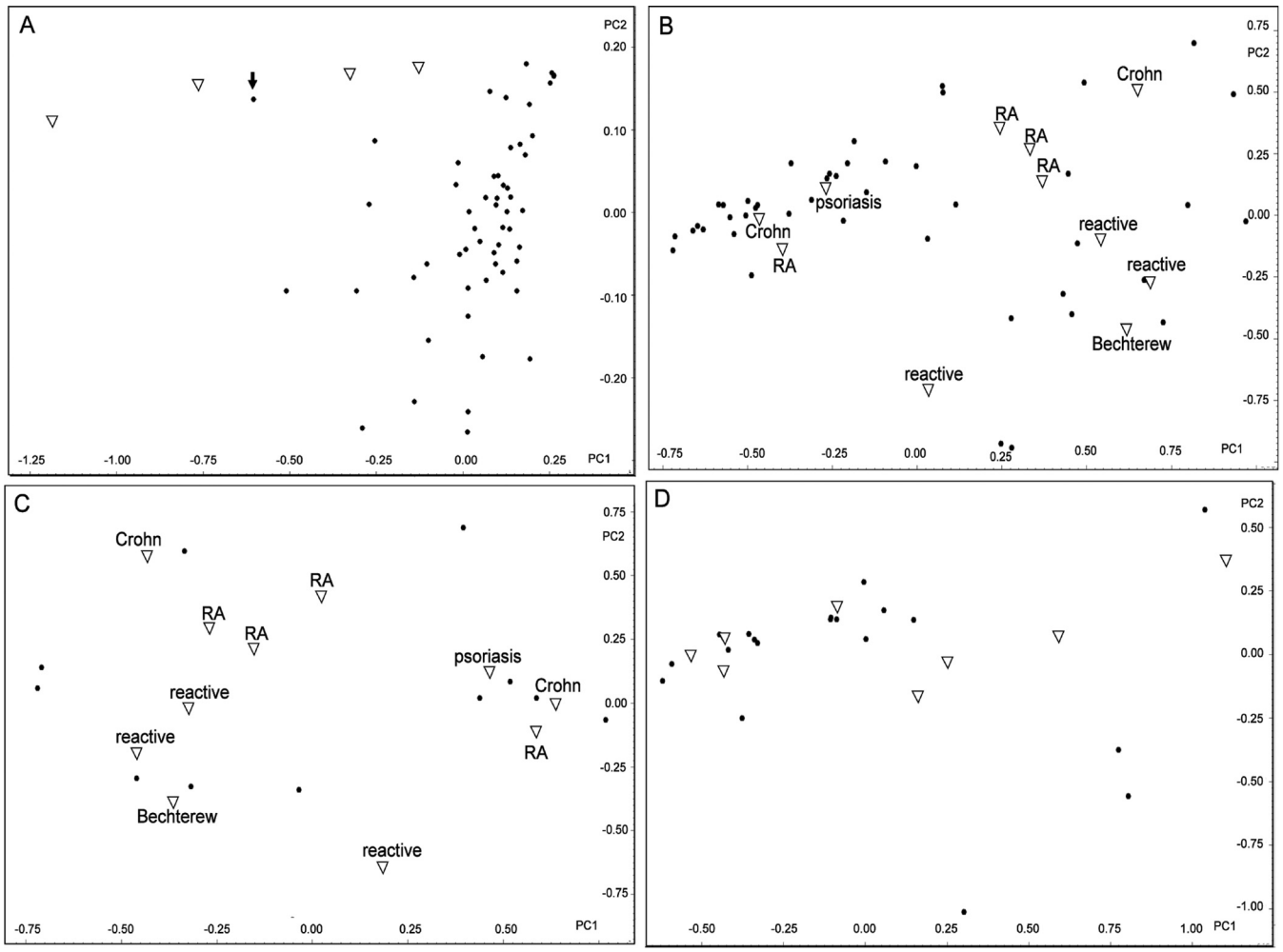

Fig. 2. Principal component scores plot. **A)** The four septic samples (indicated by open triangles) align along the first PC vector. An osteoarthritis (OA) spectrum outlying on the PC1 vector is marked with an arrow. **B**) Inflammatory forms of arthritis were compared with OA and crystal arthropathy samples (dots). Septic arthritis samples are excluded. There is no metabolic difference between RA and other forms of inflammatory arthritis, OA or crystal arthropathy, respectively. **C)** Inflammatory arthritis samples are compared to OA only (dots). No difference is seen in the PCA. **D**) Gout samples (triangle) are compared with CPPD samples (dots). There is no significant clustering of data. RA: rheumatoid arthritis; reactive: reactive arthritis; Bechterew: ankylosing spondylitis.

Most of the detected metabolites are peptide compounds which were not clearly attributable to a specific metabolic pathway and might be a product of endogenous metabolites or alimentary derived metabolites, respectively. We found hippuric acid (= N-benzoylglycine), a biomarker for occupational exposure of toluene associated with connective tissue diseases in all samples (10). 1-Methyluric acid as a metabolite of caffeine is one of the purine components in urinary calculi, but has never been described in arthritis (11). We also identified glucuronic acid, a degradation product of hyaluronic acid, which is an important synovial fluid component responsible for viscosity and elasticity of the cartilage. Furthermore, yet unidentified strong NMR-signals were observed in synovial fluid which might be of importance in the search of new biomarkers and thus will be further investigated. Characterisation by other NMR techniques such as liquid chromatography NMR will be necessary to identify these substances.

We found that the samples from patients with septic arthritis could be differentiated from synovial fluid from patients with crystal arthropathy, inflammatory arthritis or OA. The identification was mainly based on an elevated lactate concentration, reflected particularly through the well-separated lactose methyl signal. This finding is in line with previous univariate analyses of synovial fluid in patients with septic arthritis (6). In contrast to univariate analysis, however, PCA includes statistical analysis of several vectors and is therefore *a priori* more sensitive despite sensitivity and specificity could not be addressed in this study due to the low numbers of septic arthritis. Similar results have also been obtained in cerebrospinal fluid, where multivariate NMR analysis could differentiate between bacterial ventriculitis, viral infection and normal samples (12).

In this study, we could not find significant differences in the metabolic fingerprint between RA, gout, CPPD, OA and spondylarthritis by applying PCA. This indicates that the inflammatory or non-inflammatory metabolic end products of low molecular weight in the joint are similar. Alternatively, these results might support previous observations that intra-articular inflammation

also occurs in a substantial part of OA patients. However, the low numbers of included individuals especially for RA influence these results and a positive result might be detected in a larger cohort. A further limitation of this study is the variation in the length of freezing time which might have influenced the metabolism of the samples.

More advanced statistical analyses and more rapid sample processing might further improve this method and help to detect significant metabolic fingerprints in synovial fluid samples in future. Unfortunately, given the unavailability of healthy synovial fluid, the comparison to synovial fluid of healthy individuals could not be made.

In summary, NMR as a fast and high throughput analytic tool might have implications in the diagnosis of arthritis and potential in the detection of new biomarkers. The results of this pilot study warrant larger studies in order to estimate the sensitivity and specificity of NMR for the diagnosis of septic arthritis and to identify fingerprints which can be used as biomarkers in inflammatory arthritis or OA. For this, PCA will have to be compared with broader clinical features and radiological progression, respectively.

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