Imperial College London

Metabolic profiling of inflammatory synovial fluid

Thesis submitted by

Pouya Akhbari BSc, MBBS, MSc, FRCS (Tr & Orth) Eng

For the degree of Doctor of Medicine (Research)

Department of Metabolism, Digestion and Reproduction

2023

Clinical Supervision: Mr Chinmay Gupte,

Clinical Reader in Knee and Trauma Surgery, Musculoskeletal Laboratory, Imperial College London

Mr Rajarshi Bhattacharya, Clinical Director of Trauma and Orthopaedics, Imperial College Healthcare NHS Trust

Non-clinical Supervision: Dr Horace Williams

Clinical Reader in Gastrointestinal Medicine, Imperial College London

Metabolic Profiling Supervisors: Professor John Lindon, Dr Gonçalo Graça, Dr Claire Boulangé

Imperial College Phenome Centre, Department of Metabolism, Digestion and Reproduction, Imperial College London

Declaration of originality

I declare that all the work conducted in this thesis is my own, except for any ideas or quotations from the work of other people, published or otherwise, which has been fully acknowledged and appropriately referenced.

All images have been produced in this thesis by Pouya Akhbari, except for those which have been appropriately referenced.

The right of Pouya Akhbari to be identified as the author of this work has been asserted by him in accordance with the Copyright, Designs and Patent Act of 1988.

Copyright Declaration

Acknowledgement

Behind every Doctoral student, there are a whole host of people who have helped to guide, support and make a lot of the work possible.

I would like to start by thanking my friend and colleague Matthew Jaggard, who first told me about the project and has acted as an unofficial supervisor providing me with useful information, guidance and support. We have also collaborated together on a number of very good publications.

I would like to thank Mr Chinmay Gupte and Dr Horace Williams for their guidance and support during the entire four years of this research degree, including their invaluable assistance with the first author publications that this thesis has yielded. I would also like to thank Mr Gupte with his help reviewing all the chapters in this thesis.

All of the experimental work was completed alongside clinical practice, which provided much needed financial support. Therefore, thanks must also go to Mr Rajarshi Bhattacharya and the Trauma and Orthopaedic Department of Imperial College NHS Trust for their important encouragement and support.

Important thanks must go to Dr Claire Boulangé who offered constant and unrelenting operational support for the analytical aspect of the project, particularly during the first year of my involvement. She was extremely generous with her time and support.

I am also extremely grateful to Professor John Lindon for his help and support throughout this project, particularly for his helpful advice in the first author publications this project has yielded, plus his useful comments and suggestions for all the chapters in this thesis.

I would also like to thank Dr Gonçalo Graça, who in addition to Dr Claire Boulangé, has tirelessly given of his time to provide much needed support for all the analytical aspects of this project throughout the entire duration of this thesis. He also read through every draft of manuscript and thesis chapter that was written, providing extremely useful advice, comments, suggestions and feedback. Without his assistance, a lot of this work would not have been possible.

Finally, I would like to thank my mother. Throughout this research degree, she has provided unwavering support throughout some difficult times.

Oral presentations from this thesis

Differences between infected and non-infected synovial fluid: an observational study using metabolic phenotyping

Akhbari P, Jaggard M, Vaghela U, Boulangé C, Graça G, Lindon J, Williams H, Bhattacharya R, Gupte C

European Federation of Orthopaedics Research and Traumatology (virtual conference), 29/10/20

Differences between infected and non-infected synovial fluid: a nuclear magnetic resonance (NMR) spectroscopy study of metabolic profiles

Akhbari P, Jaggard M, Vaghela U, Boulangé C, Graça G, Lindon J, Williams H, Bhattacharya R, Gupte C

British Orthopaedic Association (virtual conference), 21/09/20

Differences in the synovial fluid of hips and knees: metabolic analysis using Nuclear Magnetic Resonance (NMR) Spectroscopy

Akhbari P, Jaggard M, Vaghela U, Boulangé C, Graça G, Lindon J, Williams H, Bhattacharya R, Gupte C

European Federation of Orthopaedics Research and Traumatology, Barcelona, 31/05/18

Prizes and acknowledgements from this thesis

Featured author in the Bone and Joint Research Journal in June 2020 following publication of article entitled “Can joint fluid metabolic profiling (or “metabonomics”) reveal biomarkers for osteoarthritis and inflammatory joint disease? A systematic review”.

Differences in the synovial fluid of hips and knees: metabolic analysis using Nuclear Magnetic Resonance (NMR) Spectroscopy

Akhbari P, Jaggard M, Vaghela U, Boulangé C, Graça G, Lindon J, Williams H, Bhattacharya R, Gupte C

Second place at the Lipmann Kessel Meeting, Royal College of Surgeons, London 09/03/18

Publications from this thesis

Differences between infected and non-infected synovial fluid: an observational study using metabolic phenotyping (or “metabonomics”)

Akhbari P, Jaggard M, Vaghela U, Boulangé C, Graça G, Lindon J, Williams H, Bhattacharya R, Gupte C

Bone and Joint Research 2021 Jan; 10(1): 85–95 (PMID: 33502243)

Can joint fluid metabolic profiling (or “metabonomics”) reveal biomarkers for osteoarthritis and inflammatory joint disease? A systematic review

Akhbari P, Karamchandani U, Jaggard M, Graça G, Bhattacharya R, Lindon J, Williams H, Gupte CM

Bone and Joint Research 2020; 9(3):108–119 (PMID: 32435463)

Differences in the composition of hip and knee synovial fluid in osteoarthritis: a nucelar magnetic resonance (NMR) spectroscopy study of metabolic profiles

Akhbari P, Jaggard M, Boulangé C, Vaghela U, Graça G, Bhattacharya R, Lindon J, Williams H, Gupte C

Osteoarthritis and Cartilage 2019 Dec 27(12): 1768 - 1777 (PMID: 31491490)

Abstract

Synovial fluid (SF) has unique properties regarding the maintenance of joint homeostasis. It consists mainly of proteins, but also small molecules, which are metabolic derivatives of these proteins. These molecules can be assessed by metabolic profiling using techniques such as nuclear magnetic resonance (NMR) spectroscopy and Mass Spectrometry (MS).

A systematic review identified over 200 metabolites, from which 26 were putative biomarkers in osteoarthritis, inflammatory arthropathies and trauma. The molecular composition of osteoarthritic and infected SF was examined using NMR spectroscopy. Due to ethical constraints, it was not possible to collect samples from normal joints.

It was hypothesised that SF of hip and knee joints have different metabolic characteristics. Osteoarthritic hip and knee samples were taken and assessed for differences in their metabolic composition. Four metabolites presented in significantly greater quantities in the knee compared to the hip group (*N-acetylated* molecules, *glycosaminoglycans*, *citrate*, *glutamine*). These are involved in collagen degradation, the *tricarboxylic acid* cycle and oxidative metabolism in diseased joints. These findings may represent a combination of intra and extra-articular factors.

Another promising application was to examine infected versus non-infected SF. Three classes of metabolites presented in higher relative concentrations (lipids, *cholesterol* and *N-acetylated* molecules) and 13 in lower relative concentrations in the infected group. Metabolites in significantly greater concentrations in the infected cohort represented those having a defensive role against pathogenic microorganisms, a role in lipid metabolism and the inflammatory response. Those in significantly reduced concentrations were involved in carbohydrate (*citrate*, *glucose* and *mannose*) and nucleoside metabolism (*glycine*, *glutamine* and *valine*), the *glutamate* metabolic pathway (*glutamine*, *proline* and *lysine*), increased oxidative stress in the diseased state (*dimethylsulfone*) and reduced articular cartilage breakdown (*glycosaminoglycans*).

Future studies should include larger cohorts to confirm these findings and validation of the identified metabolites using other metabolic profiling techniques with more targeted analysis,

such as MS. This may ultimately lead to identifying putative biomarkers that form the bases of new diagnostic tests.

Abbreviations

1,5-AG	*1,5-Anhydroglucitol*
AA	Amino acid
ACL	Anterior cruciate ligament
ANOVA	Analysis of Variance
Apo A-1	*Apolipoprotein A-1*
Apo B	*Apolipoprotein B*
AS	Ankylosing Spondylitis
ATP	*Adenosine Triphosphate*
B_0	External magnetic field
BCAA	Branched chain amino acid
BD	Behcet's disease
BMI	Body mass index
BMP-2	Bone morphogenic protein-2
CRP	C-Reactive Protein
COSY	^{1}H-^{1}H correlation spectroscopy
CSF	Cerebrospinal fluid
EAA	Essential amino acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay

ESOA	End-stage osteoarthritis
ESR	Erythrocyte Sedimentation Rate
F6P	*Fructose-6-phosphate*
FA	*Fatty acids*
FDR	False discovery rate
FID	Free induction decay
G6P	*Glucose-6-phosphate*
GABA	*γ-aminobutyric acid*
GAGs	*Glycosaminoglycans*
GC-MS	Gas chromatography - mass spectrometry
GC/TOF-MS	Gas chromatography/time-of-flight mass spectrometry
HA	*Hyaluronic acid*
HDL	*High-density lipoprotein*
HSF	Human synovial fluid
HSQC	Heteronuclear single-quantum correlation spectroscopy
Hyp	*4-hydroxy-L-proline*
HMDB	Human Metabolome Database
IL	Interleukin
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LC-MS	Liquid chromatography - mass spectrometry
LDL	*Low-density lipoprotein*
MC&S	Microscopy, culture and sensitivity

MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MSI	Metabolomics Standards Initiative
NAc	*N-acetylated* group
NEAA	Non-essential amino acids
NMR	Nuclear magnetic resonance
NO	Nitrous oxide
OA	Osteoarthritis
O-PLS-DA	Orthogonal Partial Least Squares- Discriminant Analysis
OSC	Orthogonal signal correction
PC	*Phosphatidylcholine*
PCA	Principal component analysis
PG	Proteoglycan
PLS	Partial least squares
PCR	Polymerase chain reaction
PJI	Periprosthetic joint infection
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analysis
PTAA	Post-traumatic arthritic ankle
PUFA	Polyunsaturated fatty acids
RA	Rheumatoid arthritis

ReA	Reactive arthritis
ROS	Reactive oxygen species
SAM	*S-adenosylmethionine*
SF	Synovial fluid
SIMCA	Soft Independent Modelling of Class Analogy
SNA	Seronegative arthritis
SPM	*Sphingomyelin*
SSA	Seronegative spondyloarthropathy
STOCSY	Statistical total correlation spectroscopy
TB	Tuberculosis
TBM	Tuberculosis meningitis
TCA	*Tricarboxylic acid*
TGF- β	Transforming growth factor-β
THR	Total hip replacement
TKR	Total knee replacement
TNF- α	Tumour necrosis factor-α
TOCSY	^{1}H-^{1}H Total correlation spectroscopy
VIP	Variable projection of importance score
VLDL	*Very low-density lipoprotein*

Table of Contents

Table of figures

1 Articular cartilage, joint disease, joint biomechanics and infection

1.1 Articular cartilage

Articular cartilage is specialised form of connective tissue unique to diarthrodial joints. It is avascular, alymphatic and aneural. Consequently, it has a limited ability to undergo intrinsic repair and healing.

1.1.1 Composition of articular cartilage

Articular or hyaline cartilage is typically 2-4mm thick and composed of cells, which are predominantly chondrocytes, and a dense extracellular matrix (ECM). The ECM itself is composed of principally of collagen, proteoglycans and water, with smaller quantities of glycoproteins and non-collagenous proteins(1, 2). There are a number of zones, which contain different amounts of the aforementioned components.

1.1.2 Zones of articular cartilage

The superficial (tangential) zone represents 10-20% of the thickness of articular cartilage. The collagen fibres (primarily type II and IX) are found in high concentrations and run parallel to the joint surface, thus helping to minimise sheer stresses. The chondrocytes themselves are found in relatively high numbers and flattened. The integrity of this layer is important to protect and maintain the deeper layers.

The middle (transitional) zone represents 40-60% of the articular cartilage. It contains thicker collagen fibrils and proteoglycans. The chondrocytes are more rounded and in fewer numbers with the collagen fibres organised obliquely. This layer helps to protect the cartilage against compressive forces.

The deep zone represents 30% of the articular cartilage, with the highest concentration of proteoglycans and the lowest concentration of water. It has the largest diameter of collagen fibres which are organised perpendicular to the articular surface, thus helping to resist compressive forces across the cartilage.

The tidemark separates the deep zone from the calcified cartilage. The calcified cartilage anchors the collagen fibres of the deep zone to the underlying subchondral bone, thus securing

the cartilage to bone. There are very few cells in the calcified cartilage and the chondrocytes are hypertrophic.

1.1.3 Chondrocytes

Chondrocytes arise from the mesenchymal cell line and contribute 2% of the articular cartilage volume(3). Their number, shape and size vary based on their location within the articular cartilage. They are each responsible for the turnover of ECM in their immediate vicinity. There is typically no cell-to-cell contact for direct communication and signal transduction between adjacent cells. However, they respond to various stimuli including hydrostatic pressures, growth factors, mechanical loads and piezoelectric forces(2). Their limited ability for replication contributes to the reduced intrinsic healing capacity of articular cartilage as a result of injury.

1.1.4 Extracellular Matrix

The ECM accounts for 65 to 80% of the total weight of articular cartilage(4). The remaining dry weight is accounted for predominantly by collagen fibres and proteoglycans.

1.1.5 Water

Water contributes 80% of the wet weight of articular cartilage. The majority of this water is located within the pore space of the matrix, with approximately 30% associated with the intrafibrillar space within the collagen(5, 6). The water concentration decreases with depth from 80% in the superficial zone to 65% in the deep zone(1).

The majority of interfibrillar water is gel-like. Movement of this fluid through the ECM can occur by compression of the articular cartilage or by applying a pressure gradient across the tissue(4, 7). The permeability of the tissue remains low as the frictional resistance against flow through the ECM is high. The combination of water pressurisation within the matrix and this frictional resistance to water flow provide the basic mechanisms, which allow articular cartilage to withstand significant loads.

1.1.6 Collagens

Collagen is responsible for 60% of the dry weight of articular cartilage. There are many types of collagen, with type II responsible for 90-95% of the collagen in ECM. Collagen types I, IV, V, VI,

IX and XI are also present in much smaller amounts. Their role is to help form and stabilise the type II collagen fibril network(8).

All types of collagen consist of a region containing three polypeptide chains (α-chains) wound into a triple helix. This structure of the polypeptide chains provides articular cartilage with important tensile and shear properties, which improve stability of the matrix (figure 1). The amino acid composition of these chains is mainly *glycine* and *proline*, with *hydroxyproline* providing stability along the length of the molecule through hydrogen bonds(9).

1.1.7 Proteoglycans

Proteoglycans account for 10-15% of the wet weight of articular cartilage. They are heavily glycosylated protein monomers consisting of a protein core "backbone" with a number of glycosaminoglycan (*GAG*) chains attached via covalent bonds (figure 1). The commonest of these proteoglycans in articular cartilage is *aggrecan*, but others include *biglycan*, *decorin* and *fibromodulin*. *Aggrecan* consist of over 100 *chondroitin* and *keratin sulfate GAG* chains attached via link proteins to a hyaluronan protein core(10, 11). *Aggrecan* is hydrophilic in nature, meaning it has an affinity for water. It occupies the interfibrillar space of the ECM, providing the cartilage with its osmotic properties, which are essential in enabling the cartilage to resist compressive loads.

1.1.8 Noncollagenous proteins and glycoproteins

There are many glycoproteins and non-collagenous proteins found within articular cartilage, but their function has yet to be clarified. A few of these molecules, such as fibronectin, may have a role in preserving and organising the macromolecular structure of the ECM.

1.1.9 Metabolism of articular cartilage

As articular cartilage is avascular and alymphatic, its nutrition takes place by diffusion from the synovial fluid across the articular surface. Chondrocytes have an important role on the synthesis of various matrix components including *GAGs* and other proteins. Their metabolic activity can be altered by a variety of factors within their surrounding environment, including proinflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF- α).

These cytokines have both anabolic and catabolic roles that are involved in the synthesis and degradation of matrix macromolecules(11).

Chondrocytes are also responsible for producing proteoglycans, with various regulatory peptides and growth factors responsible for regulating their metabolism. These include IL-1, TNF- α, transforming growth factor-β (TGF- β) and insulin like growth factors. However, little is known about how these substances exert an effect on proteoglycan metabolism.

Homeostasis of ECM metabolism is responsible for regulating both the degradation and synthesis of various macromolecules. Proteoglycan turnover itself can take up to 25 years(12).

The metalloproteinases (collagenase, stromelysin and gelatinase) and cathepsins (B and D) are the main proteinases involved in articular cartilage turnover. The metalloproteinases are involved in degrading the various collagen types and the cathepsins are involved in *aggrecan* degradation(13, 14).

Loading and motion of the joint have important roles in maintaining normal metabolism of the articular cartilage, with joint inactivity leading to cartilage degradation(1).

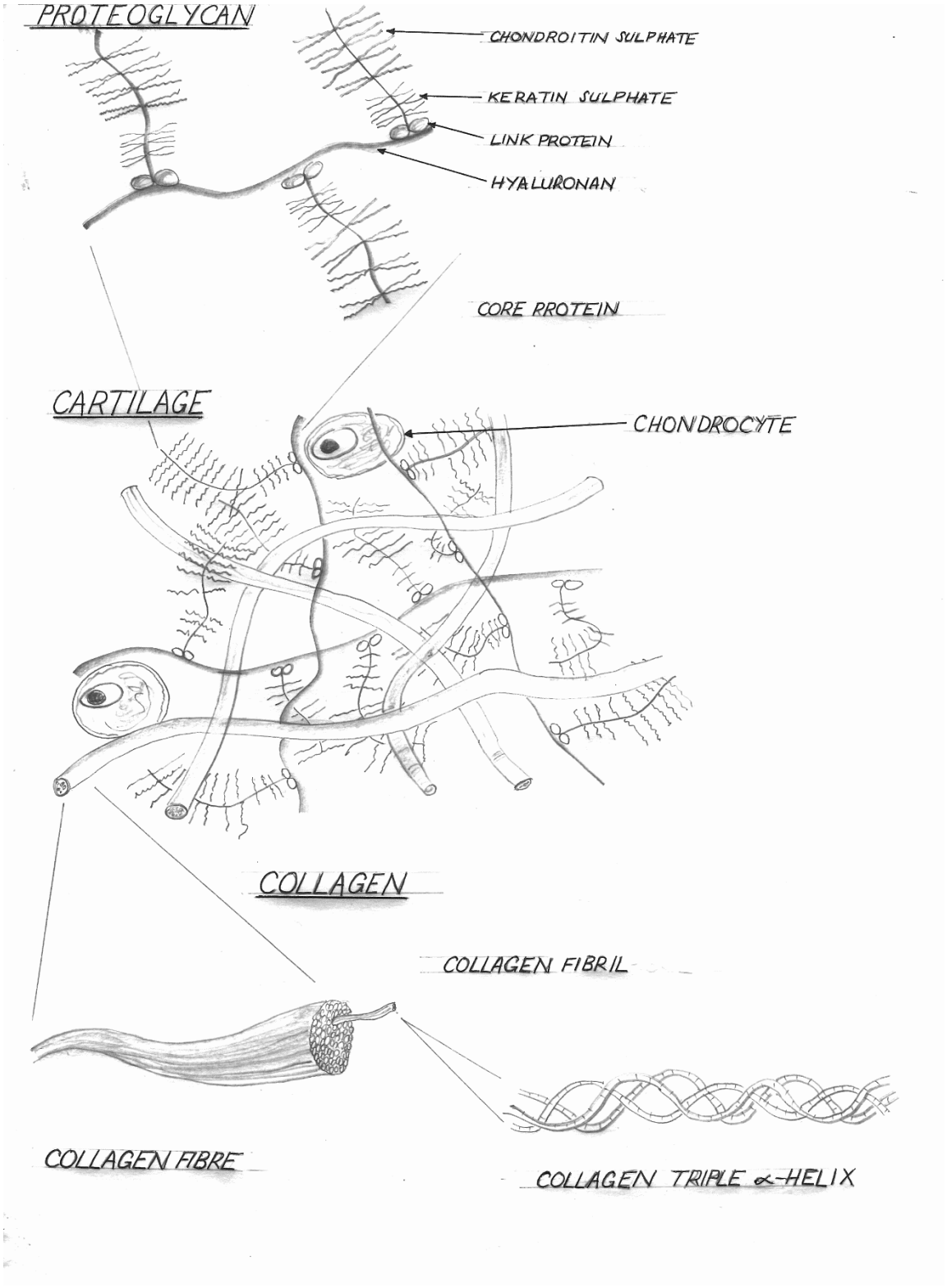


Figure 1. Illustration of cartilage and its constituent proteoglycan and collagen fibres. Image reproduced with permission by Mr Matthew Jaggard.

1.1.10 Biomechanical function of articular cartilage

The main purpose of articular cartilage is to provide a smooth, lubricated and low friction articular surface plus to facilitate load transmission to the underlying subchondral bone. It is able to withstand high cyclical loads with minimal or no evidence of degenerative changes(15, 16). The initial contact forces during joint loading results in increased interstitial fluid pressure. This leads to fluid flowing out of the ECM, which applies a large frictional drag to the ECM. The opposing bones surround the articular surfaces of the joint and confine the cartilage, thus restricting mechanical deformation. Like other biological substances, articular cartilage exhibits viscoelastic properties. These are time-dependent behavioural properties, which occur when the cartilage is subjected to deformation or a constant load(17). Articular cartilage can undergo creep and stress-relaxation. Creep is a time-dependent decrease in strain at constant stress, which occurs until an equilibrium value is reached(18). Stress-relaxation is a time-dependent decrease in stress at constant strain, which again occurs until an equilibrium value is reached(19).

1.2 Synovial fluid

The commonest joints in the human body are synovial joints and they allow low friction wear-resistant movement between opposing bone surfaces(20). These joints typically have a cavity filled with synovial fluid (SF) (figure 2). Articular cartilage covers the bone surface of the joint cavity, which also has a fibrous capsule, including the synovium (inner lining). SF has unique properties and functions regarding the maintenance of joint homeostasis, which are secondary to its molecular and cellular constituents. It is an ultrafiltrate of blood plasma with additional molecules, which are secreted by cells lying the synovium. These include hyaluronan, lubricant molecules and proteoglycan 4, also known as lubricin(21, 22). Other functions of SF include its metabolic roles of transporting nutrients, waste products and other metabolites from within the joint and synovium, plus enzymes which have a function on these tissues. SF also contains a variety of other molecules, which aid in cell communication within the joint. These include cytokines, growth factors and morphogens(23-25). Cytokines have a wide variety of molecular weights ranging from 6-70kDa. They act as modulators and mediators within highly localised environments and help with cell-to-cell communication and regulate immunological responses

to inflammatory stimuli and infectious agents(26). Morphogens describe specific signalling molecules that induce particular cellular responses by acting directly on the cell.

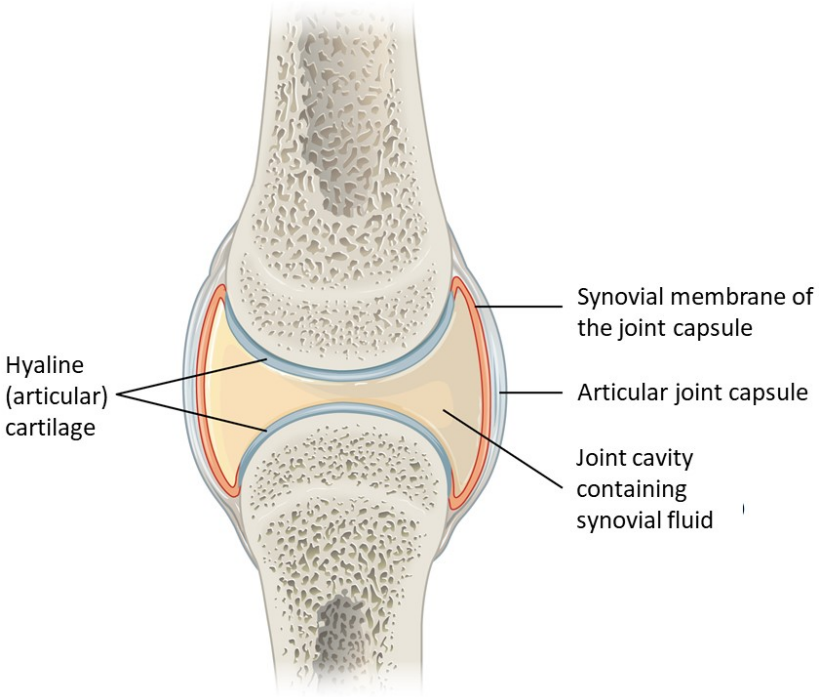


Figure 2. Schematic representation of a typical synovial joint lined with hyaline cartilage.

The synovium itself is the main barrier to molecular transport within SF and plasma. It is a thin sheet of vascularised connective tissue with macrophage-like (type A) and fibroblast-like (type B) cells within an ECM made up predominately of collagen, HA and proteoglycans. It has been modelled as a double barrier consisting of a macrovascular endothelium that limits protein transport and a synovial interstitial space that limits diffusion of small molecules(27-30).

SF is considered the best lubricant for the joints and it has unique properties that protect the bone surfaces and cartilage from high contact pressures during the majority of the patient's life. There are four major biological components making SF an efficient lubricant: Albumin, *Hyaluronic acid* (HA), globulin and mucinous glycoproteins (mostly lubricin)(31). Each of these components has been found to have a different role in SF:

Albumin: Protects the joint from articular cartilage wear(32)

HA: Increases SF viscosity(33)

Globulin: Important role in boundary lubrication(34)

Lubricin: Reduces the amount of shear at the contact interface of the joints(35)

Synovial fluid provides a film lubrication between two cartilage surfaces, but chemical and mechanical analysis has shown it to be so much more.

Table 1 lists the concentrations of the main constituents found in normal SF.

Table 1. Concentrations of the main constituents found in normal synovial fluid. HA – hyaluronic acid, PRG4 – proteoglycan 4. Modified from Martin-Alarcon and Schmidt(36).

Constituent type	**Constituent**	**Concentration (mg/ml)**	**Molecular weight (kDa)**
Glycosaminoglycans	HA	2 - 4	6000 – 7000
Glycoprotein	*PRG4*	0.035 – 0.250	220
Serum protein	Albumin	8 – 12	69
	ɣ-Globulin	2 – 7	155
Ion (electrolyte)	Na^+	3.335	
	Cl^-	3.94	
Lipid	*Phospholipids*	0.1 – 0.2	
	Cholesterol	0.07 – 0.08	
	Triglycerides	0	
Small molecule	Urea	0.082	
	Urate	0.0155	
	Glucose	0.66	

There are many similarities in the protein composition of SF and blood plasma (29, 30). The most prominent protein in SF is albumin, with various globulins forming some of the other major protein components(27). The ability of a plasma protein to be filtered through the synovial membrane and enter the SF is largely dependent on its size, which has an important bearing on the concentration of these proteins relative to the plasma concentration. For example, plasma proteins with a lower molecular weight such as *albumin* (69kDa) and transferrin (90kDa) are found in relatively higher concentrations in SF(27, 37, 38).

HA and lubricin are the main lubricant macromolecules in SF. HA has a high molecular weight (6-7MDa), an important role in maintaining SF viscosity and provides SF with viscoelastic properties beyond a simple lubricant. It is a non-sulfated *GAG* consisting of repeating disaccharide units of *D-N-acetylglucosamine* and *D-glucuronic acid*(39). Lubricin is a mucinous glycoprotein with multiple O-linked *β(1-3)Gal-GalNAc* oligosaccharides, which has an important role in mediating boundary lubrication in articular cartilage(40). Its molecular weight is 220kDa.

In cases of joint inflammation, there is an increase in the concentration and content of various proteins. This is a result of structural and functional changes within the synovium secondary to joint injury or disease, such as that which occurs in patients with osteoarthritis (OA), rheumatoid arthritis (RA) and traumatic arthritis(27, 38). This is because the ability of synovium to selectively filter and retain various proteins is affected by synovial inflammation in different diseases. There are also characteristic changes in SF lubricant macromolecules associated with these conditions with decreased concentrations of HA (41, 42). However, the effects of injury on lubricin concentrations within SF are more variable. They may be altered post-injury secondary to changes in rates of degradation, synthesis, secretion and/or loss from the joint, not to mention changes in SF volumes. At this time however, the exact effects of injury on human lubricin concentrations remains unknown(20).

1.3 Osteoarthritis

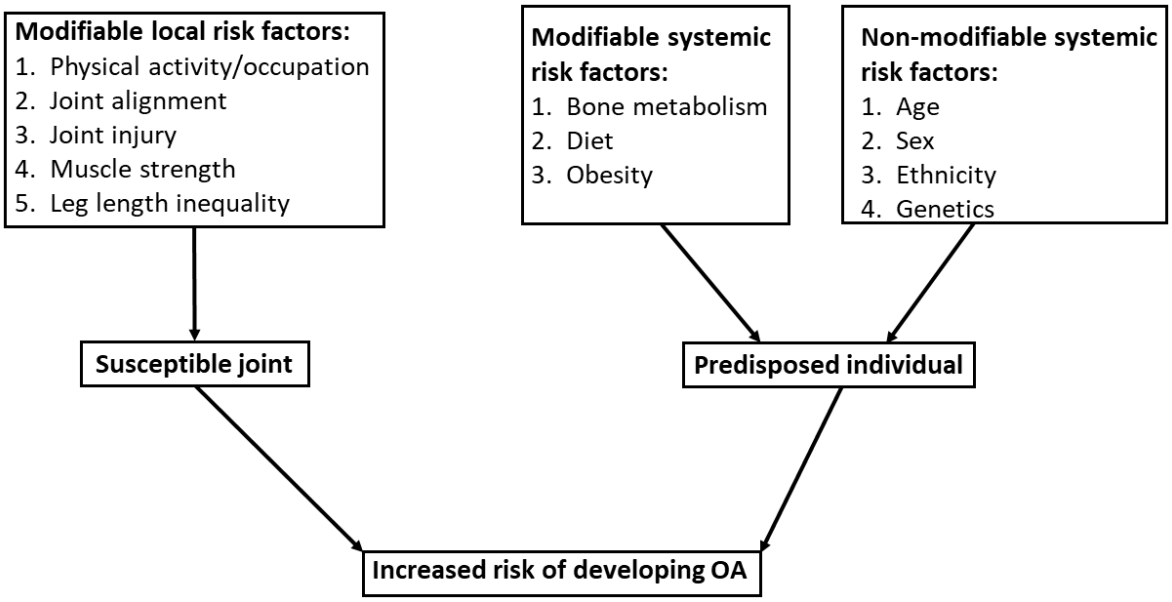
Osteoarthritis is one of the most disabling conditions in the Western world, effecting approximately 10% of the United Kingdom (UK) population and presenting a major healthcare burden. It is a heterogenous disease, which manifests in a number of different phenotypes, due

to various pathogenic factors, ultimately leading to an alteration of the whole joint structure(43). It results in progressive degradation of ligaments, cartilage and menisci, synovial inflammation and changes to the subchondral bone with common clinical and radiological manifestations(44). A number of different classification systems have been proposed, depending on the main driving force responsible for the disease, the joint involved, degree of inflammation and the patient characteristics(45-48).

The Kellgren-Lawrence (KL) classification is one of the commonest used radiological classification systems(49). It has been used for the hip and the hand as well as the tibiafemoral joint of the knee. It is graded from 0-4 as follows: 0. No radiological evidence of OA; 1. Mild joint space narrowing and possible small osteophytes; 2. Possible joint space narrowing with definite osteophyte formation; 3. Definite joint space narrowing, moderately sized osteophytes, subchondral sclerosis and possible deformity of the bony ends; 4. Bone on bone arthritis with large osteophytes, significant subchondral sclerosis and definite deformities of the bony ends.

The risk factors for OA are multifactorial and involve a complex interplay between biochemical, cellular and mechanical factors ultimately leading to the same endpoint (Table 2). Consequently, the risk factors for OA can vary amongst individuals(50).

Table 2. Possible risk factors for the development of OA. Modified from Johnson and Hunter 2014(50). OA – osteoarthritis.



1.4 Risk factors for Osteoarthritis

1.4.1 Age and Gender

One of the strongest predictors of OA is age (51), although the mechanism(s) underpinning the increased prevalence of OA with age remains poorly understood. There are a number of proposed biological changes responsible for this including cellular senescence, increased bone resorption and age-related sarcopenia, all of which reduce the ability of the joint to adapt to biomechanical insults.

There is a higher prevalence and severity of OA in females, involving the hand, foot and knee joints(52). Furthermore, the risk of radiographically severe OA of the knee is higher in women following the menopause (52). Consequently, oestrogen has been hypothesised to have a role in the pathogenesis of OA. However, the results from a number of studies have been conflicting(53, 54). Other potential causes for this gender disparity include pregnancy, differences in ligamentous laxity, neuromuscular strength, alignment and bone strength.

1.4.2 Genetics

Contribution from genetic factors account for up to 40% of knee OA and 60% of both hip and hand OA(55). A number of genes have been identified to have a potential role in OA pathophysiological pathways. These include genes for type II collagen(56), insulin-like growth factor(51), vitamin D receptors and growth differentiation factor 5. These may provide a potential drug target for future treatment.

1.4.3 Obesity

Obesity has become a global epidemic and there is evidence to suggest that it is a strong risk factor for OA in joints such as the hip and knee(57). Therefore, it stands to reason that as the incidence of obesity rises, so does the incidence of lower limb OA. Not only does obesity predate the development of OA, it is also associated with an increased risk of radiographic progression (51). A recent meta-analysis demonstrated a linear relationship between obesity and knee OA(57) with a 35% increase in the risk of knee OA for every 5 unit increase in body mass index (BMI). This association was more prominent in women. Additionally, a Swedish population study demonstrated the relative risk of knee OA to be 8.1 in patients with a BMI over 30(58).

On the other hand, the Framingham study(59) demonstrated a 50% risk reduction in knee OA when patients reduced their weight by 5kg, which is also supported by a more recent meta-analysis(60).

However, the relationship between hip OA and body weight is less consistent than for knee OA (51, 61). There may also be systemic metabolic and inflammatory effects associated with obesity, due to the fact that obesity is also associated with OA of the small bones in the hand(62).

1.4.4 Diet

Oxygen free radicals, generated intraarticularly, can cause damage to the articular cartilage(63). Furthermore, due to the role of vitamin D in regulating bone metabolism, it has been suggested that hypovitaminosis D may have a role in the increased incidence and progression of both hip and knee OA(63-65). However, the evidence surrounding this relationship is conflicting. A

number of other vitamins have also been postulated to have a role in the development of OA. For example, vitamin C intake has been shown to have a role in OA, with a reduced intake being associated with an increased risk of knee OA progression(63), and a high intake associated with reduced radiographic progression of knee OA and a lower risk of developing knee pain(66). Furthermore, vitamin K plays an important role in regulating the mineralisation of bone and cartilage. Increased rates of radiographic changes in the hand and knee associated with OA are related to low plasma levels of one of the K vitamins known as phylloquinone (<0.5nmol/l)(67).

1.4.5 Physical Activity

An increased risk of OA is associated with repetitive use of a joint. Studies have demonstrated that occupations requiring a greater deal of activity, such as kneeling and squatting, are associated with a two-fold increase in the risk of developing knee OA than occupations which are less physically demanding(68). Following the same principle, occupations involving a greater amount of manual dexterity are associated with features of hand OA(69). Prolonged standing and lifting has also been associated with hip OA(70). In essence, physical activity can be viewed as a double-edged sword. On the one hand it helps to strengthen the periarticular muscles, thus reducing the pressure on the joint, but on the other hand it could have a detrimental effect by placing unnecessary force on the joint. However, a recent review did not show a causal relationship between OA of the hip and/or knee and low- or moderate-distance running in the general population(71). The same review also failed to demonstrate a conclusive relationship between long distance running and the development of OA. They suggested that in the non-injured knee, there is no significant relationship between the development of knee or hip OA and running. However, in elite athletes an association has been shown between the development of OA and high intensity/high impact sports such as tennis and team sports. This cohort has been shown to have a greater rate of hip/knee OA when compared to an age-matched control group(72). What is not clear, is whether this is purely due to the level of activity or as a direct result of sports-related injury.

1.4.6 Post-traumatic knee injury

There are a number of factors aiding the joints ability to resist damage including the thickness of the articular cartilage, intrinsic and extrinsic ligaments, periarticular muscles and the bone

adjacent to the joint surface. Despite this, the knee remains one of the commonest injured joints. With respect to OA, the commonest injuries are those resulting in rupture of the anterior cruciate ligament (ACL). This is often accompanied by other injuries such as damage to the articular cartilage, collateral ligaments, subchondral bone and most importantly, damage to the meniscus, which is seen in 65-75% of ACL injuries(73). Such injuries have been linked to the development of OA, with radiographic and functional changes being evident by as early as 10 years following the initial injury(73, 74).

1.4.7 Muscle strength

Muscle strength has an important role in the aetiology of OA. Deficiency in muscle strength can be both a cause and consequence of OA. A recent review suggested that muscle weakness has a precipitative role in the onset and progression of OA. Conversely, disuse of a limb secondary to the pain associated with knee OA leads to reduced activation, proprioception and strength of the muscles(75). A relationship has been demonstrated between the cross-sectional area of the quadriceps muscle and the rate of asymptomatic knee OA in a cohort of women when compared to a BMI and age-matched control group(76). More severe cases have been associated with signs of muscle fibre atrophy(77), with one study demonstrating a 12% reduction in the quadriceps cross-sectional area compared to the contralateral leg, in patients undergoing a knee replacement(78). These findings suggest a relationship between loss of strength and the cross-sectional area of the quadriceps muscle in patients with OA. Furthermore, a relationship has been demonstrated between quadriceps strength and the risk of structural damage. Slemenda et al(79) showed that for every 5kg increase in extensor muscle strength there was a 20% and 29% reduction in the risk of developing radiographic and symptomatic knee OA respectively.

1.4.8 Local pathologic changes

Whereas previously OA was thought of as a primary, degenerative disorder of articular cartilage, it is now widely recognised that the development of OA involves multiple structures. Disruption of the collagen network may lead to increased tissue turgor in OA cartilage. There is also evidence to suggest upregulation of synthetic activity and cellular proliferation leading to increased concentration of chondrocytes(80). However, these cells are unable to respond to

growth factors and consequently increase the destruction of type II collagen and worsen matrix degradation(80).

Synovial inflammation may be caused by damage to the articular cartilage and bone. This leads to the release of inflammatory cytokines, matrix metalloproteinases (MMP), proteinases and aggrecanase, which in turn results in accelerated cartilage degradation(81). Together with the formation of osteophytes, these inflammatory mediators can cause pain by irritating the sensory nerve endings within the synovium.

Bone has a greater ability to modify its surrounding extracellular matrix and undergo self-repair than articular cartilage(82). During the development of OA, there are various adaptations to subchondral bone. These include subchondral sclerosis and increased plate thickness, osteophyte formation at the joint margins, narrowing of the joint space, increased cancellous bone volume, reduced matrix mineralisation and the formation of bone cysts. These changes ultimately lead to disease progression by causing changes in the adjacent joint surfaces, which itself causes a change in joint congruity(82).

1.5 Differences in hip and knee biomechanics

Degenerative changes in articular cartilage are due to biomechanical and genetic factors, as well as age progression(83, 84). The final common pathway is abnormal chondrocyte metabolism and matrix degradation. The hip has classically been thought of as a ball and socket joint with a high level of conformity (figure 3) (85). Abnormal contact stresses can lead to hip OA with contributing factors including femeroacetabular impingement and dysplasia (86, 87). The labrum increases the conformity of the hip joint but has not been shown to affect the contact pressure of the normal hip(88). However, it does have a role in the dysplastic hip, where it has been shown to provide significantly more mechanical support (89).

On the other hand, the knee joint is less conforming with greater contact stresses, due to a smaller area of contact between the tibio-femoral articulations (figure 3) (90). The cartilage of the tibio-femoral articulations is thicker in the posterior portion of the lateral compartment and anterior portion of the medial compartment. These are the areas, which are typically in contact during walking. The most important factors influencing knee OA include the mechanical axis of

the lower limb and abnormal kinematics secondary to ACL dysfunction (91, 92). Malalignment of the knee mechanical axis is one of the strongest predictors of knee OA progression, with a prospective cohort study demonstrating a strong association between abnormal alignment and increased degenerative changes in the compartment under the greatest compressive load(93). A number of studies have demonstrated a greater degree of varus deformity and articular cartilage wear in ACL deficient knees(94, 95). This gives further weight to the hypothesis that the loss of the ACL determines the antero-posterior position of the tibio-femoral contact area (96). The degenerative changes can be worsened by damage to the medial meniscus, which has been shown to have a protective role, even in an ACL deficient knee (95).

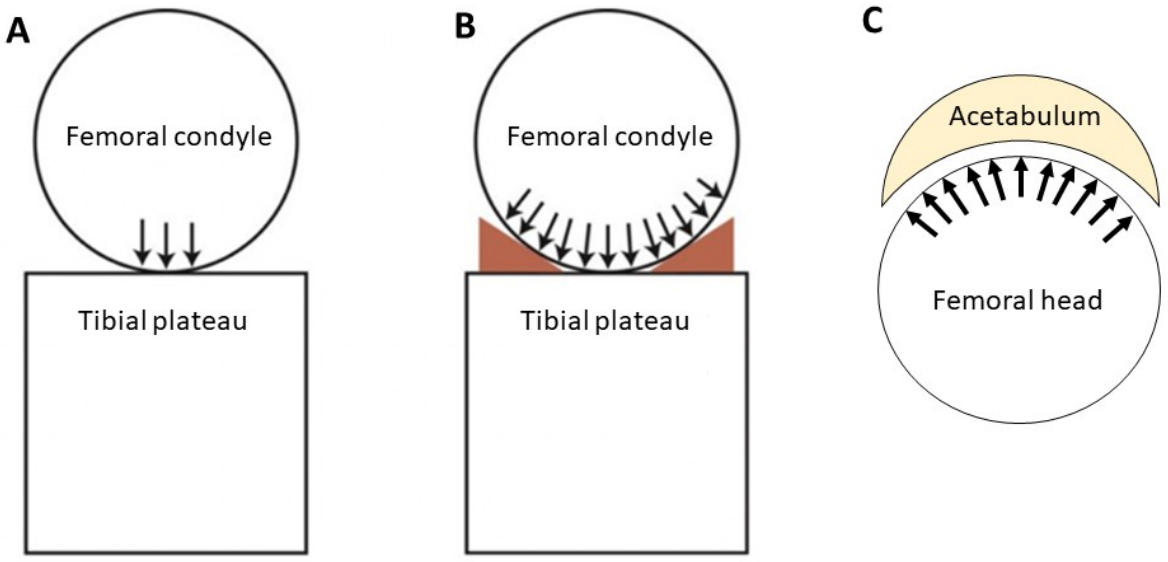


Figure 3. Illustration of conformity between the knee and hip joints. A. There is increased contact loading in an osteoarthritic knee joint where the meniscus is no longer present or has been defunctioned. B. There is a greater degree of conformity and therefore a greater dissipation of load in the knee joint where the meniscus is intact and functioning well. C. There is a greater degree of conformity in the hip joint resulting in the dissipation of load over a larger surface area.

1.6 Joint lubrication profiles

Joint lubrication can broadly be divided into boundary and fluid film lubrication(97). In boundary lubrication there is typically some surface-to-surface contact with a thin film of fluid a

few molecules thick between the two opposing articular surfaces. The lubricant surface molecules determine the associated frictional properties between the joint surfaces. It is typically the type of lubrication found in weight bearing joints at rest. However, there is decreased resistance both during steady motion and at the start-up of motion(98). In fluid film lubrication there is a thin film of fluid, which provides separation of the two opposing articular surfaces. The coefficient of friction, which is a measure of the resistance between the two opposing surfaces, is less in fluid film compared to boundary lubrication. There are many different models of fluid film lubrication (table 3).

Table 3. The mechanism of action of the different types of fluid film lubrication. HA – hyaluronic acid; SF – synovial fluid.

Type of lubrication	Mechanism of action
Hydrodynamic	A wedge of fluid is created when two non-parallel opposing forces slide against each other.
Weeping	The articular surfaces are held apart by a film of fluid, which is maintained under pressure.
Squeeze-film	As two opposing surfaces move towards each other, fluid is squeezed out of the articular cartilage creating a fluid film between the two surfaces, which can be maintained for a short time by the fluid's viscosity.
Elastohydrodynamic	The elasticity of the articular cartilage causes it to deform slightly, which taps pressurised fluid and helps maintain an adequate layer of fluid between the opposing joint surfaces.
Micro-elastohydrodynamic	Deformation of the articular cartilage causes the release of small pools of trapped SF that can then contribute to fluid film lubrication particularly at higher loads.
Boosted	Under load, much of the SF constituents are forced into the articular cartilage, increasing the HA concentration in the joint space, making it a more effective lubricant.

1.7 Infected Synovial Fluid

Infection is a problem, which can affect both the native and prosthetic joint. Septic arthritis is infection of a native joint and, if untreated, it can lead to chondrolysis with destruction of the articular cartilage secondary to the production of proteolytic enzymes. This can occur as quickly as five days from the onset of infection and long term can lead to degenerative changes in the joint. Like with other infections, it can also become systemic resulting in increased patient morbidity and a risk to life.

Periprosthetic joint infection (PJI) is one of the most devastating complications of joint arthroplasty affecting between 0.7% and 2.4% of patients undergoing joint arthroplasty(99-101). In the United States, the incidence is 1-2% and it remains the commonest indication for revision joint arthroplasty(102). Despite its low incidence, the prevalence of PJI is increasing with projections suggesting in excess of 60 to 70,000 cases in the United States by 2023. This is in part due to an increase in prevalence of diabetes mellitus, obesity and other co-morbidities in the population and the emergence of resistant infecting organisms(102).

1.7.1 Definition of periprosthetic joint infection

The first step in effectively managing PJI remains accurate and early diagnosis. In order for this to occur, there needs to be an accepted definition. In 2011, the Musculoskeletal Infection Society (MSIS) propose a "gold standard" definition, which could be universally adopted(103). Based on their criteria, PJI exists when:

1. A sinus tract exists, which communicates with the prosthesis, OR
2. A pathogen is isolated by cultures taken from two separate fluid or tissue samples obtained from the prosthetic joint, OR
3. Four out of the following six criteria are present:
    a. Raised serum Erythrocyte Sedimentation Rate (ESR>30 mm/hr) or serum C-Reactive Protein (CRP>10 mg/l) concentration
    b. Elevated synovial white blood cell (WBC) count
    c. Elevated synovial neutrophil percentage (PMN%)
    d. Purulence found in the affected joint
    e. Microorganism isolated in one culture of periprosthetic fluid or tissue, OR
    f. Greater than five neutrophils per high power field in five high power fields observed from histological analysis of periprosthetic tissue at 400 times magnification

The importance of early diagnosis is to prevent the formation of a biofilm, which can occur less than three weeks from the onset of an acute PJI. This is a complex structure comprised of sessile bacteria embedded within a multi-layered glycocalyx made up of glycolipids, polysaccharides, proteins and nucleic acid, which serves to protect the organisms from the host's immune response and is impenetrable to antibiotics(103). The phenotype of these sessile bacteria is different from their planktonic relatives due to differential gene expression, which is under the control of the "quorum sensing system". This allows the organisms to communicate within the glycocalyx(104). Extracellular DNA (eDNA) is also an important component of the biofilm. It has structural properties and is important for the initial attachment of bacteria to surfaces. It also allows for sharing of virulence factors including those responsible for antibiotic resistance and participates in the horizontal transfer of mobile genetic elements. The quorum

sensing system controls the formation of eDNA by selective lysis of bacterial subpopulations(105).

1.7.2 Markers of infection

Serological markers of infection, such as CRP and ESR, have been widely used as screening tests for PJI, due to the ease in which they can be obtained, their low cost and relatively high sensitivity. Threshold values of 1mg/dl for CRP and 30mm/hr for ESR have been widely accepted(106, 107). However, their specificity is not high, and they are often found elevated in the early postoperative period and in non-infectious inflammatory conditions. They may also be falsely low in low-grade infection secondary to biofilm-producing bacteria. Consequently, Parvizi et al questioned whether measuring the CRP of synovial fluid itself would be a more reliable method of identifying infection than serological testing, as it is closer to the source of inflammation(108). In a subsequent study, they demonstrated greater sensitivity and specificity using enzyme-linked immunosorbent assay (ELISA) (0.84 and 0.971 respectively) for quantifying synovial CRP compared with serological CRP assaying (0.76 and 0.93)(109).

Other markers of infection, such as interleukin-6 (IL-6), procalcitonin (PCT) and tumour necrosis factor alpha (TNF-α) remain under investigation as potential alternative or complementary markers of infection. IL-6 is produced by both lymphoid and non-lymphoid cells, and it has a role in the inflammatory response(110). An important advantage of IL-6 is that levels typically revert to normal 2-3 days following the index procedure(111). A recent meta-analysis demonstrated a pooled sensitivity and specificity for IL-6 of 0.72 and 0.89 in serum and 0.91 and 0.90 in synovial fluid. They concluded that although synovial fluid IL-6 has a high diagnostic value for PJI, serum IL-6 is still a useful test due to its high specificity(112).

PCT is a protein produced under physiological conditions by the parafollicular cells and neuroendocrine cells of the thyroid. Although PCT is the prehormone for calcitonin, it has distinctly different biological activities. The only recognised biological effect of calcitonin is to inhibit bone resorption, thereby reducing the serum calcium concentration(113). The main producers of PCT in sepsis are monocytes and macrophages of different organs, particularly the liver(114). PCT levels are markedly elevated as quickly as 2-4 hours following severe bacterial

infections or systemic inflammation, with levels persisting until recovery(115). Its biological half-life is approximately 24 hours giving it a distinct time point advantage compared to CRP and other acute-phase reactants(116). Despite the suggestion that a combination of CRP and IL-6 may provide the best combined sensitivity and specificity for differentiating between PJI and non-infected causes, this has yet translated into widespread clinical practice. PCT in the context of systemic bacterial infection has the greatest specificity (0.98) of any single serological test(117). However, its diagnostic value for detecting PJI remains uncertain. A recent meta-analysis, consisting of six studies, evaluated the diagnostic effectiveness of PCT in PJI. It demonstrated a pooled sensitivity and specificity of 53% and 92% respectively(118). Due to its low sensitivity, PCT is currently not a suitable biomarker for the identification of PJI.

TNF-α is a cytokine released by monocytes and macrophages, which is involved in systemic inflammation and belongs to a group of cytokines that stimulate the acute phase response, and is released in response to local infection(119). Two studies have evaluated TNF-α and demonstrated similar diagnostic parameters with low sensitivity (43% and 35%) and high specificity (94% and 86%). However, it also has a number of drawbacks including its long processing time (over two hours) and sample instability, meaning it needs to be processed within one hour of being drawn(117). Although it has yet to be demonstrated as a convincing marker in joint sepsis, it may have a role as a diagnostic marker in other forms of sepsis(120).

D-dimer is a widely available serum biomarker that is well known for its role in helping to diagnose fibrinolytic activities in thromboembolic events and has also demonstrated high sensitivity in diagnosing PJI. In an ongoing prospective cohort of 154 patients, D-Dimer has been shown to have a better sensitivity (97.7%) and specificity (93.6%) than CRP and ESR(121). However, further studies that reproduce these findings are required to confirm its superiority to conventionally used CRP and ESR.

Intercellular adhesion molecule-1 (ICAM-1) is a membrane glycoprotein with a key role in leucocyte activation and migration that has been investigated in the context of PJI. A study of 52 patients undergoing revision hip or knee surgery demonstrated significantly increased levels of ICAM-1 in PJI patients compared with noninfected patients(122). A similar study also

demonstrated increased ICAM-1 levels in PJI patients. However, they did not describe the diagnostic parameters used to confirm PJI(123). More studies are required to confirm the diagnostic importance of ICAM-1 before it can be considered a clinically relevant serum biomarker for diagnosing PJI.

1.7.3 Promising biomarkers for joint infection

The search for a single synovial fluid based diagnostic test for PJI has yielded a number of biomarkers as potential candidates, as discussed above. However, the most promising are currently Alpha-defensin (α-defensin) and leucocyte esterase.

Alpha-defensin has been claimed to be the most accurate single biomarker for PJI(118, 124). It is an antimicrobial peptide released by activated neutrophils. It then integrates and destroys the cell membrane of pathogens(125). A recent systematic review and meta-analysis performed by Wyatt et al demonstrated a pooled diagnostic sensitivity and specificity, from six studies, of 1.00 (95% confidence interval [CI] 0.82 – 1.00) and 0.96 (95% CI 0.89 – 0.99)(124). The laboratory α-defensin immunoassay (CD Diagnostics, Wynnewood, Pennsylvania) was developed from both proteomic and genomic studies, providing a qualitative result specific for synovial fluid. This has been developed into an on-table quick lateral flow test kit by Zimmer Biomet (Warsaw, Indiana). Its trade name is Synovasure both to market the laboratory immunoassay and the lateral flow test, which is used to test intra-operative synovial fluid for PJI. However, the product has been marketed using results from the laboratory-based immunoassay studies, rather than studies that have used the lateral flow test. The laboratory-based test involves centrifugation to remove cellular and particulate matter before testing, whereas the lateral flow test has a filter to remove cellular material. These methods may not be equivalent and therefore reduce the accuracy of the lateral flow test. A systematic review and meta-analysis consisting of ten studies (seven evaluating the laboratory-based immunoassay study and three looking at the lateral flow test) has been performed comparing the accuracies of these two methods(126). The pooled sensitivity and specificity of the α-defensin laboratory immunoassay was 0.953 (CI 0.87 – 0.984) and 0.965 (CI 0.943 – 0.979) respectively. Conversely, the pooled sensitivity and specificity of the lateral flow test was 0.774 (CI 0.637 – 0.87) and

0.913 (0.828 – 0.958) respectively. Therefore, the lateral flow test is less accurate than the laboratory immunoassay technique and its results must be interpreted carefully.

Leukocyte esterase is an enzyme secreted by neutrophils, which have been recruited to the cite of an infection. Since the early 1980s, a colorimetric strip test for this enzyme has been used to identify the presence of urinary tract infections(127). Parvizi et al expanded the use of this test to include PJI of the knee demonstrating a sensitivity and specificity of 81% and 100% respectively(128). More recently, Wang et al used the same colorimetric strips to test the synovial fluid of 72 patients undergoing hip or knee revision arthroplasty for aseptic loosening or PJI. They demonstrated a sensitivity and specificity of 84.2% and 97.1% respectively(129). A recent systematic review and meta-analysis of five studies demonstrated a pooled diagnostic sensitivity and specificity of 0.81 (CI 0.49 – 0.95) and 0.97 (CI 0.82 – 0.99)(124). This test will allow for improved accuracy of diagnosis and more timely management of the underlying infection. Of note, this test is also more accurate than the lateral flow test for α-defensin, based on the outcome of the meta-analysis by Suen et al outlined above(126).

1.7.4 Gold standard for diagnosing joint infection

Microbiological culture remains the current gold standard for the identification of the 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(130-132). Consequently, there has been increased awareness to optimise culture techniques to guide appropriate treatment. In order to improve the diagnostic yield and accuracy of cultures, a systematic review(133) has identified the following steps, which should be adhered to:

1. Collect a number of tissue samples (≥3) from all the affected areas
2. Incubate cultures for a minimum period of 14 days
3. Direct inoculation of synovial fluid in blood culture vials
4. Consider a broad range of bacteriological media
5. Utilise selective media to control overgrowth of certain bacteria
6. Use semi-solid media and nutrient agar to facilitate biofilm adapted phenotypes
7. Maintain strict anaerobic precautions during sample processing and transportation

1.7.5 Role of metabolic profiling in joint infection

As described above, two important biomarkers have been identified, which have been shown to be useful in the diagnosis of PJI. These are α-defensin and leukocyte esterase. However, some of the studies outlined above have shown that there is still room to develop tests with a greater sensitivity and specificity. Furthermore, although these tests are useful at identifying the presence of infection, they do not provide any information regarding the responsible organism. Consequently, there is currently an unmet need to examine the smaller molecules in SF to identify metabolites or groups of metabolites that may prove to be putative biomarkers for infection as well as potentially being organism specific.

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. Early attempts required the *a priori* selection of appropriate primers and relied on DNA amplification using polymerase chain reaction (PCR). There has been a more

recent move towards using mass spectrometry (MS) for semi-quantitative analysis of genetic elements and bacterial proteins. The Ibis T5000 Universal Biosensor based on PCR-electrospray ionisation/mass spectrometry (PCR-ESI/MS) technology is able to identify a number of different organisms in addition to bacteria, including fungi, protozoa and viruses(134). This technique is founded on the fact that each microbe produces its own unique spectrum based on its mass and charge when ionised. The microbe is then identified by comparison with an existing database. Furthermore, it is capable of detecting the bacterial genes that are responsible for antibiotic resistance (e.g. mecA for methicillin-resistant staphylococcus aureus [MRSA]) as well as having the same sensitivity for detecting biofilm cells and planktonic cells. It can detect all the bacteria within a sample in under six hours(132). A subsequent study demonstrated the ability of the Ibis T5000 in identifying a pathogen in 17 of 44 (38%) presumed aseptic failure cases in patients undergoing revision knee arthroplasty. Two of these 17 patients later developed a deep joint infection. This brings to light another potential problem, which is that some presumed aseptic joint failures may in fact be caused by low virulence organisms that are difficult to isolate(135). As the molecular techniques described above continue to be refined, they may have an important role to play in the identification of septic arthritis and PJI. However, due to the high sensitivity of these molecular techniques, care should be taken when using them as a primary detector of infection, as it may lead to an increased rate of false positive infections being identified.,Instead, they may be more useful in the identification of unknown pathogens in patients with a proven PJI(136). Palmer et al(137) looked at a cohort of 65 patients with clinical suspicion of septic arthritis (n=44) and those undergoing primary knee arthroplasty for OA (n=21). All samples were sent for routine microbiological culture and crystal analysis as well as PCR-ESI/MS to characterise the bacterial content, using the Ibis T5000 platform. They demonstrated that PCR-ESI/MS was able to identify bacteria in 50% of suspected septic arthritis cases. Furthermore, they detected bacteria in 28% (n=6/21) of patients undergoing primary arthroplasty for OA. However, the bacterial species identified in this cohort were different to the septic arthritis group and the clinical implications of this are uncertain. Conversely, routine culture identified bacteria in only 16% of the septic arthritis group and 0% of the arthroplasty group.

Nuclear magnetic resonance (NMR) spectroscopy has also been used to identify potential biomarkers for septic arthritis. Wiener et al(138) examined the synovial fluid of a cohort of 30 patients (10 with septic arthritis, 20 with osteoarthritis). Using ^{1}H magnetic resonance spectroscopy, they found a significantly greater *lactate* content in the septic arthritis cohort. However, it is unclear from the paper whether the groups were matched for age and gender. Furthermore, they included synovial fluid from three different joints (knees, hips and glenohumeral joints) and therefore the groups were not matched for this variable. These findings were confirmed in a subsequent study by Hügle et al(139). They looked at a cohort of 59 knee patients, which included 4 patients with septic arthritis. Using ^{1}H-NMR, they demonstrated a significantly greater *lactate* content in the septic arthritis cohort. However, this study was limited by only four patients with septic arthritis. Furthermore, there is no information on age, gender or other potential confounding factors, nor is the organism responsible for the underlying infection quoted in the paper. Wook et al(140) looked at a cohort of 84 patients with knee effusions. In 38 of these patients, the cause of the effusion was confirmed using clinical data, pathology reports or joint fluid analysis as follows: degenerative OA (21 patients), traumatic disease (12 patients), infectious disease (four patient) and inflammatory disease (one patient). Using ^{1}H-magnetic resonance spectroscopy, they identified three distinct lipid peaks. Although none of the lipid metabolites could significantly differentiate between the various disease states, the infection group had a large CH_2 and CH_3 lipid peak with a low CH=CH lipid peak. This may suggest different mechanisms amongst the various disease states. As with the other studies, the infection group was very small (n=4), the groups were not matched for age, gender and other confounding factors, nor were the underlying organism quoted in the paper.

1.8 Conclusion:

Synovial fluid is essential for the normal functioning of any joint, particularly weight-bearing joints such as the hip and knee. Synovial fluid consists mainly of proteins but also has small molecules, which are metabolic derivatives of these proteins as chondral tissue metabolites. These molecules can be assessed by many methods, all of which fall under the umbrella of metabolic profiling. This is a relatively novel technique used to identify the small molecule

composition of various tissues and biofluids. The methods employed are very powerful in analysing many metabolites and combinations of metabolites. They have their disadvantages particularly in signal-to-noise ratio. This technique has an important role in looking at the small molecule composition of synovial fluid. In the next chapter, we explore the basics of metabolic profiling including the various methods involved and the statistical analysis required to help interpret the large complex datasets.

2 Introduction to metabolic profiling and statistical analysis

2.1 Metabolic Profiling

Metabonomics or metabolomics has undergone fast development over the past two decades to establish itself as a resilient and well utilised technique in the analysis of biofluids or tissue. Metabonomics is defined as "the quantitative measurement of the dynamic multiparametric response of a living system to pathophysiological stimuli or genetic modification"(141). Its relationship to the -omics is demonstrated in Figure 4.

Metabolomics is the "Non-biased identification and quantification of all metabolites in a biological system"(142). These terms have occasionally been used interchangeably, leading to some confusion.

Therefore, in this thesis, the term "metabolic profiling" will be used, which is defined as "An individual's metabolic pattern that would be reflected in the constituents of their biological fluids"(143).

This technique is a top-down method of analysis, as it is looking at the metabolites, which are the end-products. Therefore, it takes into account various environmental factors, such as diet, medication, smoking, disease, etc (figure **4**). Typically, it is conducted with biofluids, the commonest of which are blood and urine. It can lead to the acquisition of a "metabolic fingerprint", which is unique to a particular biochemical perturbation, characteristic of a particular disease process or toxic stimulus amongst other things(142).

There are a number of techniques used in metabolic profiling including nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), Fourier transform infrared (IR) spectroscopy (FT-IR) and Raman spectroscopy. In this thesis, NMR spectroscopy has been used to identify all the metabolites. The principles of this technique are explained below. However, as many studies using MS have been discussed in the literature review, a briefer outline of this technique has also been provided below.

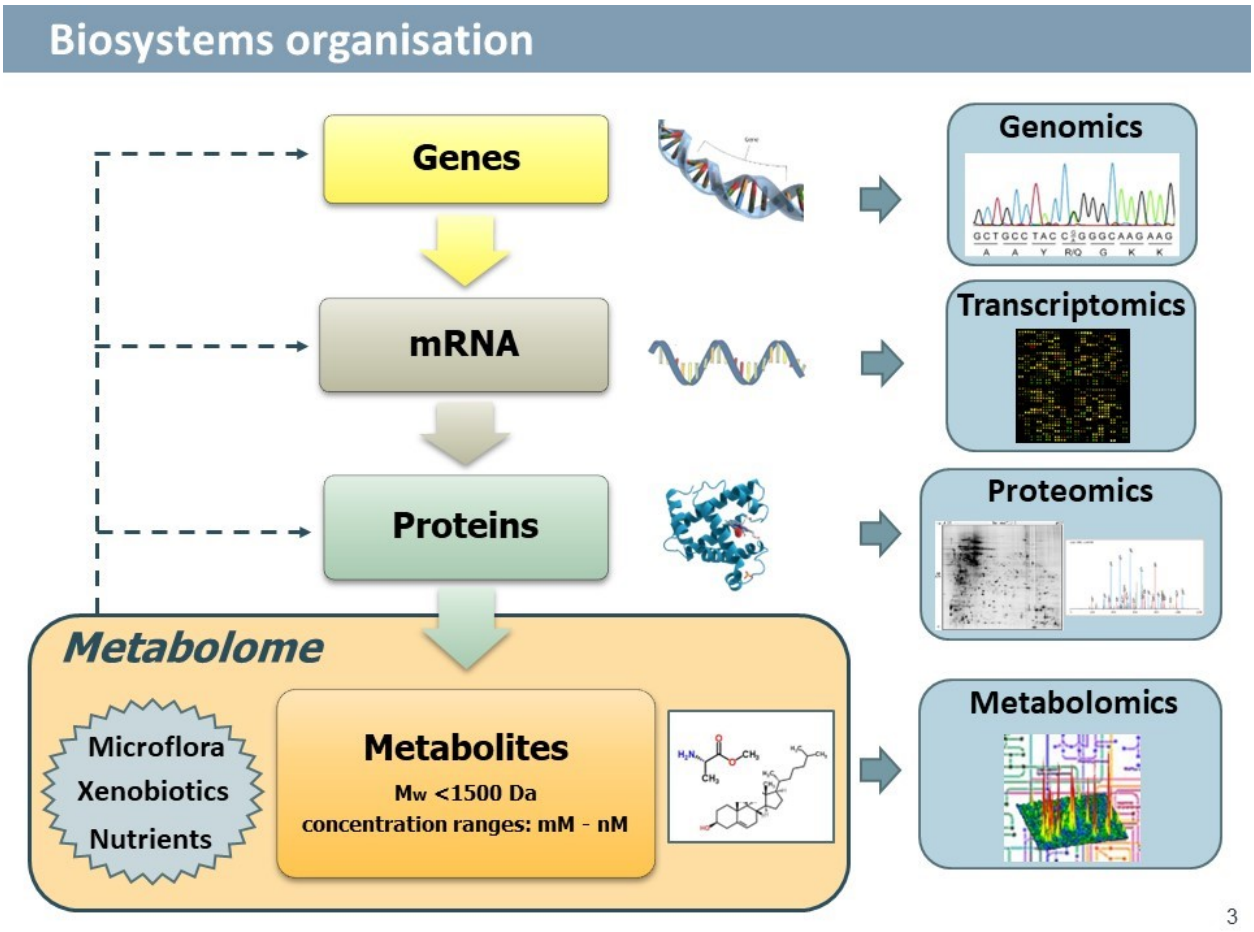


Figure 4. Biosystems organization demonstrating the role of metabolic profiling and metabolomics compared to the other "-omics". Image kindly provided by Dr Gonçalo Graça.

2.2 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a technique, whose physical principles are similar to those of magnetic resonance imaging (MRI). It utilises the magnetic properties of atomic nuclei to provide information about molecules, including their structure and dynamics. Atomic nuclei have nuclear spin (I), which may be zero, a half or a whole integer (0, ½, 1, 3/2, 2, 5/2, etc). However, only nuclei with a positive integer or half integer are NMR active, such as ^{1}H and ^{13}C. Nuclei such as ^{12}C have I=0 and are therefore not NMR active(144).

At rest, nuclei are orientated in random directions. The sample is then subjected to an external magnetic field (B_0), which causes the nuclear spins to undergo precession along an axis parallel to B_0 (Figure**5**). An excess of nuclear spins orientated with the magnetic field gives rise to the

magnetisation vector (M) (Figure **6**). A radiofrequency pulse is applied via an input coil causing a change in direction of the magnetisation (Figure **6**) and an induced voltage in a receiver radiofrequency coil(145).

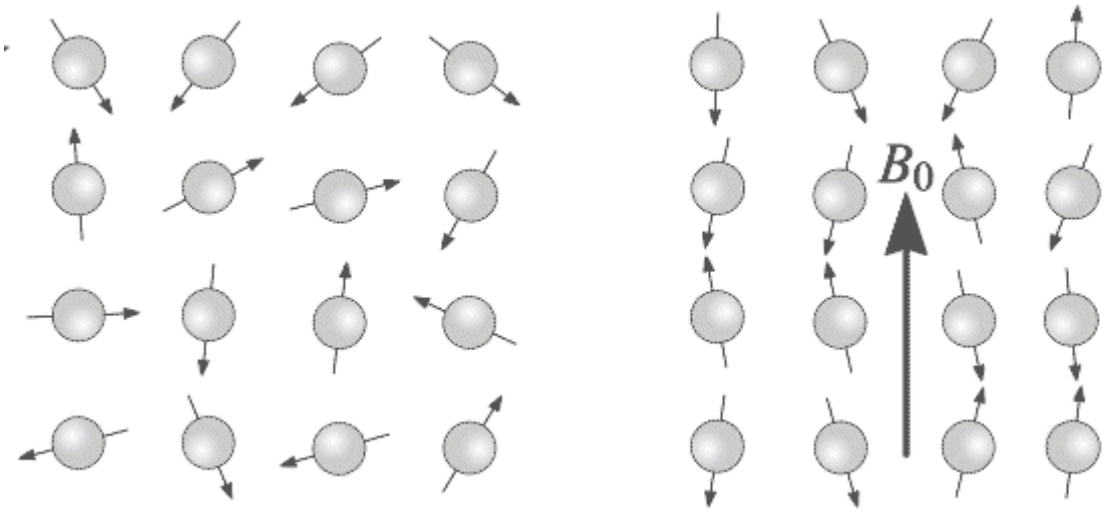


Figure 5. The effect of an external magnetic field on the alignment of nuclei. This causes the nuclei to undergo precession along an axis parallel to B_0. Adapted from an online book called "the principles of magnetic resonance imaging" http://www.pixelmeasure.io/pages/84/the-principles-of-magnetic-resonance-imaging.

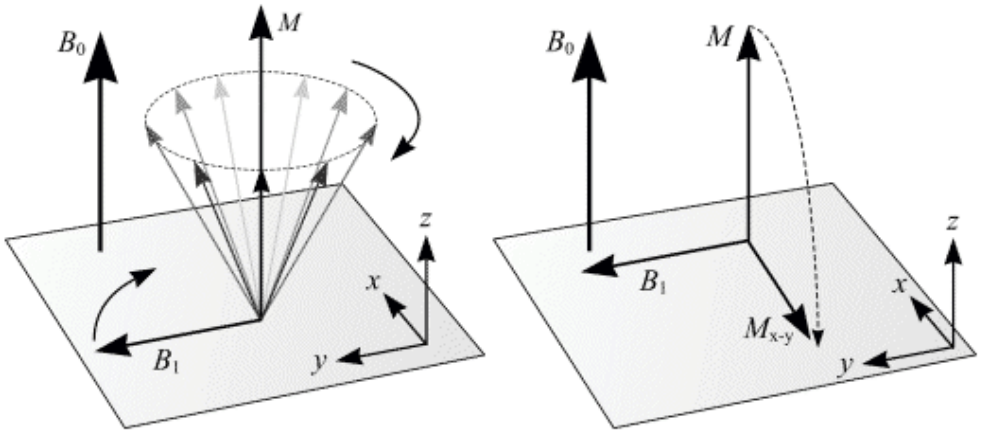


Figure 6. An excess of nuclei orientated with the external magnetic field (B_0) gives rise to the magnetisation vector (M). Adapted from Claridge (2009)(145). From an online book called "the principles of magnetic resonance imaging" http://www.pixelmeasure.io/pages/84/the-principles-of-magnetic-resonance-imaging.

Once the pulse ceases, the nuclei gradually realign themselves with B_0 and the magnetisation vector gradually returns to its original orientation. The change in magnetisation as the nuclear spins precess and return to the B_0 direction induces a current in the receiver, which is measured as a decaying oscillating signal or free induction decay (FID) (Figure 7). Providing the molecule contains an atom with at least spin ½ (e.g. ^{1}H or ^{13}C) then the FID can be converted into a spectrum of frequencies by the application of a mathematical process called Fourier transformation. The spectrum is composed of a plot of the resonance frequency of each nucleus against the intensity of the signal, which is proportional to the abundance of each nucleus. The magnitude of the radiofrequency scale depends on the B_0 field strength and so is usually presented as a relative scale and it is referenced against the resonance frequency of a standard molecule, using a unit of parts per million from the reference value. This is termed the "chemical shift" (Figure 8).

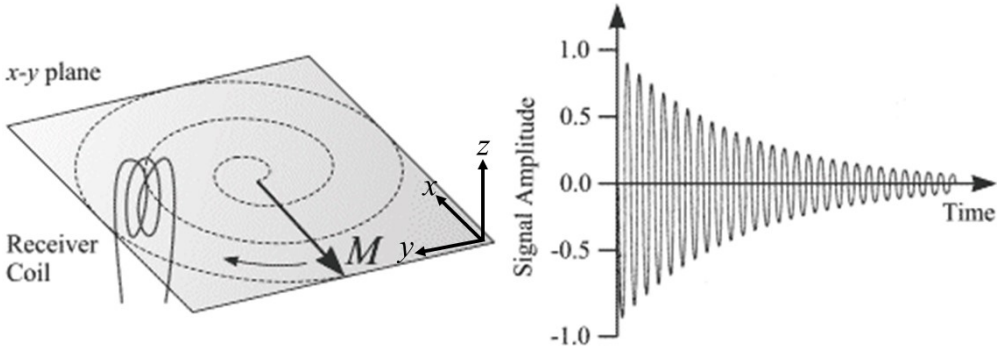


Figure 7. Following a radiofrequency pulse, the magnetisation vector (M) shifts direction from the z-axis to lie in the x-y plane, and precesses about the z-axis (left). Time domain free induction decay (FID) following the application of a radiofrequency pulse (right). Adapted from Keller, 1988(146).

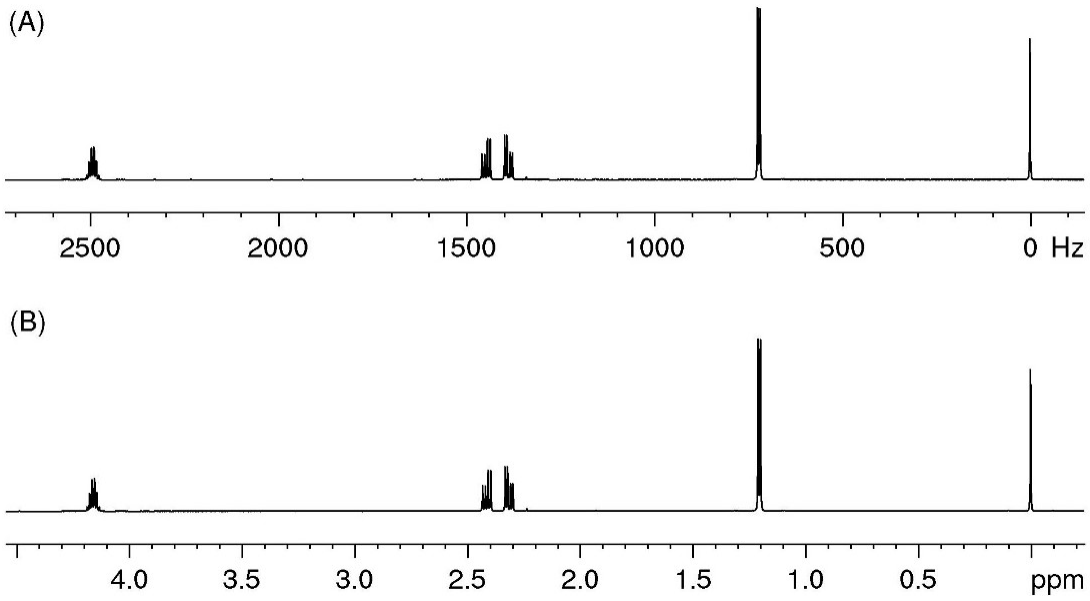


Figure 8. Illustration of the absolute and relative (chemical shift) frequency scales. The spectrum displayed is from 3-hydroxybutyrate. (A) corresponds to the absolute scale of frequencies in Hz with a field strength of 14.1 Tesla and an observation frequency of 600 MHz for ^{1}H. (B) is the same spectrum using the relative scale known as the chemical shift. The peak at 0 Hz and 0 ppm is that of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP), the internal standard. The splitting of the 3 signals from 3-hydroxybutyrate are clearly demonstrated. Hz, hertz; ppm, parts per million. Image kindly provided by Dr Gonçalo Graça.

High resolution nuclear magnetic resonance (H^1 NMR) spectroscopy is a powerful method of examining biofluids. It produces a comprehensive profile of metabolic signals without derivatization and separation(147). Its potential lies in the ability to detect hundreds of small molecules simultaneously and it is one of the commonly used techniques for metabolite identification and quantification (Figure 9). It is fast and non-destructive allowing over 100 samples to be measured in a day, and the same sample can be analysed multiple times(148). Water is typically present in large concentrations in biofluids, leading to a large peak that can obscure other molecules. Therefore, radiofrequency irradiation at the water resonance frequency is included in the pulse sequence to effectively suppress the water signal. An example of a 1D NMR spectrum of a biofluid, in this case urine, is demonstrated in Figure 10, which also illustrates the problem of overlapping peaks. The intention of this figure is to illustrate the application of ^{1}H NMR spectroscopy in the study of a complex biofluid in a broader sense.

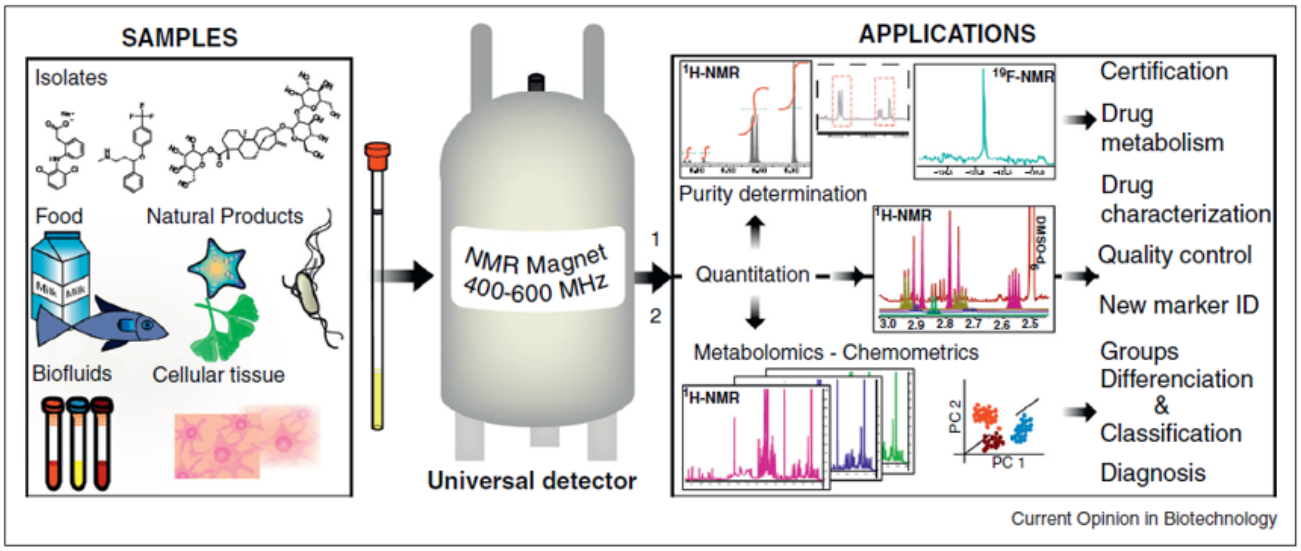


Figure 9. The role of NMR spectroscopy in metabolic profiling including its various applications. ***Image*** *reproduced with permission of the rights holder, Elsevier. From Simmler et al, Current Opinion in Biotechnology, 25, 51-59 (2014)(149).*

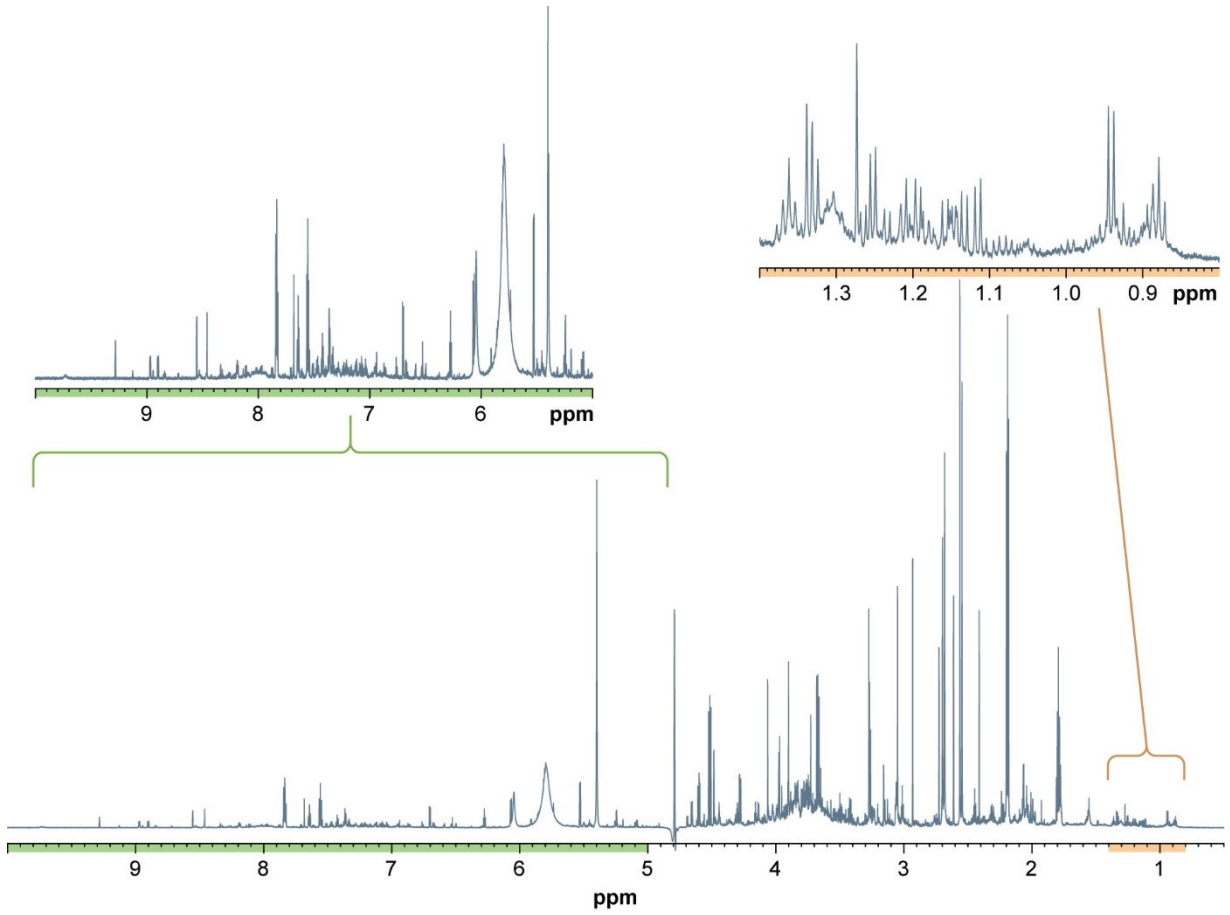


Figure 10. A 950-MHz ^{1}H NMR spectrum of human urine with expansions showing the degree of spectral complexity. Image reproduced with permission of the rights holder, Annual Reviews. From Lindon and Nicholson Annual Review of Analytical Chemistry, 1, 49-69 (2008)(148) (Annual Reviews).

In two-dimensional (2D) NMR spectra the signals are spread over two frequency dimensions to enhance spectral resolution and increase the amount of available information, helping identify biochemical substances. For proton detected NMR spectroscopy, the most commonly used 2D experiments are ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{1}H-^{1}H total correlation spectroscopy (TOCSY), which enable the identification of the signals from adjacent protons from the same molecule separated by three or more chemical bonds, respectively; and ^{1}H-^{13}C Heteronuclear Single-Quantum Correlation (HSQC) spectroscopy which enable the detection signals from protons and their directly attached ^{13}C nuclei, remembering that ^{13}C is only 1.1% naturally abundant (Figure 11).

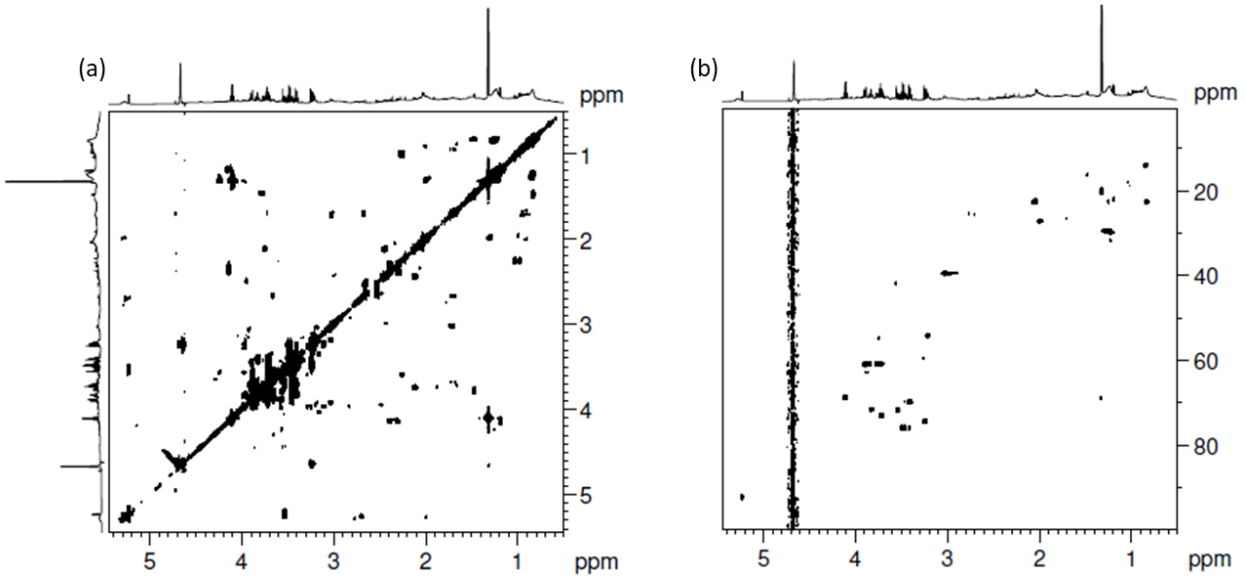


Figure 11. Two-dimensional (2D) NMR spectra of a human synovial fluid sample used for metabolite identification acquired on a 14.1 T spectrometer. (a) ^{1}H-^{1}H Total Correlation Spectrum (TOCSY); (b) ^{1}H-^{13}C Heteronuclear Single-Quantum Correlation (HSQC) spectrum. ppm – parts per million. The ^{13}C chemical shifts are on the vertical axis and the ^{1}H chemical shifts are on the horizontal axis.

2.3 Mass spectrometry

Mass spectrometry (MS) is another powerful technique used in metabolic phenotyping. Initially, it involved the detection of charged molecules whilst in their gaseous phase. Over time, new ionisation techniques were developed, which allowed direct ionisation of molecules from liquid and solid samples into the gas phase. Once ionised, the molecules are accelerated in an electric field with a high vacuum, which allows the measurement of their mass to charge ratio (m/z)(150). The advantages of MS over NMR are that it is more sensitive and requires a smaller quantity of sample material. It is able to detect compounds down to the picomolar level. Furthermore, in terms of compound concentrations, it has a greater dynamic range to allow detection(151). However, since the ions are lost after reaching the detector, the sample is consumed after a single use and it is therefore not possible to perform multiple tests on the same sample, as is the case with NMR.

The mass spectrum of a pure compound can appear very simple (Figure 12). However, when a more complex biofluid is used, the spectra can be difficult to interpret as it is often more convoluted. Aside from the presence of overlapping peaks, the more abundant ions can supress

ionisation of those ions found in smaller quantities. In order to overcome these problems, MS is typically coupled to an online compound physical separation such as liquid- or gas-chromatography. This gives rise to the techniques more commonly known as liquid- or gas-chromatography – MS (LC-MS or GC-MS respectively). LC-MS is typically used to analyse liquid and solid sample extracts, whereas GC-MS is often utilised to analyse the more volatile compounds of complex biofluids(152, 153). Ionization techniques, such as electrospray ionization (ESI), has enabled the direct ionization of molecules from the liquid into the gas phase(150). It does this by generating charged micro-particles following passing of the sample through a charged needle, thus enabling the generation of ions from liquid samples. This can be performed in positive (ESI+) or negative (ESI-) modes. Examples of MS are illustrated in Figure 12 below.

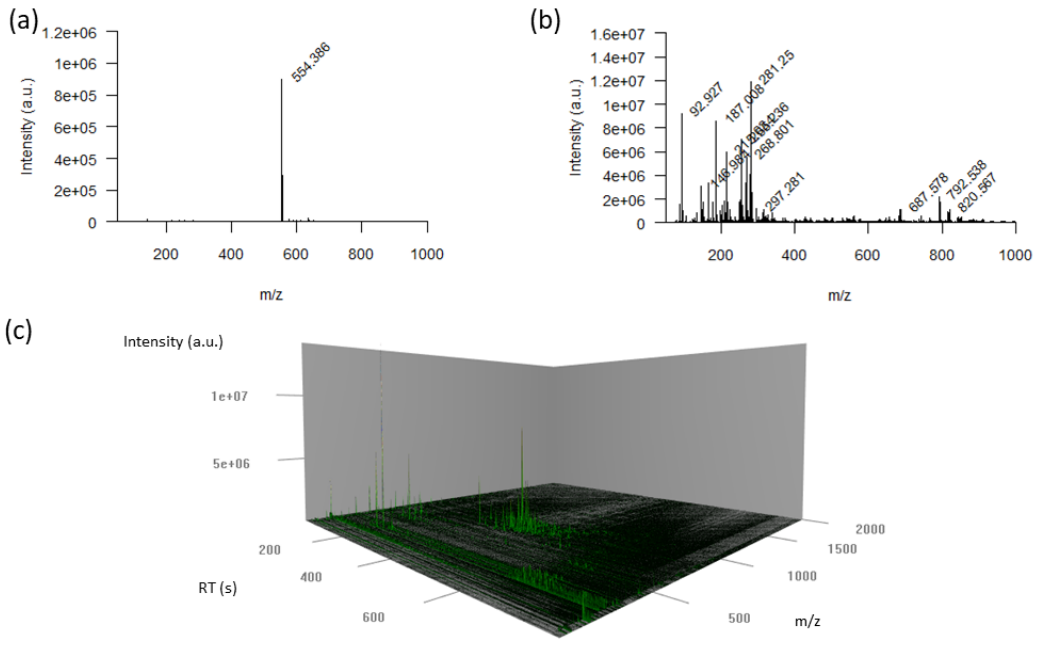


Figure 12. Examples of mass spectrometry data sets: (a) A spectrum of a peptide, Leucine enkephalin, acquired by electrospray ionization in negative ion mode (ESI–); (b) spectrum of a human blood serum lipid extract acquired in ESI–, (c) LC-MS chromatogram of human blood serum lipid extract acquired in positive ion mode (ESI+), where mass spectra is acquired continuously during chromatographic separation; ESI – electrospray ionisation; RT – retention time; m/z – mass-to-charge ratio. Image kindly provided by Dr Gonçalo Graça.

2.4 Contrasting Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry

As described above, NMR spectroscopy and MS are two complementary analytical techniques. While both techniques are used for the identification and structural characterisation of molecules, there are some key differences between them to highlight.

NMR spectroscopy is based on the magnetic properties of atomic nuclei, particularly those with a non-zero nuclear spin (e.g. 1H, 13C), while MS is based on the mass-to-charge ratio of ionized molecules. With NMR spectroscopy, the whole sample can be analysed in one measurement, whereas different LC packings and conditions are required for different classes of metabolites in MS. In other words, MS requires a more targeted approach and poor chromatographic separation can be problematic.

NMR spectroscopy typically requires a relatively large amount of sample (in the milligram to gram range) in a solvent, whilst MS can analyse much smaller amounts of sample (in the microgram to nanogram range) and can be used for the analysis of both volatile and non-volatile compounds. There is minimal sample preparation in NMR spectroscopy, which requires the addition of buffer solution, whereas sample preparation with MS may be more substantial with different LC columns and protein precipitations.

MS is generally more sensitive than NMR spectroscopy, allowing for the detection of trace amounts of analyte. However, variable ionisation and ion suppression effects may impair analyte detection in MS, which is an important limitation in its use(148). NMR spectroscopy can also cover a wide range of metabolite classes in one assay. Furthermore, analysis by MS involves destruction of the samples, thus preventing further testing of the same sample from taking place. As described above, in order to improve metabolite resolution, MS is typically coupled with prior separation of the fluid using chromatography or capillary electrophoresis, which can introduce unwanted inter-run and inter-sample variability.

Despite NMR spectroscopy having a sparser metabolite coverage and lower sensitivity than MS, it does have a number of important strengths over MS. It is widely used for small molecule struture elucidation, hence provides ease of annotation of spectral features in comparison to MS. It is a fast, non-destructive technique that is more reproducible than MS(148). NMR

spectroscopy typically has a higher spectral resolution than MS, allowing for the detection of subtle differences in molecular structure.

For these reasons, NMR spectroscopy was chosen over MS as the technique for metabolic profiling in this thesis.

2.5 Statistical analysis

The techniques involved in metabolic profiling typically lead to the formation of large and complex datasets, with hundreds or even thousands of variables. These can be difficult to summarise and interpret without appropriate tools to extract meaningful biological and biochemical information. Both univariate and multivariate techniques aim to assess group-wise differences and have important roles in identifying significant metabolic differences between groups. In this section, the various statistical techniques commonly used in metabolic profiling are discussed.

2.5.1 Univariate analysis

Univariate analysis techniques can be used to reduce a potentially large number of metabolites to only those that demonstrate the strongest response under the investigated conditions. Such techniques include Analysis of Variance (ANOVA), Mann-Whitney test and the Student's t-test, which all involve individual analysis of each variable. Examples for such techniques include a two-way ANOVA to investigate different concentrations of individual metabolites in relation to a specific medication(154) or a Wilcoxon rank-sum test combined with ANOVA to identify different disease states in an osteoarthritic joint ranging from early (mild) to late (severe) OA. However, these techniques can be impractical when looking at large metabolic datasets. They can fail to differentiate between groups if only minor differences exist on a single molecule level, even if multi-molecule combinations would differentiate them on a system level(155).

2.5.2 False discovery rate testing

The application of univariate analysis to multiple variable metabolic profiling data involves performing multiple statistical tests on the same dataset to identify metabolic differences. However, when more than one statistical test is performed on the same dataset, many statisticians and journal editors demand more stringent criteria for a result to be classed as

"statistically significant" than the conventional $p<0.05$(156). There are two main types of errors encountered with multiple statistical testing. These are referred to as type I and type II errors. If a null hypothesis is true, for example no difference is found between two treatments when no difference truly exists, there is still a chance that a significant difference ($p<0.05$) will be identified once in twenty trials. This is referred to as a type I error or α. On the other hand, a type II error or β refers to the probability of accepting the null hypothesis, when it is not true(157).

In order to reduce the risk of false positives, or type II errors, various techniques have been developed. One such technique commonly used in metabolic profiling is the false discovery rate (FDR) adjusted p-values, which can be calculated using the Benjamini-Hochberg method (158). This is performed to account for multiple testing of identified metabolic differences between groups. FDR refers to the proportion of the rejected null hypotheses, which are incorrectly rejected. In metabolic profiling, this would refer to incorrectly demonstrating particular metabolites are found in significantly different concentrations between groups, when no true difference exists.

2.5.3 Multivariate analysis

Multivariate analysis can analyse all the variables at once and also produce predictive models. The main methods employed in metabolic profiling are principal component analysis (PCA) and partial least squares (PLS)(159). These techniques can help reduce the dimensionality of complex datasets into something, which can be more easily visualised.

2.5.4 Principal component analysis

PCA allows the multivariate dataset to be represented by a lower number of variables, the principal components. It provides a summary/overview of all samples within the dataset. Furthermore, trends, clustering of data points and outliers can be visualised(160). It represents an "unsupervised" method of looking at the data. This implies there is no prior information about the sample classes for the observations being considered. For example, it is unknown which samples belong to a control or disease group. Each point on the PCA plot represents all the variables for an individual sample in lower dimensional space. The **X**-matrix represents a 2-

dimensional construct of the data. It does this by stacking each variable on top of each other. Each principal component (PC) is orthogonal to the rest. The “first principal component” (PC1) is a regression line representing the variables, which explains as much of the data variance as possible. The “second principal component” (PC2) is another regression line orthogonal to PC1, which represents the variables that incorporates the data variance not explained by PC1. Each consecutive PC explains a lower percentage of the global variation. The PC loadings represents those variables from each PC that representing the link to the original X matrix variables (148). As an example, a PCA scores plot of PC1 versus PC2 provides a two-dimensional plot that explains as much of the data variance as possible in two dimensions. This highlights the metabolic differences between the samples. The Hotelling’s T2 region is an ellipse on the scores plot and represents the 95% confidence interval of the modelled variation(161) (Figure 13). This can also help to identify outlier samples, which can be investigated and/or removed as deemed appropriate by the researcher.

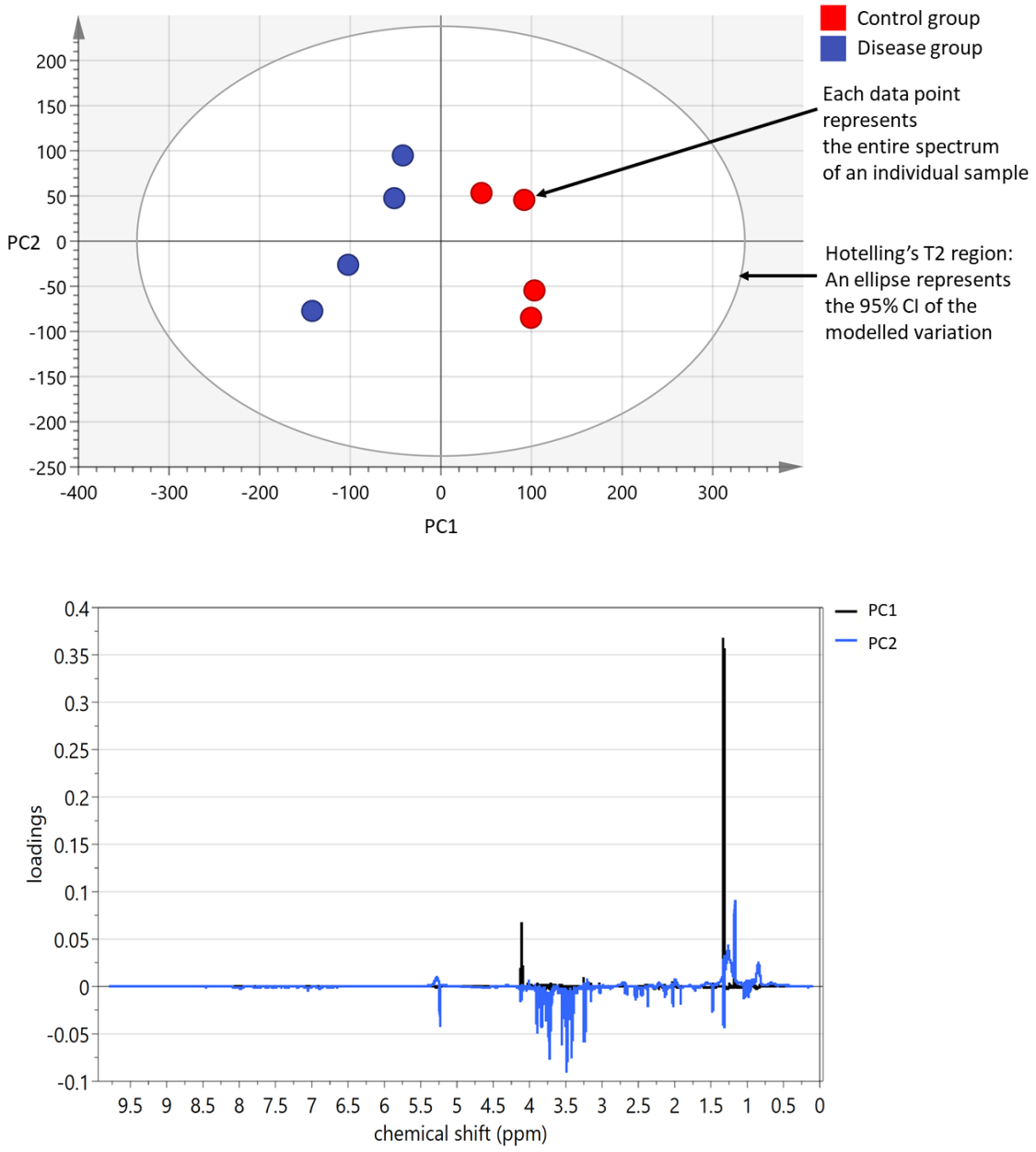


Figure 13. A) PCA scores plot of PC1 vs PC2 with each data point representing the NMR spectrum of an individual human synovial fluid sample and demonstrating separation between the control and disease groups. B) Loadings plot for PC1 and PC2 demonstrating the direction of variation of each original spectral variable in relation to the scores distribution. The percentage variation explained in figure A is 34.1% for PC1 and 17.2% for PC2. CI – confidence interval, PC – principal component.

2.5.5 Partial least squares

Following an "unsupervised" method of looking at the data, the next step would be to employ "supervised" methods. This involves assigning each sample to a particular class, such as a control or disease group, before calculating the model.

Partial least squares (PLS) is a supervised method, where a quantitative relationship between two data tables is sought. These include an **X** matrix, which represents the spectral intensity values that are independent variables from the samples, to a **Y** matrix, which is a dependent variable containing continuous quantitative values, such as BMI, or discrete values to represent different classes (159, 162). For example, the **X** matrix represents ^{1}H-NMR spectral intensities from a particular biofluid in both the control and disease group, and the **Y** matrix represents the vector of class identifiers or observations (e.g. 0 for control and 1 for disease). In this way, discriminant analysis (DA) can be combined with PLS to calculate the optimal model for class discrimination. This utilises linear regression modelling of the component axes to represent the variables. It attempts to identify the variance and therefore the metabolic differences between the two classes (e.g. control and disease groups)(159). Orthogonal signal correction (OCS) can be incorporated into PLS modelling to remove the portions of data that are not relevant and do not correlate (i.e. are orthogonal) with the Y class identifiers, or the disease trait (163). This variant of PLS is known as orthogonal partial least squares discriminant analysis (O-PLS-DA). This method can enhance the visualisation of the metabolic differences between the disease traits(164). It separates the data into the predictive components that explains the differences between the two groups and the orthogonal component that contains the remaining unrelated information (Figure 14).

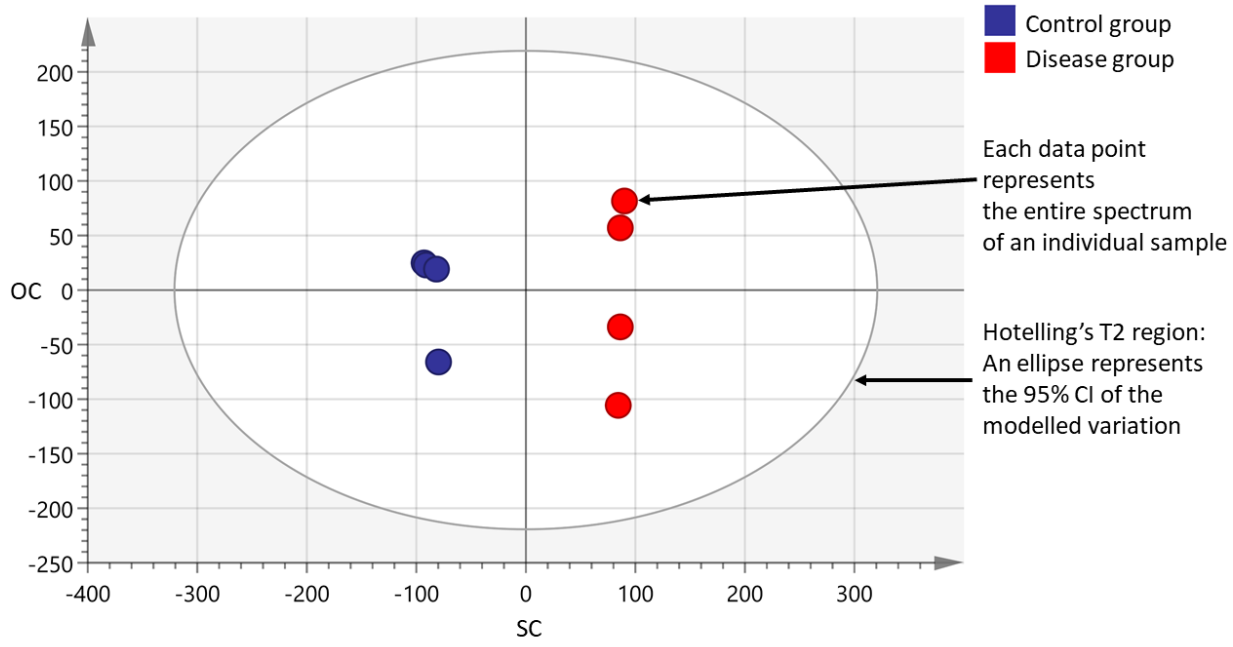


*Figure 14. Scores plot of the cross-validated O-PLS-DA model demonstrated good separation between the control and disease groups with differences in the spectra of each sample explained by whether the sample was taken from the control or disease biofluid. The **Y** matrix represents the vector of class identifiers or observations, which here would be illustrated by 0 for control and 1 for diseased biofluid. SC – separating component, OC – orthogonal component.*

2.5.6 Cross validation

Despite the rigorous statistical methods already mentioned, there is still a chance that a model may identify false positives; in other words, metabolic differences that have occurred by chance. In order to assess the model validity, cross validation should be performed. This consists of producing PLS or O-PLS models several times using the same dataset. Each time a model is calculated, a proportion of the samples is left out. The classes of left-out samples are then predicted using the obtained model. This process is repeated several times until all samples have been left out at least once. The predictions of all the models are then used to calculate the goodness of prediction of the model built on the full dataset. The goodness of prediction, or the error between the real and predicted score (Q^2Y), measures the predictability of the model. The goodness of fit (R^2Y) is the proportion of the data that explains the differences between the two groups (Y variable). Additionally, the robustness of the model can

be assessed by calculating the Q^2Y 100 times by randomly assigning the cross-validation groups (the samples predicted in each of the seven cross-validation rounds). The Q^2Y values can then be compared with Q^2Y values obtained after performing 100 permutations of the Y variable. A model is considered reliable if the Q^2Y confidence intervals of the permuted and non-permuted models do not overlap. Consequently, there is a low chance of model overfitting and false positive results. If the Q^2Y is positive and the closer it is to one, the greater the probability that the model is predictive. However, a negative or low Q^2Y suggests the model is not predictive and should be disregarded(165). A p-value can also be obtained from model permutation testing. The Q^2Y values of these permuted models are compared to the Q^2Y value of the original model with a view to validating the model if it is significantly different from the original model ($p<0.05$)(165).

2.5 Conclusion

Metabolic profiling comprises a group of relatively novel techniques, which are used to identify metabolic differences in various tissues and biofluids. It is a top-down method of analysis as it looks at the metabolites, which are the end-product. Consequently, it is useful for analysing various biofluids including SF due to its ability to identify numerous metabolites, including differing metabolite concentrations between samples and/or predetermined cohorts. It may lead to the acquisition of a unique “metabolic fingerprint”. Some of the commonest techniques employed include NMR spectroscopy and MS. Due to the complexity of the datasets, multivariate statistical analysis is required, providing a robust method to identify differences between two groups of metabolites, making it useful in the analysis of SF. Numerous studies have been performed identifying the small molecule composition of SF and suggesting potential biomarkers relating to osteoarthritis and inflammatory arthropathies. There are many metabolic profiling studies looking at human SF, but no systematic review of the literature. In the next chapter, a systematic review was performed specifically looking at metabolic profiling studies from human SF samples.

3 Literature Review

An adaptation of this chapter has been published in Bone and Joint Research in May 2020(166).

3.1 Aim and Scope of the Systematic Literature Review

The primary aim of this systematic literature review was to identify small molecule metabolites in human synovial fluid (HSF), which have been categorised by different metabolic profiling techniques, especially those including Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) Spectroscopy. The secondary aim was to identify any metabolites that may represent putative biomarkers of various orthopaedic diseases.

3.2 Method

A systematic review was undertaken in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines(167).

3.2.1 Eligibility Criteria

Inclusion criteria consisted of published articles and abstracts in English looking at the metabolites of HSF in any disease state using the techniques of MS and NMR spectroscopy. The exclusion criteria were articles not in English, patients < 18 years, review articles, expert opinions and studies using the same cohort of patients.

3.2.2 Identification of Studies

A systematic literature review was conducted of the Cochrane, Embase (Exerpta Medica Database, Amsterdam, The Netherlands), MEDLINE (Medical Literature Analysis and Retrieval System Online) and PUBMED databases without date restrictions on the 1st August 2018. The search terms used are detailed below in table 4.

Table 4. Search string for systematic review on the role of metabolic profiling in human synovial fluid.

Search term
metabo#omic*
metabolome*
metabolic adj profil*
metabolite*
biomarker*
biologic* adj marker*
exp Metabolomics/
exp Metabolome/
AND
synovia*
joint fluid*
articular fluid*
synovium
NOT
exp animals/ not humans.sh
exp child/ not (exp adult/ or exp aged/)
exp review/

3.2.3 Screening and Assessment of Eligibility

Two independent reviewers (P.A and U.K) examined the article titles identified in the preliminary literature search. If the reviewers had any disagreement about whether or not the article was relevant, it resulted in the article proceeding to the next stage of review. The abstracts of the remaining articles were then read by the same reviewers. As before, if there

was any disagreement, the articles proceeded to full-text review. Once the final articles were reviewed by the same authors, any conflict was discussed to achieve consensus.

3.2.4 Risk of Bias (Quality) Assessment

The articles were evaluated for relevance, the underlying disease process, sample numbers, quality of evidence, analytical validity, statistical power, and conclusions. The study design was evaluated using the Newcastle-Ottawa score. The relevant metabolites were highlighted, and significance was quoted when statistical analysis was available. The metabolites identified in the articles were checked and confirmed by looking at published databases including the Biological Magnetic Resonance Bank(168), the Human Metabolome Database (169) and various in-house databases.

3.3 Results

3.3.1 Literature Search

The electronic database searches identified 4477 articles. From these, 4391 articles were excluded following exclusion of any duplicates and review of the titles. The abstracts of the remaining 86 articles were reviewed and a further 14 were excluded as they contained duplicate data. Of the remaining 72 articles, 32 were excluded. Twenty-five did not meet the entry criteria, three did not involve human SF, two contained data from the same cohort and two looked specifically at synovial membranes and not SF. Of the remaining 40 articles, a further 19 were excluded. In 11, the metabolites were not clearly identified or only a portion of them were presented. In four, it was unclear from which cohort the metabolites were present in larger quantities. Two articles did not use the techniques of metabolic profiling, one had duplicate data and one investigated serum as opposed to SF. This left 21 studies (17 articles and four conference abstracts). Figure 15 summarises this process.

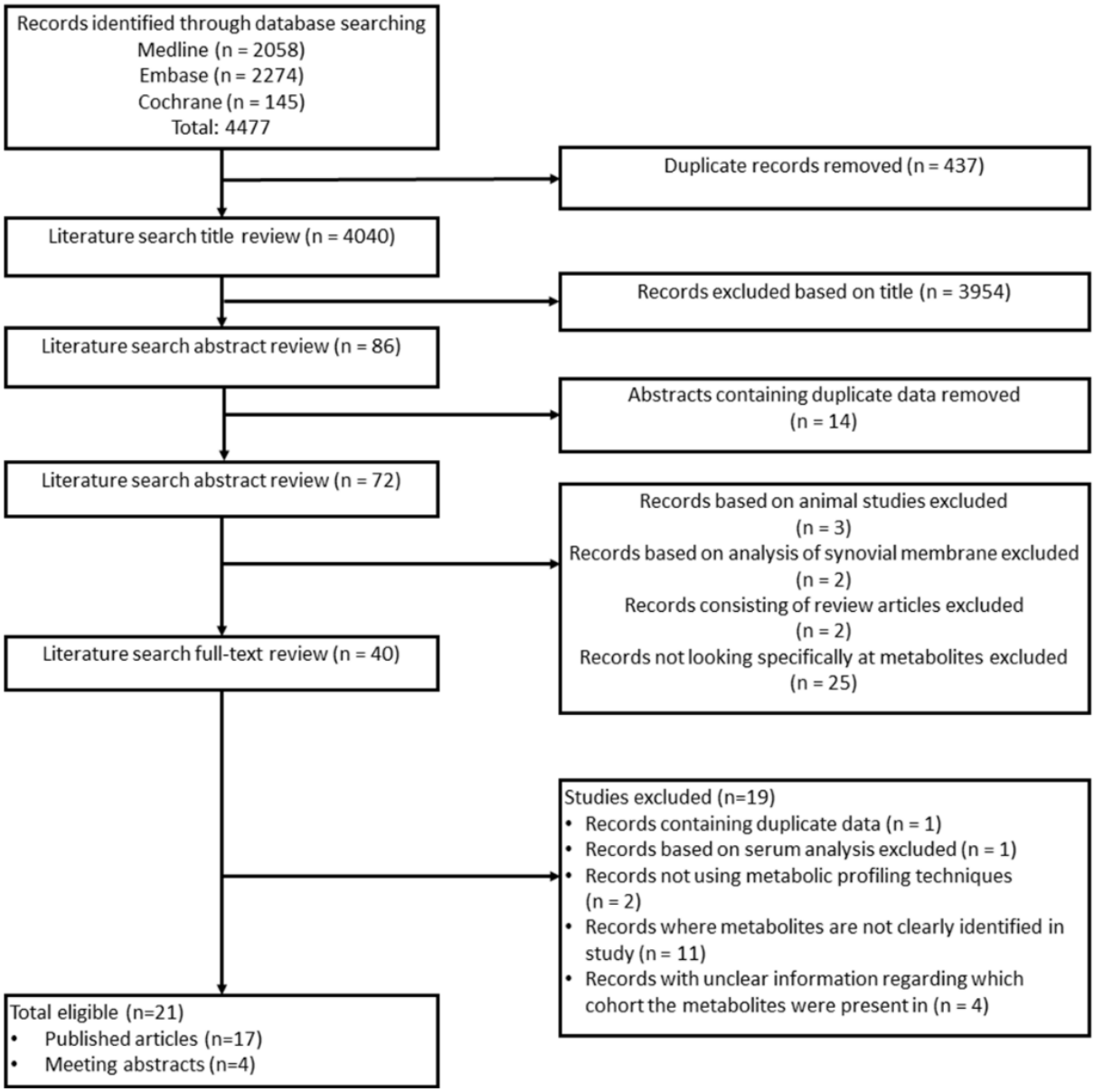


*Figure 15. Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) search and screening flowchart for the role of metabolic profiling in human synovial fluid research. *Excerpta Medica Database, Amsterdam, The Netherlands.*

3.3.2 Study Characteristics and Quality

The Cochrane criteria for bias and the Newcastle-Ottawa score were used to assess the methodology of the published studies. The designs and metabolic profiling techniques were similar in all the studies. As all studies were looking at specific disease processes, it was not possible for patient selection to be random. Furthermore, due to the nature of the studies, it

was not possible for blinding to take place at sample collection for either the researcher or the patient. The more abundant metabolites may be represented more disproportionately by multivariate statistical analysis techniques regardless of significance. Additionally, the data cannot be blinded as such supervised statistical methods require information about the sample class. There is also the possibility of reporting bias towards studies that reported p-values, as they were not consistently stated in all the articles. Furthermore, studies which assayed hundreds of metabolites may have overestimate the significance of the p-values, unless false discovery rate (FDR) or validation data sets were utilised. Table 5 lists all the identified studies. The complete list of identified metabolite can be found in appendix 2.

Table 5. Baseline description of all the studies included in this systematic review. ^{1}H-NMR, nuclear magnetic resonance spectroscopy; ACR, American College of Rheumatology; AS, ankylosing spondylitis; ASAS, Assessment of SpondyloArthritis international Society; BD, Behçet's disease; C, control group; ESOA, end-stage osteoarthritis; GC-MS, gas chromatograph-mass spectrometry; GC/TOF MS, gas chromatography/time-of-flight mass spectrometry; ISG, International Study Group; KL, Kellgren and Lawrence; LC-MS, liquid-chromatography mass spectrometry; MSU, monosodium urate; N/A, not available; NOS, Newcastle-Ottawa score; OA, osteoarthritis; RA, rheumatoid arthritis; ReA, reactive arthritis; SSA, seronegative spondyloarthropathy; TQ MS, triple quadrupole mass spectrometry; UHLC/MS/MS, ultra-high performance liquid chromatography/tandem mass spectrometry; UPLC-MS/MS, ultra-high performance liquid chromatography-tandem mass spectroscopy; UPLC-QTOF-MS, ultraperformance liquid chromatography quadruple time-of-flight mass spectrometer; UPLC-TQ-MS, ultra-high performance liquid chromatography triple quadrupole mass spectrometry; UspA, undifferentiated spondyloarthropathy.

First Author & year	Study design	Country of origin	Joint	Diagnosis	Disease staging	Sample size	Type of analysis	Validated analysis	Controls	Statistical validity	NOS
Adams et al, 2014(170)	Case control study	USA	Ankle	Radiological	Takakura grading	n=20; c=20	UHLC/MS/MS	Weak	Healthy asymptomatic patients	Adequate	7
Ahn et al, 2015 (171)	Case series	South Korea	Not stated	Clinical	N/A	n=24	GC/TOF MS	Strong	None	Adequate	3
Anderson et al, 2018 (172)	Cohort study	UK	Knee	Not stated	N/A	n=10 (OA); n=14 (RA)	1H-NMR	N/A	None	Adequate	0
Carlson et al, 2018 (173)	Case control study	USA	Not stated	Not stated	N/A	n=5 (OA); n=3 (RA); c=5	LC-MS	Weak	Post-mortem samples	Adequate	3
Chen et al, 2018 (174)	Case control study	China	Knee	Clinical & radiological	KL	n=32; c=35	UPLC-TQ-MS	Weak	Healthy asymptomatic patients	Adequate	8
Dubey et al, 2019(175)	Case series	India	Knee	Not stated	N/A	n=8	1H-NMR	N/A	None	Adequate	0
Dubey et al, 2017 (176)	Cohort study	India	Knee	Clinical	N/A	n=19(ReA); n=13 (USpA)	1H-NMR	N/A	None	Adequate	
Dubey et al, 2019 (177)	Case control study	India	Knee	Clinical	Braun's, ASAS and ACR criteria	n=52(SSA); n=29(RA); c=82	1H-NMR	Weak	Healthy asymptomatic patients	Adequate	6
Furman et al, 2017 (178)	Case control study	USA	Knee	Clinical	Not applicable	n=8; c=8	UPLC-MS/MS	N/A	Contralateral non-injured knee	Adequate	7
Hwang et al, 2013 (179)	Cohort study	South Korea	Not stated	Not stated	N/A	n=18(RA); n=11(OA)	GC/TOF MS	N/A	None	Adequate	6
Kang et al, 2015 (180)	Case series	South Korea	Knee	Clinical & radiological	KL (OA); ACR (RA)	n=10 (OA); n=10 (RA)	UPLC-QTOF-MS	Weak	None	Adequate	5
Khatib et al, 2018(181)	Case series	UK	Knee	Not stated	N/A	n=13	1H-NMR	N/A	None	Adequate	3
Kim et al, 2017(182)	Case series	South Korea	Knee	Clinical & radiological	KL	n=8 (KL1-2); n=7 (KL3-4)	GC/TOF MS	Strong	None	Adequate	4
Kim et al, 2014 (183)	Case series	South Korea	Not stated	Clinical & radiological	ACR for RA; ASAS for AS; criteria of the 1990 ISG for BD; MSU crystals in joint fluid for gout.	N=13(RA); n=7(AS); n=5(BD); n=13(gout)	GC/TOF MS	Adequate	None	Adequate	4
Leimer et al, 2017 (184)	Cohort study	USA	Ankle	Radiological	N/A	n=19; c=19	UPLC-MS/MS	Adequate	Contralateral non-injured ankle	Adequate	8
Meshitsuka et al, 1999 (185)	Case series	Japan	Knee	Clinical & radiological	ACR	n=14 (RA); n=16 (OA)	1H-NMR	Adequate	None	Adequate	2
Mickiewicz et al, 2015(186)	Cohort study	Canada	Knee	Clinical & radiological	N/A	n=55; c=13 (cadaveric – 6 bilateral & 1 unilateral sample)	1H-NMR; GC-MS	Strong	Cadaveric controls	Adequate	6
Naughton et al, 1993 (187)	Cohort study	UK	Knee	Not stated	N/A	n=22 (RA); c=6	1H-NMR	Adequate	Healthy asymptomatic patients	Adequate	5
Yang et al, 2015 (188)	Case control study	China	Knee	ACR	N/A	n=25 (RA); c=10	GC/TOF MS	Adequate	Above knee amputated patients	Adequate	6
Zhang et al, 2014 (189)	Case series	Canada	hip & Knee	ACR	ESOA	n=80	LC-MS	Adequate	None	Adequate	5
Zhang et al, 2015 (190)	Case series	Canada	Knee	Not stated	ESOA	n=69	LC-MS	Adequate	None	Adequate	5
Zhang et al, 2016 (191)	Case control study	Canada	Knee	ACR criteria & clinical judgement	ESOA	n=97	LC-MS	Adequate	No SF sample controls (only serum)	Adequate	6
Zheng et al, 2017 (192)	Cohort study	China	Knee	KL	KL2 & KL4	n=49, c=21	GC-TOF/MS & LC-MS/MS	Adequate	Asymptomatic patients	Adequate	7

In the sections below, the identified studies have been subdivided into those including healthy controls that have identified putative biomarkers and those looking at specific disease processes.

3.3.3 Studies with a healthy control group

Cytokine and metabolic differences were examined from the SF of a group of healthy and end-stage post-traumatic arthritic ankle (PTAA) joints(170). Twenty-nine metabolites were identified as being in significantly different concentrations between these two groups, with *glutamate* being the most important. They suggest an imbalance in both lipid and amino acid (AA) metabolism, amongst other factors, with the presence of a predominantly proinflammatory and oxidative environment.

Chen et al(174) investigated the metabolic changes that take place in the physiological responses of early knee OA. Twenty-two metabolites were found to be significantly different between the two groups. They suggested that OA is precipitated and accompanied by changes in AA metabolism. They based this suggestion on the fact that the majority of serum AA levels were found to be altered in the OA group compared to the healthy control group. Three potential biomarkers were identified: *γ-aminobutyric acid* (*GABA*), *alanine* and *4-hydroxy-L-proline* (*Hyp*). *Alanine* and *Hyp* were increased and *GABA* was reduced in the OA group.

The hypothesis that metabolic profiling would identify a distinctive metabolic signature of seronegative spondyloarthropathy (SSA) not affected by age and gender was explored by Dubey et al(177). They had two subgroups of healthy patients, which were stratified by age, creating a young and older control group. There were a few patient cohorts consisting of those with RA, SSA and reactive arthritis (ReA). They suggested *acetone*, *creatine*, *leucine*, *lysine*/*arginine*, *glycine*, *glucose*, *low-density lipoprotein* (*LDL*), *very low-density lipoprotein* (*VLDL*), polyunsaturated fatty acids (PUFA) and *phenylalanine* as putative biomarkers for ReA when compared to age-matched controls. Conversely, *phenylalanine*, *valine*, *leucine* and *lysine*/*arginine* were suggested as putative biomarkers for discriminating between RA and ReA.

Furman et al(178) published an abstract looking at any metabolic pathways affected by knee injury and identifying any discriminatory SF biomarkers between this cohort and a cohort of healthy knees. They demonstrated significantly increased *2-hydroxy-fatty acids* and

sphingomyelin (*SPM*) in the injury group. The authors then suggested these may be putative SF biomarkers of knee injury and possible prognostic indicators of the risk of post-traumatic arthritis.

The global metabolic profile of SF after intra-articular ankle fractures was described by Leimer et al(184). Sixteen lipid-based metabolites were identified in significantly greater concentrations following an intra-articular ankle fracture, which subsequently decreased six months post-surgery. The majority of PUFAs and long chain fatty acids (*FA*) were acutely increased in the fractured ankles at baseline compared to the control group. However, the authors did not suggest these as putative biomarkers. They did however suggest the distinctive lipid signature identified is reflective of fracture, injury and early changes associated with OA.

Naughton et al(187) qualitatively assessed the metabolic status of normal and RA SF. Increased levels of *LDL* in RA compared to the control group were identified and this was suggested to be secondary to increased synovial membrane permeability and inflammation. However, it is important to note the controls were younger (25-42 years old) and hence not age-matched compared to the RA group (40-67 years old)). As it is known from other studies that metabolic profiles can depend on age(193), this may have implications for the validity of the conclusions.

Zheng et al explored the metabolites of OA(192). They identified 6 metabolites as significantly different between the two groups. Three were found in significantly increased concentrations and three in significantly lower concentrations in the OA group. *Threonine*, *gluconic lactone* and *1,5-Anhydroglucitol* (*1,5-AG*) were in significantly increased concentrations in OA SF compared to the control group. *Tyramine*, *glutamine* and *8-aminocaprylic acid* were in significantly lower concentrations in OA SF compared to the control group. They concluded that a new diagnostic model, which combines two metabolites offers a more sensitive method of diagnosing OA than a single metabolite alone. Furthermore, as *gluconic lactone* was found in significantly different concentrations between OA and RA, it may prove to be a novel benchmark for the differential diagnosis of these conditions with a high level of sensitivity and specificity between them. Unfortunately, none of the aforementioned studies performed any analysis to account for multiple testing of the data. This may affect the validity of the results, which must be viewed

with caution. The metabolites identified in this section, which have been proposed to serve as putative biomarkers are listed in Table 6.

Table 6. Putative biomarkers identified from studies with an asymptomatic control group. All metabolites were identified from human knee synovial fluid. OA – osteoarthritis; RA – rheumatoid arthritis; ReA – reactive arthritis; FC – fold change; variable importance on projection score (VIP); N/A – not available; FDR – false discovery rate; Hyp – 4-hydroxy-L-proline; 1-5 AG – 1,5-Anhydroglucitol; GABA - ɣ-aminobutyric acid; VLDL – very low-density lipoprotein; LDL – low-density lipoprotein; PUFA – polyunsaturated fatty acids; SPM – sphingomyelin.

Underlying pathology	Metabolite	Change	Multivariate analysis
OA	*Alanine*(174)	Increased in OA	VIP 3.31, p<0.001
	Hyp(174)		VIP 1.75, p<0.001
	Gluconic lactone(192)		FC 1.54, p<0.05
	Threonine(192)		FC 2.71, p<0.05
	1,5-AG(192)		FC 1.67, p<0.05
	GABA(174)	Decreased in OA	VIP 2.61, p<0.001
	Glutamine(192)		FC 0.28, p<0.05
	Tyramine(192)		FC 0.30, p<0.05
	8-Aminocaprylic acid(192)		FC 0.27, p<0.05
Inflammatory arthropathies	*Acetone*(177)	Increased in ReA	FC 1.54, p<0.006
	Creatine(177)		FC 0.63, p<0.001
	VLDL(177)		N/A
	Glucose(177)		FC 1.12, p<0.367
	Glycine(177)		FC 1.03, p<0.02
	LDL(177)		N/A
	Leucine(177)		FC 0.83, p<0.051
	Lysine/arginine(177)		FC 0.78/1.21, p<0.002/p<0.46
	Phenylalanine(177)		FC 1.33, p<0.122
	PUFA(177)		N/A
	Leucine(177)	Increased in ReA vs RA	FC 1.88, p < 0.001
	Lysine/arginine(177)		FC 1.46/2.07, p < 0.005/ p<0.001
	Phenylalanine(177)		FC 2.56, p < 0.001
	Valine(177)		FC 1.57, p < 0.001
RA	*LDL*(187)	Increased in RA	No value
Knee injury	*SPM*(178)	Increased in knee trauma	p<0.0065 following FDR
	2-hydroxy-fatty acids(178)		p<0.0065 following FDR

3.3.4 Osteoarthritis studies

In a study looking at OA SF, Mickiewicz et al(186) identified two metabolites found in significantly greater concentrations in OA SF (*citrate* and *fructose*) and nine metabolites (*creatine*, *ethanol*, *ethanolamine*, *3-hydroxybutyrate*, *malate*, *methionine*, *O-acetylcarnitine*,

hexanoylcarnitine and *N-phenylacetylglycine*) found in lower concentrations in OA SF compared to a cadaveric control group.

The metabolite differences between early and late-stage OA were characterised by Kim et al(182). Twenty-eight metabolites were found to be significantly different between the groups, with all of these metabolites significantly increased in late-stage OA.

Zhang et al(189) looked at various metabolic markers in SF that could be used to classify patients with OA into specific subgroups and. They described many metabolites including *hexoses* (>90% was *glucose*), nine biogenic *amines*, 11 *sphingolipids*, 20 AAs, 40 *acylcarnitines*, *carnitine* and 87 *glycerophospholipids*. Following multivariate statistical analysis, they found two main OA subgroups (A and B), which differed in fat metabolism and levels of *acylcarnitine*. Group B was further subdivided into B1 and B2 based on observed differences in concentrations of *sphingomyelins* and *glycophospholipids*. However, the lack of age matched controls and correlation to clinical factors made it difficult to draw definitive conclusions.

The same authors then looked at differences between OA and type II diabetes mellitus, in a study using similar methodology. They found that *phosphatidylcholine* (*PC*) and *leucine* metabolism were influenced by both OA and diabetes mellitus. *PC* is involved in many membrane-related processes including signal transduction, regulation of membrane trafficking and forming the essential lipid bilayer of all biological membranes.

In an abstract, Khatib et al(181) looked at a group of patients with ACL deficient knees. They investigated whether mechanical loading of the joint during pivot shift revealed a profile of mechanically regulated metabolic biomarkers. Using ^{1}H-NMR spectroscopy, they found significant differences in *alanine* and *choline* between pre- and post-pivot shift testing of these ACL deficient knees. When accounting for multiple testing, these metabolites remained significant and the authors suggest they might be useful for targeting early rehabilitation or surgical intervention in patients with knee injuries who may have an increased risk of developing post-traumatic OA.

3.3.5 Inflammatory arthropathy studies

Yang et al recently analysed the metabolic profile of knee SF from RA patients and a control group to look for RA-related biochemical abnormalities(188). The control group consisted of

patients who had a “high-level” amputation. However, the reason for the amputation was not stated in the paper nor when the sample was taken in relation to the timing of the amputation, which may have critical metabolic consequences. Thirteen metabolites remained significantly different between the two groups following multivariate analysis and using a variable projection of importance score (VIP) >1 plus $p<0.05$. *Lactate* was increased and levels of *glucose*, *glucose-1-phosphate* and *D-mannose* were decreased in RA SF.

Ahn et al(171) conducted a study looking at the metabolic profile of SF in patients with Behcet’s disease (BD) with arthritis compared to those with seronegative arthritis (SNA). Eleven metabolites were identified as being significantly increased in BD with arthritis compared to SNA. These include citramalate, *glutamate*, branched chain AAs (BCAA: valine, *leucine*, and *isoleucine*), and *methionine sulfoxide*.

In an earlier study, Kim et al(183) evaluated putative biomarkers for RA. The underlying diagnosis of the patients included were BD (n=5), Ankylosing Spondylitis (AS) (n=7), RA (n=13) and gout (n=13). These patients were pooled into two larger groups, which were RA and non-RA. Following robust statistical analysis, 20 metabolites were identified as remaining significantly different between the two groups, which they proposed could be putative biomarkers. A total of 14 metabolites were found in significantly greater concentrations in the RA group and six were in greater concentrations in the non-RA group.

3.3.6 Osteoarthritis vs Rheumatoid arthritis studies

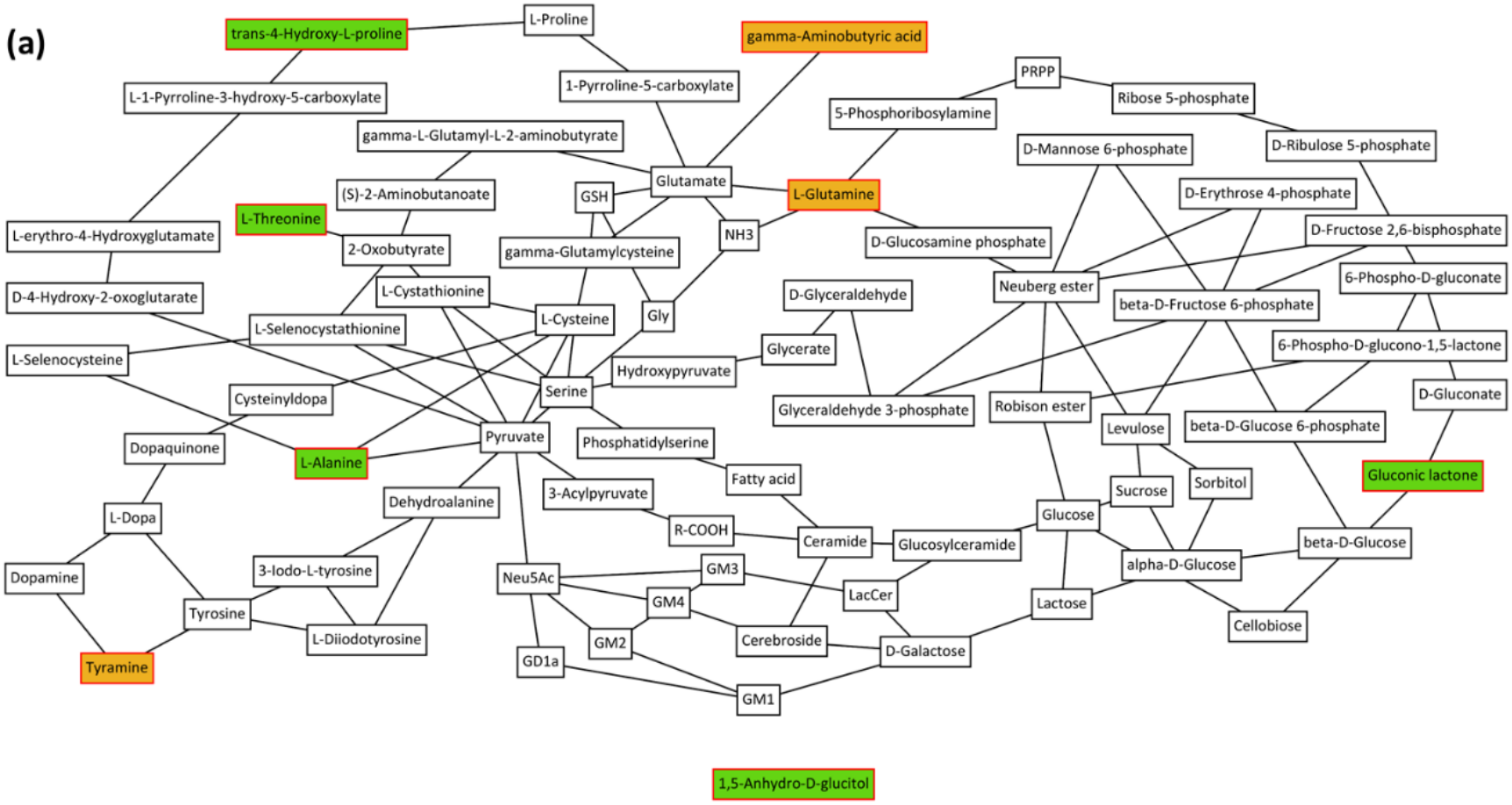
Using global liquid chromatography coupled to mass spectrometry (LC-MS), Carlson et al(173) sought to quantify biomarkers within SF. There were five purchased post-mortem samples in their control group. However, the paper does not state how long after death these samples were harvested and this time interval may have crucial metabolic consequences, making it a major confounding factor. They identified one metabolite (*L-citrulline*) that was found in greater concentrations in OA compared to RA plus controls and five metabolites (*D-lactic acid methyl ester*, *L-methionine*, *L-isoleucine*, *citric acid* and *hydroxyl-L-proline*) found in significantly lower concentrations in OA and RA SF, compared to controls. The authors also performed FDR analysis to account for multiple testing.

Anderson et al recently published an abstract exploring the role of NMR spectroscopy in producing analysable spectra from a low volume of SF taken in a clinical environment(172). Significantly different concentrations of 11 metabolites were identified between OA and RA SF. Seven of these metabolites were more abundant in OA and six were more abundant in RA SF. Their analysis suggested the metabolic pathways most impacted were *taurine* and *hypotaurine* metabolism, *glycine*, *serine* and *threonine* metabolism, *leucine* and *isoleucine* biosynthesis, nitrogen metabolism and aminoacyl-tRNA biosynthesis. Despite the authors suggesting their method could be useful for analysing low volume SF, they state in their methodology that each sample consists of approximately 100mL, a quantity more than sufficient for standard NMR spectroscopy and vastly more than needed for MS. However, this is likely a typographical error.
In a different abstract, Hwang et al attempted to identify metabolites that differentiated between OA and RA SF(179). Using gas chromatography/time-of-flight mass spectrometry (GC/TOF MS) and following multivariate analysis, 17 metabolites were found in significantly different concentrations between the two groups. Eleven were upregulated in OA (*alanine*, *asparagine*, *citrulline*, *hydroxylamine*, *glycerol*, *glutamate*, *glyceric acid*, *glycerol*, *tyrosine*, *tryptophan* and *valine*) and six were upregulated in RA (*lignoceric acid*, *maltose*, *mannitol*, *uracil*, *phosphoric acid* and *pyrophosphate*).
Twenty-one metabolites were found in significantly different concentrations between RA and OA SF in a study by Kang et al(180) . Lipid metabolites were typically found in greater concentrations in RA than OA SF, which has previously been demonstrated(194). *Tryptophan* metabolites also differed significantly in concentration between the two groups.
The ratios of *alanine* and *lactate* have also been shown to be significantly greater in RA than in OA SF(185).

3.4 Discussion

Metabolic profiling provides relatively novel and useful techniques of identifying patients with various pathologies and separating them from healthy individuals in the clinical setting. In this systematic review, various metabolites have been identified in different pathologies. Some of these metabolites have also been suggested as putative biomarkers associated with specific pathologies. Furthermore, some of the studies have shown the important role of lipid

mediators and metabolism in both OA and RA. However, its role in inflammatory arthropathies, including RA, is more significant. Furthermore, this systematic review has summarised some of the putative biomarkers identified in the published literature, although further research is required to determine their significance (Table 6). Focusing specifically on these metabolites, this review illustrates how metabolic changes may be interlinked in both OA and inflammatory arthropathies, whilst suggesting the potential metabolic pathways, which may be affected. This is illustrated in Figure 16 which shows a network of how the putative metabolic biomarkers are linked mechanistically, produced using the software Metabonetworks(195).


(a)
trans-4-Hydroxy-L-proline
L-Proline
gamma-Aminobutyric acid
L-1-Pyrroline-3-hydroxy-5-carboxylate
1-Pyrroline-5-carboxylate
PRPP
Ribose 5-phosphate
5-Phosphoribosylamine
D-Mannose 6-phosphate
D-Ribulose 5-phosphate
gamma-L-Glutamyl-L-2-aminobutyrate
Glutamate
L-Glutamine
(S)-2-Aminobutanoate
GSH
D-Erythrose 4-phosphate
L-Threonine
NH3
D-Fructose 2,6-bisphosphate
L-erythro-4-Hydroxyglutamate
2-Oxobutyrate
gamma-Glutamylcysteine
D-Glucosamine phosphate
6-Phospho-D-gluconate
D-4-Hydroxy-2-oxoglutarate
L-Cystathionine
D-Glyceraldehyde
Neuberg ester
beta-D-Fructose 6-phosphate
L-Selenocystathionine
L-Cysteine
Gly
6-Phospho-D-glucono-1,5-lactone
L-Selenocysteine
Glycerate
Hydroxypyruvate
Serine
D-Gluconate
Cysteinyldopa
Glyceraldehyde 3-phosphate
Robison ester
Levulose
beta-D-Glucose 6-phosphate
Dopaquinone
Pyruvate
Phosphatidylserine
L-Alanine
Fatty acid
Gluconic lactone
Sucrose
Sorbitol
L-Dopa
Dehydroalanine
3-Acylpyruvate
Glucose
R-COOH
Ceramide
Glucosylceramide
beta-D-Glucose
Dopamine
3-Iodo-L-tyrosine
Neu5Ac
GM3
alpha-D-Glucose
LacCer
GM4
Lactose
Tyrosine
Cellobiose
L-Diiodotyrosine
GM2
Cerebroside
D-Galactose
Tyramine
GD1a
GM1
1,5-Anhydro-D-glucitol

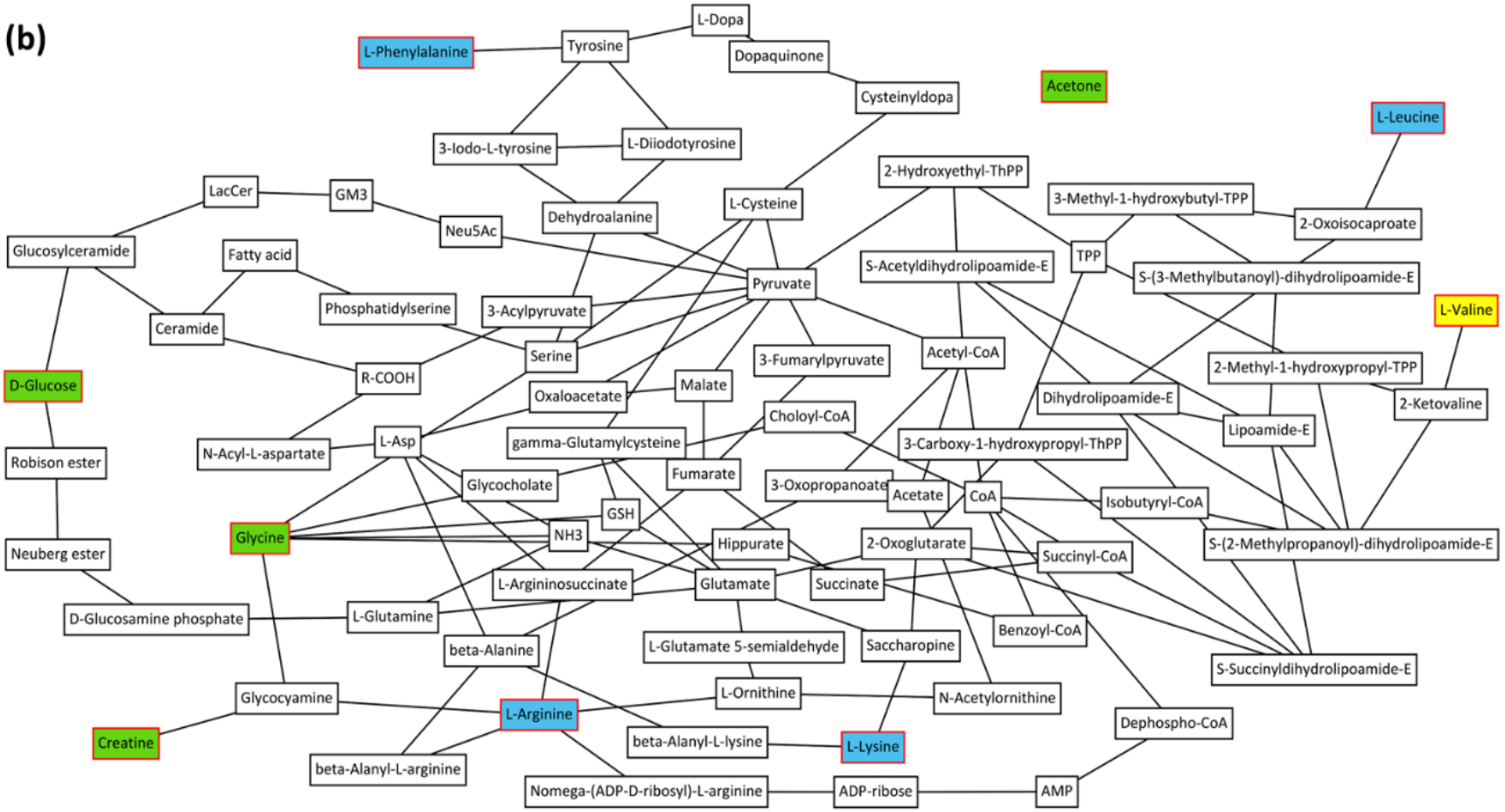


Figure 16. Metabolic network analysis of all the putative biomarkers identified in this systematic review demonstrating the associated metabolic pathways. All metabolites with a red outline were putative biomarkers. ***a****. Putative biomarkers identified in osteoarthritic synovial fluid. Those in green were raised and those in orange were reduced in osteoarthritic synovial fluid compared to an asymptomatic control group.* ***b****. Putative biomarkers identified in inflammatory arthropathies. Those in green and blue were raised in reactive arthritis compared to an asymptomatic control group; those in blue were also raised in reactive arthritis compared to rheumatoid arthritis; valine (in yellow) was raised in reactive arthritis compared to rheumatoid arthritis. GSH, reduced glutathione; Gly, glycine; Neu5Ac, N-Acetylneuraminic acid; GD1a, N-Acetylneuraminyl-D-galactosyl-N-acetyl-D-galactosaminyl-(N-acetylneuraminyl)-D-galactosyl-D-glucosylceramide; GM1, D-Galactosyl-N-acetyl-D-galactosaminyl-(N-acetylneuraminyl)-D-galactosyl-D-glucosylceramide; GM2, N-Acetyl-D-galactosaminyl-(N-acetylneuraminyl)-D-galactosyl-D-glucosylceramide; GM3, (N-Acetylneuraminyl)-D-galactosyl-D-glucosylceramide; GM4, N-Acetylneuraminyl-galactosylceramide; NH3, Ammonia; R-COOH, Carboxylic acid; LacCer, Lactosylceramide; PRPP, 5-Phosphoribosyl 1-pyrophosphate; L-Asp, L-Aspartic acid; CoA, Coenzyme A; TPP, Thiamin pyrophosphate; ADP, Adenosine 5'-diphosphate; AMP, Adenosine 5'-monophosphate; ThPP, Thiamin pyrophosphate.*

Inflammation and stress, which are often present in the disease state, can lead to metabolic abnormalities within a joint resulting in an anaerobic state. However, when contemplating the importance of diagnostic or prognostic putative biomarkers, it is important to consider whether these changes differ between the normal and diseased joints.

3.4.1 Role of the identified putative biomarkers

The identified putative biomarkers in OA can broadly be classified into two main groups: AAs plus related metabolites (*tyramine*, *threonine*, *GABA*, *glutamate*, *Hyp*, *alanine*) and sugars plus related metabolites (*1,5-AG*, *gluconic lactone*). *Hyp*, *alanine* and *threonine* are found in articular cartilage(196). Increased concentrations in osteoarthritic SF may be associated with greater catabolism of the articular cartilage. This could also represent an increase in energy consumption secondary to the increased bone turnover and subchondral sclerosis seen in OA. *1,5-AG* is a *monosaccharide* occasionally used as a short-term marker of glycaemia(197). A reduction in the *glucose* concentration of osteoarthritic SF(198) is consistent with elevation of *1,5-AG*, secondary to increased energy expenditure. Increased levels of reactive oxygen species (ROS) can result in auto-oxidation, which may be responsible for raised concentrations of *gluconic lactone* in osteoarthritic SF. Through the activation of matrix metalloproteinases (MMP), the cleaving of *aggrecan* plus collagen, ROS can directly induce cartilage degradation. Both *GABA* and *glutamate* have been suggested to have a role in increasing energy expenditure within diseased joints. *GABA* arises from *glutamic acid*(199), which is a *glucose* regulator, suggesting increased energy consumption due to less residual *glucose* in the diseased joint. *Glutamine* has a role in oxidative metabolism, with reduced levels suggesting an alteration in oxidative metabolism within the diseased joints secondary to an increase in energy expenditure(200). *Glutamine* is known to supress inflammatory cytokines(201) and also protect chondrocytes from heat stress and nitrous oxide (NO) induced apoptosis(202). As a consequence of these effects, chondrocytes may be protected from various types of stress and prevented from progressive cartilage degeneration in OA. The AA *tyrosine* give rise to *tyramine*, which has been suggested to have a role in promoting osteophyte formation. Increased levels have been demonstrated in subchondral bone(203, 204).

There are four main categories for the putative biomarkers increased in inflammatory arthropathies: AAs and related metabolites (*arginine*, *phenylalanine*, *creatine*, *glycine*, *valine*, *leucine*, *lysine*), ketone bodies (*acetone*), lipids and lipoproteins (PUFA, *VLDL*, *LDL*) and sugars (*glucose*). The identified AAs are all constituents of articular cartilage with *glycine*, *glutamic acid*, *proline* and *leucine* specifically being constituents of proteoglycans(196). The increased concentration of these metabolites suggests breakdown of the articular cartilage, which is likely related to the underlying inflammatory process. Low concentrations of *FAs* have been demonstrated in human SF. The increased levels of PUFA, *VLDL* and *LDL* are a consequence of increased inflammation and synovial membrane permeability associated with underlying inflammatory arthropathies(187).

3.4.2 Metabolic changes seen in osteoarthritis

The upregulation of *glucose phosphate* isomerase has been demonstrated to be a consequence of hypoxia, which catalyses the conversion of *glucose-6-phosphate* (*G6P*) into *fructose-6-phosphate* (*F6P*) in inflammatory arthritis(205). For this reason, a hypoxic condition within an inflamed and diseased joint has been suggested by an increased concentration on *fructose* within the joint itself. Lower concentrations of *N-phenylacetylglycine*, hexanoylcarnitine, *O-acetylcarnitine* and *ethanolamine* indicate protracted lipid and *FA* metabolism in SF of OA patients compared to controls(169). Decreased *methionine* concentrations indicate its use, where it is likely converted to *S-adenosylmethionine* (*SAM*), which is a proposed factor for inflammatory reduction and cartilage damage repair.

In a study by Kim et al(206), three unique pathways were identified, which corresponded to the metabolic differences they discovered. These were the *Tricarboxylic Acid* (*TCA*) cycle, *glycolipid* metabolism and *FA* metabolism. The authors suggested that an increasing degree of severity in OA may be associated with these pathways. Various *FA* and *glycerol* concentrations were more prominent in the late-stage OA group. Their findings suggested energy generation in late-stage OA is predominantly a result of *FA* biosynthesis. Furthermore, increased concentrations of *malate* in the early versus late-stage OA groups suggested a possible difference in the energy level between the two groups.

Furthermore, the development of OA has been shown to be associated with alterations in the composition and concentration of *phospholipids* covering articular cartilage(207).

3.4.3 Metabolic changes seen in inflammatory arthropathies

Lactic acid was increased and *glucose* decreased in RA SF(188). Levels of *D-mannose* and *glucose-1-phosphate* were also decreased. The increased energy demands caused by inflammation in RA may be responsible for these observed decreases. Furthermore, increased *lactic acid* production may be caused by the increased consumption of *glucose*. Levels of *citric acid* were decreased in RA SF. This is an important component of the *TCA* cycle and provides the complete oxidation of *acetyl-CoA* derived from carbohydrates, AAs and fats. Consequently, this results in decreased *ATP* production from aerobic oxidation. Yang et al(188) have suggested that high *lactic acid* and low *glucose* concentrations in RA SF may represent potential biomarkers of RA.

Ahn et al(171) identified greater concentrations of BCAAs, resulting in increased production of TNF-α and/or IL-1, which are typically raised in SNA and RA(208). A disturbed metabolism of *glutamate* has been suggested to be indicated by increased expression of citramalate in the setting of active inflammation(209). Elevated expression of *methionine sulfoxide* and *citrulline* were also identified in this study. This may be a reflection of neutrophil hyperactivity documented in BD(210). Ahn et al(171) suggested these metabolites may be putative biomarkers for discriminating BD with arthritis from SNA. However, their study did not have a control group.

Kim et al(183) identified a number of metabolites that are major intermediates of AA and *FA* metabolism, the urea and *TCA* cycles. The authors suggest that AA metabolism, the urea and *TCA* cycles had greater activity in the RA compared with the non-RA group. Despite the authors suggesting these metabolites may be putative biomarkers, there were no control groups and many different disease processes were being compared. Consequently, it cannot be stated with any certainty whether these metabolites are indeed putative biomarkers.

3.4.4 Metabolic changes seen between osteoarthritis and rheumatoid arthritis

Twenty-one metabolites were identified by Kang et al(180) as being in significantly different concentrations between OA and RA SF. Concentrations of lipid metabolites were typically

higher in RA than OA SF, which has previously been demonstrated(194). Furthermore, regulation of inflammation includes the roles of prostaglandins and lipid mediators. Prostaglandins and leukotrienes are vital in the development of arthritic diseases(211). Concentrations of *tryptophan* metabolites also differed significantly between the RA and OA groups. This is an essential AA, which must be provided in the diet. *Tryptophan* and its associated metabolites are involved in inflammation. One of the metabolites, *kynurenine*, has known anti-inflammatory effects that are toxic to T cells and result in apoptosis(212).

3.4.5 Limitations

Despite the identification of many metabolites in a multitude of diseases, including some putative biomarkers, there are important limitations. Only seven studies were identified in the literature with a healthy control group(170, 174, 177, 178, 184, 187, 192). Furthermore, only two studies performed a robust statistical analysis by accounting for multiple testing, and neither of these studies had a healthy control group(172, 173). Multiple confounding factors present in many of the studies was another important limitation. Not all studies accounted for age or gender and few considered medical co-morbidities. Consequently, the results need to be viewed with caution. The solution would be to conduct a large-scale epidemiological metabolic profiling study incorporating multiple confounding factors such as age, gender, medications and medical co-morbidities with the intent to address correlations between clinical features of disease, metabolism and inflammation. Furthermore, the majority of the studies reported here are untargeted metabolic profiling studies, where the identity of any putative biomarker is initially unknown. In those cases where metabolites were annotated, no level of confidence in the annotation, as those set by the Metabolomics Standards Initiative (MSI), was reported(213). Consequently, the occurrence of incorrect metabolite annotations is possible hence affecting further metabolic interpretation and biomarker validation. Another important limitation is that some of these studies did not provide quantitative changes, such as percentage or fold change, but only the direction of change. This is very important in metabolic profiling studies as without quoting fold changes, no measure can be made of sensitivity or specificity. Consequently, any study that does not demonstrate this cannot reliably identify any biomarkers. In this systematic review, only two of the studies with an asymptomatic control group that suggested putative

biomarkers (table 6) had performed fold changes to quantify their results, and neither of these studies had performed an FDR correction or used another method of accounting for multiple testing of the same dataset. Consequently, it is difficult to draw any meaningful conclusions from these studies.

3.5 Conclusion

The role of metabolic profiling in the field of orthopaedics is proving to be an invaluable method of identifying putative biomarkers unique to different pathologies. Despite many studies being performed using these techniques in human SF, there is still a need for larger studies to be performed. These should ideally include healthy controls, account for multiple confounding factors and use robust statistical analysis to identify putative biomarkers. Until fully quantified metabolic levels can be derived and measures of specificity and sensitivity are available, the utility of metabolic biomarkers is limited. The culmination of such studies may result in the development of new diagnostic techniques and possible treatment strategies. Recent advances in both genomic and proteomic studies have demonstrated the importance of these techniques in improving disease understanding and biomarker identification(214, 215). Future studies integrating proteomic, genomic and metabolic profiling techniques may provide the greatest hope for the advancement of biomarker discovery.

This thesis includes two discovery studies examining the metabolic composition of osteoarthritic and infected synovial fluid. The hope was that specific metabolites would be identified in significantly different concentrations upon which targeted analysis could be performed in the future, to investigate whether any may represent putative biomarkers for osteoarthritis and/or joint infections. In the next chapter, the methodology for these studies is explained in detail.

4 Materials and Methods

4.1 Ethical approval and constraints

Ethical approval was granted by the Imperial College London local research ethics committee (Project 15/LO/0388) and all patients gave informed consent to participate in this research project. Due to ethical constraints, it was not possible to collect SF from control patients, without any underlying pathology or joint injury. However, there was an abundance of samples from patients with end-stage osteoarthritis undergoing joint replacement surgery. Therefore, the decision was taken to look at the metabolic differences between hip and knee osteoarthritic SF. Similar osteoarthritic SF samples were used as a non-infected control group when comparing differences between infected and non-infected SF.

There were two main discovery experiments performed in this thesis and the procedures used for differences in the acquisition and preparation of samples for these experiments is detailed in the sections below. The theory of and background to the analytical methods and statistical approaches used has already been summarised in Chapters 1 and 2. All of the technical aspects and parameters used for the metabolic phenotyping experiments, using NMR spectroscopy for the processes of metabolite identification and the statistical analyses that were performed, were similar between the two investigations and are explained in this chapter. Figure 17 demonstrates a flow chart of the main methodological steps.

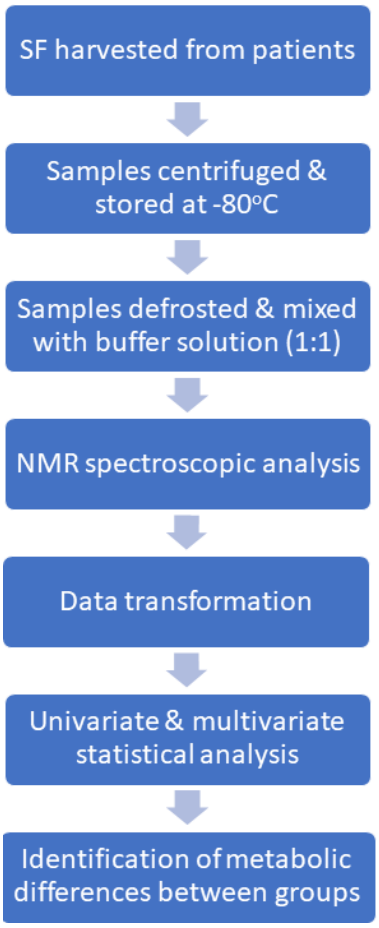


Figure 17. Flow chart illustrating the main methodological steps that took place to conduct the experimental work in this thesis.

4.2 Sample selection and preparation for spectroscopic analysis

4.2.1 Sample details for chapter 5: Metabolic differences between hip and knee synovial fluid (SF) in osteoarthritis

SF was harvested from 24 patients undergoing primary knee (12 patients) or hip (12 patients) arthroplasty for end-stage OA (ESOA) at Imperial College NHS Trust. Patients were matched exactly for gender and within five years for age and five units for body mass index (BMI). In a further attempt to reduce the number of confounding factors, patients with similar medical comorbidities were selected. ESOA was confirmed based on the patient's radiographs, symptoms and findings at the time of surgery.

A questionnaire was completed by all patients, which included information on demographics, medical co-morbidities, medications, diet, lifestyle, duration and severity of symptoms and previous intra-articular corticosteroid injections. Co-morbidities included metabolic-related diseases (ischaemic heart disease, hypertension, gout, osteoporosis, diabetes, raised cholesterol and stroke). Data on BMI was also collected from the patient's records.

Samples were collected intraoperatively using a standardised protocol. A posterior approach was used for the hip joint, whereas the knee joint was approached using a midline incision and medial parapatellar approach(216). Following skin incision but prior to knee arthrotomy or hip capsulotomy, a 14G syringe was inserted along the femoral neck of the hip or into the suprapatellar pouch of the knee and SF was aspirated. The sampling technique was similar between the groups.

4.2.2 Sample details for chapter 6: Metabolic differences between infected and non-infected synovial fluid

SF was harvested from eight patients with MC&S proven infection and eight matched patients with uninfected joints at Imperial College Healthcare NHS Trust. The infected cohort consisted of four native knees, two total hip replacements (THR) and two total knee replacements (TKR). The non-infected cohort consisted of three native knees, one native elbow, two THRs and two TKRs. Similar joints were chosen for the non-infected cohort, as differences between the metabolic profiles of osteoarthritic hip and knee HSF have been demonstrated(217). As before, all patients provided informed consent and completed a questionnaire. Exclusion criteria included patients unable to consent, those under the age of 18, pregnancy or a dry aspirate.

Samples in the septic arthritis cohort were collected during diagnostic aspiration at the bedside or intraoperatively using a standardised protocol. For bedside aspirates, the knee area was prepped with chlorhexidine and a 16G needle was inserted in a standard superolateral approach. When samples were taken intraoperatively, a posterior approach was used for the hip joint, whereas the knee joint was approached using a medial parapatellar approach through a midline incision(216) in exactly the same manner as described earlier in this section. Samples were divided into two aliquots, with the first being sent for routine MC&S.

The subsequent methodology was identical for both experiments and is detailed in the sections below.

4.3 Sample preparation and metabolic phenotyping

In line with other work carried out at Imperial College, the collected samples were left refrigerated at 4°C for no longer than four hours, as it has been shown that after this time, changes took place in the concentrations of some of the metabolites(218). All samples were then aliquoted and centrifugated at 10000 x *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 6 months before analysis (figure 18).

Figure 18. Photograph of centrifuge and -80°C freezer used to conduct the methodology in this thesis.

Samples were defrosted within one of hour of being assayed. Each sample (400 µl) was combined with an equal amount of 75 mmol/L sodium dihydrogen phosphate (NaH_2PO_4) buffer at pH 7.4 containing 6.2 mmol/L sodium azide (NaN_3) and 4.6 mmol/L of 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid sodium salt (TSP) and 20% deuterium oxide (D_2O) as published by Dona et al (figure 19) (219).

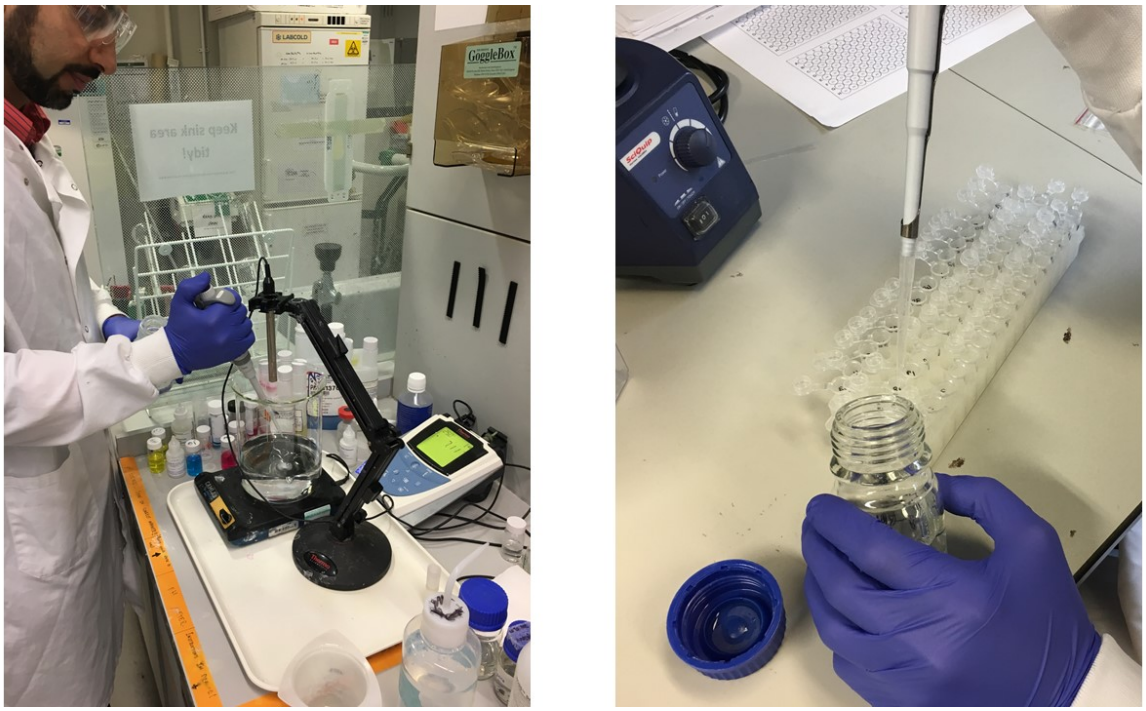


Figure 19. Photographs demonstrating preparation of the buffer solution and combining the buffer with the synovial fluid samples.

Potential chemical contaminants were identified and excluded by running control samples containing only buffer in tandem with the HSF. Such contamination may have occurred at any stage during sample preparation.

4.4 NMR spectroscopic analysis

Experiments were performed in a Bruker® Avance III 600 MHz spectrometer equipped with a Samplejet 96 well autosampler (figure 20). Standard one dimensional (1D) high resolution NMR (^{1}H-NMR) spectra were acquired for each sample at 25 ^{o}C using the standard NOESY 1D pulse sequence with optimised water presaturation, as a sum of 128 free induction decays, with 128 k complex points using a spectral width of 20 ppm, a mixing time of 10ms, a delay between the first two 90^o radiofrequency pulses of 4µs and a relaxation time of 4s. The water presaturation irradiation was applied during the relaxation delay and the mixing time.

Figure 20. Photograph showing the NMR spectroscopy racks used to analyse the samples in this thesis. NMR – nuclear magnetic resonance.

An exponential function was applied to the NMR spectroscopy free induction decays (FIDs) prior to Fourier transformation providing a line broadening of 0.3Hz. The resulting NMR spectra were automatically phased and baseline corrected and chemical shifts were internally referenced to that of H1 of *α-glucose* (at 5.23 ppm). Spectra processing was performed using Topspin 3.2 (Bruker, Germany). The NMR spectra were then imported into Matlab™ (Matlab2016b, Mathworks, Massachusetts, United States) and consisted of 27,492 data points. Spectra were aligned according to the method developed by Veselkov et al to remove any expected small changes in peak positions between spectra so as to allow comparison of peaks between samples(220). The probabilistic quotient normalisation method was used to normalise the spectra(221). Following normalisation, the data was scaled to unit variance(222).

4.5 Metabolite Identification

Metabolite assignment took place by matching the peak chemical shifts, relative peak intensities and multiplicities to metabolite NMR spectral information from the literature and databases (Human Metabolome Database (169), Biological Magnetic Resonance Bank(168)). However, it may be difficult to interpret 1D spectra alone, primarily due to peak overlap. For this reason, statistical total correlation spectroscopy (STOCSY) (223) was used to aid metabolite identification by demonstrating peaks (or variables), which show statistical correlations with other peaks (or variables) in the spectra, and thus belong to the same molecule or originate from another molecule related functionally(223). Two-dimensional NMR spectra, namely ^{1}H-^{1}H total correlation spectroscopy (TOCSY) and ^{1}H-^{13}C heteronuclear single-quantum correlation spectroscopy (HSQC), were acquired for representative samples to further confirm the identified metabolites. All metabolites were confirmed by comparing spectral signals to those of pure metabolite standards from public databases (Metabolomics Standards Initiative) via analysis of 2D NMR spectra (213).

4.6 Statistical analysis

To analyse the large dataset (27,492 variables per sample) generated by NMR spectroscopy, Principal Component Analysis (PCA) and Orthogonal Partial Least Squares-Discriminate Analysis (O-PLS-DA), are the most prominent multivariate analysis techniques (155). PCA was performed to obtain an overview of the study samples, highlight clusters and identify outliers. O-PLS-DA modelling was used to further investigate the differences between the groups under investigation. In this method, the NMR spectral data (X matrix) was regressed against a class variable (Y matrix). The Y variable consists in a binary numerical vector, which encodes the group information, for instance controls (as 0) and cases (as 1). Differences between groups under study were sought using this procedure. In order to assess the model validity, seven-fold cross validation was performed to obtain the parameters goodness of prediction (Q^2Y) and goodness of fit (R^2Y), which were evaluated as measures of model fitting. The goodness of prediction (Q^2Y) measures the predictability of the model and the goodness of fit (R^2Y) is the proportion of the NMR spectral data that explains the differences between the two groups (Y variable). The O-PLS-DA model robustness was further assessed by calculating the Q^2Y 100

times by randomly assigning samples predicted in each of the seven cross-validation rounds. The Q^2Y values thus obtained were compared with the Q^2Y values obtained by performing 100 random permutations of the Y variable. The model was considered reliable if the Q^2Y confidence intervals of the permuted and non-permuted models did not overlap.

The metabolite signals responsible for the separation between the two groups were identified from the corresponding O-PLS-DA loadings plot. Only signals with a correlation coefficient to the Y variable >0.6 were considered to have a significant impact on group separation. Multivariate analysis was performed using the SIMCA 14™ statistical software (Sartorius Stedim Biotech, Umeå, Sweden).

To better identify the peaks, back transformation of the loadings was performed by multiplying each variable by its standard deviation (obtained before scaling). The NMR spectral signals related to the predictive components are then individually analysed to demonstrate the differences between the two groups for the identified metabolites. The individual peaks of all the spectra were also plotted for visual inspection of obvious differences between the two groups. The area under each metabolite peak was obtained by integration and analysed by univariate analysis, using the Student's t-test.

The false discovery rate (FDR) adjusted p-values were calculated using the Benjamini-Hochberg method (158) and was performed to account for multiple testing of the identified metabolites. The MetaboNetworks software package (195) within Matlab™ (Matlab2016b, Mathworks, Massachusetts, United States), was used to map the identified metabolites in a network of metabolic substrates from the human metabolic pathways from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (224).

The KEGG database was designed to link genomic information with functional information by standardised gene annotations and computerised knowledge on cellular processes. The most organised portion of the KEGG database pertains to metabolism, which consists of approximately 90 diagrams that graphically represent the reference metabolic pathways. The nodes represent the metabolites, and the lines correspond to the enzyme-catalysed reactions (biotransformations) that occur between them. (224).

4.7 Conclusion

The complex methodology employed in this thesis has been explained in detail including the statistical techniques required to analyse the complex datasets. In the next chapter, the metabolic differences between osteoarthritic hip and knee SF are identified and the differences explained in detail.

5 Metabolic differences in the composition of hip and knee synovial fluid in osteoarthritis

An adaptation of this chapter has been published in Osteoarthritis and Cartilage in December 2019(217).

5.1 Summary

The hip and knee joints have important biomechanically differences with respect to fluid lubrication, contact stresses and wear patterns. There are also a number of other aetiopathological differences, which can be intra- or extra-articular. These variations may be reflected in the synovial fluid (SF) composition of the two joints, but the nature of these differences remains unknown. Chapter 3 identified a number of putative biomarkers in patients with osteoarthritis. However, to date, no studies have looked at potential metabolic variations between different joints. The objective of this study was to identify metabolic differences in osteoarthritic hip and knee SF using the techniques of metabolic phenotyping with Nuclear Magnetic Resonance (NMR) spectroscopy.

Twenty-four SF samples (12 hip, 12 knee) were collected from patients with end-stage osteoarthritis (ESOA) undergoing hip/knee arthroplasty. Samples were matched for age, gender, ethnicity and medical comorbidities. The metabolites present in each sample were analysed using NMR spectroscopy. Multivariate analysis of NMR spectra was undertaken by PCA and OPLS-DA, using the SIMCA 14^{TM} statistical software, to investigate metabolic differences between the hip and knee groups. Metabolites were identified using 2D NMR spectra, statistical spectroscopy and by comparison to entries in published databases.

This study identified significant differences in the metabolic profile between the hip and knee groups (P=0.023). Following vigorous statistical analysis, four metabolites were found in significantly greater concentrations in the knee group compared to the hip group (*citrate*, *glutamate*, *glycosaminoglycans* and *N-acetylated* molecules).

This study is the first to reveal differences in the metabolic profile of hip and knee SF in ESOA. The identified metabolites can broadly be grouped into those involved in the *tricarboxylic acid* cycle, collagen degradation and oxidative metabolism in diseased joints. These findings may

reflect differing lubrication and wear profiles between these joints, resulting from many intra- or extra-articular aetiopathalogical factors.

5.2 Introduction

Osteoarthritis (OA) most commonly involves the hip and knee joints, and its treatment results in significant socioeconomic costs (225). The aetiology is multifactorial, but the final pathway results in progressive degradation of chondral cartilage and may involve changes to subchondral bone and synovial inflammation. This wear of intra-articular tissues leads to the presence of metabolites in the synovial fluid (SF) that represent degradation and inflammatory products(226).

As discussed in chapter 1, there are differing biomechanical profiles between the hip and knee joints (85, 90). Consequently, the wear patterns differ in these joints both clinically and radiologically. As a result, there may be differences in the fluid components related to these wear patterns, although the evidence for this remains limited (226-229). A number of studies have demonstrated differences in protein, enzyme and cytokine concentrations of SF between these joints (227-229). Another possibility is that small molecule metabolite levels may be different between the SF of the two joints and contribute to the explanation of the variations in wear.

Metabolic phenotyping is a relatively novel technique, which studies the low molecular weight metabolites within a cell, tissue or biofluid using a variety of techniques. It has the ability to analyse hundreds or even thousands of small molecules simultaneously, which is what makes it so appealing. The techniques involved have been employed in several conditions to influence clinical practice (230-232). Recently, they have been used to identify metabolites within the SF, blood and urine of both human and animal models with OA (233, 234). In addition to identifying individual biomarkers for a specific pathological process, this approach has yielded unique metabolic “signatures” consisting of numerous metabolites that may identify a particular pathology based on relative concentrations of these molecules (235).

Despite recent developments in this field, with improvements in the detection of small molecules in various biological tissues and fluids, very few studies have been performed

examining the small molecule composition of human SF (HSF) (189, 191, 204, 234, 236-238). The majority of these studies have used mass spectrometry (MS). Although MS has good sensitivity, as explained in chapter 2, it is limited by variable ionisation and ion suppression effects, which may impair analyte detection (148). Furthermore, the technique involves destruction of the samples, thus preventing repeat testing from taking place. Furthermore, in order to improve metabolite resolution, MS is also typically coupled with prior separation of the fluid using capillary electrophoresis or chromatography, which can introduce unwanted inter-sample and inter-run variability.

Despite NMR spectroscopy having a sparser metabolite coverage and lower sensitivity than MS, its strengths lie in ease of quantitation, universality of detection and the ability to annotate spectral features. It is non-destructive and fast, allowing multiple samples to be measured daily, and the same sample can be analysed numerous times (148).

Previous metabolic profiling studies of OA in HSF have identified some putative OA biomarkers such as *arginine* and the ratio of branched chain amino acids (BCAAs) to *histidine* (234, 238-240). However, these studies have examined hip and knee SF as a homogeneous group (189, 191, 204, 234, 236-238). Currently, no studies in the literature have utilised NMR spectroscopy to assess the differences in the small molecule composition between hip and knee SF in patient with ESOA.

In the present study, human SF samples from the knee and hip joints in patients with ESOA have been analysed using ^{1}H NMR spectroscopy (where the technical details have been summarised in Chapter 2).

5.2 Aims and hypothesis

1. To investigate the metabolic profile of human SF (HSF) from native hip and knee joints in patients with ESOA using NMR spectroscopy.
2. To assess metabolic differences between the fluids using multivariate and univariate statistical analysis.
3. To evaluate the metabolic pathways involved that are implicated in the metabolic differences between hip and knee HSF.

The null hypothesis states that no significant differences exist in the metabolite concentrations between native hip and knee joint HSF in patients with ESOA.

5.4 Materials and Methods

The methodology used for this experiment is detailed in chapter 4. In summary, the samples were centrifuged for 15 minutes and the supernatant was aliquoted, before the samples were stored at -80^{o}C. Following defrosting, all samples were mixed with buffer solution in a one to one ratio before undergoing NMR spectroscopic analysis. Subsequently, the data was transformed before univariate and multivariate analysis took place. The metabolic differences between the hip and knee groups were then identified and the results are detailed below.

5.5 Results

5.5.1 Patient Demographics

No significant differences were identified between the two groups in terms of age (p=0.27), gender or BMI (p=0.71). Furthermore, the common medical co-morbidities also remained similar (table 7). The full list of medical co-morbidities and medications are included in table 8. It is important to note that some of the medications listed could have passed into the SF either by diffusion or through various transport mechanisms. For example, paracetamol (acetaminophen) glucuronide was found in some of the SF samples in this study. However, this did not have any significance to the metabolitic differences discussed later in this chapter. Steroid injections had previously been performed in two hip and three knee patients. All injections were performed over a year before HSF sample collection, except for one injection in the knee group, which was performed six months before the sample was collected. No synthetic *hyaluronic acid* injections had been performed in any of the patients.

Table 7. Patient demographics. There was no significant difference in age (p=0.27), gender, BMI (p=0.71) or medical co-morbidities between the groups. IHD, Ischaemic heart disease; DM, Diabetes Mellitus; CVA, Cerebrovascular Accident.

	Hip	Knee
Number of patients	12	12
Age (Mean ± SD)	67.7 (12.2)	63.3 (21.2)
Gender (Male: Female)	3:9	3:9
BMI (Mean ± SD)	30.4 (5.1)	29.9 (4.6)
Ethnicity	11 Caucasian 1 Asian	9 Caucasian 2 Afro-Caribbean 1 Asian
Disease (Number of patients per group)		
IHD	2	0
Hypertension	9	8
Hypercholesterolaemia	7	6
DM	4	2
CVA	1	0

Table 8. Demographic information for all patients including all medical co-morbidities and regular medications. BMI – body mass index; PMH – past medical history; F – female; M – male; T2 DM – type II diabetes mellitus; HTN – hypertension; COPD – chronic obstructive pulmonary disease; AVN – avascular necrosis; SLE – systemic lupus erythromatosus; CVA – cerebrovascular accident; AF – atrial fibrillation; CA – carcinoma; IHD – ischaemic heart disease; OA – osteoarthritis; GORD – gastro-oesophageal reflux disease; MI – myocardial infarction; BPH – benign prostatic hypertrophy; PVD – peripheral vascular disease; CKD – chronic kidney disease; HH – hiatus hernia.

	Gender	Age	BMI	PMH	Medications
hip	F	78	34.7	T2 DM, HTN, COPD, Hypothyroid, hypercholesterolaemia, Frey's syndrome, vitamin D deficiency	Metformin, atenolol, atorvastatin, lorartidine, gabapentin, beclomethasone, aspirin, Iron tablets, esomeprazole, paracetamol, losartan, budesomide, levothyroxine
hip	F	48	25.7	Renal transplant, AVN, asthma, SLE, depression, HTN	Tacrolimus, mycofenolate mofetil, betamethasone, citalopram, aspirin, nifedipine, simvastatin
hip	F	72	29.6	Asthma, hypercholesterolaemia, iron deficient anaemia, endometriosis	Salbutamol
hip	M	72	33.7	Asthma, CVA (2008), HTN, epilepsy, AF, iron deficient anaemia	Amlodipine, doxazosin, carbamazepine, salbutamol
hip	F	73	26	HTN, hypercholesterolaemia, breast CA	Paracetamol, naproxen, tamoxifen, amlodipine, cholecalciferol, loratadine, omeprazole
hip	F	67	24	OA	Paracetamol, cholecalciferol
hip	M	70	22.1	T2 DM, HTN, hypercholesterolaemia, CA prostate (radical prostatectomy), depression	Metformin, gliclazide, simvastatin, perindopril, citalopram
hip	F	74	40.9	GORD, MI, HTN, temporal arteritis, hypercholesterolaemia, angina, IHD	Atenolol, aspirin, Lipitor, prednisolone
hip	F	68	34.8	Lumbar spine OA, depression	Paracetamol
hip	M	85	29.6	T2 DM, HTN, hypercholesterolaemia, deafness, BPH, appendicectomy	Insulin, metformin, amlodipine, pravastatin, ramipril, paracetamol
hip	F	77	27.1	HTN, multinodular goitre, hiatus hernia	Ramipril, Bendroflumethiazide
hip	F	61	27	T2 DM, HTN, hypercholesterolaemia, PVD	Diltiazem, aspirin, atorvastatin, bendroflumethiazide, enalapril, metformin, tramadol, fentanyl, sitagliptin
knee	M	71	26.1	BPH, diverticulitis, meniscal tear, HTN	Frusemide, tamsulosin
knee	F	82	29.5	HTN, PE, breast CA, CKD stage 3, depression, spinal stenosis	Senna, sertraline, zopiclone, tolfedine, alendronic acid, Adcal D3, amitriptyline, co-codamol, perindopril, indapamide
knee	F	84	31.1	GORD, T2 DM, anaemia, anxiety, biliary cirrhosis, HTN, vit D deficiency, fibromyalgia	omeprazole, metformin, citalopram, rosuvastatin, tramadol, paracetamol, loratadine, omeprazole, ursodeoxycholic acid, propanolol
knee	M	88	29.5	HTN, hypercholesterolaemia, vit D deficiency	Gabapentin, co-dydramol, simvastatin, ramipril
knee	F	64	39.6	Radical nephrectomy (donor), HTN, GORD, hypothyroidism	Amlodipine, enalapril, thyroxine, lansoprazole, atorvastatin, aspirin
knee	F	66	28.1	Hypercholesterolaemia, cutaneous lupus, vit D deficiency, HTN	Simvastatin
knee	F	74	29.6	Sjogren's syndrome, HTN, HH, cystic lung lesion, parathyroid lesion	Prednisolone, felodipine, omeprazole
knee	F	75	26.5	T2 DM, hypercholesterolaemia, appendicitis	lactulose, metformin, gliclazide, aspirin, simvastatin, beclomethasone
knee	M	76	29.5	BPH, anaemia, renal impairment	finasteride, indapamide, atorvastatin, tamsulosin, sildenafil
knee	F	60	28	Hypothyroid, GORD, hypercholesterolaemia, GORD	omeprazole, levothyroxine, simvastatin, cetirizine, ibuprofen
knee	F	72	24	Hypothyroid, migraine, hypercholesterolaemia	alendronic acid, levothyroxine, cholecalciferol, sumatriptan
knee	F	65	32.4	HTN, hypothyroid, GORD, hypercholesterolaemia, vit D deficiency	amlodipine, losartan, omeprazole, levothyroxine, atorvastatin

5.5.2 PCA and O-PLS-DA analysis of differences between hip and knee metabolites

Figure 21 demonstrates a typical ^{1}H-NMR spectra of hip and knee SF from this cohort. Tables 9 and 10 lists the assignment of the metabolite peaks, which were consistently identified in all the samples. There is some separation evident on the PCA scores plot (figure 22) between the ^{1}H-NMR spectra in the hip and knee groups. This leads to the suggestion that metabolic differences exist between the HSF composition of the two joints. The scores plot of the first principal component (PC1) vs PC2 demonstrated that the percentage of variation in the data explained was 24% for PC1 and 15% for PC2. An additional PCA scores plot of PC1 vs PC3 revealed that a further 11% of the data variance was explained by PC3 (figure 23). There was greater variability within the hip group, whereas the knees were grouped into a tighter cluster of points. Although the two patient groups were well-matched in terms of possible confounding variables – age, gender and BMI – the PCA scores plots were also examined to investigate whether any of these factors could account for some or all of the patient group clustering. Thus, as can be seen in Figure 24a, 24b and 24c where the PCA scores plots were labelled according to age, gender or BMI respectively, there was no discernible clustering based on these parameters and this demonstrates that these factors were not responsible for the differences seen in figure 22.

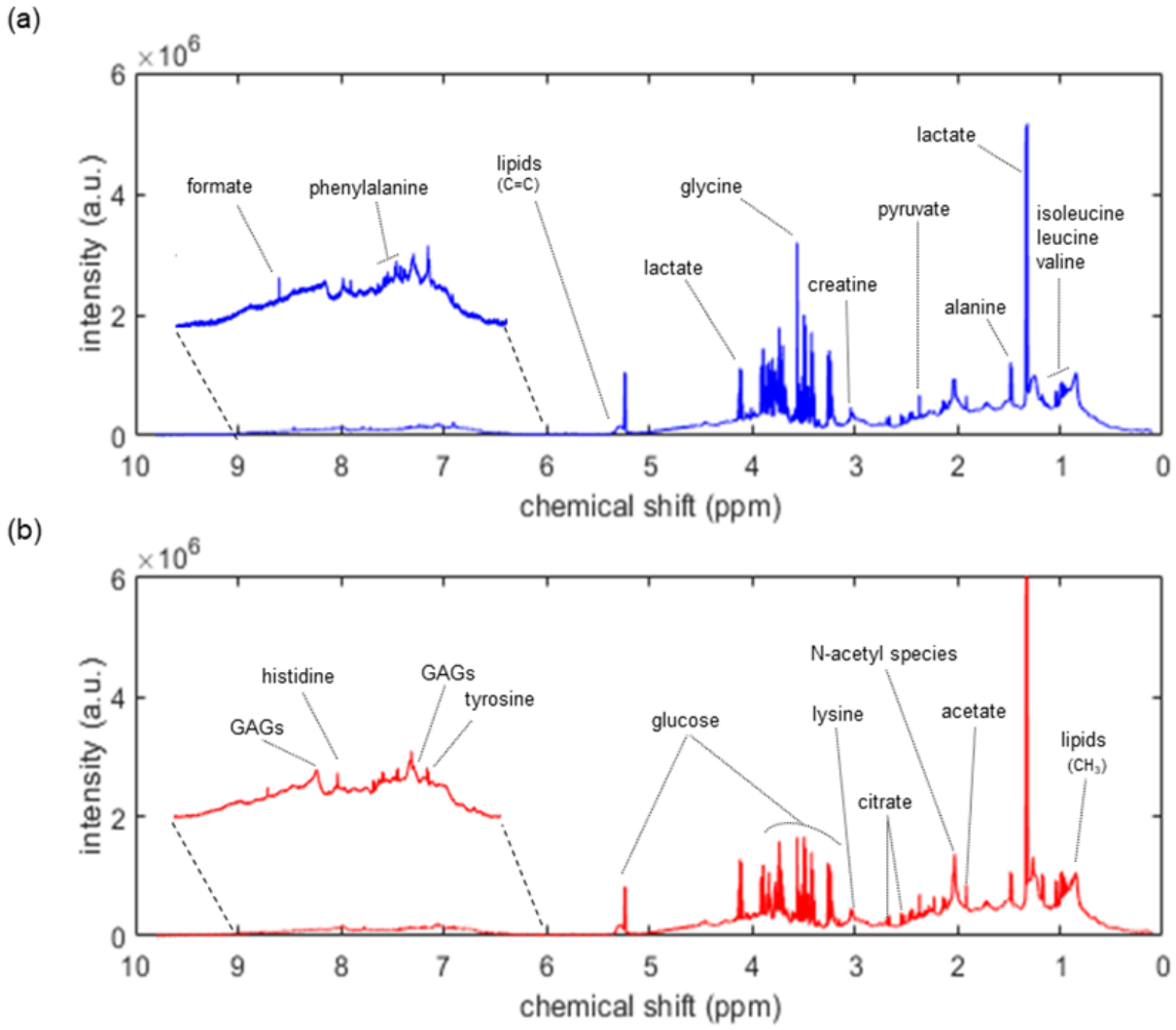


Figure 21. Typical ^{1}H-NMR spectra taken from the study cohort demonstrating some of the identified metabolites. ***A.*** *Knee synovial fluid.* ***b.*** *Hip synovial fluid. GAGs – glycosaminoglycans.*

Table 9. Metabolites from both hip and knee synovial fluid consistently identified in all samples. S – singlet; bs – broad singlet; d – doublet; t – triplet; m – multiplet; ppm – parts per million.

Metabolite	Chemical shift (ppm)
Cholesterol/cholesterol esters (C18)	0.58(bs), 0.65(bs)
CH3 (fatty acyl residues, lipids)	0.85(bs)
CH2 (fatty acyl residues, lipids)	1.25(bs)
2-Hydroxybutyrate	0.89(t), 1.66(m), 1.71(m)
Isoleucine	0.93(t), 1.00(d), 1.25(m), 1.46(m), 1.96(m), 3.66(d)
Leucine	0.95(d), 0.96(d), 2.00(m)
Valine	0.98(d), 1.03(d), 2.26(m), 3.60(d)
3-Hydroxyisobutyrate	1.06(d), 2.48(m)
2-ketoisovalerate	1.11(d), 3.00(m)
3-Hydroxybutyrate	1.19(d), 2.35(dd), 4.14(m)
Lactate	1.32(d), 4.10(q)
Threonine	1.31(d), 3.56(d), 4.24(dd)
Alanine	1.47(d), 3.78(q)
Lysine	1.47(m), 1.71(m), 1.89(m), 3.03(t), 3.72(t)
Arginine	1.69(m), 1.90(m), 3.23(t)
Acetate	1.91(s)
CH2C=C (fatty acyl chains, lipids)	1.99(bs)
N-acetyl groups (GAG and Glycoproteins)	2.03(bs)
N-acetyl groups (Glycoproteins)	2.06(bs)
Glutamate	2.06(m), 2.34(m)
Methionine	2.14(s), 2.16(m), 2.65(t)
Acetone	2.22(s)
Acetoacetate	2.27(s), 3.44(s)
Pyruvate	2.37(s)
Succinate	2.40(s)
Glutamine	2.12(m), 2.45(m), 3.74(dd)
Citrate	2.53(d), 2.67(d)
Dimethylamine	2.72(s)
Asparagine	2.82(dd), 2.93(dd), 3.97(dd)
N,N-dimethylglycine	2.92(s)
Creatine	3.03(s), 3.91(s)
Creatinine	3.04(s), 4.05(s)
Dimethylsulfone	3.14(s)
Choline	3.19(s)
Choline (Phospholipids)	3.21(s)
Glucose	3.23(dd), , 3.46(dd), 3.49(t), 3.53(dd), 3.71(t), 3.72 (dd), 3.76(dd), 3.82(m), 3.85(m), 3.90(dd), 4.64(d), 5.23(d)
Glycine	3.55(s)
Myo-inositol	3.28(t), 3.60(t), 4.06(t)
Gluconate	3.66(dd), 3.77(m), 3.82(m), 4.04(m), 4.13(d)
CH=CH (fatty acyl residues, lipids)	5.28(bs)
Tyrosine	6.89(d), 7.19(d)
Histidine	7.05(s), 7.77(s)
Paracetamol glucuronide	2.16(s), 5.09(d), 7.15(d), 7.36(d)
GAGs	2.02(s), 3.34(bs), 4.46(bs), 7.97(bs)
Hypoxanthine	8.18(s), 8.20(s)
Formate	8.45(s)

Table 10. Unknown metabolites from both hip and knee synovial fluid consistently identified in all samples. S – singlet; bs – broad singlet; d – doublet; t – triplet; m – multiplet; ppm – parts per million; UN – unknown.

Metabolite	Chemical shift (ppm)
UN	0.97(t), 1.88(m)
UN	1.12(d), 4.12
UN	1.15 (s)
UN	3.18(s)
UN	3.798 (s), 3.876(d)
UN	5.90(bs)
UN	7.24(d)

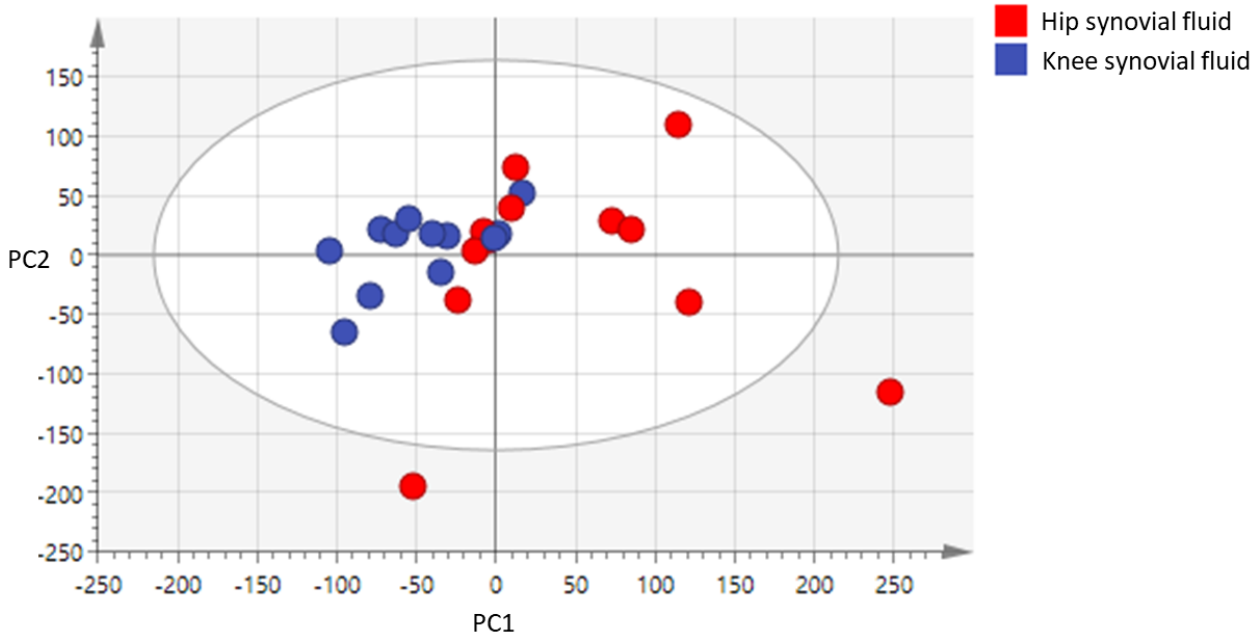


Figure 22. PCA scores plot of PC1 vs PC2 with each data point representing the NMR spectrum of an individual human synovial fluid sample and demonstrating separation between the hip and knee groups. The ellipse represents Hotelling T2 with 95% confidence interval. The percentage variation explained is 24% for PC1 and 15% for PC2. PC – principal component.

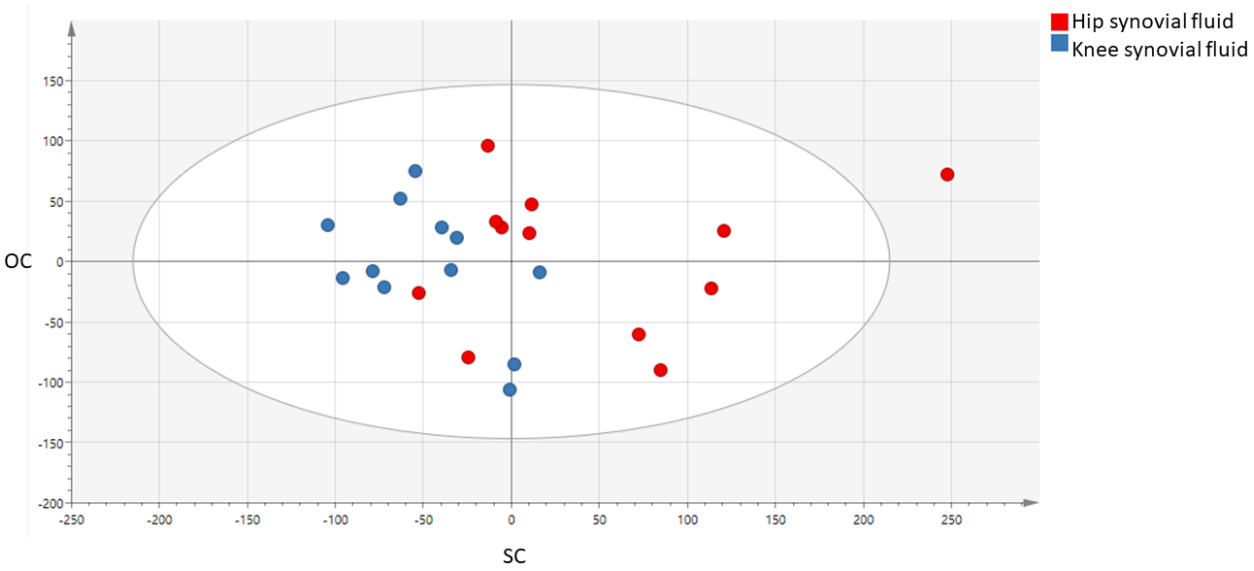


Figure 23. PCA scores plot of PC1 vs PC3 with each data point representing the NMR spectrum of an individual human synovial fluid sample and demonstrating separation between the hip and knee groups. The ellipse represents Hotelling T2 with 95% confidence interval. The percentage variation explained is 24% for PC1 and 11% for PC3. PC – principal component.

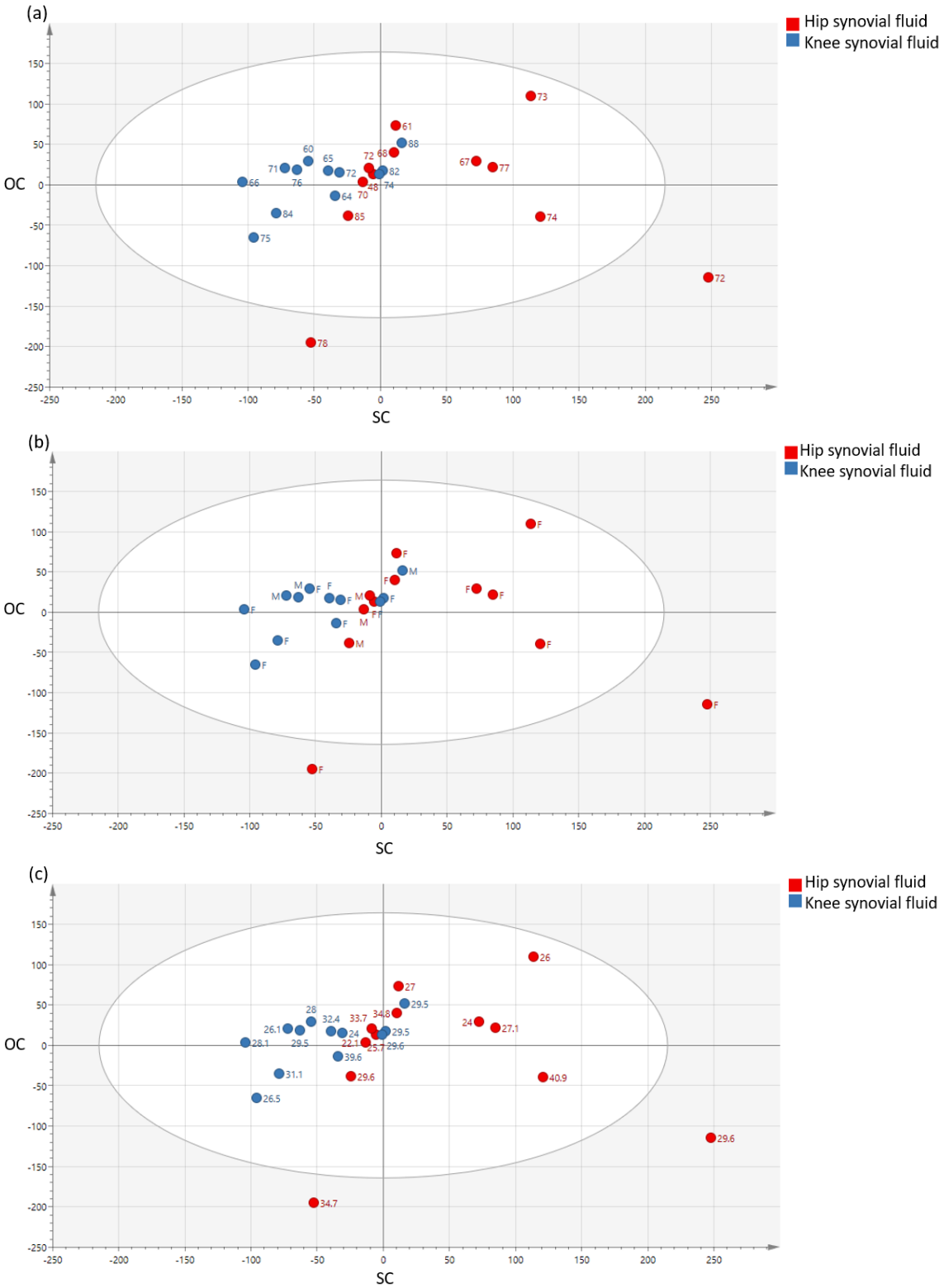


Figure 24. PCA scores plots demonstrating that age, gender and BMI are not driving the changes seen between the hip and knee groups. The ellipse represents Hotelling T2 with 95% confidence interval. ***a****. Age; numbers represent age in years.* ***B****. Gender; M – male; F – female.* ***C****. body mass index (BMI); numbers represent the BMI.*

By using seven-fold cross-validation, a robust O-PLS-DA model was obtained (Q^2Y = 0.360 [95% CI 0.348 – 0.373], R^2Y = 0.873). This was further confirmed by running permutation analyses on the model (Q^2Y -0.234 [95% CI -0.298 – -0.170] and R^2Y 0.705 [95% CI was 0.693 – 0.716]) (figure 25a). The scores from the O-PLS-DA showed separation between hip and knee HSF (figure 25b). The metabolite resonances responsible for the separation of the hip and knee groups were inspected in the back-scaled O-PLS-DA loadings plot (Figure 26a and 26b).

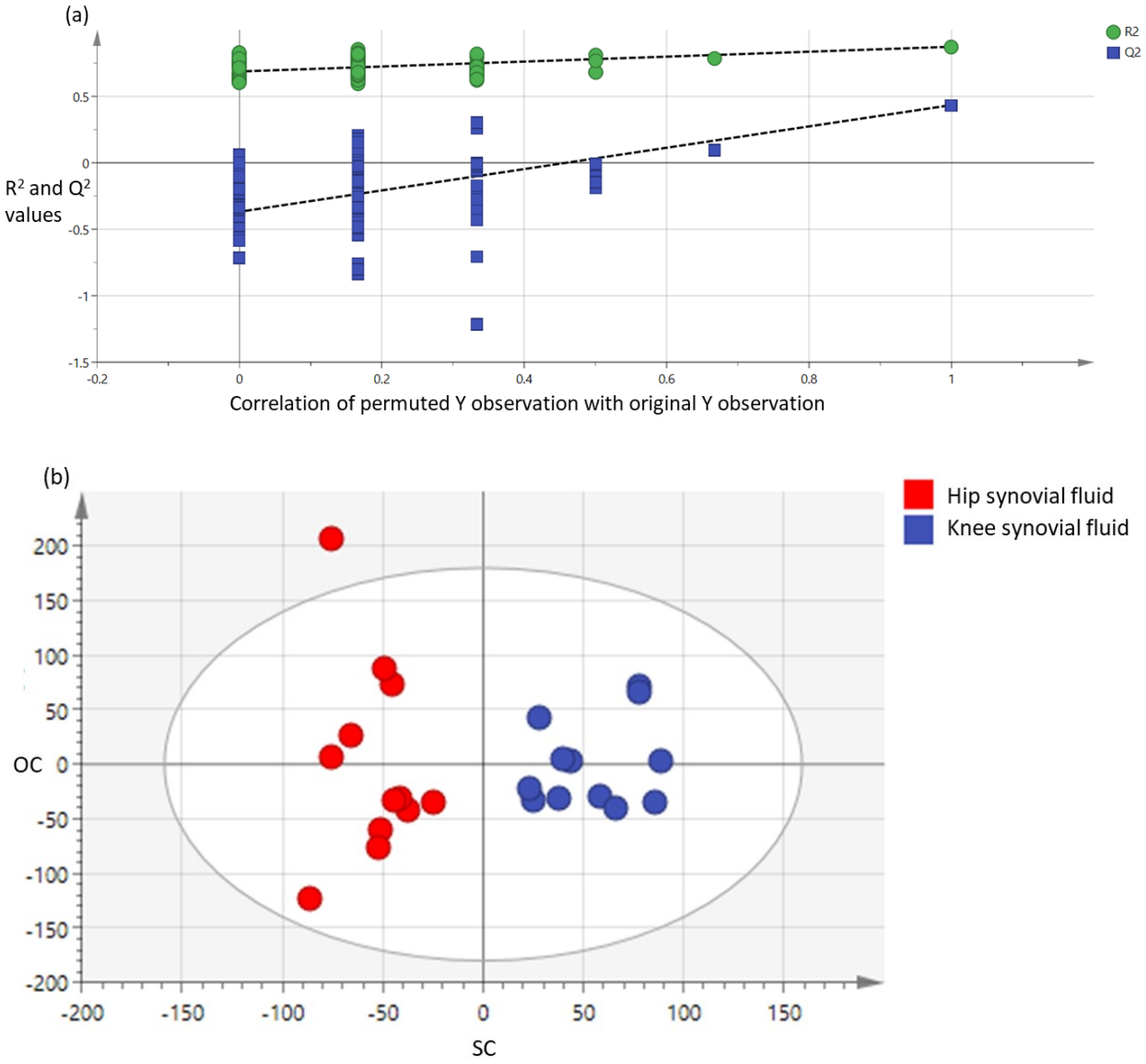


Figure 25. O-PLS-DA model of osteoarthritic SF comparing hip and knee groups. ***a.*** *Graphical representation of the permutation analysis demonstrating that all the permuted models have a Q^2Y value lower than the values obtained in the hip vs knee synovial fluid model.* ***B.*** *Scores plot of the cross-validated O-PLS-DA model demonstrated a good separation between the hip and knee groups with differences in the spectra of each sample explained by whether the fluid was taken from the hip or knee joint. The ellipse represents Hotelling T2 with 95% confidence interval. SC – separating component; OC – orthogonal component.*

HSF from the knee joint showed greater levels of *alanine*, citrate, *dimethylsulfone*, *glucose*, *glutamine*, *glycosaminoglycans* (GAGs), *histidine*, *lysine*, *N-acetylated* molecules, *pyruvate* and

valine compared to the hip group. HSF from the hip group showed greater levels of *choline* compared to the knee group (figures 26a and 26b).

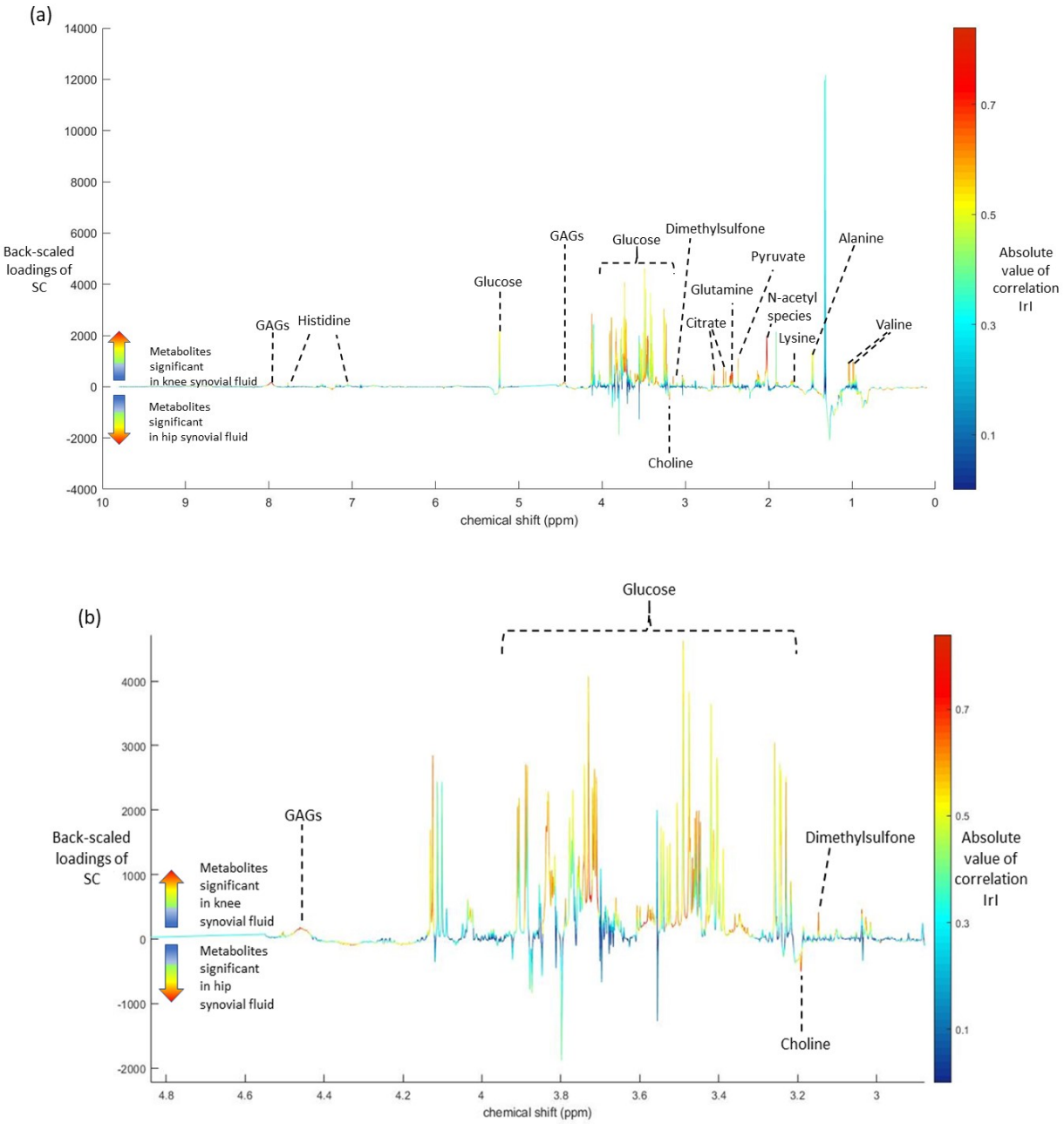


Figure 26. Back-scaled loadings plot of the SC from the O-PLS-DA model. ***A.*** *The significant metabolites are red peaks, with those pointing upwards significantly increased in knee and those pointing downwards significantly increased in hip synovial fluid.* ***b.*** *Expansion of the back-scaled loadings plot demonstrating the difference between the glucose and choline peaks. GAGs – glycosaminoglycans; SC – separating component; ppm – parts per million.*

5.5.3 Evaluation of individual spectra

All metabolic differences identified from the back-scaled loadings plot of the O-PLS-DA were confirmed by visual spectral inspection. Additionally, this revealed greater quantities of *hypoxanthine* in the hip and *tyrosine* in the knee groups (figure 27). Table 11 lists all identified metabolites that were present in different concentrations between the 2 groups. Following FDR adjustment of the p-values, four of these metabolites remained significant (table 11). These were *citrate*, *GAGs*, *glutamine* and *N-acetylated* molecules. A network analysis of all the identified metabolites was performed to identify the potential metabolic pathways (figure28).

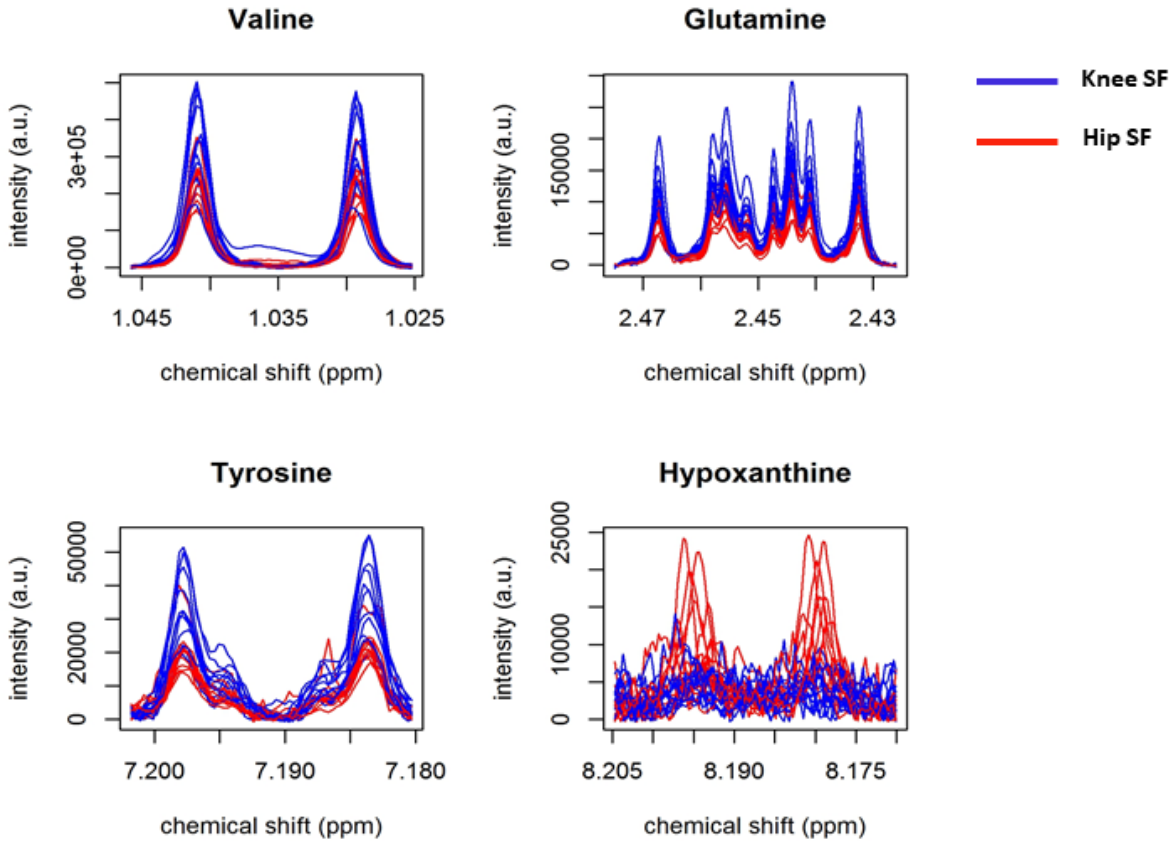
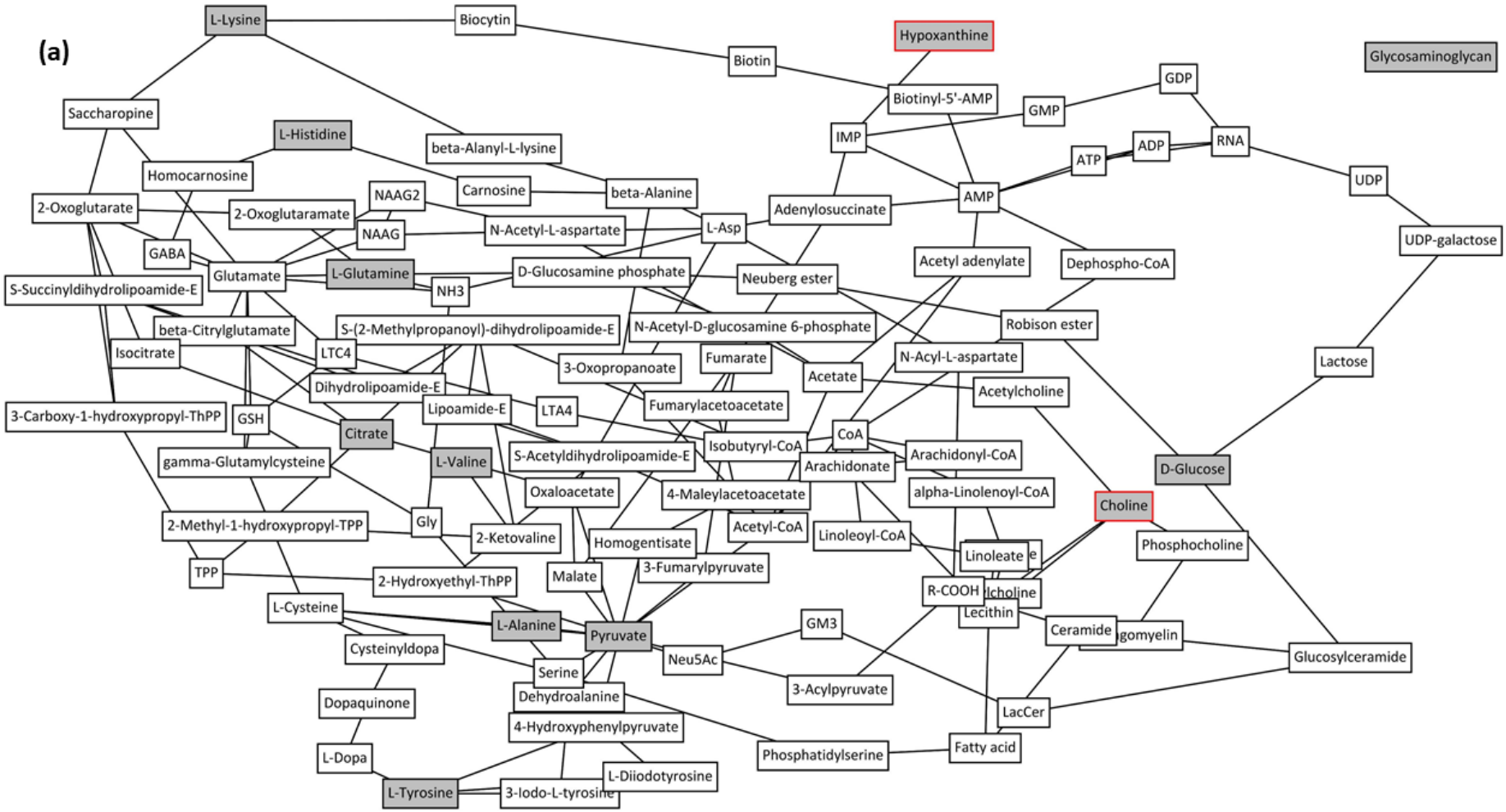


Figure 27. Metabolite analysis from spectral inspection. Figure demonstrates differences in signal intensity between the groups. Valine & glutamine were identified from the back-scaled loadings but have been inserted to demonstrate they still show a difference between the groups on spectral inspection. Tyrosine & hypoxanthine did not show significant differences on the back-scaled loadings, but the differences are apparent when comparing the individual spectra. SF – synovial fluid; ppm – parts per million.

Table 11. Metabolites identified from the back-scaled loadings, univariate analysis and confirmed by 2D NMR spectra. Following FDR adjustment, only four of the 14 identified metabolites remained significant and they were all found in greater quantities in the knee group (*). The chemical shift indicates which metabolite peaks were integrated and used for metabolite comparison between the two groups. The peak area for the two cohorts is also provided, but the units are arbitrary and hence not included. GAG – glycosaminoglycans; ppm – parts per million; 95%CIs – 95% confidence intervals; FDR – false discovery rate.

Metabolite ID	NMR spectra chemical shift (ppm)	Joint containing larger quantity of metabolite	Peak area: knee cohort (mean[95%CIs])	Peak area: hip cohort (mean[95%CIs])	*p*-value (Student's T-test)	FDR-adjusted *p*-value
N-Acetylated molecules*	2.02	knee	1674.9 [1365.6 – 1984.1]	625.7 [386.7 – 864.7]	0.000034	0.001
GAG*	7.97	knee	2615.1 [2352.4 – 2877.8]	1669.9 [1371.6 – 1968.2]	0.00012	0.004
Citrate*	2.53	knee	1243.2 [1065.2 – 1421.2]	766.1 [639.4 – 892.7]	0.00037	0.012
Glutamine*	2.45	knee	2759.4 [2366.2 – 3152.6]	1837.5 [1622.2 – 2052.8]	0.0009	0.028
Hypoxanthine	8.18	hip	33.7 [27.8 – 39.5]	54.8 [44.2 – 65.5]	0.003	0.110
Dimethylsulfone	3.15	knee	139.8 [104.0 – 175.5]	71.8 [50.1 – 93.5]	0.005	0.167
Tyrosine	7.19	knee	254.8 [207.6 – 301.9]	166.8 [139.0 – 194.6]	0.006	0.183
Lysine	3.03	knee	608.2 [540.3 – 676.0]	483.4 [444.0 – 522.8]	0.006	0.200
Pyruvate	2.37	knee	529.8 [444.3 – 615.3]	350.6 [259.4 – 441.8]	0.010	0.339
Valine	1.04	knee	1506.4 [1219.2 – 1793.7]	1047.8 [924.1 – 1171.6]	0.012	0.384
Glucose	5.23	knee	4113.4 [3448.2 – 4778.6]	2742.3 [2000.9 – 3483.6]	0.013	0.436
Choline	3.19	hip	160.5 [130.2 – 190.8]	254.8 [203.7 – 305.9]	0.014	0.461
Histidine	7.05	knee	166.3 [139.2 – 193.4]	125.7 [113.6 – 137.9]	0.017	0.566
Alanine	1.47	knee	2666.4 [2121.0 – 3211.8]	1829.8 [1452.1 – 2207.5]	0.023	0.751


(a)
L-Lysine
Biocytin
Biotin
Hypoxanthine
Glycosaminoglycan
Biotinyl-5'-AMP
GDP
GMP
IMP
RNA
ADP
ATP
Saccharopine
L-Histidine
beta-Alanyl-L-lysine
Homocarnosine
Carnosine
beta-Alanine
AMP
UDP
NAAG2
2-Oxoglutarate
2-Oxoglutaramate
NAAG
N-Acetyl-L-aspartate
L-Asp
Adenylosuccinate
UDP-galactose
GABA
Glutamate
L-Glutamine
NH3
D-Glucosamine phosphate
Neuberg ester
Acetyl adenylate
Dephospho-CoA
S-Succinyldihydrolipoamide-E
beta-Citrylglutamate
S-(2-Methylpropanoyl)-dihydrolipoamide-E
N-Acetyl-D-glucosamine 6-phosphate
Robison ester
Isocitrate
LTC4
Fumarate
N-Acyl-L-aspartate
Lactose
3-Oxopropanoate
Acetate
Dihydrolipoamide-E
Acetylcholine
3-Carboxy-1-hydroxypropyl-ThPP
GSH
Lipoamide-E
LTA4
Fumarylacetoacetate
Citrate
CoA
Isobutyryl-CoA
Arachidonyl-CoA
gamma-Glutamylcysteine
L-Valine
S-Acetyldihydrolipoamide-E
Arachidonate
D-Glucose
Oxaloacetate
4-Maleylacetoacetate
alpha-Linolenoyl-CoA
Choline
2-Methyl-1-hydroxypropyl-TPP
Gly
2-Ketovaline
Acetyl-CoA
Linoleoyl-CoA
Homogentisate
Linoleate
Phosphocholine
TPP
Malate
3-Fumarylpyruvate
2-Hydroxyethyl-ThPP
R-COOH
Lecithin
L-Cysteine
L-Alanine
Pyruvate
GM3
Ceramide
Cysteinyldopa
Neu5Ac
Glucosylceramide
Serine
3-Acylpyruvate
Dopaquinone
Dehydroalanine
LacCer
4-Hydroxyphenylpyruvate
L-Dopa
Phosphatidylserine
Fatty acid
L-Diiodotyrosine
L-Tyrosine
3-Iodo-L-tyrosine

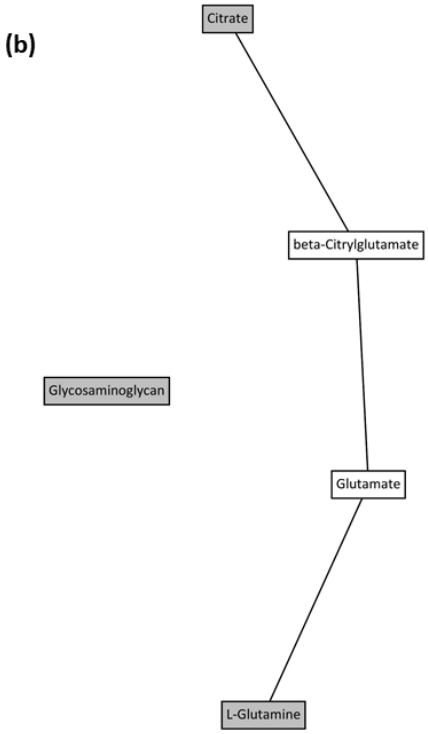


Figure 28. Network of all the identified metabolites illustrating the potential metabolic pathways. ***a.*** *Network analysis of all the identified metabolites. The two metabolites found in greater concentrations in the hip group relative to the knee group have a red border. The remaining grey metabolites were raised in the knee group compared to the hip group.* ***b.*** *Network analysis of the remaining metabolites following FDR correction. This network highlights a pathway connecting citrate to glutamate via two intermediate metabolites. Glycosaminoglycans could not be mapped to any human pathway in the KEGG database.*

5.6 Discussion

Synovial fluid of hips and knees with end-stage OA (ESOA) were examined for small metabolites using NMR spectroscopy. The metabolic profile of the 2 groups were discriminated with O-PLS-DA, and significant differences in 14 metabolites were found between the hip and knee OA groups. Twelve metabolites (*alanine*, citrate, *dimethylsulfone*, *glucose*, *glutamine*, *glycosaminoglycans* (*GAGs*), *histidine*, *lysine*, *N-acetylated* species, *pyruvate*, *tyrosine* and *valine*) were found in greater proportion in the knee group. Two metabolites (*choline* and *hypoxanthine*) were found in greater proportion in the hip group. Following FDR adjusted p-values, only 4 metabolites remained significant (*N-acetylated* molecules, *GAGs*, *citrate* and *glutamine*), which were all found in greater quantities in the knee group. This is an expected outcome related to the small number of samples in this study compared with the number of detected metabolites. These 4 metabolites, which remained significant following FDR adjustment are the most promising putative biomarkers identified in this study.

The purpose of the FDR analysis was to ensure that any differences detected are unlikely to be due to chance. However, this may lead to false negative results when the sample number is

low. Consequently, all of the 14 identified metabolites are considered in the discussion. These may also contribute to the understanding of the disease processes.

This is the first study to examine the metabolic differences in HSF between the hip and knee joints in patients with ESOA. All of the identified metabolites can broadly be grouped into those involved in collagen and proteoglycan (PG) degradation, the *tricarboxylic acid* (*TCA*) cycle, BCAA and lipid metabolism.

5.6.1 Analysis of metabolite differences following FDR correction

Table 12 lists the possible functions of the four metabolites, which remained significant following FDR adjustment.

Table 12. List of identified metabolites, the joint in which they are found in significantly greater concentrations and the function of the metabolites in osteoarthritis. GAGs – glycosaminoglycans; NO – Nitrous Oxide; PG – proteoglycan; TCA tricarboxylic acid.

Metabolite ID	Joint containing larger quantity of metabolite	Function of metabolite	Reference
N-Acetylated molecules	knee	Marker of *PG* breakdown & cartilage degradation.	Schiller et al(241)
GAGs	knee	Marker of articular cartilage breakdown.	Thompson et al(242)
Citrate	knee	Major intermediary in the *TCA* cycle, urea cycle, amino acid & *fatty acid* metabolism. Role in altered energy metabolism with elevated levels suggesting altered oxidative metabolism in diseased joints.	Berg et al(243) Kim et al(236)
Glutamine	knee	Key role in oxidative metabolism. Elevated levels suggest altered oxidative metabolism in diseased joints. It also suppresses the formation of inflammatory cytokines and protects chondrocytes from NO induced apoptosis and heat stress.	Handley et al(200) Kim et al(201) Tonomura et al(202)

GAGs are commonly found in the extracellular matrix (ECM) of articular cartilage(244). They attach to a core protein leading to the formation of *PGs*. They have an important function in helping to maintain the turgor pressure of articular cartilage(245). Therefore, the greater concentration of *GAGs* present in the knee group suggests an increased rate of articular

cartilage catabolism compared to the hip group. This results in altered mechanical and tribological responses, culminating in matrix degradation(242). Furthermore, as the principal *N-acetyl-glucosamine* containing polymer in SF is *hyaluronic acid* (HA), greater rates of *PG* and hence cartilage degradation are suggested by the greater intensity of *N-acetylated* molecule signal in the knee group.(241).

Citrate is a major intermediary in amino acid and *fatty acid* metabolism, the *TCA* and urea cycles(243). The *TCA* cycle has an important role in providing cellular energy through *Adenosine Triphosphate* (*ATP*) via a biochemical pathway, which involves oxidative conversion of carbohydrates. The majority of the carbon contributes to the *TCA* cycle through the conversion of *glucose* to *pyruvate*. Furthermore, *glutamine* provides a contribution to the *TCA* intermediary *alpha-ketoglutarate*. Since it also has an important function in oxidative metabolism, elevated levels suggest that diseased joints may have alterations in their oxidative metabolism(200). The results in this study agree with those in the published literature, demonstrating that OA patients have alterations in the metabolic profile of their SF, which suggests a possible role for altered energy metabolism in OA(236, 237, 239). Furthermore, as the *TCA* cycle functions within the mitochondria itself, the greater abundance of *TCA* intermediaries within the knee group could be related to mitochondrial dysfunction (246).

5.6.2 Analysis of the remaining metabolite differences

The functions of the remaining metabolites will be considered in this section, as they may provide useful insight into the OA disease process.

5.6.3 Metabolites with increased concentrations in knee synovial fluid

Eight further metabolites (*alanine*, *dimethylsulfone*, *glucose*, *histidine*, *lysine*, *pyruvate*, *tyrosine* and *valine*) were found in greater proportion in the knee group (table 13).

Alanine and *tyrosine* are non-essential amino acids (NEAA). *Alanine* is known to inhibit *pyruvate* kinase, thus regulating gluconeogenesis and glycolysis ultimately leading to increased *glucose* production(247). Using H^1-NMR spectroscopy, *tyrosine* has been detected in greater proportions in the SF of Ovine with early OA(203). Studies have also demonstrated that *alanine* is increased in the synovium, SF and urine of OA animal models(139, 248, 249). Recently, Yang et al demonstrated an increase of *tyrosine* in the subchondral bone of 42 OA patients (250).

Tyrosine is also thought to have a role in promoting osteophyte formation(204). Both *tyrosine* and *alanine* were found in greater levels in the knee group. This may suggest more severe subchondral sclerosis in the knee group.

Dimethylsulfone is a metabolite commonly found in plasma. An ovine study demonstrated increased levels following meniscal destabilisation(248). Its role in OA is unclear as it has previously been investigated as a potential therapeutic agent in OA(251) and has also been demonstrated to possess anti-inflammatory properties in the chondrocyte cell lineage(252).

Histidine is an essential amino acid (EAA). This study demonstrated greater levels of *histidine* in the knee group. However, a number of other studies looking specifically at knee OA patients demonstrated lower histamine levels(233, 234, 237). *Histidine* is known to be metabolised into histamine, which is responsible for stimulating the metabolism of articular chondrocytes into clusters, a known feature of osteoarthritic cartilage(253). Although the results in this chapter demonstrated increased levels of *histidine* in the knee group, this may represent its role as a precursor for articular chondrocyte metabolism. Further studies are required to confirm this hypothesis. The ratio of BCAA to *histidine* has also been suggested as a biomarker for OA(234).

Lysine is an EAA. Schiller et al performed a study looking at bovine and guinea pig nasal cartilage treated with collagenase. They demonstrated increased levels of *lysine*, along with *glutamate*, *glycine*, *hydroxyproline* and *proline*, in bovine-collagenase-treated cartilage(241). As these amino acids are abundant in collagen, their presence suggests increased collagen breakdown, and in the context of this study may represent a greater degree of articular cartilage breakdown in the knee group.

Valine is a BCAA and the results demonstrated greater levels in the knee group. This could suggest a greater rate of collagen degradation compared to the hip group. Furthermore, BCAAs increase the production of cytokines including IL-1, IL-2, interferon and tumour necrosis factor(254). As increased concentrations of cytokines have been associated with OA(255, 256), it is possible that increased levels of BCAAs lead to the production of cytokines that ultimately leads to the destruction of articular cartilage. A subsequent study demonstrated increased serum BCAA correlated with greater severity of knee OA(257). Both studies suggest a role for altered BCAA metabolism in OA that may prove to be a marker for disease progression.

Consequently, the greater levels of *valine* in the knee group may represent a greater severity of OA in this cohort.

5.6.4 Metabolites with increased concentrations in hip synovial fluid

Two metabolites (*choline* and *hypoxanthine*) were found in greater proportion in the hip group. *Choline* was found in significantly greater quantities in the hip group compared to the knee group. *Choline* is a building block of important *phospholipid* classes, namely *phosphatidylcholines* and *sphingomyelins*. Articular cartilage is covered by a thin layer of *phospholipid* that is thought to contribute to the lubrication(258) and load bearing functions of the joint(259). Lipid metabolism has significant clinical relevance with emerging evidence for a link between OA and the use of statins. OA is known to have a variable inflammatory component, but it is still unclear whether this is a response to pathological change or a driving factor in the disease process(187, 260). An equine study demonstrated increased levels of *high-density lipoprotein* (*HDL*), *choline* and unsaturated *fatty acids* in osteoarthritic SF(240). Young et al examined the metabolic profile of serum collected from patients with established rheumatoid arthritis (RA) and early OA patients. They attempted to correlate their findings with inflammatory markers, such as CRP, and demonstrated differences in *phospholipid* metabolism with elevation of *cholesterol*, *choline*, *fatty acids* and *low-density lipoproteins* (*LDLs*)(261). Together with the known inflammatory component of OA, these findings suggest a potential role for lipids as a biomarker for OA.

Hypoxanthine is an *oxypurine* and has a role in purine metabolism. Animal studies have suggested a role for *hypoxanthine* in the osteoarthritic process(203, 249). Zhang et al compared the SF of 40 patients with knee OA to 20 controls, and amongst other things, found an increase in *hypoxanthine* in the OA group(237). Studies have also demonstrated increased levels of *hypoxanthine* in the SF of RA and inflamed knee joints(262, 263).

Table 13. List of identified metabolites, which were no longer significant following FDR adjustment of the p-values. The joint in which they are found in significantly greater quantities and the function of the metabolites in osteoarthritis has been described. TCA – Tricarboxylic acid; SF – synovial fluid; RA – rheumatoid arthritis; BCAA – branched chain amino acid; EAA – essential amino acid; LDLs – low-density lipoproteins.

Metabolite ID	Joint containing larger quantity of metabolite	Function of metabolite	Reference
Dimethylsulfone	Knee	An ovine study demonstrated increased levels following meniscal destabilisation. Its role in OA is unclear as it has previously been investigated as a potential therapeutic agent in OA.	Maher et al(248) Hadipour-Jahromy et al(251)
Tyrosine	Knee	Detected in greater proportions in SF of Ovine with early OA. Thought to have a role in promoting osteophyte formation. Increased levels in subchondral bone.	Mickiewicz et al(203) Xu et al(204) Yang et al(250)
Lysine	Knee	An EAA associated with collagen & cartilage degradation.	Schiller et al(241)
Pyruvate	Knee	The majority of carbon contributes to the *TCA* cycle through the conversion of *glucose* to *pyruvate*. Elevated levels suggest altered oxidative metabolism in diseased joints.	June et al (264)
Valine	Knee	One of the BCAAs. Associated with greater severity of knee OA. Increased levels may also be secondary to collagen degradation or suggest an increased rate of protein breakdown. Also increase the production of cytokines including IL-1, IL-2, interferon and tumour necrosis factor.	Adams et al (257) Bassit et al (254)
Glucose	Knee	Major source of energy for chondrocytes. Its metabolism involves glycolysis, the pentose phosphate pathway (PPP) and the *TCA* cycle, which is responsible for a number of precursors to amino acids. The majority of carbon contributes to the *TCA* cycle through the conversion of *glucose* to *pyruvate*. Elevated levels suggest altered oxidative metabolism in diseased joints.	Peansukmanee et al (265) June et al (264)
Histidine	Knee	Metabolised into histamine, which is responsible for stimulating the metabolism of articular chondrocytes into clusters, a known feature of osteoarthritic cartilage. The ratio of BCAA to *histidine* has also been suggested as a biomarker for OA.	Tetlow et al(253) Zhai et al(234)
Alanine	Knee	Increased in the synovium, SF & urine of OA animal models. Also inhibits *pyruvate* kinase, thus regulating gluconeogenesis & glycolysis ultimately leading to increased *glucose* production(247).	Lamers et al(249) Maher et al(248) Hugle et al(139) Wu et al(247)
Hypoxanthine	Hip	Role in purine metabolism. Both animal and human studies have suggested a role for *hypoxanthine* in the osteoarthritic process. Increased levels of *hypoxanthine* have also been shown in the SF of RA and inflamed knee joints.	Lamers et al(249) Mickiewicz et al(203) Zhang et al(237) Gudbjornsson et al(262) Herbert et al(263)
Choline	Hip	Increased levels in osteoarthritic SF. OA is known to have a variable inflammatory component. Differences in *phospholipid* metabolism with elevation of *cholesterol*, *choline*, *fatty acids* and *LDLs* have been demonstrated in patients with early OA and RA.	Lacitignola et al(240) Young et al(261)

A network analysis of all the identified metabolites, including those that did not survive FDR correction, illustrated a number of different pathways involved in hip (*phospholipid* metabolism via *choline*) and knee (amino acids, *TCA* and *glucose* metabolism) osteoarthritic HSF metabolism. This correlates with the potential functions of these metabolites as described above and in table 13.

5.6.5 Possible causes of differences in hip and knee synovial fluid metabolites

It is important to remember that OA is a heterogenous syndrome with various aetiopathogenic phenotypes. Consequently, when considering the pathological processes involved, a more integrated approach is required. The same philosophy should be adopted when considering the metabolic variations between the hip and knee groups. Generally speaking, these differences may be due to or a combination of intra- and extra-articular factors.

As explained in chapter 1, the hip joint has a high level of conformity and has classically been thought of as a ball and socket joint(85). The knee joint however, is much less conforming resulting in greater contact stresses, due to a smaller area of contact between the tibio-femoral articulations. The meniscus present within the knee joint has a number of important functions. One of these functions is to increase the contact area in the knee, thus making the joint more conforming by helping to dissipate the joint forces over a larger surface area, reducing the direct femoral-tibial cartilage contact region (90). However, in severe knee OA, the meniscus loses its ability to function normally due to calcification or meniscal tears, thus reducing its ability to increase joint conformity. As a consequence, the knee joint may have greater point loading resulting in higher contact stresses due to a larger force passing through a smaller area, whereas the increased conformity seen within the hip joint produces a more volumetric wear pattern.

The surrounding tissues within the joint, as well as systemic sources, also contribute to the metabolites found in SF. Synovial inflammation present in OA may have an important role. A number of mediators and pathways may also be responsible, including matrix metalloproteinases and proinflammatory mediators, such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10), which ultimately result in changes to the metabolic profile of SF(266). Increased mitochondrial activity may also be expected in local cells (chondrocytes)

secondary to the presence of osteoarthritic SF (266). Furthermore, SF and plasma bone morphogenic protein-2 (BMP-2) levels have been positively correlated to the severity of knee OA(267). A significantly increased level of cartilage turnover has been demonstrated in the knee compared with the hip joint, suggesting different mechanisms of disease progression between these joints(268). Differences in protein turnover was thought to be the cause for this, more specifically, the attempted cartilage repair response.

Extra-articular factors, which may be responsible for identified metabolic differences include age, BMI and medical co-morbidities. Obesity has been linked to OA in both these joints, but its effect is more profound in the knee joint(269). However, in this study, patients were matched for age and BMI in an attempt to remove these as confounding factors. For the same reason, both groups in this study included patients with comparable medical co-morbidities to reduce their effects as possible confounding factors.

Despite the possibility that all the metabolic differences identified in this study may primarily be a result of the disease process itself or reflect alterations in normal cartilage and SF biology, the likelihood is that the differences observed are represented by a complex interplay of all these factors.

5.6.6 Limitations

The rigorous matching of samples in both groups for age, gender, BMI, medical comorbidities and ethnicity resulted in the overall numbers being small, with 12 patients in each group. However, despite this fact, the validated O-PLS-DA model demonstrated 14 metabolites that were significantly different between the two groups, with four remaining significant following univariate testing and FDR adjustment. Secondly, there were no non-arthritic control groups, although it would not have been possible to acquire such samples due to ethical constraints. Finally, the number of compartments in the knee affected with OA was not considered in this study. However, unpublished analysis from the metabolic profiling lab at Imperial College, London has not identified any metabolic differences between OA affecting one or more compartments of the knee joint (P Akhbari unpublished data).

5.7 Conclusion

To the author's knowledge, this discovery study is the first to indicate that differences exist in the metabolic profile of HSF between the hip and knee joints in patients with end-stage OA. Four metabolites were found in significantly greater proportions in the knee group (*citrate*, *GAGs*, *glutamine* and *N-acetylated* molecules). These metabolites have a role in the *TCA* cycle, collagen degradation and oxidative metabolism in diseased joints.

Further research with a larger cohort of patients and a non-arthritic age and gender matched control group is required to see if the metabolites identified in this study may serve as putative biomarkers for diagnosing OA of the knee, monitoring of disease progression and/or future treatment strategies.

Another area of interest was infection. Therefore, a discovery study was performed looking at the metabolic differences between infected and non-infected HSF.

6 Metabolic differences in the composition of infected and non-infected synovial fluid

An adaptation of this chapter has been published in Bone and Joint Research in January 2021(270).

6.1 Summary

Diagnosing joint infections is an inexact science using combinations of serum inflammatory markers and microscopy, culture and sensitivity of synovial fluid (SF). Small molecule metabolites in infected SF have the potential to act as infection markers that could improve the speed and accuracy of detection. The objective was to use Nuclear Magnetic Resonance (NMR) spectroscopy to identify small molecule differences between infected and non-infected human SF.

Sixteen SF samples (eight infected, eight non-infected) were collected from patients. The metabolites present in each sample were analysed using NMR spectroscopy. Both multivariate and univariate statistical analysis were undertaken to investigate metabolic differences between the two groups.

Sixteen metabolites were identified in significantly different concentrations between the two groups. Three were in higher relative concentrations (*cholesterol*, lipids, and *N-acetylated* molecules) and thirteen were in lower relative concentrations in the infected group (*citrate*, *creatinine*, *dimethylsulfone*, formate, *glucose*, *glutamine*, *glycine*, *glycosaminoglycans*, *histidine*, *lysine*, *mannose*, *proline* and *valine*).

Metabolites identified in significantly greater concentrations in the infected cohort represented those which have a role in the inflammatory response, lipid metabolism and are markers of inflammation and infection. Those identified in significantly reduced concentrations were involved in carbohydrate and nucleoside metabolism, the *glutamate* metabolic pathway, reduced articular cartilage breakdown and increased oxidative stress in the diseased state. This study is the first to reveal differences in the metabolic profile of infected and non-infected

human SF, using a non-infected matched cohort, and may represent putative biomarkers that form the basis of new diagnostic tests for infected SF.

6.2 Introduction

Periprosthetic joint infection (PJI) remains one of the most devastating complications of joint arthroplasty affecting between 0.7% and 2.4% of arthroplasty patients(100). It is also one of the commonest indication for revision arthroplasty(102). Native joint infections are also problematic and, left untreated, can lead to irreversible degenerative changes in the joint. As with other infections, it can also become systemic resulting in increased patient morbidity and a risk to life. The rapid identification of bacterial species in synovial fluid (SF) is vital for a number of reasons. It aids in the diagnostic process, provides valuable information to help identify the probable source of infection and can recognise any potential antibiotic resistance leading to suitably targeted antibiotic therapy(137).

As discussed in chapter 1, the gold standard for identifying infecting organisms in both PJI and septic arthritis remains microscopy, culture and sensitivity (MC&S). However, the increased prevalence of polymicrobial infections and PJIs secondary to biofilm producing bacteria and slow growing, fastidious organisms makes the diagnosis of the infective organism(s) increasingly challenging(130). Serological markers of infection, which include Erythrocyte Sedimentation Rate (ESR) and C-Reactive Protein (CRP), have commonly been used as screening tests for PJI, due to their low cost, relatively high sensitivity and the ease in which they can be obtained. The widely accepted threshold values for ESR and CRP are 30mm/hr and 1mg/dl respectively(106). However, they have a low specificity and are often found elevated in non-infectious inflammatory conditions and the early postoperative period.

Many other markers are still being investigated as potential complementary or alternative markers of infection. Amongst these are interleukin-6, tumour necrosis factor alpha, procalcitonin(117), D-Dimer(121) and Intercellular adhesion molecule-1(122). However, the most promising biomarkers remain Alpha-defensin (α-defensin) and leucocyte esterase, with α-defensin being claimed to be the single most accurate biomarker for PJI(124). A pooled diagnostic sensitivity and specificity, from six studies, of 1.00 (95% confidence interval [CI] 0.82

– 1.00) and 0.96 (95% CI 0.89 – 0.99) was identified in one particular systematic review and meta-analysis(124). However, mixed results have been demonstrated for Synovasure, the on-table quick lateral flow test kit by Zimmer Biomet (Warsaw, Indiana), with one meta-analysis having shown it to be significantly less accurate than the α-defensin laboratory immunoassay(126). Therefore, its results must be interpreted carefully. The Leukocyte esterase colorimetric strip test has also been shown to provide improved accuracy of diagnosis and more expeditious management of the underlying infection, with one systematic review and meta-analysis of five studies revealing a pooled diagnostic sensitivity and specificity of 0.81 (CI 0.49 – 0.95) and 0.97 (CI 0.82 – 0.99)(124).

A number of recent articles have provided useful summaries of some of the serum biomarkers for PJI and also demonstrate a shift towards proteomics and genomics as important techniques in identifying putative biomarkers(271, 272). However, the role of molecular techniques in identifying putative biomarkers in infected SF is less clearly defined. They are a promising frontier in the diagnosis of both PJI and septic arthritis and are particularly suited for diagnosing PJI caused by a biofilm. Furthermore, they 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. It has been utilised in many conditions to influence clinical practice (273). It is able to analyse hundreds or even thousands of small molecules and mobile moieties within macromolecules, such as glyco- and lipo-proteins, simultaneously, which is what gives it such great potential. Not only can this provide individual biomarkers for a specific pathological process, but also identify a unique metabolic “signature” made up of numerous metabolites that may identify a distinctive pathology based on the relative concentrations of these molecules.

In contrast to MS, NMR spectroscopy can be performed more rapidly, requires little sample preparation and does not involve destruction of the individual samples. Few NMR spectroscopy studies have been performed to date looking for biomarkers of SF infection(138-140), with all of those performed containing unmatched cohorts or results that are not statistically significant.

6.3 Aims and hypothesis

1. To investigate the metabolic profile of human SF (HSF) from infected and non-infected HSF using NMR spectroscopy.
2. To assess metabolic differences between the fluids using multivariate and univariate statistical analysis.
3. To evaluate the metabolic pathways involved that are implicated in the metabolic differences between infected and non-infected HSF.

6.4 Materials and Methods

The methodology used for this experiment is detailed in chapter 4. In summary, the samples were all centrifuged for 15 minutes and the supernatant was aliquoted, before the samples were stored at -80^{o}C. Following defrosting, all samples were mixed with buffer solution in a one to one ratio before undergoing NMR spectroscopic analysis. The data was then transformed before univariate and multivariate analysis took place. Finally, the metabolic differences between the infected and non-infected groups were identified. The results are detailed below.

6.5 Results

6.5.1 Patient Demographics

The patient demographics have been summaries in table 14. No significant differences were identified between the two groups in terms of age ($p=0.223$), gender or common medical co-morbidities.

Table 14.Patient demographics. There was no significant difference in age (p=0.22), gender, or medical co-morbidities between the groups. IHD, Ischaemic heart disease; DM, Diabetes Mellitus; CVA, Cerebrovascular Accident; TKR, total knee replacement; THR, total hip replacement; SF, synovial fluid; S aureus, staphylococcus aureus; strep, streptococcus; sp, species; coag neg, coagulase negative. There was no elbow non-infected control and therefore a knee control was used instead.

	Infected SF	Non-infected SF (control group)	P value
Number of patients	8	8	-
Joint involved	Elbow 1 Knee 3 TKR 2 THR 2	Knee 4 TKR 2 THR 2	-
Responsible organism	Coag positive *S. Aureus* (n=1) *Group B Meningococcus* (n= 2) *B-haemolytic strep group C* (n=1) *Bacillus sp* (n=1) *Rothia sp* (n=1) *Coag negative S. Aureus* (n=2)	-	-
Age (Mean ± SD)	66.6 (12.0)	58.9 (11.6)	0.223
Gender (Male: Female)	6:2	6:2	-
Ethnicity	5 Caucasian 2 Asian 1 Afro-Caribbean	3 Caucasian 2 Asian 2 Afro-Caribbean	-
Disease (Number of patients per group)			
IHD	0	1	1.00
Hypertension	3	2	1.00
Hypercholesterolemia	0	2	0.48
DM	1	2	1.00
CVA	1	0	1.00

6.5.2 PCA analysis of differences between infected and non-infected metabolites

Separation between the ^{1}H-NMR spectra of the infected and non-infected groups was demonstrated in PC1 on the PCA scores plot (figure 29), although there still remained some overlap between the groups. This led to the suggestion that metabolic differences may exist in the HSF composition between the two groups. These differences were independent of the joint type from which the fluid was taken, as was demonstrated in an additional PCA scores plot with the involved joints labelled (figure 30). Furthermore, a third PCA scores plot containing four subgroups (native and prosthetic non-infected SF plus native and prosthetic infected SF) was created (figure 31), which demonstrated separation of the infected and non-infected groups,

that appeared to be independent from the type of joint (native or prosthetic). However, the O-PLS-DA model obtained was not significant following cross-validation. This result contrasts with the PCA results and may reflect the low ratio of samples to variables and a lower strength of metabolite variation between groups. Therefore, univariate analysis was conducted to investigate the differences between the groups shown by the PCA scores plot(274).

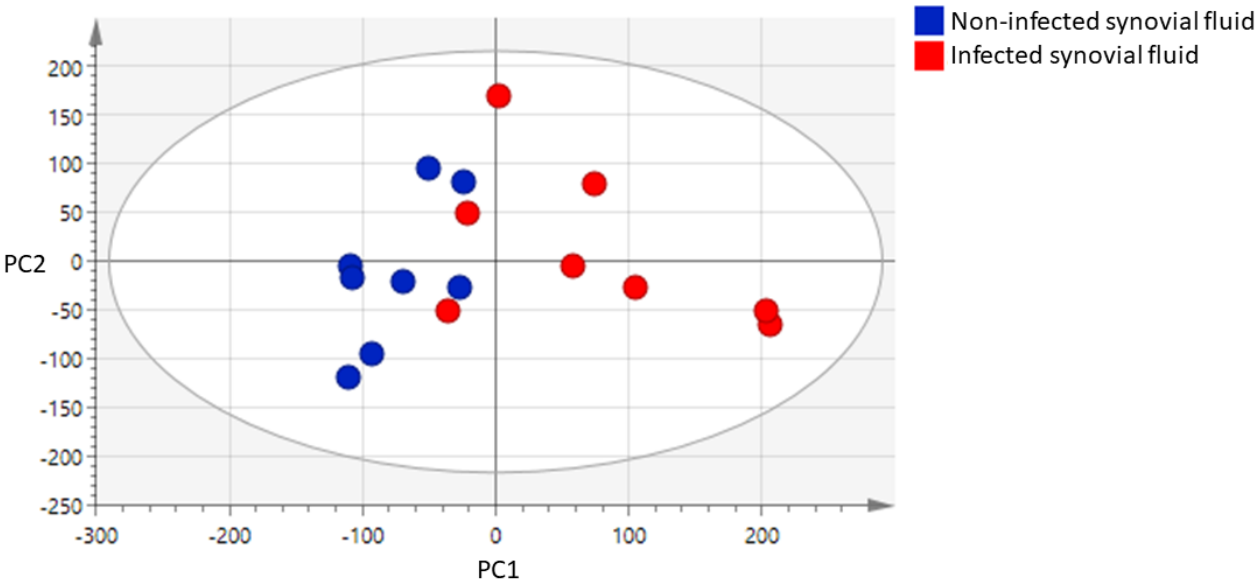


Figure 29. PCA scores plot of PC1 vs PC2 with each data point representing the NMR spectrum of an individual human synovial fluid sample and demonstrating separation between the infected and non-infected groups. Data were scaled to unit variance. The percentage variation explained is 38% for PC1 and 21% for PC2. PC – principal component.

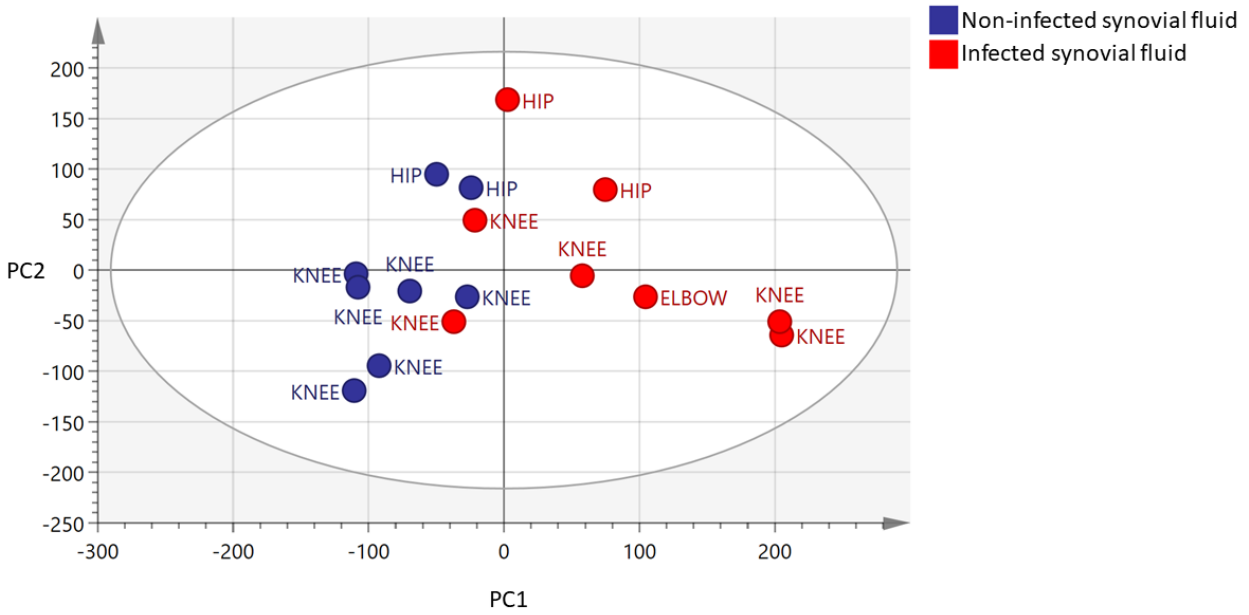


Figure 30. PCA scores plot of PC1 vs PC2 with each data point representing the NMR spectrum of an individual human synovial fluid sample and demonstrating separation between the infected and non-infected groups. Data were scaled to unit variance. The percentage variation explained is 38% for PC1 and 21% for PC2. The joint from which the synovial fluid was taken has also been labelled for each sample. This demonstrates that the separation was independent of the joint type from which the fluid was taken. PC – principal component.

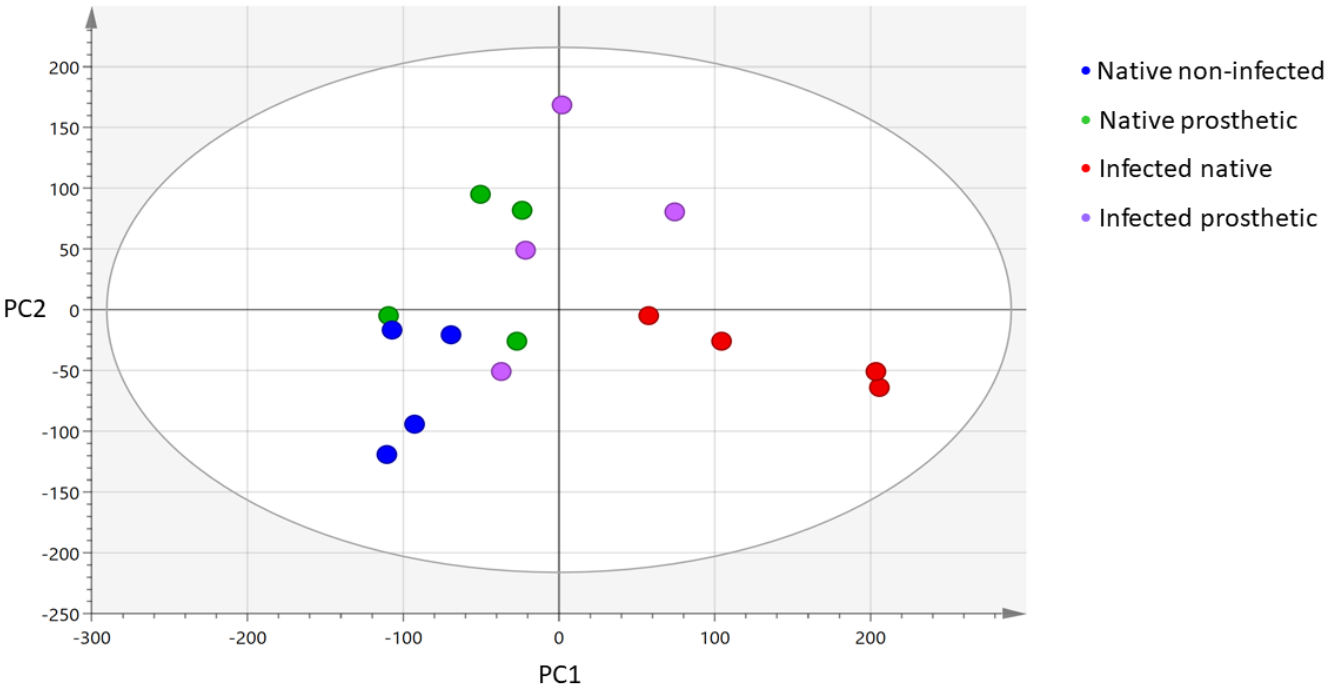


Figure 31. PCA scores plot of PC1 vs PC2 with each data point representing the NMR spectrum of an individual human synovial fluid sample and demonstrating that the tendency for separation is driven by the presence of infection rather than joint type (native or prosthetic). Data were scaled to unit variance. The percentage variation explained is 38% for PC1 and 21% for PC2. PC – principal component.

6.5.3 Univariate analysis and metabolite identification

Table 15 lists all the metabolites, which were consistently identified from the NMR spectra of all the samples. The Student's t-test was used to test the integral of each metabolite peak, which is proportional to the concentration of each metabolite. Following univariate analysis, 16 metabolites remained significantly different between the groups, and these are illustrated in figure 32. Following FDR correction, only one metabolite, *citrate* (decreased in the infected group) remained significant (Table 16). 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 (274). Therefore, all the identified metabolites were considered in the analysis. This is clearly illustrated in Figure 32, where signals from *NAc-1* and *GAGs* for example are evidently different between the groups but were no longer significant following FDR correction. Therefore, all metabolites, which remained significant following univariate analysis will be summarised and discussed.

Table 15. Metabolites from both infected and non-infected synovial fluid consistently identified in all samples. GAGs – glycosaminoglycans; S – singlet; bs – broad singlet; d – doublet; dd – doublet of doublet; q – quartet; t – triplet; m – multiplet; ppm – parts per million.

Metabolite	Chemical shift (ppm)
Cholesterol (C18)	0.58(bs), 0.65(bs)
CH3 (fatty acyl residues, lipids)	0.85(bs)
CH2 (fatty acyl residues, lipids)	1.25(bs)
Isoleucine	0.93(t), 1.00(d), 1.25(m), 1.46(m), 1.96(m), 3.66(d)
Leucine	0.95(d), 0.96(d), 2.00(m)
Valine	0.98(d), 1.03(d), 2.26(m), 3.60(d)
3-Hydroxyisobutyrate	1.06(d), 2.48(m)
3-Hydroxybutyrate	1.19(d), 2.35(dd), 4.14(m)
Lactate	1.32(d), 4.10(q)
Alanine	1.47(d), 3.78(q)
Lysine	1.47(m), 1.71(m), 1.89(m), 3.03(t), 3.72(t)
Arginine	1.69(m), 1.90(m), 3.23(t)
Acetate	1.91(s)
GAGs	2.02(s), 3.34(bs), 4.46(bs), 7.97(bs)
N-acetyl groups (Glycoproteins)	2.04(bs)
Glutamate	2.06(m), 2.34(m)
Acetone	2.22(s)
Pyruvate	2.37(s)
Glutamine	2.12(m), 2.45(m), 3.74(dd)
Citrate	2.53(d), 2.67(d)
Creatine	3.03(s), 3.91(s)
Creatinine	3.04(s), 4.05(s)
Dimethylsulfone	3.14(s)
Choline	3.19(s)
Glucose	3.23(dd), 3.46(dd), 3.49(t), 3.53(dd), 3.71(t), 3.72 (dd), 3.76(dd), 3.82(m), 3.85(m), 3.90(dd), 4.64(d), 5.23(d)
Proline	3.34 (m)
Glycine	3.55(s)
Mannose	5.18(d)
CH=CH (fatty acyl residues, lipids)	5.28(bs)
Tyrosine	6.89(d), 7.19(d)
Histidine	7.05(s), 7.77(s)
Formate	8.45(s)

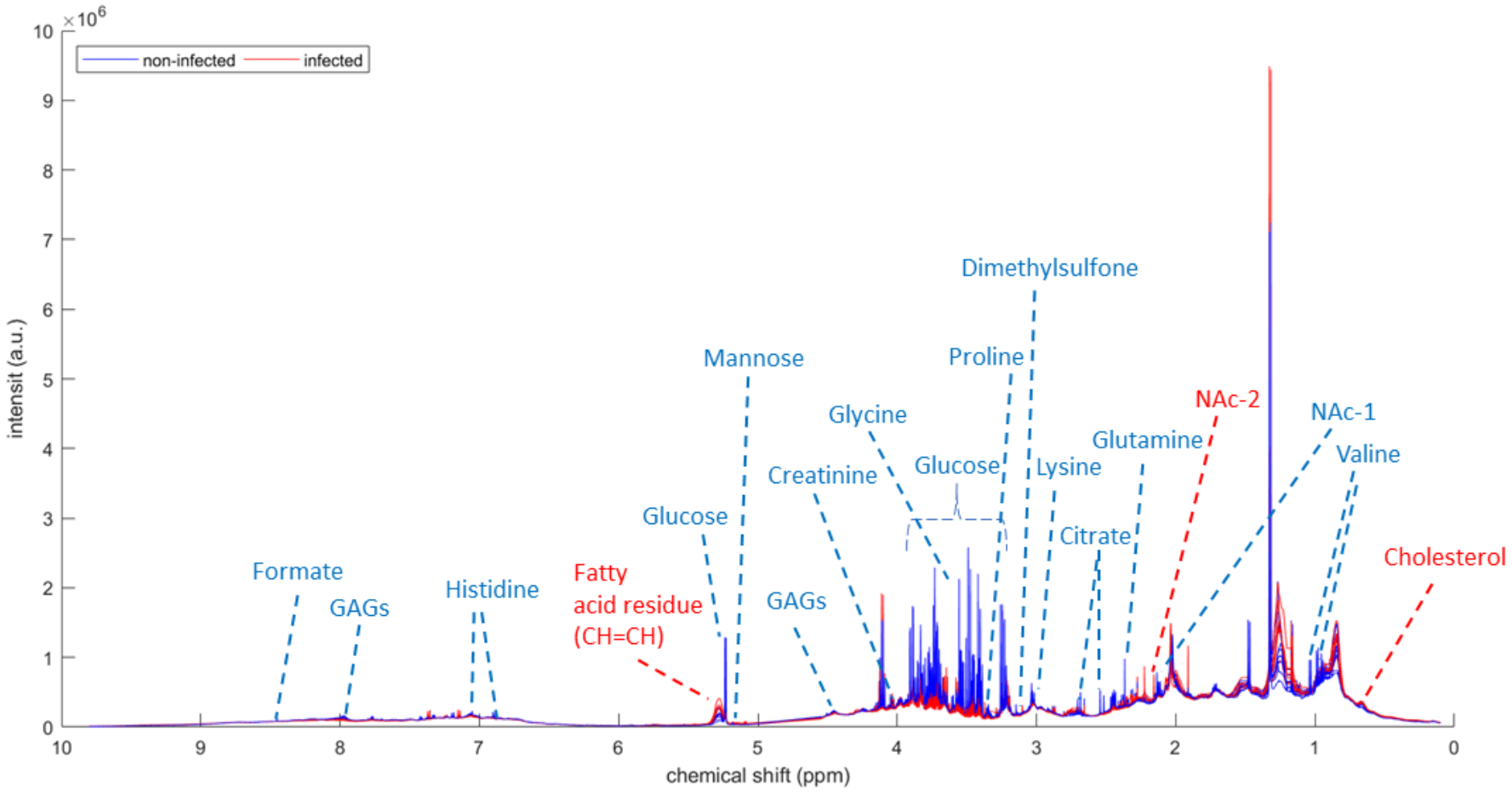


Figure 32. Stacked spectra of all sixteen samples demonstrating the significant metabolites, with those in red having a higher concentration in the infected group and those in blue having a higher concentration in the non-infected group. The CH=CH group represents protons from the unsaturated fatty acid residues. NOESY spectra acquired in a spectrometer operating at frequency of 600 MHz for ^{1}H. GAGs – glycosaminoglycans; ppm – parts per million; NAc – N-acetylated group.

Table 16. 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. HSF – human synovial fluid; GAGs – glycosaminoglycans; ppm – parts per million; NAc – N-acetylated group.

Metabolite ID	NMR spectra chemical shift (ppm)	Higher in infected/non-infected HSF	*p*-value (Student's T-test)	FDR
Citrate	2.53	Non-infected	0.00137	0.041093
Glycine	3.55	Non-infected	0.001775	0.053242
GAGs	7.97	Non-infected	0.002503	0.07508
NAc-1	2.02	Non-infected	0.004712	0.141364
Creatinine	4.05	Non-infected	0.007372	0.221156
Lysine	3.03	Non-infected	0.008382	0.251463
Histidine	7.05	Non-infected	0.010865	0.325937
Formate	8.45	Non-infected	0.011967	0.35901
Glucose	5.23	Non-infected	0.01497	0.449114
Fatty acyl residues ***(CH=CH)***	5.28	Infected	0.016659	0.499766
Cholesterol (C18)	0.65	Infected	0.022633	0.679
Proline	3.34	Non-infected	0.023079	0.692367
Valine	1.035	Non-infected	0.027658	0.829732
Dimethylsulfone	3.15	Non-infected	0.038742	1.00
Mannose	5.18	Non-infected	0.038921	1.00
Glutamine	2.45	Non-infected	0.039291	1.00
NAc-2	2.04	Infected	0.049718	1.00

Significantly higher relative concentrations of *N-acetylated groups* (based on a *NAc* peak, *NAc-2*), *cholesterol* and *cholesteryl esters* from lipoproteins (based on the C18 methyl peak) and *fatty acyl* residues (based on the CH=CH peak), were found in the infected SF group compared to the non-infected group. Conversely, significantly lower levels of *citrate*, *creatinine, dimethylsulfone*, *formate*, *glucose*, *glutamine*, *glycine*, *GAGs* (based on a broad amide resonance), *histidine*, *lysine*,

mannose, *N-acetylated groups* (based on a different *N-acetyl* peak, *NAc-1*), *proline* and *valine* were found in the infected group compared to the non-infected group.

The intensity of the broad peak at 7.97 ppm, attributed to the amide protons of *GAGs*, was highly correlated with the intensity of the *NAc-1* peak. Consequently, *NAc-1* was not considered as a separate metabolite in the subsequent analysis as it may have originated 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(275). A subgroup analysis of prosthetic and native joints in both the infected and non-infected groups revealed lower concentrations of *GAGs* in the infected group, which was independent of whether the joint was prosthetic or native (figure 33).

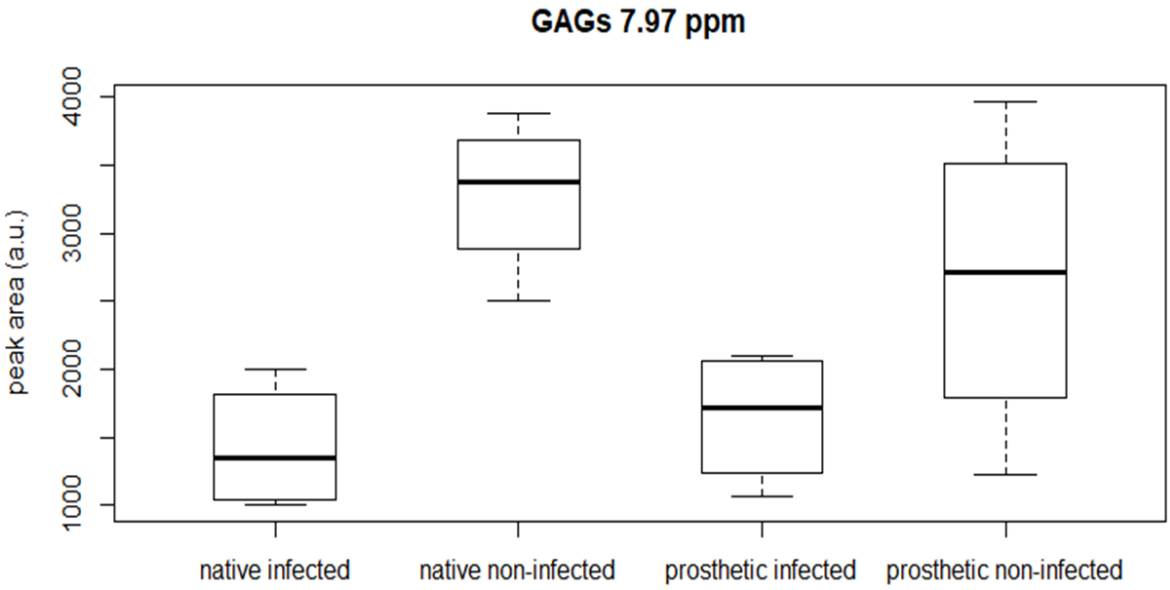


Figure 33. Boxplot of the area under the curve of the GAGs peak at 7.97 ppm. The samples are grouped according to the type of joint and presence of infection. This demonstrates lower concentrations of GAGs in the infected groups, which appears independent of whether the joint was native or prosthetic. GAGs – glycosaminoglycans.

6.5.4 Evaluation of individual spectra

Figure 34 demonstrates two identified metabolites found in significantly lower concentration in the infected group (*GAGs* and *citrate*). These can be compared to the individual spectral analysis of the three metabolites, *N-acetylated groups* (based on a *NAc* peak, *NAc-2*), *cholesterol* and *cholesteryl esters* from lipoproteins (based on the C18 methyl peak) and *fatty acyl* residues (based on the CH=CH peak) found in higher concentration in the infected group. This figure nicely demonstrates the spectral analysis of these metabolites and how their concentrations differ between the two groups.

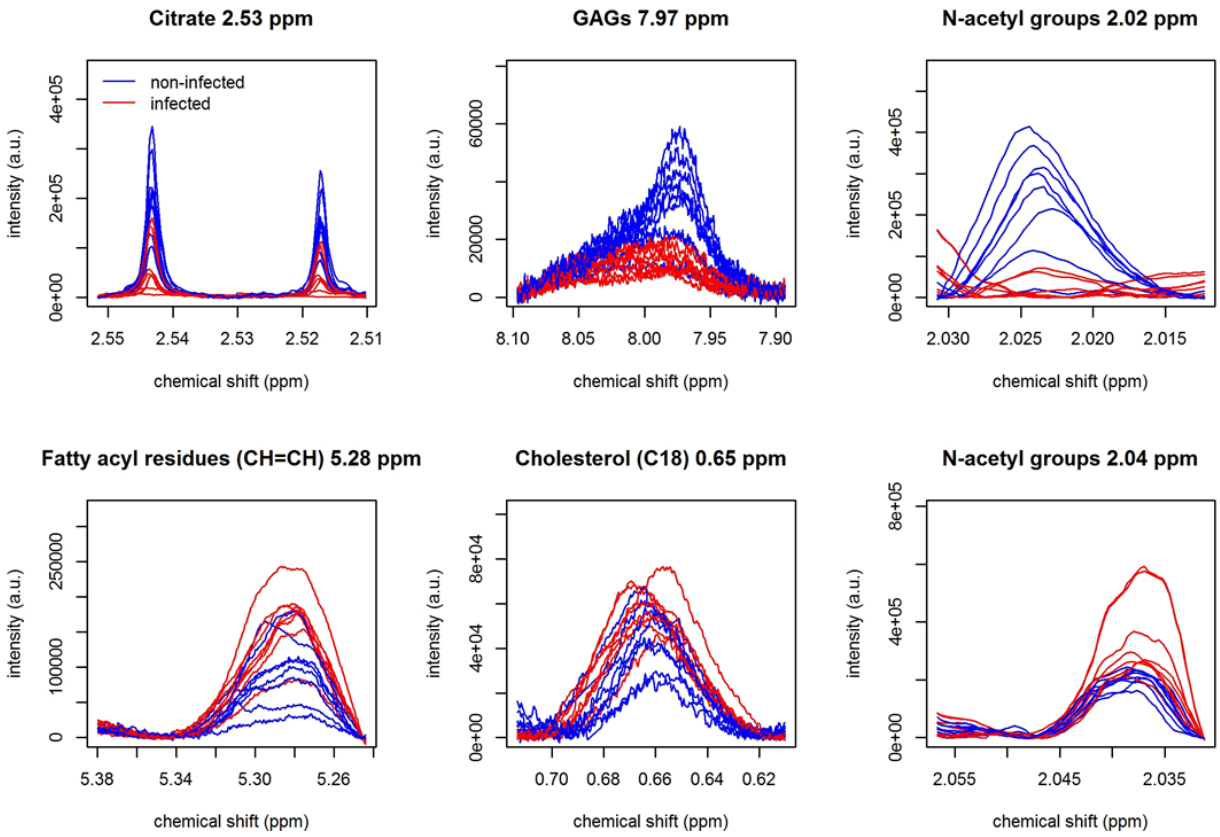


Figure 34. Metabolite analysis from spectral inspection. The figure demonstrates differences in signal intensity between the infected (red) and non-infected (blue) synovial fluid samples. Citrate and GAGs are included as examples of metabolites found in significantly lower concentrations in the infected compared to the non-infected group. GAGs were considered false negatives after FDR, although visually and following statistical testing, differences can be seen between the two groups. Fatty acyl residues, cholesterol and N-acetylated groups were found in significantly greater quantities in the infected compared to the non-infected group. FDR – false discovery rate; GAGs – glycosaminoglycans; NAc – N-acetylated group; ppm – parts per million.

The remaining *fatty acyl* residues in the stacked spectra were also pictured to identify any difference between the cohorts. The peak at 0.85 ppm from *fatty acyl* CH_3 groups had a p value of 0.068 and the peak at 1.26 ppm from *fatty acyl* CH_2 groups gave a p-value of 0.051 (Figure 35). Whilst these values remained insignificant at the arbitrarily chosen p cut-off value, they showed the same trend and reinforced the assignment as lipids (*fatty acyl* residues).

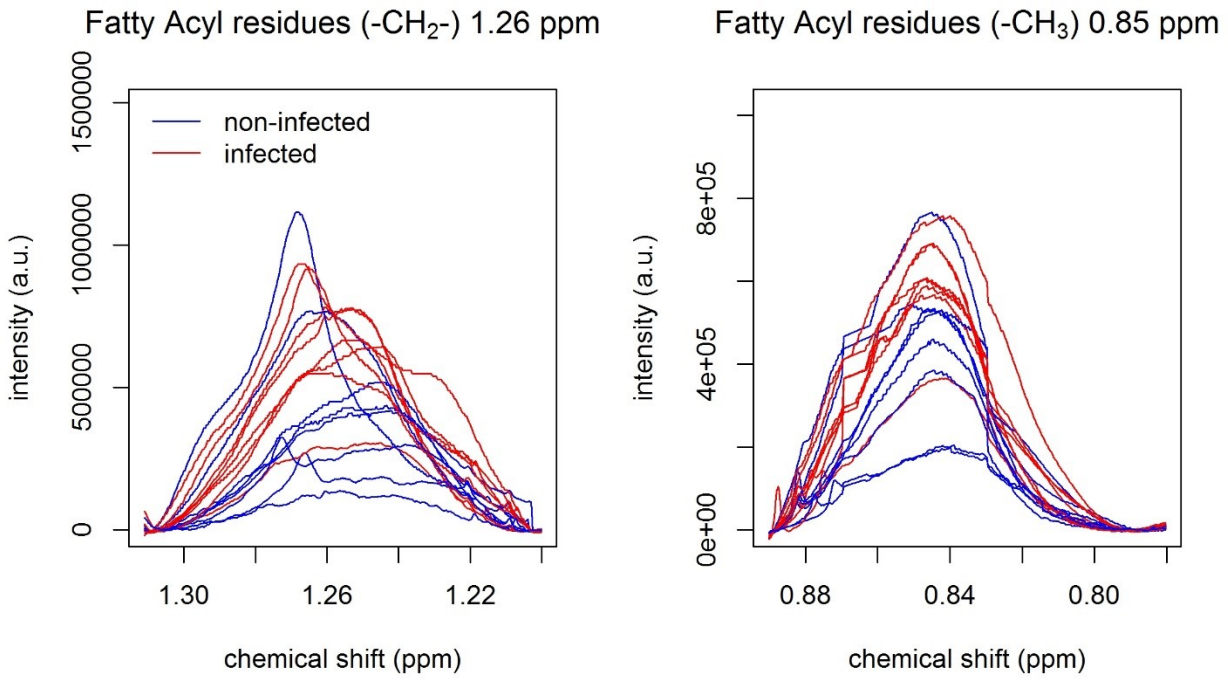


Figure 35. Further metabolite analysis from spectral inspection of the remaining fatty acyl residue regions. The figure demonstrates the same trend in peak intensities for both the infected (red) and non-infected (blue) synovial fluid samples. p values are p=0.068 (0.85 parts per million (ppm)) and p=0.051 (1.26ppm). ppm –parts per million.

Using the KEGG database, a network analysis was performed, illustrating the connections between the significantly changed metabolites in infected SF and their possible connections in the human metabolic pathway (figure 36). This network analysis demonstrates the complex relationships between all the metabolites found in significantly different concentrations between infected and non-infected SF. For example, the connections between the metabolites found in lower concentrations in infected SF (green in figure 36) demonstrate that a complex relationship may occur through several metabolic pathways.

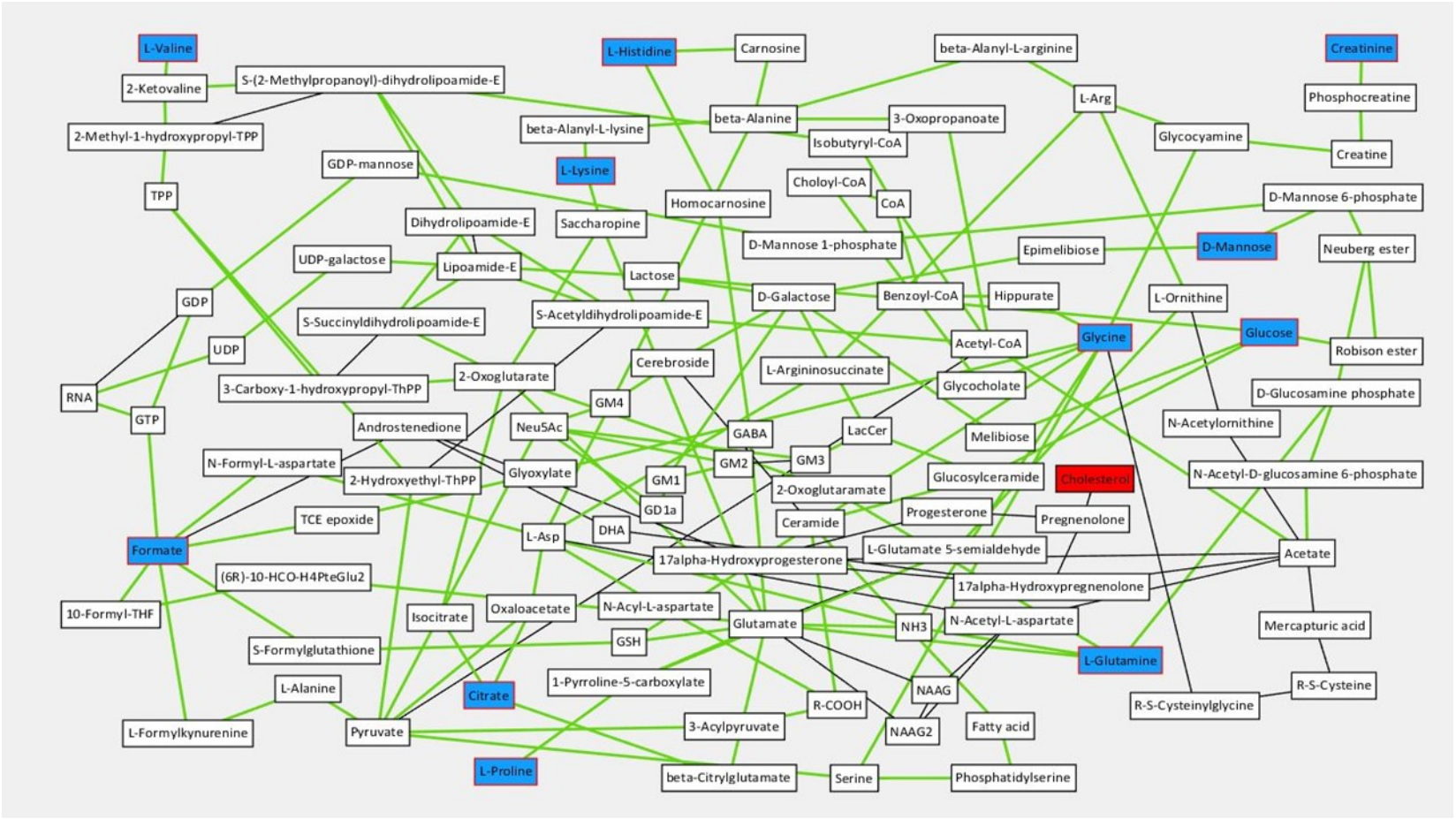


Figure 36. Network analysis of all the identified metabolites from the KEGG database illustrating the potential metabolic pathways. Metabolites found in greater concentrations in infected synovial fluid are in red. Metabolites found in lower concentrations in infected synovial fluid are in blue. Metabolic 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), GAGs and glycoproteins (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.

6.6 Discussion

The small molecule composition of HSF from patients with infected joints was analysed and compared with matched samples from patients with non-infected joints, using NMR spectroscopy. Following statistical analysis (PCA and univariate analysis), three metabolites were found in relatively higher concentrations (*NAc-2*, *cholesterol* (C18) and lipids) and thirteen in relatively lower concentrations in the infected group (*citrate*, *creatinine*, *dimethylsulfone*, *formate*, *glucose*, *glutamine*, *glycine*, *GAGs*, *histidine*, *lysine*, *mannose*, *proline* and *valine*). These may represent different metabolic pathways between infected and non-infected SF.

This discovery study is the first of its kind to examine the metabolic differences in HSF between infected and non-infected joints with the samples matched for gender, age and common medical co-morbidities. The metabolites found in significantly greater concentrations in the infected cohort can broadly be grouped into those having a role in the inflammatory response, lipid metabolism and are markers of infection and inflammation. Those found in significantly reduced concentrations in the infected cohort can broadly be grouped into those involved in carbohydrate and nucleoside metabolism, the *glutamate* metabolic pathway, reduced articular cartilage breakdown and increased oxidative stress in the diseased state.

6.6.1 Analysis of metabolites with increased concentrations in infected synovial fluid

Table 17 proposes the possible functions of the three metabolites found in significantly greater concentrations in the infected group.

Table 17. List of identified metabolites found in significantly higher concentrations in infected synovial fluid and their proposed function. PG, proteoglycan; SCFA, short chain fatty acids; HDL, high-density lipoprotein; apo A-1, apolipoprotein A-1; SF, synovial fluid; RA, rheumatoid arthritis; NAc, N-acetylated groups.

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.	Naughton et al(187)
Unsaturated lipids (CH=CH)	Increased levels of lipoprotein-associated *fatty acids* are found in inflamed joints.	Wook et al(140)
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(276) Li et al(277)

When compared with blood serum, acute phase glycoproteins, such as α1-acid glycoprotein have *N-acetyl* signals around 2.00-2.05 ppm region(275). *NAc-2* may correspond to an acute phase glycoprotein. Such acute proteins are known to be markers of infection and inflammation(187).

Lipids have an important role in energy production, both at rest and during muscle activity, acting as a crucial substrate. Consequently, measuring their peak signals may provide important information on the effusion mechanisms, thus helping to improving understanding of disease progression(278). The presence of *fatty acyl* residues and lipoproteins in SF was demonstrated in a canine study using NMR spectroscopy at the same chemical shift as that identified in this study(279). The remaining unsaturated lipids in the stacked spectra were also visualised, but no significant difference was found between the cohorts (figure 36). This may be because the unsaturated lipids with a peak at 5.28 ppm had the best resolved peak compared to the peaks at 1.26 and 0.85 ppm. However, since the p-values were close to the arbitrary level of significance, with more samples, the resolution of the peaks may improve.

In this discovery study, evidence of *cholesterol*/*cholesterol esters* originating from SF lipoproteins, increased in the infected group, was identified. Although unequivocal attribution of these signals to a particular group of lipoproteins is not trivial from 1D NMR spectral information, there is evidence of involvement of lipoproteins in the context of both infection and inflammation(280).

High-density lipoprotein (*HDL*)-associated *apolipoprotein A-1* (*apo A-1*) is a negative acute-phase protein, with acute inflammation causing a reduction in its levels by at least 25%. A number of conditions can give rise to changes in the plasma concentration of acute-phase proteins, including various infections and inflammatory conditions(281). In rheumatoid arthritis (RA), levels of circulating *apo A-1* and *HDL cholesterol* in untreated patients are less than in normal controls(282). However, in the SF of RA patients, concentrations of *apo A-1* are increased, although it is still substantially below what is found in the plasma(283). This is accompanied by increased levels of *cholesterol* in the SF giving rise to the suggestion that there is infiltration of *HDL* within inflamed joints. As explained above, *HDL*-associated *apo A-1* may function to inhibit lymphocyte/monocyte interactions in order to try and mediate the inflammatory response associated with infection. However, this remains speculation at the current time. An increase in abnormal lipid metabolism in the CSF of patients with tuberculosis meningitis (TBM) has been demonstrated in a proteomic study(276). *Apolipoprotein B* (*Apo B*), which is the major structural protein of *very-low-density lipoprotein* (*VLDL*), was found in significantly greater concentrations in the CSF of TBM patients. A similar study identified three molecules associated with lipid metabolism (*glycine*, lipoprotein and *choline*) at increased concentrations in the CSF of TBM patients, using the techniques of ^{1}H NMR spectroscopy(277). The increased *glycine* and *choline* concentrations in TBM patients was suggested by the authors to be consequences of greater *glycerolipid* and *glycophospholipid* metabolism to meet the increased energy demand in TBM.

6.6.2 Analysis of metabolites with decreased concentrations in infected synovial fluid

Table 18 proposes the possible functions of the metabolites found in significantly decreased concentrations in the infected group.

Table 18. List of identified metabolites found in significantly lower concentrations in infected synovial fluid and their proposed function. CSF – cerebrospinal fluid; TBM – tuberculosis meningitis; SF – synovial

fluid; TCA – tricarboxylic acid cycle; GAGs – glycosaminoglycans; BCAA – branched chain amino acid; OA – osteoarthritis; ESOA – end-stage osteoarthritis; NAc, N-acetylated groups.

Metabolite ID	Function of metabolite	Reference
Citrate ***Glucose*** ***Mannose***	*Citrate* is a major intermediary in the *TCA* cycle, urea cycle, amino acid and *fatty acid* metabolism. 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.	Li et al(277) Berg et al(243)
Glycine ***Glutamine*** ***Valine***	*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(277) Wu et al(247)
Glutamine ***Proline*** ***Lysine***	*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(247) Weiner et al(284) Revelles et al(285)
Creatinine ***Histidine***	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(284)
Dimethylsulfone	*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(286) Marthyn et al(287) Watson et al(288)
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 non-infected group secondary to chronic ESOA versus acute cartilage degradation in the infected group.	Thompson et al(242) Schiller et al(241) Smith and Schurman(289)
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(290)

Lower concentrations of carbohydrates including *glucose* and *mannose* plus the intermediate metabolite, *citrate*, were found in infected HSF. These changes in carbohydrate metabolism suggested increased aerobic and anaerobic energy metabolism in infected HSF compared with non-infected HSF. Although no study has previously demonstrated this in SF, similar findings have been identified in the CSF of bacterial meningitis patients(277). However, there may be the involvement of other metabolic pathways, which can cause changes in these metabolites

such as the synthesis of *glycolipids* (figure 36). Two metabolites involved in purine metabolism (*glutamine* and *glycine*) were also found in significantly reduced concentrations in infected HSF. This led to the suggestion of altered nucleoside metabolism in the presence of infection. As before, there were similar findings in the CSF of bacterial meningitis patients(277). Furthermore, as *valine* has a role in the synthesis of *glutamine*(247), it is to be expected that its concentration is reduced in infected HSF.

Lysine, *proline* and *glutamine* have a role in the metabolic pathway of *glutamate*, which accounts for the decreased concentration of *proline* in infected SF(247, 285). The *TCA* and urea cycles are bridged by *glutamate*(247). Therefore, increased energy expenditure in the infected HSF may be due to reduced concentrations of these metabolites. A similar finding was demonstrated in another study where reduced *glutamine* concentrations were identified in the serum of patients with active TB(284). Furthermore, a reduced concentration of *creatine* and *histidine* amongst other amino acids (AAs) were also identified in the serum of patients with TB. Since *creatine* is metabolised to *creatinine*, this may provide an explain for the reduced concentrations of *creatinine* in the infected HSF group. The metabolism of AAs is a complex process, involving numerous metabolites. Proteolysis, gluconeogenesis and oxidative catabolism all contribute to the balance of AAs. Furthermore, greater protein synthesis secondary to increased bacterial(291) and/or macrophage activity(292) as part of the immune response, may provide another explanation for reduced concentrations of AAs seen in infected SF.

Dimethylsulfone is derived from intestinal bacterial metabolism, endogenous human methanethiol metabolism and dietary sources(293). It is a powerful scavenger of oxygen free-radicals(286), induces macrophage apoptosis(287) and stimulates granulocyte differentiation(288). Reduced concentrations of this metabolite in infected HSF may be secondary to differentiation and induced apoptosis of macrophages plus increased oxidative stress.

Four of the patients in the matched non-infected group had ESOA of the knee. *GAGs* are markers of proteoglycan and articular cartilage breakdown(242). In vitro animal studies have

also demonstrated *GAG* breakdown with subsequent destruction of the articular cartilage secondary to *staphylococcal* infections(289). The reduced concentration of *GAGs* in the infected group may be due to increased proteoglycan and articular cartilage degradation in the matched non-infected group secondary to ESOA, which is greater than the degree of degradation occurring in the infected group. A possible explanation could be the chronicity of articular cartilage degradation secondary to ESOA in the non-infected group, compared to the acute and more short-term degradation occurring in the infected group secondary to proteolysis.

Formate is a short-chain *fatty acid* produced during BCAA catabolism. These short-chain *fatty acids* are the major end-products of bacterial metabolism in human large intestine(290). However, their role in infected SF remains unclear. The network analysis performed highlights the connection of *formate* to other metabolites involved in AA and nucleotide metabolism and therefore that those pathways are affected in SF infection (figure 36).

6.6.3 Potential of NMR spectroscopy in further analysis of SF in health and disease

As discussed in chapter 2, ^{1}H-NMR remains one of the preferred analytical techniques to study complex biological samples as it produces a comprehensive profile of metabolic signals without separation, derivatization and preselected measurement parameters (273).

Over the past decade, ^{1}H-NMR-based spectroscopy metabolic phenotyping has become a powerful tool for identifying biochemical markers and metabolites for a multitude of human disorders(273). Metabolic phenotyping of SF provides a direct representation of end stage biochemistry making metabolites good candidates for biomarker screening. The metabolites themselves are the final product of enzyme catalysis and other biotransformations as well as being much smaller in number than the proteome(273). They provide a “top down” view of a biological system, with the advantage of representing the genetic disease traits but also other secondary factors, such as environmental interactions, as well as being sensitive to gut microbiome activity(273). NMR spectroscopy provides sharp well-resolved peaks for small molecule metabolites (usually defined as < 1500 Dalton) but yields only broad unresolved bands for proteins and other macromolecules. Therefore, enzymes such as α-defensin cannot be

detected. The techniques utilised in this discovery study could be used in larger cohorts to help identify putative biomarkers in native and PJIs, acting as an adjunct to α-defensin.

6.6.4 Clinical relevance

This discovery study demonstrates the potential role of metabolic phenotyping of HSF using NMR spectroscopy to identify important metabolites in the context of joint infection. Some of these metabolites will require further validation in larger cohorts of HSF samples to determine whether they provide useful putative biomarkers of infection, leading to the tantalising prospect of developing bedside diagnostic tests for joint infection.

Future studies should utilise larger groups of HSF samples with a more targeted analysis of specific metabolites for a particular patient group or disease category. The fundamental purpose of these studies would be to identify concentrations and combinations of metabolites for individual bacterial species that would provide a "metabolic fingerprint" for the organism, thus enabling early diagnosis, appropriate antibiotic therapy and surgical treatment.

6.6.5 Limitations

Despite all the samples in each group being matched for gender, age and common medical comorbidities, the overall numbers remained small with only eight samples in each group. However, at the time this thesis was written, this was the largest cohort of infected HSF analysed by NMR spectroscopy with a non-infected comparison group of matched controls. Although, it would have been desirable to have an age/gender-matched non-arthritic, non-infected control group, ethical constraints would make acquisition of such samples difficult. Further validation of the identified metabolites could have taken place by performing additional tests, such as an enzyme-linked immunosorbent assay (ELISA). However, lack of funding and other resources meant this was not possible at the time.

6.7 Conclusion

To the author's knowledge, this discovery study is the first to demonstrate differences in the metabolic profile of infected and non-infected HSF with matched controls. Three molecules were found in significantly greater proportions in the infected cohort (*glycoproteins*, *cholesterol*/*cholesterol esters* and unsaturated lipids). These substances have a role in the

inflammatory response, lipid metabolism and are markers of inflammation and infection. There were thirteen metabolites found in significantly reduced concentrations in the infected cohort (*citrate*, *creatinine*, *dimethylsulfone*, *formate*, *glucose*, *glutamine*, *glycine*, *GAGs*, *histidine*, *lysine*, *mannose*, *proline* and *valine*). These can broadly be grouped into those involved in carbohydrate and nucleoside metabolism, the *glutamate* metabolic pathway, reduced articular cartilage breakdown and increased oxidative stress in the diseased state.

Although more studies are required with a larger cohort of HSF, these metabolites may serve as putative biomarkers for the diagnosis of native and PJIs, plus could be used as adjuncts with other recognised biomarkers, such as α-defensin.

Now that the metabolic differences have been explained between infected and non-infected human synovial fluid, in the next and final chapter of this thesis, the main findings are discussed including future studies to take this research forward and validate the results by performing larger and more complex studies.

7 Summary and future work

7.1 Introduction

Metabolic profiling, also known as metabonomics or metabolomics, is emerging as a useful method of identifying various metabolites associated with different diseases. It has also identified specific biomarkers unique to particular conditions, which play an important role in disease prediction, monitoring and early detection. In contrast to the other -omics, including genomics or proteomics, which often do not provide real biological end points, such as the benefits or adverse effects of medications on a biological system, metabolic profiling is able to achieve this outcome. It is the quantitative measurement of a living systems' metabolic response to time related genetic modification or pathophysiological stimuli. Specifically, with regards to this thesis, it can provide the time dependent metabolic response of the joints and SF to a complex interplay of aetiopathophysiological factors and the presence of infective organisms.

Simply put, it provides a top-down method of analysis, as it is looking at the metabolites, which are the end-products of the metabolic process. Consequently, it accounts for a variety of external and environmental factors, such as diet, disease, medication, smoking, etc. It has the capacity to identify hundreds and even thousands of metabolites from biofluids such as blood, urine and of course synovial fluid.

There are many complex analytical methods available, which include, but are not limited to, nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography mass spectroscopy (LC-MS), gas chromatography mass spectroscopy (GC-MS), capillary electrophoresis mass spectroscopy, Fourier transform infrared (IR) spectroscopy (FT-IR) and Raman spectroscopy. These techniques can lead to the acquisition of a "metabolic fingerprint", which may be characteristic of a particular disease process or unique to a particular biochemical perturbation.

In this thesis, the techniques of NMR spectroscopy have been employed. This has many advantages and disadvantages over the various techniques involving MS. It is fast and non-destructive allowing numerous samples to be measured daily, with the same sample analysed multiple times(148). Furthermore, many of the metabolites detected by NMR spectroscopy

have previously been identified. Consequently, less effort is required to determine the metabolites from the spectral analysis. In essence, it is where the “low hanging fruit” can be found.

The role of metabolic profiling in Orthopaedics is still in its early stages, but there is a lot of potential to identify putative biomarkers, which are disease specific. In this thesis, a literature review was conducted, which identified 21 studies (17 published articles and four meeting abstracts) that looked at the role of metabolic profiling using human SF (HSF). In addition to the many metabolites that were identified in both osteoarthritis and inflammatory arthropathies, a select few were identified as putative biomarkers, as they had been identified in studies with a normal control group(166). These have been discussed in chapter 3. However, none of these studies performed a robust statistical analysis accounting for multiple testing. Furthermore, many of these studies had multiple confounding factors affecting the validity of the results. These included age, gender and medical co-morbidities, which were not considered in all studies.

This lack of robust statistical analysis and consideration of confounding factors meant there was room for further research looking at the role of various metabolites in orthopaedic conditions. This led to the development of two discovery studies, which were conducted as part of this MD thesis.

7.2 Results

The first study, detailed in chapter 5, investigated the metabolic profile of HSF from native hip and knee joints in patients with end-stage OA (ESOA) using NMR spectroscopy. The aims were to assess metabolic differences between the fluids using multivariate and univariate statistical analysis and to evaluate the metabolic pathways involved that are implicated in the metabolic differences between hip and knee HSF.

This study accounted for a number of confounding factors including age, gender, ethnicity and common medical co-morbidities. Following both multivariate and univariate analysis, 14 metabolites were identified as being found in significantly different concentrations between hip and knee osteoarthritic SF. Twelve of these were found in significantly greater concentrations in

knee SF (*alanine*, *citrate*, *dimethylsulfone*, *glucose*, *glutamine*, *glycosaminoglycans* (*GAGs*), *histidine*, *lysine*, *N-acetylated* species, *pyruvate*, *tyrosine* and *valine*) and two in hip SF (*choline* and *hypoxanthine*).

The potential role of these metabolites is described fully in chapter 5. However, in essence, they can broadly be grouped into those responsible for branched chain amino acid catabolism, proteoglycan and articular cartilage degradation, having a role in the *tricarboxylic acid* (*TCA*) cycle and lipid metabolism.

Following false discovery rate (FDR) correction, four of these metabolites remained significant (*citrate, glutamate*, *GAGs* and *N-acetylated molecules*). These were all found in significantly greater concentrations in the knee group compared to the hip group. Their roles can broadly be grouped into those involved in the *TCA* cycle, collagen degradation and oxidative metabolism in diseased joints.

The identified differences may be due to or a combination of intra- and extra-articular factors. Intra-articular factors include anatomic and biomechanical differences between the joints plus differences in cartilage turnover and the presence of various mediators including matrix metalloproteinases and proinflammatory mediators. Extra-articular factors include age, BMI and medical co-morbidities.

However, although the metabolic differences identified in this discovery study may primarily be a result of the disease process itself or reflect alterations in normal cartilage and SF biology, the probability is that the observed differences are represented by a complex interplay of all these aetiopathalogical factors.

The second discovery study, detailed in chapter 6, investigated the metabolic profile of HSF from infected and non-infected HSF using NMR spectroscopy. The aims were to assess metabolic differences between the fluids using multivariate and univariate statistical analysis and to evaluate the metabolic pathways involved that are implicated in the metabolic differences between infected and non-infected HSF.

This study accounted for a number of confounding factors including age, gender and common medical co-morbidities. Following both multivariate and univariate analysis, 16 metabolites were identified as being found in significantly different concentrations between infected and non-infected HSF groups. Three metabolites (*cholesterol*, lipids, and *N-acetylated* molecules) were found in significantly increased concentrations and thirteen (*citrate*, *creatinine*, *dimethylsulfone*, *formate*, *glucose*, *glutamine*, *glycine*, *GAGs*, *histidine*, *lysine*, *mannose*, *proline* and *valine*) in significantly decreased concentrations in the infected compared to the non-infected HSF group.

The potential role of these metabolites is described fully in chapter 6. Broadly, those found in significantly greater concentrations in the infected cohort represented metabolites, which have a role in the inflammatory response, lipid metabolism and are markers of inflammation and infection. Those identified in significantly reduced concentrations were involved in carbohydrate and nucleoside metabolism, the *glutamate* metabolic pathway, reduced articular cartilage breakdown and increased oxidative stress in the diseased state.

Following FDR correction, only one metabolite (*citrate*) remained significant, and this was present in decreased concentrations in the infected compared to the non-infected HSF group. 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 (274). Therefore, all the identified metabolites were considered in the analysis.

7.3 Clinical Applications

Although these studies have demonstrated significant results, it is important to consider their clinical implications and how they may help to lead to a change in practice.

Chapter 5 demonstrated metabolic differences between hip and knee osteoarthritic HSF using NMR spectroscopy. With further research, these metabolites may prove to be putative biomarkers of OA that may be joint specific. They may lead to the development of a diagnostic test for OA that could help identify this condition early in the disease process, where various preventative measures could be implemented to delay the progression of the disease.

Chapter 6 demonstrates the potential role of metabolic phenotyping of HSF using NMR spectroscopy to identify important metabolites in the context of joint infection. Currently, microscopy, culture and sensitivity (MC&S) remains the gold standard for identifying joint infection. However, delays in results could lead to some patients waiting longer before being given appropriate antibiotics and/or surgical intervention. This could lead to irreversible changes to the native joint, the formation of a biofilm in prosthetic joints, and in some cases an increased mortality risk.

Some of the metabolites identified in this discovery study will require further validation in larger cohorts of HSF samples to determine whether they provide useful putative biomarkers of infection, leading to the tantalising prospect of developing bedside diagnostic tests for joint infection that may be specific to a particular organism by providing a "metabolic fingerprint".

7.4 Limitations

Whilst this thesis has yielded some useful data with important clinical implications, it is important to acknowledge that there are some inherent limitations.

Due to ethical constraints, it was not possible to collect samples from healthy volunteers. As such, neither of the discovery studies had a healthy control group for comparative purposes. This makes it more difficult to interpret the metabolic differences, as it is less clear whether the identified metabolites are secondary to the underlying disease or some other process.

The number of patients in both discovery studies was small, with 24 HSF samples in the hip and knee OA study (12 hip and 12 knee HSF samples) and 16 HSF samples in the infection study (eight infected and eight non-infected HSF samples). However, this was partly due to the rigorous matching of samples that took place to account for potential confounding factors including, age, gender BMI and common medical co-morbidities. Consequently, fewer metabolites survived FDR correction, meaning the possibility of some of the metabolic differences identified occurring by chance was greater. Having said that, as stated previously, FDR or multiple testing correction has been shown to result in a number of false discoveries in metabolomics data, particularly in the presence of a small sample size. Therefore, all the identified metabolic differences were considered in the analysis and discussion.

Another important limitation from the results in chapter 5 was that the number of compartments in the knee affected with OA was not considered. However, as discussed in the chapter itself, unpublished analysis has not identified any metabolic differences between OA of the knee joint associated with the number of compartments affected (P Akhbari unpublished data).

An important limitation to the data from chapter 6 was the lack of additional tests to further validate the identified metabolites, such as an enzyme-linked immunosorbent assay (ELISA). However, as explained, lack of funding and other resources meant this was not possible at the time.

Despite these limitations, the data produced from this thesis is still useful, and the standards employed are similar to those in the published literature. This was demonstrated from the literature review in Chapter 3. Only seven of the identified articles had matched controls. Furthermore, the majority of studies did not account for potential confounding factors, whereas the discovery studies performed in this thesis were matched for a number of other variables in an attempt to remove them as confounding factors. Therefore, the results produced in this thesis still add to the body of medical science and have already been published.

7.5 Future work

The discovery studies performed in this thesis have yielded some valuable results with important clinical implications. They have provided further insight into the role of metabolic profiling in identifying metabolites with important functions in various orthopaedic disease processes.

Further research would involve utilising a larger group of SF samples with a non-arthritic age and gender matched control group to see whether the identified metabolites from this thesis may serve as putative biomarkers for diagnosing OA of the knee, monitoring of disease progression and/or future treatment strategies.

Such larger studies with a matched control group would also allow a more targeted analysis of specific metabolites for a particular patient group or disease category. Where possible, these

studies should include other techniques to further validate the identified metabolites in order to identify concentrations and combinations of metabolites for individual bacterial species that would provide a "metabolic fingerprint" for the organism. This would enable early diagnosis, appropriate antibiotic therapy and surgical treatment, plus lead to better patient care. Correlations between microbiome and metabolites could help clarify the metabolic fingerprint of the organism. This would require a more sensitive technique such as an MS method to measure low abundance metabolites and get better insights into the changes in metabolite composition. The commonest infective organism found in human joints is Staphylococcus aureus, followed by Streptococcus species(294). Larger studies looking specifically at these organisms may yield molecular differences that lead to the identification of putative biomarkers that are organism specific.

Recent advances in both genomic and proteomic studies have demonstrated the importance of these techniques in improving disease understanding and biomarker identification(214, 215). Future studies integrating proteomic, genomic and metabolic profiling techniques may provide the greatest hope for the advancement of biomarker discovery, although this is a very big undertaking, requiring collaborations between different departments and a large amount of funding.

8 References

1. Buckwalter JA, Mankin HJ. Articular cartilage: tissue design and chondrocyte-matrix interactions. Instr Course Lect. 1998;47:477-86.

2. Woo SL-Y, Buckwalter JA. Injury and repair of the musculoskeletal soft tissues. Savannah, Georgia, June 18–20, 1987. J Orth Res. 1988;6(6):907-31.

3. Alford JW, Cole BJ. Cartilage restoration, part 1: basic science, historical perspective, patient evaluation, and treatment options. Am J Sports Med. 2005;33(2):295-306.

4. Mow VC, Ratcliffe A, Poole AR. Cartilage and diarthrodial joints as paradigms for hierarchical materials and structures. Biomaterials. 1992;13(2):67-97.

5. Maroudas A, Wachtel E, Grushko G, Katz EP, Weinberg P. The effect of osmotic and mechanical pressures on water partitioning in articular cartilage. Biochim Biophys Acta. 1991;1073(2):285-94.

6. Torzilli PA. Influence of cartilage conformation on its equilibrium water partition. J Orthop Res. 1985;3(4):473-83.

7. Mow VC, Kuei SC, Lai WM, Armstrong CG. Biphasic creep and stress relaxation of articular cartilage in compression? Theory and experiments. J Biomech Eng. 1980;102(1):73-84.

8. Fässler R, Schnegelsberg PN, Dausman J, Shinya T, Muragaki Y, McCarthy MT, Olsen BR, Jaenisch R. Mice lacking alpha 1 (IX) collagen develop noninflammatory degenerative joint disease. Proc Natl Acad Sci U S A. 1994;91(11):5070-4.

9. Sophia Fox AJ, Bedi A, Rodeo SA. The basic science of articular cartilage: structure, composition, and function. Sports health. 2009;1(6):461-8.

10. Garnero P, Rousseau J-C, Delma PD. Molecular Basis and Clinical Use of Biochemical Markers of Bone, Cartilage, and Synovium in Joint Diseases. Arthritis Rheum. 2000;43(5):953-68.

11. Hardingham T, Bayliss M. Proteoglycans of articular cartilage: changes in aging and joint disease. Seminars of Arthritis and Rheumatology. 1990;20:12-33.

12. Masuda K, Sah RL, Hejna MJ, Thonar EJ. A novel two-step method for the formation of tissue-engineered cartilage by mature bovine chondrocytes: the alginate-recovered-chondrocyte (ARC) method. J Orthop Res. 2003;21(1):139-48.

13. Poole CA, Flint MH, Beaumont BW. Chondrons in cartilage: ultrastructural analysis of the pericellular microenvironment in adult human articular cartilages. J Orthop Res. 1987;5(4):509-22.

14. Recht M, Bobic V, Burstein D, Disler D, Gold G, Gray M, Kramer J, Lang P, McCauley T, Winalski C. Magnetic resonance imaging of articular cartilage. Clin Orthop Relat Res. 2001(391 Suppl):S379-96.

15. Mankin HJ. The response of articular cartilage to mechanical injury. J Bone Joint Surg Am. 1982;64(3):460-6.
16. Buckwalter JA. Articular cartilage: injuries and potential for healing. J Orthop Sports Phys Ther. 1998;28(4):192-202.
17. Woo SL, Lee TQ, Gomez MA, Sato S, Field FP. Temperature dependent behavior of the canine medial collateral ligament. J Biomech Eng. 1987;109(1):68-71.
18. Mow VC, Holmes MH, Lai WM. Fluid transport and mechanical properties of articular cartilage: a review. J Biomech. 1984;17(5):377-94.
19. Woo SLY MV, Lai WM. Biomechanical properties of articular cartilage. In: Skalak R CS, editors. , eds, editor. Handbook of Bioengineering. New York, NY: McGraw-Hill Book Co; 1987. p. 4.1-4.44.
20. Hui AY, McCarty WJ, Masuda K, Firestein GS, Sah RL. A systems biology approach to synovial joint lubrication in health, injury, and disease. Wiley Interdiscip Rev Syst Biol Med. 2012;4:15-37.
21. Schumacher BL, Block JA, Schmid TM, Aydelotte MB, Kuettner KE. A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. Arch Biochem Biophys. 1994;311(1):144-52.
22. Jay GD, Britt DE, Cha CJ. Lubricin is a product of megakaryocyte stimulating factor gene expression by human synovial fibroblasts. J Rheumatol. 2000;27(3):594-600.
23. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nature Reviews Immunology. 2007;7:429-42.
24. Goldring MB. Osteoarthritis and cartilage: the role of cytokines. Curr Rheumatol Rep. 2000;2(6):459-65.
25. Blewis ME, Lao BJ, Schumacher BL, Bugbee WD, Sah RL, Firestein GS. Interactive cytokine regulation of synoviocyte lubricant secretion. Tissue engineering Part A. 2010;16(4):1329-37.
26. Stenken JA, Poschenrieder AJ. Bioanalytical chemistry of cytokines--a review. Anal Chim Acta. 2015;853:95-115.
27. Levick JR. Permeability of rheumatoid and normal human synovium to specific plasma proteins. Arthritis Rheum. 1981;24(12):1550-60.
28. Levick JR, McDonald JN. Ultrastructure of transport pathways in stressed synovium of the knee in anaesthetized rabbits. J Physiol. 1989;419:493-508.
29. Schmid K, Macnair MB. Characterization of the proteins of human synovial fluid in certain disease states. J Clin Invest. 1956;35(7):814-24.

30. Schmid K, Macnair MB. Characterization of the proteins of certain postmortem human synovial fluids. J Clin Invest. 1958;37(5):708-18.
31. Trunfio-Sfarghiu AM, Berthier Y, Meurisse MH, Rieu JP. Multiscale analysis of the tribological role of the molecular assemblies of synovial fluid. Case of a healthy joint and implants. Tribology International. 2007;40(10):1500-15.
32. Kitano T, Ateshian GA, Mow VC, Kadoya Y, Yamano Y. Constituents and pH changes in protein rich hyaluronan solutions affect the biotribological properties of artificial articular joints. J Biomech. 2001;34:1031-7.
33. Ghosh S, Choudhury D, Roy T, Moradi A, Masjuki HH, Pingguan-Murphy B. Tribological performance of the biological components of synovial fluid in artificial joint implants. Science and Technology of Advanced Materials. 2015;16(4):045002.
34. Trunfio-Sfarghiu A-M, Berthier Y, Meurisse M-H, Rieu J-P. Role of Nanomechanical Properties in the Tribological Performance of Phospholipid Biomimetic Surfaces. Langmuir. 2008;24(16):8765-71.
35. Klein J. Molecular mechanisms of synovial joint lubrication. Proceedings of the Institution of Mechanical Engineers, Part J: Journal of Engineering Tribology. 2006;220(8):691-710.
36. Martin-Alarcon L, Schmidt TA. Rheological effects of macromolecular interactions in synovial fluid. Biorheology. 2016;53(2):49-67.
37. Schur PH, Sandson J. Immunologic studies of the proteins of human synovial fluid. Arthritis Rheum. 1963;6:115-29.
38. Decker B, Mc KB, Mc GW, Slocumb CH. Comparative distribution of proteins and glycoproteins of serum and synovial fluid. Arthritis Rheum. 1959;2(2):162-77.
39. Lee HG, Cowman MK. An agarose gel electrophoretic method for analysis of hyaluronan molecular weight distribution. Anal Biochem. 1994;219(2):278-87.
40. Jay GD, Harris DA, Cha CJ. Boundary lubrication by lubricin is mediated by O-linked beta(1-3)Gal-GalNAc oligosaccharides. Glycoconj J. 2001;18(10):807-15.
41. Decker B, Mc GW, Mc KB, Slocumb CH. Concentration of hyaluronic acid in synovial fluid. Clin Chem. 1959;5:465-9.
42. Gomez JE, Thurston GB. Comparisons of the oscillatory shear viscoelasticity and composition of pathological synovial fluids. Biorheology. 1993;30(5-6):409-27.
43. Castaneda S, Roman-Blas JA, Largo R, Herrero-Beaumont G. Osteoarthritis: a progressive disease with changing phenotypes. Rheumatology (Oxford). 2014;53(1):1-3.
44. Poole AR. Osteoarthritis as a Whole Joint Disease. HSS J. 2012;8(1):4-6.

45. Haynes MK, Hume EL, Smith JB. Phenotypic characterization of inflammatory cells from osteoarthritic synovium and synovial fluids. Clin Immunol. 2002;105(3):315-25.
46. Knoop J, van der Leeden M, Thorstensson CA, Roorda LD, Lems WF, Knol DL, Steultjens MP, Dekker J. Identification of phenotypes with different clinical outcomes in knee osteoarthritis: data from the Osteoarthritis Initiative. Arthritis Care Res (Hoboken). 2011;63(11):1535-42.
47. Solovieva S, Kamarainen OP, Hirvonen A, Hamalainen S, Laitala M, Vehmas T, Luoma K, Nakki A, Riihimaki H, Ala-Kokko L, Mannikko M, Leino-Arjas P. Association between interleukin 1 gene cluster polymorphisms and bilateral distal interphalangeal osteoarthritis. J Rheumatol. 2009;36(9):1977-86.
48. Scanzello CR, Umoh E, Pessler F, Diaz-Torne C, Miles T, Dicarlo E, Potter HG, Mandl L, Marx R, Rodeo S, Goldring SR, Crow MK. Local cytokine profiles in knee osteoarthritis: elevated synovial fluid interleukin-15 differentiates early from end-stage disease. Osteoarthritis Cartilage. 2009;17(8):1040-8.
49. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis. 1957;16(4):494-502.
50. Johnson VL, Hunter DJ. The epidemiology of osteoarthritis. Best Pract Res Clin Rheumatol. 2014;28(1):5-15.
51. Felson DT, Lawrence RC, Dieppe PA, et al. Osteoarthritis: New insights. part 1: the disease and its risk factors. Ann Intern Med. 2000;133(8):635-46.
52. Srikanth VK, Fryer JL, Zhai G, Winzenberg TM, Hosmer D, Jones G. A meta-analysis of sex differences prevalence, incidence and severity of osteoarthritis. Osteoarthritis Cartilage.13(9):769-81.
53. Hanna FS, Wluka AE, Bell RJ, Davis SR, Cicuttini FM. Osteoarthritis and the postmenopausal woman: Epidemiological, magnetic resonance imaging, and radiological findings. Semin Arthritis Rheum.34(3):631-6.
54. Nevitt MC, Felson DT, Williams EN, Grady D. The effect of estrogen plus progestin on knee symptoms and related disability in postmenopausal women: The Heart and Estrogen/Progestin Replacement Study, a randomized, double-blind, placebo-controlled trial. Arthritis Rheum. 2001;44(4):811-8.
55. Spector TD, MacGregor AJ. Risk factors for osteoarthritis: genetics. Osteoarthritis Cartilage. 2004;12 Suppl A:S39-44.
56. Palotie A, Vaisanen P, Ott J, Ryhanen L, Elima K, Vikkula M, Cheah K, Vuorio E, Peltonen L. Predisposition to familial osteoarthrosis linked to type II collagen gene. Lancet. 1989;1(8644):924-7.
57. Jiang L, Tian W, Wang Y, Rong J, Bao C, Liu Y, Zhao Y, Wang C. Body mass index and susceptibility to knee osteoarthritis: a systematic review and meta-analysis. Joint Bone Spine. 2012;79(3):291-7.

58. Lohmander LS, Gerhardsson de Verdier M, Rollof J, Nilsson PM, Engstrom G. Incidence of severe knee and hip osteoarthritis in relation to different measures of body mass: a population-based prospective cohort study. Ann Rheum Dis. 2009;68(4):490-6.
59. Felson DT, Zhang Y, Anthony JM, Naimark A, Anderson JJ. Weight loss reduces the risk for symptomatic knee osteoarthritis in women. The Framingham Study. Ann Intern Med. 1992;116(7):535-9.
60. Christensen R, Bartels EM, Astrup A, Bliddal H. Effect of weight reduction in obese patients diagnosed with knee osteoarthritis: a systematic review and meta-analysis. Ann Rheum Dis. 2007;66(4):433-9.
61. Karlson EW, Mandl LA, Aweh GN, Sangha O, Liang MH, Grodstein F. Total hip replacement due to osteoarthritis: the importance of age, obesity, and other modifiable risk factors. Am J Med. 2003;114(2):93-8.
62. Carman WJ, Sowers M, Hawthorne VM, Weissfeld LA. Obesity as a risk factor osteoarthritis of the hand and wrist: a prospective study. Am J Epidemiol. 1994;39:119-29.
63. McAlindon TE, Jacques P, Zhang Y, Hannan MT, Aliabadi P, Weissman B, Rush D, Levy D, Felson TD. Do antioxidant micronutrients protect the development and progression of knee osteoarthritis? Arthritis & Rheumatology. 1996;39:648-56.
64. Felson DT, Niu J, Clancy M, Aliabadi P, Sack B, Guermazi A, Hunter DJ, Amin S, Rogers G, Booth SL. Low levels of vitamin D and worsening of knee osteoarthritis: results of two longitudinal studies. Arthritis Rheum. 2007;56(1):129-36.
65. Lane NE, Gore LR, Cummings SR, Hochberg MC, Scott JC, Williams EN, Nevitt MC. Serum vitamin D levels and incident changes of radiographic hip osteoarthritis: a longitudinal study. Study of Osteoporotic Fractures Research Group. Arthritis Rheum. 1999;42(5):854-60.
66. McAlindon TE, Felson DT, Zhang Y, Hannan MT, Aliabadi P, Weissman B, Rush D, Wilson PW, Jacques P. Relation of dietary intake and serum levels of vitamin D to progression of osteoarthritis of the knee among participants in the Framingham Study. Ann Intern Med. 1996;125(5):353-9.
67. Neogi T, Booth SL, Zhang YQ, Jacques PF, Terkeltaub R, Aliabadi P, Felson DT. Low vitamin K status is associated with osteoarthritis in the hand and knee. Arthritis Rheum. 2006;54(4):1255-61.
68. Messier SP, Legault C, Mihalko S, Miller GD, Loeser RF, DeVita P, Lyles M, Eckstein F, Hunter DJ, Williamson JD, Nicklas BJ. The Intensive Diet and Exercise for Arthritis (IDEA) trial: design and rationale. BMC Musculoskelet Disord. 2009;10:93.
69. Hadler NM, Gillings DB, Imbus HR, Levitin PM, Makuc D, Utsinger PD, Yount WJ, Slusser D, Moskovitz N. Hand structure and function in an industrial setting. Arthritis Rheum. 1978;21(2):210-20.

70. Croft P, Coggon D, Cruddas M, Cooper C. Osteoarthritis of the hip: an occupational disease in farmers. BMJ. 1992;304(6837):1269-72.
71. Hansen P, English M, Willick SE. Does running cause osteoarthritis in the hip or knee? PM & R : the journal of injury, function, and rehabilitation. 2012;4(5 Suppl):S117-21.
72. Hunter DJ, Eckstein F. Exercise and osteoarthritis. J Anat. 2009;214(2):197-207.
73. Slauterbeck JR, Kousa P, Clifton BC, Naud S, Tourville TW, Johnson RJ, Beynnon BD. Geographic mapping of meniscus and cartilage lesions associated with anterior cruciate ligament injuries. J Bone Joint Surg Am. 2009;91(9):2094-103.
74. Lohmander LS, Englund PM, Dahl LL, Roos EM. The long-term consequence of anterior cruciate ligament and meniscus injuries: osteoarthritis. Am J Sports Med. 2007;35(10):1756-69.
75. Bennell KL, Wrigley TV, Hunt MA, Lim BW, Hinman RS. Update on the role of muscle in the genesis and management of knee osteoarthritis. Rheum Dis Clin North Am. 2013;39(1):145-76.
76. Ikeda S, Tsumura H, Torisu T. Age-related quadriceps-dominant muscle atrophy and incident radiographic knee osteoarthritis. J Orthop Sci. 2005;10(2):121-6.
77. Fink B, Egl M, Singer J, Fuerst M, Bubenheim M, Neuen-Jacob E. Morphologic changes in the vastus medialis muscle in patients with osteoarthritis of the knee. Arthritis Rheum. 2007;56(11):3626-33.
78. Petterson SC, Barrance P, Buchanan T, Binder-Macleod S, Snyder-Mackler L. Mechanisms underlying quadriceps weakness in knee osteoarthritis. Med Sci Sports Exerc. 2008;40(3):422-7.
79. Slemenda C, Brandt KD, Heilman DK, Mazzuca S, Braunstein EM, Katz BP, Wolinsky FD. Quadriceps Weakness and Osteoarthritis of the Knee. Ann Intern Med. 1997;127(2):97-104.
80. Loeser RF. Age-related changes in the musculoskeletal system and the development of osteoarthritis. Clin Geriatr Med. 2010;26(3):371-86.
81. Brandt KD, Dieppe P, Radin EL. Etiopathogenesis of osteoarthritis. Rheum Dis Clin North Am. 2008;34(3):531-59.
82. Goldring MB, Goldring SR. Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis. Ann N Y Acad Sci. 2010;1192:230-7.
83. Borzi RM, Mazzetti I, Marcu KB, Facchini A. Chemokines in cartilage degradation. Clin Orthop Relat Res. 2004(427 Suppl):S53-61.
84. Silvestri T, Pulsatelli L, Dolzani P, Frizziero L, Facchini A, Meliconi R. In vivo expression of inflammatory cytokine receptors in the joint compartments of patients with arthritis. Rheumatol Int. 2006;26(4):360-8.

85. Yoshida H, Faust A, Wilckens J, Kitagawa M, Fetto J, Chao EY. Three-dimensional dynamic hip contact area and pressure distribution during activities of daily living. J Biomech. 2006;39(11):1996-2004.

86. Jessel RH, Zurakowski D, Zilkens C, Burstein D, Gray ML, Kim YJ. Radiographic and patient factors associated with pre-radiographic osteoarthritis in hip dysplasia. J Bone Joint Surg Am. 2009;91(5):1120-9.

87. Beck M, Kalhor M, Leunig M, Ganz R. Hip morphology influences the pattern of damage to the acetabular cartilage: femoroacetabular impingement as a cause of early osteoarthritis of the hip. J Bone Joint Surg Br. 2005;87(7):1012-8.

88. Konrath GA, Hamel AJ, Olson SA, Bay B, Sharkey NA. The role of the acetabular labrum and the transverse acetabular ligament in load transmission in the hip. J Bone Joint Surg Am. 1998;80(12):1781-8.

89. Henak CR, Abraham CL, Anderson AE, Maas SA, Ellis BJ, Peters CL, Weiss JA. Patient-specific analysis of cartilage and labrum mechanics in human hips with acetabular dysplasia. Osteoarthritis Cartilage. 2014;22(2):210-7.

90. Andriacchi TP, Koo S, Scanlan SF. Gait mechanics influence healthy cartilage morphology and osteoarthritis of the knee. J Bone Joint Surg Am. 2009;91 Suppl 1:95-101.

91. Logan M, Dunstan E, Robinson J, Williams A, Gedroyc W, Freeman M. Tibiofemoral kinematics of the anterior cruciate ligament (ACL)-deficient weightbearing, living knee employing vertical access open "interventional" multiple resonance imaging. Am J Sports Med. 2004;32(3):720-6.

92. Scarvell JM, Smith PN, Refshauge KM, Galloway HR, Woods KR. Comparison of kinematic analysis by mapping tibiofemoral contact with movement of the femoral condylar centres in healthy and anterior cruciate ligament injured knees. J Orthop Res. 2004;22(5):955-62.

93. Sharma L, Song J, Felson DT, Cahue S, Shamiyeh E, Dunlop DD. The role of knee alignment in disease progression and functional decline in knee osteoarthritis. JAMA. 2001;286(2):188-95.

94. Harman MK, Markovich GD, Banks SA, Hodge WA. Wear patterns on tibial plateaus from varus and valgus osteoarthritic knees. Clin Orthop Relat Res. 1998(352):149-58.

95. Moschella D, Blasi A, Leardini A, Ensini A, Catani F. Wear patterns on tibial plateau from varus osteoarthritic knees. Clin Biomech (Bristol, Avon). 2006;21(2):152-8.

96. Georgoulis AD, Papadonikolakis A, Papageorgiou CD, Mitsou A, Stergiou N. Three-dimensional tibiofemoral kinematics of the anterior cruciate ligament-deficient and reconstructed knee during walking. Am J Sports Med. 2003;31(1):75-9.

97. Wright V, Dowson D. Lubrication and cartilage. J Anat. 1976;121(Pt 1):107-18.

98. Schmidt TA, Sah RL. Effect of synovial fluid on boundary lubrication of articular cartilage. Osteoarthritis Cartilage. 2007;15(1):35-47.

99. Kurtz SM, Lau E, Watson H, Schmier JK, Parvizi J. Economic burden of periprosthetic joint infection in the United States. J Arthroplasty. 2012;27(8 Suppl):61-5.e1.

100. Huotari K, Peltola M, Jamsen E. The incidence of late prosthetic joint infections: a registry-based study of 112,708 primary hip and knee replacements. Acta Orthop. 2015;86(3):321-5.

101. Padegimas EM, Maltenfort M, Ramsey ML, Williams GR, Parvizi J, Namdari S. Periprosthetic shoulder infection in the United States: incidence and economic burden. J Shoulder Elbow Surg. 2015;24(5):741-6.

102. Parvizi J. INFECTION: A RECURRING SLIPPERY SLOPE. Orthopaedic Proceedings. 2015;97-B(SUPP_1):116-.

103. Parvizi J, Zmistowski B, Berbari EF, Bauer TW, Springer BD, Della Valle CJ, Garvin KL, Mont MA, Wongworawat MD, Zalavras CG. New definition for periprosthetic joint infection: from the Workgroup of the Musculoskeletal Infection Society. Clin Orthop Relat Res. 2011;469(11):2992-4.

104. Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol. 2005;21:319-46.

105. Montanaro L, Poggi A, Visai L, Ravaioli S, Campoccia D, Speziale P, Arciola CR. Extracellular DNA in biofilms. Int J Artif Organs. 2011;34(9):824-31.

106. Schinsky MF, Della Valle CD, Sporer SM. Perioperative testing for joint infection in patients undergoing revision total hip arthroplasty. Journal of Bone and Joint Surgery of America. 2008;90:1869-75.

107. Spangehl MJ, Masri BA, O'Connell JX, Duncan CP. Prospective analysis of preoperative and intraoperative investigations for the diagnosis of infection at the sites of two hundred and two revision total hip arthroplasties. J Bone Joint Surg Am. 1999;81(5):672-83.

108. Parvizi J, McKenzie JC, Cashman JP. Diagnosis of periprosthetic joint infection using synovial C-reactive protein. J Arthroplasty. 2012;27(8 Suppl):12-6.

109. Parvizi J, Jacovides C, Adeli B, Jung KA, Hozack WJ. Mark B. Coventry Award: synovial C-reactive protein: a prospective evaluation of a molecular marker for periprosthetic knee joint infection. Clin Orthop Relat Res. 2012;470(1):54-60.

110. Song M, Kellum JA. Interleukin-6. Crit Care Med. 2005;33(12 Suppl):S463-5.

111. Di Cesare PE, Chang E, Preston CF, Liu CJ. Serum interleukin-6 as a marker of periprosthetic infection following total hip and knee arthroplasty. J Bone Joint Surg Am. 2005;87(9):1921-7.
112. Xie K, Dai K, Qu X, Yan M. Serum and Synovial Fluid Interleukin-6 for the Diagnosis of Periprosthetic Joint Infection. Sci Rep. 2017;7(1):1496.
113. Gilbert DN. Use of plasma procalcitonin levels as an adjunct to clinical microbiology. J Clin Microbiol. 2010;48(7):2325-9.
114. Prucha M, Bellingan G, Zazula R. Sepsis biomarkers. Clin Chim Acta. 2015;440:97-103.
115. Pfafflin A, Schleicher E. Inflammation markers in point-of-care testing (POCT). Anal Bioanal Chem. 2009;393(5):1473-80.
116. Lee H. Procalcitonin as a biomarker of infectious diseases. The Korean Journal of Internal Medicine. 2013;28(3):285-91.
117. Bottner F, Wegner A, Winkelmann W, Becker K, Erren M, Gotze C. Interleukin-6, procalcitonin and TNF-alpha: markers of peri-prosthetic infection following total joint replacement. J Bone Joint Surg Br. 2007;89(1):94-9.
118. Xie K, Qu X, Yan M. Procalcitonin and alpha-Defensin for Diagnosis of Periprosthetic Joint Infections. J Arthroplasty. 2017;32(4):1387-94.
119. Francesconi LP, Cereser KM, Mascarenhas R, Stertz L, Gama CS, Belmonte-de-Abreu P. Increased annexin-V and decreased TNF-alpha serum levels in chronic-medicated patients with schizophrenia. Neurosci Lett. 2011;502(3):143-6.
120. Lv B, Huang J, Yuan H, Yan W, Hu G, Wang J. Tumor Necrosis Factor-α as a Diagnostic Marker for Neonatal Sepsis: A Meta-Analysis. The Scientific World Journal. 2014;2014:14.
121. Shahi A, Kheir MM, Tarabichi M, Hosseinzadeh HRS, Tan TL, Parvizi J. Serum D-Dimer Test Is Promising for the Diagnosis of Periprosthetic Joint Infection and Timing of Reimplantation. J Bone Joint Surg Am. 2017;99(17):1419-27.
122. Drago L, Vassena C, Dozio E, Corsi MM, De Vecchi E, Mattina R, Romano C. Procalcitonin, C-reactive protein, interleukin-6, and soluble intercellular adhesion molecule-1 as markers of postoperative orthopaedic joint prosthesis infections. Int J Immunopathol Pharmacol. 2011;24(2):433-40.
123. Worthington T, Dunlop D, Casey A, Lambert R, Luscombe J, Elliott T. Serum procalcitonin, interleukin-6, soluble intercellular adhesin molecule-1 and IgG to short-chain exocellular lipoteichoic acid as predictors of infection in total joint prosthesis revision. Br J Biomed Sci. 2010;67(2):71-6.

124. Wyatt MC, Beswick AD, Kunutsor SK, Wilson MJ, Whitehouse MR, Blom AW. The Alpha-Defensin Immunoassay and Leukocyte Esterase Colorimetric Strip Test for the Diagnosis of Periprosthetic Infection: A Systematic Review and Meta-Analysis. J Bone Joint Surg Am. 2016;98(12):992-1000.
125. Lehrer RI, Ganz T. Defensins: endogenous antibiotic peptides from human leukocytes. Ciba Found Symp. 1992;171:276-90; discussion 90-3.
126. Suen K, Keeka M, Ailabouni R, Tran P. Synovasure 'quick test' is not as accurate as the laboratory-based alpha-defensin immunoassay: a systematic review and meta-analysis. The bone & joint journal. 2018;100-b(1):66-72.
127. Chernow B, Zaloga GP, Soldano S, Quinn A, Lyons P, McFadden E, Cook D, Rainey TG. Measurement of urinary leukocyte esterase activity: a screening test for urinary tract infections. Ann Emerg Med. 1984;13(3):150-4.
128. Parvizi J, Jacovides C, Antoci V, Ghanem E. Diagnosis of Periprosthetic Joint Infection: The Utility of a Simple Yet Unappreciated Enzyme. The Journal of Bone and Joint Surgery. 2011;93:2242-8.
129. Wang C, Li R, Wang Q, Duan J, Wang C. Leukocyte Esterase as a Biomarker in the Diagnosis of Periprosthetic Joint Infection. Medical Science Monitor : International Medical Journal of Experimental and Clinical Research. 2017;23:353-8.
130. Huang R, Hu CC, Adeli B, Mortazavi J, Parvizi J. Culture-negative periprosthetic joint infection does not preclude infection control. Clin Orthop Relat Res. 2012;470(10):2717-23.
131. Arciola CR, Campoccia D, Speziale P, Montanaro L, Costerton JW. Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. Biomaterials. 2012;33(26):5967-82.
132. Costerton JW, Post JC, Ehrlich GD, Hu FZ, Kreft R, Nistico L, Kathju S, Stoodley P, Hall-Stoodley L, Maale G, James G, Sotereanos N, DeMeo P. New methods for the detection of orthopedic and other biofilm infections. FEMS Immunol Med Microbiol. 2011;61(2):133-40.
133. Larsen LH, Lange J, Xu Y, Schonheyder HC. Optimizing culture methods for diagnosis of prosthetic joint infections: a summary of modifications and improvements reported since 1995. J Med Microbiol. 2012;61(Pt 3):309-16.
134. Arciola CR, Montanaro L, Costerton JW. New trends in diagnosis and control strategies for implant infections. Int J Artif Organs. 2011;34(9):727-36.
135. Rasouli MR, Harandi AA, Adeli B, Purtill JJ, Parvizi J. Revision total knee arthroplasty: infection should be ruled out in all cases. J Arthroplasty. 2012;27(6):1239-43.e1-2.

136. Hansen EN, Zmistowski B, Parvizi J. Periprosthetic joint infection: what is on the horizon? Int J Artif Organs. 2012;35(10):935-50.

137. Palmer MP, Melton-Kreft R, Nistico L, Hiller NL, Kim LHJ, Altman GT, Altman DT, Sotereanos NG, Hu FZ, De Meo PJ, Ehrlich GD. Polymerase Chain Reaction–Electrospray–Time-of-Flight Mass Spectrometry Versus Culture for Bacterial Detection in Septic Arthritis and Osteoarthritis. Genet Test Mol Biomarkers. 2016;20(12):721-31.

138. Wiener E, Zanetti M, Hodler J, Pfirrmann CWA. Lactate and T 2 measurements of synovial aspirates at 1.5 T: differentiation of septic from non-septic arthritis. Skeletal Radiol. 2008;37(8):743.

139. Hugle T, Kovacs H, Heijnen IA, Daikeler T, Baisch U, Hicks JM, al. e. Synovial fluid metabolomics in different forms of arthritis assessed by nuclear magnetic resonance spectroscopy. Clin Exp Rheumatol. 2012;30:240-5.

140. Jin W, Woo DC, Jahng GH. In vivo H1 MR spectroscopy using 3 Tesla to investigate the metabolic profiles of joint fluids in different types of knee diseases. J Appl Clin Med Phys. 2016;17(2):561-72.

141. Nicholson JK, Lindon JC, Holmes E. Metabolomic analysis using optimized NMR and statistical methods. Xenobiotica. 1999;29(11):1181-9.

142. Lindon JC, Holmes E, Nicholson JK. So what's the deal with metabonomics? Anal Chem. 2003;75:384A-91A.

143. Horning EC, Horning MG. Metabolic profiles: gas-phase methods for analysis of metabolites. Clin Chem. 1971;17(8):802-9.

144. T.D.W HLMaC. Introduction to organic spectroscopy: Oxford University Press; 1999.

145. Claridge TDW. High Resolution NMR Techniques in Organic Chemistry. 2nd edition ed: Elsevier Science, Oxford; 2009.

146. P K. Basic principles of MR imaging: Milwaukee, GE Medical Medical Systems; 1988.

147. Nicholson JK, Wilson ID. High Resolution Proton Magnetic Resonance Spectroscopy Of Biological Fluids. Prog Nucl Magn Reson Spectrosc. 1989;21:449-501.

148. Lindon JC, Nicholson JK. Spectroscopic and Statistical Techniques for Information Recovery in Metabonomics and Metabolomics doi:10.1146/annurev.anchem.1.031207.113026. Annual Review of Analytical Chemistry. 2008;1(1):45-69.

149. Simmler C, Napolitano JG, McAlpine JB, Chen SN, Pauli GF. Universal quantitative NMR analysis of complex natural samples. Curr Opin Biotechnol. 2014;25:51-9.

150. Glish GL, Vachet RW. The basics of mass spectrometry in the twenty-first century. Nat Rev Drug Discov. 2003;2(2):140-50.
151. Lindon JC, Nicholson JK, Wilson ID. Directly coupled HPLC-NMR and HPLC-NMR-MS in pharmaceutical research and development. J Chromatogr B Biomed Sci Appl. 2000;748(1):233-58.
152. Emwas AH. The strengths and weaknesses of NMR spectroscopy and mass spectrometry with particular focus on metabolomics research. Methods Mol Biol. 2015;1277:161-93.
153. Rocha SM, Caldeira M, Carrola J, Santos M, Cruz N, Duarte IF. Exploring the human urine metabolomic potentialities by comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry. J Chromatogr A. 2012;1252:155-63.
154. Altmaier E, Ramsay SL, Graber A, Mewes H-W, Weinberger KM, Suhre K. Bioinformatics Analysis of Targeted Metabolomics—Uncovering Old and New Tales of Diabetic Mice under Medication. Endocrinology. 2008;149(7):3478-89.
155. Bartel J, Krumsiek J, Theis FJ. Statistical methods for the analysis of high-throughput metabolomics data. Comput Struct Biotechnol J. 2013;4:e201301009.
156. Tukey JW. Some thoughts on clinical trials, especially problems of multiplicity. Science. 1977;198(4318):679-84.
157. Rothman KJ. No adjustments are needed for multiple comparisons. Epidemiology. 1990;1(1):43-6.
158. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological). 1995;57(1):289-300.
159. Trygg J, Holmes E, Lundstedt Tr. Chemometrics in Metabonomics. J Proteome Res. 2006;6(2):469-79.
160. Lindon JC, Holmes E, Nicholson JK. Pattern recognition methods and applications in biomedical magnetic resonance. Prog Nucl Magn Reson Spectrosc. 2001;39:1-40.
161. Hotelling H. The Generalization of Student's Ratio. Ann Math Statist. 1931;2(3):360-78.
162. Wold S, Sjöström M, Eriksson L. PLS-regression: a basic tool of chemometrics. Chemometrics Intellig Lab Syst. 2001;58(2):109-30.
163. Trygg J, Wold S. Orthogonal projections to latent structures (O-PLS). J Chemometrics. 2002;16(3):119-28.

164. Fonville JM, Richards SE, Barton RH, Boulange CL, Ebbels TMD, Nicholson JK, Holmes E, Dumas ME. The evolution of partial least squares models and related chemometric approaches in metabonomics and metabolic phenotyping. J Chemometrics. 2010;24(11-12):636-49.

165. Eriksson L, Bryne T, Johansson E, Trygg J, Vikstrom E. Multi- and Megavariate Data Analysis Basic Principles and Applications. 3rd ed. Sweden: MKS Umetrics AB; 2013.

166. Akhbari P, Karamchandani U, Jaggard MKJ, Graça G, Bhattacharya R, Lindon JC, Williams HRT, Gupte CM. Can joint fluid metabolic profiling (or "metabonomics") reveal biomarkers for osteoarthritis and inflammatory joint disease? Bone & joint research. 2020;9(3):108-19.

167. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. BMJ. 2009;339:b2535.

168. Ulrich EL, Akutsu H, Doreleijers JF, Harano Y, Ioannidis YE, Lin J, Livny M, Mading S, Maziuk D, Miller Z, Nakatani E, Schulte CF, Tolmie DE, Kent Wenger R, Yao H, Markley JL. BioMagResBank. Nucleic Acids Res. 2008;36(Database issue):D402-8.

169. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, Djoumbou Y, Mandal R, Aziat F, Dong E, Bouatra S, Sinelnikov I, Arndt D, Xia J, Liu P, Yallou F, Bjorndahl T, Perez-Pineiro R, Eisner R, Allen F, Neveu V, Greiner R, Scalbert A. HMDB 3.0—The Human Metabolome Database in 2013. Nucleic Acids Res. 2013;41(Database issue):D801-D7.

170. Adams SB, Nettles DL, Jones LC, Miller SD, Guyton GP, Schon LC. Inflammatory cytokines and cellular metabolites as synovial fluid biomarkers of posttraumatic ankle arthritis. Foot Ankle Int. 2014;35(12):1241-9.

171. Ahn JK, Kim S, Kim J, Hwang J, Kim KH, Cha HS. A Comparative Metabolomic Evaluation of Behcet's Disease with Arthritis and Seronegative Arthritis Using Synovial Fluid. PLoS One. 2015;10(8):e0135856.

172. Anderson JR, Chokesuwattanaskul S, Phelan MP, Welting TJM, Lian LY, Peffers MJ, Wright HL, editors. Synovial fluid metabolite profiles differ between osteoarthritis and rheumatoid arthritis2018; Netherlands: 2018 Annual Conference of the British Society for Rheumatology. United Kingdom. 57 (Supplement 3) (pp iii152-iii153); Oxford University Press.

173. Carlson AK, Rawle RA, Adams E, Greenwood MC, Bothner B, June RK. Application of global metabolomic profiling of synovial fluid for osteoarthritis biomarkers. Biochem Biophys Res Commun. 2018;499(2):182-8.

174. Chen R, Han S, Liu X, Wang K, Zhou Y, Yang C, Zhang X. Perturbations in amino acids and metabolic pathways in osteoarthritis patients determined by targeted metabolomics analysis. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences. 2018;1085:54-62.
175. Dubey D, Chaurasia S, Guleria A, Kumar S, Modi DR, Misra R, Kumar D. Metabolite assignment of ultrafiltered synovial fluid extracted from knee joints of reactive arthritis patients using high resolution NMR spectroscopy. Magn Reson Chem. 2019;57(1):30-43.
176. Dubey D, Kumar S, Ahmed S, Chaudhary A, Chaurasia S, Guleria A, Kumar D, Misra R, editors. NMR based serum and synovial fluid metabolomics reveal similar metabolomic profile in patients with reactive arthritis and undifferentiated spondyloarthropathy2017; Netherlands: 33rd Annual Conference of the Indian Rheumatology Association, IRACON 2017. India. 12 (5 Supplement 1) (pp S24-S25); Medknow Publications.
177. Dubey D, Kumar S, Chaurasia S, Guleria A, Ahmed S, Singh R, Kumari R, Modi DR, Misra R, Kumar D. NMR-Based Serum Metabolomics Revealed Distinctive Metabolic Patterns in Reactive Arthritis Compared with Rheumatoid Arthritis. J Proteome Res. 2019;18(1):130-46.
178. Furman BD, Kimmerling KA, Ramamoorthy S, Li YJ, Wu YH, Huebner JL, Guilak F, Kraus VB, Olson SA, editors. Articular fracture upregulates lipid metabolites in human synovial fluid2017; Netherlands: 2017 Osteoarthritis Research Society International, OARSI World Congress. United States. 25 (Supplement 1) (pp S381); W.B. Saunders Ltd.
179. Hwang J, Ahn JK, Lee J, Kim I, Lee S, Jeon CH, Koh EM, Cha HS, editors. Discriminative metabolite profiling of synovial fluid in rheumatoid arthritis compared to osteoarthritis2013; Arthritis and Rheumatism. Conference: American College of Rheumatology/Association of Rheumatology Health Professionals Annual Scientific Meeting, ACR/ARHP 2013. San Diego, CA United States. Conference Publication: (var.pagings). 65 (SUPPL. 10) (pp S561); John Wiley and Sons Inc.
180. Kang KY, Lee SH, Jung SM, Park SH, Jung BH, Ju JH. Downregulation of tryptophan-related metabolomic profile in rheumatoid arthritis synovial fluid. J Rheumatol. 2015;42(11):2003-11.
181. Khatib N, Papageorgiou A, Fairhurst S, Wilson C, Mason DJ. Identifying load responsive synovial fluid metabolic markers following pivot-shift testing in ACL injury subjects. Osteoarthritis Cartilage. 2018;26:S168-S9.
182. Kim S, Hwang J, Kim J, Ahn JK, Cha HS, Kim KH. Metabolite profiles of synovial fluid change with the radiographic severity of knee osteoarthritis. Joint Bone Spine. 2017;84(5):605-10.

183. Kim S, Hwang J, Xuan J, Jung YH, Cha HS, Kim KH. Global metabolite profiling of synovial fluid for the specific diagnosis of rheumatoid arthritis from other inflammatory arthritis. PLoS One. 2014;9(6):e97501.

184. Leimer EM, Pappan KL, Nettles DL, Bell RD, Easley ME, Olson SA, Setton LA, Adams SB. Lipid profile of human synovial fluid following intra-articular ankle fracture. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2017;35(3):657-66.

185. Meshitsuka S, Yamazaki E, Inoue M, Hagino H, Teshima R, Yamamoto K. Nuclear magnetic resonance studies of synovial fluids from patients with rheumatoid arthritis and osteoarthritis. Clin Chim Acta. 1999;281:163-7.

186. Mickiewicz B, Kelly JJ, Ludwig TE, Weljie AM, Wiley JP, Schmidt TA, Vogel HJ. Metabolic analysis of knee synovial fluid as a potential diagnostic approach for osteoarthritis. J Orth Res. 2015;33(11):1631-8.

187. Naughton DP, Haywood R, Blake DR, Edmonds S, Hawkes GE, Grootveld M. A comparative evaluation of the metabolic profiles of normal and inflammatory knee-joint synovial fluids by high resolution proton NMR spectrosopy. Federation of European Biochemical Societies. 1993;332(3):221-5.

188. Yang XY, Zheng KD, Lin K, Zheng G, Zou H, Wang JM, Lin YY, Chuka CM, Ge RS, Zhai W, Wang JG. Energy metabolism disorder as a contributing factor of rheumatoid arthritis: A comparative proteomic and metabolomic study. PLoS One. 2015;10(7) (pagination):Arte Number: e0132695. ate of Pubaton: 06 Ju 2015.

189. Zhang W, Likhodii S, Zhang Y, Aref-Eshghi E, Harper PE, Randell E, Green R, Martin G, Furey A, Sun G, Rahman P, Zhai G. Classification of osteoarthritis phenotypes by metabolomics analysis. BMJ Open. 2014;4(11):e006286.

190. Zhang W, Likhodii S, ArefEshghi E, Zhang Y, Harper PE, Randell E, Green R, Martin G, Furey A, Sun G, Rahman P, Zhai G. Relationship between blood plasma and synovial fluid metabolite concentrations in patients with osteoarthritis. J Rheumatol. 2015;42(5):859-65.

191. Zhang WD, Sun G, Likhodii S, Aref-Eshghi E, Harper PE, Randell E, Green R, Martin G, Furey A, Rahman P, Zhai GJ. Metabolomic analysis of human synovial fluid and plasma reveals that phosphatidylcholine metabolism is associated with both osteoarthritis and diabetes mellitus. Metabolomics. 2016;12(2):24.

192. Zheng K, Shen N, Chen H, Ni S, Zhang T, Hu M, Wang J, Sun L, Yang X. Global and targeted metabolomics of synovial fluid discovers special osteoarthritis metabolites. J Orth Res. 2017;35(9):1973-81.

193. Yu Z, Zhai G, Singmann P, He Y, Xu T, Prehn C, Römisch-Margl W, Lattka E, Gieger C, Soranzo N, Heinrich J, Standl M, Thiering E, Mittelstraß K, Wichmann HE, Peters A, Suhre K, Li Y, Adamski J, Spector TD, Illig T, Wang-Sattler R. Human serum metabolic profiles are age dependent. Aging cell. 2012;11(6):960-7.

194. Oliviero F, Lo Nigro A, Bernardi D, Giunco S, Baldo G, Scanu A, Sfriso P, Ramonda R, Plebani M, Punzi L. A comparative study of serum and synovial fluid lipoprotein levels in patients with various arthritides. Clin Chim Acta. 2012;413(1-2):303-7.

195. Posma JM, Robinette SL, Holmes E, Nicholson JK. MetaboNetworks, an interactive Matlab-based toolbox for creating, customizing and exploring sub-networks from KEGG. Bioinformatics. 2014;30(6):893-5.

196. Miller EJ, Van Der Korst JK, Sokoloff L. Collagen of human articular and costal cartilage. Arthritis Rheum. 1969;12(1):21-9.

197. Januszewski AS, Karschimkus C, Davis KE, O'Neal D, Ward G, Jenkins AJ. Plasma 1,5 anhydroglucitol levels, a measure of short-term glycaemia: assay assessment and lower levels in diabetic vs. non-diabetic subjects. Diabetes Res Clin Pract. 2012;95(1):e17-9.

198. Tillmann K. Pathological aspects of osteoarthritis related to surgery. Inflammation. 1984;8 Suppl:S57-74.

199. Thomas-Reetz A, Hell JW, During MJ, Walch-Solimena C, Jahn R, De Camilli P. A gamma-aminobutyric acid transporter driven by a proton pump is present in synaptic-like microvesicles of pancreatic beta cells. Proceedings of the National Academy of Sciences. 1993;90(11):5317-21.

200. Handley CJ, Speight G, Leyden KM, Lowther DA. Extracellular matrix metabolism by chondrocytes 7. Evidence that L-glutamine is an essential amino acid for chondrocytes and other connective tissue cells. Biochimica et Biophysica Acta (BBA) - General Subjects. 1980;627(3):324-31.

201. Kim DS, Shin MR, Kim YS, Bae WJ, Roh DH, Hwang YS, Kim EC. Anti-inflammatory effects of glutamine on LPS-stimulated human dental pulp cells correlate with activation of MKP-1 and attenuation of the MAPK and NF-kappaB pathways. Int Endod J. 2015;48(3):220-8.

202. Tonomura H, Takahashi KA, Mazda O, Arai Y, Inoue A, Terauchi R, Shin-Ya M, Kishida T, Imanishi J, Kubo T. Glutamine protects articular chondrocytes from heat stress and NO-induced apoptosis with HSP70 expression. Osteoarthritis Cartilage. 2006;14(6):545-53.

203. Mickiewicz B, Heard BJ, Chau JK, Chung M, Hart DA, Shrive NG, Frank CB, Vogel HJ. Metabolic profiling of synovial fluid in a unilateral ovine model of anterior cruciate ligament reconstruction of the knee suggests biomarkers for early osteoarthritis. J Orthop Res. 2015;33(1):71-7.

204. Xu Z, Chen T, Luo J, Ding S, Gao S, Zhang J. Cartilaginous Metabolomic Study Reveals Potential Mechanisms of Osteophyte Formation in Osteoarthritis. J Proteome Res. 2017;16(4):1425-35.
205. Chang X, Wei C. Glycolysis and rheumatoid arthritis. Int J Rheum Dis. 2011;14(3):217-22.
206. Kim S, Hwang J, Kim J, Ahn JK, Cha H-S, Kim KH. Metabolite profiles of synovial fluid change with the radiographic severity of knee osteoarthritis. Joint Bone Spine. 2017;84(5):605-10.
207. Hills BA. Surface-active phospholipid: a Pandora's box of clinical applications. Part II. Barrier and lubricating properties. Intern Med J. 2002;32(5-6):242-51.
208. Bassit RA, Sawada LA, Bacurau RF, Navarro F, Martins E, Jr., Santos RV, Caperuto EC, Rogeri P, Costa Rosa LF. Branched-chain amino acid supplementation and the immune response of long-distance athletes. Nutrition. 2002;18(5):376-9.
209. Perlman S, Carr SA. Citramalic acid in cerebrospinal fluid of patients with bacterial meningitis. Clin Chem. 1984;30(7):1209-12.
210. Pineton de Chambrun M, Wechsler B, Geri G, Cacoub P, Saadoun D. New insights into the pathogenesis of Behcet's disease. Autoimmun Rev. 2012;11(10):687-98.
211. Chen M, Lam BK, Kanaoka Y, Nigrovic PA, Audoly LP, Austen KF, Lee DM. Neutrophil-derived leukotriene B4 is required for inflammatory arthritis. The Journal of Experimental Medicine. 2006;203:837-42.
212. Fallarino F, Grohmann U, Vacca C, Bianchi R, Orabona C, Spreca A, Fioretti MC, Puccetti P. T cell apoptosis by tryptophan catabolism. Cell Death Differ. 2002;9(10):1069-77.
213. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, Fan TW, Fiehn O, Goodacre R, Griffin JL, Hankemeier T, Hardy N, Harnly J, Higashi R, Kopka J, Lane AN, Lindon JC, Marriott P, Nicholls AW, Reily MD, Thaden JJ, Viant MR. Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics. 2007;3(3):211-21.
214. Soul J, Dunn SL, Anand S, Serracino-Inglott F, Schwartz JM, Boot-Handford RP, Hardingham TE. Stratification of knee osteoarthritis: two major patient subgroups identified by genome-wide expression analysis of articular cartilage. Ann Rheum Dis. 2018;77(3):423.
215. Kung LHW, Ravi V, Rowley L, Angelucci C, Fosang AJ, Bell KM, Little CB, Bateman JF. Cartilage MicroRNA Dysregulation During the Onset and Progression of Mouse Osteoarthritis Is Independent of Aggrecanolysis and Overlaps With Candidates From End-Stage Human Disease. Arthritis & rheumatology (Hoboken, NJ). 2018;70(3):383-95.

216. Hoppenfeld S, De Boer PG, Buckley R. Surgical exposures in orthopaedics : the anatomic approach. 2009.
217. Akhbari P, Jaggard MK, Boulangé CL, Vaghela U, Graça G, Bhattacharya R, Lindon JC, Williams HRT, Gupte CM. Differences in the composition of hip and knee synovial fluid in osteoarthritis: a nuclear magnetic resonance (NMR) spectroscopy study of metabolic profiles. Osteoarthritis Cartilage. 2019;27(12):1768-77.
218. Jaggard MKJ, Boulangé CL, Graça G, Akhbari P, Vaghela U, Bhattacharya R, Williams HRT, Lindon JC, Gupte CM. The influence of sample collection, handling and low temperature storage upon NMR metabolic profiling analysis in human synovial fluid. J Pharm Biomed Anal. 2021;197:113942.
219. Dona AC, Jiménez B, Schäfer H, Humpfer E, Spraul M, Lewis MR, Pearce JTM, Holmes E, Lindon JC, Nicholson JK. Precision High-Throughput Proton NMR Spectroscopy of Human Urine, Serum, and Plasma for Large-Scale Metabolic Phenotyping. Anal Chem. 2014;86(19):9887-94.
220. Veselkov KA, Lindon JC, Ebbels TM, Crockford D, Volynkin VV, Holmes E, Davies DB, Nicholson JK. Recursive segment-wise peak alignment of biological (1)h NMR spectra for improved metabolic biomarker recovery. Anal Chem. 2009;81(1):56-66.
221. Dieterle F, Ross A, Schlotterbeck G, Senn H. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. Anal Chem. 2006;78(13):4281-90.
222. van den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, van der Werf MJ. Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics. 2006;7(1):142.
223. Cloarec O, Dumas ME, Craig A, Barton RH, Trygg J, Hudson J, Blancher C, Gauguier D, Lindon JC, Holmes E, Nicholson J. Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic 1H NMR data sets. Anal Chem. 2005;77(5):1282-9.
224. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acid Research. 2000;28(1):27-30.
225. Chen A, Gupte C, Akhtar K, Smith P, Cobb J. The Global Economic Cost of Osteoarthritis: How the UK Compares. Arthritis. 2012;2012:698709.
226. Krasnokutsky S, Attur M, Palmer G, Samuels J, Abramson SB. Current concepts in the pathogenesis of osteoarthritis. Osteoarthritis Cartilage. 2008;16 Suppl 3:S1-3.

227. Galandakova A, Ulrichova J, Langova K, Hanakova A, Vrbka M, Hartl M, Gallo J. Characteristics of synovial fluid required for optimization of lubrication fluid for biotribological experiments. J Biomed Mater Res B Appl Biomater. 2017;105(6):1422-31.

228. Olszewska-Slonina D, Jung S, Matewski D, Olszewski KJ, Krzyzynska-Malinowska E, Braszkiewicz A, Kowaliszyn B. Lysosomal enzymes in serum and synovial fluid in patients with osteoarthritis. Scand J Clin Lab Invest. 2015;75(2):145-51.

229. Snelling SJ, Bas S, Puskas GJ, Dakin SG, Suva D, Finckh A, Gabay C, Hoffmeyer P, Carr AJ, Lubbeke A. Presence of IL-17 in synovial fluid identifies a potential inflammatory osteoarthritic phenotype. PLoS One. 2017;12(4):e0175109.

230. Fischer K, Kettunen J, Wurtz P, Haller T, Havulinna AS, Kangas AJ, Soininen P, Esko T, Tammesoo ML, Magi R, Smit S, Palotie A, Ripatti S, Salomaa V, Ala-Korpela M, Perola M, Metspalu A. Biomarker profiling by nuclear magnetic resonance spectroscopy for the prediction of all-cause mortality: an observational study of 17,345 persons. PLoS Med. 2014;11(2):e1001606.

231. Holmes E, Loo RL, Stamler J, Bictash M, Yap I, Chan Q, Ebbels T, De Iorio M, Brown I, Veselkov K, Daviglus M, Kesteloot H, Ueshima H, Zhao L, Nicholson J, Elliott P. Human metabolic phenotype diversity and its association with diet and blood pressure. Nature. 2008;453:396-400.

232. Yu B, Zheng Y, Nettleton JA, Alexander D, Coresh J, Boerwinkle E. Serum metabolomic profiling and incident CKD among African Americans. Clin J Am Soc Nephrol. 2014;9(8):1410-7.

233. Lamers R-JAN, van Nesselrooij JHJ, Kraus VB, Jordan JM, Renner JB, Dragomir AD, Luta G, van der Greef J, DeGroot J. Identification of an urinary metabolite profile associated with osteoarthritis. Osteoarthritis Cartilage. 2005;13(9):762-8.

234. Zhai G, Wang-Sattler R, Hart DJ, Arden NK, Hakim AJ, Illig T, Spector TD. Serum branched-chain amino acid to histidine ratio: a novel metabolomic biomarker of knee osteoarthritis. Ann Rheum Dis. 2010;69(6):1227-31.

235. Qiu Y, Cai G, Zhou B, Li D, Zhao A, Xie G, Li H, Cai S, Xie D, Huang C, Ge W, Zhou Z, Xu L, Jia W, Zheng S, Yen Y, Jia W. A distinct metabolic signature of human colorectal cancer with prognostic potential. Clin Cancer Res. 2014.

236. Kim S, Hwang J, Kim J, Ahn JK, Cha HS, Kim KH. Metabolite profiles of synovial fluid change with the radiographic severity of knee osteoarthritis. Joint Bone Spine. 2016.

237. Zhang Q, Li H, Zhang Z, Yang F, Chen J. Serum metabolites as potential biomarkers for diagnosis of knee osteoarthritis. Dis Markers. 2015;2015:684794.

238. Zhang W, Sun G, Likhodii S, Liu M, Aref-Eshghi E, Harper PE, Martin G, Furey A, Green R, Randell E, Rahman P, Zhai G. Metabolomic analysis of human plasma reveals that arginine is depleted in knee osteoarthritis patients. Osteoarthritis Cartilage. 2016;24(5):827-34.

239. Damyanovich AZ, Staples JR, Chan ADM, Marshall KW. Comparative Study of Normal and Osteoarthritic Canine Synovial Fluid using 500 MHz ^{1}H Magnetic Resonance Spectroscopy. Journal of Orthpaedic Research. 1999;17:223-31.

240. Lacitignola L, Fanizzi FP, Francioso E, al. e. 1H NMR investigation of normal and osteoarthritic synovial fluid in the horse. Vet Comp Orthop Traumatol. 2008;21:85-8.

241. Schiller J, Naji L, Huster D, Kaufmann J, Arnold K. 1H and 13C HR-MAS NMR investigations on native and enzymatically digested bovine nasal cartilage. MAGMA. 2001;13(1):19-27.

242. Thompson Jr RC, Oegema Jr TR. Metabolic activity of articular cartilage in osteoarthritis. An in vitro study. Journal of Bone and Joint Surgery - Series A. 1979;61(A3):407-16.

243. Berg JM TJ, Stryer L. . Amino Acids Are Made from Intermediates of the Citric Acid Cycle and Other Major Pathways. Biochemistry. Section 24.2. 5th edition ed: New York: W H Freeman; 2002.

244. Kresse H, Schonherr E. Proteoglycans of the extracellular matrix and growth control. J Cell Physiol. 2001;189(3):266-74.

245. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. FASEB J. 1992;6(3):861-70.

246. Blanco FJ, Lopez-Armada MJ, Maneiro E. Mitochondrial dysfunction in osteoarthritis. Mitochondrion. 2004;4(5-6):715-28.

247. Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids. 2009;37(1):1-17.

248. Maher AD, Coles C, White J, Bateman JF, Fuller ES, Burkhardt D, Little CB, Cake M, Read R, McDonagh MB, Rochfort SJ. 1H NMR Spectroscopy of Serum Reveals Unique Metabolic Fingerprints Associated with Subtypes of Surgically Induced Osteoarthritis in Sheep. J Proteome Res. 2012;11(8):4261-8.

249. Lamers R-JAN, DeGroot J, Spies-Faber EJ, Jellema RH, Kraus VB, Verzijl N, TeKoppele JM, Spijksma GK, Vogels JTWE, van der Greef J, van Nesselrooij JHJ. Identification of Disease- and Nutrient-Related Metabolic Fingerprints in Osteoarthritic Guinea Pigs. The Journal of Nutrition. 2003;133(6):1776-80.

250. Yang G, Zhang H, Chen T, Zhu W, Ding S, Xu K, Xu Z, Guo Y, Zhang J. Metabolic analysis of osteoarthritis subchondral bone based on UPLC/Q-TOF-MS. Anal Bioanal Chem. 2016;408(16):4275-86.

251. Hadipour-Jahromy M, Mozafary-Kermani R, Noorafshan S. Histopathological changes and therapeutic effects of glucosamine, chondroitine and methly-sulfonyl-methane in monoiodoacetate-induced osteoarthritis in knee joint of rat. Inflammation Res. 2007;56:S301-S2.
252. Kloesch B, Liszt M, Broell J, Steiner G. Dimethyl sulphoxide and dimethyl Sulphone are potent inhibitors of IL-6 and IL-8 expression in the human chondrocyte cell line C-28/I2. Life Sci. 2011;89(13-14):473-8.
253. Tetlow LC, Woolley DE. Histamine stimulates the proliferation of human articular chondrocytes in vitro and is expressed by chondrocytes in osteoarthritic cartilage. Ann Rheum Dis. 2003;62(10):991e4.
254. Bassit RA SL, Bacurau RFP, Navarro F, Costa Rosa LFBP. The effect of BCAA supplementation upon the immune response of triathletes. Med Sci Sports Exerc. 2002;32:1214 - 9.
255. Livshits G, Zhai G, Hart DJ, Kato BS, Wang H, Williams FMK, Spector TD. Interleukin-6 is a Significant Predictor of Radiographic Knee Osteoarthritis. Arthritis Rheum. 2009;60:2037-45.
256. Fernandes JC, Martel-Pelletier J, Pelletier J-P. The role of cytokines in osteoarthritis pathophysiology. Biorheology. 2002;39:237-46.
257. Adams Jr. SB, Setton LA, Kensicki E, Bolognesi MP, Toth AP, Nettles DL. Global metabolic profiling of human osteoarthritic synovium. Osteoarthritis Cartilage. 2012;20(1):64-7.
258. Hills BA. Oligolamellar nature of the articular surface. The Journal of rheumatology. 1990;17(3):349-56.
259. Oloyede A, Gudimetla P, Crawford R, Hills BA. Consolidation responses of delipidized articular cartilage. Clin Biomech (Bristol, Avon). 2004;19(5):534-42.
260. Stockwell RA, Billingham ME, Muir H. Ultrastructural changes in articular cartilage after experimental section of the anterior cruciate ligament of the dog knee. J Anat. 1983;136:425-39.
261. Young SP, Kapoor SR, Viant MR, Bryne JJ, Filer A, Buckley CD, Kitas GD, Raza K. The Impact of Inflammation on Metabolic Profiles in Patients With Arthritis. Arthritis Rheum. 2013;65(8):2015-23.
262. Gudbjornsson B, Zak A, Niklasson F, Hallgren R. Hypoxanthine, xanthine, and urate in synovial fluid from patients with inflammatory arthritides. Ann Rheum Dis. 1991;50(10):669-72.
263. Herbert KE, Scott DL, Perrett D. Nucleosides and bases in synovial fluid from patients with rheumatoid arthritis and osteoarthritis. Clin Sci (Lond). 1988;74(1):97-9.
264. June RK, Liu-Bryan R, Long F, Griffin TM. Emerging role of metabolic signaling in synovial joint remodeling and osteoarthritis. J Orthop Res. 2016;34(12):2048-58.

265. Peansukmanee S, Vaughan-Thomas A, Carter SD, Clegg PD, Taylor S, Redmond C, Mobasheri A. Effects of hypoxia on glucose transport in primary equine chondrocytes in vitro and evidence of reduced GLUT1 gene expression in pathologic cartilage in vivo. J Orthop Res. 2009;27(4):529-35.
266. de Sousa EB, Dos Santos Junior GC, Aguiar RP, da Costa Sartore R, de Oliveira ACL, Almeida FCL, Neto VM, Aguiar DP. Osteoarthritic Synovial Fluid Modulates Cell Phenotype and Metabolic Behavior In Vitro. Stem cells international. 2019;2019:8169172.
267. Liu Y, Hou R, Yin R, Yin W. Correlation of bone morphogenetic protein-2 levels in serum and synovial fluid with disease severity of knee osteoarthritis. Medical science monitor : international medical journal of experimental and clinical research. 2015;21:363-70.
268. Catterall JB, Zura RD, Bolognesi MP, Kraus VB. Aspartic acid racemization reveals a high turnover state in knee compared with hip osteoarthritic cartilage. Osteoarthritis Cartilage. 2016;24(2):374-81.
269. Reyes C, Leyland KM, Peat G, Cooper C, Arden NK, Prieto-Alhambra D. Association Between Overweight and Obesity and Risk of Clinically Diagnosed Knee, Hip, and Hand Osteoarthritis: A Population-Based Cohort Study. Arthritis & rheumatology (Hoboken, NJ). 2016;68(8):1869-75.
270. Akhbari P, Jaggard MK, Boulangé CL, Vaghela U, Graça G, Bhattacharya R, Lindon JC, Williams HRT, Gupte CM. Differences between infected and noninfected synovial fluid. Bone & joint research. 2021;10(1):85-95.
271. Ahmed SS, Haddad FS. Prosthetic joint infection. Bone & joint research. 2019;8(11):570-2.
272. Saleh A, George J, Faour M, Klika AK, Higuera CA. Serum biomarkers in periprosthetic joint infections. Bone & joint research. 2018;7(1):85-93.
273. Parkinson JA. Chapter 2 - NMR Spectroscopy Methods in Metabolic Phenotyping. In: Lindon JC, Nicholson JK, Holmes E, editors. The Handbook of Metabolic Phenotyping: Elsevier; 2019. p. 53-96.
274. Saccenti E, Hoefsloot HCJ, Smilde AK, Westerhuis JA, Hendriks MMWB. Reflections on univariate and multivariate analysis of metabolomics data. Metabolomics. 2014;10(3):361-74.
275. Otvos JD, Shalaurova I, Wolak-Dinsmore J, Connelly MA, Mackey RH, Stein JH, Tracy RP. GlycA: A Composite Nuclear Magnetic Resonance Biomarker of Systemic Inflammation. Clin Chem. 2015;61(5):714-23.
276. Mu J, Yang Y, Chen J, Cheng K, Li Q, Wei Y, Zhu D, Shao W, Zheng P, Xie P. Elevated host lipid metabolism revealed by iTRAQ-based quantitative proteomic analysis of cerebrospinal fluid of tuberculous meningitis patients. Biochem Biophys Res Commun. 2015;466(4):689-95.

277. Li Z, Du B, Li J, Zhang J, Zheng X, Jia H, Xing A, Sun Q, Liu F, Zhang Z. Cerebrospinal fluid metabolomic profiling in tuberculous and viral meningitis: Screening potential markers for differential diagnosis. Clin Chim Acta. 2017;466:38-45.

278. Prete PE, Gurakar-Osborne A, Kashyap ML. Synovial fluid lipoproteins: review of current concepts and new directions. Semin Arthritis Rheum. 1993;23(2):79-89.

279. Damyanovich AZ, Staples JR, Marshall KW. ^{1}H NMR investigation of changes in the metabolic profile of synovial fluid in bilateral canine ostroarthritis with unilateral joint denervation. Osteoarthritis Cartilage. 1999;7:165-72.

280. Burger D, Dayer J-M. High-density lipoprotein-associated apolipoprotein A-I: the missing link between infection and chronic inflammation? Autoimmun Rev. 2002;1(1):111-7.

281. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. New Engl J Med. 1999;340(6):448-54.

282. Park YB, Lee SK, Lee WK, Suh CH, Lee CW, Lee CH, Song CH, Lee J. Lipid profiles in untreated patients with rheumatoid arthritis. J Rheumatol. 1999;26(8):1701-4.

283. Ananth L, Prete PE, Kashyap ML. Apolipoproteins A-I and B and cholesterol in synovial fluid of patients with rheumatoid arthritis. Metabolism. 1993;42(7):803-6.

284. Weiner J, 3rd, Parida SK, Maertzdorf J, Black GF, Repsilber D, Telaar A, Mohney RP, Arndt-Sullivan C, Ganoza CA, Fae KC, Walzl G, Kaufmann SH. Biomarkers of inflammation, immunosuppression and stress with active disease are revealed by metabolomic profiling of tuberculosis patients. PLoS One. 2012;7(7):e40221.

285. Revelles O. E-UM. Proline and Lysine Metabolism. In: Ramos JL. (eds) Pseudomonas: Springer, Boston, MA; 2004.

286. Rosenblum W. Dimethyl sulfoxide effects on platelet aggregation and vascular reactivity in pial microcirculation. Ann N Y Acad Sci. 1983;411:110-9.

287. Marthyn P, Beuscart A, Coll J, Moreau-Gachelin F, Righi M. DMSO reduces CSF-1 receptor levels and causes apoptosis in v-myc immortalized mouse macrophages. Exp Cell Res. 1998;243(1):94-100.

288. Watson RWG, Rotstein OD, Parodo J, Bitar R, Hackam D, Marshall JC. Granulocytic differentiation of HL-60 cells results in spontaneous apoptosis mediated by increased caspase expression. FEBS Lett. 1997;412(3):603-9.

289. Smith RL, Schurman DJ. Bacterial arthritis. A staphylococcal proteoglycan-releasing factor. Arthritis Rheum. 1986;29(11):1378-86.

290. Macfarlane S, Macfarlane GT. Regulation of short-chain fatty acid production. Proc Nutr Soc. 2003;62(1):67-72.

291. R. Harwood C, Wipat A. 15 - Bacterial Protein Synthesis. In: Sussman M, editor. Molecular Medical Microbiology. London: Academic Press; 2002. p. 321-39.

292. Eichelbaum K, Krijgsveld J. Rapid temporal dynamics of transcription, protein synthesis, and secretion during macrophage activation. Mol Cell Proteomics. 2014;13(3):792-810.

293. Engelke UF, Tangerman A, Willemsen MA, Moskau D, Loss S, Mudd SH, Wevers RA. Dimethyl sulfone in human cerebrospinal fluid and blood plasma confirmed by one-dimensional (1)H and two-dimensional (1)H-(13)C NMR. NMR Biomed. 2005;18(5):331-6.

294. Shirtliff ME, Mader JT. Acute septic arthritis. Clin Microbiol Rev. 2002;15(4):527-44.

Appendix 1

List of all metabolites identified from the systematic review of small molecules found in human synovial fluid

Table 19. List of all the identified metabolites by article in human synovial fluid identified from the systematic review detailed in chapter 3. PTAA – posttraumatic ankle arthritis; OA – osteoarthritis; RA – rheumatoid arthritis; BD – Behçet's disease; SNA – seronegative arthritis; SSA – seronegative spondyloarthropathy; AS – ankylosing spondylitis; ACL – anterior cruciate ligament.

Underlying pathology	Metabolite	Change	Joint	Author & year
PTAA	*Glutamate* *DSGEGDFXAEGGGVR* *2-hydroxypalmitate* *Arachidonate* *Palmitoyl-sphingomyelin* *2-hydroxystearate* *1-stearoylglycerophosphoinositol* *1-oleoylglycerophosphoethanolamine* *Tryptophan* *1-arachidonoylglycerophosphoinositol* *Kynurenine* *Dihomo-linoleate-20-2n6* *Androsterone sulfate* *1-stearoylglycerophosphoethanolamine* *4-androsten-3beta-17beta-diol-disulfate-1-* *bilirubin E-E* *Mannose* *Cis-4-decenoyl-carnitine* *Lactate* *Pseudouridine* *Urate* *Alpha-tocopherol* *5-dodecenoate (12:1n7)* *1-arachidonoylglycerophosphoethanolamine* *Cortisol* *3-indoxyl-sulfate* *Uridine* *Carnitine* *ADSGEGDFXAEGGGVR* *Cystine*	Increased	Ankle	Adams et al 2014(170)
Behcet's disease	*Glutamate* *Citramalate* *Valine* *Leucine* *Methionine sulfoxide* *Glycerate* *Phosphate* *Lysine* *Isoleucine* *Urea* *Citrulline*	Increased in BD with arthritis compared to SNA	Not stated	Ahn et al 2015(171)

OA or RA	*Citrate* *Creatine* *Glucose* *Glutamine* *Glycerol* *Pyruvate* *Taurine*	Increased in OA compared to RA	Knee	Anderson et al 2018(172)
	3-hydroxybutarate *Acetate* *Isoleucine* *Leucine* *Sarcosine* *Threonine*	Increased in RA compared to OA		
OA or RA	*Citric acid* *D-lactic acid methyl ester* *hydroxyl-L-proline* *L-isoleucine* *L-methionine*	Decreased in OA & RA compared to controls	Not stated	Carlson et al 2018(173)
	L-citrulline	Increased in OA compared to RA & controls	Not stated	
OA	*4-hydroxy-L-proline (Hyp)* *Alanine* *Arginine* *Creatine* *Isoleucine* *Leucine* *Lysine* *Tryptophan* *Tyrosine* *Valine*	Increased in OA	Knee	Chen et al 2018(174)
	Acetyl-carnitine *Aminobutyric acid* *Asparagine* *Citrulline* *Creatinine* *Dimethylglycine* *Glutamine* *Phenylalanine* *Proline* *Serine* *Taurine* *γ-aminobutyric acid (GABA)*	Decreased in OA		
Inflammatory arthropathies	*3-HB* *Citrulline* *Glucose* *Glutamate* *Glycine* *Histidine* *Isoleucine* *Leucine*	Increased in SSA & RA	Knee	Dubey et al 2019(177)
	LDL *VLDL* *Choline* *PUFA*	Decreased in SSA & RA		

	Valine *Lysine* *Arginine* *Acetate* *Acetoacetate* *Creatinine* *Phenylalenine*	Increased in SSA		
Acute fracture	*Sphingomyelin* *2-hydroxy-fatty acids*	Increased with acute fracture	Knee	Furman et al 2017(178)
RA or OA	*Maltose* *Lignoceric acid* *Uracil* *Mannitol* *Pyrophosphate* *Phosphoric acid*	Increased in RA compared to OA	Not stated	Hwang et al 2013(179)
	Lysine *Tyrosine* *Valine* *Glyceric acid* *Alanine* *Asparagines* *Hydroxylamine* *Tryptophan* *Glycerol* *Glutamine* *Citrulline*	Increased in OA compared to RA		
Inflammatory arthropathies	*Succinic acid* *Octadecanol* *Asparagine* *Terephtalic acid* *Salicylaldehyde* *Glutamine* *Citrulline* *Tyrosine* *Uracil* *Lysine* *Phenylalanine* *Ribitol* *Tryptophan* *Xylose* *Pyrophosphate*	Increased in RA compared to other inflammatory arthropathies (AS, BD, gout)	Not stated	Hwang et al 2013(296)
	Isopalmitic acid *Glycerol* *Myristic acid* *Palmitoleic acid* *Hydroxylamine* *Ethanolamine* *Alanine* *Serine*	Decreased in RA compared to other inflammatory arthropathies (AS, BD, gout)		

OA or RA	*N6,N6,N6-Trimethyl-L-lysine* *L-Carnitine* *3-Hydroxymandelic acid* *Kynurenine* *Indolelactic acid* *Indoleacetaldehyde* *N'-Formylkynurenine* *Phenylactic acid* *Trihydroxyeicosatrienoic acid/13,14-Dihydrolipoxin A4* *12,20-Dioxoleukotriene B4* *11b-Hydroxy-3,20-dioxopregn4-en-21-oic acid* *LysoPC(18:1)*	Increased in OA compared to RA	Knee	Kang et al 2015(180)
	5-L-Glutamyltaurine *Taurine* *(S)-Ureidoglycolic acid* *Methylguanine* *CE[24:1(15Z)]* *Trimethyltridecanoic acid* *Docosapentaenoic acid* *Galactosylceramide (d18:1/16:0)*	Decreased in OA compared to RA		
ACL deficient knee (trauma)	*Alanine* *Choline*	Increased following pivot shift test in ACL deficient knee	Knee	Khatib et al 2018(181)
Early vs late OA	*Squalene* *Palmitoleic acid* *Pentadecanoic acid* *Glycerol* *Myristic acid* *Lignoceric acid* *α-tocopherol* *Heptadecanoic acid* *Oleic acid* *Linolenic acid* *Threose* *3-hydroxypropionate* *Lanosterol* *Ethanolamine* *Putrescine* *N-carbamoylaspartate* *Capric acid* *Malate* *Asparagine* *Arachidonic acid* *Pelargonic acid* *Benzoate* *Palmitic acid* *1-monostearin* *Salicylaldehyde* *Stearic acid* *Adipate* *Phenylalanine*	Increased in late OA compared to early OA	Knee	Kim et al 2017(182)

Inflammatory arthropathies	*Succinate* *Octadecanol* *Asparagine* *Terephthalate* *Salicylaldehyde* *Glutamine* *Citrulline* *Tyrosine* *Uracil* *Lysine* *Ribitol* *Tryptophan* *Xylose* *Ribose*	Increased in RA compared to other inflammatory arthropathies (AS, BD, gout)	Not stated	Kim et al 2014(183)
	Isopalmitic acid *Glycerol* *Myristic acid* *Palmitoleic acid* *Hydroxylamine* *Ethanolamine*	Decreased in RA compared to other inflammatory arthropathies (AS, BD, gout)		
Acute fracture vs control	*Oleate* *Eicosenoate* *10-heptadecenoate* *Myristoleate* *Palmitoleate* *Linoleate* *Dihomo-linoleate* *Arachidonate* *Linolenate* *Dihomo-linolenate* *Stearoyl sphingomyelin* *Palmitoly sphingomyelin* *Octadecanedioate* *5-dodecenoate* *Oleoylcarnitine* *Cis-4-decenoyl carnitine*	Increased following intra-articular ankle fracture & Decreased 6 months post-surgery	Ankle	Leimer et al 2017(184)
OA vs cadaveric controls	*Fructose* *Citrate*	Increased in OA compared to cadaveric controls	Knee	Mickiewicz et al 2015 (186)
	Malate *Methionine* *N-Phenylacetylglycine* *O-Acetylcarnitine* *Hexanoylcarnitine* *Creatine* *Ethanol* *Ethanolamine* *3-Hydroxybutyrate*	Decreased in OA compared to cadaveric controls		
RA vs healthy controls	*LDL*	Increased in RA comparted with control samples	Knee	Naughton et al 1993(187)

RA vs controls	*beta-Mannosylglycerate* *Carnitine* *Diglycerol* *Lactic acid* *Pipecolinic acid*	Increased in RA comparted with control samples	Knee	Yang et al 2015(188)
	5-Methoxytryptamine *Citric acid* *Gluconic lactone* *D-glucose* *Glucose-1-phosphate* *Mannose* *Ribitol* *L-valine*	Decreased in RA comparted with control samples		
OA vs controls	*1,5-Anhydroglucitol* *Gluconic lactone* *Threonine*	Increased in OA compared to healthy controls	Knee	Zheng et al 2017(192)
	8-Aminocaprylic acid *Glutamine* *Tyramine*	Decreased in OA compared to healthy controls		

Appendix 2

Patient information leaflet, consent form and questionnaire

Looking for Biomarkers in Human Joint Fluid

Imperial College Healthcare NHS Trust

Imperial College London

Principal Investigator: Chimnay Gupte

You are being invited to take part in a research study. Before you do so it is important that you understand the research being undertaken and what it involved.

Please take time to read the following and raise any queries you may have:
"Looking for Biomarkers in Human Joint Fluid" v1.5 date 17/04/2015

Purpose of this Study

Diagnosing joint problems remains a challenge to your doctors. We aim to investigate the human response to joint disease by studying the joint fluid. It is hoped the results may assist doctors by providing ways to detect joint diseases and predict the patients' response to treatment.

Failure of joint replacements is a widespread problem. Some patients wear out joint replacements sooner than others. We hope to study the joint fluid to help understanding of what causes early failure of joint replacements.

In patients with joint disease, our aim is to find substances released by the body which may be helpful in diagnosing joint conditions. Furthermore, we aim to improve the understanding of joint diseases.

Why have I been chosen?

We are looking to recruit patients to conduct a study about joint fluid and how it can used to improve treatment and understanding of joint diseases. Unfortunately, you require treatment for your joint problem at Imperial College Healthcare NHS Trust.

Do I have to take part?

It is entirely your decision whether or not you participate. As a patient who met certain criteria you are being asked if you wish to enter the study. If you decide to take part you will be given an information sheet to read and shall be asked to sign the consent form. You may withdraw from the study at any time and do not have to supply a reason. This will not affect your medical or legal rights. If you withdraw from the study all body fluid samples and medical information gathered will be destroyed.

What will happen to me if I take part?

If you agree to take part you will be provided with an information sheet and consent form to sign. Any questions you may have can be answered initially or at any time during the study. You will be asked to consider the information sheet. If you agree you will be asked to sign the consent form.

A small proportion of the samples you provide as part of your medical care will be taken for research purposes. This is likely to provide enough results for your study. In rare cases when this is not diagnostic and we would like to study something in more detail, we may ask if you would consent to returning and providing samples for a second occasion. We would like to stress that the chances of further samples being needed is very low.

A medical history will be taken and your medical notes will be screened for further information. You will be asked to fill in a questionnaire about your diet and lifestyle.

What do I have to do?

We would be grateful if you would complete our questionnaire.

If your samples are being taken in the operating theatre then nothing is required on your part except consent.

As part of your medical care at Imperial College NHS Trust, samples of blood, urine and joint fluid are required. We will merely be taking a few millilitres of these samples. In the vast majority of cases, no additional samples are required. In very rare cases a sample may be requested at a later date once you have recovered from your hospital treatment.

What is being tested?

We are analysing your body fluids and find body substances related to joint disease. We then hope to use these results to suggest better ways of detecting and monitoring joint conditions, thereby improving treatment.

We will also be analysing the joint fluid to try and understand what makes it a better lubricant. It is hoped this knowledge will allow us to improve the lifetime of joint replacements.

What are the possible disadvantages and risks of taking part?

The risks of taking part are minimal. Removing fluid from the joint (when not having an operation) requires an injection to numb the area and fluid to be aspirated. The numbness may persist for a few hours. On rare occasions this can cause bleeding into the joint. On very rare occasions infection can be introduced into the joint. Should this unlikely event occur then you would receive the appropriate medical treatment from the speciality teams at the most appropriate treating hospital.

We may take blood and/or urine for analysis. There are no risks of sampling urine. The taking of blood can sometimes cause a small bruise which usually resolves relatively quickly. There is a very small theoretical risk of infection after taking blood. In this unlikely event you would receive the appropriate medical treatment from the speciality teams at the most appropriate treating hospital.

What are the benefits to taking part?

There are not direct personal (including financial) benefits from participation in this study, however you may help us to further improve joint disease treatment in the future.

What will happen when the research study stops?

We aim to publish our results. Further investigation may be taken into any key body substances identified in this study.

What if something goes wrong?

If you have a complaint about conduct or any aspect of the study then it should be addressed directly to the principal investigator.

Imperial College holds Public Liability (“negligent harm”) and Clinical Trial (“non-negligent harm”) insurance policies which apply to this trial. If you can demonstrate that you experienced harm

or injury as a result of your participation in this trial, you will be eligible to claim compensation without having to prove that Imperial College is at fault. If the injury resulted from any procedure which is not part of the trial, Imperial College will not be required to compensate you in this way. Your legal rights to claim compensation for injury where you can prove negligence are not affected.

Will my taking part in this study be kept confidential?

In the analysis of data we may use any information from your medical history, diet and lifestyle. The information allows us to compare any relationship between lifestyle, medical factors and the results we generate. In order to guarantee your confidentiality, only clinicians involved in the study will have access to your medical history. Any data associated with your name and details will not leave the treating hospital or Imperial College. At a time when data is published, any reference to you personally will be removed.

What will happen to the results of the Research Study?

The study is being conducted in part as fulfilment of an educational project. Therefore, the final results may be compiled in a thesis or published.

Your results will be available to you afterwards should you wish to be informed. We are aiming to publish the results of the study. You will not be identified personally within the results.

Who is organising and funding the research?

The study is being organised and funded by Imperial College. Funding support is being sought through the Medical Research Council and various Osteoarthritis charities.

Who has reviewed the study?

This study has been reviewed and received favourable opinion by London-Chelsea Research Ethics Committee.

Who can I ask if I have more questions?

If you have any questions about the project, feel free to discuss them with your doctor and/or the research team.

The principal investigator of study can be contacted should any serious concerns arise:

Principal Investigator: Chinmay Gupte

Contact details: 02074835160

Contact details: Secretary +44 (0)203 312 1150

St Mary's Hospital

London

W2 1NY

It is important to remember that:

- Your input is voluntary and you can withdraw at any time without affecting your medical or legal rights
- All data is anonymous for the purposes of publication.
- Only clinicians directly involved in the study will have access to medical data
- No amount of money will be paid to you or us for your input. We believe this is the most ethical way of doing our research.
- We are not in a position to offer any money from treatments or tests we develop as a result of your input.
- Your participation will be appreciated and may help us to further improve joint disease treatment

This information sheet is for your information and yours to keep. You will also be supplied with a copy of the consent form for the study.

Thank you for your time and generosity in considering whether to take part in our study.

Imperial College London

Principal Investigator: Chinmay Gupte

Sponsor: Imperial College London

Looking for biomarkers in Human Joint Fluid

	Please initial
I have read the patient information sheet version	
I have had the opportunity to ask questions.	
My questions have been answered to my satisfaction.	
I understand that I may choose not to take part in this study and can withdraw at any time with my legal or medical rights being protected. I can withdraw without having to give reason and without affecting my medical or legal rights. At such a time all data and samples will be destroyed.	
I understand that data generated from this study may be published in the future, but that my identity will be protected by anonymisation.	
I give permission for my medical history to be taken for the purposes of data analysis	
I agree to take part in this study.	
I understand that sections of my research notes may be looked at by responsible individuals from Imperial College Healthcare NHS Trust or from regulatory authorities where it is relevant to my taking part in this research	

Thank you for your time.

Name of patient in block letters……..

Signature…… Date……..

Name of person obtaining consent……..

Signature…… Date……..

Looking for Biomarkers in Human Joint Fluid

Imperial College London

PLACE PATIENT STICKER HERE

PLACE RESEARCH SAMPLE LABEL HERE

Principal Investigator: Chinmay Gupte

FOR THE RESEARCHER TO COMPLETE ON BEHALF OF THE PATIENT – SECTIONS 1&5 TO BE COMPLETED WITH THE PATIENT

Patient Questionnaire: Please delete as appropriate

JOINT ASPIRATED ……..

Section 1 – Diet and Lifestyle

1. "Are you known to be?" Pre-menopause
   Experiencing menopause
   Post menopause

2. "Are you Vegetarian?" Yes
   No

3. "How much of the following have you consumed in the last 24hours and do you consume regularly in your diet?" STATE QUANTITIES

	Last 24hrs	Regularly
Red Meat		
White Meat		
Fish		
Coffee		
Black tea		
Herbal tea		
Cherries		
Cheese		
Grapefruit		
Liquorice		
Walnuts		
Vanilla		
Carbonated (fizzy) drinks		
Yoghurt		
Berries		
Alcohol (units)		

4. “What was your last meal?” ……..

5. “How many hours ago did you eat your last meal?” ……..hrs

6. “How many units of alcohol do you drink in an average week?”.....

7. “Do you smoke (including nicotine replacement)?” Yes / No

8. “How many hours of exercise do you do in an average week?” …..hrs

9. “Please list any herbal or over the counter medication you take?”.....

10. “Please list any vitamin or other food supplements you take?”.....

Section 2 – Past Medical History

11. Please list the patient’s complete past medical history – augment with notes if necessary

Section 3 – Drug History

12. Please list the patient's complete current medication history including dosages. (or attach a copy of their prescription) Please also indicate if the patient has taken his/her prescription as prescribed in the last 48hrs.

Drug	Dosage	Freq	Taken as prescribed (last 48hrs)

Section 4 – Joint Diseases

13. "Which of the following do you suffer with?"
    a. Osteoarthritis Yes / No
    b. Rheumatoid Arthritis Yes / No
    c. Psoariatic Arthritis Yes / No
    d. Septic Arthritis Yes / No
    e. Gout Yes / No

14. "Do you have pain free days from your joint?" Yes / No

15. "Is the joint painful at the moment?" Yes / No

16. "Do you find great variation in the symptoms they feel on a daily basis?"
Yes / No

17. Is your joint ever so painful that you cannot walk/get out of bed/use the joint at all?
Yes / No

18. "Have you injured the joint recently?" Yes
No **go to section 5**

19. "How long ago did you injure the joint?".....weeks

Section 5 – Injections (ONLY COMPLETE IF PATIENT HAS RECEIVED INJECTIONS OR WILL RECEIVE TODAY)

20. "How long ago did you receive the last injection?" ……..months

21. "Was the injection a Steroid?" Yes No **go to question 22**

22. "Did the steroid injection relieve any symptoms and if so how long for?".....weeks
go to question 24

23. "Was the injection an artificial lubricant (e.g. Duralane)?" Yes
No **go to section 6**

24. "Did the lubricant injection relieve your symptoms and if so how long for?".....weeks

Section 6 – The Joint Sampled (CAN BE COMPLETED WITHOUT PATIENT)

25. Did the patient receive an injection today? Yes / No

26. Please give the drug and dosage injected today ……

27. Was a large joint effusion present? Yes / No

28. Does the patient have any indications of an active synovitis from the history? Yes / No

29. Does the patient have any signs of active synovitis on examination? Yes / No

30. Is the patient suspected to have a septic joint? Yes
No **go to section 7**

31. Is the organism known and what is it? ……..

Section 7 – Prosthetic Joints (ONLY COMPLETE IF PROSTHETIC JOINT IN SITU)

32. Are there concerns or signs of excessive wear or debris from the prosthetic joint? Yes / No

33. What are the bearing surfaces?
    Metal / Metal Metal / Poly Ceramic / Poly Ceramic / Ceramic

34. How long has the prosthetic joint been in situ?.....yrs

35. What is the make and model of prosthetic joint?.....

36. Was this sample taken as part of an operation being performed to revise a joint?Yes / No

37. What was the reason for revision? Loosening Wear Dislocation Fracture

38. Was bone cement used? Yes / No

39. Is a hybrid fixation used? Yes
    No **go to section 8**

40. What is the uncemented interface? Grit blasted / Hydroxyapatite

Section 8 – Operative findings (ONLY COMPLETE IF SAMPLE TAKEN AT THE TIME OF OPERATION)

41. Please list the grade of joint destruction seen and comment which part of the joint was affected (draw diagram if helpful)?

42. Was any other joint pathology seen and what was it? …….

43. Was there any prosthetic material (not a joint) remaining in the joint from a previous procedure e.g. meniscal repair or suture material? Yes / No

Section 9 – Operative findings (ONLY COMPLETE IF SAMPLE TAKEN AT THE TIME OF OPERATION)

44. Please list the patients ethnic group

What is your ethnic group? Choose one option that best describes your ethnic group or background

White
1. English / Welsh / Scottish / Northern Irish / British
2. Irish
3. Gypsy or Irish Traveller
4. Any other White background, please describe

Mixed / Multiple ethnic groups
5. White and Black Caribbean
6. White and Black African
7. White and Asian
8. Any other Mixed / Multiple ethnic background, please describe

Asian / Asian British
9. Indian
10. Pakistani
11. Bangladeshi

12. Chinese
13. Any other Asian background, please describe

Black / African / Caribbean / Black British
14. African
15. Caribbean
16. Any other Black / African / Caribbean background, please describe

Other ethnic group
17. Arab
18. Any other ethnic group, please describe

Please ensure the samples of blood urine and joint fluid are labelled with the research label provided.

Please ensure you have placed an NHS patient sticker on the front of the questionnaire

Appendix 3

Copyright and permissions for inclusion of published work from this thesis and figures from other authors

Permission Table

Page No.	Type of work:	Name of work	Source of work	Copyright holder and contact	permission requested on	I have permission yes /no	Permission note
60	Figure	Figure 37. The role of NMR spectroscopy in metabolic profiling including its various applications.	Universal Quantitative NMR Analysis of Complex Natural Samples. *Current Opinion in Biotechnology, 25, 51-59 (2014)*	© 2013 Elsevier	04.03.20	yes	Written permission
61	Figure	Figure 38. A 950-MHz 1H NMR spectrum of human urine with expansions showing the degree of spectral complexity.	Spectroscopic and Statistical Techniques for Information Recovery in Metabonomics and Metabolomics. *Review of Analytical Chemistry, 1, 49-69 (2008)*	© 2008 Annual Reviews	15.01.21	yes	Written permission
Chapter 3 & Appendix 5	Manuscript including figures	Published manuscript: Can joint fluid metabolic profiling (or "metabonomics") reveal biomarkers for osteoarthritis and inflammatory joint disease?	*Bone and Joint Research 2020; 9(3):108–119*	Open access journal with each paper including an open access Creative Commons statement	10.02.21	yes	Written permission plus open access Creative Commons statement
Chapter 5 & Appendix 5	Manuscript including figures	Published manuscript: Differences in the composition of hip and knee synovial fluid in osteoarthritis: a nucelar magnetic resonance (NMR) spectroscopy study of metabolic profiles	*Osteoarthritis and Cartilage 2019 Dec 27(12): 1768 - 1777*	© 2019 Osteoarthritis Research Society International	15.02.21	yes	Written permission
Chapter 6 & Appendix 5	Manuscript including figures	Published manuscript: Differences between infected and noninfected synovial fluid: an observational study using metabolic phenotyping (or "metabonomics")	*Bone and Joint Research 2021 Jan;10(1):85-95*	Open access journal with each paper including an open access Creative Commons statement	10.02.21	yes	Written permission plus open access Creative Commons statement

Thank you for your order!

Dear Dr. Pouya Akhbari,
Thank you for placing your order through Copyright Clearance Center's RightsLink® service.

Order Summary

Licensee:	Dr. Pouya Akhbari
Order Date:	Mar 4, 2020
Order Number:	4781951456182
Publication:	Current Opinion in Biotechnology
Title:	Universal quantitative NMR analysis of complex natural samples
Type of Use:	reuse in a thesis/dissertation
Order Ref:	PMC3912461
Order Total:	0.00 GBP

View or print complete details of your order and the publisher's terms and conditions.

Sincerely,

Copyright Clearance Center

Tel: +1-855-239-3415 / +1-978-646-2777
customercare@copyright.com
https://myaccount.copyright.com

Copyright Clearance Center | Marketplace™

Annual Reviews, Inc. - License Terms and Conditions

This is a License Agreement between Imperial College London ("You") and Annual Reviews, Inc. ("Publisher") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Annual Reviews, Inc., and the CCC terms and conditions.

All payments must be made in full to CCC.

Order Date	15-Jan-2021	**Type of Use**	Republish in a thesis/dissertation
Order license ID	1090692-1	**Publisher**	Annual Reviews
ISSN	1936-1335	**Portion**	Image/photo/illustration

LICENSED CONTENT

Publication Title	Annual review of analytical chemistry	**Rightsholder**	Annual Reviews, Inc.
Date	01/01/2008	**Publication Type**	e-Journal
Language	English	**URL**	http://arjournals.annualreviews.org/loi/a...
Country	United States of America		

REQUEST DETAILS

Portion Type	Image/photo/illustration	**Distribution**	U.K. and Commonwealth (excluding Canada)
Number of images / photos / illustrations	1	**Translation**	Original language of publication
Format (select all that apply)	Print	**Copies for the disabled?**	No
Who will republish the content?	Academic institution	**Minor editing privileges?**	No
Duration of Use	Life of current edition	**Incidental promotional use?**	No
Lifetime Unit Quantity	Up to 499	**Currency**	GBP
Rights Requested	Main product		

NEW WORK DETAILS

Title	The role of metabolic profiling in inflammatory synovial fluid	**Institution name**	Imperial College London
Instructor name	P Akhbari	**Expected presentation date**	2021-03-22

ADDITIONAL DETAILS

Order reference number	N/A	**The requesting person / organization to appear on the license**	Imperial College London

REUSE CONTENT DETAILS

Title, description or numeric reference of the portion(s)	Figure 1. A 950-MHz 1H nuclear magnetic resonance spectrum of human urine with expansions showing the degree of spectral complexity.	**Title of the article/chapter the portion is from**	Spectroscopic and statistical techniques for information recovery in metabonomics and metabolomics
		Author of portion(s)	John C Lindon, Jeremy K Nicholson
Editor of portion(s)	Jeanne E. Pemberton	**Issue, if republishing an article from a serial**	N/A
Volume of serial or monograph	2008;1:45-69		

Bone & Joint Research

10 February 2021

To whom it may concern

We certify that Pouya Akhbari has the right to reproduce his articles from *Bone & Joint Research* as an appendix to his thesis provided the source is credited.

Akhbari P, Karamchandani U, Jaggard MKJ, et al. Can joint fluid metabolic profiling (or "metabonomics") reveal biomarkers for osteoarthritis and inflammatory joint disease? *Bone Joint Res*. 2020;9(3):108-119.

Akhbari P, Jaggard MK, Boulangé CL. Differences between infect and noninfected synovial fluid. *Bone Joint Res*. 2021;10(1):85-95.

pp. Deborah Gray

Prof. Hamish Simpson
Editor-in-Chief, Bone & Joint Research

The British Editorial Society of Bone & Joint Surgery
22 Buckingham Street, London WC2N 6ET
t: 020 7782 0010 f: 020 7839 9777

Differences in the composition of hip and knee synovial fluid in osteoarthritis: a nuclear magnetic resonance (NMR) spectroscopy study of metabolic profiles

Author: P. Akhbari,M.K. Jaggard,C.L. Boulangé,U. Vaghela,G. Graça,R. Bhattacharya,J.C. Lindon,H.R.T. Williams,C.M. Gupte

Publication: Osteoarthritis and Cartilage

Publisher: Elsevier

Date: December 2019

Appendix 4

Published papers from this thesis

BJR

SYSTEMATIC REVIEW

Can joint fluid metabolic profiling (or "metabonomics") reveal biomarkers for osteoarthritis and inflammatory joint disease?

A SYSTEMATIC REVIEW

P. Akhbari,
U. Karamchandani,
M. K. J. Jaggard,
G. Graça,
R. Bhattacharya,
J. C. Lindon,
H. R. T. Williams,
C. M. Gupte

Imperial College London, London, UK

Aims

Metabolic profiling is a top-down method of analysis looking at metabolites, which are the intermediate or end products of various cellular pathways. Our primary objective was to perform a systematic review of the published literature to identify metabolites in human synovial fluid (HSF), which have been categorized by metabolic profiling techniques. A secondary objective was to identify any metabolites that may represent potential biomarkers of orthopaedic disease processes.

Methods

A systematic review was conducted in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines using the MEDLINE, Embase, PubMed, and Cochrane databases. Studies included were case series, case control series, and cohort studies looking specifically at HSF.

Results

The primary analysis, which pooled the results from 17 published studies and four meeting abstracts, identified over 200 metabolites. Seven of these studies (six published studies, one meeting abstract) had asymptomatic control groups and collectively suggested 26 putative biomarkers in osteoarthritis, inflammatory arthropathies, and trauma. These can broadly be categorized into amino acids plus related metabolites, fatty acids, ketones, and sugars.

Conclusion

The role of metabolic profiling in orthopaedics is fast evolving with many metabolites already identified in a variety of pathologies. However, these results need to be interpreted with caution due to the presence of multiple confounding factors in many of the studies. Future research should include largescale epidemiological metabolic profiling studies incorporating various confounding factors with appropriate statistical analysis to account for multiple testing of the data.

Cite this article: *Bone Joint Res.* 2020;9(3):108–119.

Keywords: Metabonomics, Metabolic profiling, Osteoarthritis, Rheumatoid arthritis, Inflammatory arthropathies

Article focus

- To identify all metabolites in human synovial fluid (HSF), which have been categorized by metabolic profiling techniques.
- To recognize any metabolites that may represent potential biomarkers of orthopaedic disease processes.

Key messages

- Over 200 metabolites have been identified in HSF from the published literature.
- A total of 26 putative biomarkers have been demonstrated in osteoarthritis, inflammatory arthropathies, and trauma.

Correspondence should be sent to P. Akhbari;
email: pakhbari@nhs.net

doi: 10.1302/2046-3758.93.BJR-2019-0167.R1

Bone Joint Res 2020;9:108–119.

- The results should be interpreted with caution due to the presence of multiple confounding factors.

Strengths and limitations

- The study methodology was robust.
- The search criteria were broad to ensure all relevant articles were captured.
- There was notable heterogeneity between studies.

Introduction

Osteoarthritis (OA) is one of the most disabling conditions in the western world, affecting approximately 10% of the UK population and presenting a major healthcare burden. It is a heterogenous disease, which manifests in a number of different phenotypes due to various pathogenic factors, ultimately leading to an alteration of the whole joint structure.[1] It results in progressive degradation of ligaments, cartilage and menisci, synovial inflammation, and changes to the subchondral bone with common clinical and radiological manifestations.[2]

The risk factors for OA are multifactorial and involve a complex interplay between biochemical, cellular, and mechanical factors that ultimately lead to the same endpoint. Consequently, the risk factors for OA can vary among individuals.[3]

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by autoantibodies, systemic inflammation, and synovitis leading to damage of the affected joints.[4] Early diagnosis is important to delay disease progression by starting early intervention. A well-known biomarker of RA is rheumatoid factor (RF). However, this is non-specific and detected in other rheumatic and non-rheumatic conditions such as malignancy, infection, and even in some normal individuals.[5] Anticitrullinated protein antibodies (ACPAs) are other biomarkers that have been suggested as a useful tool to differentiate RA from other types of arthritis in the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria.[6] However, as not all RA patients are seropositive for ACPA more reliable diagnostic biomarkers are still required.

Various '-omics' technologies including proteomics, transcriptomics, and genomics have been increasingly utilized for the identification of disease biomarkers including those for RA. Transcriptomics has helped discover defence-related and immunity genes in RA patients and to predict the effectiveness of infliximab, the anti-tumour necrosis factor-α (TNF-α) biological agent, in RA patients.[7,8] Furthermore, genomics has demonstrated differences between ACPA-positive and ACPA-negative diseases.[9]

Metabolic profiling (also known as metabolic phenotyping, metabolomics, and metabonomics) is an increasingly used approach, which studies the low-molecular-weight metabolites within a cell, tissue, or biofluid. These terms have been used interchangeably, leading to some confusion. Therefore, in this article, the term 'metabolic profiling' will be used, which is defined as "an individual's metabolic pattern that would be reflected in the constituents of their biological fluids."[10]

Metabolic profiling is a top-down method of analysis as it is looking at the metabolites, which are the intermediate or end products of various cellular pathways.[11] Analyzing their concentrations provides a useful avenue to understanding the relationship of their cellular processes and biological reactions.[12] As well as genetic factors, this process accounts for various environmental factors such as diet, medication, smoking, and disease. Typically, it is conducted with biofluids, the most common of which are blood serum/plasma and urine. It can lead to the formation of a 'metabolic fingerprint', which is unique to a particular biochemical perturbation, characteristic of a particular disease process, or toxic stimulus among other things.[13]

Metabolic profiling has the ability to detect and potentially quantify hundreds or even thousands of small molecules simultaneously. The most common techniques employed are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). NMR spectroscopy is based on the same physical principles as MRI. It uses the magnetic property of the nuclei called spin to study the interaction of nuclei of the different atoms in a molecule, being therefore useful to determine the structures of molecules. The most commonly used nuclei is the proton (1H) due to its natural abundance in nature (close to 100%).[14] NMR spectroscopy is fast and non-destructive, allowing multiple samples to be measured daily, and the same sample can be analyzed multiple times.[15] MS is more sensitive with greater metabolite coverage than NMR spectroscopy, but it often requires prior separation of the different types of compounds using chromatography. Liquid chromatography (LC), particularly ultra-high-performance liquid chromatography (UPLC) is being more frequently used due to its increased compound resolution and higher throughput. Other techniques include gas chromatography-mass spectrometry (GC-MS), which is more useful for volatile compounds. Regarding biofluids, LC-MS is typically employed, usually with both positive and negative ion detection modes using standard protocols.[16] However, MS does involve sample consumption, thus preventing multiple testing of the same sample.[15] It has been used successfully in clinical medicine, toxicology, environmental science, and plant science.[17–21] It has also been employed in a number of conditions to influence clinical practice.[19,22,23] The various metabolic profiling techniques are often used together to provide a wider coverage of the metabolic space.

Metabolic profiling may be well suited for the purposes of orthopaedic research due to the great heterogeneity of the different disease processes including OA and inflammatory arthropathies such as RA, with recognition that no single biomarker is capable of explaining the breadth of pathological and temporal processes associated with these conditions.[24] Combining several biomarkers would

also increase its discriminatory capacity.[25] Furthermore, as metabolic perturbations occur in real time, they indicate the current disease state, thus providing a distinct advantage over other disease monitoring and diagnostic techniques such as radiography.

More recently, metabolic profiling has been used to identify metabolites within the urine, blood, and synovial fluid (SF) of both animal models and patients with OA.[26–28] Changes in joint metabolism may be a contributing factor to the pathogenesis of OA.[29] Previous metabolic analysis of SF has led to a better understanding of the metabolic processes associated with OA and to the identification of some of the biomarkers of OA.[30,31]

The aim of this systematic review was to identify metabolites in human SF (HSF), which have been categorized by metabolic profiling techniques. The secondary aim was to identify any metabolites that may represent potential biomarkers of orthopaedic disease processes.

The scope of this systematic review is to look at the role of metabolic profiling in identifying the small molecule metabolites in HSF and identify any that may represent putative biomarkers, specifically using the techniques associated with metabolic profiling including MS and NMR spectroscopy. Therefore, studies looking at macromolecules including cytokines and interleukins (ILs), plus studies utilizing the techniques of genomics, proteomics, and transcriptomics, were considered outside the scope of this article.[32–35]

Methods

A systematic review was undertaken in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines.[36]

Eligibility criteria. Inclusion criteria consisted of published articles and abstracts in English looking at small molecule metabolism of HSF in any disease state using metabolic profiling techniques. The exclusion criteria were articles not written in English, patients less than 18 years old, expert opinions, review articles, and studies using the same cohort of patients.

Identification of studies. A systematic literature review was conducted of the MEDLINE (Medical Literature Analysis and Retrieval System Online), Embase (Excerpta Medica Database, Amsterdam, The Netherlands), PubMed, and Cochrane databases without date restrictions on 1 August 2018. The search terms used are detailed in Supplementary Table i.

Screening and assessment of eligibility. Two independent reviewers (PA and UK) looked at the titles of the articles identified in the preliminary literature search. Any disagreement resulted in the article proceeding to the next stage of review. The same authors then read the abstracts of the remaining articles. Any disagreement resulted in the articles proceeding to full-text review. The full-text articles were then reviewed by the same authors and any conflict was discussed to achieve consensus.

Risk of bias (quality) assessment. The articles were evaluated for relevance, sample numbers, the underlying disease process, statistical power, analytical validity, quality of evidence, and conclusions. The Newcastle-Ottawa Scale was used to evaluate the study design. Relevant metabolites were highlighted and where statistical testing was performed, significance was quoted. The metabolites themselves were identified using various commercial software packages such as Chenomx NMR Suite (Chenomx, Edmonton, Canada), as well as by identifying them from published databases including the Human Metabolome Database,[37] the Biological Magnetic Resonance Bank,[38] and various in-house databases.

Results

Literature search. The electronic database searches identified 4,477 articles. Following exclusion of any duplicates and reviewing the titles, 4,391 articles were excluded. The abstracts of the remaining 86 articles were reviewed and a further 14 were excluded as they contained duplicate data. Of the remaining 72 articles, 25 were excluded for not meeting the entry criteria, two were removed as they contained data from the same cohort, three were excluded because they did not look at HSF, and two were removed as they looked specifically at synovial membranes and not SF. Of the remaining 40 articles, one article was excluded as it had duplicate data, one was excluded as it only looked at serum and not SF, two were excluded for not using metabolic profiling techniques, 11 were removed because the metabolites were not clearly identified or only a portion of them were presented, and four were excluded as it was unclear in these articles which cohort the metabolites were found in greater quantities. As a result, 21 studies were eventually used (17 articles and four abstracts) (Figure 1).

Study characteristics and quality. The methodology of the published studies was assessed using the Cochrane criteria for bias and the Newcastle-Ottawa Scale. The studies included had similar designs and metabolic profiling techniques. Patient selection was not random, as all studies were looking at specific disease processes. Furthermore, blinding was not possible at sample collection for either the researcher or the patient. Multivariate analysis was performed to detect patterns of changes in the metabolites detected, which did not necessarily involve significance testing. These types of analyses were performed mostly using supervised methods, which required information about the sample class, and therefore the data could not be blinded. As p-values were not consistently reported in all the studies, reporting bias may exist towards those that do so. Furthermore, studies that involve assaying hundreds of metabolites may have overestimated the significance of the p-values, unless false discovery rate (FDR) or validation datasets were utilized. All the identified studies are listed in Table I.[39–61]

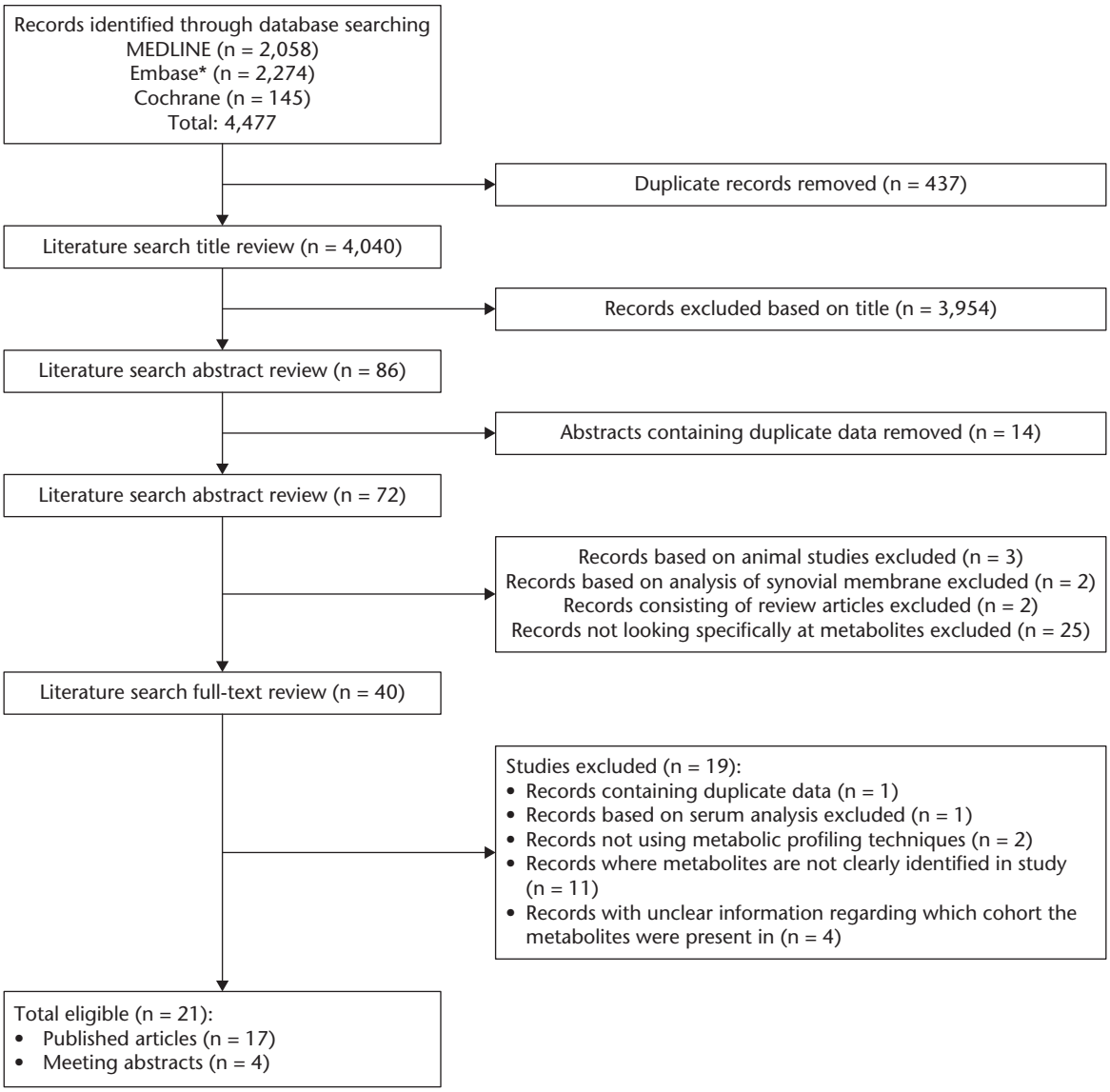


Fig. 1

Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) search and screening flowchart for the role of metabolic profiling in human synovial fluid research. *Excerpta Medica Database, Amsterdam, The Netherlands.

All the identified metabolites have been listed in Supplementary Table ii. The identified studies have been subdivided into those with healthy controls that have identified putative biomarkers and those looking at specific disease processes.

Studies with a healthy control group. Adams et al[39] examined the cytokine and metabolic differences between healthy and end-stage post-traumatic arthritic ankle (PTAA) joint SF. They identified 29 metabolites in significantly different concentrations between the PTAA and control groups, the most important of which is glutamate. Their findings suggest a mainly oxidative and pro-inflammatory environment with an imbalance in amino acid (AA) and lipid metabolism among other factors. However, there are no p-values stated in the paper and no FDR or other analysis was performed to account for multiple testing.

The metabolic changes in the physiological responses of early knee OA were performed by Chen et al.[43] They identified 22 significant metabolic differences between the two groups. Most serum AA levels were found to be altered in the OA group, suggesting that OA is accompanied and precipitated by changes in AA metabolism. They identified three potential biomarkers: alanine, γ-aminobutyric acid (GABA), and 4-hydroxy-L-proline (Hyp). Alanine and Hyp were increased in the OA group and GABA was reduced in the OA group.

Dubey et al[46] explored whether metabolic profiling would identify a distinctive metabolic signature of seronegative spondyloarthropathy (SSA) that is not influenced

Table I. Baseline description of all the studies included in this systematic review

First author/ year	Study design	Country of origin	Joint	Diagnosis	Disease staging	Sample size	Type of analysis	Validated analysis	Controls	Statistical validity	NOS
Adams et al, 2014[39]	Case control study	USA	Ankle	Radiological	Takakura grading	n = 20; c = 20	UHPLC-MS/MS	Weak	Healthy asymptomatic patients	Adequate	7
Ahn et al, 2015[40]	Case series	South Korea	N/A	Clinical	N/A	n = 24	GC-TOF-MS	Strong	None	Adequate	3
Anderson et al, 2018[41]	Cohort study	UK	Knee	N/A	N/A	n = 10 (OA); n = 14 (RA)	^{1}H-NMR	N/A	None	Adequate	0
Carlson et al, 2018[42]	Case control study	USA	N/A	N/A	N/A	n = 5 (OA); n = 3 (RA); c = 5	LC-MS	Weak	Post-mortem samples	Adequate	3
Chen et al, 2018[43]	Case control study	China	Knee	Clinical/ radiological	KL	n = 32; c = 35	UHPLC-TQ-MS	Weak	Healthy asymptomatic patients	Adequate	8
Dubey et al, 2019[44]	Case series	India	Knee	N/A	N/A	n = 8	^{1}H-NMR	N/A	None	Adequate	0
Dubey et al, 2017[45]	Cohort study	India	Knee	Clinical	N/A	n = 19 (ReA); n = 13 (USpA)	^{1}H-NMR	N/A	None	Adequate	
Dubey et al, 2019[46]	Case control study	India	Knee	Clinical	Braun's, ASAS, and ACR criteria	n = 52 (SSA); n = 29 (RA); c = 82	^{1}H-NMR	Weak	Healthy asymptomatic patients	Adequate	6
Furman et al, 2017[47]	Case control study	USA	Knee	Clinical	Not applicable	n = 8; c = 8	UHPLC-MS/MS	N/A	Contralateral non-injured knee	Adequate	7
Hwang et al, 2013[48]	Cohort study	South Korea	N/A	N/A	N/A	n = 18 (RA); n = 11 (OA)	GC-TOF-MS	N/A	None	Adequate	6
Kang et al, 2015[49]	Case series	South Korea	Knee	Clinical/ radiological	KL (OA); ACR (RA)	n = 10 (OA); n = 10 (RA)	UPLC-QTOF-MS	Weak	None	Adequate	5
Khatib et al, 2018[50]	Case series	UK	Knee	N/A	N/A	n = 13	^{1}H-NMR	N/A	None	Adequate	3
Kim et al, 2017[51]	Case series	South Korea	Knee	Clinical/ radiological	KL	n = 8 (KL1 to 2); n = 7 (KL3 to 4)	GC-TOF-MS	Strong	None	Adequate	4
Kim et al, 2014[52]	Case series	South Korea	N/A	Clinical/ radiological	ACR for RA; ASAS for AS; criteria of the 1990 ISG for BD; MSU crystals in joint fluid for gout.	n = 13 (RA); n = 7 (AS); n = 5 (BD); n = 13 (gout)	GC-TOF-MS	Adequate	None	Adequate	4
Leimer et al, 2017[53]	Cohort study	USA	Ankle	Radiological	N/A	n = 19; c = 19	UHPLC-MS/MS	Adequate	Contralateral non-injured ankle	Adequate	8
Meshitsuka et al, 1999[54]	Case series	Japan	Knee	Clinical/ radiological	ACR	n = 14 (RA); n = 16 (OA)	^{1}H-NMR	Adequate	None	Adequate	2
Mickiewicz et al, 2015[55]	Cohort study	Canada	Knee	Clinical/ radiological	N/A	n = 55; c = 13 (cadaveric - 6 bilateral/1 unilateral sample)	^{1}H-NMR; GC-MS	Strong	Cadaveric controls	Adequate	6
Naughton et al, 1993[56]	Cohort study	UK	Knee	N/A	N/A	n = 22 (RA); c = 6	^{1}H-NMR	Adequate	Healthy asymptomatic patients	Adequate	5
Yang et al, 2015[57]	Case control study	China	Knee	ACR	N/A	n = 25 (RA); c = 10	GC-TOF-MS	Adequate	Above knee amputated patients	Adequate	6
Zhang et al, 2014[58]	Case series	Canada	Hip/ knee	ACR	ESOA	n = 80	LC-MS	Adequate	None	Adequate	5
Zhang et al, 2015[59]	Case series	Canada	Knee	N/A	ESOA	n = 69	LC-MS	Adequate	None	Adequate	5
Zhang et al, 2016[60]	Case control study	Canada	Knee	ACR criteria and clinical judgement	ESOA	n = 97	LC-MS	Adequate	No SF sample controls (only serum)	Adequate	6
Zheng et al, 2017[61]	Cohort study	China	Knee	KL	KL2 and KL4	n = 49, c = 21	GC-TOF-MS and LC-MS/MS	Adequate	Asymptomatic patients	Adequate	7

^{1}H-NMR, nuclear magnetic resonance spectroscopy; ACR, American College of Rheumatology; AS, ankylosing spondylitis; ASAS, Assessment of SpondyloArthritis international Society; BD, Behçet's disease; C, control group; ESOA, end-stage osteoarthritis; GC-MS, gas chromatograph-mass spectrometry; GC-TOF-MS, gas chromatography/time-of-flight mass spectrometry; ISG, International Study Group; KL, Kellgren and Lawrence; LC-MS, liquid-chromatography mass spectrometry; MSU, monosodium urate; N/A, not available; NOS, Newcastle-Ottawa Scale; OA, osteoarthritis; RA, rheumatoid arthritis; ReA, reactive arthritis; SSA, seronegative spondyloarthropathy; TQ MS, triple quadrupole mass spectrometry; UHPLC-MS/MS, ultra-high performance liquid chromatography/tandem mass spectrometry; UPLC-QTOF-MS, ultraperformance liquid chromatography quadruple time-of-flight mass spectrometer; UHPLC-TQ-MS, ultra-high performance liquid chromatography triple quadrupole mass spectrometry; USpA, undifferentiated spondyloarthropathy.

by age and sex. Their control group consisted of two subgroups of healthy patients, who were stratified by age, creating a young and older control group. There were a number of patient cohorts consisting of those with reactive arthritis (ReA), SSA, and RA. They suggested low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), leucine, lysine/arginine, acetone, glycine, glucose, creatine, polyunsaturated fatty acids (PUFAs), and phenylalanine as putative biomarkers for ReA when compared to an age-matched control group. Conversely, leucine, lysine/arginine, phenylalanine, and valine were suggested as putative biomarkers for discriminating between ReA and RA.

An abstract published by Furman et al[47] analyzed healthy and injured knees to identify any metabolic pathways affected by the knee injury and identify any discriminatory SF biomarkers. They demonstrated significantly increased sphingomyelin (SPM) and 2-hydroxy-fatty acids in the injury group. They suggested that these may be potential SF biomarkers of knee injury and may be prognostic indicators of the risk of post-traumatic arthritis.

Leimer et al[53] attempted to characterize the global metabolic profile of SF after intra-articular ankle fractures with an emphasis on changes in the lipid profile. They identified 16 lipid-based metabolites found in significantly greater quantities following an intra-articular ankle fracture, which subsequently decreased six months post-surgery. Most long-chain fatty acids (FAs) and PUFAs were acutely elevated in the fractured ankles at baseline compared to the control group. However, none of these were suggested as potential biomarkers. They suggest the distinctive lipid signature identified is reflective of injury, fracture, and early changes associated with OA.

The metabolic status of normal and RA SF was assessed qualitatively by Naughton et al.[56] They demonstrated increased levels of LDL in RA compared to the control group and suggested this to be secondary to inflammation and increased synovial membrane permeability. However, the controls were not age-matched (25 to 42 years old) and were younger than the RA group (40 to 67 years old), which may result in important metabolic differences.

Zheng et al[61] explored the metabolites of OA. Six metabolites were significantly different between the two groups. Three were found in significantly greater concentrations in the OA group and three in the control group. Gluconic lactone, threonine, and 1,5-Anhydroglucitol (1,5-AG) were in significantly greater concentrations in OA SF compared to the control group. Glutamine, tyramine, and 8-aminocaprylic acid were in significantly lower concentrations in OA SF compared to the control group. The authors concluded that a new diagnostic model combining two metabolites provides greater sensitivity in diagnosing OA than a single metabolite alone. Furthermore, gluconic lactone may prove to be a novel benchmark for the differential diagnosis of OA from RA due to the significant differences in the concentration of this metabolite between these conditions with a high level of sensitivity and specificity between them.

Table II. Putative biomarkers identified from studies with an asymptomatic control group. All metabolites were identified from human knee synovial fluid.

Underlying pathology	Metabolite	Change	Multivariate analysis
OA	Alanine[43]	Increased in OA	VIP 3.31, $p < 0.001$
	Hyp[43]		VIP 1.75, $p < 0.001$
	Gluconic lactone[61]		FC 1.54, $p < 0.05$
	Threonine[61]		FC 2.71, $p < 0.05$
	1,5-AG[61]		FC 1.67, $p < 0.05$
	GABA[43]	Decreased in OA	VIP 2.61, $p < 0.001$
	Glutamine[61]		FC 0.28, $p < 0.05$
	Tyramine[61]		FC 0.30, $p < 0.05$
	8-Aminocaprylic acid[61]		FC 0.27, $p < 0.05$
Inflammatory arthropathies	Acetone[46]	Increased in ReA	FC 1.54, $p < 0.006$
	Creatine[46]		FC 0.63, $p < 0.001$
	VLDL[46]		N/A
	Glucose[46]		FC 1.12, $p < 0.367$
	Glycine[46]		FC 1.03, $p < 0.02$
	LDL[46]		N/A
	Leucine[46]		FC 0.83, $p < 0.051$
	Lysine/arginine[46]		FC 0.78/1.21, $p < 0.002$/$p < 0.46$
	Phenylalanine[46]		FC 1.33, $p < 0.122$
	PUFA[46]		N/A
	Leucine[46]	Increased in ReA vs RA	FC 1.88, $p < 0.001$
	Lysine/arginine[46]		FC 1.46/2.07, $p < 0.005$/$p < 0.001$
	Phenylalanine[46]		FC 2.56, $p < 0.001$
	Valine[46]		FC 1.57, $p < 0.001$
RA	LDL[56]	Increased in RA	N/A
Knee injury	SPM[47]	Increased in knee trauma	$p < 0.0065$ following FDR
	2-hydroxy-fatty acids[47]		$p < 0.0065$ following FDR

1-5 AG, 1,5-Anhydroglucitol; FC, fold change; FDR, false discovery rate; GABA, γ-aminobutyric acid; Hyp, 4-hydroxy-L-proline; LDL, low-density lipoprotein; N/A, not available; OA, osteoarthritis; PUFA, polyunsaturated fatty acid; RA, rheumatoid arthritis; ReA, reactive arthritis; SPM, sphingomyelin; VIP, variable importance on projection score; VLDL, very low-density lipoprotein.

None of these studies performed FDR or other analysis to account for multiple testing of the data. Consequently, the results must be reviewed with caution. The metabolites identified in this section, which have been proposed to serve as putative biomarkers, are listed in Table II.[43,46,47,56,61]

Osteoarthritis studies. Mickiewicz et al[62] identified two metabolites found in significantly greater concentrations in OA SF (fructose and citrate) and nine metabolites (O-acetylcarnitine, hexanoylcarnitine, N-phenylacetylglycine, ethanol, ethanolamine, methionine, malate, creatine, and 3-hydroxybutyrate) found in lower concentrations in OA SF compared to cadaveric controls.

Kim et al[51] characterized the metabolite differences between early- and late-stage OA. They identified 28 metabolites as being significantly different between the groups, with all 28 increased significantly in late-stage OA.

Zhang et al[58] examined the metabolic markers in SF that can be used to classify patients with OA into distinct

subgroups. Broadly speaking, they identified numerous metabolites included 40 acylcarnitines (one free carnitine), 20 AAs, nine biogenic amines, 87 glycerophospholipids, 11 sphingolipids, and one hexose (> 90% was glucose). Following multivariate analysis, they identified subgroups of OA, which differed in acylcarnitine levels and fat metabolism. They observed distinctions in the glycophospholipids and SPMs. However, as no age-matching and no correlation to clinical factors took place, it is difficult to draw definitive conclusions.

Using a similar methodology, Zhang et al[63] later investigated the differences between OA and type II diabetes mellitus. Of note, leucine and phosphatidylcholine (PC) metabolism were influenced by both diabetes mellitus and OA. Phosphatidylcholine is involved in many membrane-related phenomena including forming the essential lipid bilayer of all biological membranes, regulation of membrane trafficking, and signal transduction.[64]

An abstract by Khatib et al[50] investigated whether the mechanical loading of the joint during pivot shift will reveal a profile of mechanically regulated metabolic biomarkers in patients with ACL deficient knees. Using nuclear magnetic resonance spectroscopy (^{1}H-NMR), they identified a significant difference in alanine and choline between pre- and post-pivot shift testing of ACL deficient knees. These metabolites remained significant when accounting for multiple testing and the authors suggest they might be useful for rehabilitation or surgical intervention in patients with knee injuries who may be at risk of post-traumatic OA.

Inflammatory arthropathy studies. A recent study explored RA-related biochemical abnormalities by analyzing the metabolic profile of knee SF from RA patients and a control group.[57] These controls were patients who had a 'high-level' amputation. However, the paper does not state the reason for the amputation nor whether the sample was taken before or after amputation, which may have important metabolic consequences. Following multivariate analysis and using a variable projection of importance score (VIP) > 1 plus $p < 0.05$, 13 of these metabolites were significant between the two groups. Glucose was decreased and lactic acid was increased in RA SF. Levels of glucose-1-phosphate and D-mannose were also decreased.

Ahn et al[40] evaluated the metabolomic profile of SF in patients with Behçet's disease (BD) with arthritis compared to those with seronegative arthritis (SNA). They identified 11 metabolites as being significantly increased in BD with arthritis compared to SNA. These include branched-chain AAs (BCAA: valine, leucine, and isoleucine), citramalate, glutamate, and methionine sulfoxide.

In an earlier study, Kim et al[52] also evaluated potential biomarkers for RA. Their study consisted of patients with RA (n = 13), ankylosing spondylitis (AS) (n = 7), BD (n = 5), and gout (n = 13). These patients were then combined into two groups, which were RA and non-RA. They identified 20 metabolites that remained significantly different between the two groups following robust statistical analysis, which they proposed could be putative biomarkers. Of these, 14 were in significantly greater concentrations in the RA group and six were in greater concentrations in the non-RA group.

Osteoarthritis versus rheumatoid arthritis studies. Carlson et al[42] evaluated global liquid chromatography coupled to mass spectrometry (LC-MS) based metabolic profiles as a tool for quantifying biomarkers within SF. Their control group consisted of five purchased post-mortem samples. It is unclear how long after death these samples were harvested and the death to post-mortem interval is likely to be a major confounding variable, so this uncertainty might have important metabolic consequences.[65] They identified five metabolites (citric acid, D-lactic acid methyl ester, hydroxyl-L-proline, L-isoleucine, and L-methionine) found in significantly lower concentrations in OA and RA SF, compared to controls and one metabolite (L-citrulline) found in greater concentrations in OA compared to RA and controls. The authors also performed FDR analysis to account for multiple testing.

A recently published abstract explored the role of NMR spectroscopy in producing analyzable spectra from a low volume of SF taken in a clinical environment.[41] They identified 11 metabolites found in significantly different concentrations between OA and RA SF. Seven were more abundant in OA and six were more abundant in RA SF. Their analysis suggested the metabolic pathways most impacted were: aminoacyl-transfer RNA (tRNA); biosynthesis; nitrogen metabolism; valine, leucine, and isoleucine biosynthesis; glycine, serine, and threonine metabolism; and taurine and hypotaurine metabolism. The authors allude to their methodology being useful for analyzing low-volume SF. However, in their methodology they state that each sample consists of approximately 100 ml. Furthermore, although the authors mention the term "FDR < 0.05", it is unclear exactly what analysis took place.

Another abstract investigated the metabolites of SF in patients with RA and OA to identify the characteristic metabolites differentiating the two diseases.[48] Using gas chromatography/time-of-flight mass spectrometry (GC/TOF MS) and following multivariate analysis, they identified 17 metabolites as being found in significantly different concentrations between the two groups. Six were upregulated in RA (maltose, lignoceric acid, uracil, mannitol, pyrophosphate, and phosphoric acid) and 11 in OA (lysine, tyrosine, valine, glyceric acid, alanine, asparagine, hydroxylamine, tryptophan, glycerol, glutamine, and citrulline).

Kang et al[49] identified 21 metabolites as being in significantly different concentrations between RA and OA SF. Concentrations of lipid metabolites were typically higher in RA than OA SF, which has previously been demonstrated.[66] Concentrations of tryptophan metabolites also differed significantly between the two groups.

The ratios of lactate and alanine have also been shown to be significantly greater in RA than in OA SF.[54]

Discussion

Metabolic profiling is an invaluable method of differentiating patients with various pathologies from healthy individuals in the clinical setting. This systematic review has identified numerous metabolites in different pathologies. Specifically, a few putative biomarkers have been illustrated. The studies identified have further demonstrated the important role of lipid mediators and metabolism in both OA and RA. However, its role in RA and other inflammatory arthropathies is more prominent. Furthermore, this article has summarized some of the putative biomarkers identified in the literature, although further studies are required to confirm and determine their significance (Table II). Looking specifically at these metabolites, collation of these results illustrates how metabolic changes may be interlinked in OA and inflammatory arthropathies, while postulating the potential metabolic pathways that may be affected (Figure 2).

The metabolic homeostasis within a joint is often disturbed in the disease state leading to an anaerobic state secondary to stress and inflammation. Whether these changes differ between the diseased joint and the normal joint is an important question when considering the importance of diagnostic or prognostic putative biomarkers.

Role of the identified putative biomarkers. Broadly speaking, the putative biomarkers in OA can be classified into two main groups: AAs plus related metabolites (alanine, Hyp, threonine, GABA, glutamine, tyramine); and sugars plus related metabolites (gluconic lactone, 1,5-AG). Alanine, Hyp, and threonine are found in articular cartilage.[67] Their increase in OA SF could be associated with increased catabolism of the articular cartilage. This may also represent increased energy consumption to account for the increased bone turnover and subchondral sclerosis seen in OA. 1,5-AG is a monosaccharide occasionally used as a short-term marker of glycaemia.[68] Elevation of this metabolite in SF is consistent with the reduced glucose concentration in OA SF,[69] secondary to increased energy expenditure. Increased gluconic lactone in OA SF may be due to auto-oxidation induced by increased levels of reactive oxygen species (ROS). ROS are able to directly induce cartilage degradation by cleaving aggrecan and collagen plus activating matrix metalloproteinases (MMPs).[70]

GABA arises from glutamic acid,[71] which regulates glucose, also suggesting increased energy consumption in the diseased joint due to less residual glucose. Glutamine has a role in oxidative metabolism, and reduced levels suggest altered oxidative metabolism in diseased joints secondary to increased energy expenditure.[72] Glutamine has been shown to supress inflammatory cytokines[73] and protect chondrocytes from heat stress and nitrous oxide (NO)-induced apoptosis.[74] These effects may protect chondrocytes from various types of stress and prevent progressive cartilage degeneration in OA. Tyramine is derived from the AA tyrosine, which is thought to have a role in promoting osteophyte formation. Increased levels have been seen in subchondral bone.[55,75]

The putative biomarkers increased in inflammatory arthropathies can be classified into four main groups: AAs and related metabolites (creatine, glycine, leucine, lysine, arginine, phenylalanine, valine); lipids and lipoproteins (LDL, VLDL, PUFA); sugars (glucose); and ketone bodies (acetone). They identified that AAs are all constituents of articular cartilage with leucine, proline, glutamic acid, and glycine specifically being constituents of proteoglycans.[67] Their increase suggests breakdown of the articular cartilage, likely related to the underlying inflammatory process. Low concentrations of FAs have been demonstrated in HSF. The increased levels of LDL, VLDL, and PUFA identified here are secondary to increased synovial membrane permeability and inflammation associated with underlying inflammatory arthropathies.[56]

Metabolic changes seen in osteoarthritis. Fructose elevation suggests a hypoxic condition of the diseased and inflamed knee joint. Hypoxia has been shown to result in the upregulation of glucose phosphate isomerase, which catalyzes the conversion of glucose-6-phosphate (G6P) into fructose-6-phosphate (F6P) in inflammatory arthritis.[76] Lower concentrations of O-acetylcarnitine, hexanoylcarnitine, N-phenylacetylglycine, and ethanolamine indicate protracted FA and lipid metabolism in the SF of OA patients compared to controls.[37] Decreased methionine concentrations indicate its use, where it is likely converted to S-adenosylmethionine (SAM), a proposed factor for cartilage damage repair and inflammatory reduction.[77]

Kim et al[51] identified three unique pathways in their study, which corresponded to the metabolic differences they identified. These were FA metabolism, glycolipid metabolism, and the tricarboxylic acid (TCA) cycle. These pathways may be associated with an increasing degree in the severity of OA. Glycerol and various FA concentrations were more prominent in the late-stage OA group. Their findings suggest that FA biosynthesis is predominantly responsible for energy generation in late-stage OA. Furthermore, increased concentrations of malate in the late-stage OA group compared to the early-stage group suggest a possible difference in the energy level between the two groups.

Furthermore, alterations in the concentration and composition of phospholipids covering articular cartilage has been shown to be associated with the development of OA.[78]

Metabolic changes seen in inflammatory arthropathies. Glucose was decreased and lactic acid was increased in RA SF.[57] Levels of glucose-1-phosphate and D-mannose

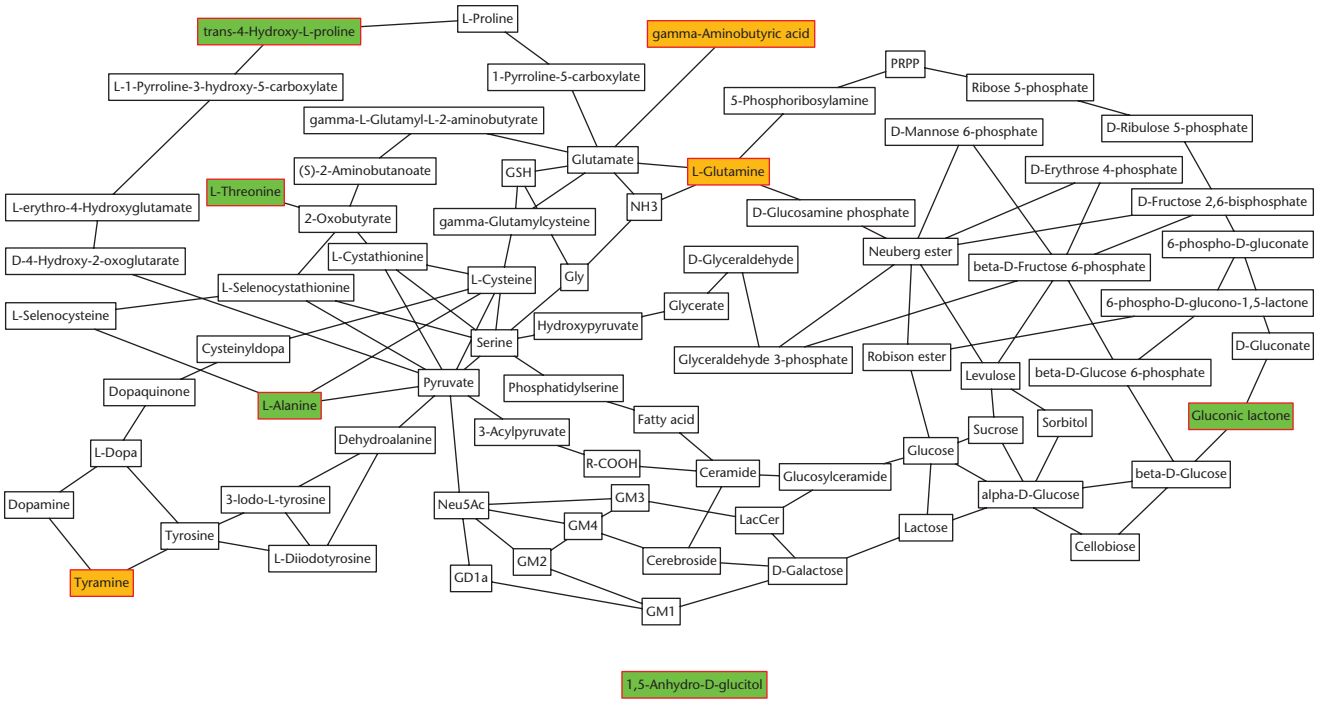


Fig. 2a

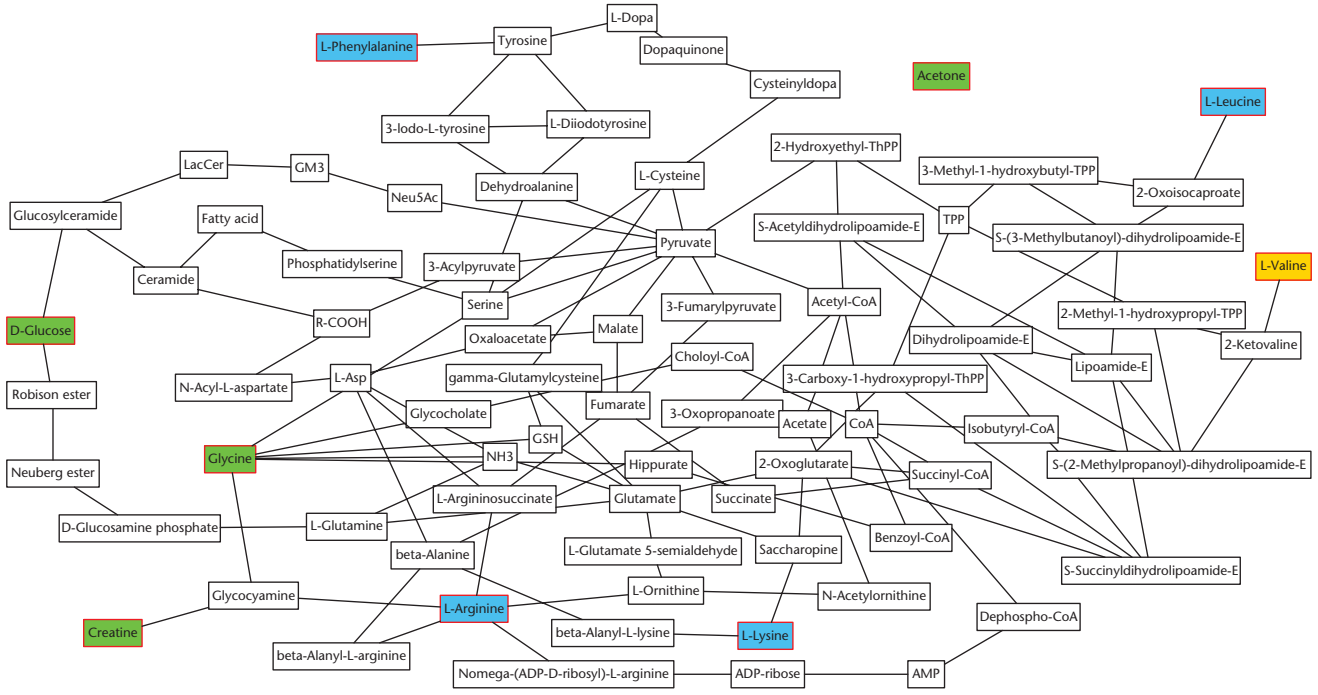


Fig. 2b

Metabolic network analysis of all the putative biomarkers identified in this systematic review demonstrating the associated metabolic pathways. All metabolites with a red outline were putative biomarkers. a) Putative biomarkers identified in osteoarthritic synovial fluid (SF). Those in green were raised and those in orange were reduced in osteoarthritic SF compared to an asymptomatic control group. b) Putative biomarkers identified in inflammatory arthropathies. Those in green and blue were raised in reactive arthritis (ReA) compared to an asymptomatic control group; those in blue were also raised in ReA compared to rheumatoid arthritis (RA); valine (in yellow) was raised in ReA compared to RA. ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; CoA, coenzyme A; GD1a, N-acetylneuraminyl-D-galactosyl-N-acetyl-D-galactosaminyl-(N-acetylneuraminyl)-D-galactosyl-D-glucosylceramide; Gly, glycine; GM1, D-galactosyl-N-acetyl-D-galactosaminyl-(N-acetylneuraminyl)-D-galactosyl-D-glucosylceramide; GM2, N-acetyl-D-galactosaminyl-(N-acetylneuraminyl)-D-galactosyl-D-glucosylceramide; GM3, (N-acetylneuraminyl)-D-galactosyl-D-glucosylceramide; GM4, N-acetylneuraminyl-galactosylceramide; GSH, reduced glutathione; LacCer, lactosylceramide; L-Asp, L-aspartic acid; Neu5Ac, N-acetylneuraminic acid; NH3, ammonia; PRPP, 5-phosphoribosyl 1-pyrophosphate; R-COOH, carboxylic acid; ThPP, thiamin pyrophosphate; TPP, thiamin pyrophosphate.

were also decreased. These decreases may be explained by the increased energy demands caused by inflammation in RA. Furthermore, the increased consumption of glucose can lead to increased lactic acid production. Levels of citric acid were decreased in RA SF. Citric acid is an important component of the TCA cycle, which provides the complete oxidation of acetyl-coenzyme A (CoA) derived from AAs, carbohydrates, and fats. Consequently, this leads to decreased adenosine triphosphate (ATP) production from the aerobic oxidation process. Yang et al[57] suggest that low glucose and high lactic acid concentrations in RA SF may represent potential biomarkers of RA.

The increased levels of BCAAs, identified in the study by Ahn et al,[40] result in increased production of IL-1 and/or TNF-α, which are typically increased in RA and SNA.[79] Elevated expression of citramalate has been suggested to indicate disturbed metabolism of glutamate in the setting of active inflammation.[80] Elevated expression of citrulline and methionine sulfoxide were also identified in this study. This may reflect neutrophil hyperactivity documented in BD.[81] Ahn et al[40] suggest that these metabolites may act as potential biomarkers for discriminating BD with arthritis from SNA. However, there are no normal controls in their study.

The metabolites identified in a study by Kim et al[52] are major intermediates of FA and AA metabolism, the TCA cycle, and the urea cycle. The authors suggest that AA metabolism, the TCA cycle, and the urea cycle were more activated in the RA group compared with those in the non-RA group. Although the authors suggest these metabolites may be potential biomarkers, there are no normal controls and several different disease processes were compared. Consequently, it is difficult to say with any certainty whether any of these metabolites are indeed potential biomarkers.

Metabolic changes seen between osteoarthritis and rheumatoid arthritis. Kang et al[49] identified 21 metabolites as being in significantly different concentrations between RA and OA SF. Concentrations of lipid metabolites were typically higher in RA than in OA SF, which has previously been demonstrated.[66] Furthermore, regulation of inflammation includes the roles of lipid mediators and prostaglandins (PGs). Leukotrienes and PGs are crucial in the development of arthritic diseases.[82] Concentrations of tryptophan metabolites also differed significantly between the OA and RA groups. This is an exogenous AA that must be provided in the diet. Tryptophan and its metabolites are involved in inflammation. One of the metabolites, kynurenine, has well known anti-inflammatory effects that are toxic to T cells and which cause apoptosis.[83]

Although this systematic review has identified many metabolites present in different disease states, including some putative biomarkers, there are some important limitations. There were only seven studies identified in the literature with healthy controls.[39,43,46,47,53,56,61] Furthermore, only two studies performed an analysis to account for multiple testing of their dataset and neither of these studies had healthy controls.[41,42] The presence of multiple confounding factors in many of the studies was another important limitation. Not all studies accounted for age or sex and certainly very few considered medical comorbidities. Consequently, the results must be viewed with caution. The solution to these limitations would be to conduct a largescale epidemiological metabolic profiling study incorporating multiple confounding factors such as age, sex, medical comorbidities, and medications with a view to addressing the correlations between clinical features of disease, inflammation, and metabolism. It should be noted that the majority of the reported works are untargeted metabolic profiling studies, where the identity of any putative biomarker is unknown at the outset. In those cases where metabolites were identified or annotated, no parameter of identification certainty, such as the Metabolomics Standards Initiative (MSI) level of identification, was reported.[84] Therefore, the occurrence of incorrect identifications is possible hence affecting further metabolic interpretation and biomarker validation. Another important limitation of some of the studies is that they do not provide quantitative percentage or fold change, but only the direction of change.

In conclusion, metabolic profiling is proving to be an invaluable method of identifying putative biomarkers in the field of orthopaedics unique to different pathologies. Although numerous studies have been performed using these techniques in human SF, larger studies are required with healthy controls accounting for multiple confounding factors and using robust statistical analysis to identify putative biomarkers. This may lead to the development of new diagnostic techniques and possible treatment strategies. Recent advances in both proteomic and genetic studies have demonstrated the importance of these techniques to improve disease understanding and identify biomarkers.[85,86] Future studies integrating genomic, proteomic, and metabolic profiling techniques may provide the greatest hope for the advancement of biomarker discovery.

Supplementary Material

Tables showing search terms used for this systematic review on the role of metabolic profiling in human synovial fluid (HSF) (Supplementary Table i), and a list of all identified metabolites by article in HSF (Supplementary Table ii).

References

1. **Castañeda S, Roman-Blas JA, Largo R, Herrero-Beaumont G.** Osteoarthritis: a progressive disease with changing phenotypes. *Rheumatology (Oxford).* 2014;53(1):1-3.
2. **Poole AR.** Osteoarthritis as a whole joint disease. *HSS J.* 2012;8(1):4-6.
3. **Johnson VL, Hunter DJ.** The epidemiology of osteoarthritis. *Best Pract Res Clin Rheumatol.* 2014;28(1):5-15.
4. **Scott DL, Wolfe F, Huizinga TW.** Rheumatoid arthritis. *Lancet.* 2010;376(9746): 1094-1108.
5. **Cammarata RJ, Rodnan GP, Fennell RH.** Serum anti-γ-globulin and antinuclear factors in the aged. *JAMA.* 1967;199(7):455-458.
6. **Humphreys JH, Symmons DP.** Postpublication validation of the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis: where do we stand? *Curr Opin Rheumatol.* 2013;25(2):157-163.
7. **Tanino M, Matoba R, Nakamura S, et al.** Prediction of efficacy of anti-TNF biologic agent, infliximab, for rheumatoid arthritis patients using a comprehensive transcriptome analysis of white blood cells. *Biochem Biophys Res Commun.* 2009;387(2):261-265.
8. **Teixeira VH, Olaso R, Martin-Magniette ML, et al.** Transcriptome analysis describing new immunity and defense genes in peripheral blood mononuclear cells of rheumatoid arthritis patients. *PLoS One.* 2009;4(8):e6803.
9. **Kallberg H, Padyukov L, Plenge RM, et al; Epidemiological Investigation of Rheumatoid Arthritis study group.** Gene-gene and gene-environment interactions involving HLA-DRB1, PTPN22, and smoking in two subsets of rheumatoid arthritis. *Am J Hum Genet.* 2007;80(5):867-875.
10. **Horning EC, Horning MG.** Metabolic profiles: gas-phase methods for analysis of metabolites. *Clin Chem.* 1971;17(8):802-809.
11. **Lee SJ, Trostel A, Adhya S.** Metabolite changes signal genetic regulatory mechanisms for robust cell behavior. *MBio.* 2014;5(1):e00972-13.
12. **Hu T, Zhang W, Fan Z, et al.** METABOLOMICS DIFFERENTIAL CORRELATION NETWORK ANALYSIS OF OSTEOARTHRITIS. *Pac Symp Biocomput.* 2016;21:120-131.
13. **Lindon JC, Holmes E, Nicholson JK.** So what's the deal with metabonomics? *Anal Chem.* 2003;75(17):384A-391A.
14. **Harwood LM, Claridge TDW.** *Introduction to Organic Spectroscopy.* Oxford: Oxford University Press. 1999.
15. **Lindon JC, Nicholson JK.** Spectroscopic and statistical techniques for information recovery in metabonomics and metabolomics. *Annu Rev Anal Chem (Palo Alto Calif).* 2008;1:45-69.
16. **Want EJ, Wilson ID, Gika H, et al.** Global metabolic profiling procedures for urine using UPLC-MS. *Nat Protoc.* 2010;5(6):1005-1018.
17. **Odunsi K, Wollman RM, Ambrosone CB, et al.** Detection of epithelial ovarian cancer using 1H-NMR-based metabonomics. *Int J Cancer.* 2005;113(5):782-788.
18. **Wang Z, Chen Z, Yang S, et al.** (1)H NMR-based metabolomic analysis for identifying serum biomarkers to evaluate methotrexate treatment in patients with early rheumatoid arthritis. *Exp Ther Med.* 2012;4(1):165-171.
19. **Yu B, Zheng Y, Nettleton JA, Alexander D, Coresh J, Boerwinkle E.** Serum metabolomic profiling and incident CKD among African Americans. *Clin J Am Soc Nephrol.* 2014;9(8):1410-1417.
20. **Athersuch TJ.** The role of metabolomics in characterizing the human exposome. *Bioanalysis.* 2012;4(18):2207-2212.
21. **Hong J, Yang L, Zhang D, Shi J.** Plant Metabolomics: An Indispensable System Biology Tool for Plant Science. *Int J Mol Sci.* 2016;17(6):E767.
22. **Fischer K, Kettunen J, Würtz P, et al.** Biomarker profiling by nuclear magnetic resonance spectroscopy for the prediction of all-cause mortality: an observational study of 17,345 persons. *PLoS Med.* 2014;11(2):e1001606.
23. **Holmes E, Loo RL, Stamler J, et al.** Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature.* 2008;453(7193):396-400.
24. **Zhang A, Sun H, Wang P, Han Y, Wang X.** Recent and potential developments of biofluid analyses in metabolomics. *J Proteomics.* 2012;75(4):1079-1088.
25. **Garnero P, Rousseau JC, Delmas PD.** Molecular basis and clinical use of biochemical markers of bone, cartilage, and synovium in joint diseases. *Arthritis Rheum.* 2000;43(5):953-968.
26. **Lamers RJ, van Nesselrooij JH, Kraus VB, et al.** Identification of an urinary metabolite profile associated with osteoarthritis. *Osteoarthritis Cartilage.* 2005;13(9): 762-768.
27. **Lamers RJ, DeGroot J, Spies-Faber EJ, et al.** Identification of disease- and nutrient-related metabolic fingerprints in osteoarthritic Guinea pigs. *J Nutr.* 2003;133(6):1776-1780.
28. **Zhai G, Wang-Sattler R, Hart DJ, et al.** Serum branched-chain amino acid to histidine ratio: a novel metabolomic biomarker of knee osteoarthritis. *Ann Rheum Dis.* 2010;69(6):1227-1231.
29. **Blanco FJ, Ruiz-Romero C.** Osteoarthritis: metabolomic characterization of metabolic phenotypes in OA. *Nat Rev Rheumatol.* 2012;8(3):130-132.
30. **Damyanovich AZ, Staples JR, Chan ADM, Marshall KW.** Comparative study of normal and osteoarthritic canine synovial fluid using 500 MHz 1H magnetic resonance spectroscopy. *J Orthop Res.* 1999;17(2):223-231.
31. **Lacitignola L, Fanizzi FP, Francioso E, Crovace A.** 1H NMR investigation of normal and osteo-arthritic synovial fluid in the horse. *Vet Comp Orthop Traumatol.* 2008;21(1):85-88.
32. **Xie K, Dai K, Qu X, Yan M.** Serum and Synovial Fluid Interleukin-6 for the Diagnosis of Periprosthetic Joint Infection. *Sci Rep.* 2017;7(1):1496.
33. **Ivy MI, Thoendel MJ, Jeraldo PR, et al.** Direct Detection and Identification of Prosthetic Joint Infection Pathogens in Synovial Fluid by Metagenomic Shotgun Sequencing. *J Clin Microbiol.* 2018;56(9).
34. **Peffers MJ, Smagul A, Anderson JR.** Proteomic analysis of synovial fluid: current and potential uses to improve clinical outcomes. *Expert Rev Proteomics.* 2019;16(4):287-302.
35. **Griswold AJ, Perez J, Nuytemans K, Strong TA, Wang L, Vance DD, et al.** Transcriptomic analysis of synovial extracellular RNA following knee trauma: A pilot study. *J Orthop Res.* 2018;36(6):1659-1665.
36. **Moher D, Liberati A, Tetzlaff J, Altman DG; PRISMA Group.** Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Int J Surg.* 2010;8(5):336-341.
37. **Wishart DS, Jewison T, Guo AC, et al.** HMDB 3.0—The Human Metabolome Database in 2013. *Nucleic Acids Res.* 2013;41(Database issue):D801-D807.
38. **Ulrich EL, Akutsu H, Doreleijers JF, et al.** BioMagResBank. *Nucleic Acids Res.* 2008;36(Database issue):D402-D408.
39. **Adams SB Jr, Nettles DL, Jones LC, Miller SD, Guyton GP, Schon LC.** Inflammatory cytokines and cellular metabolites as synovial fluid biomarkers of posttraumatic ankle arthritis. *Foot Ankle Int.* 2014;35(12):1241-1249.
40. **Ahn JK, Kim S, Kim J, Hwang J, Kim KH, Cha HS.** A Comparative Metabolomic Evaluation of Behcet's Disease with Arthritis and Seronegative Arthritis Using Synovial Fluid. *PLoS One.* 2015;10(8):e0135856.
41. **Anderson JR, Chokesuwattanaskul S, Phelan MP, et al.** Synovial fluid metabolite profiles differ between osteoarthritis and rheumatoid arthritis. 2018; 57(suppl_3):iii152-iii153.
42. **Carlson AK, Rawle RA, Adams E, Greenwood MC, Bothner B, June RK.** Application of global metabolomic profiling of synovial fluid for osteoarthritis biomarkers. *Biochem Biophys Res Commun.* 2018;499(2):182-188.
43. **Chen R, Han S, Liu X, et al.** Perturbations in amino acids and metabolic pathways in osteoarthritis patients determined by targeted metabolomics analysis. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2018;1085:54-62.
44. **Dubey D, Chaurasia S, Guleria A, et al.** Metabolite assignment of ultrafiltered synovial fluid extracted from knee joints of reactive arthritis patients using high resolution NMR spectroscopy. *Magn Reson Chem.* 2019;57(1):30-43.
45. **Dubey D, Kumar S, Ahmed S, et al.** NMR based serum and synovial fluid metabolomics reveal similar metabolomic profile in patients with reactive arthritis and undifferentiated spondyloarthropathy. Poster Presentations. *Indian Journal of Rheumatology.* 2017;12(5):S24-25.
46. **Dubey D, Kumar S, Chaurasia S, et al.** NMR-Based Serum Metabolomics Revealed Distinctive Metabolic Patterns in Reactive Arthritis Compared with Rheumatoid Arthritis. *J Proteome Res.* 2019;18(1):130-146.
47. **Furman BD, Kimmerling KA, Ramamoorthy S, et al.** Articular fracture upregulates lipid metabolites in human synovial fluid. *Osteoarthritis Cartilage.* 2017;25(Supplement 1):S381.
48. **Hwang J, Ahn JK, Lee J, et al.** Discriminative metabolite profiling of synovial fluid in rheumatoid arthritis compared to osteoarthritis. *Ann Rheum Dis.* 2013;72(Suppl 3): A548-A549.
49. **Kang KY, Lee SH, Jung SM, Park SH, Jung BH, Ju JH.** Downregulation of tryptophan-related metabolomic profile in rheumatoid arthritis synovial fluid. *J Rheumatol.* 2015;42(11):2003-2011.
50. **Khatib N, Papageorgiou A, Fairhurst S, Wilson C, Mason DJ.** Identifying load responsive synovial fluid metabolic markers following pivot-shift testing in ACL injury subjects. *Osteoarthritis Cartilage.* 2018;26 Suppl 1:S168–S169.
51. **Kim S, Hwang J, Kim J, Ahn JK, Cha HS, Kim KH.** Metabolite profiles of synovial fluid change with the radiographic severity of knee osteoarthritis. *Joint Bone Spine.* 2017;84(5):605-610.
52. **Kim S, Hwang J, Xuan J, Jung YH, Cha HS, Kim KH.** Global metabolite profiling of synovial fluid for the specific diagnosis of rheumatoid arthritis from other inflammatory arthritis. *PLoS One.* 2014;9(6):e97501.

53. **Leimer EM, Pappan KL, Nettles DL, et al.** Lipid profile of human synovial fluid following intra-articular ankle fracture. *J Orthop Res.* 2017;35(3):657-666.
54. **Meshitsuka S, Yamazaki E, Inoue M, Hagino H, Teshima R, Yamamoto K.** Nuclear magnetic resonance studies of synovial fluids from patients with rheumatoid arthritis and osteoarthritis. *Clin Chim Acta.* 1999;281(1-2):163-167.
55. **Mickiewicz B, Heard BJ, Chau JK, et al.** Metabolic profiling of synovial fluid in a unilateral ovine model of anterior cruciate ligament reconstruction of the knee suggests biomarkers for early osteoarthritis. *J Orthop Res.* 2015;33(1): 71-77.
56. **Naughton DP, Haywood R, Blake DR, Edmonds S, Hawkes GE, Grootveld M.** A comparative evaluation of the metabolic profiles of normal and inflammatory knee-joint synovial fluids by high resolution proton NMR spectroscopy. *FEBS Lett.* 1993;332(3):221-225.
57. **Yang XY, Zheng KD, Lin K, et al.** Energy Metabolism Disorder as a Contributing Factor of Rheumatoid Arthritis: A Comparative Proteomic and Metabolomic Study. *PLoS One.* 2015;10(7):e0132695.
58. **Zhang W, Likhodii S, Zhang Y, et al.** Classification of osteoarthritis phenotypes by metabolomics analysis. *BMJ Open.* 2014;4(11):e006286.
59. **Zhang W, Likhodii S, Aref-Eshghi E, et al.** Relationship between blood plasma and synovial fluid metabolite concentrations in patients with osteoarthritis. *J Rheumatol.* 2015;42(5):859-865.
60. **Zhang WD, Sun G, Likhodii S, et al.** Metabolomic analysis of human synovial fluid and plasma reveals that phosphatidylcholine metabolism is associated with both osteoarthritis and diabetes mellitus. *Metabolomics.* 2016;12(2):24.
61. **Zheng K, Shen N, Chen H, et al.** Global and targeted metabolomics of synovial fluid discovers special osteoarthritis metabolites. *J Orthop Res.* 2017;35(9):1973-1981.
62. **Mickiewicz B, Kelly JJ, Ludwig TE, et al.** Metabolic analysis of knee synovial fluid as a potential diagnostic approach for osteoarthritis. *J Orthop Res.* 2015;33(11): 1631-1638.
63. **Zhang W, Sun G, Likhodii S, et al.** Metabolomic analysis of human synovial fluid and plasma reveals that phosphatidylcholine metabolism is associated with both osteoarthritis and diabetes mellitus. *Metabolomics.* 2016;12(2):1-10.
64. **Farooqui AA, Horrocks LA, Farooqui T.** Glycerophospholipids in brain: their metabolism, incorporation into membranes, functions, and involvement in neurological disorders. *Chem Phys Lipids.* 2000;106(1):1-29.
65. **Ghauri FYK, Nicholson JK, Sweatman BC, et al.** NMR spectroscopy of human post mortem cerebrospinal fluid: distinction of Alzheimer's disease from control using pattern recognition and statistics. *NMR Biomed.* 1993;6(2):163-167.
66. **Oliviero F, Lo Nigro A, Bernardi D, et al.** A comparative study of serum and synovial fluid lipoprotein levels in patients with various arthritides. *Clin Chim Acta.* 2012;413(1-2):303-307.
67. **Miller EJ, Van der Korst JK, Sokoloff L.** Collagen of human articular and costal cartilage. *Arthritis Rheum.* 1969;12(1):21-29.
68. **Januszewski AS, Karschimkus C, Davis KE, O'Neal D, Ward G, Jenkins AJ.** Plasma 1,5 anhydroglucitol levels, a measure of short-term glycaemia: assay assessment and lower levels in diabetic vs. non-diabetic subjects. *Diabetes Res Clin Pract.* 2012;95(1):e17-e19.
69. **Tillmann K.** Pathological aspects of osteoarthritis related to surgery. *Inflammation.* 1984;8(suppl):S57-S74.
70. **Davies CM, Guilak F, Weinberg JB, Fermor B.** Reactive nitrogen and oxygen species in interleukin-1-mediated DNA damage associated with osteoarthritis. *Osteoarthritis Cartilage.* 2008;16(5):624-630.
71. **Thomas-Reetz A, Hell JW, During MJ, Walch-Solimena C, Jahn R, De Camilli P.** A gamma-aminobutyric acid transporter driven by a proton pump is present in synaptic-like microvesicles of pancreatic beta cells. *Proc Natl Acad Sci U S A.* 1993;90(11):5317-5321.
72. **Handley CJ, Speight G, Leyden KM, Lowther DA.** Extracellular matrix metabolism by chondrocytes. 7. Evidence that L-glutamine is an essential amino acid for chondrocytes and other connective tissue cells. *Biochim Biophys Acta.* 1980 ;627(3):324-331.
73. **Kim DS, Shin MR, Kim YS, et al.** Anti-inflammatory effects of glutamine on LPS-stimulated human dental pulp cells correlate with activation of MKP-1 and attenuation of the MAPK and NF-κB pathways. *Int Endod J.* 2015;48(3):220-228.
74. **Tonomura H, Takahashi KA, Mazda O, et al.** Glutamine protects articular chondrocytes from heat stress and NO-induced apoptosis with HSP70 expression. *Osteoarthritis Cartilage.* 2006;14(6):545-553.
75. **Xu Z, Chen T, Luo J, Ding S, Gao S, Zhang J.** Cartilaginous Metabolomic Study Reveals Potential Mechanisms of Osteophyte Formation in Osteoarthritis. *J Proteome Res.* 2017;16(4):1425-1435.
76. **Chang X, Wei C.** Glycolysis and rheumatoid arthritis. *Int J Rheum Dis.* 2011; 14(3):217-222.
77. **Glass GG.** Osteoarthritis. *Dis Mon.* 2006;52(9):343-362.
78. **Hills BA.** Surface-active phospholipid: a Pandora's box of clinical applications. Part II. Barrier and lubricating properties. *Intern Med J.* 2002;32(5-6):242-251.
79. **Bassit RA, Sawada LA, Bacurau RF, et al.** Branched-chain amino acid supplementation and the immune response of long-distance athletes. *Nutrition.* 2002; 18(5):376-379.
80. **Perlman S, Carr SA.** Citramalic acid in cerebrospinal fluid of patients with bacterial meningitis. *Clin Chem.* 1984;30(7):1209-1212.
81. **Pineton de Chambrun M, Wechsler B, Geri G, Cacoub P, Saadoun D.** New insights into the pathogenesis of Behçet's disease. *Autoimmun Rev.* 2012;11(10):687-698.
82. **Chen M, Lam BK, Kanaoka Y, et al.** Neutrophil-derived leukotriene B4 is required for inflammatory arthritis. *J Exp Med.* 2006;203(4):837-842.
83. **Fallarino F, Grohmann U, Vacca C, et al.** T cell apoptosis by tryptophan catabolism. *Cell Death Differ.* 2002;9(10):1069-1077.
84. **Sumner LW, Amberg A, Barrett D, et al.** Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics.* 2007;3(3):211-221.
85. **Soul J, Dunn SL, Anand S, et al.** Stratification of knee osteoarthritis: two major patient subgroups identified by genome-wide expression analysis of articular cartilage. *Ann Rheum Dis.* 2018;77(3):423.
86. **Kung LHW, Ravi V, Rowley L, et al.** Cartilage MicroRNA Dysregulation During the Onset and Progression of Mouse Osteoarthritis Is Independent of Aggrecanolysis and Overlaps With Candidates From End-Stage Human Disease. *Arthritis Rheumatol.* 2018;70(3):383-395.

Author information

- P. Akhbari, BSc, MBBS, MSc, FRCS (TR & Orth), Upper Limb Fellow and Senior Research Fellow,
- M. K. J. Jaggard, MA Hons CANTAB, BMedSci, BMBS, MRCS, Orthopaedic Registrar,
- R. Bhattacharya, MBBS, MRCS(Edin), MRCS(Glas), MSc, FRCS (Tr. & Orth), Orthopaedic Consultant,
- C. M. Gupte, MA Hons OXON, BMBCh, PhD, FRCS Orth, Clinical Reader, Orthopaedic Consultant, Department of Surgery and Cancer, Imperial College London, and Department of Orthopaedics & Trauma, Imperial College Healthcare NHS Trust, London, UK.
- U. Karamchandani, BSc (Hons), Medical Student, School of Medicine,
- G. Graça, BSc, PhD, Research Associate, Digestion and Reproduction, Imperial College London, London, UK.
- J. C. Lindon, BSc, PhD, DSc, Professor of Chemistry,
- H. R. T. Williams, BSc, PhD, FRCP, Gatroenterology Consultant, Digestive Diseases, Department of Metabolism, Digestion and Reproduction, Imperial College London, London, UK.

Author Contributions

- P. Akhbari: Assisted in designing the article search strategy, Screened articles for final inclusion, Wrote the manuscript.
- U. Karamchandani: Assisted in designing the article search strategy, Performed the article searches according to the PRISMA guidelines, Screened the articles for final inclusion.
- M. K. J. Jaggard: Assisted in writing the manuscript.
- G. Graça: Assisted in writing the manuscript.
- R. Bhattacharya: Reviewed the manuscript prior to submission.
- J. C. Lindon: Assisted in writing the manuscript.
- H. R. T. Williams: Reviewed the manuscript prior to submission.
- C. M. Gupte: Assisted in writing the manuscript, Reviewed the final manuscript prior to submission.

Funding statement

- No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

ICMJE COI statement

- None declared

Acknowledgements

- The authors would like to acknowledge Rebecca Jones for her help with performing the article search. The authors would also like to thank Dr Claire L. Boulangé for her help setting up the metabolic profiling projects conducted at Imperial College, London and for the useful discussions that took place around the topics covered in this systematic review.
- The authors also acknowledge the support of the Imperial NIHR Biomedical Research Centre (BRC).

Ethical review statement

- This study did not require ethical approval.

Osteoarthritis and Cartilage 27 (2019) 1768–1777

Osteoarthritis and Cartilage

Differences in the composition of hip and knee synovial fluid in osteoarthritis: a nuclear magnetic resonance (NMR) spectroscopy study of metabolic profiles

P. Akhbari † *, M.K. Jaggard †, C.L. Boulangé §, U. Vaghela ‖, G. Graça §, R. Bhattacharya †, J.C. Lindon §, H.R.T. Williams ‡ [a], C.M. Gupte † [a]

† Department of Orthopaedics & Trauma, Imperial College Healthcare NHS Trust, London, United Kingdom
‡ Department of Digestive Diseases, Imperial College Healthcare NHS Trust, London, United Kingdom
§ Department of Metabolism, Digestion and Reproduction, Imperial College London, London, United Kingdom
‖ School of Medicine, Imperial College London, London, United Kingdom

ARTICLE INFO

Article history:
Received 3 July 2018
Accepted 3 July 2019

Keywords:
Metabolic profiling
Metabonomics
NMR
Osteoarthritis
Synovial fluid

SUMMARY

Objective: The hip and knee joints differ biomechanically in terms of contact stresses, fluid lubrication and wear patterns. These differences may be reflected in the synovial fluid (SF) composition of the two joints, but the nature of these differences remains unknown. The objective was to identify differences in osteoarthritic hip and knee SF metabolites using metabolic profiling with Nuclear Magnetic Resonance (NMR) spectroscopy.

Design: Twenty-four SF samples (12 hip, 12 knee) were collected from patients with end-stage osteoarthritis (ESOA) undergoing hip/knee arthroplasty. Samples were matched for age, gender, ethnicity and had similar medical comorbidities. NMR spectroscopy was used to analyse the metabolites present in each sample. Principal Component Analysis and Orthogonal Partial Least Squares Discriminant Analysis were undertaken to investigate metabolic differences between the groups. Metabolites were identified using 2D NMR spectra, statistical spectroscopy and by comparison to entries in published databases.

Results: There were significant differences in the metabolic profile between the groups. Four metabolites were found in significantly greater quantities in the knee group compared to the hip group (N-acetylated molecules, glycosaminoglycans, citrate and glutamine).

Conclusions: This is the first study to indicate differences in the metabolic profile of hip and knee SF in ESOA. The identified metabolites can broadly be grouped into those involved in collagen degradation, the tricarboxylic acid cycle and oxidative metabolism in diseased joints. These findings may represent a combination of intra and extra-articular factors.

Introduction

Osteoarthritis (OA) most commonly involves the hip and knee joints, and treatment of this condition leads to significant social and economic costs[1]. The aetiology of OA is multifactorial, but the final pathway results in progressive degradation of chondral cartilage and may involve synovial inflammation and changes to the subchondral bone. This wear of intra-articular tissues results in metabolites in the synovial fluid (SF) that represent inflammatory and degradation products[2].

Hip and knee joints have different biomechanical profiles[3,4]. Thus, the wear patterns in these joints is different both clinically and radiologically. This may in turn lead to differences in the fluid metabolites related to these wear patterns, although there is

* Address correspondence and reprint requests to: P. Akhbari, Department of Trauma and Orthopaedics Imperial College NHS Trust Praed Street St Mary's Hospital Salton House London, W2 1NY United Kingdom. Tel.: 44-20-33121930; fax: 44-20-33121471.

E-mail addresses: pakhbari@nhs.net (P. Akhbari), m.jaggard@nhs.net (M.K. Jaggard), c.boulange09@imperial.ac.uk (C.L. Boulangé), uddhav.vaghela14@imperial.ac.uk (U. Vaghela), g.gomes-da-graca@imperial.ac.uk (G. Graça), rajarshi.bhattacharya@imperial.ac.uk (R. Bhattacharya), j.lindon@imperial.ac.uk (J.C. Lindon), h.williams@imperial.ac.uk (H.R.T. Williams), c.gupte00@imperial.ac.uk (C.M. Gupte).

[a] H.R.T. Williams and C.M. Gupte are joint senior authors.

https://doi.org/10.1016/j.joca.2019.07.017
1063-4584/

limited evidence of this[2,5–7]. Several studies have demonstrated differences in cytokine, enzyme and protein concentrations of SF between these joints[5–7].

Metabolic profiling is a novel technique, which studies the low molecular weight metabolites within a cell, tissue or biofluid using either mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy. Its potential lies in the ability to analyse hundreds or even thousands of small molecules simultaneously. This technique has been employed in several conditions to influence clinical practice[8–10]. More recently, it has been used to identify metabolites within the urine, blood and SF of both animal models and in humans with OA[11,12]. This has yielded not only individual biomarkers for a specific pathological process, but also unique metabolic "signatures" consisting of many metabolites that may identify a distinct pathology based on relative concentrations of these molecules[13].

Although recent developments in the field of metabolic profiling have improved the detection of small molecules in various biological tissues and fluids, there are few studies examining the small molecule composition of human SF (HSF)[12,14–19]. The majority of these have used MS, which has good sensitivity. However, MS is limited by variable ionisation and ion suppression effects, which impair analyte detection[20] and involves destruction of the samples such that repeat testing is precluded. Furthermore, in order to improve metabolite resolution, MS is usually coupled with prior separation of the fluid using chromatography or capillary electrophoresis.

Whilst NMR spectroscopy has a lower sensitivity and a sparser metabolite coverage than MS, its strengths lie in universality of detection, ease of quantitation and the ability to annotate spectral features. It is fast and non-destructive allowing multiple samples to be measured in a day, and the same sample can be analysed numerous times, unlike with MS[20].

Previous analysis of SF has led to a better understanding of the metabolic processes associated with OA and also led to the identification of some of the biomarkers of OA such as arginine and the ratio of branched chain amino acids (BCAAs) to histidine[12,19,21,22]. However, these studies have examined hip and knee SF as a homogeneous group[12,14–19]. There are currently no studies in the literature utilising NMR spectroscopy to assess the differences in small molecule composition between hip and knee SF.

Aims and hypothesis

Our hypothesis was that there were differences in the metabolic composition of SF of end-stage osteoarthritic hip and knee joints. The aim of this study was therefore to identify these differences using NMR spectroscopy.

Materials and methods

Ethical approval was granted by our local research ethics committee (Project 15/LO/0388) and all patients gave informed consent to participate in this study. From the available samples, patients were matched exactly for gender and within 5 units for body mass index (BMI) and 5 years for age. From this cohort, patients with similar medical comorbidities were selected in an attempt to remove these as confounding factors. SF was harvested from 24 patients undergoing primary hip (12 patients) or knee (12 patients) arthroplasty for end-stage osteoarthritis (ESOA) at our institution. ESOA was confirmed based on the patient's symptoms, radiographs and intraoperative findings. All patients completed a questionnaire, which included information on demographics, diet, lifestyle, medical co-morbidities, medications, duration and severity of symptoms and previous intra-articular injections. Co-morbidities included metabolic-related diseases (diabetes, hypertension, ischaemic heart disease, gout, osteoporosis, raised cholesterol and stroke). Data was also collected from the patient's records on BMI.

Sample preparation and metabolic profiling

Samples were collected intraoperatively using a standardised protocol. The knee joint was approached using a midline incision and medial parapatellar approach, whilst a posterior approach was used for the hip[23]. Following skin incision but prior to hip capsulotomy/knee arthrotomy, a 14G syringe was inserted into the suprapatellar pouch of the knee/along the femoral neck of the hip and SF was aspirated. The sampling technique was similar between the groups. Samples were aliquoted and centrifugated at 10,000 g for 15 min. The supernatant was aliquoted, thus removing any cellular material or debris. All samples were stored at −80°C for a maximum of 6 months before analysis.

Samples were defrosted no more than 1 h before being assayed. Each sample (400 μl) was combined with an equal amount of 75 mmol/L sodium dihydrogen phosphate (NaH_2PO_4) buffer at pH 7.4 containing 6.2 mmol/L sodium azide (NaN_3) and 4.6 mmol/L of 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid sodium salt (TSP) and 20% deuterium oxide (D_2O) as published by Dona et al.[24].

Control samples containing only buffer were run in tandem with the HSF samples to identify and exclude potential contamination, which may have occurred at any stage during sample preparation.

Experiments were performed using a Bruker® Avance III 600 MHz spectrometer with a Samplejet 96 well autosampler. One dimensional (1D) high resolution NMR (^{1}H-NMR) spectra were acquired for each sample using the NOESY 1D pulse sequence with optimised water presaturation, as a sum of 128 free induction decays, with 128 k complex points, a mixing time of 10 ms, a delay between the two 90 radiofrequency pulses of 4μs and a relaxation time of 4s.

NMR spectral chemical shifts were internally referenced to glucose (5.23 ppm), automatically phased and baseline corrected. An exponential function was applied to the NMR FIDs prior to being Fourier transformed providing a line broadening of 0.3 Hz, using Topspin 3.2 (Bruker, Germany). The NMR spectra were then imported into Matlab™ (Matlab2016b, Mathworks, Massachusetts, United States) and consisted of 27,492 data points. Spectra were aligned according to the method developed by Veselkov et al. to allow comparison of peaks between samples[25]. A median-fold change normalisation method was used to normalise the spectra[26]. Following normalisation, the data was scaled to unit variance[27].

Metabolite identification

Metabolite identification was achieved by matching chemical shift and peak multiplicity with information in the literature and databases (Human Metabolome Database[28], Biological Magnetic Resonance Bank[29]). Statistical total correlation spectroscopy (STOCSY)[30] was used to aid metabolite identification by

demonstrating peaks (or variables), which show statistical correlations with other peaks (or variables) in the spectra, and thus belong to the same molecule or originate from another molecule related functionally[30]. Two-dimensional NMR spectra, namely $^{1}H-^{1}H$ total correlation spectroscopy (TOCSY) and $^{1}H-^{13}C$ heteronuclear single–quantum correlation spectroscopy (HSQC), were acquired for representative samples to further confirm the identified metabolites.

Statistical analysis

To analyse the large dataset (27,492 variables per sample) generated by NMR, Principal Component Analysis (PCA) and Orthogonal Partial Least Squares-Discriminate Analysis (O-PLS-DA), are the most prominent multivariate analysis techniques[31]. The different data points of the NMR spectra can be thought of being part of a multidimensional graph representing "metabolic coordinates". A more detailed explanation can be found in the article by Lindon et al.[20] and in Appendix 1. Analysis was performed using the SIMCA 14™ statistical package (Sartorius Stedim Biotech, Umea, Sweden). PCA provides an overview of the samples, highlights clustering, and identifies outliers. O-PLS-DA modelling was used to further investigate the differences between the groups. In this method, we regress the NMR data (X matrix) against class variables for the two groups or Y variable. The Y variable is a numerical vector, which encodes the group information. Using this procedure, we identified differences between the two groups. In order to assess the model validity, seven-fold cross validation was performed. The parameters Q^2Y and R^2Y were evaluated as measures of model fitting. The goodness of prediction (Q^2Y) measures the predictability of the model. The goodness of fit (R^2Y) is the proportion of the NMR data that explains the differences between the two groups (Y variable). The robustness of the model was assessed by calculating the Q^2Y 100 times by randomly assigning the cross-validation groups (the samples predicted in each of the seven cross-validation rounds). The Q^2Y values were compared with Q^2Y values obtained after performing 100 permutations of the Y variable. The model was considered reliable if the Q^2Y confidence intervals of the permuted and non-permuted models did not overlap.

The metabolite signals responsible for the separation between the two groups were identified from the O-PLS-DA model loadings plot. The O-PLS-DA separates the data into predictive components that explains the differences between the two groups and the orthogonal component that contains the remaining unrelated information. Only signals with a correlation coefficient to the Y variable >0.6 were considered to have a significant impact on group separation. To better identify the peaks, back transformation of the loadings was performed by multiplying each variable by its standard deviation. The NMR signals related to the predictive components are then integrated to demonstrate the differences between the two groups for the identified metabolites. The individual peaks of all the spectra were also visualised to see if there were any obvious differences between the two groups. The area under each metabolite peak was analysed by univariate analysis, using the Student's t-test.

The false discovery rate (FDR) adjusted *p*-values were calculated using the Benjamini-Hochberg method[32] and was performed to account for multiple testing of the identified metabolites. Network analysis was performed on the identified metabolites to further the interpretation of metabolic changes, using the MetaboNetworks software in Matlab™[33].

Table I
Patient demographics. There was no significant difference in age ($P = 0.27$), gender, BMI ($P = 0.71$) or medical co-morbidities between the groups. IHD, Ischaemic heart disease; DM, Diabetes Mellitus; CVA, Cerebrovascular Accident

	Hip	Knee
Number of patients	12	12
Age (Mean ± SD)	67.7 (12.2)	63.3 (21.2)
Gender (Male: Female)	3:9	3:9
BMI (Mean ± SD)	30.4 (5.1)	29.9 (4.6)
Ethnicity	11 Caucasian 1 Asian	9 Caucasian 2 Afro-Caribbean 1 Asian
Disease (Number of patients per group)		
IHD	2	0
Hypertension	9	8
Hypercholesterolaemia	7	6
DM	4	2
CVA	1	0

Results

Patient demographics

There were no significant differences in age ($P = 0.27$), gender or BMI ($P = 0.71$) between the groups and the common medical co-morbidities were also similar (Table I). Two hip and three knee patients had received a steroid injection. One injection in the knee group was performed 6 months before the HSF sample was taken and the remaining four injections were performed over a year before the samples were taken. None of the patients had received a synthetic hyaluronic acid injection. The full list of medical co-morbidities and medications are included in Appendix 2.

PCA and O-PLS-DA analysis of differences between hip and knee metabolites

A typical ^{1}H-NMR spectra of hip and knee SF from the current cohort is demonstrated in Fig. 1. The PCA scores plot (Fig. 2) demonstrated some separation between the ^{1}H-NMR spectra in the hip and knee groups in PC1 (24%). This suggested that there may be metabolic differences in the HSF composition of the two joints. Although PC2 (15%) and PC3 (11%) are still associated with variability within the data, no separation is observed in the scores of those components (Fig. 2 and Appendix 3). The knee group demonstrated tighter clustering whereas the hip group showed more variability based on the NMR spectra of HSF. Further inspection of the PCA scores plots showed that the heterogeneity of the hip group is not motivated by age, gender or BMI (Appendix 4).

By using seven-fold cross-validation, an O-PLS-DA model with good predictive ability was obtained (Q^2Y = 0.360 [95% CI 0.348–0.373], $R^2Y = 0.873$). This was further confirmed by running permutation analyses on the model (Q^2Y −0.234 [95% CI -0.298 to −0.170] and R^2Y 0.705 [95% CI was 0.693–0.716]) [Fig. 3(a)]. O-PLS-DA analysis showed separation between hip and knee SF [Fig. 3(b)] and allowed identification of the metabolite resonances, which were responsible for the separation of the hip and knee

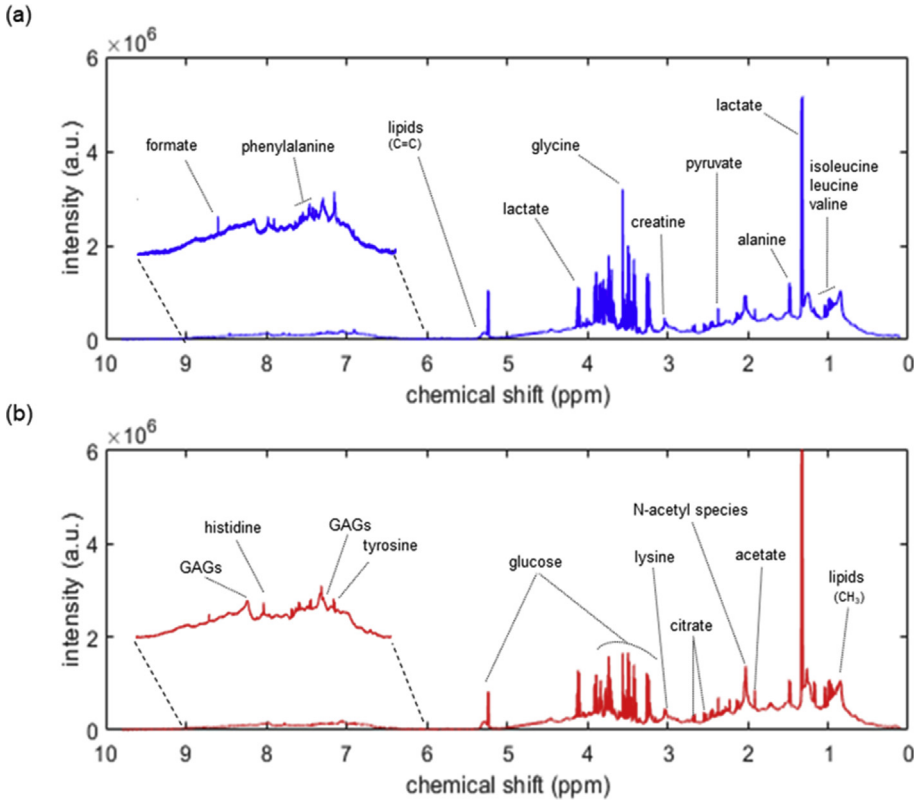


Fig. 1. Typical ^{1}H-NMR spectra taken from the study cohort demonstrating some of the identified metabolites. a. Knee synovial fluid. b. Hip synovial fluid. GAGs – glycosaminoglycans.

groups through inspection of the back-scaled O-PLS-DA loadings plot.

All the metabolites consistently identified in all samples are listed in Appendix 5. HSF from the knee group showed greater levels of alanine, citrate, dimethylsulfone, glucose, glutamine, glycosaminoglycans (GAGs), histidine, lysine, N-acetylated molecules, pyruvate and valine compared to the hip group. HSF from the hip group showed greater levels of choline compared to the knee group [Fig. 4(a) and (b)].

Evaluation of individual spectra

The individual spectral analysis confirmed the metabolic differences identified from the back-scaled loadings plot of the O-PLS-DA and additionally revealed greater quantities of tyrosine in the knee and hypoxanthine in the hip group (Fig. 5). All the metabolites identified that were found in different quantities between the 2 groups are listed in Table II. Following FDR adjustment of the *p*-values, four of these metabolites remained significant (Table II).

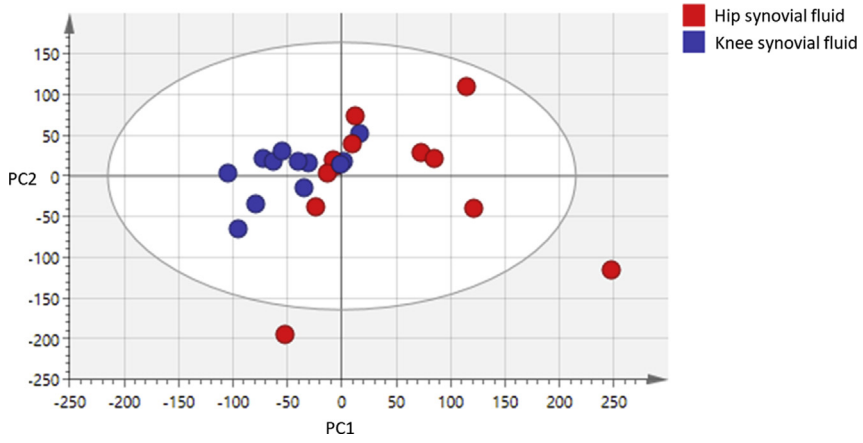


Fig. 2. PCA scores plot of PC1 vs PC2 with each data point representing the NMR spectrum of an individual human synovial fluid sample and demonstrating separation between the hip and knee groups. The ellipse represents Hotelling T2 with 95% confidence interval. The percentage variation explained is 24% for PC1 and 15% for PC2. PC – principal component.

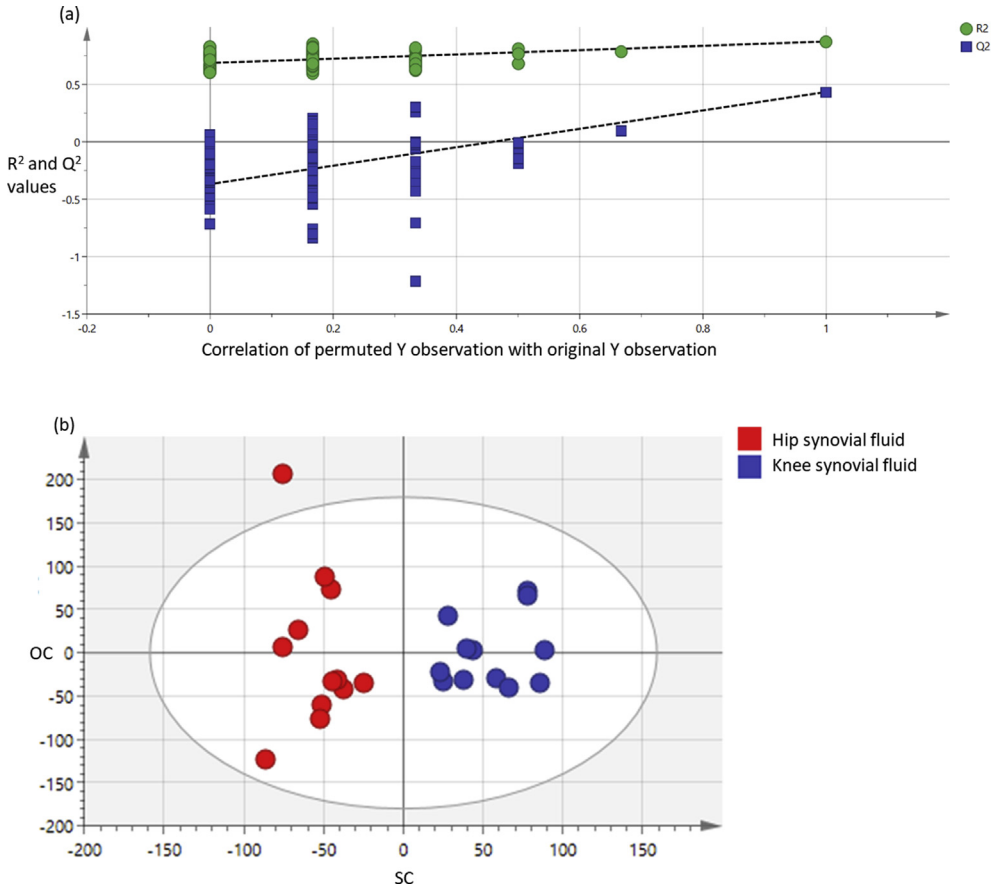


Fig. 3. O-PLS-DA model of osteoarthritic SF comparing hip and knee groups. a. Graphical representation of the permutation analysis demonstrating that all the permuted models have a Q2Y value lower than the values obtained in the hip vs knee synovial fluid model. b. Scores plot of the cross-validated O-PLS-DA model demonstrated a good separation between the hip and knee groups with differences in the spectra of each sample explained by whether the fluid was taken from the hip or knee joint. The ellipse represents Hotelling T2 with 95% confidence interval. SC – separating component; OC – orthogonal component.

Table II
Metabolites identified from the back-scaled loadings, univariate analysis and confirmed by 2D-NMR spectra. Following FDR adjustment, only four of the 14 identified metabolites remained significant and they were all found in greater quantities in the knee group (*). The chemical shift indicates which metabolite peaks were integrated and used for metabolite comparison between the two groups. The peak area for the two cohorts is also provided, but the units are arbitrary and hence not included. GAG – glycosaminoglycans; ppm – parts per million; 95%CIs – 95% confidence intervals; FDR – false discovery rate

Metabolite ID	NMR chemical shift (ppm)	Joint containing higher concentration of metabolite	Peak area: knee cohort (mean [95%CIs])	Peak area: hip cohort (mean [95%CIs])	*P*-value (Student's *t*-test)	FDR-adjusted *P*-value
N-Acetylated molecules*	2.02	knee	1,674.9 [1365.6–1984.1]	625.7 [386.7–864.7]	0.000034	0.001
GAG*	7.97	knee	2,615.1 [2,352.4–2,877.8]	1,669.9 [1371.6–1968.2]	0.00012	0.004
Citrate*	2.53	knee	1,243.2 [1065.2–1421.2]	766.1 [639.4–892.7]	0.00037	0.012
Glutamine*	2.45	knee	2,759.4 [2,366.2–3,152.6]	1,837.5 [1622.2–2052.8]	0.0009	0.028
Hypoxanthine	8.18	hip	33.7 [27.8–39.5]	54.8 [44.2–65.5]	0.003	0.110
Dimethylsulfone	3.15	knee	139.8 [104.0–175.5]	71.8 [50.1–93.5]	0.005	0.167
Tyrosine	7.19	knee	254.8 [207.6–301.9]	166.8 [139.0–194.6]	0.006	0.183
Lysine	3.03	knee	608.2 [540.3–676.0]	483.4 [444.0–522.8]	0.006	0.200
Choline	3.19	hip	160.5 [130.2–190.8]	254.8 [203.7–305.9]	0.006	0.200
Pyruvate	2.37	knee	529.8 [444.3–615.3]	350.6 [259.4–441.8]	0.010	0.339
Valine	1.04	knee	1,506.4 [1219.2–1793.7]	1,047.8 [924.1–1171.6]	0.012	0.384
Glucose	5.23	knee	4,113.4 [3,448.2–4,778.6]	2,742.3 [2000.9–3,483.6]	0.013	0.436
Histidine	7.05	knee	166.3 [139.2–193.4]	125.7 [113.6–137.9]	0.017	0.566
Alanine	1.47	knee	2,666.4 [2,121.0–3,211.8]	1,829.8 [1452.1–2,207.5]	0.023	0.751

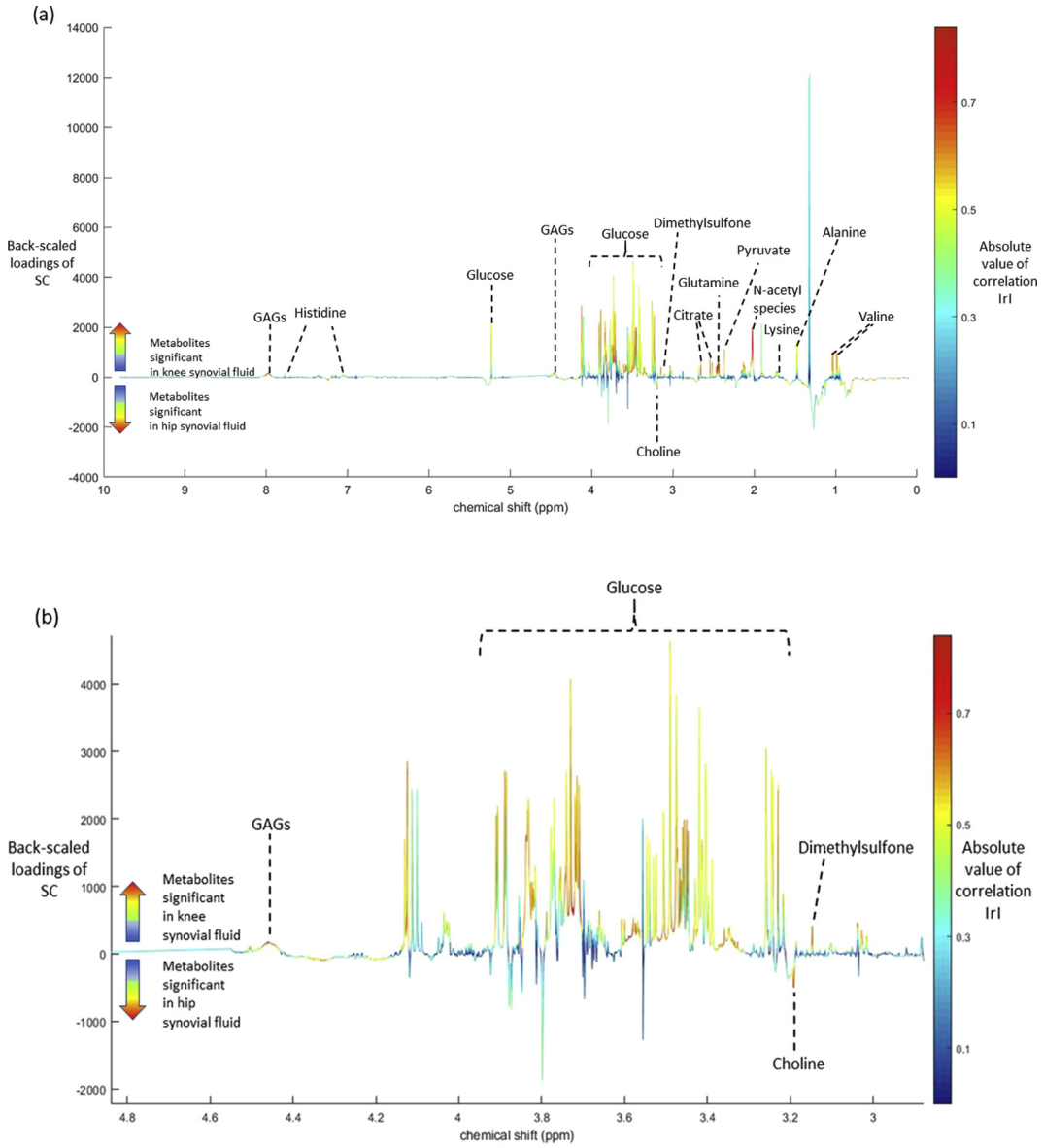


Fig. 4. Back-scaled loadings plot of SC from the O-PLS-DA model. a. The significant metabolites are red peaks, with those pointing upwards significantly increased in knee and those pointing downwards significantly increased in hip synovial fluid. b. Expansion of the Back-scaled loadings plot demonstrating the difference between the glucose and choline peaks. GAGs – glycosaminoglycans; SC – separating component; ppm – parts per million.

These were N-acetylated molecules, GAGs, citrate and glutamine. A network analysis of all the identified metabolites was created and is included in Appendix 6.

Discussion

SF of hips and knees with end-stage OA (ESOA) was examined for small metabolites using NMR spectroscopy. After appropriate statistical analysis (PCA, O-PLS-DA, FDR adjustment of *P*-values), there were significant differences in four metabolites (N-acetylated molecules, GAGs, citrate and glutamine), which were found in greater quantities in knee SF.

FDR analysis ensures that detected differences are unlikely to be due to chance, but this can lead to false negatives when small numbers of samples are used. For this reason, the remaining metabolites are also considered in the discussion. Eight further metabolites (alanine, dimethylsulfone, glucose, histidine, lysine, pyruvate, tyrosine and valine) were found in greater proportion in the knee group and two metabolites (choline and hypoxanthine) were found in greater proportion in the hip group. The possible functions of these metabolites are listed in Appendix 7.

This is the first study to examine the metabolic differences in HSF between the hip and knee joints in patients with ESOA. All the identified metabolites can broadly be grouped into those involved in proteoglycan (PG) and collagen degradation, the tricarboxylic acid (TCA) cycle, lipid metabolism and BCAA catabolism.

Analysis of metabolite differences

Table III lists the possible functions of the four metabolites, which remained significant following FDR adjustment.

GAGs are typically found in the extracellular matrix (ECM)[34]. They attach to a core protein leading to the formation of PGs. Due to their important role in maintaining the turgor pressure of articular

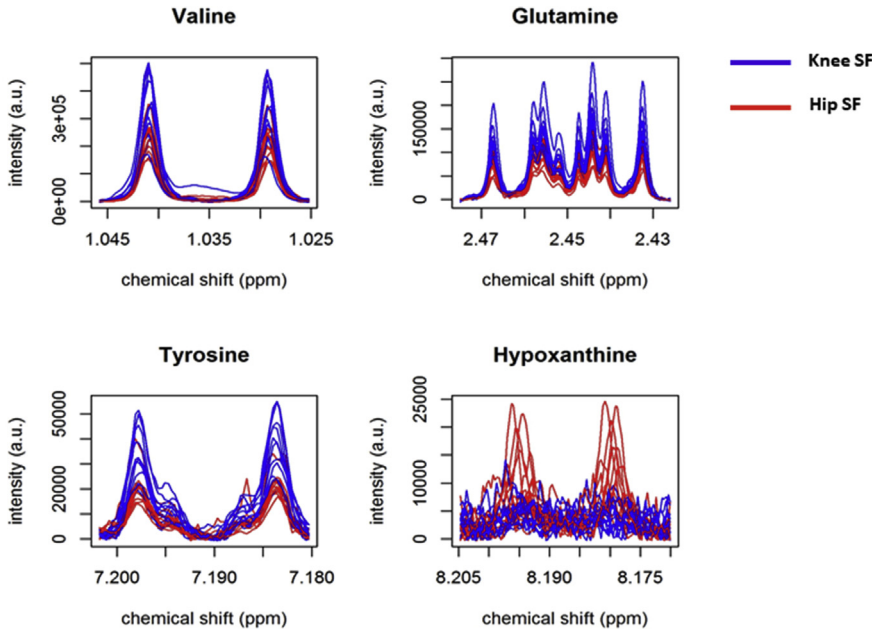


Fig. 5. Metabolite analysis from spectral inspection. Figure demonstrates differences in signal intensity between the groups. Valine & glutamine were identified from the back-scaled loadings but have been inserted to demonstrate they still show a difference between the groups on spectral inspection. Tyrosine & hypoxanthine did not show significant differences on the back-scaled loadings, but the differences are apparent when comparing the individual spectra. SF – synovial fluid; ppm – parts per million.

cartilage[35], the greater proportion of GAGs in the knee group suggests a greater rate of articular cartilage breakdown compared to the hip group. This results in altered tribological and mechanical responses, ultimately leading to matrix degradation[36]. As hyaluronic acid (HA) is the principal N-acetyl-glucosamine containing polymer in SF, a greater intensity of the N-acetylated molecule signal in the knee group suggests greater PG and hence cartilage degradation[37].

Citrate is a major intermediary in the TCA cycle, urea cycle, amino acid and fatty acid metabolism[38]. The TCA cycle is a key biochemical pathway that provides cellular energy in the form of Adenosine Triphosphate (ATP) by oxidative conversion of carbohydrates. Most of carbon contributes to the TCA cycle through the conversion of glucose to pyruvate, but there is also a contribution of glutamine to the TCA intermediary alpha-ketoglutarate. Glutamine has an important role in oxidative metabolism, and therefore elevated levels suggest altered oxidative metabolism in diseased joints[39]. Our results agree with other studies, demonstrating that alterations in the metabolic profile of SF from OA patients also suggests a role for altered energy metabolism in OA[15,17,21]. The greater abundance of TCA intermediaries in the knee group could also be related to mitochondrial dysfunction, where the TCA cycle functions[40].

A network analysis of all the identified metabolites was performed, including those that did not survive FDR correction (Appendix 6). This illustrates several different pathways involved in hip (phospholipid metabolism via choline) and knee (amino acids, TCA and glucose metabolism) osteoarthritic HSF metabolism. This correlates with the potential functions of these metabolites described above and in Appendix 7.

Possible causes of differences in hip and knee synovial fluid metabolites

It is important to realise that OA is a heterogenous syndrome with various aetiopathogenic phenotypes. Therefore, a more integrated approach is required when thinking about the pathological processes involved in this condition. This remains true when considering the metabolic differences between the hip and knee groups. Broadly speaking, these differences may be due to or a combination of intra and extra-articular factors.

The hip has classically been thought of as a ball and socket joint with a high level of conformity[3]. On the other hand, the knee joint is less conforming with greater contact stresses, due to a smaller area of contact between the tibio-femoral articulations. One of the functions of the meniscus is to increase the contact area in the knee to help distribute joint forces to a larger surface area than the direct tibio-femoral cartilage contact region[4]. However, in severe knee OA, the meniscus does not always function normally due to meniscal tears or calcification, thus reducing its ability to act in this capacity. Consequently, there may be greater point loading in the knee resulting in a larger force through a smaller area, whereas in the hip there is a more volumetric wear pattern due to the greater conformity. In addition, SF incorporates metabolites from the surrounding tissues within the joint, as well as systemic sources. Synovial inflammation in OA may have an important role. There are numerous pathways and mediators, which may be responsible, including proinflammatory mediators, such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), and matrix metalloproteinases, ultimately resulting in changes to the metabolic profile of SF[41]. Mitochondrial activity has also been shown to increase when exposed to osteoarthritic SF[41]. Furthermore, plasma and SF bone morphogenic protein-2 (BMP-2) levels have been positively correlated to the severity of knee OA[42]. Cartilage turnover has been demonstrated to be significantly greater in the knee than the hip, suggesting different mechanisms of disease progression between these joints[43]. This was felt to be due to differences in protein turnover, more specifically, the attempted cartilage repair response.

Extra-articular factors include age, BMI and medical comorbidities. Obesity has been linked to OA in both the hip and knee, but its effect is more profound in the knee joint[44]. However, in this study, patients were matched for age and BMI in attempt to

Table III
List of identified metabolites, the joint in which they are found in significantly greater quantities and the function of the metabolites in osteoarthritis. GAGs – glycosaminoglycans; NO - Nitric Oxide; PG – proteoglycan; TCA tricarboxylic acid

Metabolite ID	Joint containing larger quantity of metabolite	Function of metabolite	Reference
N-Acetylated molecules	knee	Marker of PG breakdown & cartilage degradation.	Schiller et al.[34]
GAGs	knee	Marker of articular cartilage breakdown.	Thompson et al.[35]
Citrate	knee	Major intermediary in the TCA cycle, urea cycle, amino acid & fatty acid metabolism. Role in altered energy metabolism with elevated levels suggesting altered oxidative metabolism in diseased joints.	Berg et al.[36] Kim et al.[16]
Glutamine	knee	Key role in oxidative metabolism. Elevated levels suggest altered oxidative metabolism in diseased joints. It also suppresses the formation of inflammatory cytokines and protects chondrocytes from NO induced apoptosis and heat stress.	Handley et al.[37] Kim et al.[38] Tonomura et al.[39]

remove these as confounding factors. Similarly, there were comparable medical co-morbidities in both groups to reduce their effects as potential confounding factors.

Although the metabolic differences identified in this study may soley be due to the disease process or reflect differences in normal cartilage and SF biology, it is more likely to be represented by a complex interplay of all these factors.

Potential of NMR and MS in further analysis of SF in health and disease

There are a few spectroscopic methods available to analyse metabolites and generate metabolic datasets for complex biological samples. ^{1}H-NMR spectroscopy produces a comprehensive profile of metabolic signals without derivatization, separation and preselected measurement parameters[45].

In the past decade, ^{1}H-NMR-based metabolic profiling has developed into a powerful tool for the identification of metabolites and biochemical markers for a variety of human disorders[46]. Metabolic profiling of SF provides a direct representation of what is taking place in the joint and yields the most accurate and joint specific metabolic profile that is relevant to OA[47].

There is some evidence in the literature that biomarkers exist in SF for numerous conditions including OA degradation products[12,14,17–19,21,22], prosthetic joint infection[48,49], and bacterial infection in native joints[50]. The techniques utilised in this study could be used in larger groups to further analyse the relevance of previously found biomarkers in joint disease and to identify new metabolic patterns of diseases such as OA and inflammatory arthritis.

Our study demonstrates the potential of metabolic profiling of joint fluid using NMR spectroscopy to identify important metabolites in these conditions.

At present the above speculations remain little more than hypotheses with a modicum of evidence already in the literature. Further studies should utilise a larger group of fluid samples with a more targeted analysis of individual metabolites for a specific patient group or disease category.

Limitations

Despite the samples in both groups being matched for age, gender, BMI, with similar medical comorbidities and ethnicity, the overall numbers were small (12 in each group). However, the validated O-PLS-DA model demonstrated 14 metabolites that were significantly different between the hip and knee groups, four of which remained significant following univariate testing and FDR adjustment. Secondly, there was no age/gender-matched non-arthritic control groups, although such samples would be difficult to acquire because of ethical constraints. Furthermore, this study did not take into account the number of compartments in the knee affected with OA. However, our unpublished analysis has not demonstrated any metabolic differences between OA affecting one or more compartments.

Conclusion

To our knowledge, this is the first study to indicate differences in the metabolic profile of HSF between the hip and knee joints in patients with end-stage OA. There were four metabolites found in significantly greater proportions in the knee group (N-acetylated molecules, GAGs, citrate and glutamine). These metabolites have a role in collagen degradation, the TCA cycle and oxidative metabolism in diseased joints.

Further research is required with a larger group of patients and a control group to see if these metabolites may serve as putative biomarkers for the diagnosis of knee OA, monitoring of disease progression and/or future treatment strategies.

Author contributions

All authors made substantial contributions to the conception and design of the study, data acquisition and analysis, drafting/revision and final approval of the manuscript.

Competing interests

Professor John Lindon declares a shareholding in the company “Metabometrix Ltd” which is contracted to perform small molecule studies. Dr Claire Boulangé has received funding from “Metabometrix Ltd”. No other authors disclose any competing interests.

Source of funding

No external funding was received for this study.

Acknowledgements

This article is independent research funded by the National Institute for Health Research (NIHR) and Imperial Biomedical Research Centre (BRC). The views expressed in this publication are those of the authors and not necessarily those of the National Health Service (NHS), the NIHR or the Department of Health.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2019.07.017.

References

1. Chen A, Gupte C, Akhtar K, Smith P, Cobb J. The global economic cost of osteoarthritis: how the UK compares. Arthritis 2012;2012:698709.
2. Krasnokutsky S, Attur M, Palmer G, Samuels J, Abramson SB. Current concepts in the pathogenesis of osteoarthritis. Osteoarthr Cartil 2008;16(Suppl 3):S1–3.

3. Yoshida H, Faust A, Wilckens J, Kitagawa M, Fetto J, Chao EY. Three-dimensional dynamic hip contact area and pressure distribution during activities of daily living. J Biomech 2006;39:1996–2004.
4. Andriacchi TP, Koo S, Scanlan SF. Gait mechanics influence healthy cartilage morphology and osteoarthritis of the knee. J Bone Joint Surg Am 2009;91(Suppl 1):95–101.
5. Galandakova A, Ulrichova J, Langova K, Hanakova A, Vrbka M, Hartl M, *et al*. Characteristics of synovial fluid required for optimization of lubrication fluid for biotribological experiments. J Biomed Mater Res B Appl Biomater 2017;105:1422–31.
6. Olszewska-Slonina D, Jung S, Matewski D, Olszewski KJ, Krzyzynska-Malinowska E, Braszkiewicz A, *et al*. Lysosomal enzymes in serum and synovial fluid in patients with osteoarthritis. Scand J Clin Lab Investig 2015;75:145–51.
7. Snelling SJ, Bas S, Puskas GJ, Dakin SG, Suva D, Finckh A, *et al*. Presence of IL-17 in synovial fluid identifies a potential inflammatory osteoarthritic phenotype. PLoS One 2017;12, e0175109.
8. Fischer K, Kettunen J, Wurtz P, Haller T, Havulinna AS, Kangas AJ, *et al*. Biomarker profiling by nuclear magnetic resonance spectroscopy for the prediction of all-cause mortality: an observational study of 17,345 persons. PLoS Med 2014;11, e1001606.
9. Holmes E, Loo RL, Stamler J, Bictash M, Yap I, Chan Q, *et al*. Human metabolic phenotype diversity and its association with diet and blood pressure. Nature 2008;453:396–400.
10. Yu B, Zheng Y, Nettleton JA, Alexander D, Coresh J, Boerwinkle E. Serum metabolomic profiling and incident CKD among African Americans. Clin J Am Soc Nephrol 2014;9:1410–7.
11. Lamers R-JAN, van Nesselrooij JHJ, Kraus VB, Jordan JM, Renner JB, Dragomir AD, *et al*. Identification of an urinary metabolite profile associated with osteoarthritis. Osteoarthritis Cartilage 2005;13:762–8.
12. Zhai G, Wang-Sattler R, Hart DJ, Arden NK, Hakim AJ, Illig T, *et al*. Serum branched-chain amino acid to histidine ratio: a novel metabolomic biomarker of knee osteoarthritis. Ann Rheum Dis 2010;69:1227–31.
13. Qiu Y, Cai G, Zhou B, Li D, Zhao A, Xie G, *et al*. A Distinct Metabolic Signature of Human Colorectal Cancer with Prognostic Potential. Clinical Cancer Research; 2014.
14. Zhang WD, Sun G, Likhodii S, Aref-Eshghi E, Harper PE, Randell E, *et al*. Metabolomic analysis of human synovial fluid and plasma reveals that phosphatidylcholine metabolism is associated with both osteoarthritis and diabetes mellitus. Metabolomics 2016;12:24.
15. Kim S, Hwang J, Kim J, Ahn JK, Cha HS, Kim KH. Metabolite profiles of synovial fluid change with the radiographic severity of knee osteoarthritis. Jt Bone Spine 2017 October;84:605–10.
16. Xu Z, Chen T, Luo J, Ding S, Gao S, Zhang J. Cartilaginous metabolomic study reveals potential mechanisms of osteophyte formation in osteoarthritis. J Proteome Res 2017;16:1425–35.
17. Zhang Q, Li H, Zhang Z, Yang F, Chen J. Serum metabolites as potential biomarkers for diagnosis of knee osteoarthritis. Dis Markers 2015;2015:684794.
18. Zhang W, Likhodii S, Zhang Y, Aref-Eshghi E, Harper PE, Randell E, *et al*. Classification of osteoarthritis phenotypes by metabolomics analysis. BMJ Open 2014;4, e006286.
19. Zhang W, Sun G, Likhodii S, Liu M, Aref-Eshghi E, Harper PE, *et al*. Metabolomic analysis of human plasma reveals that arginine is depleted in knee osteoarthritis patients. Osteoarthr Cartil 2016;24:827–34.
20. Lindon JC, Nicholson JK. Spectroscopic and Statistical Techniques for Information Recovery in Metabonomics and Metabolomics, doi:10.1146/annurev.anchem.1.031207.113026. Annual Review of Analytical Chemistry 2008; 1: 45-69.
21. Damyanovich AZ, Staples JR, Chan ADM, Marshall KW. Comparative study of normal and osteoarthritic canine synovial fluid using 500 MHz ^{1}H magnetic resonance spectroscopy. J Orthpaedic Res 1999;17:223–31.
22. Lacitignola L, Fanizzi FP, Francioso E. 1H NMR investigation of normal and osteoarthritic synovial fluid in the horse. Vet Comp Orthop Traumatol 2008;21:85–8.
23. Hoppenfeld S, De Boer PG, Buckley R. Surgical Exposures in Orthopaedics : The Anatomic Approach 2009.
24. Dona AC, Jiménez B, Schäfer H, Humpfer E, Spraul M, Lewis MR, *et al*. Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping. Anal Chem 2014;86:9887–94.
25. Veselkov KA, Lindon JC, Ebbels TM, Crockford D, Volynkin VV, Holmes E, *et al*. Recursive segment-wise peak alignment of biological (1)h NMR spectra for improved metabolic biomarker recovery. Anal Chem 2009;81:56–66.
26. Dieterle F, Ross A, Schlotterbeck G, Senn H. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. Anal Chem 2006;78:4281–90.
27. van den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, van der Werf MJ. Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics 2006;7:142.
28. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, *et al*. HMDB 3.0—the human Metabolome database in 2013. Nucleic Acids Res 2013;41:D801–7.
29. Ulrich EL, Akutsu H, Doreleijers JF, Harano Y, Ioannidis YE, Lin J, *et al*. BioMagResBank. Nucleic Acids Res 2008;36:D402–8.
30. Cloarec O, Dumas ME, Craig A, Barton RH, Trygg J, Hudson J, *et al*. Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic 1H NMR data sets. Anal Chem 2005;77:1282–9.
31. Bartel J, Krumsiek J, Theis FJ. Statistical methods for the analysis of high-throughput metabolomics data. Comput Struct Biotechnol J 2013;4, e201301009.
32. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B 1995;57:289–300.
33. Posma JM, Robinette SL, Holmes E, Nicholson JK. MetaboNetworks, an interactive Matlab-based toolbox for creating, customizing and exploring sub-networks from KEGG. Bioinformatics 2014;30:893–5.
34. Kresse H, Schonherr E. Proteoglycans of the extracellular matrix and growth control. J Cell Physiol 2001;189:266–74.
35. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. FASEB J 1992;6:861–70.
36. Thompson Jr RC, Oegema Jr TR. Metabolic activity of articular cartilage in osteoarthritis. An in vitro study. Journal of Bone and Joint Surgery - Series A 1979;61:407–16.
37. Schiller J, Naji L, Huster D, Kaufmann J, Arnold K. 1H and 13C HR-MAS NMR investigations on native and enzymatically digested bovine nasal cartilage. Magma 2001;13:19–27.

38. Berg JMTJ, Stryer L. Amino acids are made from intermediates of the citric acid cycle and other major pathways. In: Biochemistry. New York: W H Freeman; 2002.
39. Handley CJ, Speight G, Leyden KM, Lowther DA. Extracellular matrix metabolism by chondrocytes 7. Evidence that L-glutamine is an essential amino acid for chondrocytes and other connective tissue cells. Biochim Biophys Acta Gen Subj 1980;627:324–31.
40. Blanco FJ, Lopez-Armada MJ, Maneiro E. Mitochondrial dysfunction in osteoarthritis. Mitochondrion 2004;4:715–28.
41. de Sousa EB, Dos Santos Junior GC, Aguiar RP, da Costa Sartore R, de Oliveira ACL, Almeida FCL, *et al.* Osteoarthritic synovial fluid modulates cell phenotype and metabolic behavior in vitro. Stem Cell Int 2019;2019:8169172.
42. Liu Y, Hou R, Yin R, Yin W. Correlation of bone morphogenetic protein-2 levels in serum and synovial fluid with disease severity of knee osteoarthritis. Med Sci Monit : international medical journal of experimental and clinical research 2015;21:363–70.
43. Catterall JB, Zura RD, Bolognesi MP, Kraus VB. Aspartic acid racemization reveals a high turnover state in knee compared with hip osteoarthritic cartilage. Osteoarthr Cartil 2016;24:374–81.
44. Reyes C, Leyland KM, Peat G, Cooper C, Arden NK, Prieto-Alhambra D. Association between overweight and obesity and risk of clinically diagnosed knee, hip, and hand osteoarthritis: a population-based cohort study. Arthritis & rheumatology (Hoboken, N.J.) 2016;68:1869–75.
45. Nicholson JK, Wilson ID. High resolution proton magnetic resonance spectroscopy of biological fluids. Prog Nucl Magn Reson Spectrosc 1989;21:449–501.
46. Nagana Gowda GA, Raftery D. Can NMR solve some significant challenges in metabolomics? J Magn Reson 2015;260:144–60.
47. Adams Jr SB, Setton LA, Nettles DL. The role of metabolomics in osteoarthritis research. J Am Acad Orthop Surg 2013;21:63–4.
48. Parvizi J, Jacovides C, Antoci V, Ghanem E. Diagnosis of periprosthetic joint infection: the utility of a simple yet unappreciated enzyme. J Bone Jt. Surg 2011;93:2242–8.
49. Di Cesare PE, Chang E, Preston CF, Liu CJ. Serum interleukin-6 as a marker of periprosthetic infection following total hip and knee arthroplasty. J Bone Joint Surg Am 2005;87:1921–7.
50. Lenski M, Scherer MA. Diagnostic potential of inflammatory markers in septic arthritis and periprosthetic joint infections: a clinical study with 719 patients. Infectious Diseases 2015;47:399–409.

Follow us @BoneJointRes

Freely available online OPEN ACCESS

BJR

SYNOVIUM

Differences between infected and noninfected synovial fluid

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

P. Akhbari,
M. J. Jaggard,
C. L. Boulangé,
U. Vaghela,
G. Graça,
R. Bhattacharya,
J. C. Lindon,
H. R. T. Williams,
C. M. Gupte

From Imperial College NHS Trust and Imperial College, London, UK

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.

Cite this article: *Bone Joint Res* 2021;10(1):85–95.

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.

Correspondence should be sent to Pouya Akhbari; email: pakhbari@nhs.net

doi: 10.1302/2046-3758.101.BJR-2020-0285.R1

Bone Joint Res 2021;10(1):85–95.

- 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 I. 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 *Staphylococcus aureus*	1	N/A	
Group B *Meningococcus*	2	N/A	
B-haemolytic *Streptococcus* group C	1	N/A	
Bacillus species	1	N/A	
Rothia species	1	N/A	
Coag-negative *Staphylococcus aureus*	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.

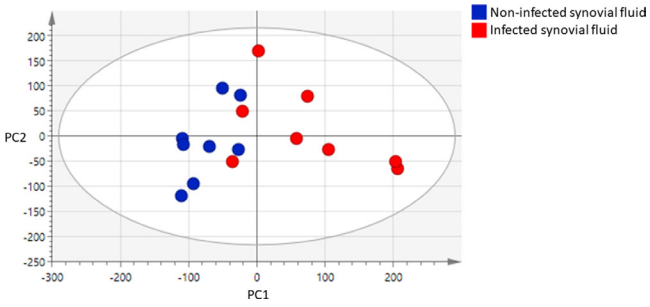


Fig. 1

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.

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.

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 arthrotomy/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-tetradeuteropropionic 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) ^{1}H-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

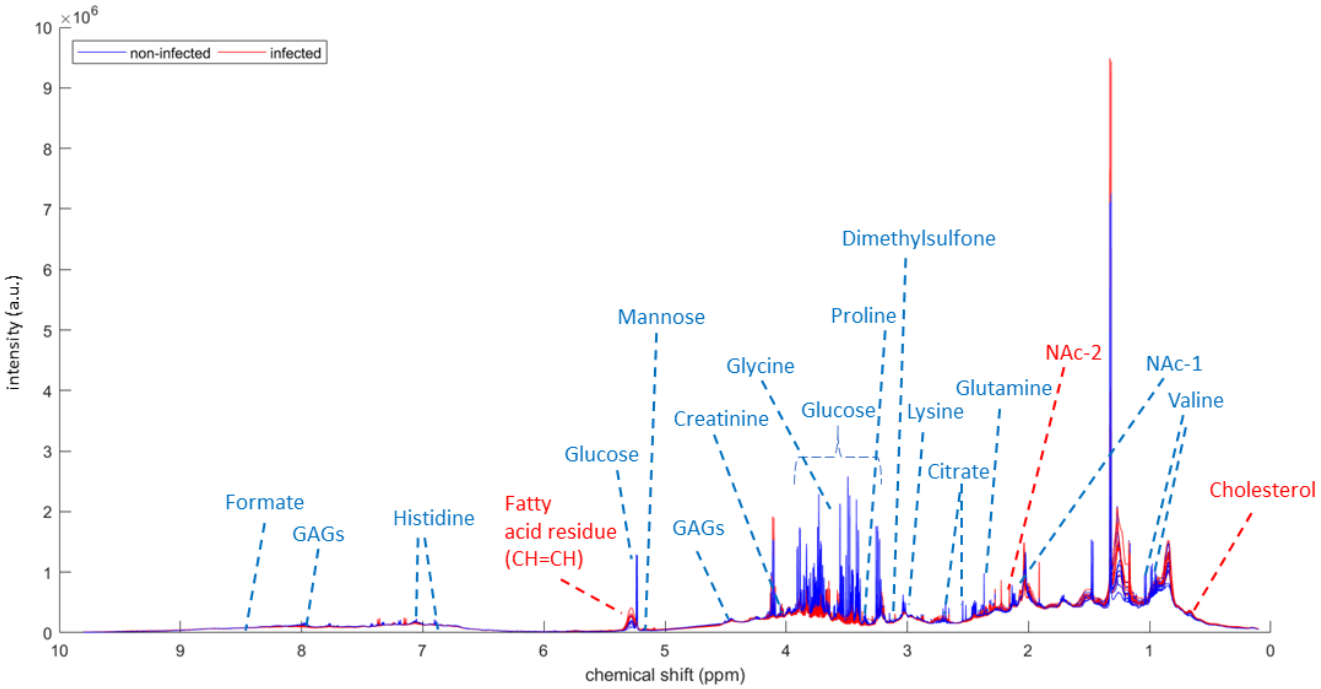


Fig. 2

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 μs, 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 methods[21] 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 ^{1}H-^{1}H total correlation spectroscopy (TOCSY) and ^{1}H-^{13}C 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.

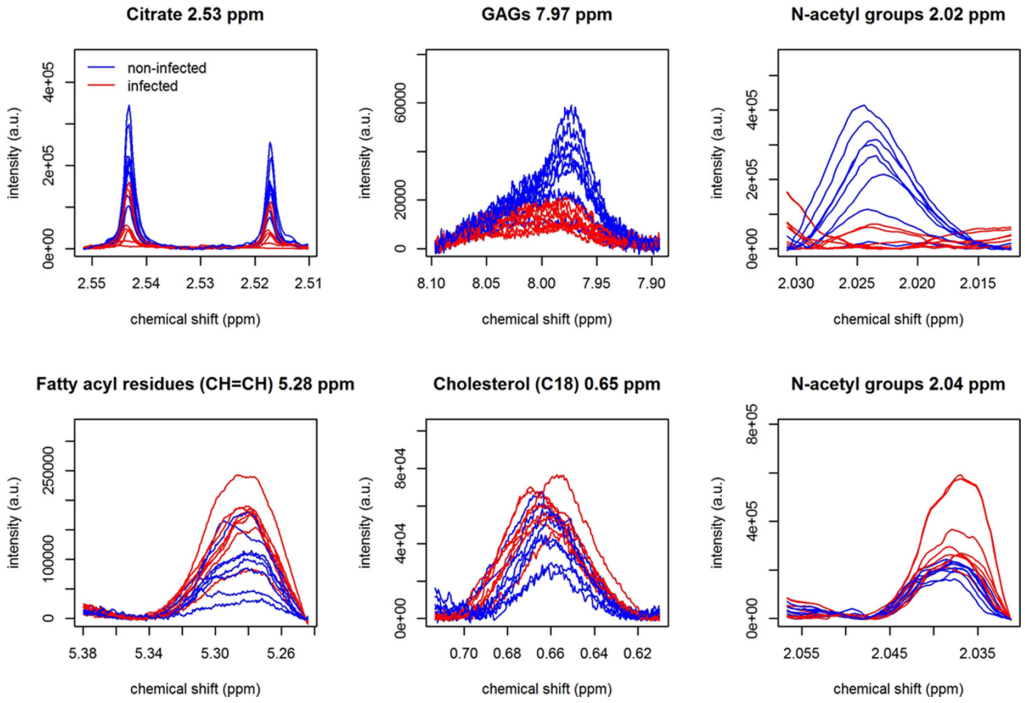


Fig. 3

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 ^{1}H-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 CH_2 groups gave a p-value of 0.051 (independent-samples *t*-test), and the peak at 0.85 ppm from fatty acyl CH_3 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

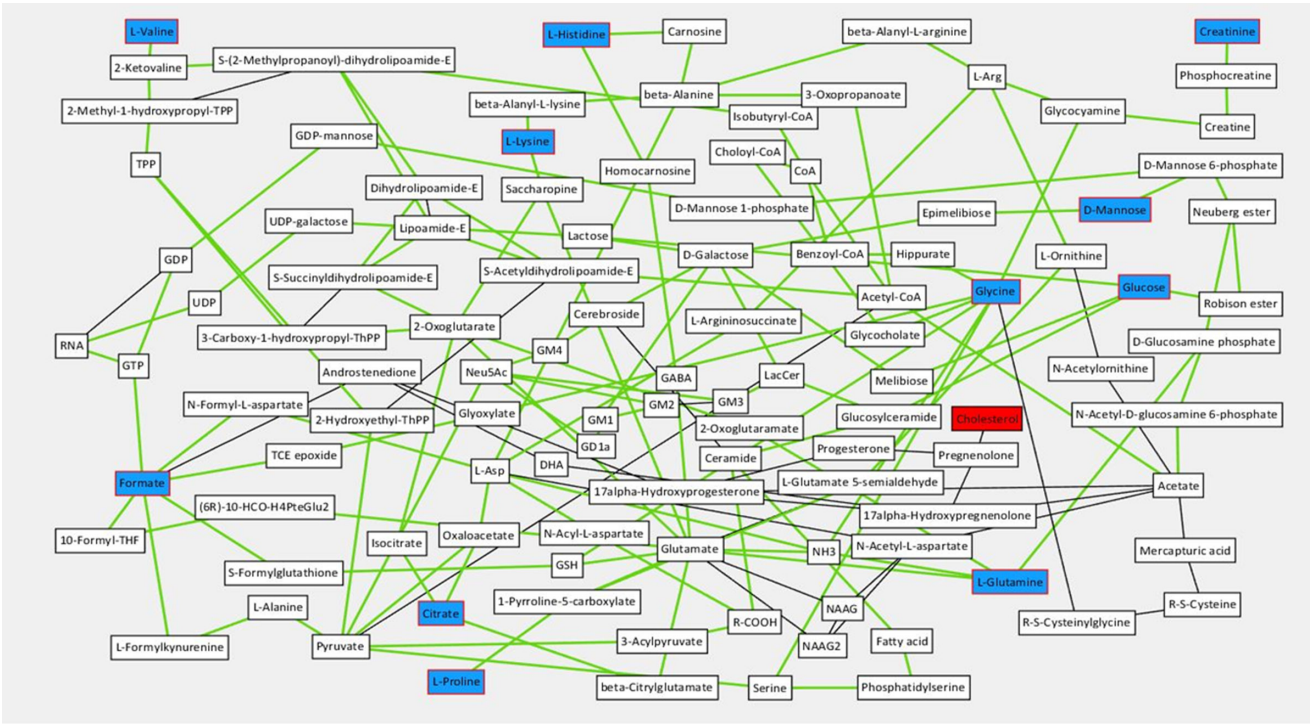


Fig. 4

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 α1-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 Glucose Mannose	Citrate is a major intermediary in the TCA cycle, urea cycle, and amino acid and fatty acid metabolism.[40] 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.	Li et al[33] Berg et al[40]
Glycine Glutamine Valine	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 Proline Lysine	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]
Creatinine Histidine	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]
Dimethylsulfone	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]
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 al[31] 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 ^{1}H 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 glycophospholipid 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 bacterial[51] and/or macrophage activity[52] 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. ^{1}H 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, ^{1}H-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

Follow P. Akhbari on Instagram at doctor.pouya

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.

References

1. **Huotari K**, **Peltola M**, **Jämsen E**. The incidence of late prosthetic joint infections: a registry-based study of 112,708 primary hip and knee replacements. *Acta Orthop.* 2015;86(3):321–325.
2. **Parvizi J**. Infection: a recurring slippery slope. *Orthopaedic Proceedings.* 2015:116.
3. **Palmer MP**, **Melton-Kreft R**, **Nistico L**, **et al**. Polymerase chain Reaction-Electrospray-Time-of-Flight mass spectrometry versus culture for bacterial detection in septic arthritis and osteoarthritis. *Genet Test Mol Biomarkers.* 2016;20(12):721–731.
4. **Huang R**, **Hu C-C**, **Adeli B**, **Mortazavi J**, **Parvizi J**. Culture-Negative periprosthetic joint infection does not preclude infection control. *Clin Orthop Relat Res.* 2012;470(10):2717–2723.
5. **Schinsky MF**, **Della Valle CJ**, **Sporer SM**, **Paprosky WG**. Perioperative testing for joint infection in patients undergoing revision total hip arthroplasty. *J Bone Joint Surg Am.* 2008;90-A(9):1869–1875.
6. **Bottner F**, **Wegner A**, **Winkelmann W**, **Becker K**, **Erren M**, **Götze C**. Interleukin-6, procalcitonin and TNF-alpha: markers of peri-prosthetic infection following total joint replacement. *J Bone Joint Surg Br.* 2007;89-B(1):94–99.
7. **Shahi A**, **Kheir MM**, **Tarabichi M**, **Hosseinzadeh HRS**, **Tan TL**, **Parvizi J**. Serum D-dimer test is promising for the diagnosis of periprosthetic joint infection and timing of reimplantation. *J Bone Joint Surg Am.* 2017;99-A(17):1419–1427
8. **Drago L**, **Vassena C**, **Dozio E**, **et al**. Procalcitonin, C-reactive protein, interleukin-6, and soluble intercellular adhesion molecule-1 as markers of postoperative orthopaedic joint prosthesis infections. *Int J Immunopathol Pharmacol.* 2011;24(2):433–440.
9. **Wyatt MC**, **Beswick AD**, **Kunutsor SK**, **Wilson MJ**, **Whitehouse MR**, **Blom AW**. The alpha-defensin immunoassay and leukocyte esterase colorimetric strip test for the diagnosis of periprosthetic infection: a systematic review and meta-analysis. *J Bone Joint Surg Am.* 2016;98-A(12):992–1000.
10. **Suen K**, **Keeka M**, **Ailabouni R**, **Tran P**. Synovasure 'quick test' is not as accurate as the laboratory-based α-defensin immunoassay: a systematic review and meta-analysis. *Bone Joint J.* 2018;100-B(1):66–72.
11. **Ahmed SS**, **Haddad FS**. Prosthetic joint infection. *Bone Joint Res.* 2019;8(11):570–572.
12. **Saleh A**, **George J**, **Faour M**, **Klika AK**, **Higuera CA**. Serum biomarkers in periprosthetic joint infections. *Bone Joint Res.* 2018;7(1):85–93.
13. **Parkinson JA**. NMR Spectroscopy Methods in Metabolic Phenotyping. In: Lindon JC, Nicholson JK, Holmes E, eds. *The Handbook of Metabolic Phenotyping*: Elsevier, 2019:53–96.
14. **Wiener E**, **Zanetti M**, **Hodler J**, **Pfirrmann CW**. Lactate and T (2) measurements of synovial aspirates at 1.5 T: differentiation of septic from non-septic arthritis. *Skeletal Radiol.* 2008;37(8):743–748.
15. **Hügle T**, **Kovacs H**, **Heijnen IAFM**, **et al**. Synovial fluid metabolomics in different forms of arthritis assessed by nuclear magnetic resonance spectroscopy. *Clin Exp Rheumatol.* 2012;30(2):240–245.
16. **Jin W**, **Woo D-C**, **Jahng G-H**. In vivo H1 MR spectroscopy using 3 tesla to investigate the metabolic profiles of joint fluids in different types of knee diseases. *J Appl Clin Med Phys.* 2016;17(2):561–572.
17. **Akhbari P**, **Jaggard MK**, **Boulangé CL**, **et al**. Differences in the composition of hip and knee synovial fluid in osteoarthritis: a nuclear magnetic resonance (NMR) spectroscopy study of metabolic profiles. *Osteoarthritis Cartilage.* 2019;27(12):1768–1777.
18. **Hoppenfeld S**, **De Boer PG**, **Buckley R**. *Surgical exposures in orthopaedics: the anatomic approach.* 4th Edition: Lippincott Williams and Wilkins, 2009.
19. **Dona AC**, **Jiménez B**, **Schäfer H**, **et al**. Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping. *Anal Chem.* 2014;86(19):2014/10/07):9887–9894.
20. **Veselkov KA**, **Lindon JC**, **Ebbels TMD**, **et al**. Recursive segment-wise peak alignment of biological (1 h) NMR spectra for improved metabolic biomarker recovery. *Anal Chem.* 2009;81(1):56–66.
21. **Dieterle F**, **Ross A**, **Schlotterbeck G**, **Senn H**. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. application in 1H NMR metabonomics. *Anal Chem.* 2006;78(13):4281–4290.
22. **Wishart DS**, **Jewison T**, **Guo AC**, **et al**. HMDB 3.0--The Human Metabolome Database in 2013. *Nucleic Acids Res.* 2013;41(Database issue):D801–D7.
23. **Cloarec O**, **Dumas M-E**, **Craig A**, **et al**. Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic 1H NMR data sets. *Anal Chem.* 2005;77(5):1282–1289.
24. **Sumner LW**, **Amberg A**, **Barrett D**, **et al**. Proposed minimum reporting standards for chemical analysis chemical analysis Working Group (CAWG) metabolomics standards initiative (MSI). *Metabolomics.* 2007;3(3):211–221.

25. **Lindon JC**, **Nicholson JK**. Spectroscopic and statistical techniques for information recovery in metabonomics and metabolomics. *Annual Review of Analytical Chemistry*. 2008;1(1):45–69.
26. **Benjamini Y**, **Hochberg Y**. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B*. 1995;57(1):289–300.
27. **Posma JM**, **Robinette SL**, **Holmes E**, **Nicholson JK**. MetaboNetworks, an interactive Matlab-based toolbox for creating, customizing and exploring sub-networks from KEGG. *Bioinformatics*. 2014;30(6):893–895.
28. **Saccenti E**, **Hoefsloot HCJ**, **Smilde AK**, **Westerhuis JA**, **Hendriks MMWB**. Reflections on univariate and multivariate analysis of metabolomics data. *Metabolomics*. 2014;10(3):361–374.
29. **Otvos JD**, **Shalaurova I**, **Wolak-Dinsmore J**, **et al**. GlycA: a composite nuclear magnetic resonance biomarker of systemic inflammation. *Clin Chem*. 2015;61(5):714–723.
30. **Naughton DP**, **Haywood R**, **Blake DR**, **Edmonds S**, **Hawkes GE**, **Grootveld M**. A comparative evaluation of the metabolic profiles of normal and inflammatory knee-joint synovial fluids by high resolution proton NMR spectroscopy. *FEBS Lett*. 1993;332(3):221–225.
31. **Wikoff WR**, **Pendyala G**, **Siuzdak G**, **Fox HS**. Metabolomic analysis of the cerebrospinal fluid reveals changes in phospholipase expression in the CNS of SIV-infected macaques. *J Clin Invest*. 2008;118(7):2661–2669.
32. **Mu J**, **Yang Y**, **Chen J**, **et al**. Elevated host lipid metabolism revealed by iTRAQ-based quantitative proteomic analysis of cerebrospinal fluid of tuberculous meningitis patients. *Biochem Biophys Res Commun*. 2015;466(4):689–695.
33. **Li Z**, **Du B**, **Li J**, **et al**. Cerebrospinal fluid metabolomic profiling in tuberculous and viral meningitis: screening potential markers for differential diagnosis. *Clinica Chimica Acta*. 2017;466:38–45.
34. **Prete P**, **Gurakar-Osborne A**, **Kashyap M**. Synovial fluid lipoproteins: review of current concepts and new directions. *Semin Arthritis Rheum*. 1993;23(2):79–89.
35. **Damyanovich AZ**, **Staples JR**, **Marshall KW**. 1H NMR investigation of changes in the metabolic profile of synovial fluid in bilateral canine osteoarthritis with unilateral joint denervation. *Osteoarthritis and Cartilage*. 1999;7(2):165–172.
36. **Burger D**, **Dayer J-M**. High-Density lipoprotein-associated apolipoprotein A-I: the missing link between infection and chronic inflammation? *Autoimmun Rev*. 2002;1(1-2):111–117.
37. **Gabay C**, **Kushner I**. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med*. 1999;340(6):448–454.
38. **Park YB**, **Lee SK**, **Lee WK**, **et al**. Lipid profiles in untreated patients with rheumatoid arthritis. *J Rheumatol*. 1999;26(8):1701–1704.
39. **Ananth L**, **Prete PE**, **Kashyap ML**. Apolipoproteins A-I and B and cholesterol in synovial fluid of patients with rheumatoid arthritis. *Metabolism*. 1993;42(7):803–806.
40. **Berg JM TJ**, **Stryer L**. Amino Acids Are Made from Intermediates of the Citric Acid Cycle and Other Major Pathways. *Biochemistry*. 5th edition. New York: W H Freeman, 2002.
41. **Wu G**. Amino acids: metabolism, functions, and nutrition. *Amino Acids*. 2009;37(1):1–17.
42. **Weiner J**, **Parida SK**, **Maertzdorf J**, **et al**. Biomarkers of inflammation, immunosuppression and stress with active disease are revealed by metabolomic profiling of tuberculosis patients. *PLoS One*. 2012;7(7):e40221.
43. **Revelles O**, **Espinosa-Urgel M**. Proline and Lysine Metabolism. In:Ramos JL, ed. *Pseudomonas*. Boston, Massachusetts: Springer, 2004:273–292.
44. **Rosenblum W**. Dimethyl sulfoxide effects on platelet aggregation and vascular reactivity in pial microcirculation. *Ann N Y Acad Sci*. 1983;411(1 Biological Ac):110–119.
45. **Marthyn P**, **Beuscart A**, **Coll J**, **Moreau-Gachelin F**, **Righi M**. Dmso reduces CSF-1 receptor levels and causes apoptosis in v-myc immortalized mouse macrophages. *Exp Cell Res*. 1998;243(1):94–100.
46. **Watson RW**, **Rotstein OD**, **Parodo J**, **Bitar R**, **Hackam D**, **Marshall JC**. Granulocytic differentiation of HL-60 cells results in spontaneous apoptosis mediated by increased caspase expression. *FEBS Lett*. 1997;412(3):603–609.
47. **Thompson RC**, **Oegema TR**. Metabolic activity of articular cartilage in osteoarthritis. An in vitro study. *J Bone Joint Surg Am*. 1979;61-A(3):407–416.
48. **Schiller J**, **Naji L**, **Huster D**, **Kaufmann J**, **Arnold K**. 1H and 13C HR-MAS NMR investigations on native and enzymatically digested bovine nasal cartilage. *MAGMA*. 2001;13(1):19–27.
49. **Smith RL**, **Schurman DJ**. Bacterial arthritis. A staphylococcal proteoglycan-releasing factor. *Arthritis Rheum*. 1986;29(11):1378–1386.
50. **Macfarlane S**, **Macfarlane GT**. Regulation of short-chain fatty acid production. *Proc Nutr Soc*. 2003;62(1):67–72.
51. **Harwood C R**, **Wipat A**. 15 - Bacterial Protein Synthesis. In: *Molecular Medical Microbiology* ed. Sussman M. London: Academic Press, 2002:321–339.
52. **Eichelbaum K**, **Krijgsveld J**. Rapid temporal dynamics of transcription, protein synthesis, and secretion during macrophage activation. *Mol Cell Proteomics*. 2014;13(3):792–810.
53. **Engelke UFH**, **Tangerman A**, **Willemsen MAAP**, **et al**. Dimethyl sulfone in human cerebrospinal fluid and blood plasma confirmed by one-dimensional (1)H and two-dimensional (1)H-(13)C NMR. *NMR Biomed*. 2005;18(5):331–336.

Author information:

- P. Akhbari, BSc MBBS MSc FRCS (Tr & Orth)Eng, Upper Limb Fellow and Senior Research Fellow
- M. J. Jaggard, MA Hons CANTAB, BMedSci, BMBS, MRCS, Orthopaedic Registrar
- R. Bhattacharya, MBBS, MRCS(Edin), MRCS(Glas), MSc, FRCS (Tr. & Orth), Orthopaedic Consultant
  Department of Trauma and Orthopaedics, Imperial College Healthcare NHS Trust, London, UK.
- C. L. Boulangé, BSc, MSc, PhD, Honorary Research Associate
- G. Graça, PhD, Research Associate
- J. C. Lindon, BSc, PhD, DSc, Professor of Chemistry
  Department of Metabolism, Digestion and Reproduction, Imperial College London, London, UK.
- U. Vaghela, BSc (Hons), MBBS, Foundation Year 1 Doctor, Department of Surgery and Cancer, Imperial College London, London, UK.
- H. R. T. Williams, BSc, PhD, FRCP, Gastroenterology Consultant, Department of Digestive Diseases, Imperial College London, London, UK.
- C. M. Gupte, MA Hons OXON, BMBCh, PhD, FRCS Orth, Consultant Orthopaedic Surgeon, Clinical Reader, Department of Trauma and Orthopaedics, Imperial College Healthcare NHS Trust, London, UK; Department of Surgery and Cancer, Imperial College London, London, UK.

Author contributions:

- P. Akhbari: Conceptualized and designed the work, Contributed to the sample collection, assembly of data, and statistical analysis, Drafted the article.
- M. J. Jaggard: Contributed substantially to the conception and design of the work, Contributed to the sample collection, assembly of data, and statistical analysis.
- C. L. Boulange: Contributed substantially to the conception and design of the work, Collected the spectral data, Contributed to the statistical analysis.
- U. Vaghela: Contributed substantially to the conception and design of the work, Contributed to the sample collection and assembly of data.
- G. Graça: Contributed to the statistical analysis, Drafted the article.
- R. Bhattacharya: Contributed substantially to the conception and design of the work, Assisted with interpretation of the data, Arranged the logistical and funding support, Critically reviewed the manuscript before final approval.
- J. C. Lindon: Contributed substantially to the conception and design of the work, Drafted the article, Assisted with the interpretation of the data, Arranged the logistical and funding support, Critically reviewed the manuscript before final approval.
- H. R. T. Williams: Contributed substantially to the conception and design of the work, Assisted with the interpretation of the data, Arranged the logistical and funding support, Critically reviewed the manuscript before final approval.
- C. M. Gupte: Contributed to the sample collection and assembly of data, Contributed to the statistical analysis, Drafted the article, Assisted with the interpretation of the data, Arranged the logistical and funding support, Critically reviewed the manuscript before final approval.

- J. C. Lindon, H. R. T. Williams, and C. M. Gupte are joint senior authors.

Funding statement:

- This article is independently funded by the National Institute for Health Research (NIHR) and Imperial Biomedical Research Centre (BRC). Infrastructure support for this research was also provided by the NIHR Imperial BRC. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

ICMJE COI statement:

- J. C. Lindon is employed by Imperial College London, and reports royalties from Elsevier and John Wiley unrelated to this study. C. L. Boulangé reports funding from Metabometrix Ltd, a company contracted to perform small molecule studies, unrelated to this study.

Acknowledgements:

- The views expressed in this publication are those of the authors and not necessarily those of the NHS, the National Institute for Health Research (NIHR), or the Department of Health.

Ethical review statement:

- Ethical approval was granted by our local research ethics committee (Project 15/LO/0388).